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Characteristics of bioactive molecules with antioxidant and biological activities of some medicinal and edible plants

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Characteristics of bioactive molecules with antioxidant and biological activities of some medicinal and edible plants

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Dedication

I dedicate this dissertation to everyone who, directly or indirectly, contributed to the achievement and success of this work.

I am grateful for the huge love and unyielding support of all my family members who never allowed me to give up on the pursuit of my dreams.

A special feeling of gratitude goes to my loving parents who always stressed the importance of education. You did not only give me moral and emotional support but also instilled in me tireless work ethic and persistent determination to be whatever I wanted to be in life without limitation. Your support helped me to overcome the troubling emotions I experienced and obstacles I faced.

I also dedicate this dissertation to my caring brothers, lovely sister and sister-in-law, as well as my sweet nieces and nephews. Thank you so much for being there for me throughout the entire period I was far from you. I will always appreciate all what you have done for me.

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Thank you all so much. You put a smile in my heart.

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Thank you so much sincerely.

Abstract

The present work focused on the study of two edible plants, with attributable medicinal values, named *Anthyllis vulneraria* L. (*A.vulneraria*) and *Azadirachta indica* L. (*A.indica*) collected in Tunisia and India, respectively. The aim of the work was to determine the chemical composition of *A.vulneraria* leaf and flower extracts and *A.indica* leaf extract and to investigate their antioxidant, antibacterial and anti-proliferative (against specific cancer cell lines) activities in order to design formulations for use in the food and cosmetic industries.

The chemical composition of the extracts was estimated by spectrophotometric assays and quantified by High Performance Liquid Chromatography coupled to Mass Spectrometry (HPLC-MS) and diode array (HPLC-DAD). The radical scavenging activity of the extracts was evaluated *in vitro* by different analytical methods. The antioxidant capacity of *A. vulneraria* and *A. indica* samples in oil-in-water emulsions and in raw minced beef was also studied to evaluate their preservative and quality evolution effect, in general, and the shelf life of these food products during the storage period. In addition, the antibacterial activity of the different extracts was tested against pathogenic microorganisms causing food poisoning and the anti-proliferative activity was evaluated against several human cancer cell lines.

The results obtained revealed that: (**1**) aqueous EtOH extracts of *A.vulneraria* and *A.indica* showed better extraction yields, higher content of phenolic compounds and higher anti-radical activity than pure EtOH extracts. (**2**) *A. vulneraria* flowers showed higher biological activities than leaves. (**3**) In *Model Food System*, at 0.25% (*v/v*), *A.vulneraria* flowers extracted in 50%-aqueous EtOH protected oil-in-water emulsions against oxidation and prolonged shelf life better than leaf extract and even better than the sample with gallic acid (under the same conditions). Moreover, freeze-dried *A.vulneraria* flower sample, added at 0.5% (*w/w*) to minced beef, showed better results in protecting against lipid oxidation and in maintaining its organoleptic properties than freeze-dried leaf sample. The preservative effect of freeze-dried *A. vulneraria* leaf and flower was slightly lower than that of the synthetic preservative (0.5%, *w/w*). Furthermore, the combination of *A. indica* and *Capsicum baccatum* showed a strong synergistic effect against the spoilage of meat products even better than the synthetic preservative (0.7%, *w/w*). (**4**) In the study of the antibacterial activity of the extracts, it was found that, on the one hand, the flower extract of *A. vulneraria* (100 µg/mL) showed better inhibition of the growth of most of the tested bacterial strains than leaf extract. On the other hand, leaf extract of *A. indica* (100 µg/mL) had better antibacterial activity than penicillin.

(**5**) The study of the anti-proliferative activity of extracts against human cancer cells showed that, at 1% (*v/v*), *A. vulneraria* leaf extract was more effective in preventing the proliferation of cancer cells than *A.vulneraria* flower extract, especially HepG2 cells. At 5% (*v/v*), both extracts (leaf and flower) showed potent anti-proliferative activity against the three cancer cell lines tested. Moreover, *A. indica* leaf extract at 7% (*v/v*) showed a more potent reduction of breast and cervical adenocarcinoma-derived cell lines viability (MCF-7 and HeLa) than hepatocellular carcinoma derived cells (HepG2). Different compounds that could be responsible for the antioxidant, antibacterial and anti-proliferative activities of the different extracts investigated, belonging to the phenolic acid and flavonoid families, were identified and quantified by HPLC-MS.

Keywords: *Anthyllis vulneraria*, antibacterial activity, anti-proliferative activity, *Azadirachta indica*, emulsion, HPLC-MS, meat products, oxidative stability, radical scavenging activity, synergistic effect.

Resumen

El presente trabajo se ha centrado en el estudio de dos plantas comestibles, con valores medicinales atribuibles, denominadas *Anthyllis vulneraria* L. (*A. vulneraria*) y *Azadirachta indica* L. (*A. indica*) recolectadas en Túnez e India, respectivamente. El objetivo del trabajo fue determinar la composición química de los extractos de hojas y flores de *A. vulneraria* y del extracto de hojas de *A. indica* e investigar sus actividades antioxidantes, antibacterianas y anti-proliferativas (frente a específicas líneas celulares cancerígenas) para diseñar formulaciones que permitan su uso en la industria alimentaria, farmacéutica y cosmética.

La composición química de los extractos se estimó mediante ensayos espectrofotométricos y se cuantificó por cromatografía líquida de alta resolución acoplada a espectrometría de masas (HPLC-MS) y diodo array (HPLC-DAD). La actividad de eliminación de radicales de los extractos se evaluó *in vitro* mediante diferentes métodos analíticos. También se estudió la capacidad antioxidante de los extractos en emulsiones de aceite en agua y en carne de vacuno cruda picada para evaluar tanto el efecto conservador y de evolución de la calidad, en general, como la vida útil de estos productos alimentarios durante el periodo de almacenamiento. Además, se probó la actividad antibacteriana de los extractos frente a microorganismos patógenos causantes de intoxicaciones alimentarias. La actividad anti-proliferativa se evaluó frente a varias líneas celulares cancerosas humanas.

Los resultados obtenidos revelaron que: (**1**) los extractos acuosos de EtOH de *A. vulneraria* y *A. indica* muestran mejores rendimientos de extracción, mayor contenido de compuestos fenólicos y mayor actividad antirradicalaria que los extractos de EtOH puro. (**2**) las flores de *A. vulneraria* presentan actividades biológicas mejor que las hojas. (**3**) en emulsiones de aceite en agua, el extracto floral de *A. vulneraria* al 50% de EtOH y concentración 0.25% (*v/v*) protege frente a la oxidación y prolonga la vida útil mejor que el extracto de hojas e, incluso, mejor que la muestra con ácido gálico. Por otra parte, el extracto de flores de *A. vulneraria* liofilizado y añadido al 0.5% (*w/w*) en carne de ternera picada presentó mejores resultados en la protección frente a la oxidación de los lípidos y en mantener sus propiedades organolépticas que el extracto de hojas liofilizado. En ambos casos el efecto conservante fue ligeramente inferior al del conservante sintético (0.5%, *w/w*). Además, la combinación de *A. indica* y *Capsicum baccatum* mostró un potente efecto sinérgico frente al deterioro de los productos cárnicos incluso con mejores resultados que el conservante sintético (0.7%, *w/w*). (**4**) En el estudio de la actividad anti-bacteriana de los extractos se comprobó que con el extracto

de flores de *A. vulneraria* (100 µg/mL) se obtiene más inhibición del crecimiento de la mayoría de las cepas bacterianas ensayadas que con el de hojas de *A. vulneraria*. Asimismo, el extracto de hoja de *A. indica* (100 µg/mL) tuvo mejor actividad anti-bacteriana que la penicilina. (**5**) En el estudio de la actividad anti-proliferativa, el extracto de hoja de *A. vulneraria* al 1% (*v/v*) fue más eficaz para impedir el crecimiento de las células cancerosas que el extracto de flor de *A. vulneraria*, especialmente las células HepG2. A concentración 5% (*v/v*) ambos extractos (hoja y flor) mostraron una actividad anti-proliferativa en las tres líneas celulares cancerosas ensayadas. Por otra parte, el extracto de hoja de *A. indica* al 7% (*v/v*) mostró una potente reducción de las líneas celulares derivadas de adenocarcinoma de mama y cuello uterino (MCF-7 y HeLa) mayor que frente a las células derivadas de carcinoma hepatocelular (HepG2). Se identificaron y cuantificaron por HPLC-MS diferentes compuestos que podrían ser responsables de dichas actividades antioxidantes, antibacterianas y anti-proliferativas de los extractos investigados, perteneciente a las familias de los ácidos fenólicos y de los flavonoides.

Palabras clave: actividad anti-bacteriana, actividad anti-proliferativa, actividad antirradical, *Anthyllis vulneraria*, *Azadirachta indica*, efecto sinérgico, emulsión, estabilidad oxidativa, HPLC-MS, productos cárnicos.

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AAPH	2, 2-azobis (2-amidinopropane) dihydrochloride
ABTS	2, 20 azino-bis (3-ethyl-benzothiazoline-6-sulfonicacid)
Ac	Acetone
AIDS	Acquired Immuno-Deficiency Syndrome
$AlCl_3$	Aluminium chloride
DMEM	Dulbecco's Modified Eagle Medium
DMPO	5, 5-Dimethyl-1-pyrroline N-oxide
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphényl-1-picrylhydrazyl
EDTA	Ethylenediaminetetra-acetic acid
EtOH	Ethanol
$FeCl_2$	Iron (II) chloride
$FeCl_3$	Iron (III) chloride
$FeSO_4$	Iron (II) sulfate
GSH	Glutathione (reduced)
GS-SG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
$K_2S_2O_8$	Potassium persulfate
KDa	Kilo Dalton
MDA	Malondialdehyde
MeOH	Methanol
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mW	Milliwatt
Na_2CO_3	Sodium carbonate
NADPH	Nicotinamide adenine dinucleotide phosphate
NOS	Nitric Oxide Synthases
PBS	Phosphate-Buffered Saline
SDS	Sodium Dodecyl Sulfate
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TPTZ	2, 4, 6-Tris (2-pyridyl)-s-triazine

TRX	Thioredoxin

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1 Foreword

1 Foreword

Every living being, whether animal or plant, is permanently exposed to various forms of stress and aggression that can cause temporary disturbances in organism functioning. Faced with these occasional and abnormal situations, living beings response rapidly to adapt quickly and effectively in order to protect themselves [1]. However, in the presence of an unusual extreme stress, normal adaptive mechanisms may not be sufficient to respond to the new situation, which leads to the induction of oxidative stress [2]. Oxidative stress is a pathophysiological mechanism characterized by an imbalance between the production of Reactive Oxygen Species (ROS) and the body's defence systems [3]. This oxidative pressure becomes abnormal when the cells of the living organism are either overwhelmed by the quantity of ROS or do not have sufficient antioxidant resources to eradicate them [4].

The ROS are highly reactive and unstable oxygen derivatives [5]. When they are in excess, they are considered toxic agents responsible for the dysfunction and death of cells, protein ageing, lipid peroxidation, and DNA damage. They are also considered as inflammatory mediators involved in various chronic and degenerative diseases [6], such as certain cancers [7], Alzheimer [8] and cardiovascular diseases [9]. The ROS elements include free radicals, for example singlet oxygen (O_2), superoxide ($O_2^{\bullet -}$), hydroxyl ($HO^{\bullet}$), hydro-peroxyl ($HO_2^{\bullet}$) etc., and non-radicals, such as hydrogen peroxide (H_2O_2), hypobromous acid (HOBr), hypochlorous acid (HOCl), nitric oxide (NO) and hypochlorite (OCl^-) etc., [10].

Radicals are group of atoms with one or more unpaired electrons. More precisely, free radicals are molecules that contain one or more unpaired electrons and thus are paramagnetic [11]. Their high reactivity allows them to take electrons from other compounds and attain stability. Thus, the attacked molecule loses its electron and becomes a free radical itself, hence the beginning of a chain reaction cascade, which finally damages the living cell [12] (**Figure 1**).

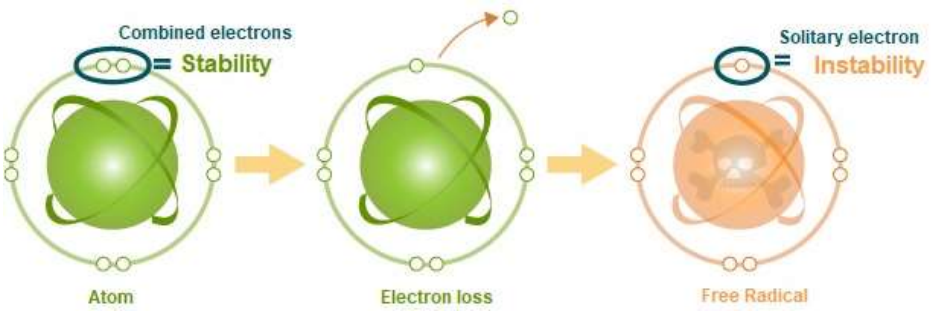

Figure 1. Mechanism of free radical generation [423]

The control of the generation level of free radicals is ensured by their elimination [13]. Indeed, the majority of living organisms have complex antioxidant systems to eliminate free radicals or to limit their negative effects [14]. For example, to protect themselves, plants respond to biotic (microbial invasion, insect pests and herbivores etc.) and abiotic (cold, frost and drought, etc.) attacks by synthesizing a variety of bioactive substances [15], which play also a crucial role when consumed by human beings to prevent and cure diseases related to the oxidative stress [16].

Different environmental factors can induce the production of these bioactive substances in plants. In fact, in response to external changes, plants can vary their chemical composition qualitatively and quantitatively and this explains the variation of their metabolites during a given season and, consequently, their richness in biological activities [15]. Among the biological activities that characterizes the bioactive substances produced by plants is their antioxidant activity [17]. The term "antioxidant" means a substance, which even if present in a low concentration relative to an oxidizable substrate, delays or prevents its oxidation [18]. In fact, a highly effective antioxidant, having an excess of electron, neutralize free radicals by giving them an electron and transforming them into stable molecules [11] (**Figure 2**).

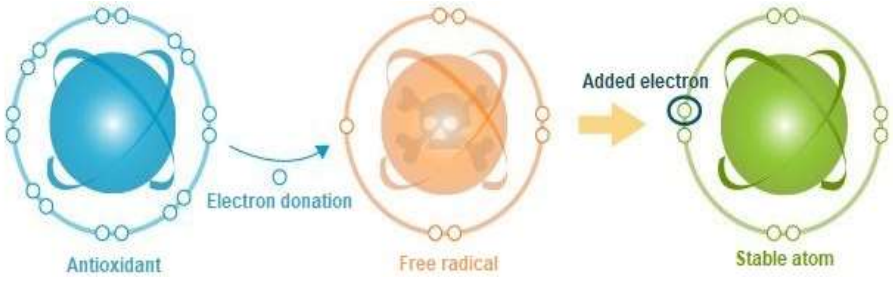

Figure 2. Mechanism of free radical stabilization [423]

Natural antioxidants, such as vitamins and phenolic compounds, are generally received by human organism as food or supplement components [14]. Phenolic compounds are secondary metabolites widely distributed in the plant kingdom and constitute a class of molecules with significant biological actions [19]. Plants rich in phenolic compounds have immense pharmacological potential due to their antioxidant power allowing them to absorb, neutralize and eliminate free radicals [20] in addition to their therapeutic potential that gives them the ability to fight against certain cancers infections, inflammation and cardiovascular disorder [21, 22, 23].

The last decade has been characterized by a significant increase in the incidence rates of cancer and bacterial diseases. Recently, the search for active compounds in natural sources has become an important issue for the pharmaceutical industry.

Food and cosmetic industries are as well paying particular attention to active compounds from plants. For instance, several studies have shown that the lipid oxidation, in the food and the cosmetic products, is one of the most important causes of the organoleptic quality degradation of products. These reactions of oxidation cause universal changes in the taste, smell and colour, resulting in a shortened shelf life of products [24].

Despite the common use of synthetic antioxidants, such as butylated hydroxy-anisole (BHA) and butylated hydroxytoluene (BHT) as additives in food and cosmetic products to prevent them from oxidation, plant kingdom remains the main supplier for obtaining natural antioxidant molecules. For this reason, the use of natural additives in food may be a good alternative to substitute synthetic preservatives suspected to have side effects on human health in the long term, especially when consumed at high concentrations [25, 26]. This alternative strategy can prevent products from deterioration and, at the same time, gives them extra nutritional value as well as safety and benefits for consumer's health [16, 27].

If this premise is accepted, the questions that arise are therefore the following:

- Which are the main active compounds derived from plants responsible for their different biological and therapeutic activities?
- How can this active compounds be used in food and cosmetic products to replace synthetic additives?
- To what extent are they put into practice by the pharmaceutical, cosmetic and food industries?

2 Introduction

2 Introduction

Owing to its richness and diversity, the plant kingdom represents a true and inexhaustible source of active compounds capable of synthesizing bioactive molecules to cope with biotic and abiotic stresses. These molecules are very useful for human well-being on account of their biological activities and present a concrete response to a problem that has been raised for years in the pharmaceutical, cosmetic and food industries [28]. This problem is about the harmful effect of oxidative processes in the rapid deterioration of food and cosmetic products during storage on the one hand, and the genesis of an increasing number of serious and fatal diseases on the other hand.

2.1 Free radicals, antioxidants and functional foods

Molecular Oxygen (O_2) is the most abundant chemical element in terms of mass in the air and plays an important role in cellular functioning and the maintenance of living being's life [29]. However, in some cases, the O_2 can be harmful when it generates, excessively, chemical species called reactive oxygen species [30].

2.1.1 Reactive oxygen species

The oxidation is a part of a redox reaction that transfers electrons from a substance to an oxidizing agent. This reaction produces reactive chemical species containing oxygen called Reactive Oxygen Species (ROS). They include free radical and certain non-radical species and participate in harmful reactions of oxidation causing serious damages that affect the biological molecules [31] (**Figure 3**).

In the human body, the generation of ROS is an inevitable consequence of cellular respiration, which consumes O_2. Low concentrations of ROS exert beneficial effects on human health by regulating cell signalling cascades [32]. However, the over-production of ROS can be responsible for DNA and cell membrane damages [33] and can promote serious diseases, such as cancers, diabetes, neurodegenerative, respiratory and digestive diseases, in addition to other health disorders accompanied by an inflammatory process that is often one of the warning signs for complications [34].

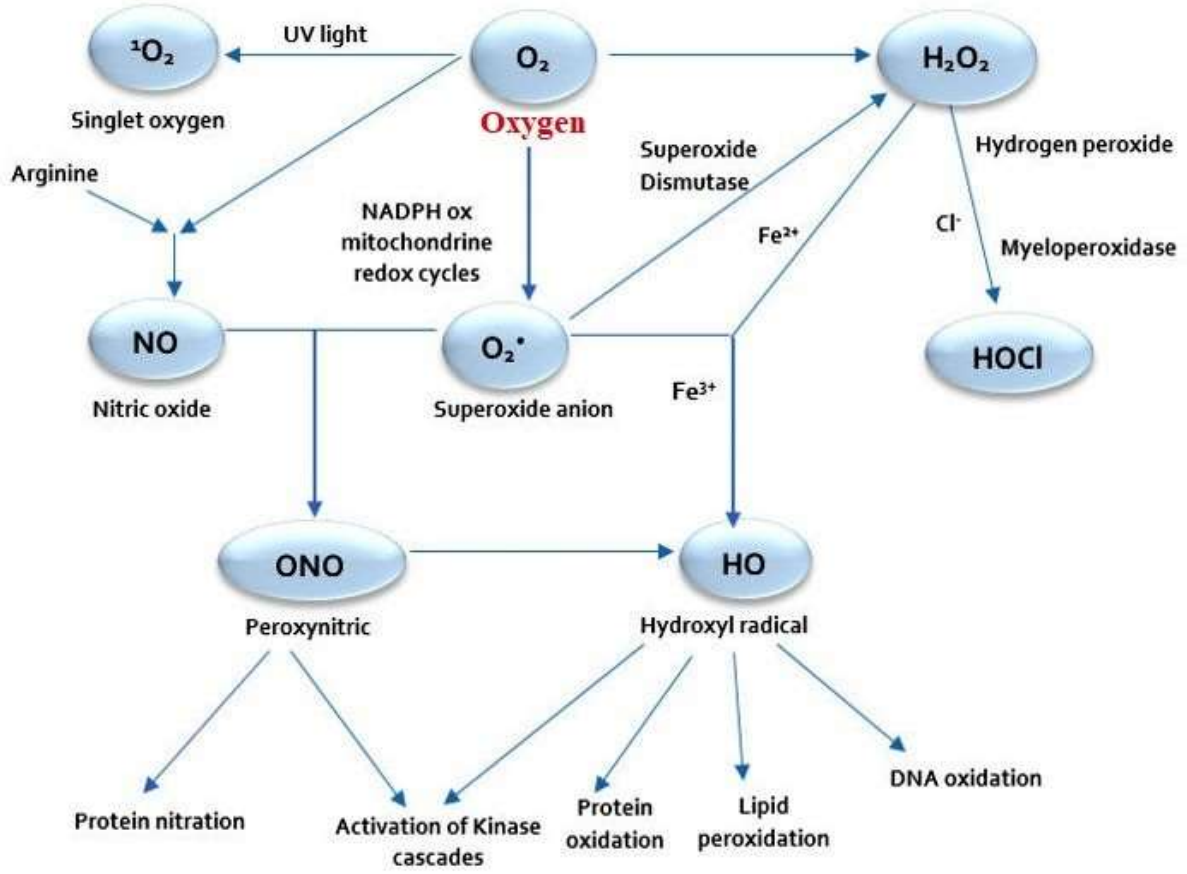

Figure 3. Schematic representation of the main reactive oxygen species origin [424]

2.1.1.1 Superoxide ion

In the mitochondria and during cellular respiration, the superoxide ion ($O_2^{\bullet}$) results from the monovalent reduction of oxygen. In addition, during oxidative stress, enzymes, such as NADPH-oxidase and xanthine oxidase can produce $O_2^{\bullet}$ radicals [35]. The Superoxide Dismutase (SOD) contributes to the elimination of $O_2^{\bullet}$ through its dis-mutation following the reaction cited below:

$$O_2^{\bullet} + O_2^{\bullet} + 2\ H^- \xrightarrow{\textbf{SOD}} H_2O_2 + O_2$$

This reaction leads to the formation of hydrogen peroxide (H_2O_2), which is metabolized to water (H_2O) by the Catalase (CAT) or the Glutathione Peroxidase (GPx).

The main targets of $O_2^{\bullet}$ are cytochrome C, ascorbic acid (vitamin C) and especially the SOD. The $O_2^{\bullet}$ is unstable in aqueous solution but stable in lipid ones, which allow it to de-esterify the lipid molecules and particularly the phospholipid membranes [40, 41].

2.1.1.2 Hydroxyl radical

The hydroxyl radical ($OH^{\bullet}$) is the most powerful oxidant present in nature. In the mitochondrial respiratory chain, H_2O_2 can directly react with the iron (Fe) and the copper (Cu) through the Fenton's reaction leading to the formation of $OH^{\bullet}$:

$$Fe^{2+}{}_{(aq)} + H_2O_2 \longrightarrow Fe^{3+}{}_{(aq)} + OH^-{}_{(aq)} + OH^{\bullet}$$

The Fe^{2+}/H_2O_2 couple is called Fenton's reagent and plays a major role in lipid peroxidation and genetic material destruction [38].

2.1.1.3 Nitric oxide

Nitric oxide ($NO^{\bullet}$) is a chemical compound produced in the organism by the oxidation of one of the N-terminal atoms of L-arginine. This reaction is catalysed by the Nitric Oxide Synthase (NOS) according to the following reaction [39].

$$O_2 + \text{Arginine} + NADPH \xrightarrow{\textbf{NOS}} NO^{\bullet} + \text{citrulline} + H_2O + NADP^+$$

This physiological production plays a major role in neurotransmission, blood pressure regulation, defence mechanism, muscle relaxation and immune regulation [40]. However, at a high concentration, $NO^{\bullet}$ becomes deleterious for the cells when it reacts with superoxide anions ($O_2^{\bullet}$) to form the peroxynitrite ($ONOO^{\bullet}$); a powerful oxidizing agent that can secondarily decompose into other oxidants, such as nitrogen dioxide (NO_2) and hydroxyl radical ($OH^{\bullet}$) [41].

2.1.1.4 Hydrogen peroxide

The hydrogen peroxide (H_2O_2) is obtained from the $O_2^{\bullet}$ by spontaneous dismutation or by the SOD and metabolized by the CAT and the GPx. The H_2O_2 is not a radical in the proper sense but allows the formation of the $OH^{\bullet}$ in presence of transition metals (Fenton's reaction) [45, 40].

In the human body, the over-production of ROS is strongly influenced by external factors, like poor diet [43], environmental pollution [44], alcohol [45] and smoking [46] etc. In foods, most of the edible products and by-products degrade under the action of the O_2. This degradation can be accelerated by the light and the high temperature so the products undergo changes in consistency, taste, colour, odour and quality [47]. To prevent or limit oxidation damages, molecules named "antioxidants", whether exogenous or endogenous, play a crucial role in the stabilization of free radicals to protect the body from several health problems,

maintain the quality of cosmetic products and increase the shelf life of both natural and processed foods [48].

2.1.2 Antioxidants: Generalities

Antioxidants are redox agents that react with oxidants and stop or slow down the oxidation process, and thus regulate the cellular redox balance. They are molecules able to neutralize free radicals in an oxidation reaction by giving them electrons and transforming them into stable molecules. They are therefore the perfect antidote to the harmful effects of ROS and free radicals [49].

In a living organism, the maintenance of a non-cytotoxic level of ROS is ensured by antioxidant systems, which are substances generated by the organism capable, at relatively low concentrations, to neutralize or reduce free radical damages [50].

Cells use many antioxidant strategies and consume a lot of energy to control their levels of ROS (**Figure 4**). The nature of antioxidant systems differs among tissues and cell types as well as intracellular and extracellular media [51].

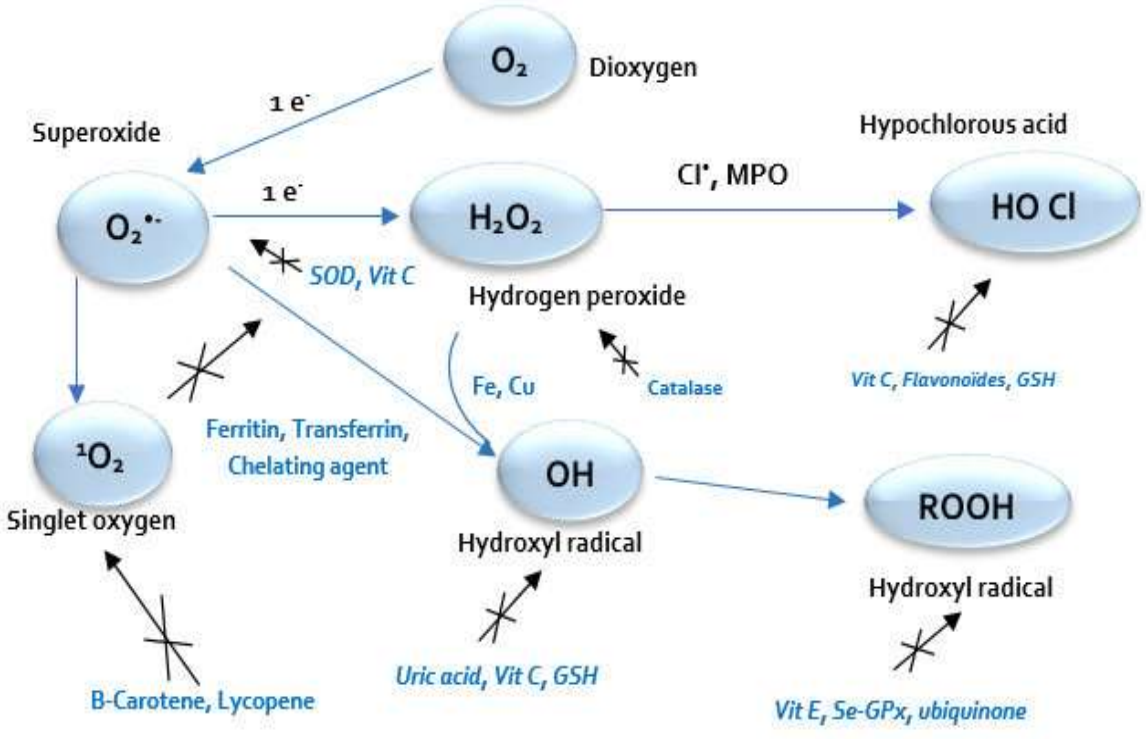

Figure 4. Regulation of reactive oxygen species generation by antioxidant systems [425]

In food and cosmetic industries, antioxidants are widely used in products as synthetic or natural additives that provide great benefits against oxidative rancidity and add a value to the products to maintain their quality [55, 56].

« *The European Parliament and Council Directive No.1333/2008 of 16 December 2008 on food additives* » controls the use of antioxidant food additives and the food in which they can be added. Their indication on the packaging is compulsory, and they may be mentioned by their name or by a code composed of the letter "E" (European Union) or the three digits "INS" (International Numbering System) [54].

2.1.3 Antioxidants classification

The nature and type of antioxidants suggest two options among others that provide a basis for their classification. From a natural point of view, antioxidants can be classified into two main groups, including synthetic and natural antioxidants [55]. Typically, natural antioxidants can be as well classified as exogenous (enzymatic) and endogenous (non-enzymatic) antioxidants [56] (**Figure 5**).

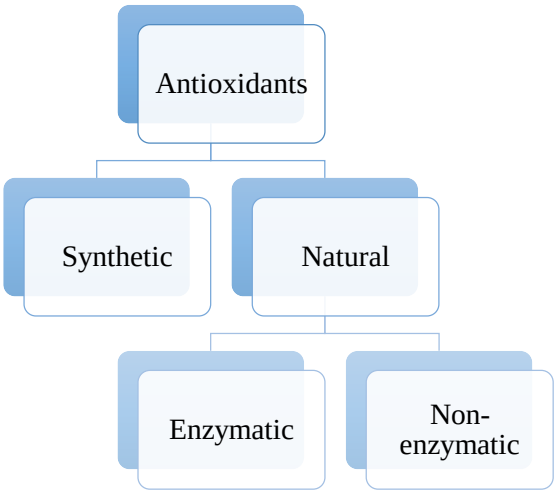

Figure 5. General classification of antioxidants

2.1.3.1 Synthetic antioxidants

Among the known synthetic antioxidants, only few of them are used in the food and cosmetic industries given the doubt that revolves around the risk of their toxicity, the carcinogenic activity of their oxidized forms and their reaction with the constituents of the

product [57]. For instance, the butylated hydroxy-anisole (BHA), the butylated hydroxytoluene (BHT) and the tert-butylhydroquinone (TBHQ) are the most used synthetic additives in several cosmetic and food products to stabilize fats and oils [58].

The **BHA** and the **BHT** are two synthetic antioxidants that has the code E320 and E321, respectively on products in the European Union and are generally in the form of a white or yellowish powder.

- The **BHA** (**Figure 6**) is insoluble in water but have a good solubility in lipid medium. In food industry, the BHA is very effective in preventing the oxidation of fats and oils, especially those of animal origin [59].

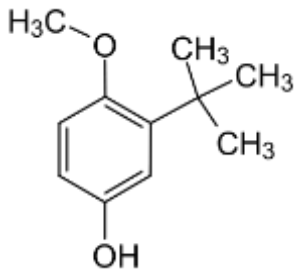

Figure 6. Chemical structure of the butylated hydroxy-anisole (BHA) [426]

- The **BHT** (**Figure 7**) is a powerful synthetic antioxidant widely used in the food and cosmetic industry. This water-soluble additive is in the form of a colourless to pale yellow powder or crystals and is resistant to high temperatures that can be reached during the manufacture of products. The BHT is found particularly in formulations containing unsaturated fats and it is stable under the operating conditions of most industrial processes [59].

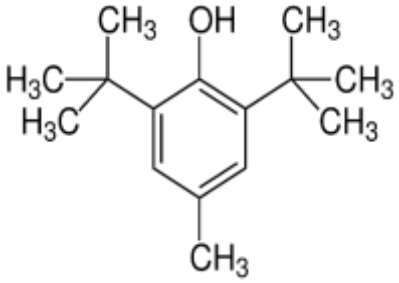

Figure 7. Chemical structure of the butylated hydroxytoluene (BHT) [427]

- The **TBHQ** (**Figure 8**) is an organic solid in the form of a light-brown crystalline powder that is combustible, soluble in water and has a faint odour. It is perfectly soluble in fats and is very effective in vegetable oils. It is stable at high temperatures and has low volatility. The presence of chelating agents, such as citric acid can considerably increase the antioxidant properties of the TBHQ [60].

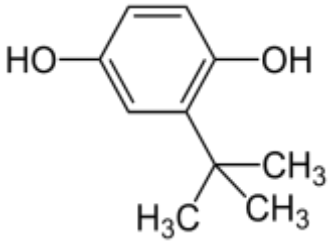

Figure 8. Chemical structure of the tert-butylhydroquinone (TBHQ) [426]

Although the fact that synthetic antioxidants are highly effective and economical, many countries have adopted currently regulations similar to the United States regarding their use because of their toxicological and cancer risks [61]. For example, the BHA and the BHT are suspected to have various adverse human health effects and can produce allergic reactions in the skin. Recent studies showed that they affect the nervous system and increase the risk of asthma [1] and dermatitis [2] [62]. Consequently, and given the desire of consumers to return to the use of natural products, the search for natural sources of antioxidants has caught the interest of researchers and industries [63].

2.1.3.2 Natural antioxidants

Natural antioxidants are present in many forms and can help prevent the formation of free radicals and participate in their elimination [64]. There are two types of natural antioxidants: endogenous and exogenous (**Figure 9**). Endogenous natural antioxidants are mainly enzymes generally produced by the organism such as SOD, CAT and GPx [65], while exogenous antioxidants are brought by eating food and dietary products rich in vitamins, minerals, carotenoids, phenolic compounds etc., [69, 57].

[1] A respiratory condition marked by attacks of spasm in the bronchi of the lungs, causing difficulty in breathing.

[2] A medical condition in which the skin becomes red, swollen, and sore, sometimes with small blisters, resulting from direct irritation of the skin by an external agent.

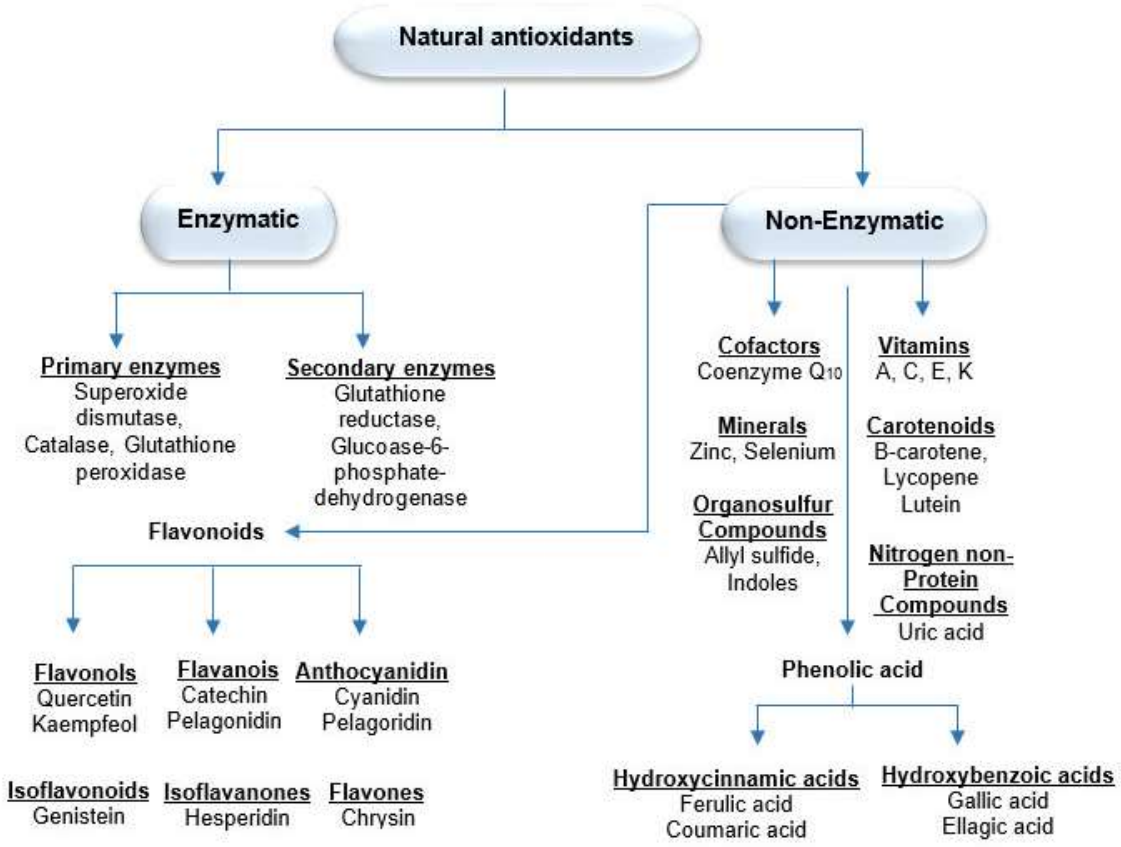

Figure 9. Classification of natural antioxidants [428]

2.1.3.2.1 Enzymatic antioxidants

All cells, whether animal or plant, are equipped with antioxidant enzymes, which represent a very effective defence system. This line of defence consists of the SOD, CAT and GPx etc., that eliminate primary free radicals [67].

- **SOD** is one of the first lines of defence against ROS. It is a metallo-enzyme that uses metals as co-factors. The SOD neutralizes oxygenated free radicals by converting them into non-toxic molecules. Its preferred target is the $O_2^{\bullet}$ that it converts into a stable H_2O_2. The SOD plays different functions according to the metal with which it is associated. When intracellular SOD bounds to Cu and zinc (Zn), it protects polyunsaturated fatty acids from oxidation and when it bounds to manganese (Mn), it transforms oxygenated free radicals and protects polyunsaturated fatty acids from apoptosis. Moreover, when extracellular SOD bounds to Cu and Zn, it has a protective role for proteins in the cell matrix [68].

- **CAT** is one of the most important antioxidant enzymes and its main target is H_2O_2 [69]. The activity of the CAT is coordinated with two H_2O_2 molecules, which are broken down into one molecule of oxygen as described in the reaction below:

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

CAT deficiency or malfunctioning is associated with different diseases, such as diabetes, hypertension, anaemia, bipolar disorder, schizophrenia, cardiovascular and Alzheimer's diseases etc. [73, 70].

- **GPx** is present in the extracellular fluids and in the cytosol and mitochondria of cells. It limits the propagation of free-radical chain reactions by reducing unstable peroxides to hydroxylated fatty acids [72].

$$H_2O_2 + 2GSH \xrightarrow{\textbf{GPx}} 2H_2O + GS\text{-}SG$$

GPx is implicated in the prevention of many common and complex diseases, including cancer and cardiovascular diseases [73].

2.1.3.2.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants are exogenous natural molecules present in numerous sources of the plant kingdom like fruits, vegetables and wild plants. They contain several antioxidant substances, such as vitamins, minerals (trace elements), carotenoids and phenolic compounds, etc. [74].

- **Vitamins** are antioxidant organic substances necessary for the metabolism of a living organism even in a small quantity. The human organism is not able to synthesize vitamins (except vitamin D that is synthesized by the skin under the effect of UV light), hence their supply through food is necessary [71]. For instance, the **vitamin C** is a powerful reducing agent capable of donating electrons and preventing the oxidation of other compounds. It donates two electrons from a double bond between the second and third carbon atoms of the 6-carbon molecule [75]. The vitamin C is involved in the synthesis of collagen and certain neuro-mediators as well as the absorption of Fe. It also has a role in strengthening immunity [76]. The vitamin C can be found in cabbage, pepper, parsley, citrus and kiwi fruits. The **vitamin E** (tocopherols) also is a very active antioxidant. It exhibits antioxidant activity by donating phenolic hydrogen to lipid free radicals, thereby retarding autocatalytic lipid peroxidation processes [77]. It protects against degenerative processes, such as cancer and cardiovascular

diseases [82, 83]. The vitamin E can be found in vegetable oils (peanuts, soybean, and sunflower) , almonds, milk, eggs and green leafy vegetables [80].

- **Minerals**, such as Cu, Mn, selenium (Se), Fe and Zn, are micronutrients provided by food and involved in energy homeostasis. They are indispensable co-factors for metabolic reactions of antioxidant enzymes, such as the SOD, CAT and GPx to protect the body from radicals [81]. Several researches demonstrated the anti-inflammatory property of the Zn and its ability to decrease oxidative stress biomarkers [82]. Moreover, other researches confirmed the antioxidant activity of the Fe and its capacity to decrease risk of heart disease and cancer [83].

- **Polyphenols** are molecules specific to the plant kingdom. A huge number of molecules have been isolated and identified from plants. According to their structural characteristics, polyphenols are divided into eleven chemical classes characterized by the presence, in their structure, of at least one 6-carbon aromatic ring carrying a variable number of hydroxyl (OH) functions [84]. According to their structure, polyphenols are classified into phenolic acids, flavonoids, tannins, stilbenes, lignans, etc. (**Figure 10**). The more important are phenol acids, flavonoids and tannins [85].

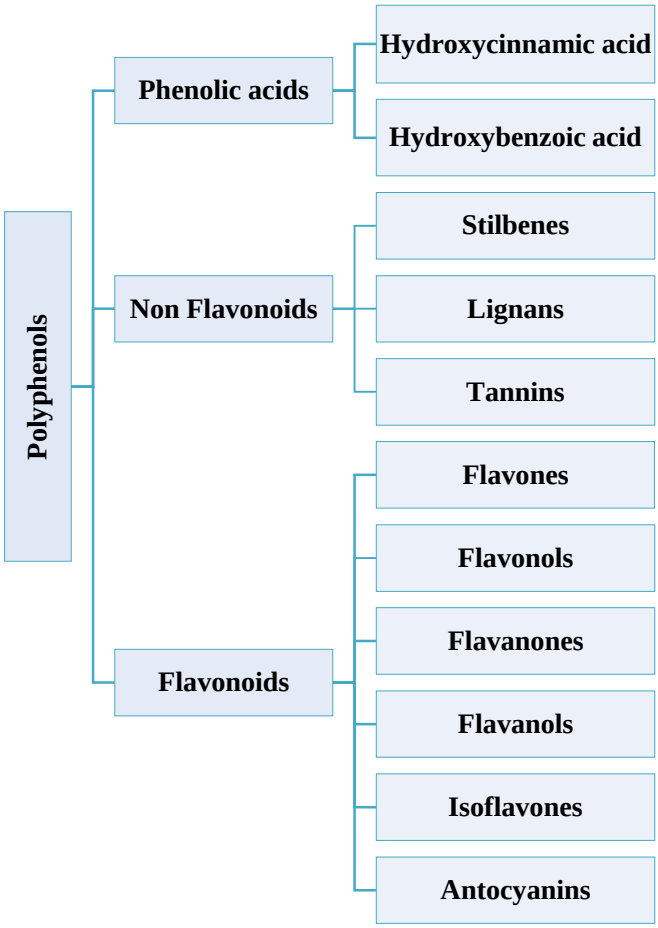

Figure 10. Classification of phenolic compounds [429]

The daily intake average of polyphenols depends on the dietary habits. They are present in different amounts in the roots, stems, flowers and leaves of plants. The main food sources are fruits and vegetables, beverage (red wine, tea, coffee, juice) , cereals and oil seeds [86].

The biosynthetic origin of polyphenols is shikimic acid [87]. Series of reactions (Shikimate pathway) convert phosphoenolpyruvate and erythrose-4-phosphate into chorismic acid, a common precursor of aromatic amino acids, such as phenylalanine and tyrosine. Subsequently, chorismic acid serves as a precursor for cinnamic acid derivatives. Mainly benzoic acids are synthesized from chorismic acid by oxidative and non-oxidative pathways, while isochorismic acid serves as a precursor to protocatechuic acid [88] (**Figure 11**).

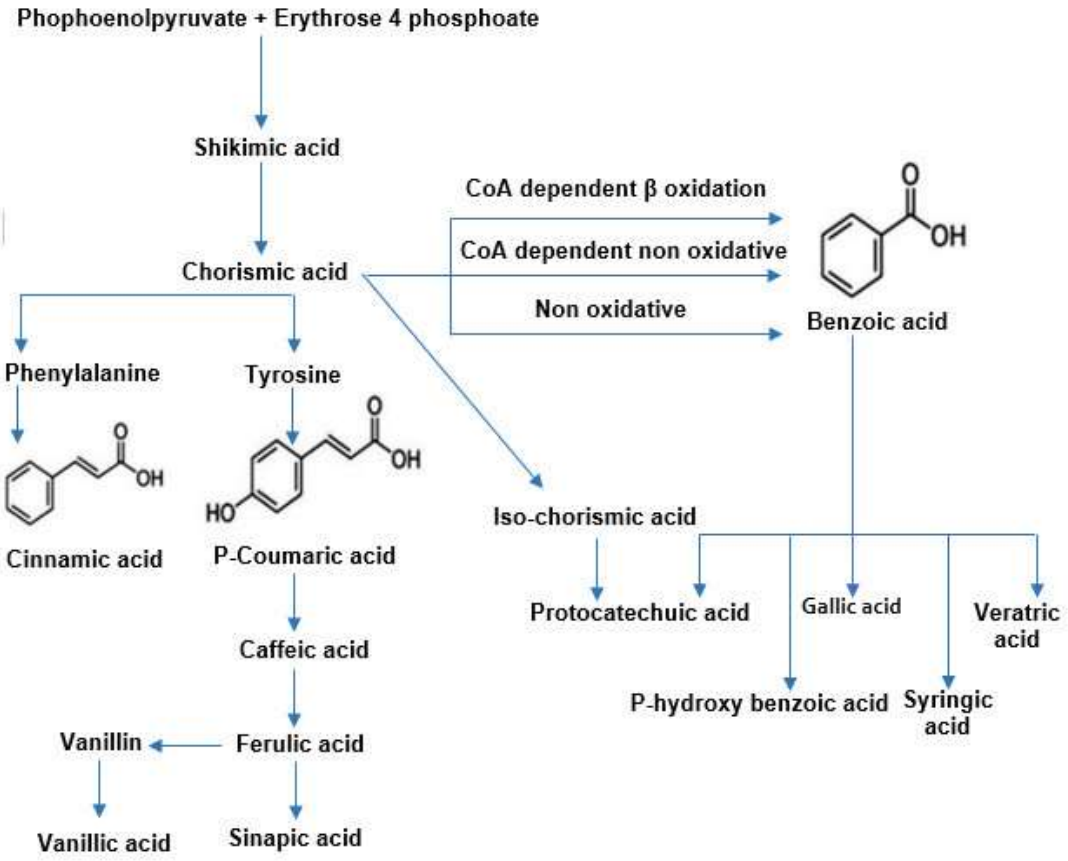

Figure 11. Biosynthesis of the main distributed polyphenols by Shikimate pathway [430]

Recent researches about polyphenols, in particular flavonoids, are very advanced due to their various and important physiological properties, such as anti-allergic, anti-inflammatory, antimicrobial, antiviral, antibacterial, anti-carcinogenic, antithrombotic (against thrombosis [3]),

[3] The formation or presence of a blood clot within a blood vessel.

cardio-protective and vasodilator activities [89]. These activities are attributed to their antioxidant effect, which is due to their redox properties by playing an important role in oxidative destruction through free radical neutralization, scavenging of oxygen or decomposition of peroxides [94, 95]. The beneficial effects of polyphenols are of a particular interest in the pharmaceutical, cosmetic and food industries. For instance, according to several studies demonstrating the positive impact of polyphenol consumption on health and prevention from diseases, manufacturers are currently marketing polyphenol-enriched foods and dietary supplements [92]. In addition, their antioxidant activity ensures better preservation of cosmetic and food products by preventing lipid peroxidation [93]. For example, in the cosmetic industry, polyphenols are added in cosmetic products owing to their antioxidant, anti-inflammatory and antimicrobial properties, as well as their wound healing and anti-aging activities [94].

2.1.3.2.3 Phenolic acid

Phenolic acids are simple compounds formed by a single phenolic ring and at least one carboxylic acid group. They are considered having antioxidant, antiradical and anti-inflammatory activities. The more important phenolic acids are caffeic acid, gallic acid and chlorogenic acid (**Figure 12**) [95]. For example, caffeic acid is very effective against oxidation, microbial infections and colon cancer [96]. Moreover, gallic acid is characterised by its antioxidant, antimicrobial, anti-inflammatory, anticancer, cardio, gastro and neuro-protective effects [101, 102].

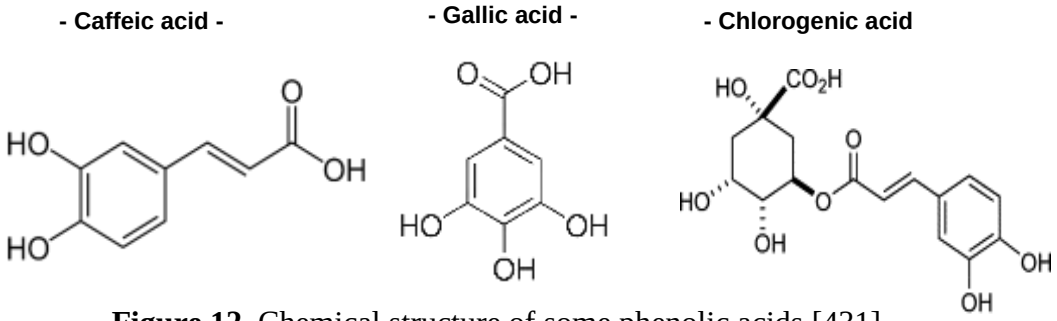

Figure 12. Chemical structure of some phenolic acids [431]

2.1.3.2.4 Flavonoids

Flavonoids (**Figure 13**) are the most representative group of polyphenols. These molecules have varied chemical structures and characteristics. They are ubiquitous in fruits, vegetables, seeds, beverages, such as tea and red wine and other parts of the plant [99].

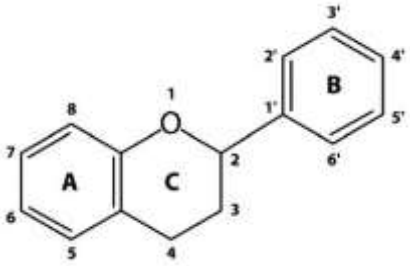

Figure 13. General chemical structure of flavonoids [432]

Flavonoids are considered pigments in almost universal plants that can participate in photosynthetic processes, gene regulation and growth metabolism [100]. Currently, a huge number of flavonoids are known, and they all have the same basic skeleton with fifteen atoms of carbons, which are arranged in a C6-C3-C6 configuration of the phenyl-2-benzopyran structure [101]. The chemical nature of flavonoids depends on their structural class, degree of hydroxylation and methoxylation, degree of polymerization, substitutions and conjugations on the C-ring (presence of C2-C3 double bonds, of the 3-O group) and the 4-oxo function [99].

Based on their skeleton, flavonoids can be divided into different classes (**Figure 14**).

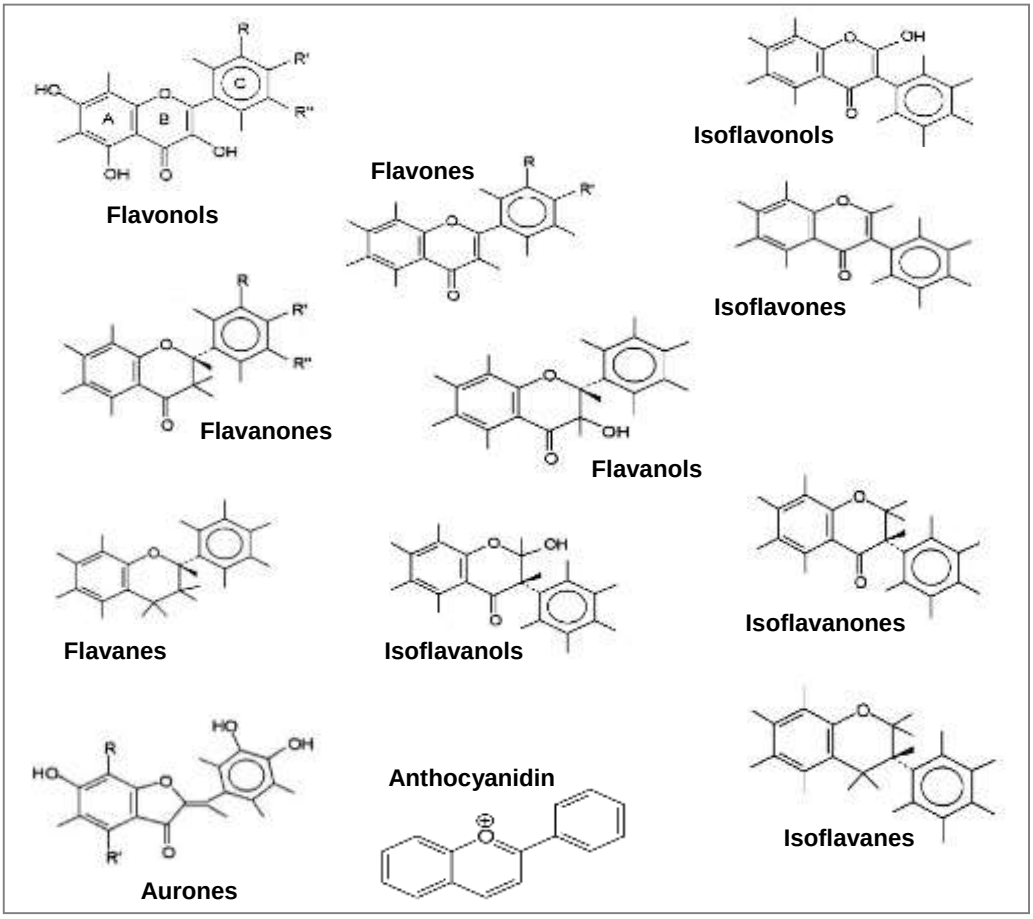

Figure 14. Basic skeletal chemical structures of flavonoids [101]

Flavonoids can prevent oxidative damage by different mechanisms [102]:

- The caption of hydroxyl, superoxide and peroxide radicals.
- The chelation of metals (Fe and Cu) that are of a major importance in initiating free radical reactions.
- The inhibition of the enzymes responsible for the generation of free radicals.

They also play a very important role in the treatment of diabetes, inflammation, hepatitis [4], tumors, hypertension, thrombosis, and bacterial infections [103].

2.1.3.2.5 Tannins

Tannins are vegetable substances, most often water-soluble, that have the ability to precipitate proteins, alkaloids and polysaccharides from their aqueous solution [104]. They have some astringent power, through which explains their vasculoprotective, healing, anti-diarrheal and antitumor properties [105]. Tannins can be classified according to their chemical structure (**Figure 15**) and have remarkable effect on wound healing and collagen synthesis [106].

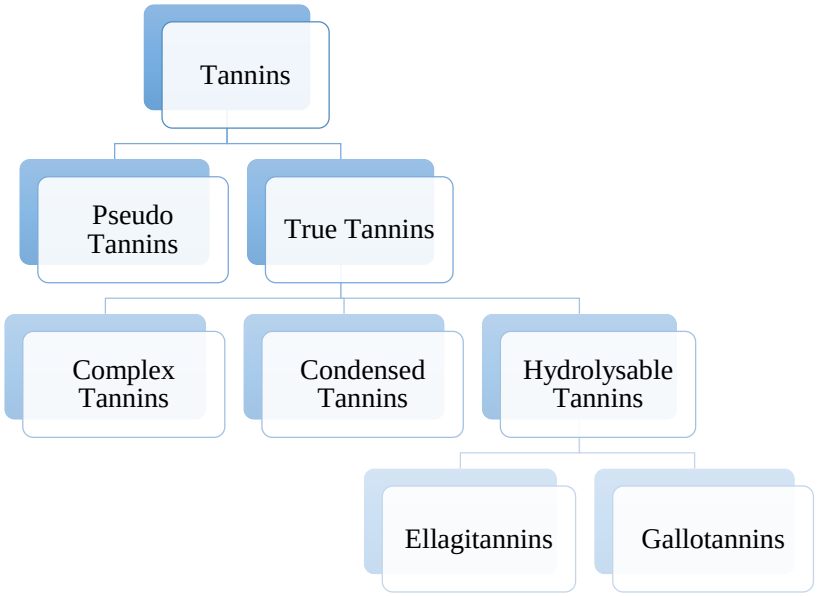

Figure 15. General classification of tannins [105]

2.1.4 Bioactive compounds as functional food ingredients and food preservative

Bioactive substances are molecules derived from a natural biological source with beneficial properties offering a wide range of applications, such as nutritional additives, foods,

[4] Inflammation of the liver tissue

cosmetic and pharmaceutical supplements in order to enhance the nutritional and health properties of the products [107].

Natural additives have had a growing interest both in the food industry and in scientific researches thanks to their richness in active compounds, such as flavonoids, terpenoids, carotenoids and their different biological properties, including antioxidant and antimicrobial activities, which allow them to compete with the synthetic additives currently used [61].

Natural additives used in food have been characterized by having an excellent effect against rancidity and by adding a value to the product [54]. In the food industry, a small number of plants are exploited for their bioactive substances. For this reason, studies have been conducted in the search for safe antioxidants to maintain products quality. For example, different plants, such as Tara (*Caesalpinia spinosa*) and Moringa (*Moringa oleifera*), rich in polyphenols, are considered good sources of antioxidant and antimicrobial agents in meat products able to maintain their safety and improve their quality [112, 113]. Previous studies showed that using natural additives, such as plant extracts, fruits, vegetables and edible plants in foods, clearly delays the lipids peroxidation process, which makes them a good alternative to synthetic additives that in some cases could be harmful to the human health in the long term [114, 115, 116].

2.1.4.1 Lipid peroxidation

Lipid peroxidation is recognized as a serious problem during the preservation or processing of food and cosmetic products. It is a reaction between unsaturated lipids and molecular oxygen, forming unstable hydro-peroxides responsible for the changes in colour and texture of the products and the appearance of a rancid odour and flavour. In addition, the generation of hydro-peroxides and other free radicals causes several human pathologies like cancer, inflammation and aging processes [113].

Lipid peroxidation mainly concerns polyunsaturated fatty acids and takes place in three steps, including **initiation**, **propagation** and **termination** (**Figure 16**).

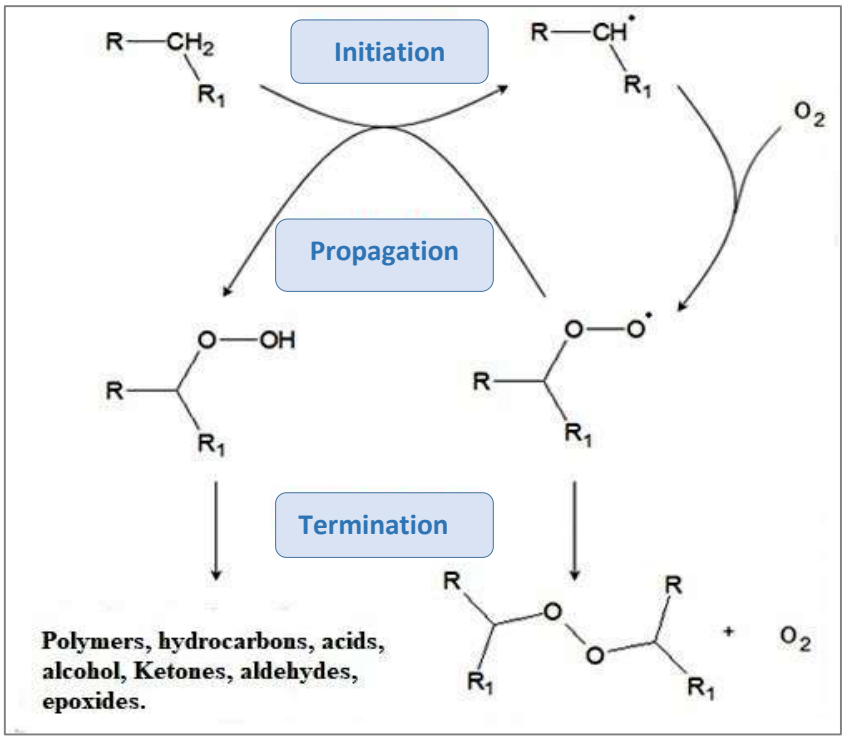

Figure 16. Oxidation process of polyunsaturated fatty acids [433]

- **Initiation**- In the presence of ROS comprising a radical with oxygen or a radical derivative not containing oxygen (an initiator, for example a hydroxyl radical $OH^{\bullet}$), the unsaturated lipids (RH) lose a hydrogen atom coming from the methylene group (CH_2) of the fatty acid to form alkyl radicals ($R^{\bullet}$) or a lipid free radicals:

$$OH^{\bullet} + RH \longrightarrow H_2O + R^{\bullet}$$

The more easily the unsaturated fatty acids are oxidized, the greater their degree of unsaturation [118, 119].

- **Propagation**- Free radicals of the unsaturated fatty acid formed (comprising unstable hydrogen), react very rapidly with O_2 to form unstable peroxyl radicals ($ROO^{\bullet}$). This $ROO^{\bullet}$ reacts with another lipid molecule (RH) to form a hydro-peroxide (ROOH) and a new alkyl radical ($R^{\bullet}$), which ensures the propagation of the reaction.

$$R^{\bullet} + O \longrightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet}$$

Hydro-peroxides are the most important products of the initial lipid oxidation reaction. They are unstable species of a very transitory nature, which propagate the oxidation initiators within the matrices [116].

- **Termination**- At atmospheric pressure, the termination begins with the combination of two peroxyl radicals (ROO•) and the formation of an unstable tetroxide intermediate compound followed rapidly by its decomposition to form non-radical products.

$$ROO^{\bullet} + ROO^{\bullet} \longrightarrow (ROOOOR) \longrightarrow \text{non- radical products} + O_2$$

The termination phase leads to the formation of various final-products, including volatile compounds (aldehydes, alcohols, ketones, furans, etc.) and non-volatile compounds (oxy-monomer and oxy-dimer). Nature and proportion of the various volatile compounds formed depend on the nature of the peroxidized fatty acids, the type of oxidation, the environmental conditions (temperature, pH, presence of iron, etc.) and the initiating agent [121, 122].

2.1.5 Antioxidant behavior of bioactive compounds in a *Model Food System*

A *Model Food System* is a system based on the formulation and processing of a real food by adding active compounds with biological properties and studying their functionality in order to assess how sensitive the characteristics of the formulated food are against the different added ingredients and processing steps [119].

2.1.5.1 Antioxidant behavior of bioactive compounds in emulsion

An emulsion is a heterogeneous mixture of two immiscible liquid substances forming different phases. One of the phases is continuous, and the other one is discontinuous and is dispersed in the first phase in the form of small droplets [120]. Emulsions are often composed of an oily/aqueous phase. An Oil-in-Water (O/W) emulsion is composed of an oily phase dispersed in a water phase, while a Water-in-Oil (W/O) emulsion is composed of an aqueous phase dispersed in an oily phase (**Figure 17**).

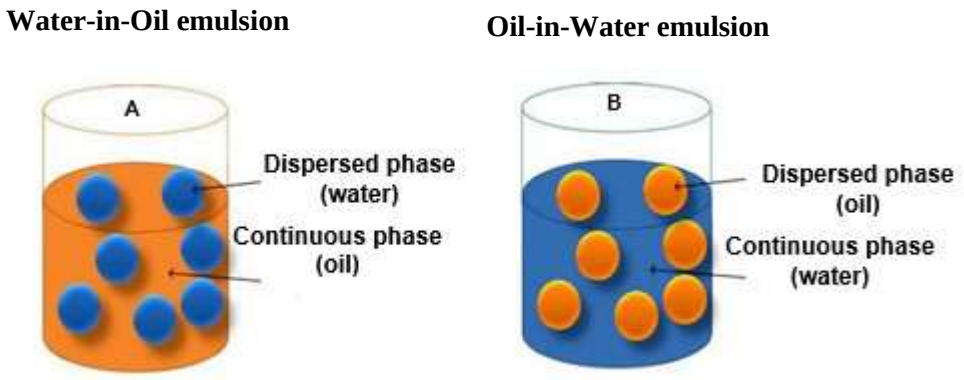

Figure 17. Concept of two-phase emulsion

A W/O emulsion is greasier to the touch, as the touch corresponds mainly to the nature of the external phase. Multiple emulsion, like Oil-in-Water-in-Oil (O/W/O), Water-in-Oil-in-Water (W/O/W) (**Figure 18**), Oil-in-Water-in-Water (O/W/W) and Water-in-Oil-in-Oil (W/O/O) emulsions can also be found in food, pharmaceutical and cosmetic applications [121].

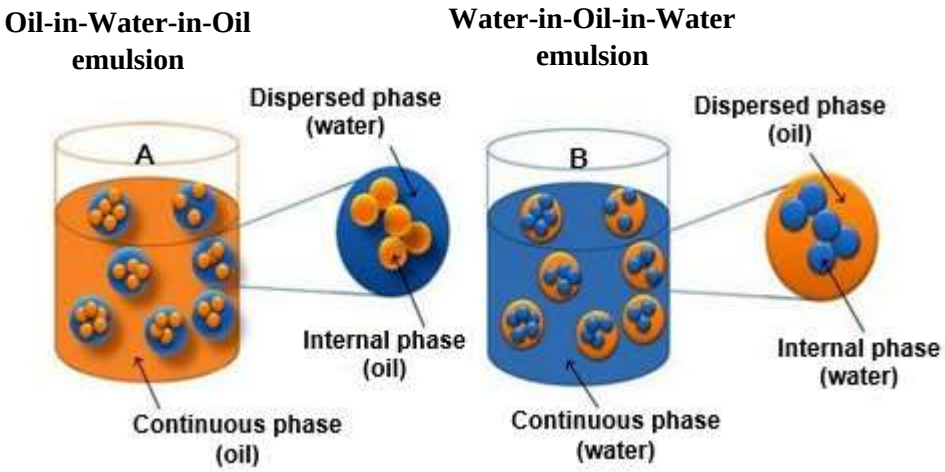

Figure 18. Concept of three phases emulsion

The emulsion allows the following of the lipid's oxidation. In the first phase of lipid's oxidation, free radicals react with the oxygen, hence the formation of hydro-peroxides. The hydro-peroxides are components that have a bad effect on the organoleptic characteristics of the emulsion. These primary oxidation products oxidize again and form secondary oxidation products (ketones, aldehydes, alcohols and acids) that negatively affect the taste, aroma, nutritional value and the overall sensory quality of the emulsions by decreasing their pH. Polyphenols and other natural antioxidants in oil improve the stability of the emulsion owing to their ability to give a hydrogen to the free radical and thus prevent it from oxidizing [122]. Among the food products in emulsion form that exist in supermarkets, we can find milk, yoghurt, mayonnaise and margarine, etc.

2.1.5.2 Antioxidant behavior of bioactive compounds in meat products

The oxidation of the major muscle compounds (lipids and proteins) is the most serious cause of the sensory and nutritional quality degradation in meat and meat products during refrigerated storage [123]. These reactions of oxidation cause irreversible changes in the taste, flavour, colour and texture of products resulting in a reduction in their shelf life. Oxidation in meat and meat products is mainly associated with the presence of free radicals, which cause the

production of different components, such as aldehydes and ketones, responsible for the development of unpleasant flavours and colour changes [124].

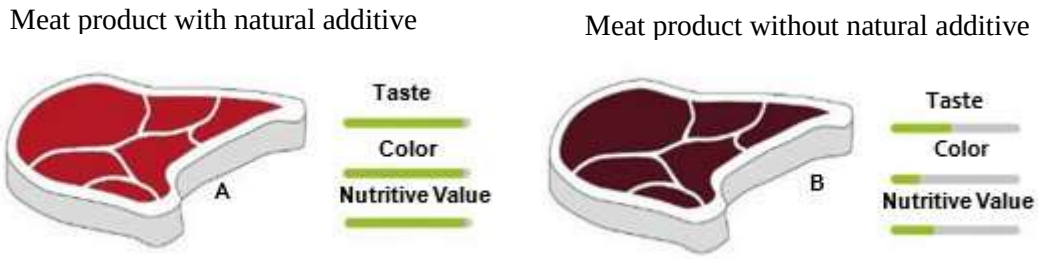

Figure 19. Effect of natural additives on the meat quality

During technological processing or during the preservation stages, meat undergoes different changes. Some operations, such as splitting (cutting, grinding and mincing) and blending promote reactions between O_2 and unsaturated fatty acids, in addition to other factors, like the high temperatures, which favor the oxidation reactions [113]. Several analyses to measure lipid oxidation are useful for the development of effective antioxidant strategies. For example, changes in muscle composition, by adding natural bioactive compounds from plants, improve the nutritive quality of meat products and extend their shelf life [125].

2.1.5.3 Antioxidant behavior of bioactive compounds in food packaging

In addition to the direct incorporation of antioxidants in food, there is a growing trend to design edible films, used as food packaging, produced naturally from a wide variety of products, such as proteins, lipids and polysaccharides [126], that maintain the quality and freshness of food products over time. The most common sources are animals (collagen, gelatin, whey, albumin and casein, etc.) but also can be derived from plants, such as corn and wheat gluten or soy protein. These films are good cracks against O_2 and carbon dioxide (CO_2) and provide mechanical stability as they serve as a vehicle for incorporating antioxidants [131, 132].

A packaged product is considered a ternary system consisting of three important factors: **Environment**, **foodstuff** and **package**.

The interactions between the three factors correspond to three different phenomena, including **permeation**, **absorption** and **migration** (**Figure 20**) that can lead to the contamination and alteration of the nutritional, gustatory and olfactory qualities of the product due to the substances present in the package.

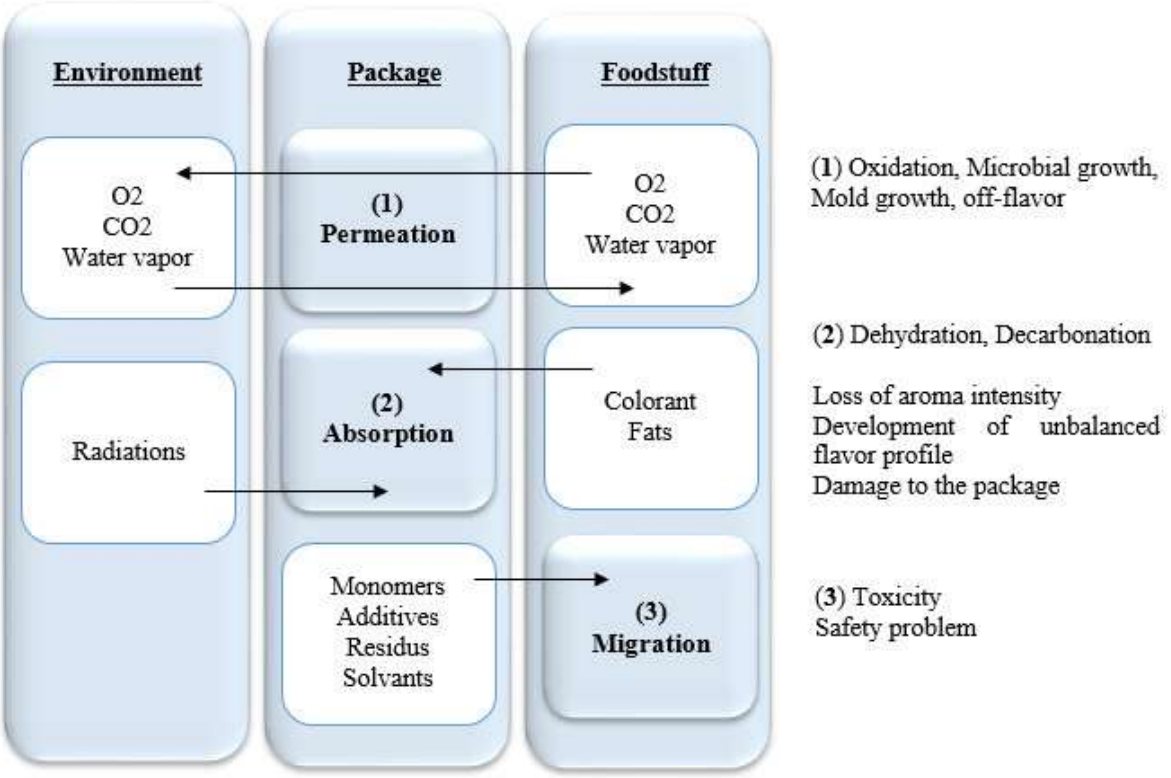

Figure 20. Possible interactions between the environment, package and foodstuff [434]

The **permeation** phenomena corresponds to the penetration of one or several constituents of the food deep into the polymeric matrix. It concerns, in particular, highly volatile substances, such as gases, including water vapor. Permeation takes place in both directions (foodstuff-environment and environment-foodstuff) through the package, which can cause reactions of oxidation, microbial and mold growth, dehydration and decarbonisation, etc. [129].

The **absorption** operation takes place in two steps:

- Physio-absorption (or surface adsorption), which is mainly the result of a charge attraction between a compound present in the foodstuff and the inner surface of the packaging. During this step, there are losses of aroma intensity and development of unbalanced flavour profile.
- Chemio-absorption (or absorption of products present in the foodstuff), which within the polymer results in a dissolution more or less important in relation to the coefficients of sharing the products under consideration, which may lead to the swelling of the plastic material and thus the damage to the package [130].

The **migration** is the step, in which components from the package migrate into the foodstuff and cause its toxicity. The number of components that can migrate depends on the concentration, molecular weight and solubility of the foodstuff and package [129].

Several studies have been carried out in the search for packaging materials with the addition of antioxidants that maintain/extend the quality of food products [131]. Synthetic antioxidants, such as the BHA and the BHT are mostly incorporated [132]. However, growing concern about the potential health risks caused by these additives has led to renew the interest in the use of naturally occurring antioxidants [133].

The incorporation of active products extracted from plants, fruits and vegetables in films is an effective strategy in food industry. The film can also serve as a carrier of active compounds with antimicrobial and antioxidant activities as well as flavour enhancer and texture preservative [134]. Many researchers have incorporated natural extracts into films and evaluated how they retarded the oxidative process of the food during storage. The incorporation of plants, such as rosemary, oregano, green tea, turmeric, grape extracts in active films have been shown to increase the stability of different meat products and extend their shelf life [139, 140]. Experiments have already been carried out on fish and chicken. For example, the addition of lemon essential oil in film of carrageenan [5] made it possible to reduce microbial growth and inhibit lipid oxidation of trout fillets. In addition, the use of thyme essential oil in films made it possible to extend the shelf life of beef meat [137].

2.2 Antimicrobial activity of plant bioactive compounds against microbial pathogens

2.2.1 Microbial infection

Infectious diseases are the leading cause of death worldwide. They are diseases caused by pathogenic microorganisms, such as bacteria, viruses, parasites or fungi and affect millions of people worldwide [138]. Over decades, microbial resistance has evolved and currently this resistance represents a major global public health problem, especially multi-drug resistant microorganisms that are difficult to treat [139].

There are only a few antimicrobial agents that can be used against some pathogens and unfortunately, this number continues to decline. For example, common multidrug-resistant pathogens, such as *Staphylococcus aureus* [6] and *Pseudomonas aeruginosa* [7] show resistance

[5] A polysaccharide extracted from red algae used as a thickening and stabilizing agent in the food industry.
[6] A Gram-positive responsible for food poisoning.
[7] A Gram-negative responsible for disease in plants and animals, including humans.

against many drugs [140]. The resistance is an adaptive response, in which microorganisms begin to tolerate the concentration of a drug that would normally be inhibitory [141]. Microbial resistance depends on several factors, such as microbial characteristics, selection pressures and social and technology changes, etc. It can be acquired when a spontaneous mutation occurs in their genes or when there is a transfer of new genes from another species [142]. The resistance can also be present in microorganisms even before they are exposed to drugs because microbes can naturally contain the genetic information that leads to the resistance. Microbes may be naturally impermeable to the antimicrobial agent, alter it, alter its target and expel it, or the target of the antimicrobial agent may simply be absent in the microbe [143].

2.2.2 Antimicrobial agents

An antimicrobial agent is a natural or synthetic substance that destroys or blocks the growth of microorganisms. There are more than 22500 biologically active compounds obtained from microorganisms, 45% of them come from actinomycetes, 38% from fungi and 17% from other bacteria. Approximately 5000 antibiotics have been identified from cultures of Gram-negative and Gram-positive bacteria as well as fungi filamentous [144]. An antimicrobial agent can treat microbial infections by the:

- Inhibition of cell wall synthesis
- Inhibition of protein synthesis
- Inhibition of nucleic acid replication and transcription
- Injury to plasma membrane
- Inhibition of synthesis of essential metabolites [145].

Recently, it has been found that the number of multi-drug resistant microorganisms is increasing and researchers are worried that several antimicrobial agents become ineffective [146]. When resistant microbes are exposed to antimicrobial agents, they are the only ones that survive and reproduce in the population. Therefore, the discovery and development of new antimicrobial agents is highly sought after. The development of microbial resistance to antibiotics has led researchers to tap into the plant kingdom and particularly medicinal and culinary plants in search of natural molecules that are effective and devoid of any adverse effects [147].

2.2.3 Microbial food poisoning

Food preservation is one of the main concerns of the food industry. Food is a favorable environment for the growth of microorganisms responsible for the loss of quality and food-borne diseases [148]. In order to ensure consumer health, it is necessary to take into account the transformations and incidents in original foods. For this reason, the control of food processing and conservation is based on the market quality and hygiene of the products [149]. Food spoilage is a significant problem in food industry. It can occur at any stage of production, transportation, storage or preparation.

During food poisoning, microorganisms multiply in food and produce toxins, which affect the health of the consumer [150]. Several bacteria are able to contaminate many food products and causing spoilage. This can induce serious illness for the consumer through their pathogenicity [151]. However, other bacteria are beneficial for human beings and are used by the food industry [152].

2.2.4 Microbiology of food products

Processed and unprocessed food products are excellent mediums for the growth of microorganisms. The contamination of food products with microorganisms causes their deterioration and the loss of their organoleptic and commercial characteristics and sometimes induces serious poisoning and toxic infections [153].

Food products can undergo several degradations of physical, chemical, enzymatic and/or microbiological natures. The microbial spoilage of food depends on several environmental factors, the nature of the food, the nature of the product and the nature of the environment, as well as the food and the microorganisms responsible of the spoilage [154].

The contamination of food products can cause an alteration of the product, resulting in a change in its organoleptic characteristics and its sanitary quality. Bacterial activity has a major impact on the quality intrinsic and therefore commercial nature of the products, which can be improved or lowered on the hygienic quality [155].

The microbial modification of foods is varied and affects the characteristics of the physicochemical, nutritive and organoleptic properties of the product. All foods can be the seat of microbial proliferation leading to changes that are often unfavorable to the colour, texture and flavour (smell and taste). The uncontrolled proliferation of microorganisms in foods can cause problems at the industrial and sanitary levels [156].

Many foods contain naturally occurring antimicrobial agents that inhibit the growth of certain microorganisms. Herbs and spices often contain important antimicrobial substances [157]. For example, sage and rosemary present a great antimicrobial activity [158]. Coumarins, found in fruits and vegetables, also act as effective antimicrobial agents [159].

2.2.5 Origin and nature of the microbial flora of food products

Various intrinsic and extrinsic factors can influence microbial growth in foods (**Figure 21**). The microbial community and foods undergo successive changes over time.

Microorganisms do not only discolour food, degrade it or make it very unpleasant to smell and eat, but can also represent serious dangers to public health. Microorganisms in food products are either from raw materials, ingredients used or from contamination. The means by which these microorganisms contaminate food are different and depend on both, the organisms present and the food product that supports them. The ability of these microorganisms to grow and create damage depends on the intrinsic properties of food and extrinsic factors applied to it. Visible microbial spoilage can take different forms, like discoloration, pigmentation, surface thickening and appearance disorder or decomposition [198, 203].

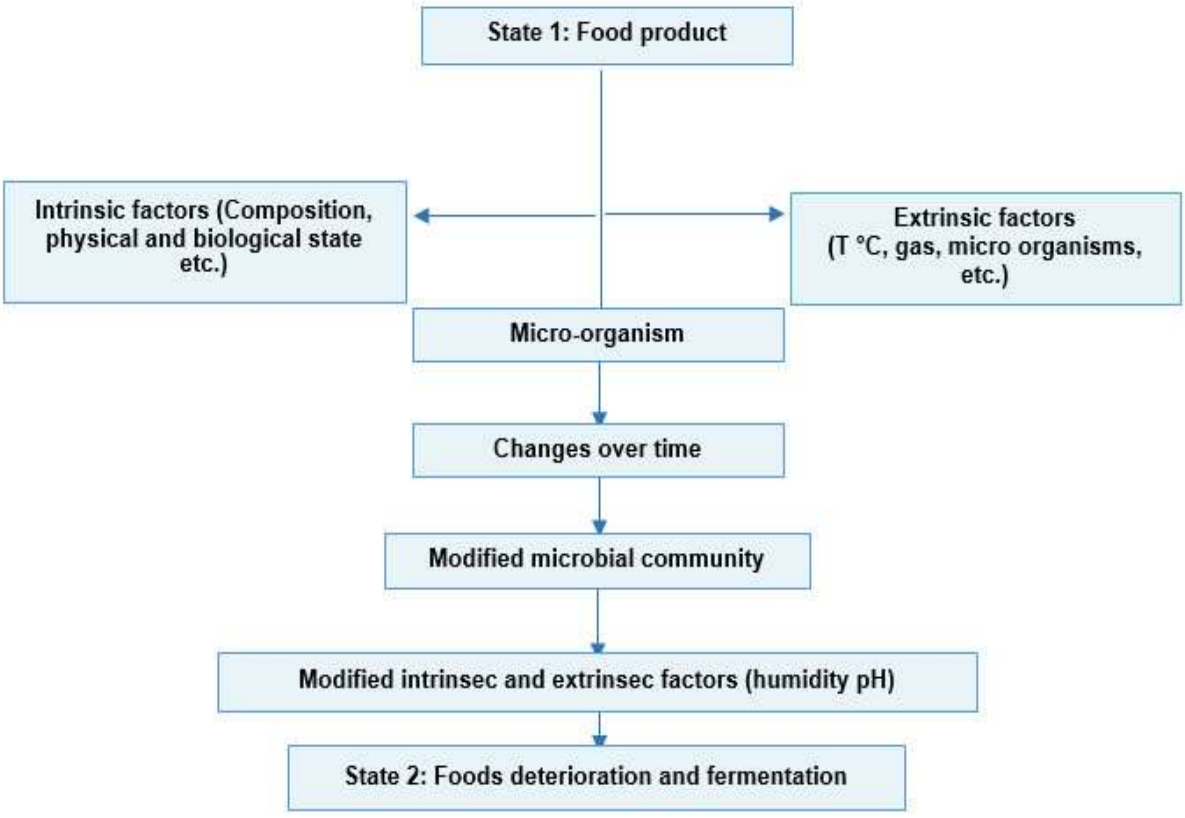

Figure 21. Intrinsic and extrinsic factors applied to food products [435]

2.2.6 Antimicrobial agents

The role of antimicrobial agents is to inhibit the growth of microorganisms or destroy them. The control of microorganisms growth can be physical, chemical and biological [161].

2.2.6.1 Natural antimicrobial agents

Plants have long been an important source of natural health products. For this reason, many scientists looking for new antimicrobial agents are paying particular attention to the plant kingdom [162].

Polyphenols are endowed with significant antimicrobial activity, probably due to their structural diversity. The sites and number of hydroxyl groups on the phenolic constituents are assumed to be related to their relative toxicity to microorganisms with evidence that the rate of hydroxylation is directly proportional to toxicity [206, 207]. Their activity is probably due to their ability to complex with extracellular proteins and to make complexes with bacterial cell walls. The most lipophilic quinones and flavonoids can also disrupt microbial membranes. The flavan-3-ols, flavonols and tannins have received more attention due to their broad spectrum and high antimicrobial activity and their ability to suppress a number of microbial virulence factors, such as the neutralization of bacterial toxins, as well as their capacity to act synergistically with some antibiotics [165].

One of the important functions of polyphenols, mainly flavonoids, is protecting plants from microbial invasion. This involves their accumulation as phytoalexins in response to microbial attacks. Owing to their ability to inhibit photogenic spore germination in plants, polyphenols have also been proposed for use against human fungal pathogens [166]. Several studies report the regular presence of antimicrobial activity in flavonoids. For instance, flavonoids have an antimicrobial effect against *Staphylococcus aureus* strain that is resistant to some antibiotics [167]. The majority of flavonoids, recognized as antifungal constituents are isoflavonoids, flavones and flavanones [168]. Moreover, the antiviral activity of flavonoids has been recently explored against the HIV immunodeficiency virus, which is the causative agent of the AIDS [169].

2.3 Anticancer potentials of bioactive compounds from plants

Since ancient civilizations, the therapeutic virtues of plants have been used to cure human illnesses and their use has evolved with the history of humans. The search for new active pharmacological agents through the screening of natural resources has resulted in the discovery

of a large number of useful drugs that play a major role in the treatment of many human diseases, such as cancer [142, 143].

2.3.1 Cancer: Generalities

The cancer is a malignant tumor characterized by an abnormal large cell proliferation leading to the invasion of the surrounding tissues with unlimited cell growth and metastasis [144, 145]. The cancer is the second leading cause of death worldwide, particularly in low and middle income countries where it represents 70% of death cases, accounting for 9.6 million deaths in 2018 [174]. Approximately one-third of cancer deaths are due to different behavioral and dietary risk factors, such as smoking, which presents the most important risk factor responsible for approximately 22% of total deaths [175].

2.3.2 Overview on some types of cancer

2.3.2.1 Breast cancer

Breast cancer is a malignant tumor of the mammary gland that affects women. This tumor originates in the cell units that secrete milk. Some risk factors for breast cancer are not modifiable, others are closely linked to lifestyle and can be avoided or at least limited. Mammography is the primary method for early detection of possible breast cancer in women over 50 years old. When breast cancer is detected at an early stage, treatment is often simpler and the chances of survival are generally higher [148, 149]. To find a treatment for this type of cancer, cell lines are needed for use in laboratory research. For instance, the MCF-7 (**Figure 22**) is a breast cancer cell line that has been widely used for 40 years as *in vitro* models in cancer research.

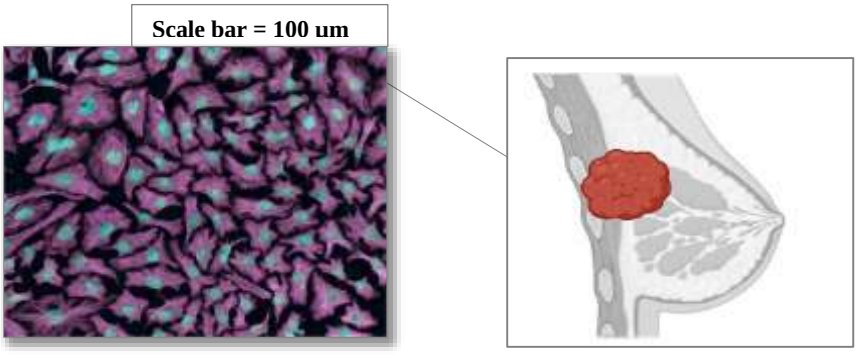

Figure 22. Confocal microscopy image of human breast cancer cell line (Sigma Aldrich)

2.3.2.2 Cervical cancer

Cervical cancer is the result of the malignant tumor development of the cervix lining. This cancer is considered a slowly progressing tumor disease of an infectious origin. In the majority of cases, cervical cancer occurs after prolonged exposure to human papillomavirus (HPV) because the long-term presence of the virus in the mucous membrane has a deleterious effect on healthy cells, which can develop into cancerous cells [151, 152]. In laboratories, HeLa cancer cell lines (**Figure 23**) are derived from cervical cancer cells and used *in vitro* scientific researches since 1951 [153, 154].

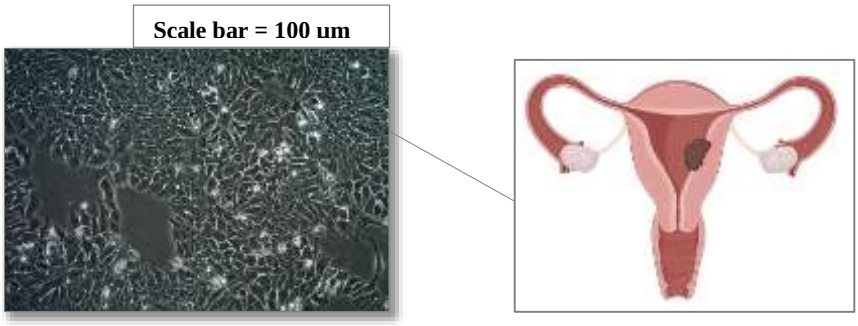

Figure 23. Confocal microscopy image of cervical cancer cell line (National Institutes of Health)

2.3.2.3 Liver cancer

Liver cancer develops from a cell called a hepatocyte, which is the most common cell in the liver. The hepatocyte can undergo a transformation that makes it cancerous. This phenomenon leads to its anarchic multiplication forming a malignant tumor of the liver or hepatocellular carcinoma [182]. Hepatitis G2 (HepG2) are human liver cancer cell lines derived from the human liver tissue with a well-differentiated hepatocellular carcinoma (**Figure 24**). These cells are suitable *in vitro* models for the study of polarized human hepatocytes [183].

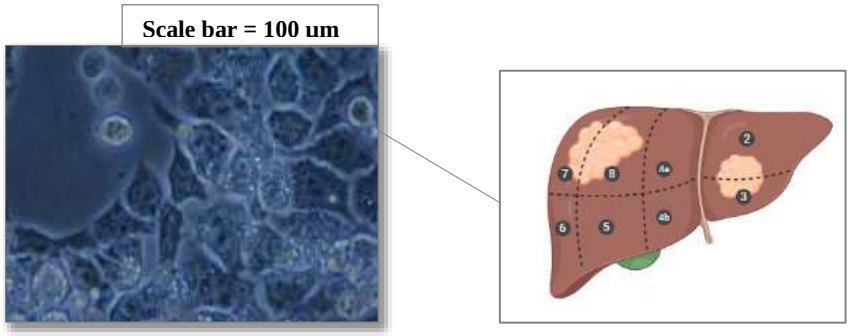

Figure 24. Confocal microscopy image of liver cancer cell line (Sigma Aldrich)

2.3.3 Relationship between the production of ROS and cancer

Oxidative stress, resulting from the imbalance between oxidant and antioxidant agents created by the excessive production of ROS, is involved in the development and progression of various human pathologies, including cancers [184]. Indeed, normal cells are hypersensitive to ROS if they are not sufficiently protected by physiological mechanisms of antioxidant defence, which can lead to several complications [185].

The first experiments about the role of ROS in tumor initiation have hypothesized that oxidative stress is involved in DNA mutations and genome instability [7] (**Figure 25**).

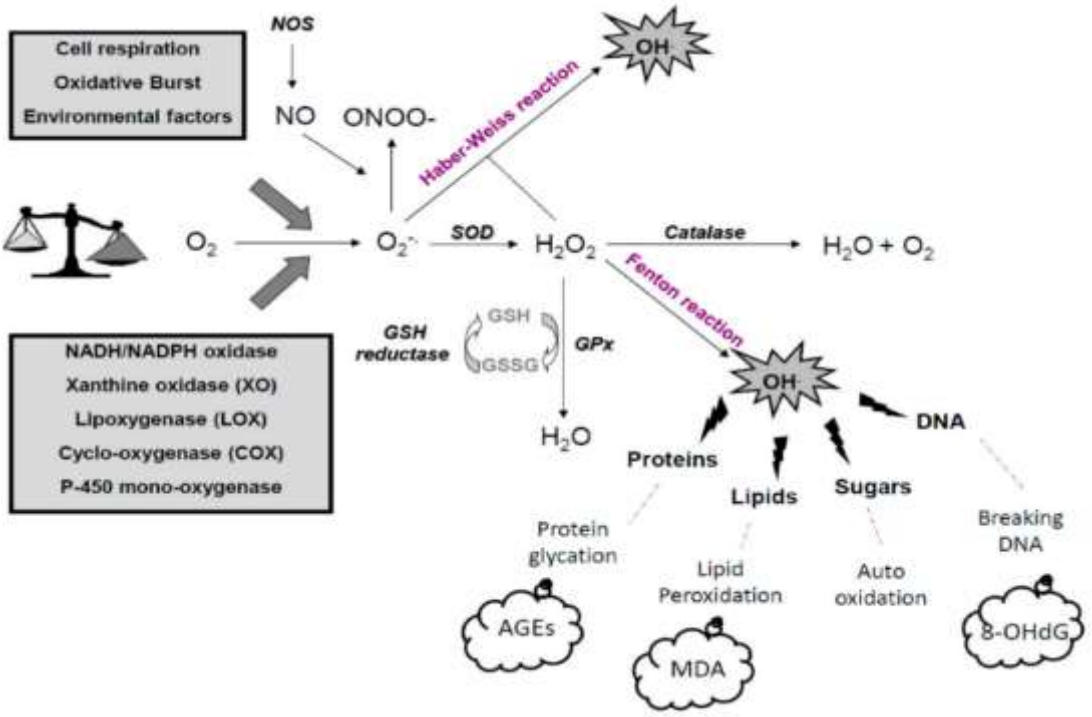

Figure 25. Oxidative stress and implication of ROS in cells damage [436]

Recent studies have shown that, in addition to inducing genomic instability, ROS can specifically activate certain signaling pathways and thus contribute to tumor development through the regulation of cell proliferation, angiogenesis [8] and metastasis [159].

ROS also can act as secondary messengers by altering the redox regulation of the GSH in cells. These results in the activation of the TRX, which activates the Nuclear Factor-Kappa B (NF-κB), a transcription factor from the super family of proteins involved in the immune and

[8] The development of new blood vessels.

cellular stress response [187]. Once activated, the NF-κB migrates into the nucleus of the cell where it can activate target genes. The deregulation of the NF-κB factors activity via the production of abnormal forms of NF-κB or a defect in their transcriptional activity is found recurrently in solid tumors [188]. More recently, it has been shown that NF-κB factors contribute to tumor development by activating apoptosis-resistance genes, making some tumors resistant to chemotherapeutic and radio-therapeutic treatments [189].

In recent years, considerable research has shown that ROS are also involved in the link between chronic inflammation and cancer. In fact, one of the most important characteristics of tumor promoters is their ability to recruit inflammatory cells and stimulate them to generate ROS. The longer the inflammation persists, the greater is the risk of cancer [190].

2.3.4 Cancer inhibition (chemotherapy treatment)

There are several effective treatments for cancer, such as radiotherapy [9] and the main treatment used is chemotherapy. Chemotherapy is the use of certain chemicals to treat a disease. These treatment technique aims to prevent cancer cells from multiplying, invading, metastasizing and killing the host (patient) [191].

The majority of chemotherapeutic substances stop the cell nucleus division called mitosis. Generally, these substances are called cytotoxic because they can damage cells. However, some molecules present fewer side effects than others [192].

Drugs in chemotherapy can be subdivided into different types, such as alkylating agent, topoisomerase inhibitors and plant alkaloids, which all affect the mitosis of the DNA.

2.3.4.1 Alkylating agents and topoisomerase inhibitors

Alkylating agents are compounds able to add alkyl groups to various electronegative groups under conditions within cells. Some of them are used to stop tumor growth by cross-linking the guanine bases of DNA, which prevents its replication and therefore stop the cell's division.

Topoisomerase inhibitors are enzymes that control the topological structure of the DNA by generating transient breaks in it and catalyzing the passage of its segments through these breaks before closing them. In particular, they enable negative supercoil [10] to be introduced or

[9] Therapy using ionizing radiation to control or kill malignant cells.

[10] A helical structure formed from a number of protein or nucleic acid chains.

removed from the DNA molecules. These enzymes are currently the subject of intense research activity because they are the pharmacological targets of important anti-cancer agents [193].

Alkaloids are cyclic organic compounds containing a nitrogen atom or atoms in a negative oxidation state and having a limited distribution among living beings. Initially, these natural substances were mainly isolated from plants, but currently they are isolated from most living organisms [194]. With over 5000 known compounds, the alkaloids represent the most structurally diverse class of secondary metabolites.

Over the past years, plants have been the largest source of metabolites with the advantage of having a great chemical diversity and anticancer activity [195]. For instance, the camptothecin is an alkaloid isolated from *Camptotheca acuminate* (*C. acuminate*) (**Figure 26**) that possesses high cytotoxic activity in a variety of cell lines and is used in chemotherapeutic treatment [196].

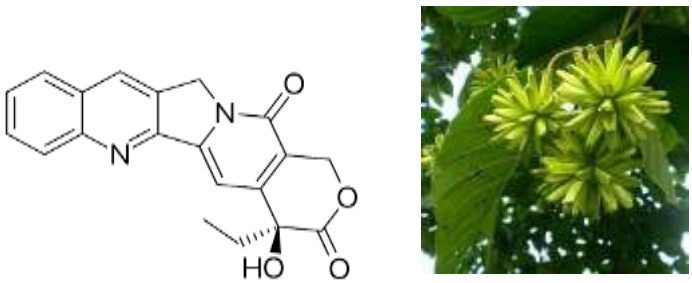

Figure 26. Chemical structure of the camptothecin [437] from *C. acuminate*

The taxols are also diterpenes widely used as chemotherapeutic agents to treat breast cancer [197]. They are extracted from *Taxus brevifolia* (*T. brevifolia*) (**Figure 27**).

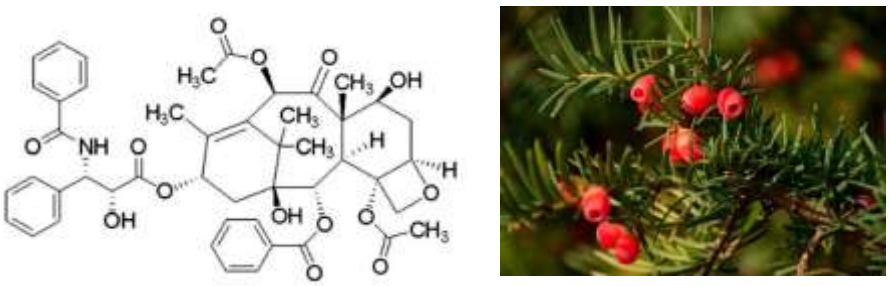

Figure 27. Chemical structure of the taxol from *T. brevifolia* [197]

In addition, epipodophyllotoxins are lignans naturally contained in the roots of *Podophyllum peltatum* (*P. peltatum*) (**Figure 28**) used in the treatment of childhood cancer [198].

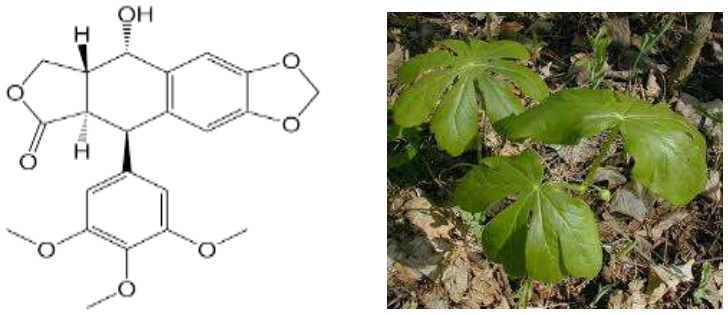

Figure 28. Chemical structure of epipodophyllotoxins from *P. peltatum* [198]

Moreover, vincristine and vinca-alkaloids are alkaloids extracted from *Catharanthus roseus* (*C. roseus*) (**Figure 29**) used as a chemotherapy treatment to fight cancer [173, 174].

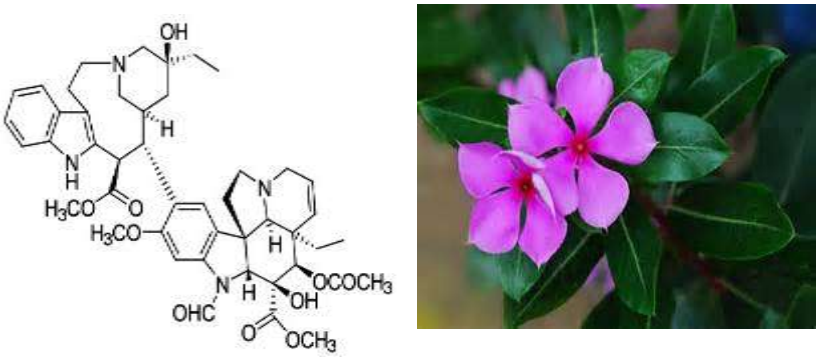

Figure 29. Chemical structure of the vincristine [438] from *C. roseus*

There are other examples of derivative products that are currently under investigation in order to test their anticancer activity, such as flavopridol, β-lapachone, and combrestatin. For example, flavopiridol is a synthetic flavone derived from the alkaloid rohitukin, which was isolated from the leaf and stem of *Amoora rohituka* (*A. rohituka*) [175,176] (**Figure 30**).

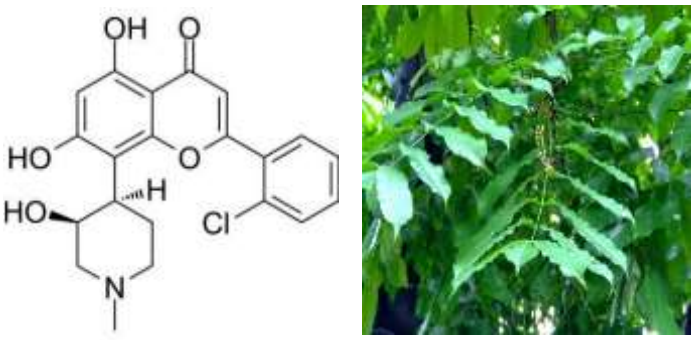

Figure 30. Chemical structure of flavopiridol [439] from *A. rohituka*

The β-lapachone is, as well, a quinone obtained from the bark of *Tabebuia avellanedae* (*T. avellanedae*) tree (**Figure 31**). It induces a slowing down of the cycle in phase G1 (growth phase) or phase S (synthesis phase) before inducing apoptosis or necrosis in many cancer cell lines [203].

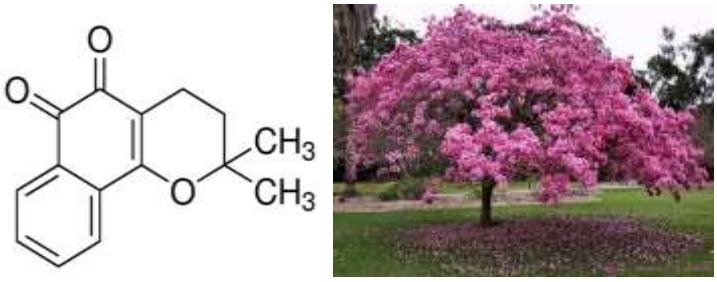

Figure 31. Chemical structure of β-lapachone [440] from *T. avellanedae*

In addition, the combretastatin A4 is isolated from the trunk of the South African tree *Combretum caffreum* (*C. caffreum*) (**Figure 32**). It inhibits the growth of blood capillaries causing cancer and necrosis of cells [178, 179].

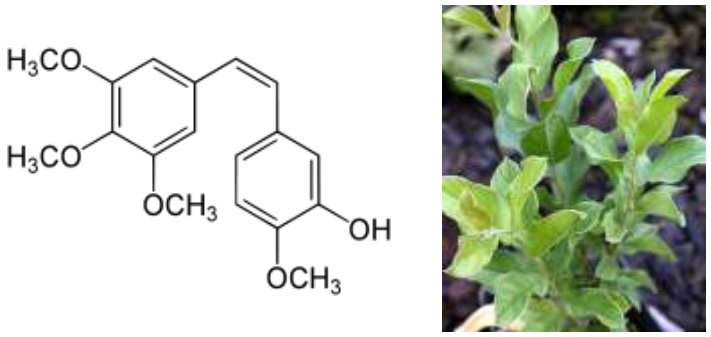

Figure 32. Chemical structure of the Combretastatin [441] from *C. caffreum*

2.4 Literature reviews of the plants studied

2.4.1 Woundwort (*Anthyllis vulneraria* L.)

Anthyllis vulneraria L. (*Anthyllidis* flos, herba; *Papilionaceae*) (**Figure 33**), commonly named "Woundwort" [206] is a Mediterranean medicinal plant that belongs to the fabaceae family. The genus *Anthyllis* comes from the Greek words "*Anthos*" and "*ioulos*", which mean "flowers" and "downy", respectively (as are the undersides of leaves), while the species *vulneraria* in Latin is "*vulnus*", which means "injury" referring generally to the healing of wounds [207].

Figure 33. *Anthyllis vulneraria*

2.4.1.1 Botanical description and geographical distribution

A. vulneraria is a perennial plant that blooms between April and May. It is characterized by an ordinarily robust stem that can reach up to 50 cm in height with hairy leaves, a terminal leaflet, large and often globular capitulates involved by one or two long egg-shaped leaves, a royal fusiform calyx of 15 to 17 cm and an oval pod containing one or two seeds (**Figure 34**). The *A. vulneraria* is widespread in the pastures of mountainous regions in Europe (Britain and Ireland), North Africa (Tunisia and Algeria), South Africa and Southeast Asia [208].

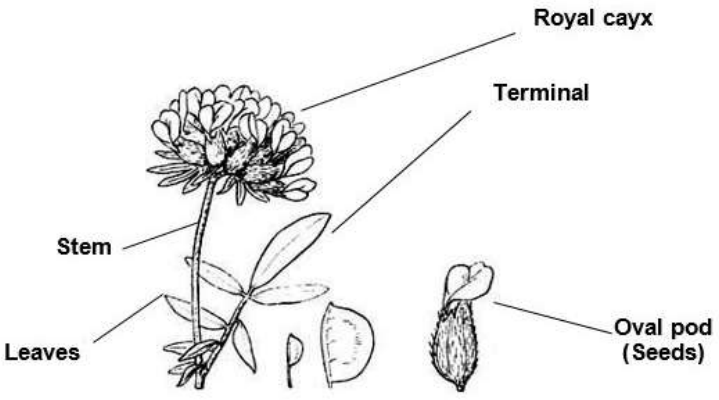

Figure 34. *Anthyllis vulneraria* anatomy

2.4.1.2 Scientific classification

According to Rola [209], the classification of *A. vulneraria* is as follow:

- **Kingdom**: Plantae
- **Branch**: Spermatophytes
- **Sub-branch**: Angiosperms
- **Family**: Fabaceae
- ***Genus***: *Anthyllis*
- ***Species***: *Anthyllis vulneraria*

2.4.1.3 Traditional and therapeutic uses

As its name suggests, *A. vulneraria* is a popular remedy for burns and skin rashes. In the traditional medicine, *A. vulneraria* flowers were used to heal wounds, to low the high blood pressure, to treat inflammation, vomiting, acne and to purify the body by promoting the elimination of toxins [206]. They were also used to heal mouth and throat pain, to limit hair loss and promote its growth [210].

Therefore, more investigations in food industry, pharmaceutical and cosmetic fields are needed to demonstrate the importance and efficiency of *A. vulneraria* active components to be used as substituents for the synthetic ones while ensuring the consumers health.

2.4.2 Neem (*Azadirachta indica* L.)

The neem, known in Latin as *Azadirachta indica* (*A.Juss*. syn. *Melia azadirachta* L.) (**Figure 35**) is a medium sized tree that is indigenous to Burma [211]. It is popularly known as "Yavan Priya", which means "beloved of Muslims". Then, *A. indica* was introduced into India [212].

Figure 35. *Azadirachta indica*

2.4.2.1 Botanical description and geographical distribution

The neem is a fast-growing tree that can reach a height of 15-40 m. It is evergreen and its small white flowers bloom from May to August (**Figure 36**). It has a straight trunk and its brown bark is hard rough and scaly, fissured even in small trees. The leaves of the neem are alternate and consist of several leaflets with serrated edges (**Figure 37**) [213].

Figure 36. *Azadirachta indica* flowers

Figure 37. *Azadirachta indica* leaves

The neem is native to Burma and India and nowadays it is wildly cultivated in several continents like Asia (Bangladesh, Sri Lanka, Malaysia and Pakistan and Kingdom of Saudi Arabia) and Africa, as well as Caribbean [214].

2.4.2.2 Scientific classification

The scientific classification of *A. indica* was described by Saleem et al [215] as follow:

- **Kingdom**: Plantae
- **Branch**: Spermatophytes
- **Sub-branch**: Angiosperms
- **Family**: Meliaceae
- ***Genus***: *Azadirachta*
- ***Species***: *Azadirachta indica*

2.4.2.3 Traditional and therapeutic use

The neem is one of the most useful plant in traditional medicine in the Indian culture owing to its therapeutic benefits [216]. The documented medicinal virtues of the neem showed that its different parts (leaves, flowers, seeds, fruits, roots and barks) were used to treat several human ailments, such as inflammation, diarrhea, bacterial infection, constipation [217] and cancer [218].

Despite the investigations done to demonstrate the biological and therapeutic benefits of neem's different parts, its effect in the food field remains unclear given the poorness of research regarding its use in food preservation and direct exploitation of its antioxidant and antimicrobial properties in food products.

2.4.3 Red pepper (*Capsicum baccatum* L.)

Capsicum baccatum L. (**Figure 38**), commonly named "Red pepper" is a pepper that belongs to the solanaceae family and is cultivated for its fruits with a particularly pungent flavour. Red pepper can be eaten freshly or processed for use as a spice. It can also be used as a source of extracts for use in various pharmaceutical or cosmetic products [219].

Figure 38. *Capsicum baccatum* fruits

2.4.3.1 Botanical description and geographical distribution

Capsicum baccatum (*C. baccatum*) is a perennial, annual, sometimes biennial shrub, a few meters high (2 meters). Its stem is green in colour with frail branches, angular and straight. The leaves are oval, serrated, and long-stalked and the fruit, chili, is a small fleshy berry, elongated and swollen. It has a glossy skin and is about 5 to 6 centimeters long. The colour of *C. baccatum* changes depending on the harvest period. It is initially green before turning bright

red. *C. baccatum* is very widespread in South America, northern Argentina, Brazil, Bolivia, Peru, Ecuador and Colombia and it is marketed almost all over the world [219].

2.4.3.2 Scientific classification

According to Crinquist [220] the classification of *C. baccatum* is as follow:

- **Kingdom**: Plantae
- **Branch**: Spermatophytes
- **Sub-branch**: Angiosperms
- **Family**: Solanaceae
- ***Genus***: *Capsicum*
- ***Species***: *Capsicum baccatum*

2.4.3.3 Traditional and therapeutic uses

In addition to its use as a spice or food, *C. baccatum* is known for its antioxidant and anti-inflammatory activity [221]. *C. baccatum* is also a good source of pro-vitamin A, vitamins E and C, carotenoids, and phenolic compounds, such as luteolin and quercetin, which are characterized by their antioxidant and biological properties, especially the capsaicinoids and dihydrocapsaicin whose antimicrobial activity have been demonstrated in several researches [222].

C. baccatum fruits were used in this work to study their preservative effect in one *Model Food System* (refrigerated minced beef) and compare them with *A. indica* leaves.

3 Hypothesis & Objectives

3 Hypothesis and objectives

The hypothesis of the present thesis is that *A. vulneraria* and *A. indica* may be rich in phenolic compounds, which can exhibit biological properties with antioxidant and anti-radical effect beneficial to human health on the one hand, and which can preserve food and cosmetic products against deterioration by the extension of their shelf life on the other hand. These phenolic compounds also can inhibit bacterial growth causing food poisoning as they can prevent the proliferation of cancer cells. Thus, the bioactive compounds of *A. vulneraria* and *A. indica* may be relevant as functional ingredients in cosmetic and pharmaceutical products and natural additives in foods to replace synthetic ones.

To prove this hypothesis, the antioxidant, antibacterial and antiproliferative activities of *A. vulneraria* and *A. indica* were determined *in vitro* separately by different methods in order to study the possibility of using them in the pharmaceutical, cosmetic and food industry as substitutes for synthetic additives.

Hence, the objectives of the present work are:

A. Analysis of the chemical composition of *A. vulneraria* and *A. indica* extracts.

A.1. To determine the extraction yield and quantify, spectrophotometrically, the different phenolic compounds (total polyphenol, flavonoid and condensed tannins) of the extracts.

A.2. To identify and quantify the main phenolic compounds in each extract using HPLC-MS.

B. Determination of the radical scavenging activity of *A. vulneraria* and *A. indica* extracts.

B.1. To measure the radical scavenging capacity and antioxidant activity of the extracts using different *in vitro* analytical methods, including TAC, FRAP, TEAC, ORAC, DPPH and HPS.

B.2. To measure the radical scavenging activity of *A. vulneraria* and *A. indica* extracts against methoxy radical by the spin trap method and Electron Paramagnetic Resonance (EPR) spectroscopy technique using ferulic acid as antioxidant pattern.

C. Evaluation of the preservative effect of *A. vulneraria* and *A. indica* samples against the oxidation of *Model Food System*

C.1. To study the antioxidant effect of extracts on the oxidative stability of Oil-in-Water (O/W) emulsion by following the formation of primary and secondary oxidation products.

C.2. To assess the antioxidant effect of powdered samples directly incorporated into minced beef meat and evaluate their effectiveness against the lipid oxidative during refrigerated storage by studying the chemical, microbial and sensory analyses of meat.

C.3. To analyze the combined effect of the powdered *A. indica* leaves with red pepper (*C. baccatum*) on the oxidative stability of meat products and the extension of their shelf life.

D. Determination of the antibacterial activity of *A. vulneraria* and *A. indica* extracts against several pathogenic microorganisms causing food poisoning by measuring the inhibitory zones and the minimum inhibitory concentration.

E. Assessment of the antiproliferative activity of *A. vulneraria* and *A. indica* extracts against different human cancer cells (HepG2, HeLa and MCF-7) using MTT assay.

4 Materials & Methods

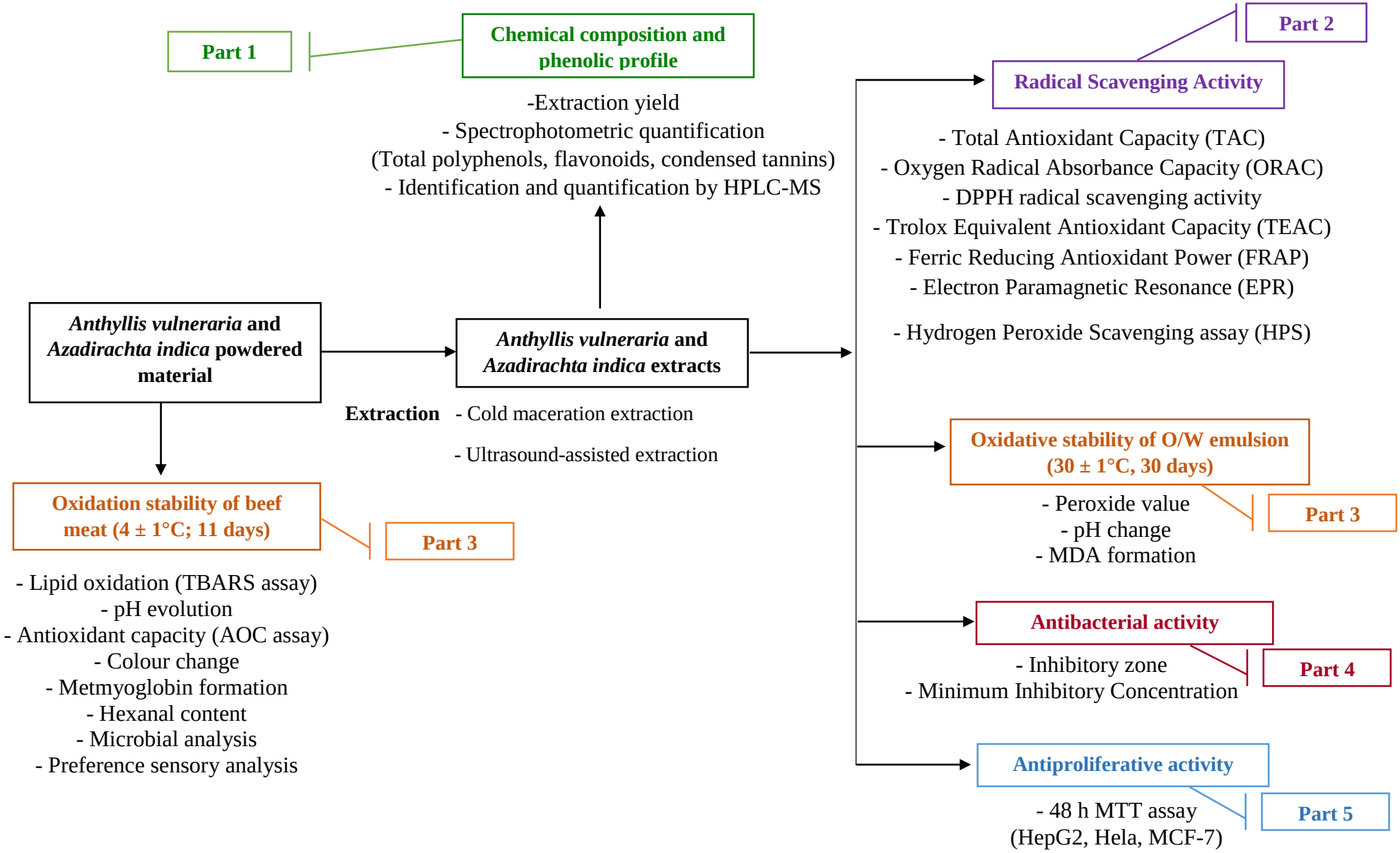

Figure 39. Schematic overview of the working plan

4 Materials and methods

4.1 Reagents and chemicals

AAPH, ABTS, acetate buffer, acetonitrile, $AlCl_3$, alumina, ammonium molybdate, ascorbic acid, catechin, DMPO, DMSO, DPPH, EtOH, $FeCl_2$, $FeCl_3$, ferulic acid, $FeSO_4$, formic acid, FRAP, gallic acid, H_2O_2, hexanal, MDA, MeOH, MTT formazan, Na_2CO_3, phosphate buffer, $K_2S_2O_8$, sodium phosphate, sodium succinate, sulfuric acid, TBA, TPTZ, trolox, tween-20 and vanillin are purchased from Sigma–Aldrich Company Ltd. (Gill Ingham, UK). Acetic acid, Ac, ammonium thiocyanate, EDTA, fluorescein, Folin–Ciocalteu reagent, HCl, PBS, phosphor-molybdenum reagent, quercetin and TCA are acquired from Panreac Química S.L.U (Barcelona, Spain).

The synthetic preservative used as a positive control in the determination of the plants antioxidant effect on raw beef patty quality is “Food preservative CAMPA no. 3 (A), code 403600”, elaborated by “La Campana, Barcelona, Spain” for burger meat and it is composed of: dextrose, preservatives: (sulfur dioxide 5.7%), E-224 (sulfite) and antioxidants (E-301 and E-331 iii). DMEM, fetal bovine serum and *Penicillin streptomycin* are bought from Thermo Fisher Scientific, GIBCO (Barcelona, Spain). Nutrient broth and Muller Hinton agar are from Thermo Fisher Scientific, OXOID (Basingstoke, UK).

4.2 Plant materials and sampling process

A. vulneraria was collected at the flowering stage (in April) from the mountains of Zaghouan (**Figure 40**) located in the North of the Tunisian ridge (36° 21' 07" north, 10° 06' 43" east), whereas *A. indica* was collected in March from Punjab in the North of India (**Figure 41**).

Figure 40. *Anthyllis vulneraria* from Zaghouan, Tunisia

Figure 41. *Azadirachta indica* from Punjab, India

A. vulneraria leaves and flowers and *A. indica* leaves were dried in the air until the achievement of constant weights and then ground using an electric grinder (*KRUPS F203, Barcelona, Spain*). The powdered dry vegetable matters obtained were conserved in crystal amber bottle and stored in a desiccator for further use.

4.3 Spectrophotometric determination of phenolic compounds

4.3.1 Extract preparation

The preparation of *A. vulneraria* leaf and flower extracts was assessed using two different methods, including cold maceration extraction and ultrasound assisted extraction, while *A. indica* leaves were extracted using cold maceration extraction method.

To perform the cold maceration extraction, 1 g of each dry sample was mixed with 10 mL of different concentration of EtOH and extracted during 24 h at 4°C under stirring using a multi-position magnetic stirrer (*Ovan, MM90E, Barcelona, Spain*). Then, the samples were centrifuged (*Orto Alresa Mod. Consul, Ajlvir, Madrid, Spain*) at 1500×g for 10 min. The different supernatants were concentrated using a sample concentrator (*Techne FSC496D sample concentrator, Madrid, Spain*) under a jet of moderate nitrogen gas, then lyophilised using a freeze dryer (*Unicryo MC2L, UniEquip Laborgerätebau & Vertr. GmbH, Munich, Germany*) for 2 days.

The ultrasound assisted extraction method also was carried out using different concentration of EtOH. Shortly, 1 g from each powdered plant material was mixed with 10 mL of EtOH. The different mixtures were stirred using a vortex (*FALC Instruments, A121498, Italy*) for 1 min and extracted in an ultrasonic bath (*COXO Medical Instrument CO., LTD, DB4820,*

Medical World Company) for 15 min at 25°C at a frequency of 40 KHz and a power of 100 W. Then, the extracts were filtered with filter paper (Wattman no. 4) and the supernatants obtained were concentrated then lyophilised for 2 days.

A. vulneraria leaf and flower freeze-dried samples were dissolved in absolute EtOH, 75%-aqueous EtOH and 50%-aqueous EtOH and *A. indica* leaf freeze-dried sample was dissolved in absolute EtOH and 80%-aqueous EtOH and stored in darkness at 4°C until their use.

4.3.2 Extraction yield

The Extraction Yield (EY) of *A. vulneraria* and *A. indica* extracts was determined. The final dry weight of each lyophilised sample was used to calculate the EY according to the following formula:

$$\text{EY (\%)} = \frac{W1}{W2} \times 100$$

Where W_1 represents the sample weight after the lyophilisation and W_2 represents the dry weight of the sample.

4.3.3 Total polyphenol content

Total Polyphenol Content (TPC) was determined according to the method of Singleton et al [223] reported by Segovia Gómez & Almajano Pablos [224]. The method is based on the Folin-Ciocalteu used as reagent. This method was developed for the first time in 1927 by Folin and Ciocalteu and is considered one of the simplest methods available for the measurement of phenolic content in plant matters and products.

The principle of this method strongly relies on the reduction of the mixture heteropolyphosphotungsates–molybdate (yellow coloured) by the phenolic compound, which results in the formation of blue coloured chromogen measured by spectrophotometry [225] (**Figure 42**). The intensity of the blue colour produced is proportional to the amount of polyphenols present in the extracts analysed and has a maximum absorption at 765 nm [226].

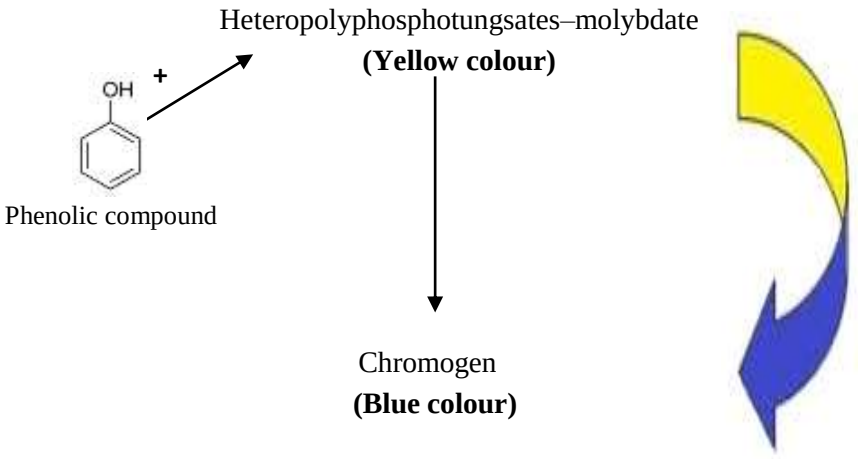

Figure 42. Reaction mechanism of Folin–Ciocalteu reagent [442]

To determine TPC in *A. vulneraria* and *A. indica* extracts, 20 µL from each diluted extract were added in each well of a white micro-titer 96-well plate and mixed with 80 µL of Folin-Ciocalteu reagent (10 N) and 80 µL of Na_2CO_3 (20%, *w/w*). The plate was stirred for 2 min and then incubated in darkness at room temperature. After 1 h, 80 µL of Milli-Q water were added to each well, and the absorbance was measured at 765 nm using a multimode micro-plate reader (*FLUOstar Omega, Ortenberg, Germany*). The measurements were based on a calibration curve made with gallic acid at different concentrations ranging from 100 to 1700 µM (R^2 = 0.992). The results are expressed as milligram of Gallic Acid Equivalent per gram of Dry Weight (GAE/g DW).

4.3.4 Total flavonoids content

Total Flavonoids Content (TFC) was determined using the aluminum chloride colorimetric method as described by Skowyra et al [227]. The quantification of flavonoids using $AlCl_3$ as a reagent is one of the most common methods because flavonoids are almost universal and, generally, contribute to antioxidant activity by scavenging free radicals [228].

This method was originally proposed by Christ and Muller in 1960 and was subsequently modified several times. Its principle consists in the formation of a yellow complex due to the reaction of the $AlCl_3$ with the C-4 keto group and hydroxyl groups of the ring C and/or the ring A and/or the ring B [229] (**Figure 43**).

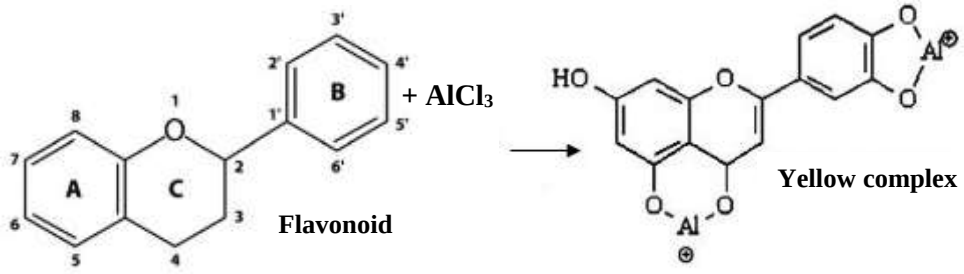

Figure 43. Complex-forming reaction between flavonoid and $AlCl_3$ [443]

To determine TFC in *A. vulneraria* and *A. indica* extracts, 150 µL from each diluted extract were added in each well of a white micro-titer 96-well plate and mixed with 50 µL of $AlCl_3$ (20 mg/mL in 5% acetic acid: MeOH (*v/v*)). The plate was stirred for 2 min, and then kept in darkness at room temperature for 30 min. The absorbance was measured at 405 nm, and the different measurements obtained were compared to a calibration curve prepared with quercetin at different concentrations ranging from 50 to 500 µM (R^2 = 0.998). Results are expressed as milligram of Quercetin Equivalent per gram of Dry Weight (mg QE/g DW).

4.3.5 Total condensed tannin content

Total Condensed Tannin Content (TCTC) was determined using the vanillin method described by Julkunen-Tiitto [230]. This method depends on the reaction of vanillin with the condensed tannin present in the extract to form a red complex (**Figure 44**).

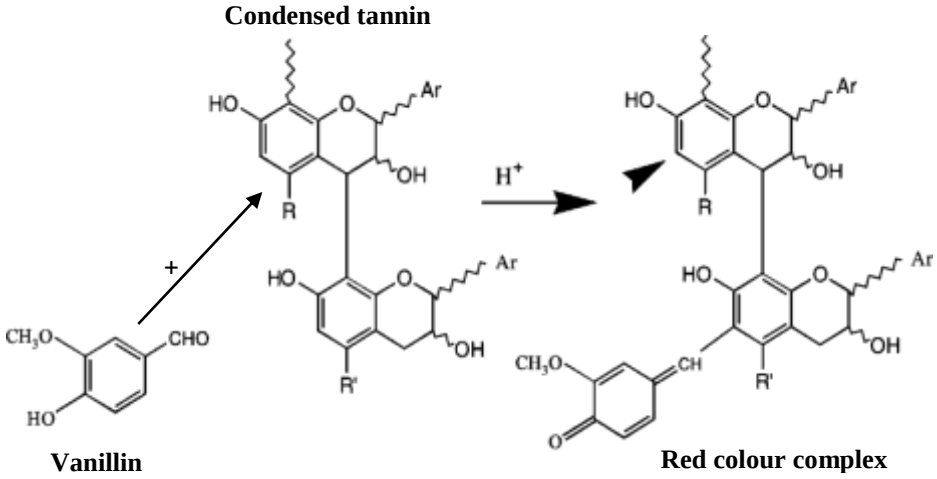

Figure 44. Complex-forming reaction between condensed tannin and vanillin [444]

To determine TCTC, 50 µL of *A. vulneraria* and *A. indica* extracts were mixed vigorously with 1500 µL of 4% vanillin/MeOH solution (*w/v*). Then, 750 µL of concentrated HCl was added. The mixtures obtained were left to react at ambient temperature for 20 min. The absorbance of the samples was measured at 550 nm against a blank. The calibration curve was prepared with Catechin at different concentrations ranging from 1 to 1000 µg/mL ($R^2 = 0.997$). Results are expressed as milligram of Catechin Equivalent per gram of Dry Weight (mg CE/g DW).

4.4 Determination of free radical scavenging activity

4.4.1 Total antioxidant capacity

Total Antioxidant Capacity (TAC) is a technique based on the reduction of MoO_4^{2-} ions to in the presence of the plant extract to form a green complex of phosphate/MoO^{2+} at and acidic pH [231].

TAC was determined by phosphor-molybdenum method as reported by Zengin et al [232], 50 µL from each extract were mixed with 1 mL of phosphor-molybdenum reagent containing sulfuric acid (0.6 M), sodium phosphate (28 mM) and ammonium molybdate (4 mM). After incubation at 95°C for 90 min, the absorbance of the mixtures obtained was measured at 695 nm. The calibration curve was prepared with ascorbic acid at different concentrations ranging from 1 to 100 µg/mL ($R^2 = 0.998$). TAC results are expressed as milligram of Ascorbic Acid Equivalent per gram of Dry Weight (mg AAE/g DW).

4.4.2 Ferric reducing antioxidant power assay

Ferric Reducing Antioxidant Power (FRAP) method was introduced by Benzie & Strain, [233] at the University of Ulster at Colerain in Northern Ireland. This method has been modified by Tsao et al [234] to be suitable for the 96 well micro-plates. The method is based on the reduction of a yellow Fe^{3+}/TPTZ complex to a blue Fe^{2+}/TPTZ complex by electron-donating antioxidant in an acidic medium [235] (**Figure 45**). The Fe^{2+} cations formed interact with the TPTZ giving an absorbance measurable at 593 nm.

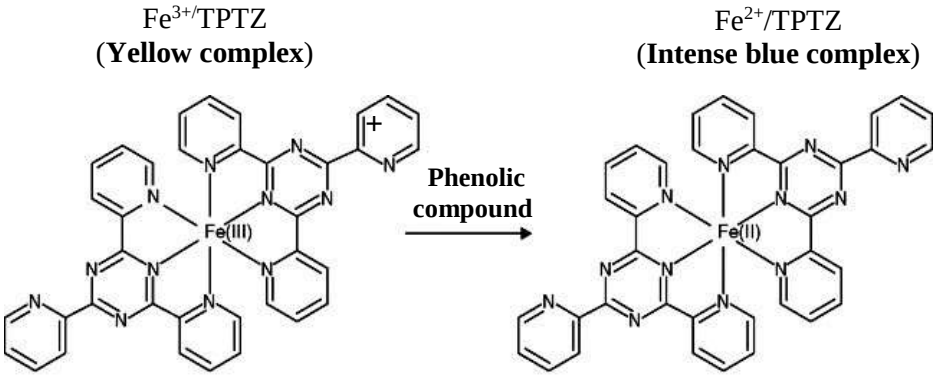

Figure 45. Reduction of Fe^{3+}/TPTZ to Fe^{2+}/TPTZ by a phenolic compound [445]

To perform FRAP assay, 20 µL of each diluted extract were allowed to react with 200 µL of the FRAP reagent (300 mM acetate buffer (pH = 3.6); 10 mM TPTZ; 20 mM $FeCl_3$, mixed in the ratio 10:1:1 (*v/v/v*), respectively). The absorbance was recorded at t = 15 min and t = 30 min using the multimode micro-plate reader at 37°C at 593 nm and FRAP values were determined from a calibration curve prepared with Trolox at different concentrations varying between 3 and 20 µM (R^2 = 0.989). The results are expressed as milli-mole of Trolox Equivalent per gram of Dry Weight (mM TE/g DW).

4.4.3 Oxygen radical absorbance capacity assay

Oxygen Radical Absorbance Capacity (ORAC) method is largely used to quantify antioxidant capacity in plant extracts and is the only method, in which inhibition time and degree of inhibition into a single sample are combined [236]. This assay consists in the inhibition of peroxyl radicals induced by thermal decomposition of AAPH [237].

ORAC assay was performed according to Azman et al [238] method using the multimode micro-plate reader, in which the temperature was set at 37°C. In each well of a black 96-well plate, 40 µL of diluted extract were mixed with 120 µL of fluorescein. An initial reading of 2 min at an excitation wavelength of 485 nm was taken, then 40 µL of AAPH (0.081 g/mL PBS) were added to the mixture and the measurement continued for 2 h at a wavelength of 535 nm. The loss of fluorescence over time was an indication of the damage caused by the peroxyl radicals and was quantified according to the following formula:

$$AUC = \frac{(0.5 + \sum_{t_c}^{N_c} f_n)}{f_i}$$

Where AUC represents the area under the sample curve in the well; f_i represents the fluorescence reading at the initiation of the reaction; f_n represents the last measurement, N_c

represents the number of cycles and t_c represents the time of each cycle (2 min). The formula cited below is specific for determining the Decrease in Fluorescence (DF).

$$DF = AUC - AUC_{Bl}$$

Where AUC_{Bl} expresses the area under the blank curve [225]. ORAC values were determined from a calibration curve made with Trolox at different concentrations ranging from 4 to 40 µM (R^2 = 0.998). Results obtained are expressed in millimole of Trolox Equivalent per gram of Dry Weight (mM TE/g DW).

4.4.4 Trolox equivalent antioxidant capacity assay

Trolox Equivalent Antioxidant Capacity (TEAC) assay, or ABTS radical cation decolorisation assay, was proposed by Miller et al [239], and then was improved by Re et al, [240]. The method is based on the neutralization of the by phenolic compounds and convert it back to its colourless neutral form [241] (**Figure 46**).

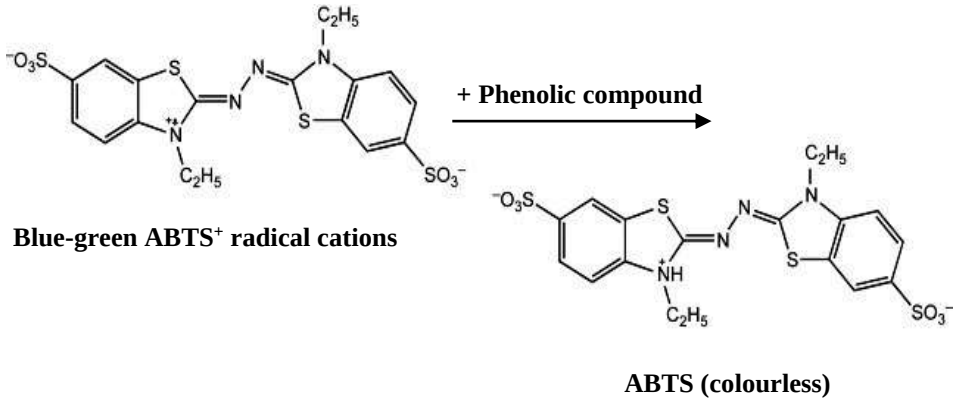

Figure 46. Reaction of $ABTS^+$ with phenolic compounds [446]

TEAC assay was assessed according to the method described by Gallego et al [242]. $ABTS^+$ radical solution (7 mM) was prepared with $K_2S_2O_8$ (24.24 mM) and kept in darkness. After 16 h of incubation, the solution was diluted with 10 mM PBS (pH = 7.4 at 30 ºC) and adjusted at the absorbance ranging between 0.9 and 1 at λ= 734 nm. The assay was performed in a white micro-titer 96-well plate by mixing 200 µL of ABTS radical solution with 20 µL from each extract in an appropriate dilution. The absorbance was measured at 734 nm using the micro-plate reader, in which the temperature was fixed at 30 ºC. The Inhibition Percentage (IP) of $ABTS^+$ radical cation was determined according to the following formula:

$$IP = \left(\frac{t_{0_s} - t_{5_s}}{t_{0_s}}\right) - \left(\frac{t_{0_{bl}} - t_{5_{bl}}}{t_{0_{bl}}}\right) \times 100$$

Where t_{0_s} represents the absorbance value of the sample at the initial time t = 0 min; t_{5_s} represents the absorbance value of the sample at t = 5 min; $t_{0_{bl}}$ represents the absorbance value of the blank at the initial time and $t_{5_{bl}}$ represents the absorbance value of the blank at t = 5 min. TEAC values were determined from a calibration curve made with Trolox at different concentrations ranging from 2 to 32 μM ($R^2 = 0.995$) and the results are expressed in millimole of Trolox Equivalent per gram of Dry Weight (mM TE/g DW).

4.4.5 Electron paramagnetic resonance spectroscopy technique

Electron Paramagnetic Resonance (EPR) spectroscopy, known also as Electron Spin Resonance (ESR) spectroscopy, is an extension of the experiment carried out by Otto Stern and Walther Gerlach in 1922 and developed in 1944 by the Soviet physicist Yevgeny Zavoisky in Kazan State University [243] and then by Brebis Bleaney at the University of Oxford. The antioxidant activity of the extracts is measured in a competitive spin trapping reaction, in which an unstable radical reacts with a spin trap [11] (DMPO) to form a much more stable radical adduct, detected by EPR spectroscopy. The antioxidant activity of natural extracts is measured by the decrease of the intensity of the radical adduct spectra [244]. EPR technique is commonly used to analyse most complex food, cosmetic, biological and pharmaceutical samples [248, 249].

To perform EPR spectroscopy assay, different amounts of each freeze-dried sample were dissolved in absolute MeOH, and then the samples were filtered through safe filters (0.22 μm) (*Teknokroma Analítica S.A. Barcelona, Spain*). Aqueous-EtOH was substituted by absolute MeOH due to the low solubility of ferulic acid in water.

A spin-trapping reaction mixture was prepared by adding 300 μL of DMPO (35 mM) to 150 μL of H_2O_2 (10 mM), 150 μL of $FeSO_4$ (2 mM) and 150 μL of the extract. Ferulic acid and deoxygenated MeOH were used as positive and negative controls, respectively. Then, 125 μL of the solution obtained were passed through a narrow quartz tube (diameter = 2 mm) and introduced into the cavity of the EPR spectrometer [247].

The spectrum was recorded with a spectrometer (*Bruker EMX 10/12-Plus, Madrid, Spain*) just 10 min after the addition of the $FeSO_4$ solution according to the following conditions (**Table 1**).

[11] Compounds used to react covalently with radical products and form more stable adduct that will also have paramagnetic resonance spectra detectable by EPR spectroscopy.

Table 1. Measurement conditions of EPR spectroscopy technique

Microwave Power	20.00 mW
Magnetic Field Amplitude Modulation	1.86 G
Frequency Modulation	100 KHz
Time Constant	10.24 ms
Conversion Time	50 ms
Scanning Time	51.20 sec
Field Width	100 G
Gain	7.10×10^3
Resolution	1024 points

4.4.6 DPPH radical scavenging activity assay

DPPH assay is a spectrophotometric method used to determine the anti-radical activity of natural products [248]. The method consists in quenching stable DPPH radicals, hence the discoloration of the radical solution from a purple colour to a pale yellow one [249]. The **Figure 47** illustrated below explains the reaction mechanism of the DPPH with antioxidants.

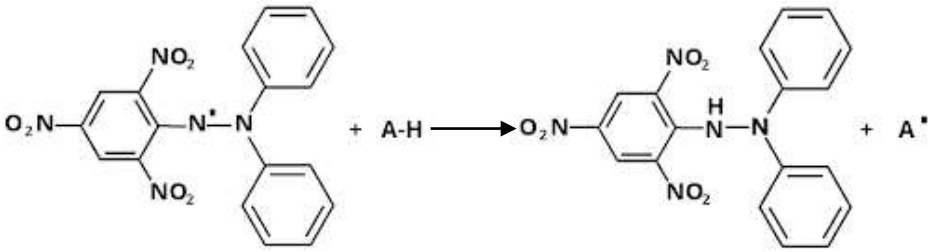

Figure 47. Reaction mechanism of DPPH with an antioxidant [447]

Where A-H is an antioxidant (donor of hydrogen atoms). A˙ is the newly formed radical, which interact with other molecules to form stable radicals [250].

The ability of the extracts to inhibit DPPH radicals was determined as described by Shalaby and Shanab [251]. Shortly, 20 µL of each diluted extract were mixed with 200 µL of DPPH radical solution (0.04 mg/mL MeOH) in each well of a black micro-titer 96-well plate. After the agitation of the plate, the absorbance was measured at 517 nm every 15 min for a total of 75 min. DPPH values were determined from a calibration curve made with Trolox at different concentrations ranging from 0.5 to 5 mM ($R^2 = 0.998$). Results are expressed in millimole of Trolox Equivalent per gram of Dry Weight (mM TE/g DW).

4.4.7 Hydrogen peroxide scavenging assay

Hydrogen Peroxide Scavenging (HPS) assay was determined as described by Ruch et al [252]. A solution of 43 mM H_2O_2 was prepared with phosphate buffer (0.1 M, pH = 7.4), then 100 µL from each extract were mixed with 0.6 mL of H_2O_2 solution. The absorbance was measured at 230 nm and the percentage of H_2O_2 scavenging effect (HPS effect) was calculated using the following formula:

$$\text{HPS effect (\%)} = \left(1 - \frac{As}{Ac}\right) \times 100$$

Where Ac is the absorbance of the blank (containing sodium phosphate buffer without H_2O_2) and As is the absorbance of the sample.

4.5 Determination of the extracts phenolic profile by HPLC-MS

The identification and quantification of the phenolic compounds present in *A. vulneraria* and *A. indica* extracts were carried out using an Agilent 1200 Series HPLC-MS equipment. The equipment consists of an automatic sample injection system, two high-pressure isocratic pumps, a degasser and a chromatographic oven. The components were separated by a C18 column (*100 mm × 2.1 mm, 3.5 m, Zorbax Eclipse, Agilent, Madrid, Spain*) connected to a C18 pre-column (*4 mm × 2 mm, Phenomenex, Torrance, CA, USA*). The HPLC-MS conditions were set as follow (**Table 2**):

Table 2. Optimised HPLC-MS conditions

Mobile Phases	Phase A: ultrapure water acidified with 0.11% formic acid Phase B: acetonitrile acidified with 0.11% formic acid
Gradients	0-2 min, 3% B 25-27 min, 100% B 28-38 min, 3% B
Flow Rate	0.2 mL/min
Injection Volume	10 µL
Column Temperature	30°C

Different commercial standards (**Table 3**) were subsequently used in order to identify the compounds detected by HPLC-MS analysis. The identification of the components was confirmed by matching the Retention Time (RT) and the fragment ions (m/z) to those of the corresponding authentic standard compounds.

Table 3. Chemical standards used

Standards	PubChem Compound CID
Chlorogenic acid	1794427
Caffeic acid	689043
Syringic acid	10742
Epicatechin	72276
P-coumaric acid	637542
Sinapinic acid	637775
Ferulic acid	445858
Ellagic acid	5281855
Rutin	5280805
Myricetin	5281672
Quercetin	5280343

4.6 Evaluation of the antioxidant effect of the plant samples on *Model Food System*

A *Model Food System* is based on the formulation and processing of a real food by adding natural products to improve its nutritional quality and prolong its shelf life. In the present work, two different *Model Food System*, including O/W emulsion and mined beef meat were developed. *A. vulneraria* and *A. indica* samples were tested in these *Model Food Systems* to investigate their ability to protect them from deterioration and to determine whether they altered the characteristics of the final product, as well as to assess the sensitivity of the product characteristics to the added plants and the processing steps.

4.6.1 Evaluation of the antioxidant effect of the extracts on O/W emulsion

4.6.1.1 Preparation of mother emulsion

A set amount of alumina was activated in the oven at 200°C for 24 h, and then kept in a desiccator until it reached room temperature. Sunflower oil was purified two times through the activated alumina in absolute darkness to exclude tocopherols (vitamin E). To prepare O/W mother emulsion, 10% of the purified sunflower oil were added, drop by drop, to an aqueous mixture containing 1% of tween-20 and Milli-Q water under sonication using an ultrasonic homogenizer (*Hielscher, UP200S, Teltow, Germany*) in an ice bath (0°C) for 10 min.

4.6.1.2 Emulsion sampling and storage conditions

The mother emulsion was divided evenly into vials. In each vial, a different extract was added to have at the end different emulsion samples (**Table 4**).

Table 4. Emulsion samples

Samples	Abbreviations	Composition
Control	E-CTR	Emulsion without antioxidant
Synthetic preservative	E-GA	Emulsion containing gallic acid [a]
A. vulneraria leaves	E-AVL	Emulsion containing *A. vulneraria* leaves extract [b]
A. vulneraria flowers	E-AVF	Emulsion containing *A. vulneraria* flower extract [c]
A. indica leaves	E-AI	Emulsion containing *A. indica* leaves extract [d]

[a] Gallic acid added to the emulsion at 0.25% and 0.5% (*v/v*), [b] *A. vulneraria* leaves extract added to the emulsion at 0.25% (*v/v*), [c] *A. vulneraria* flowers extract added to the emulsion at 0.25% (*v/v*), [d] *A. indica* leaves extract added to the emulsion at 0.25% and 0.5% (*v/v*).

All the emulsion samples were prepared in triplicate and allowed to oxidize in the oven during 30 days at 30 ± 1°C in darkness and under constant slow agitation.

4.6.1.3 Peroxide value and pH measurement

The Peroxide Value (PV) is the most widely used method to determine rancidity in unsaturated fats and oils by the detection of peroxides at the initial stage of the lipid oxidation [253]. In the present study, primary oxidation in the different emulsion samples was determined by the PV method [254].

In short, an emulsion drop, weighing between 0.0070 and 0.0130 g, was added to 1 mL of absolute EtOH and well mixed using a vortex (*Ovan Vibra Mix R, Model VM3E, Barcelona, Spain*) until complete homogenization. Then, in a plastic cuvette, 900 µL of each emulsion sample were mixed with 3100 µL of absolute EtOH, 75 mL of Iron (II) chloride reactive (37% HCl, $FeCl_2$) and 75 mL of ammonium thiocyanate reactive (302.5 g/L). The blank contained only 4000 µL of absolute EtOH and 75 mL of each reactive. The PV values were determined from a calibration curve made with H_2O_2 at different concentrations ranging from 0 to 20 mg (R^2=0.996). The absorbance was measured using a UV–vis spectrophotometer (*Zuzi spectrophotometer 4201/20, Auxilab, Navarra, Spain*) at 500 nm and the results are expressed as milli-equivalent of hydro-peroxide per kilogram of emulsion (meq hydro-peroxide/kg emulsion).

The pH of the different emulsion samples was measured in triplicate using a pH-meter (*GLP21, Criston Instruments, Barcelona, Spain*) to determine its correlation with the peroxide values.

4.6.1.4 Malondialdehyde measurement by TBARS assay

The oxidation of primary oxidation products results in the formation of secondary oxidation products, such as malondialdehyde (MDA) that can be measured by the TBARS assay [255]. The MDA reacts with TBA and gives a pink/red product (**Figure 48**) measured at 531 nm [256].

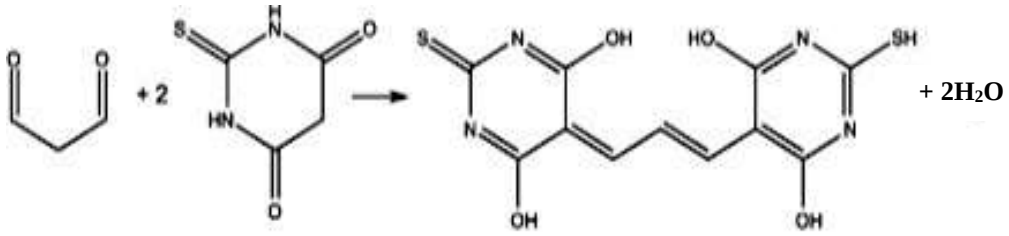

Figure 48. Reaction of MDA with two molecules of TBA [448]

To measure MDA content, 0.3 g of each emulsion sample was mixed with 3 mL of TABRS reactive solution (15% TCA, 2.1% HCl and 0.37% TBA) in assay tubes and sonicated in ultrasound water bath for 10 min. After cooling the tubes, the absorbance of each supernatant was measured [227]. Results are expressed as milligram of MDA per kilogram of emulsion (mg MDA/kg emulsion).

4.6.2 Evaluation of the antioxidant effect of the powdered samples on meat products

4.6.2.1 Formulation, processing and storage conditions of minced beef meat

Three different pieces of ground meat, taken from the round part of three different beeves, were purchased freshly in the same day from a local butcher. Each piece was mixed with salt (1.5%, *w/w*) and divided into different parts, and then each part was mixed, to homogeneity, with different compounds to obtain at the end different meat samples as mentioned in **Table 5**. The synthetic preservative and *Capsicum baccatum* (*C. baccatum*) fruits were used as positive controls.

Table 5. Raw beef patty samples

Samples	Abbreviations	Composition
Control	CTR	Patties without antioxidant
Synthetic preservative	BHT	Patties formulated with BHT [a]
A. vulneraria leaves	AVL	Patties formulated with *A. vulneraria* leaves [b]
A. vulneraria flowers	AVF	Patties formulated with *A. vulneraria* flowers [c]
A. indica leaves	A.I	Patties formulated with *A. indica* leaves [d]
C. baccatum	C.B	Patties formulated with *C. baccatum* fruits [e]
A. indica and C. baccatum	A.I + C.B	Patties formulated with *A. indica* leaves and *C. baccatum* fruits [f]

[a] BHT added to beef patties at 0.5% and 0.7% (*w/w*), [b] powdered *A. vulneraria* leaves added to beef patties at 0.5% (*w/w*), [c] powdered *A. vulneraria* flowers added to beef patties at 0.5% (*w/w*), [d] powdered *A. indica* leaves added to beef patties at 0.7% (*w/w*), [e] powdered *C. baccatum* fruits added to beef patties at 0.7% (*w/w*), [f] powdered *A. indica* leaves and powdered *C. baccatum* fruits added to beef patties at 0.7% (*w/w*).

After 3 min of kneading, each blend was flattened and formed into patties of 3 to 4 g of weight, 4 cm of diameter and 0.5 cm in thickness, using a round cutter. Then, beef patty samples were placed in plastic trays, covered with a food film and finally stored in the refrigerator at 4 ± 1°C for 11 days.

4.6.2.2 TBARS values and pH measurement

The determination of TBARS in minced beef meat was assessed according to the method described by *Azman* et al [257]. Briefly, in a glass test tube, 1 g of each raw beef patty sample was homogenized with 0.5 mL of EDTA (4 mg/mL) and 5 mL of TBARS reagent for 1 min using an Ultra Turrax (*T25 BASIC, IKA, Staufen im Breisgau, Germany*). Then, the samples were filtered through Whatman filter paper no. 1 in glass test tube and kept in boiling water bath (100°C) for 5 min. After cooling, the absorbance of each sample was measured at 531 nm. TBARS results are expressed as milligram of MDA per kilogram of meat sample (mg MDA/kg meat sample).

The pH measurement of the minced beef meat was determined using a pH meter with a meat specific electrode (*Orion 3-Star, Thermo Fisher Scientific, Waltham, MA, USA*) by introducing the electrode directly into the beef patty. The pH reading was taken three times in three different zones from the same beef patty sample.

4.6.2.3 Antioxidant capacity measurement

The Antioxidant Capacity (AOC) of the powdered *A. vulneraria* leaves and flowers in minced beef meat was determined by the hydrophilic and lipophilic FRAP assays, while the AOC of the powdered *A. indica* leaves was determined by the hydrophilic and lipophilic DPPH assays. The preparation of the samples was conducted as described by Gallego et al, [111] using hydrophilic (Milli-Q water) and lipophilic (Ac, EtOH, Milli-Q water; 5:4:1 *v/v/v*) solvents, used for the extraction of hydrophilic and lipophilic antioxidants, respectively. The different extracts were used to perform FRAP and DPPH assays as described previously. The results are expressed as micromole of Trolox Equivalent per milliliter of sample (µmol TE/mL sample).

4.6.2.4 Colour change measurement

The colour of the meat and meat products is a determining factor in the purchase decision of the consumer. In fact, meat colour is more or less responsive to the consumer expectations by its intensity, homogeneity and perfection. Moreover, the red colour is instinctively associated with the notion of meat and meat products freshness [258].

The effect of the powdered samples on the colour stability of minced beef meat was evaluated according to the method described by Gallego et al [111] using a reflectance colorimeter (*Konica Minolta Sensing, CM-3500d, INC., Milton Keynes, UK*) and it was expressed against the scale of L* (lightness), a* (redness) and b* (yellowness) in the CIELab colour space system. Three measurements from three different locations on the beef patties surface were taken during the days of analysis. Fat zones in beef patties were avoided in order to obtain correct measurements.

4.6.2.5 Metmyoglobin measurement

The colour that meat takes is partly determined by the degree of oxidation of the myoglobin (Mb), which is a protein containing heme iron that gives meat its red colour. The fresher the meat is, the more it contains Mb and the brighter the red colour remains. However, when the Mb oxidizes, it loses an electron and turns into metmyoglobin (MetMb) that gives the meat a brown pigment [259] (**Figure 49**).

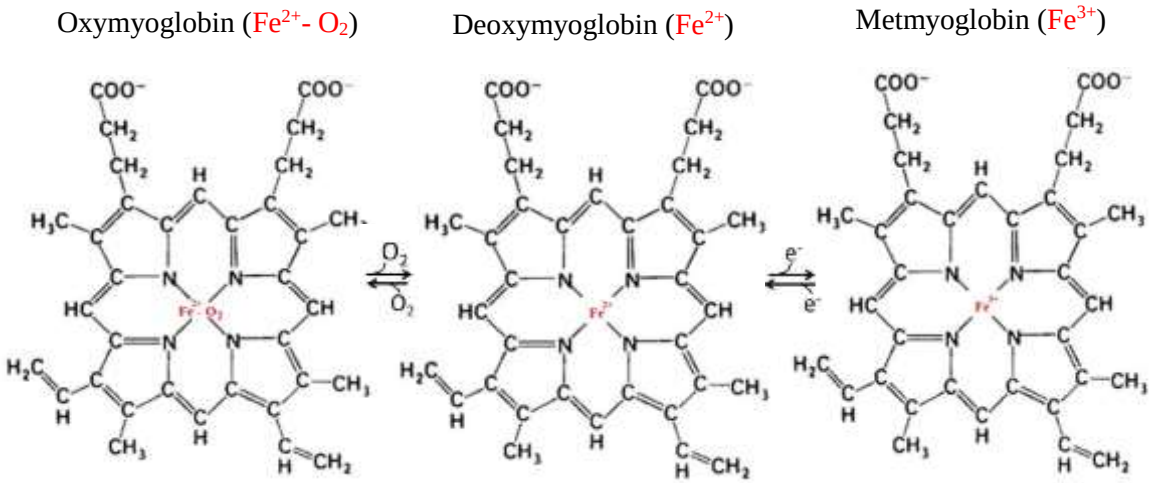

Figure 49. Oxidation of oxymyoglobin to metmyoglobin in fresh meat system [449]

To determine MetMb content, 5 g of each minced beef sample were homogenized with 25 mL of phosphate buffer 0.04 M (pH = 6.8) for 10 sec using the Ultra Turrax. The homogenized mixture was stored for 1h at 4°C and centrifuged (*2-16PK Sigma Laborzentrifugen, Germany*) at 1500×g for 20 min at 4°C. The absorbance of the supernatant was read at 572, 565, 545 and 525 nm [111]. The percentage of MetMb was determined using Krzywicki formula cited bellow:

$$MetMb\ (\%) = [2.514\ (A_{572}/A_{525}) + 0.777\ (A_{565}/A_{525}) + 0.8\ (A_{545}/A_{525}) + 1.098] \times 100$$

Where A_{572} represents the absorbance at 572 nm, A_{525} represents the absorbance at 525 nm, A_{565} represents the absorbance at 565 nm and A_{545} represents the absorbance at 545 nm.

4.6.2.6 Hexanal content (Headspace Gas Chromatography-Mass Spectroscopy)

When the lipid fraction of meat oxidizes, a large number of volatile compounds, such as hexanal, occur and spoil the meat flavour [260].

To determine hexanal content, 1.5 mL of Milli-Q water were added to 500 mg of each beef patty sample in a headspace vial. Then, each vial was sealed air-tight with a PTFE septum. The calibration curve was prepared with hexanal at different concentrations ranging between 0.005 and 0.250 ppm. To determine hexanal content, the different samples were incubated at 80°C for 30 min. The analysis was performed using a Headspace Gas Chromatography coupled to Mass Spectroscopy (HS-GC-MS) by injecting 1 mL of vapour phase through a syringe kept at 85°C [261].

The equipment used consisted of a Trace gas chromatograph with a Head Space Tri-plus auto-sampler coupled to a DSQII mass spectrometer with TRB-624 column (60 m, 0.32 mm, 1.8 mm) and 1.8 mL/min helium flow. The injector temperature was 220°C with split mode injection (split flow 20 mL/min). The temperature program was set at 60°C and held for 2 min, and then raised to 220°C at the rate of 8°C/min (5 min). The interface temperature was 260°C and ionization source temperature was 230°C. Results are expressed in milligram of hexanal per gram of meat sample (mg hexanal/g meat sample).

4.6.2.7 Microbial analysis

Meat products are sensitive to contamination caused by different factors that support the growth of microorganisms involved in spoilage of their flavour [262].

The antimicrobial evaluation in raw beef patty samples was determined according to the method described by Lan et al [263] slightly modified. In stomacher bags (*Stomacher Lab System, Barcelona, Spain*), 90 mL of Ringer solution were added to 10 g of the different beef patty samples of the day 0 (initial day of the experiment), day 5 (the middle day of the experiment) and day 11 (last day of the experiment), and then homogenized using a stomacher (*Stomacher 80 Lab Blender, Galileo Equipos, Madrid, Spain*) for 2 to 3 min. A serial dilution was prepared, and 100 μL of each dilution were plotted on a standard count agar plate, then the plates were incubated at 37°C and the microbial colonies were counted after 48 h. The procedure was done near the flame in a vertical laminar flow apparatus, which was pre-sterilized by ultraviolet irradiation. The results are reported as log10 of Colony Forming Units per gram of meat sample (log10 CFU/g meat).

4.6.2.8 Sensory analysis

A non-trained panel composed of students between 18 and 22 years old were selected from the ETSEIB. At the initial day (day 0), the different beef patty samples were cooked in a hamburger grill (*Tristar, GR-2843, Barcelona, Spain*) at full power for 3 min and presented directly to the panelists. The sensory analysis was assessed by two different tests; including the discriminative triangle test and the traditional sensory test. In both tests, water, apples and biscuits were provided for cleaning the palate after tasting each sample.

The sensory characteristics of *A. vulneraria* cooked beef patty samples were evaluated by the discriminative triangle test. Each sample was coded with a randomly selected three-digit

number. In the triangle test, each panelist chose the odd beef patty sample (two similar samples and a different one) [264].

The sensory characteristics of *A. indica* cooked beef samples were determined using the traditional sensory test, by which the beef samples were assigned an overall quality grade using a hedonic scale ranging from 0 (dislike extremely) to 9 (like extremely) [265] and based on the global appearance, colour, aroma, palatability, initial hot spicily, permanence hot spicily, acidity and acceptability. The purchase intention was evaluated by a different scale, in which 0 means "certainly would not buy the product" and 9 means "certainly would buy the product".

4.7 Antibacterial activity of extracts against bacterial strains

The antibacterial activity of the different extracts against the bacterial strains tested was determined following the disc diffusion method and the minimum inhibitory concentration assay.

4.7.1 Bacterial strains tested

Six different bacterial strains (**Table 6**), causing infective and toxic food poisoning, were provided by the "Departament de Biologia, Sanitat i Medi Ambient" of the Universitat de Barcelona to be tested.

Table 6. Bacterial strains used to test the antimicrobial activity

	Organisms	Abbreviations	Strains
(Gram$^+$)	*Staphylococcus aureus*	*S. aureus*	ATCC 25923
	Bacillus cereus	*B. cereus*	ATCC 11778
	Listeria monocytogenes	*L. monocytogenes*	ATCC 15313
	Micrococcus luteus	*M. luteus*	ATCC 4698
(Gram$^-$)	*Escherichia coli*	*E. coli*	ATCC 25922
	Salmonella paratyphi	*S. paratyphi*	ATCC 9150

4.7.2 Inhibitory zone assay (disc diffusion method)

Briefly, 15 mL of Mueller Hinton agar was inoculated with 200 µL of bacterial suspension then kept in culture dishes until solidification. Oxford discs were placed in the inoculated plates and impregnated with each extract (100 µg/mL) and then the plates were incubated at 30°C. The penicillin (100 µg/mL) was used as a positive control, while the sterile EtOH was used as a negative control [266]. After 24 h of incubation, the inhibition zones were measured using a caliper as judged by the naked eye with the plate held about 30 cm of the

eyes. The plates were read from the back against a dark background. The diameter of the zone inhibition measured in millimeter (mm) correlates to the sensitivity of the strain to the extract.

4.7.3 Minimum inhibitory concentration assay (broth dilution method)

The Minimum Inhibitory Concentration (MIC) of the extracts was determined using the broth dilution method reported by Manandhar et al [267] with some modifications. Two-fold serial dilutions of the antibiotic (penicillin) and the different extracts were prepared, then 0.2 mL of the different bacterial suspensions were added to each test tube except the negative control (CTR^-) and tubes were incubated for 24 h at 37°C. The MIC was calculated following the formula described below:

$$MIC\ (mg/mL) = \frac{Lc\ + Hc}{2}$$

Where *Lc* represents the sample's lowest concentration inhibiting the growth of microbial strains and *Hc* represents the sample's highest concentration allowing the growth of microbial strains.

4.8 Viability-reducing activity of extracts against human cancer cell lines

4.8.1 Extracts preparation

Freeze-dried *A. vulneraria* and *A. indica* samples were dissolved in PBS (5 mg/mL) and then filtered through 0.22 µm diameter sterile filters (*Teknokroma Analítica S.A. Barcelona, Spain*).

4.8.2 Cancer cell lines tested

The viability-reducing activity of the extracts was determined against three different human cancer cell lines obtained from ATCC (ATCC nos.: CCL-2, HB-8065 and HTB-22, respectively); including MCF-7 (derived from breast cancer), HeLa (derived from cervical cancer), HepG2 (derived from a hepatocellular carcinoma) cell lines, respectively.

The HeLa, HepG2 and MCF-7 cancer cell lines were cultured at 37 ˚C and 5% CO_2 using DMEM supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin and 10% (*v/v*) of heat inactivated fetal bovine serum [268].

4.8.3 Determination of viability-reducing activity by the MTT assay

The cancer cell viability-reducing activity of *A. vulneraria* and *A. indica* extracts was determined using the colorimetric MTT assay according to the method described by Kchaou et

al [269] slightly modified. In a sterile 24-well micro-plate, the cancer cells (4.7×10^4 cells/mL) were seeded in a cell growth medium for 72 h. Then, the extracts were added at different concentrations in each well and the cells were cultured for 48 h. After the treatment, the cell growth medium was removed from the plate wells, and 120 µL of MTT reagent (2.5 mg/mL) and 420 µL of sodium succinate (6 g/mL) were added to 1 mL of a fresh culture medium in each well. After 3 h of incubation at 37°C and 5% CO_2, the medium was removed and formazan was re-suspended in a DMSO containing 0.57% acetic acid and 10% SDS. Negative controls consisted of non-treated cells and cells treated with solvent (PBS). The absorbance of the different samples was measured at 570 nm in a UV spectrophotometer (*Dinko, UV2310, Barcelona, Spain*) and the viability-reducing activity is expressed in percentage (%).

4.9 Data analysis

Statistical analyses were conducted by the Minitab statistical software (*Version 18, München, Germany*). In all the analysis, each sample was analyzed in triplicate (n = 3) and reported as means ± standard deviation (SD). Tukey's test was applied to determine significant differences among formulations ($p < 0.05$).

5 Results Analysis

Part 1

Chemical composition of *Anthyllis vulneraria* and *Azadirachta indica* extracts

In this part the chemical composition of the leaf and flower extracts of *Anthyllis vulneraria* and leaf extract of *Azadirachta indica* was determined initially by spectrophotometric methods, then the main phenolic compounds were identified and quantified by High-performance liquid chromatography coupled to mass spectrometry (HPLC-MS).

5 Results analysis

5.1 Part 1. Chemical composition of *A. vulneraria* and *A. indica* extracts

5.1.1 Determination of the extraction yield of extracts

The extraction solvent and method efficiency to extract phenolic compounds from *A. vulneraria* leaf and flower extracts was determined and the results are represented in **Table 7**.

Table 7. Extraction yield of *A. vulneraria* leaf and flower extracts

Extract	Extraction yield (%)					
	Maceration Extraction			Ultrasound Assisted Extraction		
	EtOH	75% EtOH	50% EtOH	EtOH	75% EtOH	50% EtOH
Leaf	8.61 ^{b}	14.21 ^{b}	22.72 ^{b}	12.72 ^{b}	34.41 ^{b}	58.15 ^{b}
Flower	16.48 ^{a}	27.12 ^{a}	46.14 ^{a}	24.98 ^{a}	49.26 ^{a}	66.19 ^{a}

Results represent the mean of three replicates (n = 3) and are expressed as mean value ± SD. Different lowercase letters indicate significant differences between samples at $p < 0.05$.

Extraction yield values were significantly different ($p< 0.05$) depending on the extraction method and the solvent concentration used for the extraction. From the perspective of the extraction method, the highest extraction yields were found in the leaf and flower samples extracted using ultrasound assisted extraction method, which were 1.5-, 2.4- and 2.5-folds higher in leaf extract and 1.5, 1.8- and 1.4- in flower extract compared with leaf and flower samples extracted using cold maceration method when extracted with absolute EtOH, 75% and 50%-aqueous EtOH, respectively.

The results depicted in **Table 7** also revealed a significant influence of the extraction power of the solvent on the yield. Hydro-ethanolic solvent was the most suitable for better extraction of phytochemical components and flower sample presented the highest extraction yield. The leaves and flowers extracted in 50%-aqueous EtOH showed better extraction yield estimated at 58.15% and 66.19% when extracted by ultrasound assisted extraction method and 22.72% and 46.14% when extracted by cold maceration method, respectively, followed by the leaves and flowers extracted with 75%-aqueous EtOH. Extracts prepared with absolute EtOH presented the lowest extraction yield values.

The extraction yield was also determined in *A. indica* leaf extract and the results obtained are in **Table 8**.

Table 8. Extraction yield of *A. indica* leaf extract

Leaf extracts	Extraction yield (%)
EtOH	29.74 [b]
80% EtOH	61.47 [a]

Results represent the mean of three replicates (n = 3) and are expressed as mean value ± SD. Different lowercase letters indicate significant differences between samples at $p < 0.05$.

The results obtained showed that the best extraction yield of *A. indica* leaf extract was observed with the 80%-aqueous EtOH solvent, where the extraction yield was 61.47% compared to leaves extracted with absolute EtOH, which extracted less than the half as much.

The choice of the extraction method and the solvent is a very important step to isolate and recover the maximum of phytochemical compounds from plant materials [270]. The cold maceration (conventional extraction) and the ultrasound assisted extraction (alternative extraction) are considered to be effective methods of phenolic compounds extraction [271]. In terms of extraction efficiency, ultrasound assisted extraction method allowed better extraction and therefore higher extraction yield to be obtained in a very short extraction time compared to the extraction by cold maceration method. Similar results, showing a significant variation in extraction yield values depending on the extraction technique used, were reported by several studies. In line with our findings, Oroian et al [272] compared cold maceration and ultrasound assisted extraction methods in order to achieve a high extraction of phenolic compounds from Propolis using 70%-aqueous EtOH and found that ultrasound assisted extraction method allowed to obtain an extraction yield higher than cold maceration method. Moreover, Medina-Torres et al [273] also showed that, compared to conventional methods, ultrasound assisted extraction is an efficient technique for extracting phenolic compounds while using non-toxic organic solvents and reducing energy consumption and process time. Momchev et al [274] compared as well cold maceration and ultrasound assisted extraction methods to extract phenolic acids from the aerial part of *Echinacea purpurea* and reported that ultrasound assisted extraction method allowed to obtained better extraction yield of phenolic acids in shorter extraction time than cold maceration method.

Similarly, the addition of MilliQ-water to EtOH remarkably increased the values of extraction yield, which reveals the effectiveness of mixed solvents in improving the extraction of particular phenolic compounds. This may be explained by the fact that the solubility of phenolic compounds depends on the polarity and the properties of the extraction solvent [343, 344]. Some phenolic compounds are better extracted in inorganic solvents like water than in organic solvents

such as alcohols. Other polyphenols are most soluble in organic solvents less polar than water [345, 278]. Thus, the mixing of water with an organic solvent increased the extraction efficiency of some target compounds soluble in both solvents. Similar result was found by Dhanani et al [279] showing that *Withania somnifera* samples extracted by hydro-ethanolic solvent enable better extraction of phenolic compounds than samples extracted with absolute EtOH.

5.1.2 Spectrophotometric determination of phenolic compounds of extracts

The different phenolic compounds were determined in the 50%-aqueous EtOH extracts of *A. vulneraria* leaves and flowers extracted by the ultrasound assisted extraction and the results obtained are depicted in **Table 9.**

Table 9. Phenolic compounds contents of *A. vulneraria* leaf and flower extracts

50%-EtOH extract	TPC (mg GAE/g DW)	TFC (mg QE/g DW)	TCTC (mg CE/g DW)
Leaf	93.27 ± 0.21 ^{b}	37.88 ± 0.18 ^{b}	22.72 ± 0.11 ^{b}
Flower	147.77 ± 0.11 ^{a}	48.83 ± 0.37 ^{a}	24.24 ± 0.14 ^{a}

Results represent the mean of three replicates (n = 3) and are expressed as mean value ± SD. Different lowercase letters indicate significant differences between samples at $p < 0.05$.

The phenolic compound contents found in *A. vulneraria* leaf and flower extracts varied between samples with significant differences at $p < 0.05$. The highest phenolic compound contents were found in the flower extract and were estimated at 147.77 mg GAE/g DW for TPC, 48.83 mg QE/g DW for TFC and 24.24 mg CE/g DW for TCTC, while the leaf extract contained TPC and TFC 1.6- and 1.3-folds, respectively lower and a TCTC broadly similar to that determined in the flower extract.

Several previous investigations focused on the quantification of phenolic compounds in *A. vulneraria* extracts and showed contents lower than those determined in the present study. For instance, Csepregi et al [280] showed that the leaf extract of *A. vulneraria* from Transylvania in Romania contained only 6.8 mg GAE/ g DW of TPC, 4.5 mg GAE/ g DW of TFC and 1.7 mg GAE/ g DW of TCTC. Additionally, Tusevski et al [281] studied the chemical composition of *A. vulneraria* collected from Macedonia and found that the aerial part contains low TPC (12.02 mg GAE/g DW) and TFC (2.22 mg CE/g DW). Godevac et al [282] also demonstrated that the extract of *A. vulneraria* flower collected from Planine in Eastern Serbia contained less TPC estimated at 79.34 mg GAE/g DW. Contrariwise, Moradi et al [283] found higher TPC and TFC in the leaf extracts of *A. vulneraria* from Iran estimated at 146.75 mg GAE/g DW and 49.7 mg RUT/g DW, respectively. Different other medicinal plants from the fabaceae family, such as *Albizia julibrissin*,

Desmodium caudatum, *Lespedeza bicolor*, *Pueraria lobata*, *Robinia pseudoacacia*, *Sophora flavescens*, *S. japonica* and *Erythrina stricta Roxb* contained high contents of phenolic compounds [284]. Furthermore, Skowyra and Gallego [285] reported high TPC and TFC in Tara (*Caesalpinia spinosa*) extract estimated at 460.2 mg GAE/g DW and 2.93 mg CE/g DW, respectively. Similarly, Gallego et al [286] determined the phenolic compounds contents in Tara and Mauritius (*Caesalpinia decapetala*) and found that Tara leaf extract contained higher TPC compared with the results of the present study, while Mauritius contained lower TPC estimated at 63.8 mg GAE/g DW. Similarly, Al-Dabbagh et al [287] quantified TPC and TFC in the leaf extract of *Trigonella foenum-graecum* and *Cassia acutifolia* extracted with 70%-aqueous EtOH and found TPC lower than *A. vulneraria* extracts estimated at 9.7 and 10.5 mg GAE/ g DW, respectively and TFC almost similar to the TFC found in our study estimated at 14.6 and 20.8 mg QE/g, respectively. Chen et al [288] also quantified TPC and TFC in the flowers of *Pueraria lobate* and *Sophora japonica* and reported TPC of 23.99 and 81.17 mg GAE/ g DW, respectively and TFC of 14.59 and 42.88 mg RE/g DW, respectively.

Table 10 shows the phenolic compound contents of *A. indica* leaves extracted with absolute and 80%-aqueous EtOH.

Table 10. Phenolic compound contents of *A. indica* leaf extract

Leaf extracts	TPC (mg GAE/g DW)	TFC (mg QE/g DW)	TCTC (mg CE/g DW)
EtOH	21.62 ± 0.03 [b]	9.25 ± 0.12 [b]	7.48 ± 0.13 [b]
80% EtOH	47.47 ± 0.03 [a]	15.37 ± 0.12 [a]	11.23 ± 0.13 [a]

Results represent the mean of three replicates (n = 3) and are expressed as mean value ± SD. Different lowercase letters indicate significant differences between samples at $p < 0.05$.

The different phenolic compound contents varied in *A. indica* leaf extracts and it was found that 80%-aqueous EtOH extracted more phenolics. Total polyphenol content was 47.47 mg GAE/g DW and total flavonoid content value was higher than the total condensed tannin content.

A wide range of phenolic contents in *A. indica* extracts was observed in several previous studies. For example, Ghimeray et al [289] reported similar TPC values to those reported in the present study in *A. indica* leaves from Nepal. However, Datta et al [290] reported lower TPC in *A. indica* leaf extracts than those obtained in our study, whereas Khamis Al-Jadidi and Hossain [291] observed higher total phenolic compounds content in the leaf extracts of *A. indica* collected from

Oman and reported values of TPC ranging from 22.27 and 107.29 mg GAE/g DW and values of TFC varying between 63 and 525.5 mg QE/g DW.

5.1.3 Qualitative and quantitative analysis of phenolic compounds by HPLC-MS

The phenolic profile of *A. vulneraria* leaf and flower extracts and *A. indica* leaf extract was determined. The data collected from the HPLC-MS were analysed, and the different compounds detected in the different extracts were identified by comparing their Retention Time (RT) and parent ion fragmentation pattern (m/z) with standards of each compound detected, and by the comparing the fragmentation pattern with the database of chemical molecules and polyphenols (PubChem and Phenol Explorer).

HPLC-MS data and the identification of the different compounds present in *A. vulneraria* leaf and flower extracts using positive and negative ionization modes are summarized in **Table 11** and **Table 12**, respectively.

Table 11. HPLC-MS data and identification of the different compounds present in *A. vulneraria* leaf extract

Peak no.	Tentative Identification	Chemical Formula	RT (min)	Molecular Weight	Ionization Mode	Fragment Ions (m/z)		Polyphenol Class	Content *	Ref.
						Theoretical (m/z)	Observed (m/z)			
L1	Pyrogallol	$C_6H_6O_3$	6.96	126.1100	[M + H]+	127.0390	127.0391	Other polyphenols	136.94	[292]
L2	Chlorogenic acid	$C_{16}H_{18}O_9$	7.29	354.3087	[M − H]−	353.0878	353.0880	Phenolic acids	1504.62	Std/ [293]
L3	4-Hydroxybenzaldehyde	$C_7H_6O_2$	7.43	122.1213	[M − H]−	121.0295	121.0306	Other polyphenols	1265.74	[292]
L4	Caffeic acid	$C_9H_8O_4$	8.34	180.1574	[M − H]−	179.0345	179.0350	Phenolic acids	5868.65	Std/ [293]
L5	3,4-Dihydroxyphenylglycol	$C_8H_{10}O_4$	11.05	170.1626	[M − H]−	169.0506	169.0503	Other polyphenols	57.96	[292]
L6	2,3-Dihydroxybenzoic acid	$C_7H_6O_4$	11.22	154.1201	[M − H]−	153.0193	153.0203	Phenolic acids	89.73	[292]
L7	p-coumaric acid	$C_9H_8O_3$	11.46	164.1580	[M − H]−	163.0401	163.0393	Phenolic acids	106.52	Std/ [293]
L8	p-Anisaldehyde	$C_8H_8O_2$	11.74	136.1479	[M − H]−	135.0451	135.0456	Other polyphenols	2547.22	[292]
L9	Ferulic acid	$C_{10}H_{10}O_4$	13.09	194.1840	[M − H]−	193.0506	193.0502	Phenolic acids	7985.14	Std/ [293]
L10	Sinapinic acid	$C_{11}H_{12}O_5$	13.38	224.2100	[M − H]−	223.0612	223.0603	Phenolic acids	3477.81	Std/ [294]
L11	4-Hydroxy-2-phenylacetic acid	$C_8H_8O_3$	14.72	152.1473	[M − H]−	151.0400	151.0408	Phenolic acids	1069.51	[292]
L12	Cinnamic acid	$C_9H_8O_2$	17.34	148.1586	[M + H]+	149.0597	149.0587	Phenolic acids	7842.12	[292]
L13	2-Hydroxybenzoic acid	$C_7H_6O_3$	19.02	138.1207	[M − H]−	137.0244	137.0249	Phenolic acids	142.44	[292]
L14	Coumarin	$C_9H_6O_2$	20.44	146.1427	[M + H]+	147.0441	147.0429	Other polyphenols	651.23	[295]
L15	p-Coumaroyl tartaric acid	$C_{13}H_{12}O_8$	21.88	296.2296	[M − H]−	295.0459	295.0468	Phenolic acids	49.58	[294]
L16	4-Hydroxyphenylpropionic acid	$C_9H_{10}O_3$	35.16	166.1739	[M + H]+	165.0557	165.0569	Phenolic acids	133.42	[292]
L17	Kaempferol-3-O-rutinoside	$C_{15}H_{10}O_6$	37.52	286.2363	[M − H]−	285.0404	285.0404	Flavonoids	6314.85	[295]

* Expressed as microgram per g of Dry Weigh (µg/ g DW)
"Std" indicates identification of components confirmed by a standard
"L" refers to leaf

Table 12. HPLC-MS data and identification of the different compound present in *A. vulneraria* flower extract

Peak no.	Tentative Identification	Chemical Formula	RT (min)	Molecular Weight	Ionization Mode	Fragment Ions (m/z)		Polyphenols Class	Content *	Ref.
						Theoretical (m/z)	Observed (m/z)			
F1	Chlorogenic acid	$C_{16}H_{18}O_9$	7.29	354.3087	[M − H]−	353.0878	353.0880	Phenolic acids	318.55	Std/ [293]
F2	Caffeic acid	$C_9H_8O_4$	8.34	180.1574	[M − H]−	179.0345	179.0350	Phenolic acids	5568.44	Std/ [293]
F3	Syringic acid	$C_9H_{10}O_5$	8.68	198.1727	[M − H]−	198.05282	197.0453	Phenolic acids	102.209	Std/ [296]
F4	(-)-Epicatechin	$C_{15}H_{14}O_6$	10.15	290.2681	[M − H]−	289.0717	289.0717	Flavonoids	1178.12	Std/ [294]
F5	p-Coumaric acid	$C_9H_8O_3$	11.46	164.1580	[M − H]−	163.0401	163.0393	Phenolic acids	5326.11	Std/ [293]
F6	Ferulic acid	$C_{10}H_{10}O_4$	13.09	194.1840	[M − H]−	193.0506	193.0502	Phenolic acids	418.63	Std/ [293]
F7	Sinapinic acid	$C_{11}H_{12}O_5$	13.38	224.2100	[M − H]−	223.0612	223.0603	Phenolic acids	7699.18	Std/ [294]
F8	2,3-Dihydroxybenzoic acid	$C_7H_6O_4$	13.45	154.1201	[M − H]−	153.0193	153.0203	Phenolic acids	533.36	[294]
F9	Quercetin	$C_{15}H_{10}O_7$	14.50	302.2357	[M + H]+	303.0500	303.0487	Flavonoids	101.41	[294]
F10	Myricetin	$C_{15}H_{10}O_8$	18.27	318.2351	[M + H]+	319.0449	319.0427	Flavonoids	4382.05	Std/ [292]
F11	Quercetin	$C_{15}H_{10}O_7$	21.32	302.2357	[M − H]−	301.0354	301.0375	Flavonoids	1154.11	Std/ [292]
F12	p-Coumaroyl tartaric acid	$C_{13}H_{12}O_8$	22.17	296.2296	[M − H]−	295.0459	295.0468	Phenolic acids	41.77	[297]
F13	Delphinidin 3-O sambubioside	$C_{26}H_{29}O_{16}$	25.71	597.4989	[M − H]−	596.1383	596.1363	Flavonoids	64.24	[292]
F14	Rutin	$C_{27}H_{30}O_{16}$	27.14	610.5175	[M − H]−	609.1525	609.1509	Flavonoids	4982.14	Std/ [298]
F15	Kaempferol-3-O-rutinoside	$C_{15}H_{10}O_6$	37.52	286.2363	[M − H]−	285.0404	285.0404	Flavonoids	6314.85	[295]

* Expressed as microgram per g of Dry Weigh (µg/ g DW)
"Std" indicates identification of components confirmed by a standard
"F" refers to flower

Seventeen phenolic compounds were identified in *A. vulneraria* leaf extract, mainly phenolic acids (e.g., chlorogenic acid (L2); caffeic acid (L4); 2,3-dihydroxybenzoic acid (L6); p-coumaric acid (L7); ferulic acid (L9); sinapinic acid (L10); 4-hydroxy-2-phenylacetic acid (L11); cinnamic acid (L12); 2-hydroxybenzoic acid (L13); p-coumaroyl tartaric acid (L15) and 4-hydroxyphenylpropionic acid (L16)), flavonoids (e.g., kaempferol-3-O-rutinoside (L17)) and other polyphenols (e.g., pyrogallol (L1); 4-hydroxybenzaldehyde (L3); 3,4-dihydroxyphenylglycol (L5); p-anisaldehyde (L8) and coumarin (L14)). Five compounds (e.g., compounds L2, L4, L7, L9 and L10) were identified by commercial standards and data from literature and the rest of compounds were characterized by comparing their chromatographic behavior and m/z with literature-based data. A total of nine phenolic acids was identified in *A. vulneraria* leaves extract using a negative mode of ionization. The compound no. L2 with $[M-H]^-$ at m/z 353.0878 and 353.0880 was characterized as chlorogenic acid. The compound no. L4 with $[M-H]^-$ at m/z 179.0345 and 179.0350 was identified as a caffeic acid. The compound no. L6 with $[M-H]^-$ at m/z 153.0193 and 153.0203 was represented as 2, 3-dihydroxybenzoic acid. The compound no. L7 with $[M-H]^-$ at m/z 163.0401 and 163.0393 was identified as p-coumaric acid. The compound no. L9 with $[M-H]^-$ at m/z 193.0506 and 193.0502 was represented as ferulic acid. The compound no. L10 with $[M-H]^-$ at m/z 223.0612 and 223.06603 was identified as sinapinic acid. The compound no. L11 with $[M-H]^-$ at m/z 151.0400 and 151.0408 was characterized as 4-hydroxy-2-phenylacetic acid and the compound no. L13 with $[M-H]^-$ at m/z 137.0244 and 137.0249 was described as 2-hydroxybenzoic acid. Only two phenolic acids were identified using a positive mode of ionization; including the compound no. L12 with $[M+H]^+$ at m/z 149.0597 and 149.0587 identified as cinnamic acid and the compound no. L16 with $[M+H]^+$ at m/z 165.0557 and 165.0509 assigned as 4-hydroxyphenylpropionic acid. The compound no. L17 was detected in *A. vulneraria* leaf extract as a flavonoid with $[M-H]^-$ at m/z 285.0404 and 285.0404 and identified as kaempferol-3-O-rutinoside. The rest of compounds were represented as other polyphenols with $[M+H]^+$, including compound no. L1 identified as pyrogallol at m/z 127.0390 and 127.0391 and compound no. L14 assigned as coumarin at m/z 147.0441 and 147.0429, in addition to other polyphenols with $[M-H]^-$, mainly compound no. L3 identified as 4-hydroxybenzaldehyde at m/z 121.0295 and 121.0306, compound no. L5 characterized as 3, 4-dihydroxyphenylglycol at m/z 169.0506 and 169.0503 and compound no. L8 assigned as p-anisaldehyde at m/z 135.0451 and 135.0456.

In *A. vulneraria* flower extract, ten compounds (e.g., compounds F1, F2, F3, F4, F5, F6, F7, F10, F11 and F12) were identified by commercial standards and data from literature and

the rest of compounds were characterized by comparing their chromatographic behavior and m/z with literature-based data.

All phenolic acids were identified in *A. vulneraria* flower extract using a negative mode of ionization, mainly compound no. F1 with $[M-H]^-$ at m/z 353.0878 and 353.0880 identified as chlorogenic acid, compound no. F2 with $[M-H]^-$ at m/z 179.0345 and 179.0350 assigned as caffeic acid, compound no. F3 with $[M-H]^-$ at m/z 198.05282 and 197.0453 represented as syringic acid, compound no. F5 with $[M-H]^-$ at m/z 163.0401 and 163.0393 characterized as p-coumaric acid, compound no. F6 with $[M-H]^-$ at m/z 163.0401 and 163.0393 assigned as ferulic acid, compound no. F7 with $[M-H]^-$ at m/z 223.0612 and 223.0603 identified as sinapinic acid, compound no. F8 with $[M-H]^-$ at m/z 153.0193 and 153.0203 represented as 2,3-dihydroxybenzoic acid and compound no. F12 with $[M-H]^-$ at m/z 295.0459 and 295.0468 determined as p-coumaroyl tartaric acid. From a total of seven flavonoids, only two were identified using a positive mode of ionization; including compound no. F9, which yielded a $[M+H]^+$ at m/z equal to 303.05 and 303.0487 and was identified as quercetin and compound no. F10, which gave a $[M+H]^+$ at m/z of 319.0449 and 319.0427 and was assigned as myricetin, while five flavonoids were identified using a negative mode of ionization; mainly compound no. F4 with $[M-H]^-$ at m/z 289.0717 and 289.0717 represented as (-)-epicatechin, compound no. F11 with $[M-H]^-$ at m/z 301.0354 and 301.0375 characterized as quercetin, compound no. F13 with $[M-H]^-$ at m/z 596.1383 and 596.1363 assigned as delphinidin 3-O sambubioside, compound no. F14 with $[M-H]^-$ at m/z 609.1525 and 609.1509 represented as rutin and compound no. F15 with $[M-H]^-$ at m/z 285.0404 and 285.0404 identified as kaempferol-3-O-rutinoside.

The contents of the different phenolic compounds identified were determined and the results showed that *A. vulneraria* leaf extract was considered to be the most abounded source of phenolic acids, of which the contents ranged between 49.58 (p-coumaroyl tartaric acid) and 7985.14 (ferulic acid) µg/g DW, whereas the contents of flavonoids in *A. vulneraria* flower extract were higher and varied between 64.24 (delphinidin 3-O sambubioside) and 6314.85 (kaempferol-3-O-rutinoside) µg/g DW.

HPLC-MS data and the identification of the different compounds present in *A. indica* leaf extract using positive and negative ionization modes are summarized in **Table 13**.

Table 13. HPLC-MS data and identification of the different compounds present in *A. indica* leaves extract.

Peak N°	Tentative Identification	Chemical Formula	RT (min)	Molecular Weight	Ionization Mode	Fragment Ion (m/z)		Polyphenol Class	Content *	Ref.
						Theoretical (m/z)	Observed (m/z)			
1	Chlorogenic acid	$C_{16}H_{18}O_9$	7.29	354.3087	[M − H]−	353.0878	353.0880	Phenolic acids	1504.62	Std/ [302]
2	Caffeic acid	$C_9H_8O_4$	8.34	180.1574	[M − H]−	179.0345	179.0350	Phenolic acids	5568.44	Std/ [293]
3	Syringic acid	$C_9H_{10}O_5$	8.68	198.1727	[M − H]−	198.05282	197.0453	Phenolic acids	102.209	Std/ [296]
4	(-)-Epicatechin	$C_{15}H_{14}O_6$	10.15	290.2681	[M − H]−	289.0717	289.0717	Flavonoids	1178.12	Std/ [294]
5	p-Coumaric acid	$C_9H_8O_3$	11.46	164.1580	[M − H]−	163.0401	163.0393	Phenolic acids	5326.11	Std/ [293]
6	Ferulic acid	$C_{10}H_{10}O_4$	13.09	194.1840	[M − H]−	193.0506	193.0502	Phenolic acids	418.63	Std/ [293]
7	Sinapinic acid	$C_{11}H_{12}O_5$	13.38	224.2100	[M − H]−	223.0612	223.0603	Phenolic acids	7699.18	Std/ [294]
8	Myricetin	$C_{15}H_{10}O_8$	18.27	318.2351	[M + H]+	319.0449	319.0427	Flavonoids	4382.05	Std/ [292]
9	Quercetin	$C_{15}H_{10}O_7$	21.32	302.2357	[M − H]−	301.0354	301.0375	Flavonoids	1154.11	Std/ [292]
10	Luteolin C-hexoside I	$C_{21}H_{20}O_{11}$	22.47	448.3769	[M − H]−	448.1006	447.0935	Flavonoids	3618	[296]
11	Cyanidin 3-O-galactoside	$C_{21}H_{21}O_{11}$	23.11	449.3848	[M − H]−	448.1011	448.0982	Flavonoids	2845	[292]

* Expressed as microgram per g of Dry Weigh (µg/ g DW)
"Std" indicates identification of components confirmed by a standard

In *A. indica* leaf extract, eleven phenolic compounds were identified; mainly phenolic acids (e.g., chlorogenic acid (1); caffeic acid (2); syringic acid (3); p-coumaric acid (5); ferulic acid (6) and sinapinic acid (7)) and flavonoids (e.g., (-)-Epicatechin (4); myricetin (8); quercetin (9); luteolin C-hexoside I (10) and cyanidin 3-O-galactoside (11)). The first nine compounds were identified by commercial standards and data from literature and the rest of the compounds were characterized by comparing their chromatographic behavior and m/z with literature-based data.

The six phenolic acids detected in *A. indica* leaves extract were identified using a negative mode of ionization. The compound no. 1 with $[M-H]^-$ at m/z 353.0878 and 353.0880 was characterized as chlorogenic acid. The compound no. 2 with $[M-H]^-$ at m/z 179.0345 and 179.0350 was identified as a caffeic acid. The compound no. 3 with $[M-H]^-$ at m/z 198.05282 and 197.0453 was represented as syringic acid. The compound no. 5 with $[M-H]^-$ at m/z 163.0401 and 163.0393 was identified as p-coumaric acid. The compound no. 6 with $[M-H]^-$ at m/z 193.0506 and 193.0502 was represented as ferulic acid and the compound no. 7 with $[M-H]^-$ at m/z 223.0612 and 223.0603 was identified as sinapinic acid.

Only the compound no. 8 was detected in *A. indica* leaf extract as a flavonoid using a positive mode of ionization with $[M+H]^+$ at m/z 319.0449 and 319.0427 and identified as myricetin. The rest of the compounds were represented as flavonoids using a negative mode of ionization, mainly compound N° 4 identified as (-)-epicatechin at m/z 289.0717 and 289.0717, compound no. 9 assigned as quercetin at m/z 301.0354 and 301.0375, compound no. 10 identified as luteolin C-hexoside I at m/z 448.1006 and 447.0935 and compound no. 11 characterized as cyanidin 3-O-galactoside at m/z 448.1011 and 448.0982.

The contents of the different phenolic compounds identified were determined and the results showed that *A. indica* leaf extract was rich in phenolic acids with contents ranging between 102.209 (syringic acid) and 7699.18 (sinapinic acid) µg/g DW, whereas the contents of flavonoids were lower and estimated between 64.23 (delphinidin 3-O sambubios Cyanidin 3-O-galactoside) and 4382.05 (myricetin) µg/g DW.

Part 2

Radical scavenging activity of *Anthyllis vulneraria* and *Azadirachta indica* extracts

In this part the radical scavenging activity of the leaf and flower extracts of *Anthyllis vulneraria* and leaf extract of *Azadirachta indica* was determined initially *in vitro* by different analytical methods, then by the spin trap method and Electron Paramagnetic Resonance (EPR) spectroscopy to measure, for the first time, the radical scavenging activity of *Anthyllis vulneraria* and *Azadirachta indica* extracts against the methoxy radical, using ferulic acid as antioxidant pattern.

5.2 Part 2. Radical scavenging activity of *A. vulneraria* and *A. indica* extracts

5.2.1 Anti-radical activity of extracts

The evaluation of the anti-radical activity of *A. vulneraria* leaf and flower extracts was carried out *in vitro* by different analytical methods and the results are in **Table 14**.

Table 14. Radical scavenging activity of *A. vulneraria* leaf and flower extracts

	Leaf extract	Flower extract
FRAP (mM TE/g DW)	1.90 ± 0.01 [b]	3.30 ± 0.01 [a]
TEAC (mM TE/g DW)	0.82 ± 0.002 [b]	1.42 ± 0.01 [a]
ORAC (mM TE/g DW)	0.98 ± 0.003 [b]	1.64 ± 0.07 [a]
TAC (mg AAE/g DW)	219.7 ± 0.02 [b]	293.3 ± 0.01 [a]
DPPH (mM TE/g DW)	0.39 ± 1.08 [b]	0.64 ± 0.96 [a]
HPS (%)	27 ± 0.02 [b]	38 ± 0.08 [a]

Results represent the mean of three replicates (n = 3) and are expressed as mean value ± SD. Different lowercase letters indicate significant differences between samples at $p < 0.05$.

Radical scavenging activity was significantly higher in *A. vulneraria* flower extract than in leaf extract ($p < 0.05$). On the one hand, the flower extract showed a significantly higher TAC ($p < 0.05$) compared to the leaf extract estimated at 293.3 mg AAE/g DW. Similarly, ORAC assay allowed to find a value of 1.64 mM TE/g DW in the flower extract, while the leaf extract presented only 0.98 mM TE/g DW. On the other hand, FRAP and TEAC values were also higher in the flower extract and were estimated at 3.30 and 1.42 mM TE/g DW, respectively compared to their values determined in the leaf extract, which both were 1.7-folds lower. Furthermore, the ability of the flower extract to scavenge DPPH free radicals was higher compared to the leaf extract and was equal to 0.64 mM TE/g DW. The flower extract of *A. vulneraria* exhibited as well a HPS activity 1.4-fold higher than the leaf extract.

Various assays had been described to estimate overall radical scavenging activity of *A. vulneraria* extracts. For instance, Tusevski et al [281] found that the aerial part of *A. vulneraria* from Macedonia exhibited lower TEAC and DPPH values estimated at 0.113 and 0.068 mM TE/g DW. Moreover, Moradi et al [283] reported as well a potent DPPH activity of *A. vulneraria* leaf extract from Iran. The *A. vulneraria* extracts from Romania also had strong antioxidant activity determined by FRAP and TEAC assays [280] as well as DPPH and ORAC assays [299]. High radical scavenging activity had also been found in different extracts from plants belonging to fabaceae family, such as extracts from the pods of *Caesalpinia cacalaco*

[300], *Acacia pennatula* [301], extracts from the flowers and roots of *Onobrychis armena* [302] and extracts from the seeds of *Trigonella foenum-graecum* [303].

Different spectrometric (FRAP, TEAC, TAC, DPPH and HPS) and fluorometric (ORAC) assays were also used to offer a complete profile of the antiradical activity of *A. indica* leaf extracts. The results obtained are in **Table 15**.

Table 15. Anti-radical activity of *A. indica* leaf extracts

	EtOH	80%-aqueous EtOH
TAC (mg AAE/g DW)	114.85 ± 1.48 ^{b}	231.39 ± 2.04 ^{a}
FRAP (mM TE/g DW)	1.59 ± 0.02 ^{b}	2.30 ± 0.01 ^{a}
TEAC (mM TE/g DW)	1.17 ± 0.01 ^{b}	1.68 ± 0.08 ^{a}
ORAC (mM TE/g DW)	0.87 ± 0.002 ^{b}	1.33 ± 0.07 ^{a}
DPPH (mM TE/g DW)	0.13 ± 0.01 ^{b}	0.37 ± 0.01 ^{a}
HPS (%)	22 ± 0.02 ^{b}	41 ± 0.03 ^{a}

Results represent the mean of three replicates (n=3) and are expressed as mean value ± SD. Different lowercase letters indicate significant differences between samples at $p < 0.05$.

The evaluation of the anti-radical activity of *A. indica* revealed that the leaf extracts have potent antiradical capacity, in particular the 80%-aqueous EtOH extract. The ability of *A. indica* extracts to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), evaluated using the FRAP method, was higher in 80%-aqueous EtOH extract than in EtOH extract, which presented ferric reducing ability of 2.30 and 1.59 mM TE/g DW, respectively. The results obtained by TAC, TEAC and ORAC assays also confirmed the antioxidant potential of *A. indica* leaf extracts. Indeed, the three assays performed showed that 80%-aqueous EtOH extract had a higher capacity to scavenge free radicals with values equal to 231.39 mg AAE/g DW, 1.68 and 1.33 mM TE/g DW, respectively, compared with EtOH extract, which exhibited TAC, TEAC and ORAC activities 2-, 1.4- and 1.5-times lower. Moreover, 80%-aqueous extract of *A. indica* leaves appeared to be a stronger inhibitor of DPPH radicals than EtOH extract with antiradical activity equal to 0.37 mM TE/g DW. Similarly, the ability of 80%-aqueous EtOH extract to scavenge H_2O_2 radicals doubled the scavenging ability of EtOH extract, resulting in percentages of inhibition estimated at 41% and 22%, respectively.

The results of the present study corroborate the work of Fong Lores et al [304] revealing a high antioxidant activity of leaf extract of *A. indica* from Cuba determined by TAC assay and estimated at 215.01 mg AAE/g DW. Indeed, Sithisarn et al [305] showed that EtOH extracts of leaves, flowers and stem bark of *A. indica* exhibited a strong free radical scavenging effect against DPPH radicals with 50% scavenging activity at 26.5, 27.9 and 30.6 µg/mL, respectively, while TAC values of the extracts were found to be 0.959, 0.988 and 1.064 mM TE/g DW, respectively.

5.2.2 Spin trap and Electron Paramagnetic Resonance (EPR) spectroscopy

In addition to the traditional radical scavenging activity assays previously determined, the antiradical activity of *A. vulneraria* leaf and flower extracts was also evaluated against the methoxy ($CH_3O^{\cdot}$) radicals by an EPR spectroscopy assay in the presence of DMPO used as a spin trip and the results obtained were compared to ferulic acid. This method has a higher detection sensitivity comparing to the traditional radical scavenging assays and consists in detecting very unstable transient radicals that react with DMPO to form stable radical adducts [306]. The ferulic acid was used as an antioxidant pattern due to its powerful antioxidant property that makes it more and more demanded in food and cosmetic industries as it protects against harmful free radicals [307].

The Double Integration (DI) values of the spectrum signal in the presence of *A. vulneraria* leaf and flower extracts or ferulic acid gave a measure of the antiradical activity of each sample. The values of the DI at different concentrations of the ferulic acid are shown in **Table 16** and showed that the DI decreased with the increase of the ferulic acid concentration.

Table 16. Methoxy radical scavenging activity of ferulic acid

Ferulic acid (mg/mL)[a]	DI EPR signal [b]
0	36.74
1	32.11
2	28.33
3	25.24
6	19.35
10	14.39
15	10.45
20	9.25

[a] Final concentration values of ferulic acid in 250 µL

[b] Double integration of the EPR signal.

Decrease of the spectrum intensity of the spin adduct of $CH_3O^{\cdot}$ radicals with DMPO depending on the increasing concentration of ferulic acid is presented in **Figure 50**. The best-fitting function is an exponential curve with the equation given inside the graphic and expressed as gram per Litter (g/L).

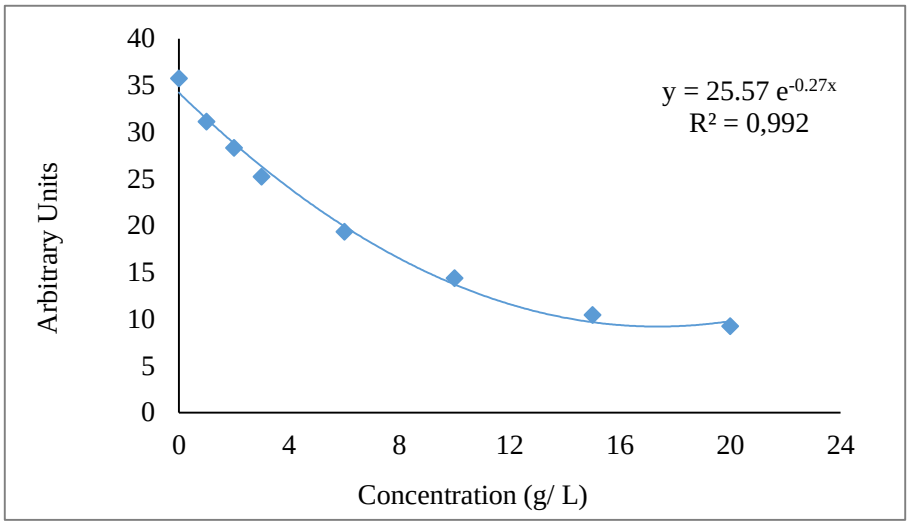

Figure 50. Decrease of the spectrum intensity of the spin adduct of methoxy radicals with the increase of ferulic acid concentration

Figure 51 and **Figure 52** represent the decrease of the spectrum intensity of the spin adduct of the $CH_3O^{\bullet}$ radicals with DMPO depending on the increasing concentration of *A. vulneraria* leaf and flower extracts, respectively.

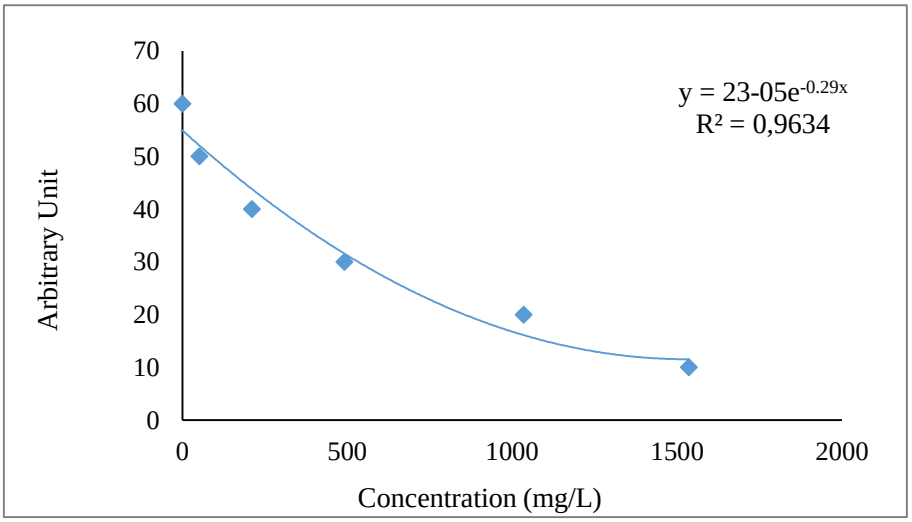

Figure 51. Decrease of the spectrum intensity of the spin adduct of $CH_3O^{\bullet}$ with the increase of *A. vulneraria* leaf concentration

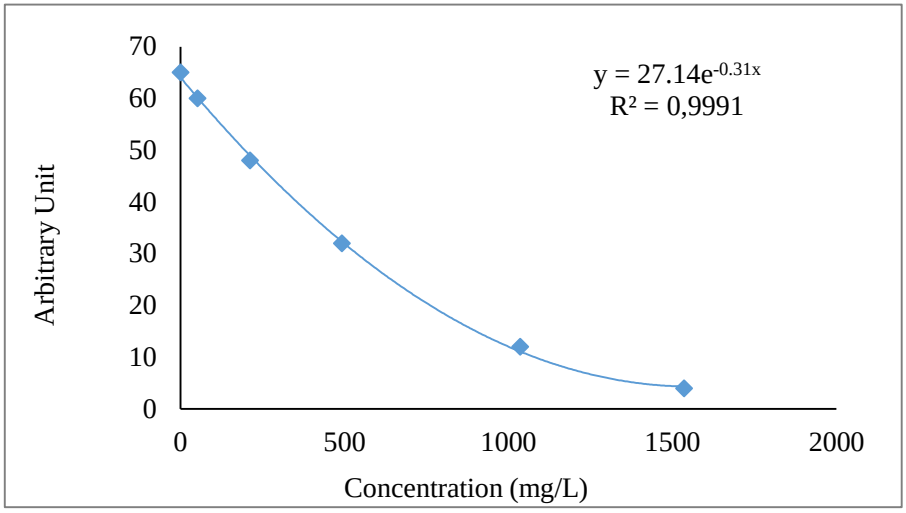

Figure 52. Decrease of the spectrum intensity of the spin adduct of $CH_3O^{\bullet}$ with the increase of *A. vulneraria* flower concentration

The $CH_3O^{\bullet}$ radical was identified by its ability to form a stable adduct with DMPO (DMPO–OCH_3), which can be determined by the DI values of the EPR signal. The best fit with the EPR signal strength was shown as an exponential function (**Figure 51** and **Figure 52**) where the x-axis represents *A. vulneraria* extract concentrations expressed as milligram per Litter (mg/L) and the y-axis represents the arbitrary units obtained from the integrated EPR signal.

Figure 51 and **Figure 52** indicated that the exponential value of the spectrum signal has decreased with the increasing of the leaf (**Figure 51**) and flower (**Figure 52**) extract's concentration. The lower is the arbitrary unit value, the higher is the antiradical activity of the extract. At the concentration 1g/L, the flower extract showed better antiradical activity with an arbitrary unit estimated at 12, followed by the leaf extract and ferulic acid with arbitrary unit equal to 20 and 32.1, respectively. The obtained results are consistent with the results obtained by TEAC, ORAC, DPPH and FRAP assays and confirmed that the scavenging activity of *A. vulneraria* extracts could be measured by decreasing the intensity of the spectral bands of the DMPO-OCH_3 adduct in the EPR spectrum with the number of antioxidant and that flower extract had better antiradical activity than leaf extract.

There are no previous publications about the use of EPR technique to determine the antiradical activity of *A. vulneraria* extracts. However, several other studies measured the radical scavenging activity of different medicinal plants extract's from fabaceae and other families and found similar results. For example, Azman et al [308] measured the radical scavenging activity of white tea extract against $CH_3O^{\bullet}$ radicals using ferulic acid as antioxidant

pattern and found that the white tea compounds had a better antiradical activity against $CH_3O^{\bullet}$ radical than ferulic acid. Another study conducted by Azman et al [238] showed that *Convolvulus arvensis* ethanol extract exhibited potent scavenging activity measured with EPR method against $CH_3O^{\bullet}$ radical.

The anti-free radical potential *A. indica* leaf extract was also determined by EPR spectroscopy assay to investigate scavenging of $CH_3O^{\bullet}$ generated in the Fenton reaction by *A. indica* leaf extract. The free radical scavenging activity of *A. indica* leaf extract was studied by competitive reaction in the presence of DMPO. The DMPO–OCH_3 formed was detected by the DI value of the EPR signal, from which the values of arbitrary units are obtained (**Figure 53**) and where x-axis represents *A. indica* leaf extract concentration expressed as mg/L and y-axis represents the arbitrary units obtained from the integrated EPR signal.

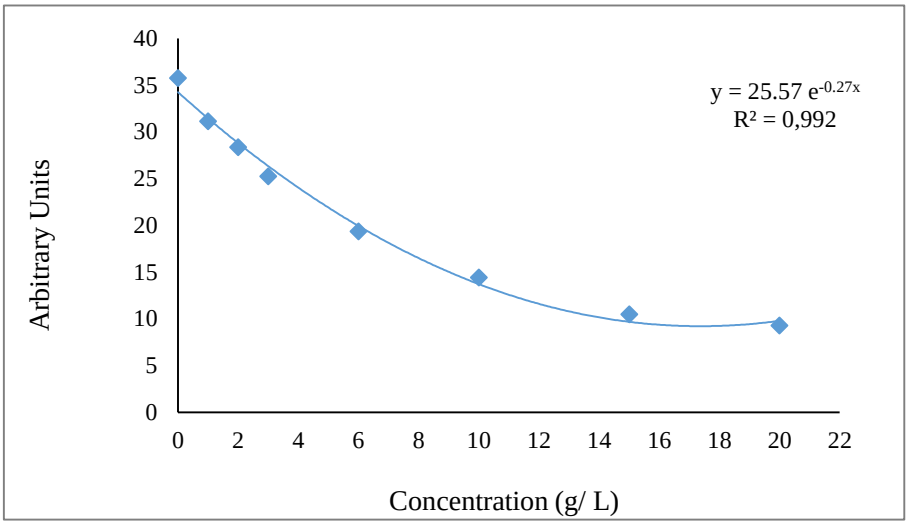

Figure 53. Decrease of the spectrum intensity of the spin adduct of $CH_3O^{\bullet}$ with the increase of *A. indica* leaf extract concentration.

The results obtained showed that *A. indica* leaf extract had a potent antiradical activity against $CH_3O^{\bullet}$ radical. The leaf extract at different concentrations competed with the spin trap in the scavenging of $CH_3O^{\bullet}$ radicals. Thus, the antioxidant effect of the leaf extract reduced the concentration of radical adducts and, accordingly, reduced the intensity of EPR signal. At the concentration 1g/L, *A. indica* leaf extract showed better antiradical activity with a lower signal intensity value estimated at 15 than ferulic acid, which showed a value 2-folds higher. These results are consistent with the results of the antiradical activity determined by the traditional spectrometric and fluorometric assays.

These results are consistent with the antiradical activity determined by traditional spectrometric and fluorometric assays. The same trend was observed in a study by Tippel et al [309], where an antioxidant effect towards hydrophilic and hydrophobic radicals was observed by an EPR test with *Quillaja saponin* extract.

The potent anti-radical activity of *A. vulneraria* leaf and flower extracts and *A. indica* leaf extract depends on the concentration of phenolic compounds. In fact, the values obtained varied proportionally, the higher the polyphenol content, the stronger the antioxidant activity. This agrees with the result found by Kaviarasan et al [310] and M. Gallego et al [311] who demonstrated that phenolic compounds are indeed responsible for the antioxidant activity of plant extracts [312] due to their ability to scavenge free radicals, by quenching ROS, inhibiting oxidative enzymes and chelating transition metals [46,47].

Part 3

Evaluation of the preservative effect of *Anthyllis vulneraria* and *Azadirachta indica* samples against oxidation of *Model Food System*

In this part the antioxidant effect of *Anthyllis vulneraria* leaves and flowers and *Azadirachta indica* leaves was studied, for the first time, in two different *Model Food System*; including Oil-in-Water (O/W) emulsion and raw beef meat. The antioxidant effect of extracts on the oxidative stability of O/W emulsion was studied by following the formation of primary and secondary oxidation products, while the antioxidant effect of powdered samples, directly incorporated into raw beef patties, was evaluated during refrigerated storage by studying the chemical, microbial and sensory analyses of beef meat. The combined effect of the powdered *A. indica* leaves with red pepper (*C. baccatum*) was studied as well to determine their synergistic antioxidant effect on the oxidative stability of meat and the extension of its shelf life.

5.3 Part 3. Evaluation of the preservative effect of *A. vulneraria* and *A. indica* samples against oxidation of *Model Food System*

5.3.1 Antioxidant effect of extracts on the oxidative stability of O/W emulsion

Given their high content of phenolic compounds and strong antioxidant activity, *A. vulneraria* 50%-aqueous EtOH and *A. indica* 80%-aqueous EtOH extracts were added to an O/W emulsion to study their preservative effect. *A.vulneraria* 50%-aqueous EtOH extract was incorporated into an O/W emulsion at the concentrations 0.25% (*v/v*), while *A. indica* 80%-aqueous EtOH extract was added at 0.25% and 0.5% (*v/v*). The oxidative stability of the different emulsion samples was investigated during 30 days of storage at 30 ± 1°C and compared to samples containing gallic acid added to the emulsion at the same concentrations.

5.3.1.1 Peroxide values

The evolution of the primary oxidation in O/W emulsion samples conserved with *A. vulneraria* leaf and flower extracts at 0.25% (*v/v*) and gallic acid at 0.25% (*v/v*) was evaluated by the determination of the peroxide value during storage at 30 ± 1°C and the results obtained are shown in **Figure 54**.

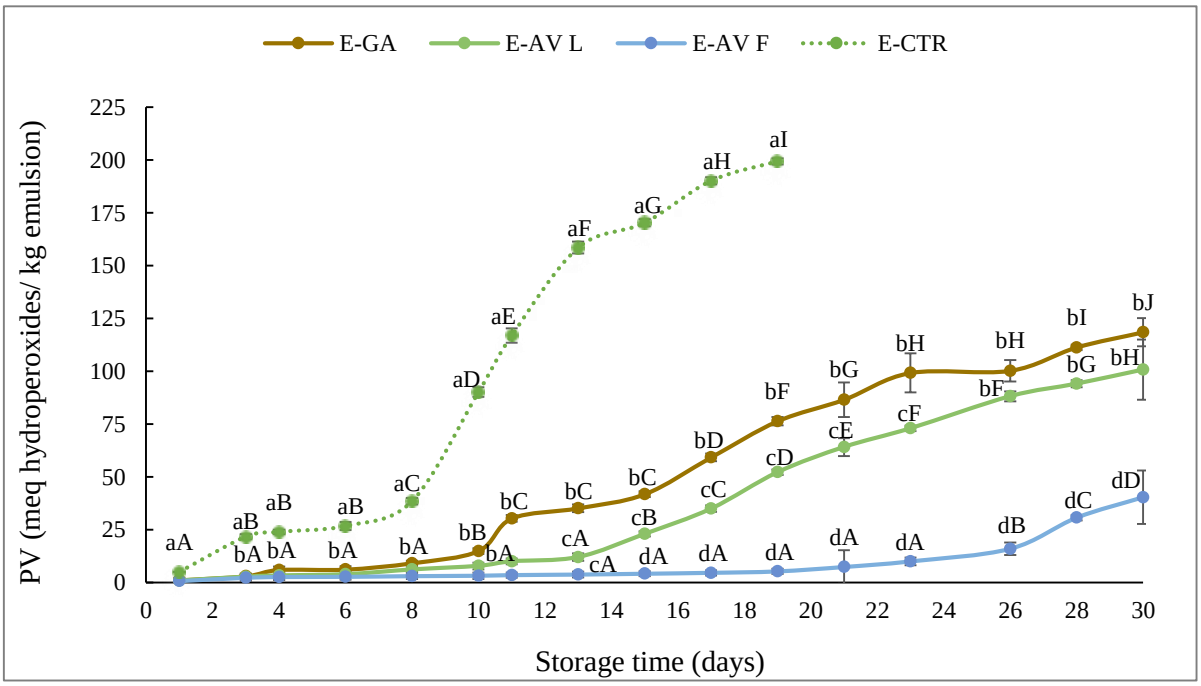

Figure 54. Peroxide values (PV) of emulsions conserved with *A. vulneraria* leaf and flower extracts during storage. Error bars represent the standard deviation (n=3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same emulsion sample at $p < 0.05$

Taking into consideration the maximum PV value estimated by the *Codex Alimentarius* at 10 meq hydroperoxides/ kg of oil [242], the formation of hydroperoxides was progressively faster in E-CTR emulsion sample, which showed a speedy increase in PV from the second day of storage and exceeded 10 meq hydroperoxides/kg of oil as described in **Table 17**, and reached a maximum of hydroperoxides content estimated at 199.37 meq hydroperoxides/kg emulsions after only 19 days of storage.

Table 17. Number of days to reached 10 meq hydroperoxides/kg of oil

Emulsion samples	Number of days to reach 10 meq hydroperoxides/ kg of oil
E-CTR	< 2
E- GA (0.25%, *v/v*)	< 8
E- AVL (0.25%, *v/v*)	< 11
E- AVF (0.25%, *v/v*)	< 23

Compared with E-CTR emulsion sample, E-GA and E-AVL emulsion samples (0.25%, *v/v*) kept their stability against lipid oxidation until 8 days (9.14 meq hydroperoxides/ kg of oil) and 11 days (9.99 meq hydroperoxides/ kg of oil), respectively and then exhibited a faster oxidation, unlike E-AVF emulsion sample (0.25%, *v/v*), which showed stable PV until 23 days (9.84 meq hydroperoxides/kg of oil). At the last day of storage, E-GA and E-AVL emulsion samples (0.25%, *v/v*) were completely oxidised and showed the highest PV equal to 188.47 and 100.78 meq hydroperoxides/ kg emulsion, respectively, in contrast to E-AVF emulsion sample (0.25%, *v/v*), which showed the best preservative effect against the lipid oxidation with the lowest PV estimated at 40.35 meq hydroperoxides/ kg emulsion.

The study of *A. vulneraria* effect against lipid oxidation in O/W emulsion has not been sufficiently investigated. However, several recent studies about the protective role of plants from the fabaceae and different other families against food emulsion deterioration were carried out. The leaf and flower extracts of *A. vulneraria* studied in the present study were more effective in retarding the lipid oxidation compared with several previous researches about the antioxidant effect of herbs on protecting emulsions from lipid oxidation. For instance, Martillanes et al [315] found that the rice bran (*Oryza sativa* L.) extracts incorporated in the mayonnaise-type emulsion at 0.5% and 2% (*v/v*) presented significantly lower PV ($p<0.05$) compared to CTR and emulsion sample containing BHT after 7 days of storage at 20°C. Similarly, Gallego et al [286] reported that *C. spinosa* extract added at 0.5% (*v/v*) and *C. decapetala* extract added at 0.5% (*v/v*) were successful to delay the emulsion oxidation during

the storage period and reached the limit allowed for products containing edible fats after 18 days of storage with PV equal to 6.7 and 18.2 meq hydroperoxides/kg emulsion, respectively. Moreover, Skowyra et al [227] investigated the antioxidant effect of *Artemisia annua* extract to maintain O/W emulsion stability and reported that *Artemisia annua* extract was also effective in slowing down the formation of hydroperoxides and reached 10 meq hydroperoxides /kg oil after 28 days. Additionally, *Gentiana lutea* extract added at 0.5% (*v/v*) was able to delay the lipid oxidation throughout storage time and reached the limit for fat products after 10 days of storage [316].

Changes in PV during storage at 30 ± 1 °C in O/W emulsions incorporated with 0.25% and 0.5% (*v/v*) of *A. indica* leaf extract are shown in **Figure 55**.

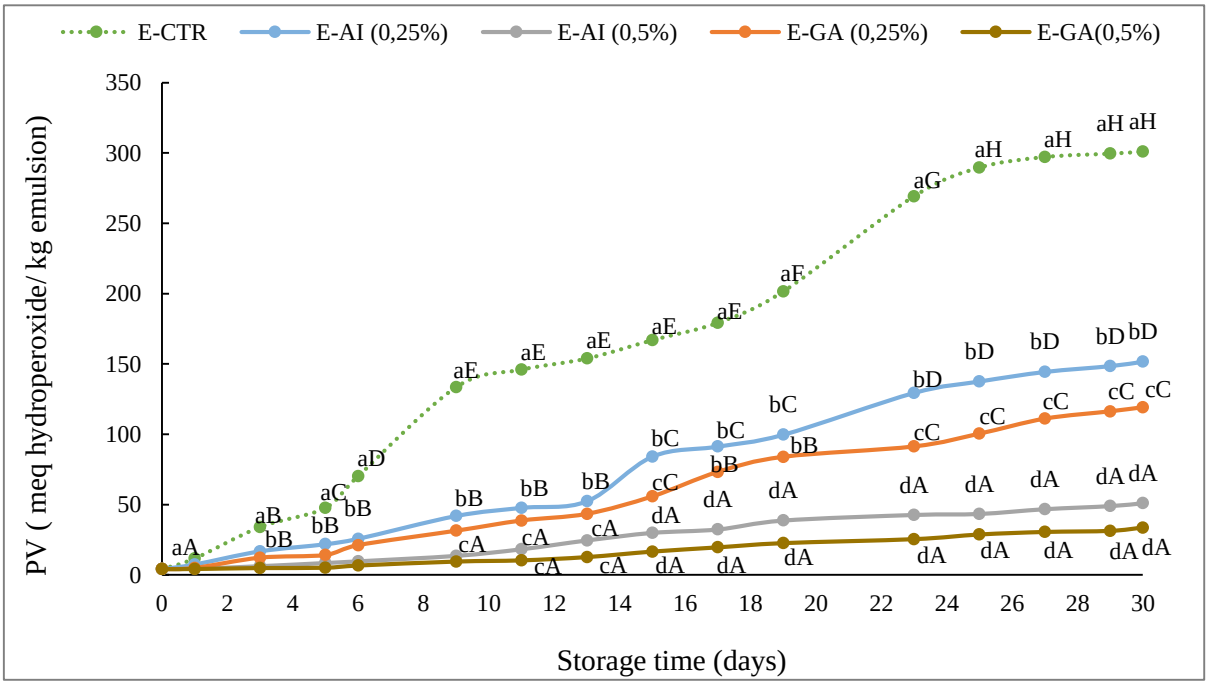

Figure 55. Peroxide values (PV) of emulsions conserved with *A. indica* leaf extract during storage. Error bars represent the standard deviation (n=3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same emulsion sample at $p < 0.05$

The formation of hydroperoxides was significantly faster in the E-CTR emulsion sample, which showed an increase of the primary oxidation values from the first day of storage. The E-GA (0.25%, *v/v*) and E-AI (0.25%, *v/v*) emulsion samples remained stable against lipid oxidation for 6 days and then exhibited a higher degree of oxidation, whereas E-GA (0.5%, *v/v*) and E-AI (0.5 %, *v/v*) emulsion samples were stable against lipid oxidation and exceeded 10 meq hydroperoxides/ kg of oil only after 9 days of storage. At the end of the storage period (30

days), the E-GA (0.25%, *v/v*) and E-AI (0.25%, *v/v*) emulsion samples deteriorated with maximum PV of 119.14 and 151.63 meq hydroperoxides/kg emulsion, respectively. The E-GA (0.5%, *v/v*) and E-AI (0.5%, *v/v*) emulsion samples presented the best protective effect against the formation of the primary oxidation products with values estimated at 33.58 and 51.02 meq hydroperoxides/kg emulsion, respectively at the end of the storage period.

A. indica leaf extract used in the present study was more effective at retarding the lipid oxidation compared with other plant extracts previously investigated to determine their antioxidant effect on protecting emulsions from lipid oxidation. For example, Fruehwirth et al [317] studied the influence of rosemary and green tea extracts on the oxidative stability of margarine heated at 80°C for 1 h and found that the extracts preserved the margarine from oxidation. Skowyra et al [318] found that *P. frutescens* extracts had good antioxidant properties in 10% sunflower O/W emulsions during storage at 32°C and were as effective as the synthetic preservative butylated hydroxyanisole (BHA).

5.3.1.2 pH changes

Since several antioxidants are less effective in acidic medium [319], the pH of emulsion samples conserved with *A. vulneraria* leaf and flower extracts at 0.25% (*v/v*) and *A. indica* leaf extract at 0.25% and 0.5% (*v/v*) was also measured as a potential indicator of O/W emulsion oxidation.

The pH changes of the emulsion samples conserved with *A. vulneraria* leaf and flower extracts were determined during storage period and the results obtained are represented in the **Figure 56**.

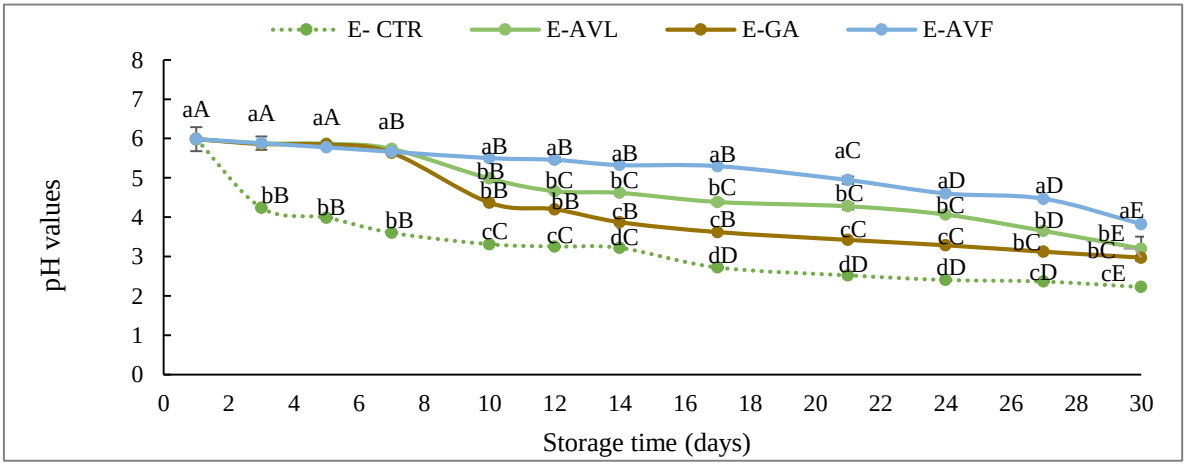

Figure 56. pH values of emulsions conserved with *A. vulneraria* leaf and flower extracts during storage. Error bars represent the standard deviation (n=3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same emulsion sample at $p < 0.05$

The initial average pH value of all the emulsion samples was pH= 5.98. E-CTR emulsion sample showed the first decrease of pH values from pH= 5.98 to pH= 3.89 after only 3 days of storage. E-GA and E-AVL emulsion samples (0.25%, *v/v*) behaved similarly and their pH remained stable at around pH = 5.51 for 8 days, then decreased to pH values of 2.97 and 3.20, respectively at the end of the storage time, whereas E-AVF emulsion sample (0.25%, *v/v*) was the only sample, in which the pH remained stable at pH= 5 for more than 17 days before decreasing to pH = 3.82 at the end of the storage period.

The pH of emulsion samples conserved with *A. indica* leaf extract at 0.25% and 0.5% (*v/v*) was also measured and the results obtained are illustrated in **Figure 57**.

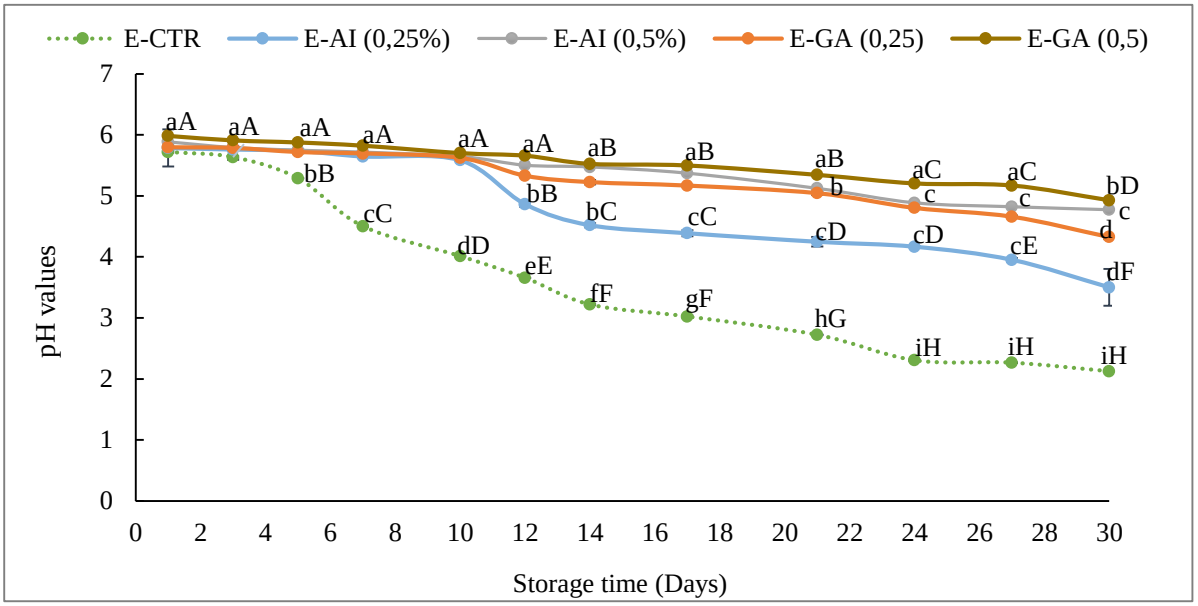

Figure 57. pH values of emulsions conserved with *A. indica* leaf extract during storage. Error bars represent the standard deviation (n=3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same emulsion sample at $p < 0.05$

All the emulsion samples started with an initial average pH value of 5.98. The first decrease of the pH values was observed in E-CTR emulsion sample from 5.98 to 4.50 after only 7 days of storage. E-GA (0.5%, *v/v*) and E-AI (0.5%, *v/v*) were the only emulsion samples that remained stable at around pH= 5 for more than 17 days before decreasing to 4.92 and 4.82, respectively at the end of the storage time. E-GA (0.25%, *v/v*) emulsion sample had a similar behavior as E-AI (0.25%, *v/v*). Their pH remained stable at around pH= 5.6 for 10 days, then decreased to pH values of 4.77 and 3.50, respectively, at the end of the storage time.

From the results obtained, it can be seen that the pH values are proportional to the peroxide values. The higher the peroxide values, the more acidic the pH of the emulsion samples. The results of the present work are in line with the finding of Skowyra et al [318] who studied the effect of *Perilla frutescens* extract on the oxidative stability of 10% O/W emulsion and found that the pH decreased when the PV increased from pH= 5.8 to pH= 4.05. Moreover, Zhou and Elias [320] and Sorensen et al [321] showed that the pH played an important role in controlling the oxidative stability of O/W emulsion. The addition of phenolic compounds to food emulsions showed antioxidant activity at high pH = 5 and 7, respectively.

The change of the pH during lipid oxidation is probably due to the decrease in the effectiveness of the phenolic compounds present in the extracts to prevent the formation of oxidation products, including hydroperoxides and their acidic degradation products [322]. The decrease in pH is often considered a factor favoring oxidation due to the increase of H^+ ions concentration in emulsion samples caused by the decrease in the effectiveness of antioxidant extracts throughout the storage period [323]. Decker et al [324] also reported that the acidic pH can affect the redox state of metals as well as the solubility, stability, and chelation capacity of antioxidants as it can influence the distribution of antioxidants between the aqueous and the lipid phases.

5.3.1.3 Malondialdehyde formation

During the development of oxidation reactions, very unstable primary products appear, which rapidly decompose into secondary products such as the malondialdehyde (MDA). This compound is considered to be one of the end products of the polyunsaturated fatty acids oxidation and responsible of the bad taste, rancid odour and unwanted taste of oxidised fats [319]. In the present study, the evolution of MDA formation in O/W emulsion samples conserved with *A. vulneraria* leaf and flower extracts and *A. indica* leaf extract was monitored by the measurement of TBARS values and the results obtained are shown in **Figure 58** and **Figure 59**, respectively.

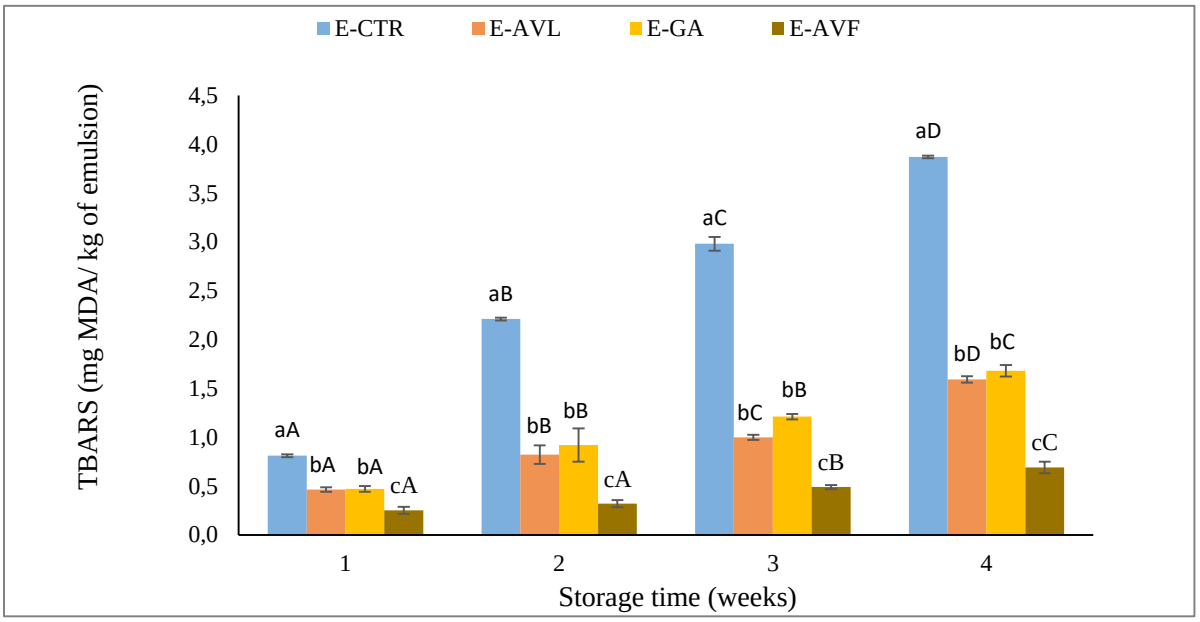

Figure 58. TBARS values of emulsions conserved with *A. vulneraria* leaf and flower extracts during storage. Error bars represent the standard deviation (n=3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same emulsion sample at $p < 0.05$

At every storage week, TBARS values were significantly different between emulsion samples ($p < 0.05$) and increased steadily over storage time. From the first week of storage, TBARS values of E-CTR emulsion sample were higher than the rest of the emulsion samples and increased rapidly from 0.81 mg MDA/kg emulsion to 3.87 mg MDA/kg emulsion at the last week of storage. E-AG and E-AVL emulsion samples added at 0.25% (*v/v*) showed lower TBARS values than E-CTR emulsion sample estimated at 1.68 and 1.59 mg MDA/kg emulsion, respectively at the fourth storage week, while E-AVF sample added at 0.25% (*v/v*) was the most effective against oxidation and presented the lowest TBARS value of 0.69 mg MDA/kg emulsion at the end of the storage process.

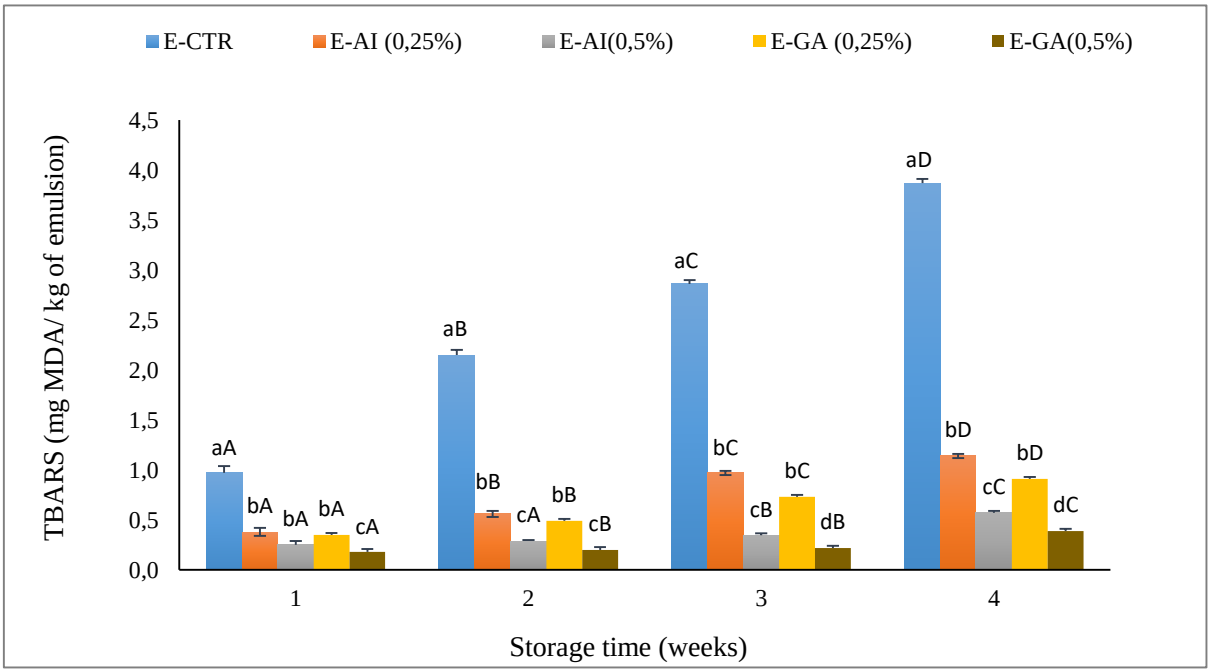

Figure 59. TBARS values of emulsions conserved with *A. indica* leaf extract during storage. Error bars represent the standard deviation (n=3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same emulsion sample at $p < 0.05$

The TBARS values obtained were significantly different between emulsion samples ($p < 0.05$) and increased steadily over storage time. After 4 weeks, TBARS values of E-CTR emulsion sample were higher than the values of the rest of samples and increased rapidly from 0.97 mg MDA/kg emulsion at the first week of storage to 3.87 mg MDA/kg emulsion at the last week. E-AG (0.25%, *v/v*) and E-AI (0.25%, *v/v*) emulsion samples presented lowest TBARS values than E-CTR emulsion sample estimated at 0.91 and 1.14 mg MDA/kg emulsion, respectively, while E-GA (0.5%, *v/v*) and E-AI (0.5%, *v/v*) were the most effective emulsion samples against oxidation and presented the lowest TBARS values estimated at 0.39 and 0.57 mg MDA/kg emulsion, respectively at the last week of the storage period.

Different researches have confirmed the antioxidant power of medicinal plants against the formation of the oxidation by-products in emulsion. Similar finding reported by Altunkaya et al [325] where the highest anti-oxidative capacity and the lowest content of MDA were found in a mayonnaise containing the highest percentage of grape seed extract added to the mayonnaise emulsion at 0.15% (*v/v*). El-Guendouz et al [326] showed as well that the thyme extract prevented the hydroperoxides formation and the accumulation of MDA in emulsions. Poyato et al [327] also found that *Melissa officinalis* dried leaves seemed to be more efficient delaying the formation of MDA in O//W emulsions than the BHA.

5.3.2 Antioxidant effect of powdered samples on the oxidative stability of minced beef meat during refrigerated storage

The effect of the powdered *A. vulneraria* leaves and flowers at 0.5 % (*w/w*) and *A. indica* leaves at 0.7% (*w/w*) on the oxidative stability of the raw beef patties was studied during 11 days of refrigerated storage at 4 ± 1°C. Moreover, the combination of powdered *A. indica* leaves with powdered red pepper (*C. baccatum*) fruits was assessed to study the synergistic effect of the different active components present in the two plant matters. *C. baccatum* was chosen owing to its strong antioxidant [228, 363] and antimicrobial [364, 365] activity demonstrated by different previous researches.

The preservative effect of powdered *A. vulneraria*, *A. indica* and *C. baccatum* samples was evaluated by the determination of the lipid peroxidation and pH evolution, the antioxidant capacity (AOC), colour changes and metmyoglobin formation, hexanal content, as well as antimicrobial and sensory analysis of treated raw beef patties and was compared to raw beef patties containing synthetic preservative (BHT) added at the same concentrations.

5.3.2.1 Lipid oxidation

The evolution of the lipid oxidation of raw beef patties containing powdered *A. vulneraria* leaves and flowers at 0.5% (*w/w*) during 11 days of refrigerated storage (4 ± 1°C) are depicted in **Figure 60**.

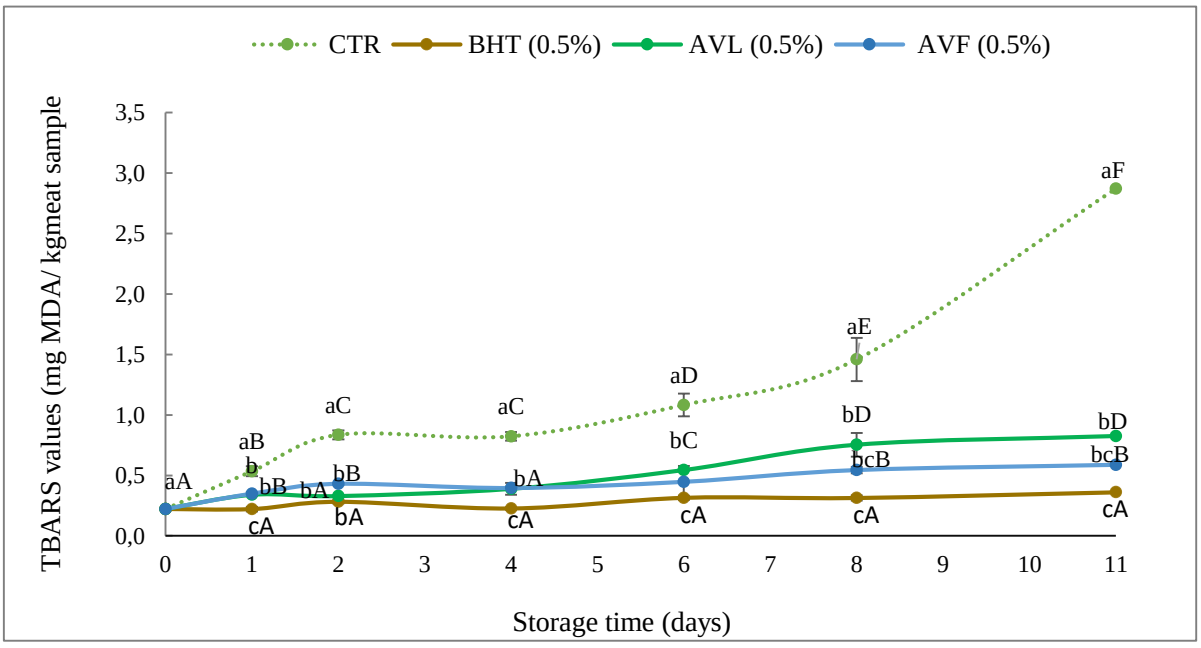

Figure 60. TBARS values of raw beef patties conserved with powdered *A. vulneraria* during refrigerated storage at 4 ± 1°C. Error bars represent the standard deviation (n = 3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same meat sample at $p < 0.05$

TBARS values of the different raw beef patty samples increased continually ($p < 0.05$) despite the low temperature of storage. CTR (without antioxidant) was the first sample, which started to oxidize after only 2 days of storage until the day 8, in which the lipid oxidation had become faster and higher. After 11 days of refrigerated storage, CTR presented the higher TBARS values estimated at 2.87 mg MDA/kg meat compared with the treated samples. Contrariwise, the most effective treatment was the BHT at 0.5% (*w/w*), which showed the lowest TBARS value of 0.36 mg MDA/ kg meat sample at the end of the experiment. AVL (0.5%, *w/w*) and AVF (0.5%, *w/w*) almost behaved in the same way and remained stable until the day 4 of storage, then AVF showed lower TBARS values estimated at 0.45 mg MDA/kg meat sample and reached a final oxidation value at the end of the storage process equal to 0.59 mg MDA/kg meat sample.

The **Figure 61** shows the evolution of the lipid oxidation of raw beef patties containing powdered *A. indica* leaves and powdered *C. baccatum* at 0.7% (*w/w*) during 11 days of refrigerated storage (4 ± 1°C).

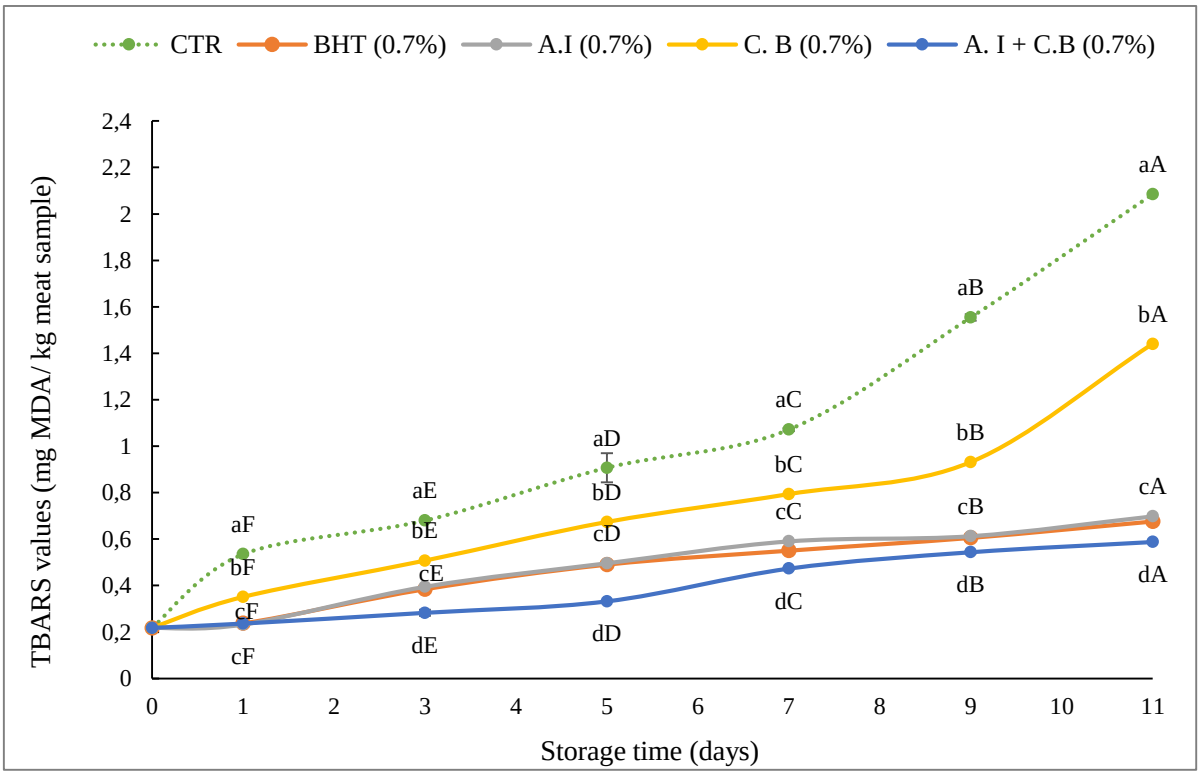

Figure 61. TBARS values of raw beef patties conserved with powdered *A. indica* during refrigerated storage at 4 ± 1°C. Error bars represent the standard deviation (n = 3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same meat sample at $p < 0.05$

The lipid oxidation of raw beef patties significantly increased during refrigerated storage ($p < 0.05$). CTR had the highest TBARS value estimated at 2.08 mg MDA/kg meat compared with A.I + C.B, which presented a synergistic antioxidant effect in raw beef patties and produced a combined inhibitory effect of MDA formation greater than the rest of formulated beef patties and reached a value of 0.59 mg MDA /kg meat by the end of the storage period. TBARS values of A.I (0.7%, *w/w*), BHT (0.7%, *w/w*) and C.B (0.7%, *w/w*) also increased progressively during storage time. A.I (0.7%, *w/w*) showed an effective antioxidant effect against lipid degradation almost similar to BHT (0.7%, *w/w*) and reached 0.68 and 0.70 mg MDA/kg meat, respectively, while C.B (0.7%, *w/w*) presented a higher TBARS value estimated at 1.44 mg MDA/kg meat. TBARS values recorded in A.I and A.I + C.B were considered to be a good sign of their efficiency against lipid oxidation since they did not exceed 1.5 mg MDA/kg meat. This value is considered as an indicator of lipid degradation in meat as reported by Martínez et al [331].

The preservative effect of *A. vulneraria* and *A. indica* on the oxidative stability of raw beef patties during refrigerated storage was investigated for the first time in this study and our findings about the effectiveness of edible plants rich in phenolic compounds in limiting lipid oxidation and extending the shelf-life of meat products are in agreement with other previous studies. For instance, Villasante et al [332] found that 2% (*w/w*) of Pecan shell and Roselle flowers were enough to delay the oxidation of raw beef patties (0.45 and 0.35 mg MDA/kg meat) after 12 days of refrigerated storage compared with the control meat sample (1.31 mg MDA/kg meat). Similarly, the antioxidant effect of roasted coffees added to refrigerated ground pork over 21 days was determined by Hashimoto et al [333] who found TBARS values in treated meat samples lower than those of control. The results obtained in the present work also consistent with those reported by Monika et al [109] and Azman et al [238] who found that raw meat patties formulated with powdered *Caesalpinia spinosa* and *Convolvulus arvensis* were more stable than the control meat sample and meat samples formulated with BHT and retarded lipid oxidation during chilled storage. Additionally, Fan et al [266] investigated the effects of *Portulaca oleracea* L. on lipid oxidation of pork meat during refrigerated storage and obtained results supporting the hypothesis that the addition of natural products enriched in polyphenols extends the shelf life of fresh meat and delays lipid oxidation.

The antioxidant effect of powdered *A. indica* leaves and *C. baccatum* on oxidative stability could possibly be associated with their wealth of phenolic compounds, which present

strong antioxidant activity allowing them to scavenge hydroperoxides, whose decomposition results in secondary oxidation products responsible for the deterioration of meat quality [242].

5.3.2.2 pH changes

The evolution of the pH values of raw beef patties formulated with the powdered *A. vulneraria* leaves and flowers at 0.5% (*w/w*) during the refrigerated storage are in **Table 18**.

Table 18. Changes in pH values of the raw beef patties conserved with powdered *A. vulneraria* leaves and flowers at 0.5% (*w/w*) during storage at 4 ± 1°C

Days	1	2	4	6	8	11
CTR	5.86 ± 0.03 ^{aA}	5.89 ± 0.02 ^{aA}	5.92 ± 0.01 ^{aC}	5.97 ± 0.02 ^{aB}	6.01 ± 0.02 ^{aC}	6.19 ± 0.03 ^{aD}
BHT	5.69 ± 0.01 ^{cA}	5.70 ± 0.11 ^{bA}	5.72 ± 0.02 ^{cB}	5.74 ± 0.01 ^{bB}	5.77 ± 0.01 ^{cC}	5.78 ± 0.02 ^{cC}
AVL	5.76 ± 0.01 ^{bA}	5.78 ± 0.03 ^{abA}	5.82 ± 0.03 ^{bB}	5.87 ± 0.1 ^{abC}	5.93 ± 0.1 ^{abD}	6.01 ± 0.05 ^{bD}
AVF	5.71 ± 0.01 ^{cA}	5.74 ± 0.02 ^{bB}	5.79 ± 0.05 ^{bcC}	5.81 ± 0.03 ^{abC}	5.84 ± 0.03 ^{bcD}	5.86 ± 0.02 ^{cD}

Results represent the mean of three replicates (n=3) and are expressed as mean value ± SD. Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same meat sample at $p < 0.05$.

The pH values were significantly different between samples ($p < 0.05$) but not in the same sample during the 11 days of refrigerated storage and increased continually with storage time from pH= 5.86; 5.69; 5.76 and 5.71 on the first day to pH= 6.19; 5.78; 6.01 and 5.86 on day 11 (last day) for the CTR, BHT, AVL and AVF, respectively. At the end of the experiment, CTR had the highest pH= 6.19, whereas BHT had the lowest pH= 5.78 and AVF had lower pH values compared with the control and AVL.

Similarly, the pH values of the raw beef patties formulated with powdered *A. indica* leaves and *C. baccatum* throughout the refrigerated storage were evaluated to determine their correlation with TBARS values and the results obtained are shown in **Figure 62**.

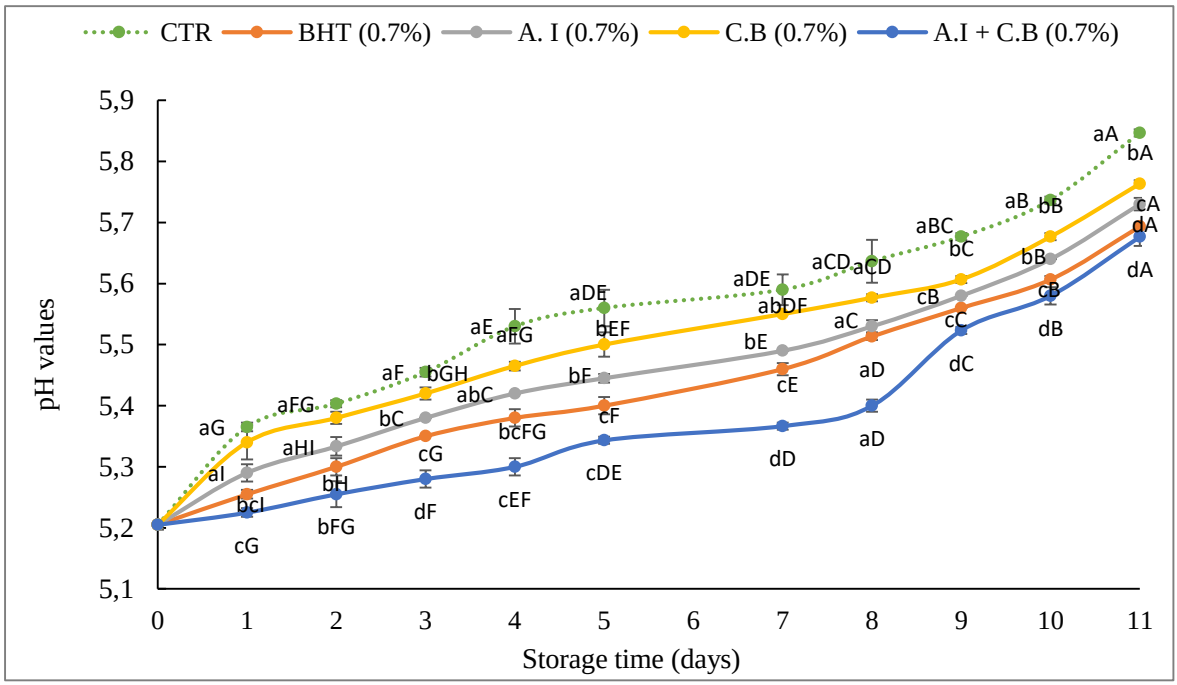

Figure 62. pH values of raw beef patties conserved with powdered *A. indica* during refrigerated storage at 4 ± 1°C. Error bars represent the standard deviation (n = 3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same meat sample at $p < 0.05$

At every storage day, the pH values were significantly different between raw beef patty samples ($p < 0.05$) and increased steadily over storage time. The highest pH values were recorded in CTR, which increased from 5.21 at day 1 to 5.85 at day 11 followed by the pH of C.B (0.7%, *w/w*) presenting pH values ranging from 5.21 to 5.76 at day 1 and day 11, respectively. BHT, A.I and A.I + C.B kept their pH relatively low, especially A.I + C.B, which presented the best pH values ranging from 5.21 in the initial day to 5.37 in the final day 11 of chilled storage.

The increase in pH values was proportional to the susceptibility of the beef patties to oxidise. A similar trend was observed in the research of Villasante et al [332] where the pH values of Pecan shell and Roselle flower added to raw meat at 2% (*w/w*) increased progressively during refrigerated storage period from pH= 5.64 and pH= 5.42, respectively at the first day of the experiment to pH= 7.31 and pH= 5.59, respectively at the final experimentation day. López-Romero et al [334] demonstrated that pH values of pork patties formulated with *Agave angustifolia* stored under refrigeration at 4°C during 10 days increased significantly ($p < 0.05$) with the increase of storage time.

For reasons of hygiene and taste, an acidic pH value of meat and meat products is always an advantage. However, the increase in pH during the storage process directly influences the quality of the meat [335]. Muela et al [336] addressed the decrease in acidity to enzymes that degrade meat proteins and produce ammonia, amines and other toxic compounds that are formed rapidly when meat starts to decompose. The increase in pH values also may be associated with bacterial spoilage [314, 315].

5.3.2.3 Antioxidant Capacity (AOC)

The antioxidant capacity of powdered *A. vulneraria* leaves and flowers added to raw beef meat at 0.5% (*w/w*) was determined by FRAP-water (hydrophilic FRAP) and FRAP-lipid (lipophilic FRAP) assays on the initial and the final days of the refrigerated storage at 4 ± 1°C and the results obtained are illustrated in **Figure 63** and **Figure 64**, respectively.

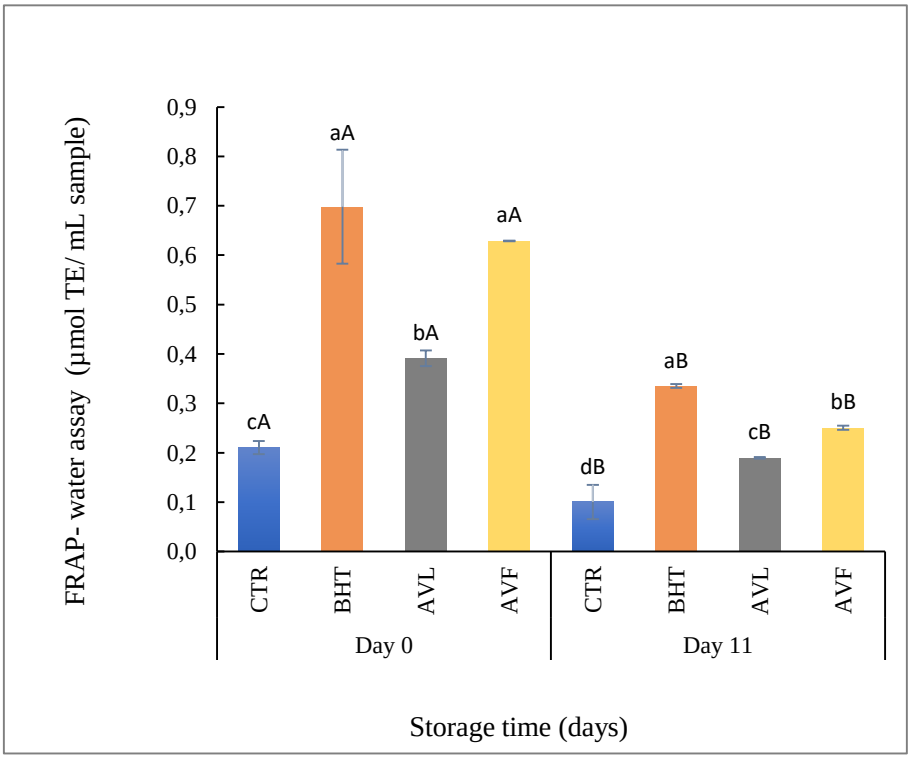

Figure 63. Antioxidant Capacity of raw beef patties conserved with powdered *A. vulneraria* leaves and flowers during refrigerated storage determined by FRAP-water assays. Error bars represent the standard deviation (n=3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same meat sample at $p < 0.05$

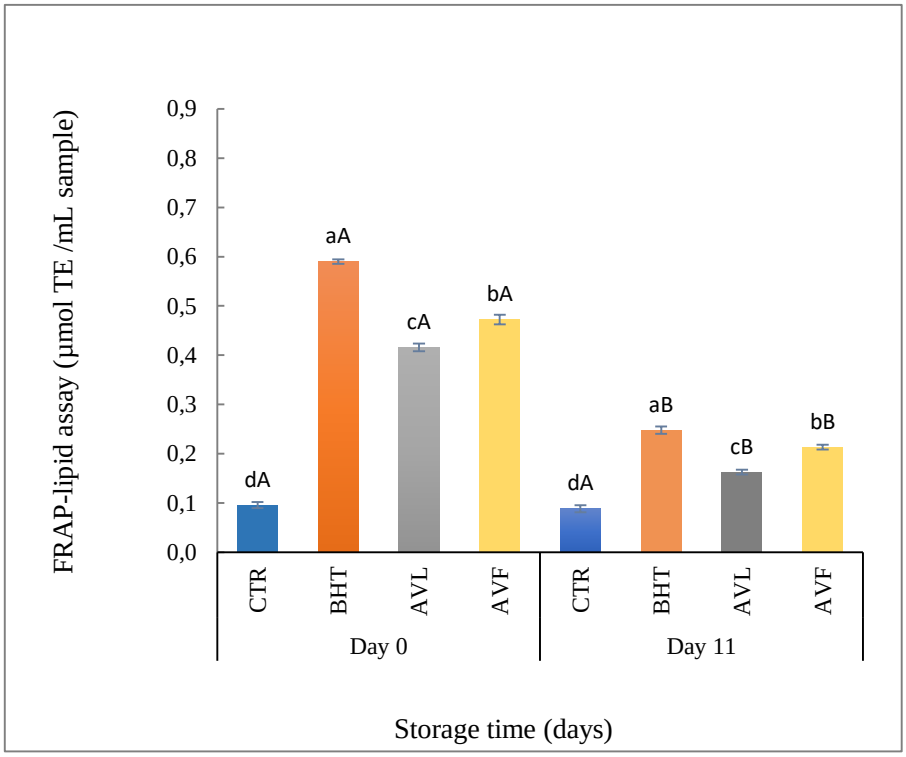

Figure 64. Antioxidant Capacity of raw beef patties conserved with powdered *A. vulneraria* leaves and flowers during refrigerated storage determined by FRAP-lipid assays. Error bars represent the standard deviation (n=3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same meat sample at $p < 0.05$.

The antioxidant capacity of the different beef patty samples was higher in the initial day of storage (day 0) than the final day (day 11) (**Figure 63**). The significant highest value ($p < 0.05$) of the hydrophilic FRAP assay was recorded in BHT (0.5%, *w/w*) and was estimated at 0.7 µmol TE/ mL sample at day 0 and 0.34 µmol TE/ mL sample at day 11, followed by AVF (0.5%, *w/w*) with 0.63 µmol TE/ mL sample at day 0 and 0.25 µmol TE/ mL sample at the last day of storage, and then AVL (0.5%, *w/w*) with 0.39 µmol TE/ mL sample at the first day and 0.19 µmol TE/ mL sample at the last day, while CTR showed the lowest antioxidant activity estimated at 0.21 and 0.10 µmol TE/ mL sample at the beginning and the end of the storage period, respectively.

Likewise, for the lipophilic FRAP assay (**Figure 64**), the highest values were recorded at the day 0. BHT (0.5%, *w/w*) presented the highest AOC value with 0.59 µmol TE/ mL sample at day 0 before to decrease to 0.25 µmol TE/ mL sample at the final day, followed by AVF (0.5%, *w/w*), which exhibited a higher AOC value of 0.47 µmol TE/ mL sample at the initial day than the final day, in which it exhibited only 0.21 µmol TE/ mL sample, then AVL (0.5%,

w/w) with 0.42 and 0.16 µmol TE/ mL sample at day 0 and day 11, respectively, while CTR presented the lowest antioxidant activity estimated at 0.1 and 0.09 µmol TE/ mL sample at day 0 and day 11, respectively.

Similarly, the antioxidant capacity of powdered *A. indica* leaves and *C. baccatum* added to raw beef meat at 0.7% (*w/w*) was determined by the DPPH-water (hydrophilic DPPH) and DPPH-lipid (lipophilic DPPH) assays in the initial day of experiment (day 0) and the final day (day 11) and the results obtained are in **Figure 65** and **Figure 66**, respectively.

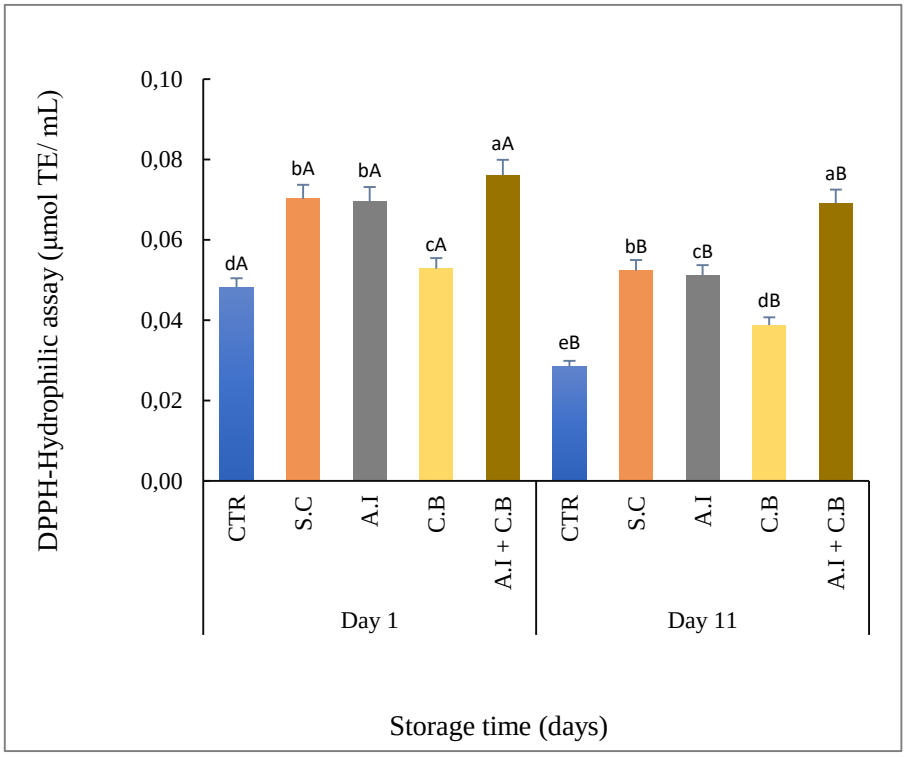

Figure 65. Antioxidant Capacity of raw beef patties conserved with powdered *A. indica* leaves during refrigerated storage determined by FRAP-water assays. Error bars represent the standard deviation (n=3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same meat sample at $p < 0.05$

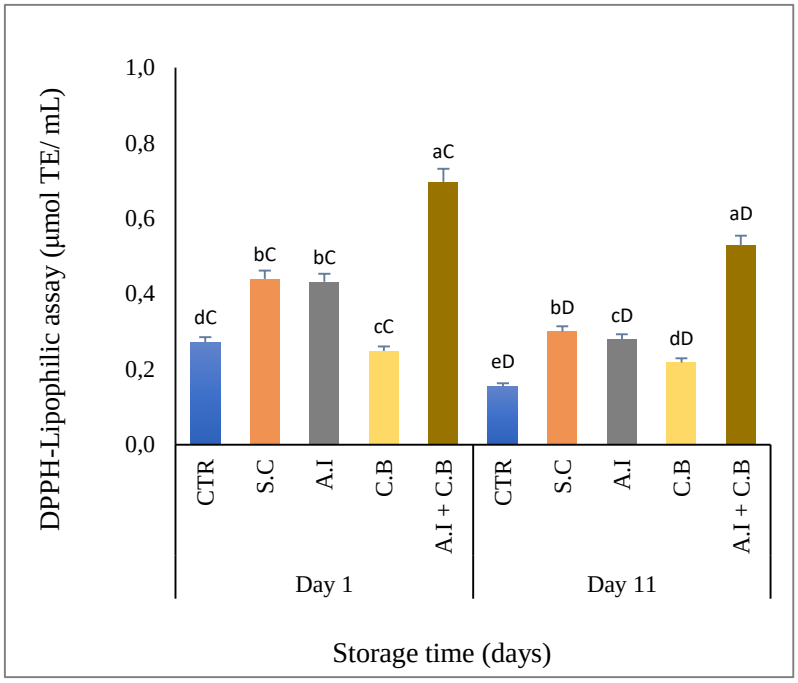

Figure 66. Antioxidant Capacity of raw beef patties conserved with powdered *A. indica* leaves during refrigerated storage determined by FRAP-lipid assays. Error bars represent the standard deviation (n=3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same meat sample at $p < 0.05$

According to the hydrophilic DPPH assay (**Figure 65**), the result obtained showed that there are significant differences ($p < 0.05$) between beef patties samples on each day of analysis. The different meat samples exhibited stronger anti-radical activity at day 1 than day 11. The highest value was recorded in the meat sample formulated with A.I + C.B ranging from 0.08 to 0.07 µmol TE/mL, followed by BHT and A.I, which had almost the same anti-radical activity ranging from 0.07 to 0.05 µmol TE/mL and had no significant difference at day 1. CTR and C.B presented the lowest AOC values estimated at 0.05 and 0.03 µmol TE/mL at day 1 and 0.05 and 0.04 µmol TE/mL at day 11, respectively.

AOC values determined by lipophilic DPPH assay (**Figure 66**) also presented significant differences between samples ($p < 0.05$). CTR and C.B had the lowest anti-radical activity estimated at 0.27 and 0.25 µmol TE/mL in day 1 and 0.16 and 0.22 µmol TE/mL at day 11. The significantly highest values were recorded in A.I + C.B sample (0.70 and 0.53 µmol TE/mL at day 1 and 11, respectively), followed by BHT and A.I (with no significant difference at day 1) ranging from 0.44 to 0.30 µmol TE/mL at day 1 and 0.43 to 0.28 µmol TE/mL at day 11, respectively.

Lipophilic (tocopherols and carotenoids) and hydrophilic (ascorbic acid and the majority of phenolic compounds) antioxidants are two different groups of antioxidants that contribute to protecting the treated meat products against oxidation [339]. Several studies demonstrated the capacity of antioxidants to improve the quality and nutrition value of meat products. For instance, Gallego et al [111] achieved similar results and found that the *Caesalpinia decapetala* can be a good source of natural antioxidants since it had higher antioxidant capacity determined with hydrophilic and lipophilic FRAP assays as they found that hydrophilic FRAP values are higher than lipophilic ones.

5.3.2.4 Colour changes

The effect of the powdered *A. vulneraria* leaves and flowers (0.5%, *w/w*) and powdered *A. indica* leaves on raw beef patties colour was determined using a reflectance colorimeter and expressed against the scale of L* (lightness), a* (redness) and b* (yellowness) in the CIELab colour space system. Colour changes measured on the surface of the different treated beef patties formulated with powdered *A. vulneraria* leaves and flowers during 11 days of refrigerated storage are illustrated in **Table 19**.

Table 19. Colour changes of raw beef patties conserved with powdered *A. vulneraria* at 0.5% (*w/w*) during storage at 4 ± 1°C

Traits	Days	CTR	BHT (0.5%)	AVL (0.5%)	AVF (0.5%)
redness (a*)	1	36.67 ± 2.37 ^{aD}	52.32 ± 0.98 ^{bA}	43.75 ± 4.28 ^{abC}	49.46 ± 0.37 ^{abB}
	2	33.80 ± 0.37 ^{aC}	49.26 ± 2.56 ^{bA}	39.96 ± 1.23 ^{abB}	48.01 ± 0.05 ^{abA}
	4	27.95 ± 0.71 ^{aD}	48.91 ± 1.63 ^{bA}	38.21 ± 0.95 ^{abC}	43.07 ± 0.03 ^{abB}
	6	27.76 ± 0.84 ^{aD}	48.77 ± 0.69 ^{bA}	38.02 ± 0.47 ^{abC}	41.80 ± 1.85 ^{abB}
	8	25.14 ± 0.12 ^{aD}	43.68 ± 0.81 ^{bA}	34.09 ± 0.83 ^{abC}	31.22 ± 0.18 ^{abB}
	11	24.89 ± 1.23 ^{aB}	37.31 ± 1.09 ^{bA}	26.84 ± 1.46 ^{abB}	25.74 ± 1.03 ^{abB}
yellowness (b*)	1	10.30 ± 0.17 ^{aB}	15.35 ± 0.02 ^{bA}	10.45 ± 1.24 ^{aB}	14.34 ± 0.57 ^{bA}
	2	10.28 ± 1.92 ^{aB}	14.63 ± 1.19 ^{bA}	09.93 ± 2.51 ^{aB}	13.32 ± 0.11 ^{bA}
	4	09.58 ± 1.93 ^{aB}	13.43 ± 0.41 ^{bA}	09.88 ± 0.15 ^{aB}	13.29 ± 0.04 ^{bA}
	6	09.46 ± 0.55 ^{aB}	13.45 ± 0.10 ^{bA}	08.78 ± 0.04 ^{aB}	12.11 ± 0.24 ^{bA}
	8	05.63 ± 0.07 ^{aC}	11.79 ± 1.24 ^{bA}	08.10 ± 1.00 ^{aB}	10.79 ± 0.53 ^{bA}
	11	05.17 ± 1.03 ^{aC}	11.13 ± 0.46 ^{bA}	05.52 ± 0.95 ^{aC}	09.92 ± 0.14 ^{bB}
lightness (L*)	1	56.18 ± 0.14 ^{aC}	70.80 ± 2.54 ^{abA}	68.36 ± 2.09 ^{abB}	70.99 ± 2.19 ^{bA}
	2	55.42 ± 3.07 ^{aC}	61.86 ± 1.62 ^{abB}	65.07 ± 2.12 ^{abA}	65.09 ± 4.12 ^{bA}
	4	55.30 ± 0.25 ^{aC}	60.61 ± 0.33 ^{abB}	61.52 ± 1.80 ^{abA}	63.70 ± 3.63 ^{bA}
	6	55.04 ± 1.60 ^{aC}	56.78 ± 2.78 ^{abB}	58.64 ± 1.97 ^{abB}	62.18 ± 040 ^{bA}
	8	52.59 ± 0.50 ^{aC}	56.65 ± 1.80 ^{abB}	55.54 ± 1.72 ^{abB}	59.53 ± 0.23 ^{bA}
	11	43.63 ± 2.98 ^{aC}	49.39 ± 0.04 ^{abB}	49.74 ± 0.21 ^{abB}	55.61 ± 4.65 ^{bA}

Results represent the mean of three replicates (n=3) and are expressed as mean value ± SD. Error bars represent the standard deviation (n=3). Lowercase letters indicate significant differences between meat samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same meat sample at $p < 0.05$.

The redness (a*) values decreased in all the beef patties samples during the period of storage at 4 ± 1°C. Compared with AVL (0.5%, *w/w*), redness (a*) values of AVF (0.5%, *w/w*) were significantly higher ($p < 0.05$) but slightly lower than BHT (0.5%, *w/w*). The redness (a*) values of AVF decreased from 49.46 in the first day of analysis to 25.74 in the last day. The lowest values of redness (a*) were recorded in CTR, which considerably decreased during chilled storage from 36.67 in the first day to 24.89 in the last day of the experiment. Beside the redness (a*) values, the powdered *A. vulneraria* leaves and flowers (0.5%, *w/w*) enhanced the yellowness (b*) values of raw beef patties. The yellowness (b*) values of CTR and the treated beef patty samples (BHT, AVL and AVF) decreased significantly ($p< 0.05$) during refrigerated storage. The lowest yellowness (b*) values were observed in CTR and the values increased with the addition of the powdered *A. vulneraria* leaves and flowers. Higher values were recorded in AVF than in AVL meat samples and were close to the values recorded in BHT. Likewise, the addition of the powdered *A. vulneraria* leaves and flowers presented a significant effect on the lightness (L*) values ($p <0.05$) which were lower in the treated meat samples (0.5%, *w/w*) when compared with CTR.

Likewise, colour changes of the treated beef patties conserved with powdered *A. indica* leaves and *C. baccatum* added at 0.7% (*w/w*) during 11 days of refrigerated storage are represented in **Table 20**.

Table 20. Colour changes of raw beef patties conserved with powdered *A. indica* at 0.7% (*w/w*) during storage at 4 ± 1°C

Traits	Days	CTR	BHT (0.7%)	A.I (0.7%)	C.B (0.7%)	AI + CB
redness (*a**)	1	39.27 ± 2.37 ^{aA}	50.12 ± 0.77 ^{bA}	42.65 ± 2.16 ^{cA}	48.36 ± 0.37 ^{dA}	45.19 ± 1.26 ^{cA}
	2	33.32 ± 0.37 ^{aB}	49.14 ± 1.32 ^{bB}	39.43 ± 0.96 ^{cB}	47.02 ± 0.05 ^{bB}	38.77 ± 2.14 ^{cB}
	3	30.15 ± 0.26 ^{aC}	48.89 ± 1.52 ^{bB}	38.73 ± 1.03 ^{cC}	45.07 ± 0.33 ^{dC}	38.23 ± 2.45 ^{cB}
	4	27.65 ± 0.71 ^{aD}	48.62 ± 0.51 ^{bB}	38.31 ± 0.65 ^{cC}	43.47 ± 0.23 ^{dD}	37.45 ± 1.45 ^{cC}
	5	27.12 ± 0.31 ^{aD}	45.56 ± 0.21 ^{bC}	38.04 ± 0.27 ^{cC}	42.11 ± 0.15 ^{bE}	37.01 ± 1.32 ^{cC}
	7	25.88 ± 0.17 ^{aE}	41.96 ± 0.23 ^{bD}	36.01 ± 0.25 ^{cD}	37.85 ± 1.22 ^{cF}	36.04 ± 1.23 ^{cD}
	8	25.24 ± 0.12 ^{aE}	39.78 ± 1.02 ^{bE}	34.29 ± 0.63 ^{cC}	31.11 ± 0.47 ^{dG}	35.77 ± 1.09 ^{cE}
	9	23.99 ± 0,13 ^{aF}	39.02 ± 1.01 ^{bE}	31.12 ± 0.16 ^{cF}	29.63 ± 0.23 ^{dH}	35.19 ± 1.47 ^{dE}
	10	23.06 ± 0.19 ^{aF}	38.45 ± 0.98 ^{bF}	28.29 ± 0.77 ^{cG}	27.03 ± 0.11 ^{cI}	30.44 ± 2.52 ^{dF}
	11	22.89 ± 1.23 ^{aG}	37.21 ± 1.00 ^{bG}	26.64 ± 1.26 ^{cH}	25.24 ± 1.14 ^{cJ}	30.01 ± 0.99 ^{dF}
yellowness (*b**)	1	11.28 ± 0.11 ^{aA}	15.25 ± 0.11 ^{bA}	14.14 ± 0.67 ^{bA}	10.55 ± 1.21 ^{dA}	12.25 ± 1.01 ^{cA}
	2	10.56 ± 1.32 ^{aB}	14.43 ± 1.14 ^{bB}	13.49 ± 1.26 ^{bB}	9.83 ± 2.01 ^{dB}	12.04 ± 1.61 ^{cA}
	3	9.89 ± 1.22 ^{aC}	13.98 ± 1.22 ^{bC}	13.32 ± 1.25 ^{bB}	9.78 ± 0.55 ^{aB}	11.78 ± 1.44 ^{cB}
	4	9.54 ± 1.03 ^{aC}	13.43 ± 0.11 ^{bC}	12.87 ± 0.42 ^{cC}	8.66 ± 1.06 ^{dC}	11.59 ± 1.25 ^{dB}
	5	9.43 ± 0.15 ^{aC}	13.25 ± 0.25 ^{bC}	12.44 ± 0.17 ^{cC}	8.51 ± 0.09 ^{dC}	11.23 ± 1.09 ^{eB}
	7	7.45 ± 0.18 ^{aD}	12.12 ± 0.13 ^{bD}	11.01 ± 0.44 ^{cD}	7.88 ± 0.15 ^{aD}	10.76 ± 1.21 ^{dC}
	8	5.33 ± 0.07 ^{aE}	11.69 ± 1.09 ^{bE}	10.82 ± 0.73 ^{cE}	7.06 ± 0.45 ^{dD}	10.66 ± 1.56 ^{cC}
	9	5.24 ± 0.04 ^{aE}	11.23 ± 0.33 ^{bE}	10.12 ± 0.01 ^{cE}	6.59 ± 0.16 ^{dE}	10.09 ± 1.33 ^{cC}
	10	5.17 ± 1.22 ^{aE}	10.76 ± 0.26 ^{bE}	9.72 ± 0.59 ^{cF}	6.13 ± 0.19 ^{dE}	9.44 ± 2.47 ^{cD}
	11	5.02 ± 1.18 ^{aE}	10.27 ± 0.11 ^{bF}	9.42 ± 0.19 ^{cF}	5.32 ± 1.69 ^{aF}	9.21 ± 2.89 ^{cD}
lightness (*L**)	1	57.37 ± 0.74 ^{aA}	70,79 ± 2.03 ^{bA}	68,70 ± 2,24 ^{cA}	65.16 ± 2.00 ^{dA}	66.15 ± 2.20 ^{dA}
	2	56.32 ± 2.17 ^{aB}	67.09 ± 3.02 ^{bB}	66.46 ± 1,42 ^{cB}	65.17 ± 2.02 ^{dA}	66.01 ± 2.09 ^{cA}
	3	56.01 ± 1.85 ^{aB}	65.33 ± 1.23 ^{bC}	64.09 ± 1.74 ^{cC}	63.46 ± 1.46 ^{dB}	65.19 ± 2.33 ^{bB}
	4	55.84 ± 1.40 ^{aC}	64.70 ± 3.53 ^{bC}	62,61 ± 0,33 ^{bD}	61.43 ± 1.52 ^{cC}	61.23 ± 2.40 ^{cC}
	5	54.98 ± 1.78 ^{aD}	63.12 ± 2.10 ^{bD}	58.12 ± 0.78 ^{cE}	59.87 ± 1.96 ^{cD}	54.54 ± 2.10 ^{aD}
	7	53.13 ± 1.45 ^{aE}	60.15 ± 1.64 ^{bE}	56.45 ± 1.77 ^{cF}	56.16 ± 1.69 ^{cE}	52.33 ± 2.44 ^{dE}
	8	52.41 ± 0.50 ^{aF}	59.23 ± 0.23 ^{bF}	54.15 ± 1.26 ^{cG}	55.14 ± 1.62 ^{cF}	51.46 ± 2.30 ^{dF}
	9	51. 26 ± 1.45 ^{aG}	56.54 ± 0.19 ^{bG}	52. 49 ± 1.65 ^{cH}	53.75 ± 1.36 ^{dG}	50.73 ± 2.96 ^{eG}
	10	47.46 ± 1.85 ^{aH}	54.16 ± 0.15 ^{bH}	50.46 ± 1.87 ^{cI}	51.36 ± 1.64 ^{dH}	49.16 ± 2.23 ^{eH}
	11	43.23 ± 2.96 ^{aI}	54.41± 2.65 ^{bF}	49.22± 0.04 ^{cJ}	48.04 ± 0.21 ^{cI}	47.36 ± 2.08 ^{dI}

Results represent the mean of three replicates (n = 3) and are expressed as mean value ± SD; different letters in the same day indicate significant difference between samples at $p < 0.05$, different capital letters indicate significant difference between storage days at $p < 0.05$ for the same sample.

The lightness (L*) values of the different treated beef patties decreased significantly ($p < 0.05$) over storage time. The results showed that, on the one hand, the beef patties formulated with BHT (0.7%, *w/w*) had the highest redness (a*) values ranging from 50.12 at day 1 to 37.21 at day 11, followed by the sample containing A.I + C.B, while CTR and C.B (0.7%, *w/w*)

presented the lowest values during storage period. On the other hand, CTR and C.B (0.7%, *w/w*) presented the lowest values during storage period. A.I (0.7%, *w/w*) showed better red colour values than CTR despite the dark colour that the powdered leaves attributed to the beef patties. In addition to redness (a*), BHT (0.7%, *w/w*) enhanced the yellowness (b*) of raw beef patties during storage period and presented lower values ranging from 15.25 at day 1 to 10.27 at day 11, compared to the yellowness (b*) values of CTR and C.B (0.7%, *w/w*), which decreased significantly ($p < 0.05$) during refrigerated storage and presented the lowest yellowness (b*) values.

Similar results were observed in fresh beef patties formulated with olive cake powder during storage period at 4°C [112]. These findings were as well consistent with the results obtained by do Prado et al [340] suggesting that the decrease in the redness (a*) and yellowness (b*) values over time indicates a change of the meat colour, from bright red to tan or brown. An increase in yellowness (b*) values was also observed in raw ground pork patties containing *Moringa oleifera* leaves [108]. Moreover, in previous studies conducted by Gallego et al [111] and Esmer et al [341] slight changes in lightless (L*) values in meat during chilled storage. According to Mancini and Hunt [342], the reduction of redness (a*) values during storage is probably due to the oxidation of myoglobin and the formation of metmyoglobin.

5.3.2.5 Metmyoglobin formation

The stability monitoring of the red colour of raw beef patties formulated with the powdered *A. vulneraria* leaves and flowers (0.5%, *w/w*) and *A. indica* leaves (0.7%, *w/w*) was also estimated by measuring the formation of metmyoglobin (MetMb) in beef patties.

The formation of MetMb in raw beef patties conserved with powdered *A. vulneraria* leaves and flowers (0.5%, *w/w*) was measured and the results obtained are in **Figure 67**.

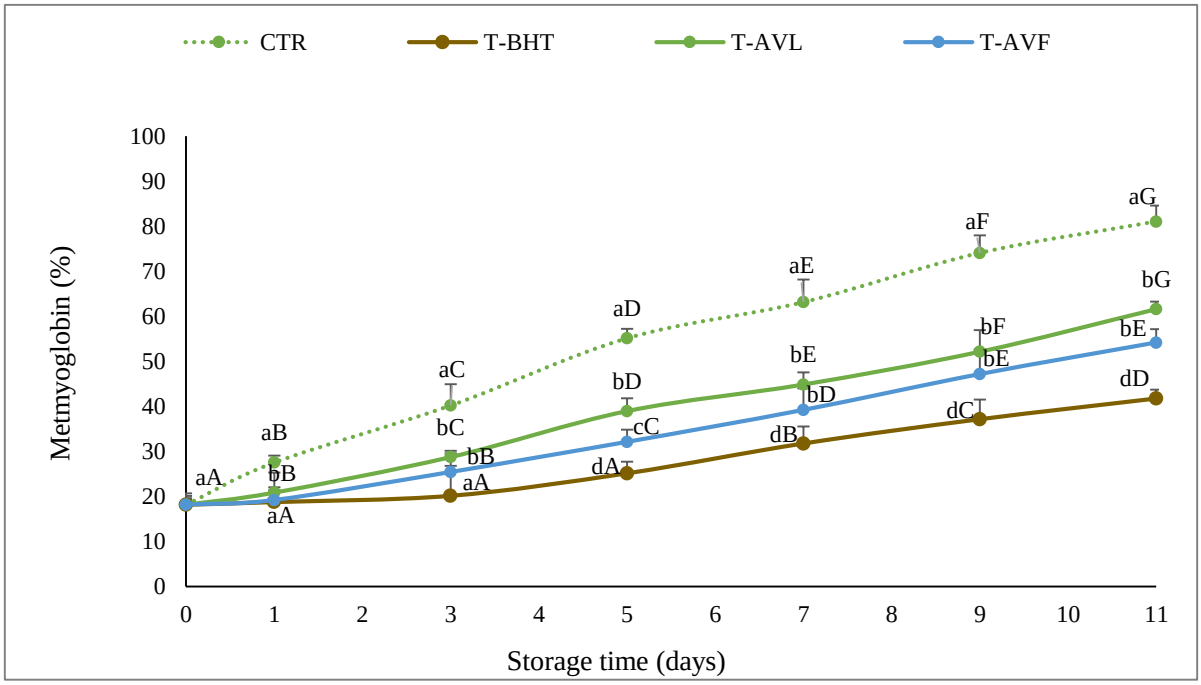

Figure 67. Metmyoglobin content in raw beef patties conserved with powdered *A. vulneraria* during refrigerated storage at 4 ± 1°C. Error bars represent the standard deviation (n = 3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same meat sample at $p < 0.05$

The MetMb in beef patty samples increased progressively and significantly ($p < 0.05$) over storage time. CTR presented the highest MetMb percentage equal to 18.2% in the initial day of storage and 81.07 % at the end of the experiment. Contrariwise, BHT (0.5%, *w/w*) presented the lowest MetMb percentage ranging from 18.2% to 41.75% during the 11 days of refrigerated storage. The formation of MetMb in the beef patty samples formulated with the powdered leaves and flowers of *A. vulneraria* (0.5%, *w/w*) was higher than BHT (0.5%, *w/w*) and lower than CTR and did not exceed 61.6 % and 54.19 %, respectively after 11 days of the refrigerated storage.

The formation of MetMb in raw beef patties conserved with powdered *A. indica* leaves and *C. baccatum* (0.7%, *w/w*) was measured and the results are depicted in **Figure 68**.

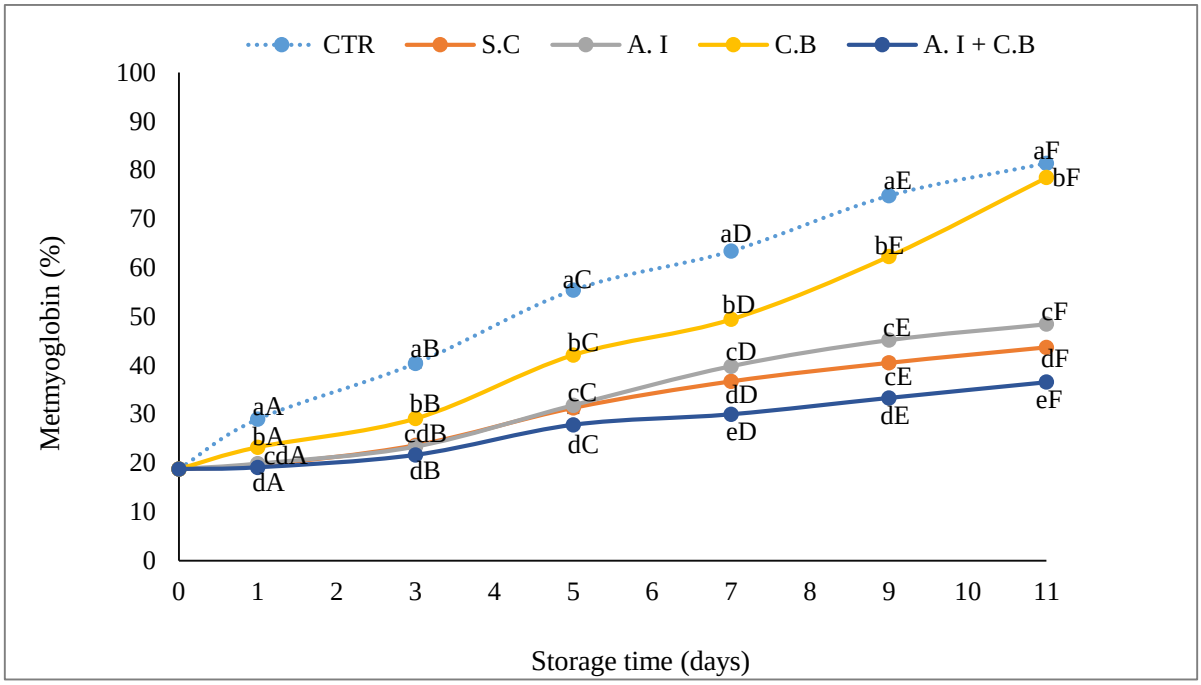

Figure 68. Metmyoglobin content in raw beef patties conserved with powdered *A. indica* during refrigerated storage at 4 ± 1°C. Error bars represent the standard deviation (n = 3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same meat sample at $p < 0.05$

The MetMb formation increased ($p < 0.05$) progressively in all the beef patty samples as the treatment time was further prolonged. CTR presented the highest percentage estimated at 18.73% in the initial day of storage and 81.39% in the end of the storage period, while A.I + C.B beef patty samples presented the lowest percentage that did not exceed 36.55% after 11 days of chilled storage. Beef patty samples treated with BHT (0.7%, *w/w*) and A.I (0.7%, *w/w*) exhibited almost the same MetMb percentage recorded at 43.69% and 48.42%, respectively, at the end of storage time. However, C.B (0.7%, *w/w*) increased gradually and had higher MetMb percentage ranging from 18.73% to 78.47%.

Different studies reported similar results and suggested that free radicals produced during lipid oxidation may damage the structure of muscle fibers and reduce pigmentation [266]. The fading of red colour of beef patties during storage is related to the oxidation of myoglobin from Fe(II) of myoglobin to Fe(III) giving the MetMb [318, 322]. Zahid et al [344] investigated the effects of the clove on beef patties during 7 days of cold storage at 4°C and found that MetMb formation increased with the increase of the storage time from 14.57% at day 1 to 19.93% at day 7. Gahruie et al [62] also observed an increase in MetMb formation over storage time and

reported that the presence of chirazi thyme, cinnamon and rosemary delayed the formation of MetMb in raw beef burgers.

5.3.2.6 Hexanal content

Just like colour, aroma is an important criterion that influences the decision of customers to buy meat and meat products [345]. The reactions of oxidation cause the creation of volatile compounds, such as the hexanal.

Hexanal content in raw beef patties conserved with powdered *A. vulneraria* leaves and flowers added at 0.5% (*w/w*) at 4 ± 1°C was determined at the first, middle and last day of the experiment and the results obtained are represented in **Figure 69**.

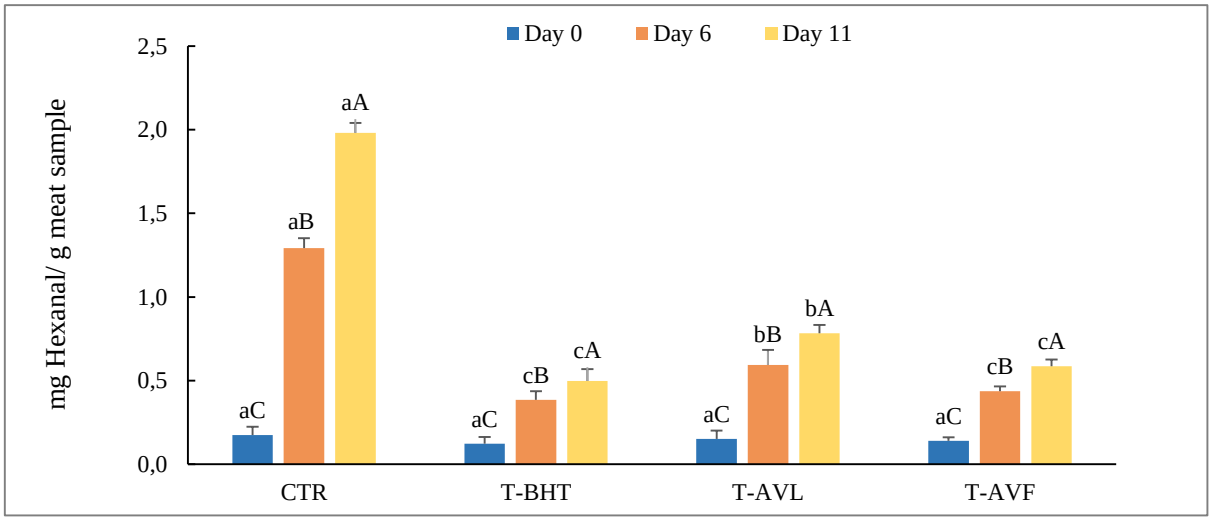

Figure 69. Hexanal content in raw beef patties conserved with powdered *A. vulneraria* during refrigerated storage at 4 ± 1°C. Error bars represent the standard deviation (n = 3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same meat sample at $p < 0.05$

The hexanal content in the different raw beef patties increased as the treatment time was further prolonged. At the first day of the experiment (day 1) no significant difference between samples was observed. From day 6, the content of hexanal increased progressively with significant differences between the samples ($p < 0.05$). The highest hexanal content was observed in CTR with 1.29 mg hexanal/ g meat sample, while the lowest hexanal content was observed in BHT (0.5%, *w/w*) with 0.39 mg hexanal/ g meat sample. AVF (0.5%, *w/w*) presented a hexanal content slightly higher than BHT and lower than AVL (0.5%, *w/w*) and estimated at 0.59 mg hexanal/ g meat sample. At the end of the storage period (day 11), hexanal

content of CTR was 1.5-fold higher than its content at day 6, while BHT, AVL and AVF (0.5%, *w/w*) were, respectively 1.3-fold higher than their hexanal contents at day 6.

Hexanal content in raw beef patties conserved with powdered *A. indica* leaves and *C. baccatum* added at 0.7% (*w/w*) at 4 ± 1°C was determined at day 1, day 6 and day 11 of the experiment and the results obtained are represented in **Figure 70**.

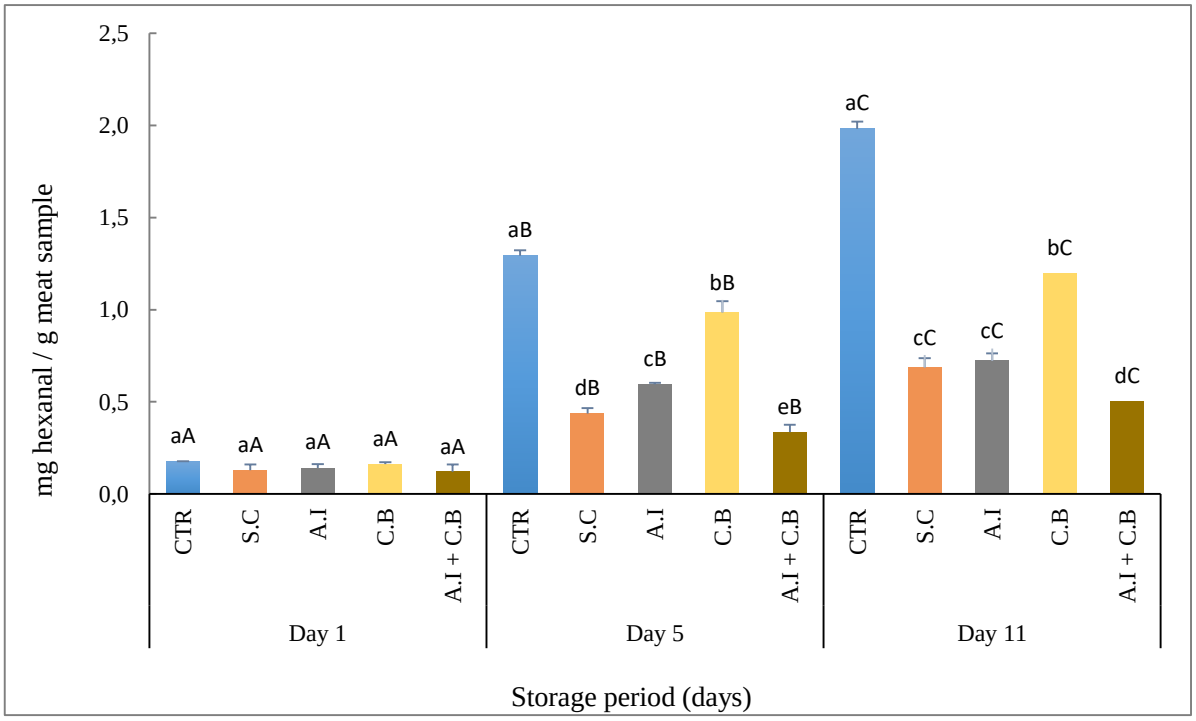

Figure 70. Hexanal content in raw beef patties conserved with powdered *A. indica* leaves during refrigerated storage at 4 ± 1°C. Error bars represent the standard deviation (n = 3). Lowercase letters indicate significant differences between emulsion samples in the same day at $p < 0.05$ and uppercase letters indicate significant differences between days of storage for the same meat sample at $p < 0.05$

The hexanal content of all the raw beef patty samples increased significantly over storage time. At the first day of storage, there were no significant differences observed between BHT (0.7%, *w/w*) and A.I (0.7%, *w/w*), whereas CTR, C.B (0.7%, *w/w*) and A.I + C.B showed significant differences ($p < 0.05$). After 5 days of chilled storage, the content of hexanal increased significantly in all the samples. The highest content of hexanal was observed in CTR and C.B (0.7%, *w/w*) with 1.29 and 0.99 mg hexanal/g meat sample, respectively, while the lowest hexanal content was observed in A.I + C.B with 0.34 mg hexanal/g meat sample. BHT (0.7%, *w/w*) and A.I (0.7%, *w/w*) exhibited almost the same effect and presented hexanal contents estimated at 0.436 and 0.594 mg hexanal/g meat sample, respectively. At the end of the storage period, CTR presented the highest content of hexanal at 1.98 mg hexanal/g meat

sample while A.I + C.B presented the lowest hexanal content estimated at 0.50 mg hexanal/g meat sample.

The analysis of volatile compounds content is a good indicator of the oxidation state of the meat products [278]. The hexanal is predominant in the volatile fractions of meat products and can cause rancid flavours and odours [111]. Similar observations were made by Juntachote et al [346] about Holy basil and Galangal in pork meat. Gallego et al [111] also reported similar results about *Caesalpinia decapetala* showing that natural antioxidants exhibited better antioxidant effect than that shown by the synthetic preservative, when assessed by hexanal formation. García-Lomillo et al [347] also demonstrated that Wine pomace inhibited the formation of hexanal, revealing their potential capacity to delay the formation of rancid odours during storage of in refrigerated beef patties.

5.3.2.7 Antimicrobial analysis

The presence of Colony Forming Units (CFU) was evaluated in raw beef patties conserved with powdered *A. vulneraria* leaves and flowers at 0.5% (*w/w*) and the results obtained are represented in **Table 21**.

Table 21. Effect of powdered *A. vulneraria* (0.5%, *w/w*) on the microbial growth in raw beef patties during refrigerated storage at 4 ± 1°C

Aerobic mesophilic bacteria	Refrigerated storage period		
	day 0	day 6	day 11
CTR	-	+	+
BHT (0.5%)	-	-	-
AVL (0.5%)	-	-	+
AVF (0.5%)	-	-	-

The sign (-) indicates a number of aerobic mesophilic bacteria less than 10^4 CFU/g of sample of aerobic mesophilic bacteria in meat samples. The sign (+) indicates a number of aerobic mesophilic bacteria between 10^4 and 10^5 CFU/g.

The different beef patty samples stored at the initial day of the experiment presented a number of mesophilic bacteria less than 10^4 CFU/g sample. However, CTR sample conserved at 4 ± 1°C for 6 days presented a number of mesophilic bacteria estimated at 3.7×10^4 CFU/g, while BHT, AVL and AVF (0.5%, *w/w*) maintained their effective antibacterial property. At the day 11, the effectiveness of the antimicrobial activity of AVL (0.5%, *w/w*) decreased and the number of aerobic mesophilic bacteria exceeded 10^4 CFU/g sample.

The presence of CFU was evaluated in raw beef patties conserved with powdered *A. indica* leaves and *C. baccatum* added at 0.7% (*w/w*) and the results obtained are represented in **Table 22**.

Table 22. Effect of powdered *A. indica* leaves on the microbial growth in raw beef patties during refrigerated storage at 4 ± 1°C

Aerobic mesophilic bacteria	Refrigerated storage period		
	day 1	day 5	day 11
CTR	-	+	+
BHT (0.7%)	-	-	-
A.I (0.7%)	-	-	-
C.B (0.7%)	-	-	+
A.I + C.B (0.7%)	-	-	-

The sign (-) indicates a number of aerobic mesophilic bacteria less than 10^4 CFU/g of sample of aerobic mesophilic bacteria in meat samples. The sign (+) indicates a number of aerobic mesophilic bacteria between 10^4 and 10^5 CFU/g.

The number of mesophilic bacteria present in all the beef patty samples at the first day of incubation was less than 10^4 CFU/g sample. After 5 days of incubation, the number of mesophilic bacteria present in CTR increased to 4.2×10^4 CFU/g, while the rest of the samples kept their effective antibacterial properties. At the last day of the experiment, the antimicrobial activity of C.B (0.7%, *w/w*) became weak, hence the increase in the number of bacteria.

Our findings are consistent with previous reports in which bioactive compounds from plants were successfully used to disinfect meat samples [265, 348, 358]. Aksoy et al [350] studied the antimicrobial effect of Hibiscus and Oregano on the shelf life of beef and found that on the first day of storage, total mesophilic bacteria count in the Hibiscus and Oregano beef samples were found to be under the detectable limit (< 10 log CFU/ g). After 7 days of chilled storage, the beef patties formulated with Hibiscus showed lower total mesophyll bacteria count (4.82 log CFU/ g) than CTR and Oregano beef samples (11.42 and 8.40 log CFU/ g, respectively). Similar results were reported by Villasante et al [332] showing the effect of Pecan shell, Roselle flower and Red pepper on the microbial growth in beef patties during chilled storage.

5.3.2.8 Sensory analysis

The sensory analysis of the different cooked beef patties was carried out using a triangle test in order to know if the cooked beef patties formulated with powdered *A. vulneraria* leaves and flowers at 0.5% (*w/w*) were identical to CTR (without additive). The results presented in **Table 23** shows the sensory evaluation of the cooked beef patties.

Table 23. Sensory analysis of cooked beef patties formulated with the powdered leaves and flowers of *A. vulneraria* at 0.5% (*w/w*)

Meat sample	Assessors			Smokers/ No smokers		Odd sample identified		Level of Significance
	Male	Female	Total	Smokers	No smokers	Correct (+)	Incorrect (-)	
AVL	18	7	25	1	24	23	2	0.1%
AVF	18	12	30	3	27	23	7	0.1%

Among 25 assessors, 23 agreed that cooked AVL had a different taste compared to CTR. Furthermore, 23 assessors from 30 agreed also that AVF (0.5%, *w/w*) had a different taste than CTR beef patty sample. According to the table used to interpret the triangular test results, if the number of assessors is 23 and 30 and the number of correct answers is 15 and 19 respectively, the level of significance is equal to 0.1%. Thus, we conclude from these results that there is a significant difference between AVL and AVF (0.5%, *w/w*) and CTR. In other words, the taste of the beef patties formulated with *A. vulneraria* samples is significantly different from CTR.

Likewise, to know the total acceptability of the different cooked beef patties formulated with powdered *A. indica* leaves at 0.7% (*w/w*) by consumers, a grade sensorial analysis was made. The results are shown in **Figure 71**.

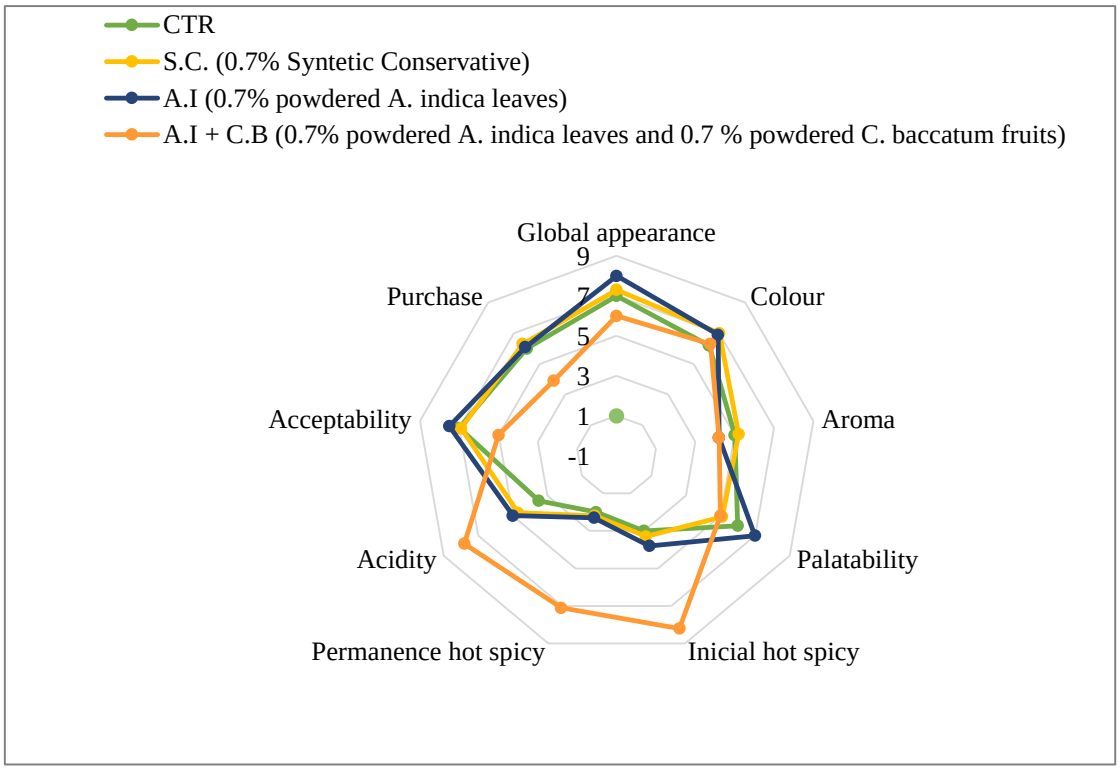

Figure 71. Sensorial attributes and purchase intention of beef patties of cooked CTR and cooked treated beef patties

The most acceptable samples were A.I and the synthetic preservative “SC” (BHT, 0.7%, *w/w*) samples. The consumer intention to purchase beef patties was highest for CTR, A.I and BHT (0.7%, *w/w*) samples, while it was the lowest for A.I + C.B sample. Generally, it should be noted that there are no major differences between the cook beef patty samples, except for the one that incorporates *C. baccatum* sample that is perceived as extremely spicy.

The same trend was observed in the study of Villasante et al [332] who found that the beef patties formulated with the synthetic preservative presented the best results, while patties formulated with hot pepper presented the worst ones possibly because the panelists are not used to eat spicy products.

Part 4

Determination of the antibacterial activity of *Anthyllis vulneraria* and *Azadirachta indica* extracts against several pathogenic microorganisms causing food poisoning

In this part the antibacterial activity of *Anthyllis vulneraria* leaf and flower extracts and *Azadirachta indica* leaf extract was studied against several pathogenic microorganisms causing food poisoning by measuring the inhibitory zones and the minimum inhibitory concentration.

5.4 Part 4. Determination of the antibacterial activity of *A. vulneraria* and *A. indica* extracts against several pathogenic microorganisms causing food poisoning

5.4.1 Inhibitory zone assay (disc diffusion method)

The sensitivity of bacterial strains against *A. vulneraria* leaf and flower extracts was determined after 48h of incubation at 37°C. The area developed around the discs treated with extracts was measured and the results obtained are represented in **Table 24**.

Table 24. Diameters of the inhibitory zones (mm) developed around discs treated with penicillin and *A. vulneraria* leaf and flower extracts (100 µg/mL)

	Inhibitory zone (mm)				
	Microorganism	**50% EtOH**	**penicillin**	**Leaf**	**Flower**
Gram +	*S. aureus*	NS	24 ± 1.8^{c}	10 ± 0.6^{a}	18 ± 1.4^{b}
	M. luteus	NS	19 ± 1.2^{c}	12 ± 0.2^{a}	15 ± 0.5^{b}
	L. monocytogenes	NS	15 ± 0.9^{b}	10 ± 0.3^{a}	11 ± 0.6^{a}
	B. cereus	NS	10 ± 0.3^{a}	9 ± 0.2^{a}	14 ± 0.8^{b}
Gram -	*S. paratyphi*	NS	30 ± 1.6^{b}	NS	4 ± 1.5^{a}
	E. coli	NS	27 ± 1.8^{b}	5 ± 1^{a}	NS

Results are means of three different experiments (n=3). Means in the same row with different letters are significantly different ($p < 0.05$). Penicillin (100 µg/mL) was used as a positive control. Sterile 50%-aqueous EtOH was used as negative control. No inhibition zone is indicated by NS (Not Sensitive).

According to the results obtained, the leaf and flower extracts of *A. vulneraria* (100 µg/mL) showed a significant inhibitory effect against the bacteria growth ($p < 0.05$). The flower extract showed better antibacterial activity than the leaf extract. The best antibacterial activity of the flower extract was against *S. aureus* strain with an inhibition zone estimated at 18 mm, while the best antibacterial activity of the leaf extract was observed against *M. luteus* strain with an inhibitory zone of 12 mm. However, the leaf and flower extracts did not show any antimicrobial activity against *S. paratyphi* and *E. coli* strains, respectively as compared with the penicillin (100 µg/mL), which showed inhibition zones of 30 and 27 mm, respectively.

The inhibition zones (mm) developed around the discs treated with *A. indica* leaf extract were measured as well and the results obtained are represented in **Table 25**.

Table 25. Diameters of the inhibitory zones (mm) developed around discs treated with penicillin (100 µg/mL) and *A. indica* leaf extract (100 µg/mL)

	Microorganisms	Strains	Inhibitory zone (mm)		
			A. indica	**penicillin**	**80% EtOH**
Gram (+)	*S. aureus*	ATCC 25423	19	24	-
	M. luteus	ATCC 4698	12	17	-
	L. monocytogenes	ATCC 15313	-	15	-
	B. cereus	ATCC 11778	-	10	-
Gram (-)	*S. paratyphi*	ATCC 9150	10	30	-
	E. coli	ATCC 25022	21	27	-

Results are means of three different experiments (n=3). Means in the same row with different letters are significantly different ($p < 0.05$). Penicillin (100 µg/ mL) was used as a positive control. Sterile 80% EtOH was used as negative control. No inhibition zone is indicated by NS (Not Sensitive).

A. indica 80%-aqueous EtOH extract (100 µg/ mL) had antimicrobial activity against the majority of bacterial strains tested. The best antimicrobial activity was against *E. coli* strain with an inhibition zone of 21 mm, followed by *S. aureus*, *M. luteus* and *S. paratyphi* strains with inhibition zones equal to 19, 12 and 10 mm, respectively. However, *A. indica* leaf extract had no activity against *L. monocytogenes* and *B. cereus* strains, which had inhibition diameters of 15 and 10 mm, respectively, in the presence of penicillin (100 µg/ mL).

Previous studies showed that the antibacterial activity of plant's extracts depends mainly on their richness on polyphenol contents [351]. Anjarasoa et al [352] reported that the antimicrobial activity of *Albizia masikororum* extract (fabaceae) may be due to the richness of the extract with different chemical groups known for their antimicrobial properties like saponins and phenolic compounds. The antimicrobial activity of *A. indica* extract observed in the present work against *S. aureus* strain was higher than the activity found in the study of Mistry et al [353], where *A. indica* extract presented an inhibition zone of 14.33 mm against *S. aureus* strain. Moreover, Chaturvedi et al [354] observed lower antimicrobial activity of *A. indica* leaf extract against *S. aureus* and *E. coli* strains with inhibitory zones of 12 mm and 14 mm, respectively. However, Al Akeel et al [212] and Koona et al [348] found better antimicrobial activity of *A. indica* extract against *S. paratyphi* strain with an inhibitory zone equal to 20 mm and *M. luteus* with an inhibitory zone of 33 mm, respectively.

5.4.2 Minimum inhibitory concentration (broth dilution method)

The Minimum Inhibitory Concentration (MIC) assay was assessed for only the bacterial strains that showed a sensitivity to *A. vulneraria* leaf and flower extracts and *A. indica* leaf extract (100 µg/mL) in the disc diffusion method previously performed.

Table 26. Turbidity of the penicillin, *A. vulneraria* leaf and flower inoculations after 24 h of incubation at 37°C

Samples	**penicillin**					***A. vulneraria* leaf extract**					***A. vulneraria* flower extract**					**CTR +**	**CTR-**
Cc (mg/mL)	**0.062**	**0.125**	**0.25**	**0.5**	**1**	**0.062**	**0.125**	**0.25**	**0.5**	**1**	**0.062**	**0.125**	**0.25**	**0.5**	**1**		
S. aureus	+	+	-	-	-	+	+	+	-	-	+	+	-	-	-	+	-
M. luteus	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	-
L. monocytogenes	+	+	+	-	-	+	+	+	-	-	+	+	-	-	-	+	-
B. cereus	+	+	-	-	-	+	+	+	-	-	+	+	-	-	-	+	-
S. paratyphi	+	+	-	-	-	NT					+	+	-	-	-	+	-
E. coli	+	+	+	-	-	+	+	+	+	+	NT					+	-

(+) Turbidity indicating bacterial growth, (-) No turbidity indicating no bacterial growth and NT indicates microbial strain not tested. “Cc” (concentration).

Table 26 shows the turbidity of the penicillin, *A. vulneraria* leaf and flower inoculations, and positive (CTR^+) and negative (CTR^-) controls after 24h of incubation. The CTR^+ containing the broth nutrient, bacterial culture and antibiotic/ *A. vulneraria* extracts showed turbidity (bacterial growth) after the 24h of incubation and was used to test the growing ability of the medium, while the CTR^- containing only the broth nutrient and the antibiotic/ *A. vulneraria* extract did not show turbidity (no bacterial growth) after the 24h of incubation and was used to test the sterility of the medium and equipment.

Bacterial growth (indicated by the presence of turbidity in the inoculum) was observed for all the bacterial strains tested at the concentrations of penicillin 0.062 and 0.125 mg/mL and only for *L. monocytogenes* and *E. coli* at the penicillin concentration 0.25 mg/mL. In one hand, the penicillin at 0.5 and 1 mg/mL inhibited totally the growth of all the bacterial strains. Likewise, *A. vulneraria* leaf extract at 0.062, 0.125 and 0.25 mg/mL was not enough to inhibit the growth of all the bacterial strains, except for *M. luteus*, which showed resistance at 0.25 mg/mL. No bacterial growth was observed at 0.5 and 1 mg/mL of *A. vulneraria* leaf extract, except for *E. coli*. In the other hand, *A. vulneraria* flower extract showed better antimicrobial activity than the penicillin and *A. vulneraria* leaf extract by inhibiting the growth of all the bacterial strains without exception at 0.25 mg /mL.

Based on results of **Table 26**, MIC of the different samples tested against the different bacterial strains were calculated and the results obtained are represented in **Table 27**.

Table 27. MIC values of the penicillin and *A. vulneraria* extracts against the different bacterial strains

	MIC (mg/mL)		
	penicillin	**Leaf**	**Flower**
S. aureus	0.625	0.75	0.625
M. luteus	0.625	0.625	0.625
L. mmonocytogenes	0.75	0.75	0.625
B. cereus	0.625	0.75	0.625
S. paratyphi	0.625	NT	0.625
E. coli	0.75	*	NT

* indicates the MIC is higher than the highest concentration of the antibiotic/extract in the first tube, NT indicates microbial strain not tested.

A. vulneraria flower extract was considered to have better antibacterial activity than penicillin and leaf extract. The flower extract of *A. vulneraria* had the lowest MIC values equal to 0.625 mg/mL to inhibit the growth of all the bacterial strains tested compared with the

penicillin that showed similar results except for *L. monocytogenes* and *E. coli*, which showed sensitivity at a MIC value of 0.75 mg/mL. The leaves of *A. vulneraria* presented the lowest antimicrobial effect with MIC values higher than those recorded in the penicillin and *A. vulneraria* flowers inoculums estimated at 0.75 mg /mL against *S. aureus, L. monocytogenes* and *B. cereus* strains, except for *M. luteus* strain (0.625 mg/mL) and *E. coli* where MIC value was higher than the highest concentration of the penicillin used (≥ 1 mg/mL).

Similarly, the turbidity of the penicillin and *A. indica* inoculations, the positive control (CTR^+) and the negative control (CTR^-) after 24 h of incubation are shown in **Table 28**.

Table 28. Turbidity of the penicillin and *A. indica* leaf inoculations after 24 h of incubation at 37°C

Samples	**penicillin**					***A. indica* leaves**					**CTR $^+$**	**CTR$^-$**
Cc (mg/mL)	**0.062**	**0.125**	**0.25**	**0.5**	**1**	**0.062**	**0.125**	**0.25**	**0.5**	**1**		
S. aureus	+	+	-	-	-	+	+	+	+	-	+	-
M. luteus	+	+	-	-	-	+	+	+	-	-	+	-
L. monocytogenes	+	+	+	-	-	NT					+	-
B. cereus	+	+	-	-	-	NT					+	-
S. paratyphi	+	+	-	-	-	+	+	+	-	-	+	-
E. coli	+	+	+	-	-	+	+	+	+	-	+	-

(+) Turbidity indicating bacterial growth, (-) No turbidity indicating no bacterial growth and NT indicates microbial strain not tested. Cc (concentration).

A. indica leaf extract showed antibacterial activity against *M. luteus* and *S. paratyphi* strains at 0.5 mg/mL, while the rest of the microbial strains showed resistance. At 1 mg/mL, no bacterial growth was observed for all the microbial strains tested.

Taking in consideration the results obtained in **Table 28**, MIC of *A. indica* leaf extract was calculated and the results obtained are summarized in **Table 29**.

Table 29. MIC values of the penicillin and *A. indica* leaf extract against the different bacterial strains

	MIC (mg/mL)	
	penicillin	*A. indica* extract
S. aureus	0.625	0.5
M. luteus	0.625	0.75
L. monocytogenes	0.75	NT
B. cereus	0.625	NT
S. paratyphi	0.625	0.75
E. coli	0.75	0.5

NT indicates microbial strain not tested.

A. indica leaf extract was considered to have better antibacterial activity against *S. aureus* and *E. coli* strains than penicillin with MIC value equal to 0.5 mg/mL, while penicillin showed better antimicrobial activity against *M. luteus* and *S. paratyphi* strains with MIC values equal to 0.625 mg/mL.

Previous studies investigated the antibacterial activity of *A. vulneraria* and *A. indica* extracts For instance, Csepregi et al [299] reported that extracts of *A. vulneraria* from Romania showed moderate antimicrobial activity against *S. aureus* and did not have an antibacterial effect against *E. coli*. Moreover, Chibuzo et al [355] studied the antimicrobial activity of *A. indica* leaf extract against *S. aureus* and *E. coli* stains and found lower antimicrobial activity with higher MIC results estimated at 1.562 mg/mL against both strains. Additionally, Ali et al [356] found a lower antimicrobial activity of *A. indica* extract against *S. paratyphi* strain (12.5 mg/mL).

Part 5

Determination of the antiproliferative activity of *Anthyllis vulneraria* and *Azadirachta indica* extracts against several human cancer cell lines.

In this part the antiproliferative activity of *Anthyllis vulneraria* leaf and flower extracts and *Azadirachta indica* leaf extract at different concentrations was studied against several human cancer cell lines by the MTT assay.

5.5 Part 5. Determination of the antiproliferative activity of *A. vulneraria* and *A. indica* extracts against several human cancer cell lines

5.5.1 Viability-reducing activity

The viability of the HepG 2, HeLa and MCF-7 cancer cell lines after being treated with *A. vulneraria* leaf and flower extracts for 48 h was measured by MTT assay and the results obtained are shown in **Figure 72**.

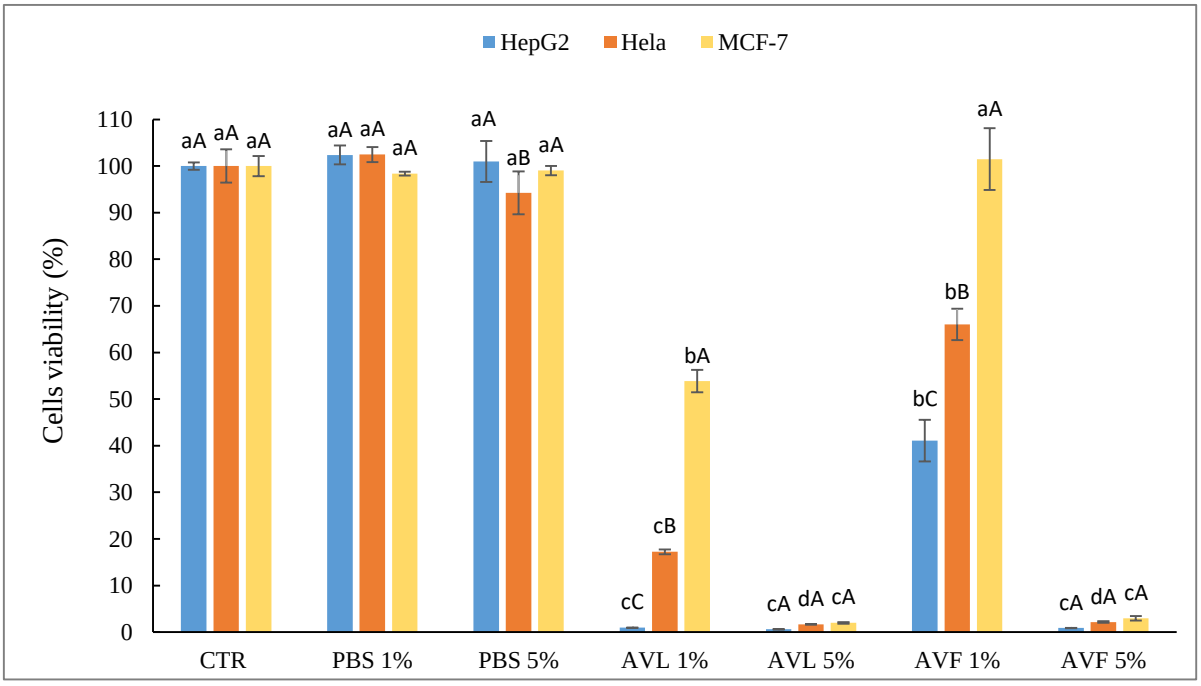

Figure 72. Effect of *A. vulneraria* leaf (AVL; 1% and 5%, *v/v*) and flower (AVF; 1% and 5%, *v/v*) extracts on cells viability assayed 48 h after treatment. CTR, non-treated cells, PBS, control cells incubated in the presence of solvent without extract. Error bars represent the standard deviation (n=3). Lowercase letters indicate significant differences between the three cancer cell lines with the same treatment at $p < 0.05$ and uppercase letters indicate significant differences between the same cancer cell line with different treatments at $p < 0.05$

CTR samples (without treatments) represented 100% of cell viability. No significant difference was observed between CTR and cancer cells treated with PBS (1% and 5%, *v/v*). AVL (1% and 5%, *v/v*) showed better antiproliferative activity against the three cancer cell lines tested than AVF (1% and 5%, *v/v*). The percentage of cancer cell viability decreased significantly ($p < 0.05$) with the increasing of the extract's concentration and all the cancer cells treated with AVL and AVF at 5% (*v/v*) showed the lower cancer cell viability than the cancer cells treated with AVL and AVF at 1% (*v/v*). AVL and AVF extracts were more effective against HepG2, followed by HeLa and MCF-7 cancer cell lines. AVL at 1% (*v/v*) extract was effective to reduce the viability of HepG2 cancer cells by 99.04%, but was less efficient against

HeLa and MCF-7 and reduced their viability by only 82.75% and 46.13%, respectively. However, AVL extract at 5% (*v/v*) showed a potent antiproliferative activity against all the cancer cells and reduced the viability of HepG2, HeLa and MCF-7 cells by 99.37%, 98.35% and 98.3%, respectively. Similarly, AVF extract at 1% (*v/v*) was effective at reducing the viability of HepG2, HeLa and MCF-7 cells by 58.93%, 33.97% and 46.13%, respectively and at 5% (*v/v*), AVF extract showed results slightly lower than AVL extract (5%, *v/v*) and eliminated 99.11%, 97.85% and 97% of HepG2, HeLa and MCF-7 cancer cells, respectively.

The evaluation of the antiproliferative activity of *A. vulneraria* has not been sufficiently studied, in particular, the antiproliferative activity against HeLa, HepG2 and MCF-7 cancer cell lines. However, several other plants from fabaceae family have been reported to exhibit antiproliferative activity or to have bioactive constituents against malignant cells [333, 329], such as isoflavones, lectins, saponins and phenolic compounds, which are characterized by their cytotoxic activity and can be used in the treatment and prevention of cancer [359]. By way of example, Souza et al [360] evaluated the cytotoxicity of flavonoid isolated from *Macroptilium martii* and observed a lower antiproliferative activity (20.96 %) against HeLa cancer cell lines than that determined in the present study. Salem et al [361] examined *in vitro* the cytotoxic activity of Egyptian *Astragalus sieberi* extract against MCF-7 culture (IC_{50} of 69.6 µg/mL) and observed that the percentage of dead cancer cells increased with the increase of the extract concentration. Silva et al [362] found as well that the *in vitro* antiproliferative activity of the leaf essential oil of *Bauhinia cheilantha* was active against MCF-7 cell lines with IC_{50} values of 18.3mg/mL. Furthermore, Ribeiro et al [363] found that *Bauhinia holophylla* extract significantly reduced HepG2 cells proliferation and this reduction was concentration-dependent.

Likewise, the antiproliferative activity of *A. indica* leaf extract against the HepG 2, HeLa and MCF-7 cancer cell lines was measured and the results obtained are in **Figure 73**.

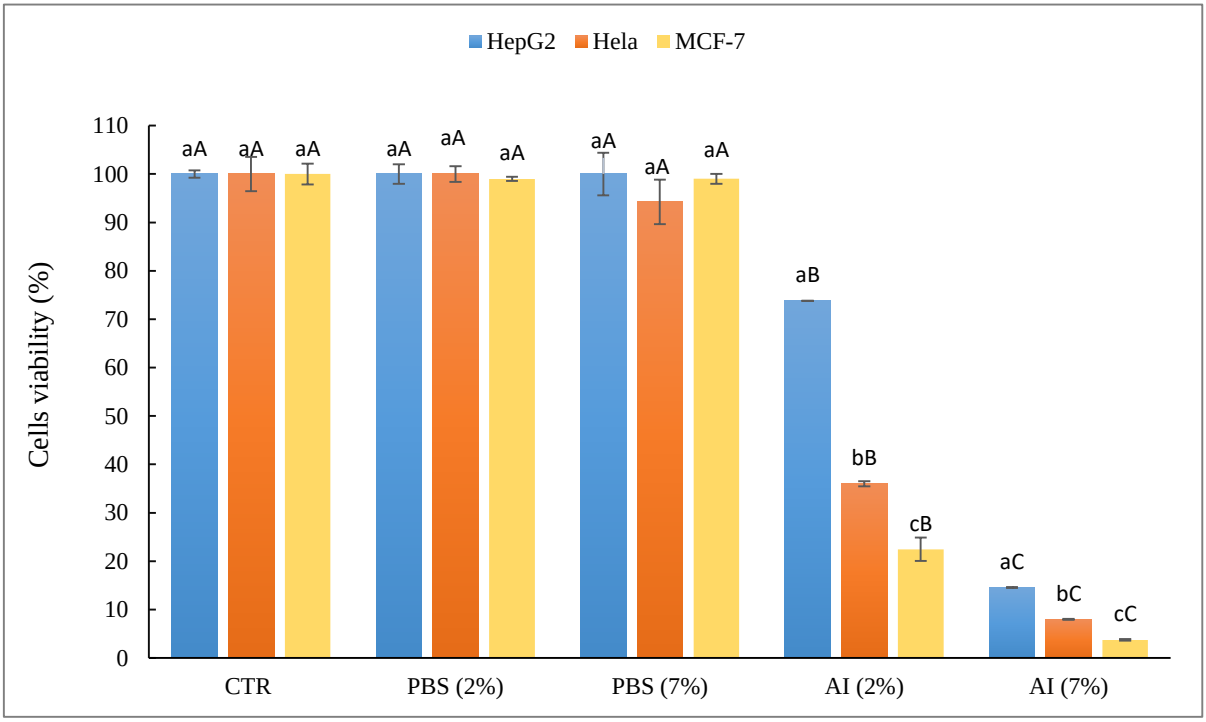

Figure 73. Effect of *A. indica* leaf (AI; 2% and 7%, *v/v*) extract on cells viability assayed 48 h after treatment. CTR, non-treated cells, PBS, control cells incubated in the presence of solvent without extract. Error bars represent the standard deviation (n=3). Lowercase letters indicate significant differences between the three cancer cell lines with the same treatment at $p < 0.05$ and uppercase letters indicate significant differences between the same cancer cell line with different treatments at $p < 0.05$

No significant difference was recorded between the CTR sample and cancer cells treated with the solvent (PBS 2% and 7%, *v/v*). The percentage of cell viability decreased significantly ($p < 0.05$) with increasing extract concentration. *A. indica* (AI) extract at 7%, *v/v*, showed increased antiproliferative activity against the three cancer cell lines tested than the AI at 2%, *v/v*. At both 2% and 7%, *v/v*, the AI extract was more effective in reducing the viability of cell lines derived from breast and cervix adenocarcinoma (MCF-7 and HeLa) than hepatocellular carcinoma-derived cells (HepG2). In HepG2 cells incubated in the presence of AI extract at 2% (*v/v*), reduction of viable cells was 26.22% compared to non-treated cells, while reduction of viable cells with AI at 7% (*v/v*) was 85.43%. Concerning HeLa cells, the AI extract at 2% (*v/v*) and 7% (*v/v*) decreased cancer cells by 64.01% and 85.22%, respectively. Likewise, the AI extract at 7% (*v/v*) was more effective on MCF-7 cancer cells than at 2% (*v/v*), and reduced by 96.26% viable cells.

Extracts of *A. indica* have been used for centuries as a natural remedy against cancer with effects attributed to the bioactive compounds present in the bark, leaves, flowers and seeds with

significant anti-carcinogenic potential against adenocarcinoma and gynecological cancers, such as breast and cervical cancers [364]. Several studies proved the anticancer effect of *A. indica* extracts. For instance, Braga et al [365] evaluated the antiproliferative activity of EtOH extracts of *A. indica* leaves collected from Brazil against MCF-7 cells using the MTT assay and found that 48 h of treatment with *A. indica* extract at a concentration of 1 µg/mL (*v/v*) reduced the viability of MCF-7 cells. Sharma et al [366] evaluated the cytotoxic effect of *A. indica* extracts on MCF-7 and HeLa cells at varying concentrations and reported that *A. indica* extract get rid of more than 40% of MCF-7 cells and 60% of HeLa cells at concentrations of 350 µg/mL and 175 µg/mL, respectively. Other studies also confirmed the sensitivity of MCF-7 and HeLa cancer cells to *A. indica* extract [75, 76, 77]. Leaf extract of *A. indica* was also shown to induce apoptosis in HepG2 cells [369].

6 General Discussion

6 General Discussion

Nature is closely related to the daily life of human beings and the plant kingdom is always a source of inspiration for food and, increasingly, plant species are now used as bioreactors to produce active substances with ever-increasing biological value [370].

Plants frequently accumulate metabolites, mainly phenolic compounds that can be used by humans for different purposes. These important molecules, such as phenolic acids, flavonoids, stilbenes and lignans, etc., are a very wide range of chemical compounds and are characterized by an unequal qualitative and quantitative distribution. One of the main properties of these compounds is their antioxidant activity, which consists in neutralizing free radicals that are harmful for living organisms. Beyond the antioxidant property, phenolic compounds are characterized by their powerful anti-microbial and anti-cancer properties as well as many other biological activities [371].

In the present work, *A. vulneraria* and *A. indica* have been studied since they were widely used in folk medicine for centuries owing to their varied chemical composition and active ingredients. Several previous investigations have focused on the biological properties of *A. vulneraria* and *A. indica* and have demonstrated their efficiency to prevent and treat different diseases. For instance, the consumption of *A. vulneraria* aerial part as an infusion has been shown to treat vomiting, diabetes and stomach disorder [299]. The aerial part also have been used to treat wounds and swelling by direct application [282]. Moreover, *A. vulneraria* leaves have been proven to be effective in inhibiting human herpes-virus and poliovirus [372]. The flowers as well have a potent therapeutic efficacy against wounds, high blood pressure, heart failure, portal hypertension, vomiting, inflammation, acne and throat pain [206]. The *A. indica* is also considered a miraculous tree due to its multiple therapeutic qualities. The documented medicinal virtues of *A. indica* showed that its different parts (leaves, flowers, seeds, roots and bark) have been used to treat several human ailments such as inflammation, diarrhea, bacterial infection, constipation and even cancer [217]. However, although the several researches carried out to demonstrate the biological and therapeutic benefits of the different parts of *A. vulneraria* and *A. indica*, their application in the food sector remains uncertain given the paucity of researches concerning their use in the preservation of foods against deterioration. Therefore, more researches about the effect of the two investigated plants on oxidative stability and shelf life of foods are needed to show the importance and effectiveness of their active compounds and to study their use instead of synthetic additives in products containing fats.

The objectives of the present research work were to determine the chemical composition of *A. vulneraria* leaf and flower extracts and *A. indica* leaf extract, study their antioxidant, antibacterial and antiproliferative properties and evaluate their capacity in delaying lipid oxidation and deterioration of *Model Food Systems* over storage period, as well as identifying and quantifying the main phenolic group responsible for exerting these biological activities through HPLC-MS.

To the best of our knowledge, the present dissertation is the first to determine the antiradical capacity of *A. vulneraria* leaf and flower extract and *A. indica* leaf extract against methoxy radicals using electron paramagnetic resonance spectroscopy technique and the first to study the preservative effect of *A. vulneraria* leaves and flowers against lipid oxidation in *Model Food System* as well as the antiproliferative property against HepG2, HeLa and MCF-7 cancer cell lines.

The chemical composition of *A. vulneraria* leaf and flower and *A. indica* leaf extracts was firstly measured spectrophotometrically in order to determine total polyphenols, flavonoids and condensed tannins contents. The optimization of polyphenols extraction from the different dried samples was assessed by cold maceration and ultrasound assisted extraction methods (solid/liquid, *w/v*) using EtOH solvent at different concentrations. EtOH solvent was chosen to extract phenolic compounds since it is considered one of the most powerful pure natural solvents that does not present a danger for human consumption. In addition, EtOH is labelled by the *Food and Drug Administration* (FDA) as *Generally Recognized As Safe* (GRAS) food substance products [373], hence the possibility of its safe use in pharmaceutical, food and cosmetic products without fear of intoxication.The results obtained showed that, for *A. vulneraria*, the best extraction yield value and the highest phenolics contents were observed in the flowers extracted with 50%-aqueous EtOH by ultrasound assisted extraction method, while the best extraction yield of *A. indica* leaf extract was observed with 80%-aqueous EtOH solvent. Being such an efficient and widely used extraction method on the industrial scale, the ultrasound assisted extraction method saves time as the chemical compounds, such as carotenoids and polyphenols, etc., diffuse more rapidly into the extraction medium, while producing high quality extracts that can be used for foods, supplements and pharmaceutical products [274, 275, 276]. Moreover, Do et al [270] showed that increasing the water concentration in EtOH solvent improves the extraction efficiency and facilitates the extraction of chemical compounds that are soluble in both inorganic and organic solvents [377] and thus, the extraction of the maximum contents of phenolic compounds. The results obtained also

showed a significant difference ($p < 0.05$) in phenolics contents between the different samples. The variability in phenolic compound contents observed in the different plant's parts may be due to biological (vegetative stage), environmental (climate) and technical (methods and extraction solvents polarity) factors [384,73,74]. In our investigation, the estimated phenolic compounds varied significantly with respect to the used plant part and the extraction method and solvent, which is in line with the findings of Zengin et al [232] and Villasante et al [381].

Research about phenolic compounds, particularly phenolic acids and flavonoids, seems to be very advanced by reason of their various physiological properties, such as anti-allergic, anti-inflammatory, antimicrobial, antiviral, antibacterial, anti-carcinogenic, antithrombotic, cardio-protective and vasodilator activities [89]. The beneficial effects of polyphenols are of a particular interest in pharmaceutical, cosmetic and food industry. According to several studies about the positive impact of polyphenol consumption on health and prevention of diseases, manufacturers are now marketing polyphenol-enriched foods and dietary supplements [92]. In addition, their antioxidant activity ensures better preservation of cosmetic and food products by preventing lipid peroxidation [93]. In cosmetic industry, phenolic compounds are added in cosmetic products owing to their well-recognized properties, such as antioxidant, anti-inflammatory, antimicrobial, anti-mutagens and anti-aging activities, as well as their emollients, humectant, wound healing, protective agents against UV-B damage and reducing skin discoloration effects [94].

Over the few last decades, several different analytical methods have been developed and improved in order to measure the antioxidant and antiradical activities of plant's extracts [382]. In the present work, the radical scavenging activity of *A. vulneraria* and *A. indica* extracts was assessed by different analytical methods, including TAC, FRAP, ORAC, DPPH, TEAC and HPS assays and the results obtained showed as well a significant difference ($p < 0.05$) in antiradical assays between the different samples. The results followed the same trend as the phenolics contents and showed that, for *A. vulneraria*, the strongest radical scavenging activities were exhibited by the flowers extracted with 50%-aqueous EtOH by ultrasound assisted extraction method, while for *A. indica* the best antiradical activities were observed with 80%-aqueous EtOH solvent. The significant difference observed between the values obtained by the different antiradical methods can be explained by the type of the methods and their chemical backgrounds. For instance, TAC, FRAP, TEAC and DPPH are spectrometric methods that directly measure antioxidant activity at a specific wavelength, while ORAC is a fluorimetric method that requires a sample to bound with a fluorescent reagent [383]. Moreover,

TEAC and DPPH assays are based on single electron transfer reaction, while ORAC assay is based on hydrogen atom transfer [384].

The determination of the antioxidant and antiradical activities using traditional antioxidant methods was not enough to evaluate the scavenging capacity of the different extracts of *A. vulneraria* and *A.indica* because of their limitation. For this reason, an EPR spectroscopy technique, commonly used in food analysis studies to identify chemical species with very specific unpaired electrons [385], has been appealed to detect one of the most harmful ROS, which is $CH_3O^{\bullet}$ radicals, generated in the Fenton's reaction, via spin trapping and using ferulic acid as antioxidant pattern. The data obtained emphasized the radical scavenging activity found using traditional antiradical techniques and showed that *A. vulneraria* flower extract had better antiradical activity against $CH_3O^{\bullet}$ radicals than *A. vulneraria* leaf extract and ferulic acid. The data also showed that *A. indica* leaf extract had potent scavenging activity of $CH_3O^{\bullet}$ radicals higher than ferulic acid. The variation of the anti-radical activity observed in the different extracts of the two plants investigated depends on the concentration of phenolic compounds. In fact, the values obtained varied proportionally, the higher the polyphenol content, the stronger the antioxidant activity. This agrees with the result found by Kaviarasan et al [310] and Gallego et al [311] who demonstrated that phenolic compounds are indeed responsible for the antioxidant activity of plant extracts [312] due to their ability of scavenging free radicals, by quenching ROS, inhibiting oxidative enzymes and chelating transition metals [393, 394].

Scientific researches were not limited to the extraction and quantification of phenolic compounds but also to the identification of each compound partly responsible for the different biological activities. In this work, the different phenolic compounds in *A. vulneraria* leaf and flower extracts and *A. indica* leaf extract were identified and quantified by HPLC-MS. The HPLC-MS chemical analysis of *A. vulneraria* extracts indicated the presence of different phenolic compounds in the leaf extract, especially phenolic acids, mainly chlorogenic acid, caffeic acid, 2,3-dihydroxybenzoic acid, p-coumaric acid, ferulic acid, sinapinic acid, 4-hydroxy-2-phenylacetic acid, cinnamic acid, 2-hydroxybenzoic acid, p-coumaroyl tartaric acid and 4-hydroxyphenylpropionic acid with different contents, while flavonoids, principally (-)-epicatechin, myricetin, quercetin, delphinidin 3-O sambubioside, rutin and kaempferol-3-O-rutinoside were more present in the flowers extract. Generally, plant leaves contain higher levels of phenolic acids with significant variations observed between different species [386], while flowers contain more flavonoids [387]. Likewise, different phenolic acids, such as chlorogenic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, sinapinic acid, and

flavonoids like (-)-epicatechin, myricetin, quercetin, luteolin C-hexoside and cyanidin 3-O-galactoside were identified in *A. indica* leaf extract at different contents (µg/ g DW). Phenolic acids are one of the main classes of plant phenolic compounds and possess a wide range of biological properties [388]. For example, cinnamic acid and benzoic acid derivatives showed antibacterial, antiviral and antifungal properties [389], as well as antioxidant activity against different type of free radicals by preventing the overproduction of reactive species [406, 407, 408]. Moreover, ferulic acid is considered one of the most common phenolic compound with multiple biological and pharmaceutical properties, such as anti-inflammatory, antimicrobial, and anticancer activities [392] and especially its potent antioxidant capacity due to its phenolic nucleus and unsaturated side chain that can easily form resonance stabilized phenoxy radical. Sinapic acid also showed a strong effect in various pathological conditions such as infections [351], inflammation [393], cancer [394], diabetes [395] and neuro-degeneration [396]. The antioxidant power of caffeic acid has also been confirmed by radical scavenging studies [397]. In addition, chlorogenic acid is one of the most important phenolic acids with several therapeutic effects, such as antioxidant activity, antibacterial, hepatoprotective, cardio-protective, anti-inflammatory, antipyretic, neuro-protective, anti-obesity, antiviral, anti-microbial and anti-hypertension activities [398].

Likewise, flavonoids also have strong biological and therapeutic effects. For instance, quercetin is known by its antioxidant, anti-inflammatory, antibacterial, antiviral, radical-scavenging, gastro-protective and immune-modulatory activities [415, 416]. In addition, myricetin is one of the key ingredients of various foods and beverages well recognized for its nutraceutical value and strong anti-oxidant, anticancer, anti-diabetic and anti-inflammatory activities [401]. Rutin also is commonly used in food industry as an antioxidant thanks to its stability to processing conditions and its possible interactions with other food components [397]. Furthermore, recent research reported that a high consumption of epicatechin is associated with a decreased risk of cardiovascular mortality [402]. All these phenolic compounds constitute useful elements for the treatment and prevention of human health diseases but also strongly participate in the preservation of the quality criteria of food and cosmetics products.

In the present study, the order of antioxidant activity relative to Trolox is FRAP> TEAC> ORAC> DPPH. The order of activity reflects the fact that the major components of the phenolic extract are sinapinic acid, myricetin, luteolin-C-hexoside and cyanidin 3-O-galactoside. The latter 3 compounds contribute to reducing and metal chelating activity due to the presence of

o-dihydroxy phenolic substituents, whereas Trolox does not have this structure. In addition, myricetin has the metal chelating effect of the flavonols structure with the carbonyl group in the C ring forming the chelate ring structure with the phenolic group in the A ring. Hence, the FRAP value is high. The ORAC value is more dependent on radical scavenging, so the value is closer to one. DPPH values are also strongly dependent on radical scavenging, but the radical is a nitrogen radical, and nitrogen is more electronegative than carbon so scavenging is reduced by oxygen containing substituents close to the phenolic hydroxyl group due to the repulsion between the electronegative atoms.

The antioxidant capacity of *A. vulneraria* and *A. indica* samples was not only analysed *in vitro*, but also in *Model Food System*. The oxidation and degradation of food products present great problems for the food industry. Food alterations may occur during the production of foods through to consumption, during the storage of living raw materials or the storage of finished products. A number of physical (colour change), chemical (oxidation), biochemical (destruction of vitamins) and microbiological alterations (fermentation, development of pathogenic microorganisms, and production of toxins) may occur [426, 427]. A wide range of food products, such as oil-in-water emulsions (milk, cream, mayonnaise, margarine, etc.) and meat products (sausages and patties) are easily exposed to deterioration during processing and conservation steps, highlighting the importance of their analysis to find effective solutions and extend their shelf life [405]. The addition of natural antioxidant additives has been successful to preserve food products from oxidation and at the same time improve their nutritional value while ensuring consumer's safety. In the present study, *A. vulneraria* flower extract at 0.25% (*v/v*) was more effective at delaying lipid oxidation in oil-in-water emulsion samples than leaf extract (0.25%, *v/v*) and attained 10 meq hydroperoxides/kg emulsion (standard value fixed by the Codex Alimentarius to measure the stability of products containing edible fats) after 23 days of storage, as it presented higher pH values and lower MDA contents, followed by leaf extract (0.25%, *v/v*) and gallic acid emulsion samples (0.25%, *v/v*), which had a potent preservative effect only at the beginning of the oxidation process. The results showed as well that the gallic acid and *A. indica* added to emulsion samples at 0.5% (*v/v*) had the best protective effect against the formation of the primary oxidation products and were the only samples that remained stable with a pH= 5 for more than 17 days before decreasing to 4.92 and 4.82, respectively at the end of the storage time. Moreover, gallic acid (0.5%, *v/v*) and *A. indica* (0.5%, *v/v*) were the most effective against the formation of secondary oxidation products and presented the lowest TBARS values at the last week of the storage period. The ability of *A. vulneraria* and *A. indica*

extracts to maintain oxidative stability of the emulsion is probably due to the potent antiradical capacity of their phenolic compounds, which causes deactivation of ROS and interruption of the radical propagation chain reaction, chelation of transition metals, such as Fe and Cu, which catalyze the lipid oxidation, as well as reduction in the partial pressure of oxygen [399, 400, 401].

The control and minimization of meat and meat products deterioration is also of great interest to the food industry. Meat products are sensitive to lipid oxidation and microbial spoilage, which are the main reasons for the alteration of their quality during storage. Initially, the oxidation of meat leads to the appearance of volatile chemical species (aldehyde and ketones), which give oxidized meat bad flavour and rancidity and progresses with the change of the red colour and microbial spoilage occurring [409]. The direct incorporation of powdered *A. vulneraria* leaves and flowers at 0.5% (*w/w*) into raw beef patties showed that after 11 days of refrigerated storage, the more effective treatments were raw beef patties formulated with BHT (0.5%, *w/w*), followed by the patties conserved with the powdered *A. vulneraria* flowers (0.5%, *w/w*) and *A. vulneraria* leaves (0.5%, *w/w*) with a potent hydrophilic and lipophilic antioxidant capacity, lower pH values and hexanal contents compared with control sample and TBARS values of 0.36, 0.59 and 0.83 mg MDA/ kg, respectively. In addition, the decrease of (a*), (b*) and (L*) values and the MetMb formation followed a similar trend to that of TBARS values (BHT > *A. vulneraria* flowers > *A. vulneraria* leaves > control). Similarly, powdered *A. indica* leaves and BHT added to raw meat at 0.7% (*w/w*) behaved in the same way and conserved the raw beef patties from lipid oxidation, colour change as well as MetMB, hydroperoxides and hexanal formation. This slow-down of the oxidation process in beef patties formulated with *A. vulneraria* and *A. indica* is at the origin of polyphenols promising substances against meat oxidation. Moreover, the results of the microbial analysis showed that raw beef patties samples conserved with powdered *A. vulneraria* flowers (0.5%, *w/w*) and powdered *A. indica* (0.7%, *w/w*) did not present a microbial growth after 11 days of refrigerated storage, while the antimicrobial effectiveness of *A. vulneraria* leaves (0.5%, *w/w*) decreased and the number of aerobic mesophilic bacteria exceeded 10^4 CFU/g sample. Furthermore, one of the most important criteria that reflects the quality of the food product and encourages the consumer's purchasing desire is its taste. The sensory or "organoleptic" properties of foods cover the appearance, colour, flavour, odour or aroma as well as the texture. In the case of beef patties mixed with the powdered *A. vulneraria* leaves and flowers, the sensory evaluation was discriminative in order to highlight a difference or a similarity between the control and the

formulated patties and the non-trained panel noticed the difference in taste between cooked beef patties formulated with powdered *A. vulneraria* leaves and flowers and the control. However, in the case of beef patties formulated with the powdered *A. indica* leaves, the sensory evaluation was descriptive and hedonic in order to describe the degree of attraction and acceptance of the product and the consumer preference. The consumer's intention to purchase beef patties was high for *A. indica* leaves cooked beef patties (6.1/9 points) and this confirms that the conservation of beef patties with *A. indica* is a successful strategy that guarantees the safety and protection of the by-products from damage without constituting an obstacle to its marketing by food industries and purchase by consumers.

A potent synergistic effect was observed in beef patties formulated with powdered *A. indica* leaves (0.7%, *w/w*) and *C. baccatum* fruits (0.7%, *w/w*). The combination of the two vegetable matters prevented meat deterioration and conserved beef patties from physical, chemical and microbial alterations better than the synthetic preservative (BHT 0.7%, *w/w*), *A. indica* leaves (0.7, *w/w*) and *C. baccatum* fruits (0.7%, *w/w*). This can be explained by the fact that the combination of plants samples is in fact a combination of different phenolic compounds that exist in both plants and therefore the strengthening of the antioxidant and antimicrobial capacity, which ensures better protection of the formulated products. However, during sensory analysis this combination was not appreciated by the panelists possibly because they are not used to eat spicy products. In this sense, the combination of natural additives in meat products depends on the taste and what the consumer is looking for in meat by-products. Its incorporation may be positive, but not always.

A strong link between the consumption of processed meat and the risk of cancer has been shown by various research. Indeed, according to the *World Health Organization* (WHO), an analysis of data from 10 studies has calculated that each 50 g of processed meat consumed every day increases the risk of cancer by about 18% [410]. Several recent researches have evaluated and shown the beneficial effects of extracts rich of phenolic compounds to inhibit cancer cells proliferation. In the present study, *A. vulneraria* leaf and flower extracts at 1% and 5% (*v/v*) and *A. indica* leaf extract at 2% and 7% (*v/v*) showed promising antiproliferative effect against all the cancer cell lines tested. Contrary to what was found in the previous analysis concerning the antioxidant and antiradical activity, *A. vulneraria* leaf extract exhibited a better antiproliferative activity than the flower extract, and showed a potent reducing viability effect against hepatocellular carcinoma-derived cells (HepG2) cancer cells than breast and cervix adenocarcinoma (HeLa and MCF-7). 1% (*v/v*) of *A. vulneraria* leaf extract was enough to

reduce the viability of HepG2, HeLa and MCF-7 cells by 90%, 83% and 46%, respectively, compared to the flower extract (1%, *v/v*), which showed lower antiproliferative effect. The concentration 5% (*v/v*) of *A. vulneraria* leaf and flower extracts reduced the viability of almost all the cancer cells. Conversely, *A. indica* leaf extract showed better reducing viability effect against HeLa and MCF-7 cancer cells than HepG2 and the antiproliferative activity was concentration dependent. In fact, 2% (*v/v*) of *A. indica* leaf extract get rid of only 26.22%, 64.01% and 78.56% of HepG2, HeLa and MCF-7 cells, respectively, while 7%, (*v/v*) of the same extract showed higher reducing viability effect and reduced the viability of all cancer cells, respectively by 85.43%, 92.02% and 96.26%. Several studies have evaluated the capacity of extracts rich in phenolic compounds to inhibit cancer cell proliferation. For instance, Ghasemzadeh and Jaafar [411] reported that extracts of *Pandanus amaryllifolius* are rich in cinnamic and ferulic acids, which has a potent effect on inhibiting breast cancer cell lines *in vitro.* Kurata et al [412] identified caffeic acid in the leaves of the sweet potatoes inhibited the growth of stomach cancer, colon cancer and promyelocytic leukemia. The higher anticancer activity of *A. vulneraria* leaf extract than flower extract may be due to the presence of high content of chlorogenic acid (5-folds higher in leaf extract than in flower extract) and ferulic acid (19-folds higher in leaf extract than in flower extract) known by its antitumor activity and potent capacity to inhibit metastasis in breast cancer cells by regulating epithelial to mesenchymal transition [413]. Chlorogenic acid is also able to inhibit he autophagy and induce cell cycle arrest in human cervical carcinoma cells [414] as well as protecting against DNA damage through increased formation of an amino acid derivative (S-adenosyl-L-homocysteine) in MCF-7 cells [415]. Besides phenolic acids, flavonoids may also contribute to a potent anticancer effect. For instance, a recent study conducted by Lim et al [416] showed the inhibitory effects of delphinidin on the proliferation of ovarian cancer cells. Myricetin is also able to suppress cancer cell invasion and metastasis as well as to induce cell cycle arrest [417].

Bearing in mind that phenolic compounds are only beneficial for health if the bioactive molecules are well absorbed and transported to sites affected by cancer without being metabolized into inactive molecules [433]. The importance of phenolic compounds in preventing cancer requires more in-depth researches.

In addition to chronic diseases caused by processed foods rotten or contaminated, foodborne infections caused by bacteria or their toxins, viruses or parasites or unconventional agents is considered one of the serious problems that threaten food industries and consumer's health. A strong correlation between phenolic compounds and antibacterial activity has been

found to be significant in several recent studies [351]. In this research work, the antimicrobial analysis revealed that *A. vulneraria* leaf extract inhibited the growth of *Staphylococcus aureus*, *Micrococcus luteus, Listeria monocytogenes, Bacillus cereus* and *Escherichia coli*, but it did not show any antimicrobial activity against *Salmonella paratyphi* strain, while flower extract had an antimicrobial activity against all the bacterial strains tested except *Escherichia coli* strain. Moreover, *A. indica* leaf extract inhibited the growth of *Staphylococcus aureus*, *Micrococcus luteus, Escherichia coli* and *Salmonella paratyphi*, while it did not show any antimicrobial activity against *Listeria monocytogenes* and *Bacillus cereus* strains. Polyphenols are endowed with significant antimicrobial activity. Their activity is probably due to their ability to complex with extracellular proteins and make complexes with bacterial cell membrane. One of the most important functions of phenolic compounds, mainly flavonoids, is their role in protective effect against microbial invasion. This involves their accumulation as phytoalexins in response to microbial attack. Due to their ability to inhibit photogenic spore germination in plants, they have also been proposed for use against fungal pathogens in humans [166]. Several studies reported the regular presence of antimicrobial activity in flavonoids. The majority of flavonoids, recognized as antifungal constituents, are isoflavonoids, flavones and flavanones [183]. Moreover, recent researches showed that Gram-positive bacteria membrane is rich in proteins, while Gram-negative strains membrane is mainly assembled into lipopolysaccharides (LPS). Owing to their negative surface charges, LPS prevent the diffusion of hydrophobic molecules, which make Gram-negative bacteria cell membrane a strong barrier with an effective permeability. Contrariwise, proteins in Gram-positive membrane exclude the passage of hydrophilic molecules with high molecular weight, which make Gram-positive bacteria less protected from antibacterial agents [419]. This explains why inhibitory zones for Gram-negative strains are smaller than inhibitory zone for Gram-positive strains. The absence of antimicrobial activity in *A. vulneraria* and *A. indica* extracts against some bacterial strains could be as well explained by the fact that these strains developed resistance mechanisms. Many studies have shown that phenotypic variability may be a strategy put in place by certain microorganisms to resist certain compounds [374, 405]. It is also possible that the solvent used during the extraction may not have been able to extract the desired molecules because of its polarity or concentration. Furthermore, several reports suggested that the antimicrobial activity of herbal extracts required high concentrations [422]. This suggests that the extract concentration used in the present study was lower than that required for antimicrobial activity.

7 Conclusions & Perspectives

7 Conclusions and perspectives

7.1 Conclusions

Part 1

- *A. vulneraria* flowers extracted in 50%-aqueous EtOH by ultrasound assisted extraction method showed higher extraction yield and total polyphenol, flavonoid and condensed tannin contents than leaf extract, while *A. indica* leaves extracted in 80%-aqueous EtOH showed better extraction yield and total polyphenols, flavonoids and condensed tannin contents than EtOH extract.

- Seventeen phenolic compounds were identified in *A. vulneraria* leaf extract by HPLC-MS, while fifteen compounds were characterized in *A. vulneraria* flower extract. The main compounds detected in the leaf extract were phenolic acid, such as chlorogenic acid, benzoic acid derivatives, caffeic acid, p-coumaric acid, p-anisaldehyde, ferulic acid, sinapinic acid, 4-hydroxy-2-phenylacetic acid, cinnamic acid, p-coumaroyl tartaric acid and 4-hydroxyphenylpropionic, and the main compounds detected in the flower extract were principally flavonoids like (-)-epicatechin, quercetin, myricetin, delphinidin 3O sambubioside, rutin and kaempferol-3-O-rutinoside.

- *A. vulneraria* leaf extract was the most abounded source of phenolic acids, of which the contents ranged between 49.58 (p-coumaroyl tartaric acid) and 7985.14 (ferulic acid) µg/ g DW, whereas the contents of flavonoids in *A. vulneraria* flower extract were higher and varied between 64.24 (delphinidin 3-O sambubioside) and 6314.85 (kaempferol-3-O-rutinoside) µg/ g DW.

- Eleven phenolic acid and flavonoids were identified in *A. indica* extract by HPLC-MS. The main phenolic acids detected were chlorogenic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid and sinapic acid, and the flavonoids identified were (-)-epicatechin, myricetin, quercetin, luteolin C-hexoside I and cyaniding 3-O- galactoside.

- *A. indica* leaf extract was rich in phenolic acids with contents ranging between 102.209 (syringic acid) and 7699.18 (sinapinic acid) µg/ g DW, whereas the contents of flavonoids were lower and estimated between 64.23 (delphinidin 3-O sambubios Cyanidin 3-O-galactoside) and 4382.05 (myricetin) µg/ g DW.

Part 2

- *A. vulneraria* flower extract exhibited better antiradical activity that was 1.7-, 1.6-, 1.7, 1.3-, 1.6- and 1.4-folds higher for FRAP, ORAC, TEAC, TAC, DPPH and HPS, respectively than leaf extract. Similarly, *A. vulneraria* 50%-aqueous extracts had effective antiradical activity against $CH_3O^{\cdot}$ radicals measured by EPR spectroscopy assay in the presence of DMPO used as a spin trip and the results obtained emphasized the antiradical capacity determined by the traditional antioxidant activities previously assessed in this study (FRAP, ORAC, TEAC, TAC, DPPH and HPS) and showed that *A. vulneraria* extracts were more efficient than ferulic acid and that flower extract had the best antiradical capacity compared with leaf extract.

- Compared to *A. indica* EtOH extract, the free radical-scavenging capacity of *A. indica* 80%-aqueous EtOH extract was 1.4-folds higher for FRAP and TEAC assays, 1.5-folds higher for ORAC assay, 2.8-better for DPPH assay and was the double for TAC and HPS assays. Moreover, *A. indica* extract showed better antiradical activity against $CH_3O^{\cdot}$ radicals measured by an EPR spectroscopy assay in the presence of DMPO (spin trip) than ferulic acid.

Part 3

- The incorporation of O/W emulsion with *A. vulneraria* leaf and flower extracts at 0.25% (*v/v*) maintained the oxidative stability of emulsion samples and prevented the formation of secondary oxidation products (MDA), and the flower extract (0.25%, *v/v*) showed better preservative effect than leaf extract (0.25%, *v/v*). Likewise, the incorporation of O/W emulsion with *A. indica* leaf extract at 0.25% and 0.5% (*v/v*) maintained the oxidative stability of emulsion samples and prevented the formation of secondary oxidation products and *A. indica* leaf extract (0.5%, *v/v*) showed preservative effect similar to BHT (0.5%, *v/v*).

- The direct addition of powdered *A. vulneraria* leaves and flowers at 0.5% (*w/w*) to ground raw beef provided potent protection against lipid oxidation and maintained the pH acidity and fresh colour stability, as it prevented the metmyoglobin and hexanal formation and inhibited the microbial growth. The powdered flowers (0.5%, *w/w*) exhibited better preservative effect than powdered leaves (0.5, *w/w*). Similarly, powdered *A. indica* leaves at 0.7% (*w/w*) were more effective to prevent raw beef patties deterioration than BHT (0.7%, *w/w*), followed by *C. baccatum* (0.7%, *w/w*).

- The combination of powdered *A. indica* leaves (0.7%, *w/w*) and powdered *C. baccatum* fruits (0.7%, *w/w*) presented a synergistic antioxidant effect in raw beef patties and produced a

combined inhibitory effect of lipid oxidation and formation of secondary products of oxidation in meat greater than the control and the rest of the formulated beef patties.

Part 4

- *A. vulneraria* leaf extract (100 µg/ mL) inhibited the growth of *Staphylococcus aureus*, *Micrococcus luteus, Listeria monocytogenes*, *Bacillus cereus* and *Escherichia coli*, while it did not show any antimicrobial activity against *Salmonella paratyphi* strain, while flower extract had an antimicrobial activity against all the bacterial strains tested except against *E. coli* strain. Moreover, *A. vulneraria* flower extract had better antibacterial activity than leaf extract by having the lowest MIC values.

-*A. indica* leaf extract (100 µg/ mL) inhibited the growth of *Staphylococcus aureus*, *Micrococcus luteus, Escherichia coli* and *Salmonella paratyphi*, while it did not show any antimicrobial activity against *Listeria monocytogenes* and *Bacillus cereus* strains. Moreover, *A. indica* leaf extract had similar antibacterial activity as penicillin (100 µg/ mL) against *Staphylococcus aureus* strain, while it had lowest antimicrobial activity against the rest of the bacterial strains tested.

Part 5

- *A. vulneraria* leaf and flower extracts were more effective in reducing the viability of HepG2 cancer cells, followed by HeLa and MCF-7 cancer cell lines. *A. vulneraria* leaf extract showed better reducing viability effect against all the cancer cells tested than the flower extract. *A. vulneraria* leaf extract at 1% (*v/v*) reduced the viability of HepG2, HeLa and MCF-7 cells by 90%, 83% and 46%, respectively, compared with the flower extract at 1% (*v/v*), which showed lower reducing viability effect. *A. vulneraria* leaf and flower extracts at 5% (*v/v*) get rid of almost all the cancer cells.

- *A. indica* leaf extract had better anticancer activity against HeLa and MCF-7 cancer cells than HepG2 cancer cells. *A. indica* leaf extract at 2% (*v/v*) reduced the viability of HepG2, HeLa and MCF-7 cells by 26.22%, 64.01% and 78.56%, respectively, compared with A. *indica* leaf extract at 7% (*v/v*), which showed higher reducing viability effect get rid of 85.43%, 92.02% and 96.26% of cancer cells respectively.

To summarize, *A. vulneraria* leaves and flowers and *A. indica* leaves had relevant biological properties from a health point of view and represent a great potential as a food additive allowing the preservation of food products and the improvement of their nutritional values.

7.2 Perspectives

For more exploitation of *A. vulneraria* and *A. indica* biological properties it is to be considered as a perspective to develop packaging and encapsulation processes and analyze their possible application in food models and to separate and identify the main bioactive molecule responsible for the antiproliferative activity.

8 References

8 References

[1] V.I. Lushchak, Adaptive response to oxidative stress: Bacteria, fungi, plants and animals, Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 153 (2011) 175–190. https://doi.org/10.1016/j.cbpc.2010.10.004.

[2] G.J. Burton, E. Jauniaux, Oxidative stress., Best Pract. Res. Clin. Obstet. Gynaecol. 25 (2011) 287–299. https://doi.org/10.1016/j.bpobgyn.2010.10.016.

[3] G. Fink, Stress: Physiology, Biochemistry, and Pathology, Volume 3, Academic Press, Australia, 2019.

[4] B. Poljsak, D. Šuput, I. Milisav, Achieving the balance between ROS and antioxidants: When to use the synthetic antioxidants, Oxid. Med. Cell. Longev. 2013 (2013). https://doi.org/10.1155/2013/956792.

[5] H. Yang, R.M. Villani, H. Wang, M.J. Simpson, M.S. Roberts, M. Tang, X. Liang, The role of cellular reactive oxygen species in cancer chemotherapy, J. Exp. Clin. Cancer Res. 37 (2018) 1–10. https://doi.org/10.1186/s13046-018-0909-x.

[6] A.A. Dayem, M.K. Hossain, S. Bin Lee, K. Kim, S.K. Saha, G.M. Yang, H.Y. Choi, S.G. Cho, The role of reactive oxygen species (ROS) in the biological activities of metallic nanoparticles, Int. J. Mol. Sci. 18 (2017) 1–21. https://doi.org/10.3390/ijms18010120.

[7] U.S. Srinivas, B.W.Q. Tan, B.A. Vellayappan, A.D. Jeyasekharan, ROS and the DNA damage response in cancer, Redox Biol. 25 (2019) 101084. https://doi.org/10.1016/j.redox.2018.101084.

[8] S. Manoharan, G.J. Guillemin, R.S. Abiramasundari, M.M. Essa, M. Akbar, M.D. Akbar, The Role of Reactive Oxygen Species in the Pathogenesis of Alzheimer's Disease, Parkinson's Disease, and Huntington's Disease: A Mini Review, Oxid. Med. Cell. Longev. 2016 (2016). https://doi.org/10.1155/2016/8590578.

[9] D. Moris, M. Spartalis, E. Spartalis, G.S. Karachaliou, G.I. Karaolanis, G. Tsourouflis, D.I. Tsilimigras, E. Tzatzaki, S. Theocharis, The role of reactive oxygen species in the pathophysiology of cardiovascular diseases and the clinical significance of myocardial redox, Ann. Transl. Med. 5 (2017) 1–11. https://doi.org/10.21037/atm.2017.06.27.

[10] B. Halliwell, Reactive Species and Antioxidants. Redox Biology Is a Fundamental Theme of Aerobic Life, Plant Physology. 141 (2006) 312–322. https://doi.org/10.1104/pp.106.077073.312.

[11] V. Lobo, A. Patil, A. Phatak, N. Chandra, Free radicals, antioxidants and functional foods: Impact on human health, Pharmacogn. Rev. 4 (2010) 118–126. https://doi.org/10.4103/0973-7847.70902.

[12] A. Phaniendra, D.B. Jestadi, L. Periyasamy, Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases, Indian J. Clin. Biochem. 30 (2015) 11–26. https://doi.org/10.1007/s12291-014-0446-0.

[13] K. Rahman, Studies on free radicals, antioxidants, and co-factors., Clin. Interv. Aging. 2 (2007) 219–236.

[14] V.I. Lushchak, Free radicals, reactive oxygen species, oxidative stress and its classification, Chem. Biol. Interact. 224 (2014) 164–175. https://doi.org/10.1016/j.cbi.2014.10.016.

[15] L. Yang, K.S. Wen, X. Ruan, Y.X. Zhao, F. Wei, Q. Wang, Response of plant secondary metabolites to environmental factors, Molecules. 23 (2018) 1–26. https://doi.org/10.3390/molecules23040762.

[16] V. Sales de Oliveira, I.M. Augusta, M.V. da C. Braz, C.J. Riger, E.R. Prudêncio, A.C.H.F. Sawaya, G.R. Sampaio, E.A.F. da S. Torres, T. Saldanha, Aroeira fruit (Schinus terebinthifolius Raddi) as a natural antioxidant: Chemical constituents, bioactive compounds and in vitro and in vivo antioxidant capacity, Food Chem. 315 (2020) 126274. https://doi.org/10.1016/j.foodchem.2020.126274.

[17] D. Stagos, Antioxidant activity of polyphenolic plant extracts, Antioxidants. 9 (2020). https://doi.org/10.3390/antiox9010019.

[18] P. Santhosh, Advances in Structure and Activity Relationship of Coumarin Derivatives, 1st Editio, Academic Press, India, 2015.

[19] A.N. Li, S. Li, Y.J. Zhang, X.R. Xu, Y.M. Chen, H. Bin Li, Resources and biological activities of natural polyphenols, Nutrients. 6 (2014) 6020–6047. https://doi.org/10.3390/nu6126020.

[20] A. Yashin, Y. Yashin, X. Xia, B. Nemzer, Antioxidant activity of spices and their impact on human health: A review, Antioxidants. 6 (2017) 1–18. https://doi.org/10.3390/antiox6030070.

[21] K.B. Pandey, S.I. Rizvi, Plant polyphenols as dietary antioxidants in human health and disease, Oxid. Med. Cell. Longev. 2 (2009) 270–278. https://doi.org/10.4161/oxim.2.5.9498.

[22] B. Ncube, J.F. Finnie, J. Van Staden, Quality from the field: The impact of environmental factors as quality determinants in medicinal plants, South African J. Bot. 82 (2012) 11–20. https://doi.org/10.1016/j.sajb.2012.05.009.

[23] A.M. Mileo, P. Nisticò, S. Miccadei, Polyphenols: Immunomodulatory and therapeutic implication in colorectal cancer, Front. Immunol. 10 (2019) 1–9. https://doi.org/10.3389/fimmu.2019.00729.

[24] S. Chaa, M.Y. Boufadi, S. Keddari, A.H. Benchaib, J. Soubhye, P. Van Antwerpen, A. Riazi, Chemical composition of propolis extract and its effects on epirubicin-induced hepatotoxicity in rats, Brazilian J. Pharmacogn. 29 (2019) 294–300. https://doi.org/10.1016/j.bjp.2019.01.005.

[25] F.S. Ferreira, G.R. Sampaio, L.M. Keller, A.C.H.F. Sawaya, D.W.H. Chávez, E.A.F.S. Torres, T. Saldanha, Impact of Air Frying on Cholesterol and Fatty Acids Oxidation in Sardines: Protective Effects of Aromatic Herbs, J. Food Sci. 82 (2017) 2823–2831. https://doi.org/10.1111/1750-3841.13967.

[26] V. Sales de Oliveira, F.S. Ferreira, M.C.R. Cople, T. da S. Labre, I.M. Augusta, O.D. Gamallo, T. Saldanha, Use of Natural Antioxidants in the Inhibition of Cholesterol Oxidation: A Review, Compr. Rev. Food Sci. Food Saf. 17 (2018) 1465–1483. https://doi.org/10.1111/1541-4337.12386.

[27] T.M. Olajide, T. Liu, H. Liu, X. Weng, Antioxidant properties of two novel lipophilic derivatives of hydroxytyrosol, Food Chem. 315 (2020) 126197. https://doi.org/10.1016/j.foodchem.2020.126197.

[28] O. Sytar, I. Hemmerich, M. Zivcak, C. Rauh, M. Brestic, Comparative analysis of bioactive phenolic compounds composition from 26 medicinal plants, Saudi J. Biol. Sci. 25 (2018) 631–641. https://doi.org/10.1016/j.sjbs.2016.01.036.

[29] A.C. Bylund-Fellenius, P.M. Walker, A. Elander, S. Holm, J. Holm, T. Scherstén, Energy metabolism in relation to oxygen partial pressure in human skeletal muscle during exercise, Biochem. J. 200 (1981) 247–255. https://doi.org/10.1042/bj2000247.

[30] B.C. Tripathy, R. Oelmüller, Reactive oxygen species generation and signaling in plants, Plant Signal. Behav. 7 (2012) 37–41. https://doi.org/10.4161/psb.22455.

[31] G. Pizzino, N. Irrera, M. Cucinotta, G. Pallio, F. Mannino, V. Arcoraci, F. Squadrito, D. Altavilla, A. Bitto, Oxidative Stress: Harms and Benefits for Human Health, 2017.

https://doi.org/10.1155/2017/8416763.

[32] S. Di Meo, T.T. Reed, P. Venditti, V.M. Victor, Harmful and beneficial role of ROS 2017, Oxid. Med. Cell. Longev. 2018 (2018). https://doi.org/10.1155/2018/5943635.

[33] M. Nita, A. Grzybowski, The Role of the Reactive Oxygen Species and Oxidative Stress in the Pathomechanism of the Age-Related Ocular Diseases and Other Pathologies of the Anterior and Posterior Eye Segments in Adults, Oxid. Med. Cell. Longev. 2016 (2016). https://doi.org/10.1155/2016/3164734.

[34] F. Collin, Chemical basis of reactive oxygen species reactivity and involvement in neurodegenerative diseases, Int. J. Mol. Sci. 20 (2019). https://doi.org/10.3390/ijms20102407.

[35] A. Whaley-connell, H. Zhang, C. Zhang, J.R. Sowers, Diabetic Vascular Disease, First Edit, Elsevier Inc., 2012. https://doi.org/10.1016/B978-0-12-381510-1.00100-9.

[36] R. Champy, D. Mitrovic, P. Collin, A. Lomri, Reactive oxygen species and superoxide dismutases : Role in joint diseases, Jt. Bone Spine. 74 (2007) 324–329. https://doi.org/10.1016/j.jbspin.2007.02.002.

[37] Y. Chen, M.B. Azad, S.B. Gibson, Superoxide is the major reactive oxygen species regulating autophagy, Cell Death Differ. 16 (2009) 1040–1052. https://doi.org/10.1038/cdd.2009.49.

[38] B. Lipinski, Hydroxyl Radical and Its Scavengers in Health and Disease, Hindawi Publ. Corp. Oxidative Med. Cell. Longev. 2011 (2011) 9. https://doi.org/10.1155/2011/809696.

[39] J.A. Stuart, J. Fonseca, F. Moradi, C. Cunningham, B. Seliman, C.R. Worsfold, S. Dolan, J. Abando, L.A. Maddalena, How supraphysiological oxygen levels in standard cell culture affect oxygen-consuming reactions, Oxid. Med. Cell. Longev. 2018 (2018). https://doi.org/10.1155/2018/8238459.

[40] K.M. Naseem, The role of nitric oxide in cardiovascular diseases, Mol. Aspects Med. 26 (2005) 33–65. https://doi.org/10.1016/j.mam.2004.09.003.

[41] S. Habib, A. Ali, Biochemistry of nitric oxide, Indian J. Clin. Biochem. 26 (2011) 3–17. https://doi.org/10.1007/s12291-011-0108-4.

[42] C. Deby, R. Goutier, New Perspectives On The Biochemistry Of Superoxide Anion And The Efficiency Of Superoxide Dismutases, Biochem. Phormaco/Ogy. 39 (1990) 399–405.

[43] Z. Liu, Z. Ren, J. Zhang, C.C. Chuang, E. Kandaswamy, T. Zhou, L. Zuo, Role of ROS and nutritional antioxidants in human diseases, Front. Physiol. 9 (2018) 1–14. https://doi.org/10.3389/fphys.2018.00477.

[44] P.S.J. Lakey, T. Berkemeier, H. Tong, A.M. Arangio, K. Lucas, U. Pöschl, M. Shiraiwa, Chemical exposure-response relationship between air pollutants and reactive oxygen species in the human respiratory tract, Sci. Rep. 6 (2016) 1–6. https://doi.org/10.1038/srep32916.

[45] L.R. Hoyt, M.J. Randall, J.L. Ather, D.P. DePuccio, C.C. Landry, X. Qian, Y.M. Janssen-Heininger, A. van der Vliet, A.E. Dixon, E. Amiel, M.E. Poynter, Mitochondrial ROS induced by chronic ethanol exposure promote hyper-activation of the NLRP3 inflammasome, Redox Biol. 12 (2017) 883–896. https://doi.org/10.1016/j.redox.2017.04.020.

[46] Z. Chen, D. Wang, X. Liu, W. Pei, J. Li, Y. Cao, J. Zhang, Y. An, J. Nie, J. Tong, Oxidative DNA damage is involved in cigarette smoke-induced lung injury in rats, Environ. Health Prev. Med. 20 (2015) 318–324. https://doi.org/10.1007/s12199-015-0469-z.

[47] R. Mian, R. Galen, Impact of particle size and other ingredients on extruded foods and feeds, in: Extrus. Probl. Solved, 2012: p. 184.

[48] K. khan Muhammad, L. Hassan, S. Paniwnyk, Polyphenols as Natural Antioxidants: Sources,

Extraction and Applications in Food, Cosmetics and Drugs, in: Plant Based “Green Chem. 2.0, 2019: pp. 197–235.

[49] B. Salehi, M. Martorell, J.L. Arbiser, A. Sureda, N. Martins, P.K. Maurya, M. Sharifi-Rad, P. Kumar, J. Sharifi-Rad, Antioxidants: Positive or negative actors?, Biomolecules. 8 (2018) 1–11. https://doi.org/10.3390/biom8040124.

[50] M.M. Berger, Nutritional manipulation of oxidative stress: Review of the evidence, Nutr. Clin. Metab. 20 (2006) 48–53. https://doi.org/10.1016/j.nupar.2005.12.005.

[51] J. Goudable, A. Favier, Radicaux libres oxygénés et antioxydants., Nutr. Clin. Métabolisme. 11 (1997) 115–120.

[52] D.W. Wilson, P. Nash, H. Singh, K. Griffiths, R. Singh, F. De Meester, R. Horiuchi, T. Takahashi, The role of food antioxidants, benefits of functional foods, and influence of feeding habits on the health of the older person: An overview, Antioxidants. 6 (2017) 1–20. https://doi.org/10.3390/antiox6040081.

[53] S.E. Turnbull, Cosmetics, An Overv. FDA Regul. Prod. From Drugs Cosmet. to Food Tob. (2018) 217–229. https://doi.org/10.1016/B978-0-12-811155-0.00010-7.

[54] S.C. Lourenço, M. Moldão-Martins, V.D. Alves, Antioxidants of natural plant origins: From sources to food industry applications, Molecules. 24 (2019) 14–16. https://doi.org/10.3390/molecules24224132.

[55] I.D. Thorat, D.D. Jagtap, D. Mohapatra, D.C. Joshi, R.F. Sutar, S.S. Kapdi, Antioxidants, their properties, uses in food products and their legal implications, Int. J. Food Stud. 2 (2013) 81–104. https://doi.org/10.7455/ijfs/2.1.2013.a7.

[56] E. Čolak, S. Ignjatović, A. Radosavljević, L. Žorić, The association of enzymatic and non-enzymatic antioxidant defense parameters with inflammatory markers in patients with exudative form of age-related macular degeneration, J. Clin. Biochem. Nutr. 60 (2017) 100–107. https://doi.org/10.3164/jcbn.16.

[57] K.H.G. Freitas, O. Fatibello-Filho, Simultaneous determination of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in food samples using a carbon composite electrode modified with Cu3(PO4)2 immobilized in polyester resin, Talanta. 81 (2010) 1102–1108. https://doi.org/10.1016/j.talanta.2010.02.004.

[58] S. Fereidoon, Handbook of antioxidants for food preservation, 1st Editio, 2015.

[59] M.H. Gordon, The Mechanism of Antioxidant Action in Vitro, BJF Hudson, London, 1990.

[60] N. Prasad, B. Siddaramaiah, M. Banu, Effect of antioxidant tertiary butyl hydroquinone on the thermal and oxidative stability of sesame oil (sesamum indicum) by ultrasonic studies, J. Food Sci. Technol. 52 (2015) 2238–2246. https://doi.org/10.1007/s13197-014-1276-z.

[61] M. Taghvaei, S.M. Jafari, Application and stability of natural antioxidants in edible oils in order to substitute synthetic additives, J. Food Sci. Technol. 52 (2015) 1272–1282. https://doi.org/10.1007/s13197-013-1080-1.

[62] H.H. Gahruie, S.M.H. Hosseini, M.H. Taghavifard, M.H. Eskandari, M.T. Golmakani, E. Shad, Lipid oxidation, color changes, and microbiological quality of frozen beef burgers incorporated with shirazi thyme, cinnamon, and rosemary extracts, J. Food Qual. 2017 (2017). https://doi.org/10.1155/2017/6350156.

[63] M. Damechki, S. Sotiropoulou, M. Tsimidou, Antioxidant and pro-oxidant factors in oregano and rosemary gourmet olive oils, Grasas y Aceites. 52 (2001) 207–213. https://doi.org/10.3989/gya.2001.v52.i3-4.359.

[64] K. V. Ramana, A.B.M. Reddy, N. V. Ravi Kumar Majeti, S.S. Singhal, Therapeutic potential of

natural antioxidants, Oxid. Med. Cell. Longev. 2018 (2018). https://doi.org/10.1155/2018/9471051.

[65] J.S. Jeeva, J. Sunitha, R. Ananthalakshmi, S. Rajkumari, M. Ramesh, R. Krishnan, Enzymatic antioxidants and its role in oral diseases, J. Pharm. Bioallied Sci. 7 (2015) S331–S333. https://doi.org/10.4103/0975-7406.163438.

[66] A. Valavanidis, T. Vlachogianni, K. Fiotakis, S. Loridas, Pulmonary oxidative stress, inflammation and cancer: Respirable particulate matter, fibrous dusts and ozone as major causes of lung carcinogenesis through reactive oxygen species mechanisms, Int. J. Environ. Res. Public Health. 10 (2013) 3886–3907. https://doi.org/10.3390/ijerph10093886.

[67] O.M. Ighodaro, O.A. Akinloye, First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid, Alexandria J. Med. 54 (2018) 287–293. https://doi.org/10.1016/j.ajme.2017.09.001.

[68] Y. Wang, R. Branicky, A. Noë, S. Hekimi, Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling, J. Cell Biol. 217 (2018) 1915–1928. https://doi.org/10.1083/jcb.201708007.

[69] N.A. Anjum, P. Sharma, S.S. Gill, M. Hasanuzzaman, E.A. Khan, K. Kachhap, A.A. Mohamed, P. Thangavel, G.D. Devi, P. Vasudhevan, A. Sofo, N.A. Khan, A.N. Misra, A.S. Lukatkin, H.P. Singh, E. Pereira, N. Tuteja, Catalase and ascorbate peroxidase—representative H2O2-detoxifying heme enzymes in plants, Environ. Sci. Pollut. Res. 23 (2016) 19002–19029. https://doi.org/10.1007/s11356-016-7309-6.

[70] L.K. Habib, M.T.C. Lee, J. Yang, Inhibitors of catalase-amyloid interactions protect cells from β-amyloid-induced oxidative stress and toxicity, J. Biol. Chem. 285 (2010) 38933–38943. https://doi.org/10.1074/jbc.M110.132860.

[71] J.-L. Schlienger, Nutrition clinique pratique chez l'adulte, l'enfant et la personne âgée, 3rd editio, 2018.

[72] I.I. Ozyigit, E. Filiz, R. Vatansever, K.Y. Kurtoglu, I. Koc, M.X. Öztürk, N.A. Anjum, Identification and comparative analysis of H2O2-scavenging enzymes (ascorbate peroxidase and glutathione peroxidase) in selected plants employing bioinformatics approaches, Front. Plant Sci. 7 (2016) 1–23. https://doi.org/10.3389/fpls.2016.00301.

[73] E. Lubos, J. Loscalzo, D.E. Handy, Glutathione peroxidase-1 in health and disease: From molecular mechanisms to therapeutic opportunities, Antioxidants Redox Signal. 15 (2011) 1957–1997. https://doi.org/10.1089/ars.2010.3586.

[74] P. Chaudhary, A. Sharma, B. Singh, A. Kaur, Bioactivities of phytochemicals present in tomato, J. Food Sci. Technol. (2018). https://doi.org/10.1007/s13197-018-3221-z.

[75] R.F. Cathcart, A unique function for ascorbate, Med. Hypotheses. 35 (1991) 32–37. https://doi.org/10.1016/0306-9877(91)90080-I.

[76] O. Fain, Carences en vitamine C, La Rev. Médecine Interne. 25 (2004) 872–880. https://doi.org/10.1016/j.revmed.2004.03.009.

[77] E. Niki, Evidence for beneficial effects of vitamin E, Korean J. Intern. Med. 30 (2015) 571–579. https://doi.org/10.3904/kjim.2015.30.5.571.

[78] G.W. Burton, M.G. Traber, Vitamin E: Antioxidant activity, biokinetics, and bio availability, Annu. Rev. Nutr. 10 (1990) 357–382.

[79] D.C. Schwenke, Does lack of tocopherols and tocotrienols put women at increased risk of breast cancer?, J. Nutr. Biochem. 13 (2002) 2–20. https://doi.org/10.1016/S0955-2863(01)00207-8.

[80] S.E. Duncan, H.H. Chang, Implications of Light Energy on Food Quality and Packaging Selection, 1st ed., Elsevier Inc., 2012. https://doi.org/10.1016/B978-0-12-394598-3.00002-2.

[81] F.Y. Leung, Trace elements that act as antioxidants in parenteral micronutrition, J. Nutr. Biochem. 9 (1998) 304–307. https://doi.org/10.1016/S0955-2863(98)00018-7.

[82] A.S. Prasad, Zinc is an Antioxidant and Anti-Inflammatory Agent: Its Role in Human Health, Front. Nutr. 1 (2014) 1–10. https://doi.org/10.3389/fnut.2014.00014.

[83] Z.K. Roughead, L.K. Johnson, J.R. Hunt, Dietary Copper Primarily Affects Antioxidant Capacity and Dietary Iron Mainly Affects Iron Status in a Surface Response Study of Female Rats Fed Varying Concentrations of Iron, Zinc and Copper, J. Nutr. 129 (1999) 1368–1376. https://doi.org/10.1093/jn/129.7.1368.

[84] L. Bravo, Polyphenols: Chemistry, Dietary Sources, Metabolism, and Nutritional Significance, Nutr. Rev. 56 (2009) 317–333. https://doi.org/10.1111/j.1753-4887.1998.tb01670.x.

[85] N. Balasundram, K. Sundram, S. Samman, Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses, Food Chem. 99 (2006) 191–203. https://doi.org/10.1016/j.foodchem.2005.07.042.

[86] W. Li, B. Trust, Food Sources of Phenolics Compounds, in: Nat. Prod., Springer, Berlin, Heidelberg, 2013: pp. 2527–2558.

[87] A. Sharma, B. Shahzad, A. Rehman, R. Bhardwaj, M. Landi, B. Zheng, Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress, Molecules. 24 (2019) 1–22. https://doi.org/10.3390/molecules24132452.

[88] T. Vogt, Phenylpropanoid biosynthesis, Mol. Plant. 3 (2010) 2–20. https://doi.org/10.1093/mp/ssp106.

[89] I.M. Generali, D. Skroza, I. Ljubenkov, V. Katalini, Š. Vida, Antioxidant and Antimicrobial Potential of Phenolic Metabolites from Traditionally Used Mediterranean, Foods. 8 (2019) 16.

[90] A.M. Pisoschi, A. Pop, The role of antioxidants in the chemistry of oxidative stress: A review, Eur. J. Med. Chem. 97 (2015) 55–74. https://doi.org/10.1016/j.ejmech.2015.04.040.

[91] G. Gabriela, H. Minna, A. María Pilar, Stability of O/W emulsions packed with PLA film with incorporated rosemary and thyme, Eur. Food Res. Technol. (2016) 1–11. https://doi.org/10.1007/s00217-016-2829-3.

[92] K.R. Martin, C.L. Apple, Polyphenols as dietary supplements: A double-edged sword, Nutr. Diet. Suppl. (2009) 12. https://doi.org/10.2147/nds.s6422.

[93] E. Chang, C.Y. Kim, Lipid Peroxidation and Antioxidant Activities of the Aqueous Rhizome Extract of Rheum officinale Baillon, J. Food Qual. 2018 (2018). https://doi.org/10.1155/2018/5258276.

[94] N. Halla, I.P. Fernandes, S.A. Heleno, P. Costa, Z. Boucherit-Otmani, K. Boucherit, A.E. Rodrigues, I.C.F.R. Ferreira, M.F. Barreiro, Cosmetics preservation: A review on present strategies, Molecules. 23 (2018) 1–41. https://doi.org/10.3390/molecules23071571.

[95] N. Kumar, N. Goel, Phenolic acids: Natural versatile molecules with promising therapeutic applications, Biotechnol. Reports. 24 (2019) e00370. https://doi.org/10.1016/j.btre.2019.e00370.

[96] C. Magnani, V.L.B. Isaac, M.A. Correa, H.R.N. Salgado, Caffeic acid: A review of its potential use in medications and cosmetics, Anal. Methods. 6 (2014) 3203–3210. https://doi.org/10.1039/c3ay41807c.

[97] S. Choubey, L.R. ache. Varughese, V. Kumar, V. Beniwal, Medicinal importance of gallic acid and its ester derivatives: a patent review, Pharm. Pat. Anal. 4 (2015) 305–315.

https://doi.org/10.4155/ppa.15.14.

[98] N. Kahkeshani, F. Farzaei, M. Fotouhi, S.S. Alavi, R. Bahramsoltani, R. Naseri, S. Momtaz, Z. Abbasabadi, R. Rahimi, M.H. Farzaei, A. Bishayee, Pharmacological effects of gallic acid in health and disease: A mechanistic review, Iran. J. Basic Med. Sci. 22 (2019) 225–237. https://doi.org/10.22038/ijbms.2019.32806.7897.

[99] D.I. Tsimogiannis, V. Oreopoulou, The contribution of flavonoid C-ring on the DPPH free radical scavenging efficiency. A kinetic approach for the 3′,4′-hydroxy substituted members, Innov. Food Sci. Emerg. Technol. 7 (2006) 140–146. https://doi.org/10.1016/j.ifset.2005.09.001.

[100] Y. Mukohata, S. Nakabayashi, M. Higashida, Quercetin, an energy transfer inhibitor in photophosphorylation, North-Holl. Biomed. Press. 85 (1978) 215–218.

[101] B.H. Havsteen, The biochemistry and medical significance of the flavonoids, Pharmacol. Ther. 96 (2002) 67–202. https://doi.org/10.1016/S0163-7258(02)00298-X.

[102] M. Higuchi, Antioxidant Properties of Wheat Bran against Oxidative Stress, Elsevier, 2014. https://doi.org/10.1016/B978-0-12-401716-0.00015-5.

[103] A.N. Panche, A.D. Diwan, S.R. Chandra, Flavonoids: an overview, J. Nutr. Sci. 5 (2017) 15. https://doi.org/10.1017/jns.2016.41.

[104] K.T. Chung, T.Y. Wong, C.I. Wei, Y.W. Huang, Y. Lin, Tannins and human health: A review, Crit. Rev. Food Sci. Nutr. 38 (1998) 421–464. https://doi.org/10.1080/10408699891274273.

[105] G. Hussain, J. Huang, A. Rasul, H. Anwar, A. Imran, J. Maqbool, A. Razzaq, N. Aziz, E. ul H. Makhdoom, M. Konuk, T. Sun, Putative roles of plant-derived tannins in neurodegenerative and neuropsychiatry disorders: An updated review, Molecules. 24 (2019). https://doi.org/10.3390/molecules24122213.

[106] X. Su, X. Liu, S. Wang, B. Li, T. Pan, D. Liu, F. Wang, Y. Diao, K. Li, Wound-healing promoting effect of total tannins from Entada phaseoloides (L.) Merr. in rats, Burns. 43 (2017) 830–838. https://doi.org/10.1016/j.burns.2016.10.010.

[107] N. Mahfoudhi, R. Ksouri, S. Hamdi, Nanoemulsions as potential delivery systems for bioactive compounds in food systems: preparation, characterization, and applications in food industry, Elsevier Inc., 2016. https://doi.org/10.1016/b978-0-12-804306-6.00011-8.

[108] M. Muthukumar, B.M. Naveena, S. Vaithiyanathan, A.R. Sen, K. Sureshkumar, Effect of incorporation of Moringa oleifera leaves extract on quality of ground pork patties, J. Food Sci. Technol. 51 (2014) 3172–3180. https://doi.org/10.1007/s13197-012-0831-8.

[109] Monika, U. Janiewicz, A.M. Salejda, G. Krasnowska, M.P. Almajano, Effect of tara (Caesalpinia spinosa) pod powder on the oxidation and colour stability of pork meat batter during chilled storage, Food Technol. Biotechnol. 53 (2015) 419–427. https://doi.org/10.17113/ftb.53.04.15.3932.

[110] N. Aini, M. Azman, M.G. Gallego, L. Juliá, L. Fajari, M. Apilar Almajano, M.G. Miguel, J.O. Rocha, E.A. Abourashed, The Effect of Convolvulus arvensis Dried Extract as a Potential Antioxidant in Food Models, Antioxidants. 4 (2015) 170–184. https://doi.org/10.3390/antiox4010170.

[111] M.G. Gallego, M.H. Gordon, F.J. Segovia, M.P. Almajano, Caesalpinia decapetala Extracts as Inhibitors of Lipid Oxidation in Beef Patties, Molecules. 20 (2015) 13913–13926. https://doi.org/10.3390/molecules200813913.

[112] M.D. Hawashin, F. Al-Juhaimi, I.A.M. Ahmed, K. Ghafoor, E.E. Babiker, Physicochemical, microbiological and sensory evaluation of beef patties incorporated with destoned olive cake powder, Meat Sci. 122 (2016) 32–39. https://doi.org/10.1016/j.meatsci.2016.07.017.

[113] R. Domínguez, M. Pateiro, M. Gagaoua, F.J. Barba, W. Zhang, J.M. Lorenzo, A comprehensive review on lipid oxidation in meat and meat products, Antioxidants. 8 (2019) 1–31. https://doi.org/10.3390/antiox8100429.

[114] A. Catalá, An overview of lipid peroxidation with emphasis in outer segments of photoreceptors and the chemiluminescence assay, Int. J. Biochem. Cell Biol. 38 (2006) 1482–1495. https://doi.org/10.1016/j.biocel.2006.02.010.

[115] H. Yin, L. Xu, N.A. Porter, Free radical lipid peroxidation: Mechanisms and analysis, Chem. Rev. 111 (2011) 5944–5972. https://doi.org/10.1021/cr200084z.

[116] A. Ayala, M.F. Muñoz, A. Sandro, Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal Antonio, Oxid. Med. Cell. Longev. (2014) 31. https://doi.org/10.1007/978-3-211-33303-7_2.

[117] H. Rubbo, S. Parthasarathy, S. Barnes, M. Kirk, B. Kalyanaraman, B.A. Freeman, Nitric oxide inhibition of lipoxygenase-dependent liposome and low-density lipoprotein oxidation: Termination of radical chain propagation reactions and formation of nitrogen-containing oxidized lipid derivatives, Arch. Biochem. Biophys. 324 (1995) 15–25. https://doi.org/10.1006/abbi.1995.9935.

[118] S. Jongberg, C.U. Carlsen, L.H. Skibsted, Peptides as antioxidants and carbonyl quenchers in biological model systems, Free Radic. Res. 43 (2009) 932–942. https://doi.org/10.1080/10715760903137101.

[119] W. James Harper, Model food systems and protein functionality, in: Milk Proteins, 1st ED, 2008: p. 552.

[120] L. Mao, Y.H. Roos, C.G. Biliaderis, S. Miao, Food emulsions as delivery systems for flavor compounds: A review, Crit. Rev. Food Sci. Nutr. 57 (2017) 3173–3187. https://doi.org/10.1080/10408398.2015.1098586.

[121] A.K. Goyal, T. Garg, S. Bhandari, G. Rath, Advancement in pulmonary drug delivery systems for treatment of tuberculosis, Elsevier Inc., 2017. https://doi.org/10.1016/b978-0-323-46143-6.00022-1.

[122] Aparicio, Hardwood, Manual del Aceite de Oliva, 1st editio, 2003.

[123] L. Purriños, R. Bermúdez, D. Franco, J. Carballo, J.M. Lorenzo, Development of Volatile Compounds during the Manufacture of Dry-Cured "Lacón," a Spanish Traditional Meat Product, J. Food Sci. 76 (2011). https://doi.org/10.1111/j.1750-3841.2010.01955.x.

[124] C.F. Ross, D.M. Smith, Use of volatiles as indicators of lipid oxidation in muscle foods, Compr. Rev. Food Sci. Food Saf. 5 (2006) 18–25. https://doi.org/10.1111/j.1541-4337.2006.tb00077.x.

[125] J. Jiang, Y.L. Xiong, Natural antioxidants as food and feed additives to promote health benefits and quality of meat products: A review, Meat Sci. 120 (2016) 107–117. https://doi.org/10.1016/j.meatsci.2016.04.005.

[126] I.A.W. Tan, A.L. Ahmad, B.H. Hameed, Adsorption isotherms, kinetics, thermodynamics and desorption studies of 2,4,6-trichlorophenol on oil palm empty fruit bunch-based activated carbon, J. Hazard. Mater. 164 (2009) 473–482. https://doi.org/10.1016/j.jhazmat.2008.08.025.

[127] K.M. Chan, E.A. De Cker, W.J. Means, Extraction and Activity of Carnosine, a Naturally Occurring Antioxidant in Beef Muscle, J. Food Sci. 58 (1993) 1–4. https://doi.org/10.1111/j.1365-2621.1993.tb03199.x.

[128] H. Chen, J. Wang, Y. Cheng, C. Wang, H. Liu, H. Bian, Y. Pan, J. Sun, W. Han, Application of protein-based films and coatings for food packaging: A review, Polymers (Basel). 11 (2019) 1–32. https://doi.org/10.3390/polym11122039.

[129] R.W.G. Van Willige, Effects of flavour absorption on foods and their packaging materials, 2002. https://library.wur.nl/WebQuery/wurpubs/318502.

[130] C.H. Mannheim, N. Passy, Interaction between packaging materials and foods, Packag. Technol. Sci. 3 (1990) 127–132. https://doi.org/10.1002/pts.2770030303.

[131] N. Khazaei, M. Esmaiili, Z. Emam-Djomeh, Effect of active edible coatings made by basil seed gum and thymol on oil uptake and oxidation in shrimp during deep-fat frying, Carbohydr. Polym. 137 (2016) 249–254. https://doi.org/10.1016/j.carbpol.2015.10.084.

[132] L. Barbosa-Pereira, I. Angulo, J.M. Lagarón, P. Paseiro-Losada, J.M. Cruz, Development of new active packaging films containing bioactive nanocomposites, Innov. Food Sci. Emerg. Technol. 26 (2014) 310–318. https://doi.org/10.1016/j.ifset.2014.06.002.

[133] J.W. Hilton, Les antioxydants: rôles, types et nécessités dans les aliments pour animaux de compagnie., Can. Vet. J. = La Rev. Vet. Can. 30 (1989) 834–837.

[134] S.Y. Cheng, B.J. Wang, Y.M. Weng, Antioxidant and antimicrobial edible zein/chitosan composite films fabricated by incorporation of phenolic compounds and dicarboxylic acids, LWT - Food Sci. Technol. 63 (2015) 115–121. https://doi.org/10.1016/j.lwt.2015.03.030.

[135] M. Calatayud, C. López-De-Dicastillo, G. López-Carballo, D. Vélez, P. Hernández Muñoz, R. Gavara, Active films based on cocoa extract with antioxidant, antimicrobial and biological applications, Food Chem. 139 (2013) 51–58. https://doi.org/10.1016/j.foodchem.2013.01.097.

[136] F. Liu, J. Antoniou, Y. Li, J. Yi, W. Yokoyama, J. Ma, F. Zhong, Preparation of Gelatin Films Incorporated with Tea Polyphenol Nanoparticles for Enhancing Controlled-Release Antioxidant Properties, J. Agric. Food Chem. 63 (2015) 3987–3995. https://doi.org/10.1021/acs.jafc.5b00003.

[137] Z.K. Emiroğlu, G.P. Yemiş, B.K. Coşkun;, K. Candoğan, Antimicrobial activity of soy edible films incorporated with thyme and oregano essential oils on fresh ground beef patties, Meat Sci. 86 (2010) 283–288. https://doi.org/10.1016/j.meatsci.2010.04.016.

[138] K. Rock, S. Brand, J. Moir, M.J. Keeling, Dynamics of infectious diseases, Reports Prog. Phys. 77 (2014). https://doi.org/10.1088/0034-4885/77/2/026602.

[139] B. Anne-Sophie, G. Benoit, Multidrug resistant (or antimicrobial-resistant) pathogens - Alternatives to new antibiotics?, Swiss Med. Wkly. 147 (2017). https://doi.org/10.4414/smw.2017.14553.

[140] S. Basak, P. Singh, M. Rajurkar, Multidrug Resistant and Extensively Drug Resistant Bacteria: A Study, J. Pathog. 2016 (2016) 1–5. https://doi.org/10.1155/2016/4065603.

[141] M.S. Alwash, N. Ibrahim, W.Y. Ahmad, Identification and mode of action of antibacterial components from Melastoma Malabathricum linn leaves, Am. J. Infect. Dis. 9 (2013) 46–58. https://doi.org/10.3844/ajidsp.2013.46.58.

[142] J. Li, S. Xie, S. Ahmed, F. Wang, Y. Gu, C. Zhang, X. Chai, Y. Wu, J. Cai, G. Cheng, Antimicrobial activity and resistance: Influencing factors, Front. Pharmacol. 8 (2017) 1–11. https://doi.org/10.3389/fphar.2017.00364.

[143] J.L. Martínez, Natural antibiotic resistance and contamination by antibiotic resistance determinants: The two ages in the evolution of resistance to antimicrobials, Front. Microbiol. 3 (2012) 2010–2012. https://doi.org/10.3389/fmicb.2012.00001.

[144] G. Gebreyohannes, F. Moges, S. Sahile, N. Raja, Isolation and characterization of potential antibiotic producing actinomycetes from water and sediments of Lake Tana, Ethiopia, Asian Pac. J. Trop. Biomed. 3 (2013) 426–435. https://doi.org/10.1016/S2221-1691(13)60092-1.

[145] F.C. Tenover, Mechanisms of Antimicrobial Resistance in Bacteria, Am. J. Med. 119 (2006).

https://doi.org/10.1016/j.amjmed.2006.03.011.

[146] R.H. Dahal, D.K. Chaudhary, Microbial Infections and Antimicrobial Resistance in Nepal: Current Trends and Recommendations, Open Microbiol. J. 12 (2018) 230–242. https://doi.org/10.2174/1874285801812010230.

[147] J. Davies, D. Davies, Origins and Evolution of Antibiotic Resistance, 74 (2010) 417–433. https://doi.org/10.1128/MMBR.00016-10.

[148] L. Trugo, P. Finglas, Preservation of food, in: B. Caballero (Ed.), Encycl. Food Sci. Nutr., 2nd Editio, 2003: p. 600.

[149] F. Barba, A. de Souza, Sant'Ana, O. Vibeke, M. Koubaa, Effects of Innovative Processing Technologies on Microbial Targets Based on Food Categories, in: Innov. Technol. Food Preserv., 2017: p. 326.

[150] C.M. Galanakis, Food Quality and Shelf Life, in: C.M. Galanakis (Ed.), Nat. Antimicrob. Agents to Improv. Foods Shelf Life, 2019: p. 420.

[151] H. Smith, The role of microbial interactions in infectious disease., Philos. Trans. R. Soc. Lond. B. Biol. Sci. 297 (1982) 551–561. https://doi.org/10.1098/rstb.1982.0060.

[152] J.M. Lorenzo, P.E. Munekata, R. Dominguez, M. Pateiro, J.A. Saraiva, D. Franco, Main groups of microorganisms of relevance for food safety and stability: General aspects and overall description, 2018. https://doi.org/10.1016/B978-0-12-811031-7.00003-0.

[153] J.H.J.H.I. Huis In't Veld, Microbial and biochemical spoilage of foods: An overview, Int. J. Food Microbiol. 33 (1996) 1–18. https://doi.org/10.1016/0168-1605(96)01139-7.

[154] H. Siddig, Factors Affecting the Growth of Microorganisms in Food, in: Prog. Food Preserv., 2012: p. 720.

[155] A. Bevilacqua, M.R. Corbo, M. Sinigaglia, The Microbiological Quality of Food: Foodborne Spoilers, 2017.

[156] J.. Guiraud, Microbiologie alimentaire, Paris, 1998.

[157] A. Lucera, C. Costa, A. Conte, M.A. Del Nobile, Food applications of natural antimicrobial compounds, Front. Microbiol. 3 (2012) 1–13. https://doi.org/10.3389/fmicb.2012.00287.

[158] B.I.B. Ozin, N.E.D.A.M.I. Ukic, I.S.S. Amojlik, E.M.J. Ovin, Antimicrobial and Antioxidant Properties of Rosemary and Sage (Rosmarinus officinalis L . and Salvia officinalis L ., Lamiaceae) Essential Oils, J. Agricutural Food Chem. 55 (2007) 7879–7885.

[159] D. Tamene, M. Endale, Antibacterial Activity of Coumarins and Carbazole Alkaloid from Roots of Clausena anisata, Adv. Pharmacol. Sci. 2019 (2019). https://doi.org/10.1155/2019/5419854.

[160] L.. Prescottl, J.. Harley, D. Klein, Microbiologie, 2nd French, Paris, 2003.

[161] S.C. Burnett-Boothroyd, B.J. McCarthy, Antimicrobial treatments of textiles for hygiene and infection control applications: an industrial perspective, Text. Hyg. Infect. Control. (2011) 196–209. https://doi.org/10.1533/9780857093707.3.196.

[162] M.C. Marjorie, Plant Products as Antimicrobial Agents, Clin. Microbiol. Rev. 12 (1999) 564–582. https://doi.org/10.1590/s0006-87052004000200004.

[163] C. Cueva, M.V. Moreno-Arribas, P.J. Martín-Álvarez, G. Bills, M.F. Vicente, A. Basilio, C.L. Rivas, T. Requena, J.M. Rodríguez, B. Bartolomé, Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria, Res. Microbiol. 161 (2010) 372–382. https://doi.org/10.1016/j.resmic.2010.04.006.

[164] M. Nabil, Flore d ela Tunisie, intech ope, 2012.

[165] T.P.T. Cushnie, A.J. Lamb, Antimicrobial activity of flavonoids, Int. J. Antimicrob. Agents. 26 (2005) 343–356. https://doi.org/10.1016/j.ijantimicag.2005.09.002.

[166] M.H. Cho, S.W. Lee, Phenolic phytoalexins in rice: Biological functions and Biosynthesis, Int. J. Mol. Sci. 16 (2015) 29120–29133. https://doi.org/10.3390/ijms161226152.

[167] L.E. Alcaráz, S.E. Blanco, O.N. Puig, F. Tomás, F.H. Ferretti, Antibacterial activity of flavonoids against methicillin-resistant Staphylococcus aureus strains, J. Theor. Biol. 205 (2000) 231–240. https://doi.org/10.1006/jtbi.2000.2062.

[168] Y. Xie, W. Yang, F. Tang, X. Chen, L. Ren, Antibacterial Activities of Flavonoids: Structure-Activity Relationship and Mechanism, Curr. Med. Chem. 22 (2014) 132–149. https://doi.org/10.2174/09298673216661409161134 43.

[169] S. Pasetto, V. Pardi, R.M. Murata, Anti-HIV-1 activity of flavonoid myricetin on HIV-1 infection in a dual-chamber in vitro model, PLoS One. 9 (2014) 1–18. https://doi.org/10.1371/journal.pone.0115323.

[170] S.M.K. Rates, Plants as source of drugs, Toxicon. 39 (2001) 603–613. https://doi.org/10.1016/S0041-0101(00)00154-9.

[171] N. Gm, D. and Cragg, Natural Products as Sources of New Drugs over the 30 Years, J. Nat. Prod. 75 (2012) 311–335. https://doi.org/10.1021/np200906s.Natural.

[172] A. Tokumitsu, S. Wakitani, M. Takagi, Noninvasive discrimination of human normal cells and malignant tumor cells by phase-shifting laser microscopy, J. Biosci. Bioeng. 109 (2010) 499–503. https://doi.org/10.1016/j.jbiosc.2009.10.020.

[173] C.C. Chang, W.C. Chen, T.F. Ho, H.S. Wu, Y.H. Wei, Development of natural anti-tumor drugs by microorganisms, J. Biosci. Bioeng. 111 (2011) 501–511. https://doi.org/10.1016/j.jbiosc.2010.12.026.

[174] HealthOrganizationWorld, Cancer 12, Cancer. (2018). https://www.who.int/news-room/fact-sheets/detail/cancer.

[175] P. Jha, The hazards of smoking and the benefits of cessation: A critical summation of the epidemiological evidence in high-income countries, Elife. 9 (2020) 1–47. https://doi.org/10.7554/eLife.49979.

[176] R. Reilly, Breast cancer, in: S.J. Enna, D.B. Bylund (Eds.), XPharm Compr. Pharmacol. Ref., 2007.

[177] A. Romm, S. Mills, M. L. Hardy, Breast cancer, in: Bot. Med. Women's Heal., 2010: p. 720.

[178] Y. Chen, J. Ma, F. Wang, J. Hu, A. Cui, C. Wei, Q. Yang, F. Li, Amygdalin induces apoptosis in human cervical cancer cell line HeLa cells, Immunopharmacol. Immunotoxicol. 35 (2013) 43–51. https://doi.org/10.3109/08923973.2012.738688.

[179] X.M. Li, X.G. Luo, J.F. He, N. Wang, H. Zhou, P.L. Yang, T.C. Zhang, Induction of apoptosis in human cervical carcinoma hela cells by active compounds from Hypericum ascyron L., Oncol. Lett. 15 (2018) 3944–3950. https://doi.org/10.3892/ol.2018.7812.

[180] W.F. Scherer, J.T. Syverton, G.O. Gey, Studies on the propagation in vitro of poliomyelitis viruses: IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain hela) derived from an epidermoid carcinoma of the cervix, J. Exp. Med. 97 (1953) 695–710. https://doi.org/10.1084/jem.97.5.695.

[181] A. Braue, C. Dolianitis, G. Varigos, Spontaneous resolution of facial papular mucinosis in a transplant patient, Australas. J. Dermatol. 49 (2008) 164–166. https://doi.org/10.1111/j.1440-0960.2008.00462.x.

[182] L. Gravitz, Liver cancer, Nature. 516 (2014) S1. https://doi.org/10.1038/516S1a.

[183] G.H. Qiu, X. Xie, F. Xu, X. Shi, Y. Wang, L. Deng, Distinctive pharmacological differences between liver cancer cell lines HepG2 and Hep3B, Cytotechnology. 67 (2014) 1–12. https://doi.org/10.1007/s10616-014-9761-9.

[184] S. Saikolappan, B. Kumar, G. Shishodia, S. Koul, H.K. Koul, Reactive oxygen species and cancer: A complex interaction, Cancer Lett. 452 (2019) 132–143. https://doi.org/10.1016/j.canlet.2019.03.020.

[185] H. Truna, M. Parihar, Reactive Oxygen Species and Oxidative Stress, Indian J Physiol Pharmacol. 42 (1998) 440–452. https://doi.org/10.1016/B978-0-12-386525-0.00069-X.

[186] F. Virgili, M. Marino, Regulation of cellular signals from nutritional molecules: a specific role for phytochemicals, beyond antioxidant activity, Free Radic. Biol. Med. 45 (2008) 1205–1216. https://doi.org/10.1016/j.freeradbiomed.2008.08.001.

[187] M.J. Morgan, Z.G. Liu, Crosstalk of reactive oxygen species and NF-κB signaling, Cell Res. 21 (2011) 103–115. https://doi.org/10.1038/cr.2010.178.

[188] Y. Xia, S. Shen, I.M. Verma, NF-κB, an active player in human cancers, Cancer Immunol. Res. 2 (2014) 823–830. https://doi.org/10.1158/2326-6066.CIR-14-0112.

[189] P. Godwin, A.M. Baird, S. Heavey, M. Barr, K. O'Bryrne, K. Gately, Targeting nuclear factor-kappa B to overcome resistance to chemotherapy, Front. Oncol. 3 (2013) 1–10. https://doi.org/10.3389/fonc.2013.00120.

[190] S. Reuter, S.C. Gupta, M.M. Chaturvedi, B.B. Aggarwal, Oxidative stress, inflammation, and cancer: How are they linked?, Free Radic. Biol. Med. 49 (2010) 1603–1616. https://doi.org/10.1016/j.freeradbiomed.2010.09.006.

[191] R. Skeel, S. Khleif, Handbook of cancer chemotherapy, Eighth Edi, Lippincott Williams and Wilking, a Wolters Kluwer business, 2011.

[192] M. Qaiser Baig, Practical radiotherapy and chemotherapy planning, The health sciences publisher, 2016.

[193] G. Goldberg, R. Airley, Cancer chemotherapy: basic science to the clinic, John Wiley, 2020.

[194] M. Hesse, Alkaloids - Nature's Curse or Blessing?, Wiley-VCH, Weinhein, 2002.

[195] J. Mann, S. Davidson, D. Banthorpe, J. Hobbs, J. Harborne, Natural Products: Their Chemistry and Biological Significance, Longman Scientific & Technical, 1994.

[196] V.J. Venditto, E.E. Simanek, Cancer therapies utilizing the camptothecins: A review of the in vivo literature, Mol. Pharm. 7 (2010) 307–349. https://doi.org/10.1021/mp900243b.

[197] W.J. Gradishar, Taxanes for the treatment of metastatic breast cancer, Breast Cancer Basic Clin. Res. 6 (2012) 159–171. https://doi.org/10.4137/BCBCR.S8205.

[198] G.K. Rivera, C.H. Pui, V.M. Santana, C.B. Pratt, W.M. Crist, Epipodophyllotoxins in the treatment of childhood cancer, Cancer Chemother. Pharmacol. 34 (1994) 89–95. https://doi.org/10.1007/BF00684870.

[199] M. Moudi, R. Go, C.Y.S. Yien, M. Nazre, Vinca alkaloids, Int. J. Prev. Med. 4 (2013) 1131–1135. https://doi.org/10.2165/00128415-200711380-00080.

[200] A.T. Mohammad, A.B.N. Mohammad, M.B.A. Mohammad, M. Mohammad, Vinca alkaloid-the second most used alkaloid for cancer treatment- A review, Int. J. Physiol. Nutr. Phys. Educ. 2 (2017) 723–727. https://www.researchgate.net/publication/321727597_Vinca_alkaloid-_the_second_most_used_alkaloid_for_cancer_treatment-_A_review.

[201] S. Nobili, D. Lippi, E. Witort, M. Donnini, L. Bausi, E. Mini, S. Capaccioli, Natural compounds for cancer treatment and prevention, Pharmacol. Res. 59 (2009) 365–378. https://doi.org/10.1016/j.phrs.2009.01.017.

[202] V.B. Cetintas, E. Acikgoz, G. Yigitturk, K. Demir, G. Oktem, F. Oltulu, H. Aktug, Effects of fl avopiridol on critical regulation pathways of CD133, Medicine (Baltimore). 95 (2016) 1–9.

[203] A. Pardee, Y. Li, C. Li, Cancer Therapy with ß-Lapachone, Curr. Cancer Drug Targets. 2 (2005) 227–242. https://doi.org/10.2174/1568009023333854.

[204] C.H. Shen, J.J. Shee, J.Y. Wu, Y.W. Lin, J. Der Wu, Y.W. Liu, Combretastatin A-4 inhibits cell growth and metastasis in bladder cancer cells and retards tumour growth in a murine orthotopic bladder tumour model, Br. J. Pharmacol. 160 (2010) 2008–2027. https://doi.org/10.1111/j.1476-5381.2010.00861.x.

[205] M.E. Nik, A.A. Momtazi-Borojeni, P. Zamani, J.G. Navashenaq, M. Iranshahi, M.R. Jaafari, B. Malaekeh-Nikouei, Targeted-nanoliposomal combretastatin A4 (CA-4) as an efficient antivascular candidate in the metastatic cancer treatment, J. Cell. Physiol. 234 (2019) 14721–14733. https://doi.org/10.1002/jcp.28230.

[206] J. Nartowska, I. Wawer, H. Strzelecka, Triterpenoid sapogenin from Anthyllis vulneraria L., Acta Pol. Pharm. - Drug Res. 58 (2001) 289–291.

[207] M. Halabalaki, A. Urbain, A. Paschali, S. Mitakou, F. Tillequin, A.L. Skaltsounis, Quercetin and kaempferol 3-O-[α-L-rhamnopyranosyl-(→2)-α- L-arabinopyranoside]-7-O-α-L-rhamnopyranosides from Anthyllis hermanniae: Structure determination and conformational studies, J. Nat. Prod. 74 (2011) 1939–1945. https://doi.org/10.1021/np200444n.

[208] M. Nabli, Flore de la Tunisie, 1st Ed, Tunis, 2011.

[209] K. Rola, A morphometric study on Anthyllis vulneraria (Fabaceae) from Poland and its taxonomic implications, Biologia (Bratisl). 67 (2012) 296–309. https://doi.org/10.2478/s11756-012-0013-1.

[210] N. Menković, K. Šavikin, S. Tasić, G. Zdunić, D. Stešević, S. Milosavljević, D. Vincek, Ethnobotanical study on traditional uses of wild medicinal plants in Prokletije Mountains (Montenegro), J. Ethnopharmacol. 133 (2011) 97–107. https://doi.org/10.1016/j.jep.2010.09.008.

[211] J. Chen, X. Fan, J. Zhu, L. Song, Z. Li, F. Lin, R. Yu, H. Xu, J. Zi, Limonoids from seeds of Azadirachta indica A. Juss. and their cytotoxic activity, Acta Pharm. Sin. B. 8 (2018) 639–644. https://doi.org/10.1016/j.apsb.2017.12.009.

[212] R. Al Akeel, A. Mateen, K. Janardhan, V.C. Gupta, Analysis of anti-bacterial and anti oxidative activity of Azadirachta indica bark using various solvents extracts, Saudi J. Biol. Sci. 24 (2017) 11–14. https://doi.org/10.1016/j.sjbs.2015.08.006.

[213] I.P. Ogbuewu, Y.U. Odoemenam, H.O. Obikaonu, M.N. Opara, O.O. Emenalom, M.C. Uchegbu, I.C. Okoli, B.O. Esonu, M.U. Iloeje, The growing importance of neem (Azadirachta indica A. Juss) in agriculture, industry, medicine and environment: A review, Res. J. Med. Plant. 5 (2011) 230–245. https://doi.org/10.3923/rjmp.2011.230.245.

[214] W. Sujarwo, A.P. Keim, G. Caneva, C. Toniolo, M. Nicoletti, Ethnobotanical uses of neem (Azadirachta indica A.Juss.; Meliaceae) leaves in Bali (Indonesia) and the Indian subcontinent in relation with historical background and phytochemical properties, J. Ethnopharmacol. 189 (2016) 186–193. https://doi.org/10.1016/j.jep.2016.05.014.

[215] S. Saleem, | Gulzar Muhammad, M.A. Hussain, | Syed, N.A. Bukhari, S. Nasir, A. Bukhari, A comprehensive review of phytochemical profile, bioactives for pharmaceuticals, and pharmacological attributes of Azadirachta indica, Phyther. Res. (2018) 1–32.

https://doi.org/10.1002/ptr.6076.

[216] V. Kumar, S. Ranjan, V. Akhouri, M.S. Siddiqui, B. Sharma, Anti-hyperglycemic and antioxidant action of Azadirachta indica leaf in alloxan induced type-i diabetes mellitus in experimental diabetic swiss albino mice: A histopathological investigation, Int. J. Pharm. Sci. Res. 9 (2018) 1673–1682. https://doi.org/10.13040/IJPSR.0975-8232.9(4).1673-82.

[217] A. Duangjai, N. Nuengchamnong, L.H. Lee, B.H. Goh, S. Saokaew, N. Suphrom, Characterisation of an extract and fractions of Azadirachta indica flower on cholesterol lowering property and intestinal motility, Nat. Prod. Res. 6419 (2017) 1–4. https://doi.org/10.1080/14786419.2017.1416386.

[218] R. Paul, M. Prasad, N.K. Sah, Anticancer biology of Azadirachta indica L (neem): A mini review, Cancer Biol. Ther. 12 (2011) 467–476. https://doi.org/10.4161/cbt.12.6.16850.

[219] W.L. Morris, M.A. Taylor, The Solanaceous Vegetable Crops: Potato, Tomato, Pepper, and Eggplant, in: Encycl. Appl. Plant Sci., 2nd Editio, Reference Module in Life Sciences, 2017: pp. 55–58.

[220] A. Cronquist, An Integrated System of Classification of Flowering Plants, 1981.

[221] A.R. Zimmer, B. Leonardi, D. Miron, E. Schapoval, J.R. De Oliveira, G. Gosmann, Antioxidant and anti-inflammatory properties of Capsicum baccatum: From traditional use to scientific approach, J. Ethnopharmacol. 139 (2012) 228–233. https://doi.org/10.1016/j.jep.2011.11.005.

[222] G.E.S. Batiha, A. Alqahtani, O.A. Ojo, H.M. Shaheen, L. Wasef, M. Elzeiny, M. Ismail, M. Shalaby, T. Murata, A. Zaragoza-Bastida, N. Rivero-Perez, A.M. Beshbishy, K.I. Kasozi, P. Jeandet, H.F. Hetta, Biological properties, bioactive constituents, and pharmacokinetics of some capsicum spp. And capsaicinoids, Int. J. Mol. Sci. 21 (2020) 1–35. https://doi.org/10.3390/ijms21155179.

[223] V.L. Singleton, J.A. Rossi, J. Jr, Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, Am J Enol Vitic. 16 (1965) 144–158.

[224] F. Segovia Gómez, M.P. Almajano Pablos, Pineapple Waste Extract for Preventing Oxidation in Model Food Systems, J. Food Sci. 81 (2016) C1622–C1628. https://doi.org/10.1111/1750-3841.13341.

[225] R.L. Prior, X. Wu, K. Schaich, Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements, J. Agric. Food Chem. 53 (2005) 4290–4302. https://doi.org/10.1021/jf0502698.

[226] S. Georgé, P. Brat, P. Alter, M.J. Amiot, Rapid determination of polyphenols and vitamin C in plant-derived products, J. Agric. Food Chem. 53 (2005) 1370–1373. https://doi.org/10.1021/jf048396b.

[227] M. Skowyra, M.G. Gallego, F. Segovia, M.P. Almajano, Antioxidant properties of artemisia annua extracts in model food emulsions, Antioxidants. 3 (2014) 116–128. https://doi.org/10.3390/antiox3010116.

[228] D. Mammen, M. Daniel, A critical evaluation on the reliability of two aluminum chloride chelation methods for quantification of flavonoids, Food Chem. 135 (2012) 1365–1368. https://doi.org/10.1016/j.foodchem.2012.05.109.

[229] A. Pękal, K. Pyrzynska, Evaluation of Aluminium Complexation Reaction for Flavonoid Content Assay, Food Anal. Methods. 7 (2014) 1776–1782. https://doi.org/10.1007/s12161-014-9814-x.

[230] R. Julkunen-Tiitto, Phenolic Constituents in the Leaves of Northern Willows: Methods for the Analysis of Certain Phenolics, J. Agric. Food Chem. 33 (1985) 448.

[231] P. Prieto, M. Pineda, M. Aguilar, Spectrophotometric Quantitation of Antioxidant Capacity

through the Formation of a Phosphomolybdenum Complex : Specific Application to the Determination of Vitamin E 1, Anal. Biochem. 269 (1999) 337–341.

[232] G. Zengin, S. Uysal, R. Ceylan, A. Aktumsek, Phenolic constituent, antioxidative and tyrosinase inhibitory activity of Ornithogalum narbonense L. from Turkey: A phytochemical study, Ind. Crops Prod. 70 (2015) 1–6. https://doi.org/10.1016/j.indcrop.2015.03.012.

[233] I.. Benzie, J.. Strain, The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay., Anal. Biochem. 239 (1996) 70–6. https://doi.org/10.1006/abio.1996.0292.

[234] R. Tsao, R. Yang, J.C. Young, Antioxidant Isoflavones in Osage Orange, Maclura pomifera (Raf.) Schneid, J. Agric. Food Chem. 51 (2003) 6445–6451. https://doi.org/10.1021/jf0342369.

[235] A. Wojdyło, J. Oszmiański, R. Czemerys, Antioxidant activity and phenolic compounds in 32 selected herbs, Food Chem. 105 (2007) 940–949. https://doi.org/10.1016/j.foodchem.2007.04.038.

[236] K. Thaipong, U. Boonprakob, K. Crosby, L. Cisneros-Zevallos, D. Hawkins Byrne, Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts, J. Food Compos. Anal. 19 (2006) 669–675. https://doi.org/10.1016/j.jfca.2006.01.003.

[237] G. Cao, H.M. Alessio, R.G. Cutler, Oxygen-radical absorbance capacity assay for antioxidants, Free Radic. Biol. Med. 14 (1993) 303–311. https://doi.org/10.1016/0891-5849(93)90027-R.

[238] A. Azman, M. Gallego, L. Julià, L. Fajari, M.P. Almajano, The effect of convolvulus arvensis dried extract as a potential antioxidant in food models, Antioxidants. 4 (2015) 170–184. https://doi.org/10.3390/antiox4010170.

[239] N.J. Miller, J. Sampson, L.P. Candeias, P.M. Bramley, C.A. Rice-Evans, Antioxidant Properties of Carotenes and Xanthophylls, FEBS Lett. 384 (1996) 240–242.

[240] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radic. Biol. Med. 26 (1999) 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3.

[241] J. Sochor, M. Ryvolova, O. Krystofova, P. Salas, J. Hubalek, V. Adam, L. Trnkova, L. Havel, M. Beklova, J. Zehnalek, I. Provaznik, R. Kizek, Fully automated spectrometric protocols for determination of antioxidant activity: Advantages and disadvantages, Molecules. 15 (2010) 8618–8640. https://doi.org/10.3390/molecules15128618.

[242] G. Gallego, M. Gordon, F. Segovia, M. Skowyra, Antioxidant Properties of Three Aromatic Herbs (Rosemary , Thyme and Lavender) in Oil-in-Water Emulsions, JAOCS, J. Am. Oil Chem. Soc. (2013) 1559–1568. https://doi.org/10.1007/s11746-013-2303-3.

[243] E.K. Zavoisky, Paramagnetic relaxation of liquid solutions for perpendicular fields, J. Phys. 9 (1945) 211–216. http://chiralqubit.eu/zavoisky-JoP-1945-IX-211.

[244] M. Polovka, EPR spectroscopy: A tool to characterize stability and antioxidant properties of foods, J. Food Nutr. Res. 45 (2006) 1–11.

[245] P.T. Gardner, D.B. McPhail, A. Crozier, G.G. Duthie, Electron spin resonance (ESR) spectroscopic assessment of the contribution of quercetin and other flavonols to the antioxidant capacity of red wines, J. Sci. Food Agric. 79 (1999) 1011–1014. https://doi.org/10.1002/(SICI)1097-0010(19990515)79:7<1011::AID-JSFA320>3.0.CO;2-Y.

[246] S. Zang, S. Tian, J. Jiang, D. Han, X. Yu, K. Wang, D. Li, D. Lu, A. Yu, Z. Zhang, Determination of antioxidant capacity of diverse fruits by electron spin resonance (ESR) and UV–vis spectrometries, Food Chem. 221 (2017) 1221–1225. https://doi.org/10.1016/j.foodchem.2016.11.036.

[247] N.A.M. Azman, S. Peirò, L. Fajarí, L. Juliá, M.P. Almajano, N.A.M. Azman, S. Peirò, L. Fajarí, L. Julià, M. Pilar, Radical Scavenging of White Tea and of its Flavonoid Constituents by EPR Spectroscopy Radical Scavenging of White Tea and of its Flavonoid Constituents by EPR Spectroscopy, (2014). https://doi.org/10.1021/jf501707p.

[248] W. Sujarwo, A.P. Keim, Spondias pinnata (L. f.) Kurz. (Anacardiaceae): Profiles and Applications to Diabetes, 2nd ed., Elsevier Inc., 2019. https://doi.org/10.1016/b978-0-12-813822-9.00027-8.

[249] N. Paixão, R. Perestrelo, J.C. Marques, J.S. Câmara, Relationship between antioxidant capacity and total phenolic content of red, rosé and white wines, Food Chem. 105 (2007) 204–214. https://doi.org/10.1016/j.foodchem.2007.04.017.

[250] M.E. Brand-Williams, Cuvelier, C. Berset, Use of a Free Radical Method to Evaluate Antioxidant Activity, Leb. u.-Technol. 30 (1995) 25–30.

[251] E. Shalaby, S.M.M. Shanab, Comparison of DPPH and ABTS assays for determining antioxidant potential of water and methanol extracts of Spirulina platensis, Indian J. Geo-Marine Sci. 42 (2013) 556–564.

[252] R.J. Ruch, S. jun Cheng, J.E. Klaunig, Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from chinese green tea, Carcinogenesis. 10 (1989) 1003–1008. https://doi.org/10.1093/carcin/10.6.1003.

[253] S. Dermiş, S. Can, B. Doru, Determination of peroxide values of some fixed oils by using the mFOX method, Spectrosc. Lett. 45 (2012) 359–363. https://doi.org/10.1080/00387010.2012.666702.

[254] M.J. Del Bano, J. Lorente, J. Castillo, O. Benavente-Garcia, J.A. Del Rio, A. Ortuno, K.-W. Quirin, D. Gerard, Phenolic Diterpenes, Flavones, and Rosmarinic Acid Distribution during the Development of Leaves, Flowers, Stems, and Roots of Rosmarinus officinalis. Antioxidant Activity., J. Agric. Food Chem. 51 (2003) 4247–4253.

[255] A. Ayala, M.F. Muñoz, S. Argüelles, Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal, Oxid. Med. Cell. Longev. 2014 (2014). https://doi.org/10.1155/2014/360438.

[256] M.A. Ghani, C. Barril, D.R. Bedgood, P.D. Prenzler, Development of a method suitable for high-throughput screening to measure antioxidant activity in a linoleic acid emulsion, Antioxidants. 8 (2019). https://doi.org/10.3390/antiox8090366.

[257] Azman, M.H. Gordon, M. Skowyra, F. Segovia, M.P. Almajano, Use of lyophilised and powdered Gentiana lutea root in fresh beef patties stored under different atmospheres, J. Sci. Food Agric. 95 (2015) 1804–1811. https://doi.org/10.1002/jsfa.6878.

[258] R.A. Mancini, D.H. Kropf, M.C. Hunt, D.E. Johnson, Effects of endpoint temperature, pH, and storage time on cooked internal color reversion of pork longissimus chops, J. Muscle Foods. 16 (2005) 16–26. https://doi.org/10.1111/j.1745-4573.2004.07103.x.

[259] Q.P. Yu, D.Y. Feng, J. Xiao, F. Wu, X.J. He, M.H. Xia, T. Dong, Y.H. Liu, H.Z. Tan, S.G. Zou, T. Zheng, X.H. Ou, J.J. Zuo, Studies on meat color, myoglobin content, enzyme activities, and genes associated with oxidative potential of pigs slaughtered at different growth stages, Asian-Australasian J. Anim. Sci. 30 (2017) 1739–1750. https://doi.org/10.5713/ajas.17.0005.

[260] I.K. Karabagias, Volatile profile of raw lamb meat stored at 4 ± 1 ◦ C: The potential of specific aldehyde ratios as indicators of lamb meat quality, Foods. 7 (2018) 1–11. https://doi.org/10.3390/foods7030040.

[261] V. Sirocchi, F. Devlieghere, N. Peelman, G. Sagratini, F. Maggi, S. Vittori, P. Ragaert, Effect of Rosmarinus officinalis L. essential oil combined with different packaging conditions to extend

the shelf life of refrigerated beef meat, Food Chem. (2016). https://doi.org/10.1016/j.foodchem.2016.11.054.

[262] T.. A.. Dutson and Pearson, Quality Attributes and their Measurement in Meat, Poultry and Fish Products, 1994th ed., Springer, Boston, MA, 1994. Springer, Boston, MA.

[263] Y. Lan, Y. Shang, Y. Song, Q. Dong, Changes in the quality of superchilled rabbit meat stored at different temperatures, Meat Sci. 117 (2016) 173–181. https://doi.org/10.1016/j.meatsci.2016.02.017.

[264] T.L. Wheeler, L.S. Papadopoulos, R.K. Miller, Research guidelines for cookery, sensory evaluation, and instrumental tenderness measurements of meat., Second edi, 2016.

[265] M. Everitt, Consumer-Targeted Sensory Quality, Glob. Issues Food Sci. Technol. (2009) 117–128. https://doi.org/10.1016/B978-0-12-374124-0.00008-9.

[266] X.J. Fan, S.Z. Liu, H.H. Li, J. He, J.T. Feng, X. Zhang, H. Yan, Effects of Portulaca oleracea L. extract on lipid oxidation and color of pork meat during refrigerated storage, Meat Sci. 147 (2019) 82–90. https://doi.org/10.1016/j.meatsci.2018.08.022.

[267] S. Manandhar, S. Luitel, R.K. Dahal, In Vitro Antimicrobial Activity of Some Medicinal Plants against Human Pathogenic Bacteria, J. Trop. Med. 2019 (2019). https://doi.org/10.1155/2019/1895340.

[268] A. Gallego, I. Metón, I. V. Baanante, J. Ouazzani, E. Adelin, J. Palazon, M. Bonfill, E. Moyano, Viability-reducing activity of Coryllus avellana L. extracts against human cancer cell lines, Biomed. Pharmacother. 89 (2017) 565–572. https://doi.org/10.1016/j.biopha.2017.02.046.

[269] W. Kchaou, F. Abbès, R. Ben Mansour, C. Blecker, H. Attia, S. Besbes, Phenolic profile, antibacterial and cytotoxic properties of second grade date extract from Tunisian cultivars (Phoenix dactylifera L.), Food Chem. 194 (2016) 1048–1055. https://doi.org/10.1016/j.foodchem.2015.08.120.

[270] Q.D. Do, A.E. Angkawijaya, P.L. Tran-Nguyen, L.H. Huynh, F.E. Soetaredjo, S. Ismadji, Y.H. Ju, Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of Limnophila aromatica, J. Food Drug Anal. 22 (2014) 296–302. https://doi.org/10.1016/j.jfda.2013.11.001.

[271] M.M. Meregalli, B.M.S. Puton, F.D.M. Camera, A.U. Amaral, J. Zeni, R.L. Cansian, M.L. Mignoni, G.T. Backes, Conventional and ultrasound-assisted methods for extraction of bioactive compounds from red araçá peel (Psidium cattleianum Sabine), Arab. J. Chem. 13 (2020) 5800–5809. https://doi.org/10.1016/j.arabjc.2020.04.017.

[272] M. Oroian, F. Dranca, F. Ursachi, Comparative evaluation of maceration, microwave and ultrasonic-assisted extraction of phenolic compounds from propolis, J. Food Sci. Technol. 57 (2020) 70–78. https://doi.org/10.1007/s13197-019-04031-x.

[273] N. Medina-Torres, T. Ayora-Talavera, H. Espinosa-Andrews, A. Sánchez-Contreras, N. Pacheco, Ultrasound assisted extraction for the recovery of phenolic compounds from vegetable sources, Agronomy. 7 (2017). https://doi.org/10.3390/agronomy7030047.

[274] P. Momchev, P. Ciganović, M. Jug, E. Marguí, J. Jablan, M. Zovko Končić, Comparison of Maceration and Ultrasonication for Green Extraction of Phenolic Acids from Echinacea purpurea Aerial Parts, Molecules. 25 (2020). https://doi.org/10.3390/molecules25215142.

[275] S.W. Chan, C.Y. Lee, C.F. Yap, W.M. Wan Aida, C.W. Ho, Optimisation of extraction conditions for phenolic compounds from limau purut (Citrus hystrix) peels, Int. Food Res. J. 16 (2009) 203–213.

[276] B. Sultana, F. Anwar, M. Ashraf, Effect of extraction solvent/technique on the antioxidant

activity of selected medicinal plant extracts, Molecules. 14 (2009) 2167–2180. https://doi.org/10.3390/molecules14062167.

[277] C.W.I. Haminiuk, M.S.V. Plata-Oviedo, G. de Mattos, S.T. Carpes, I.G. Branco, Extraction and quantification of phenolic acids and flavonols from Eugenia pyriformis using different solvents, J. Food Sci. Technol. 51 (2014) 2862–2866. https://doi.org/10.1007/s13197-012-0759-z.

[278] R. Bosse, M. Wirth, A. Konstanz, T. Becker, J. Weiss, M. Gibis, Determination of volatile marker compounds in raw ham using headspace-trap gas chromatography, Food Chem. 219 (2017) 249–259. https://doi.org/10.1016/j.foodchem.2016.09.094.

[279] T. Dhanani, S. Shah, N.A. Gajbhiye, S. Kumar, Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of Withania somnifera, Arab. J. Chem. 10 (2017) S1193–S1199. https://doi.org/10.1016/j.arabjc.2013.02.015.

[280] R. Csepregi, T. Bencsik, N. Papp, Examination of secondary metabolites and antioxidant capacity of Anthyllis vulneraria, Fuchsia sp., Galium mollugo and Veronica beccabunga, Acta Biol. Hung. 67 (2016) 442–446. https://doi.org/10.1556/018.67.2016.4.10.

[281] O. Tusevski, A. Kostovska, A. Iloska, L. Trajkovska, S.G. Simic, Phenolic production and antioxidant properties of some Macedonian medicinal plants, Cent. Eur. J. Biol. 9 (2014) 888–900. https://doi.org/10.2478/s11535-014-0322-1.

[282] D. Godevac, G. Zdunić, K. Šavikin, V. Vajs, N. Menković, Antioxidant activity of nine Fabaceae species growing in Serbia and Montenegro, Fitoterapia. 79 (2008) 185–187. https://doi.org/10.1016/j.fitote.2007.10.001.

[283] M.T. Moradi, A. Karimi, S. Alidadi, L. Hashemi, In vitro anti-herpes simplex virus activity, antioxidant potential and total phenolic compounds of selected iranian medicinal plant extracts, Indian J. Tradit. Knowl. 17 (2018) 255–262.

[284] S.Y. Son, N.K. Kim, S. Lee, D. Singh, G.R. Kim, J.S. Lee, H. sun Yang, J. Yeo, S. Lee, C.H. Lee, Metabolite fingerprinting, pathway analyses, and bioactivity correlations for plant species belonging to the Cornaceae, Fabaceae, and Rosaceae families, Plant Cell Rep. 35 (2016) 1917–1931. https://doi.org/10.1007/s00299-016-2006-y.

[285] M. Skowyra, G. Gallego, Antioxidant properties of aqueous and ethanolic extracts of tara (Caesalpinia spinosa) pods in vitro and in model food emulsions ´ a, J Sci Food Agric. 94 (2013) 911–918. https://doi.org/10.1002/jsfa.6335.

[286] M.G. Gallego, T. Rodriguez, I. Rodriguez, M.P. Almajano, Analytical Characterization of Polyphenols from Tara and Caesalpinia decapetala as Stabilizers of O/W Emulsions, J. Food Sci. 81 (2016) C2676–C2685. https://doi.org/10.1111/1750-3841.13502.

[287] B. Al-Dabbagh, I.A. Elhaty, A. Al Hrout, R. Al Sakkaf, R. El-Awady, S.S. Ashraf, A. Amin, Antioxidant and anticancer activities of Trigonella foenum-graecum, Cassia acutifolia and Rhazya stricta, BMC Complement. Altern. Med. 18 (2018) 1–12. https://doi.org/10.1186/s12906-018-2285-7.

[288] G.L. Chen, S.G. Chen, Y. Xiao, N.L. Fu, Antioxidant capacities and total phenolic contents of 30 flowers, Ind. Crops Prod. 111 (2018) 430–445. https://doi.org/10.1016/j.indcrop.2017.10.051.

[289] A.K. Ghimeray, C.W. Jin, B.K. Ghimire, H.C. Dong, Antioxidant activity and quantitative estimation of azadirachtin and nimbin in Azadirachta Indica A. Juss grown in foothills of Nepal, African J. Biotechnol. 8 (2009) 3084–3091. https://doi.org/10.5897/AJB09.1842.

[290] A. Datta, I.U. Grün, M.T. Kwasniewski, L.N. Fernando, Comparison of Two Adsorbent Based de-Bittering Procedures for Neem (Azadirachta indica A. Juss) Tea- Effect on Polyphenols, Anti-Oxidant Capacity, Color and Volatile Profile, Plant Foods Hum. Nutr. 72 (2017) 88–95. https://doi.org/10.1007/s11130-016-0595-9.

[291] H.S. Khamis Al-Jadidi, M.A. Hossain, Studies on total phenolics, total flavonoids and antimicrobial activity from the leaves crude extracts of neem traditionally used for the treatment of cough and nausea, Beni-Suef Univ. J. Basic Appl. Sci. 4 (2015) 93–98. https://doi.org/10.1016/j.bjbas.2015.05.001.

[292] J. Tang, F.R. Dunshea, H.A.R. Suleria, LC-ESI-QTOF/MS Characterization of Phenolic Compounds from Medicinal Plants (Hops and Juniper Berries) and Their Antioxidant Activity, Foods. 9(1) (2020) 1–25.

[293] Z. Qun-Qun, D. Xin, L. Xin-Guang, G. Wen, L. Ping, Y. Hua, Rapid separation and identification of multiple constituents in Danhong Injection by ultra-high performance liquid chromatography coupled to electrospray ionization quadrupole time-of-flight tandem mass spectrometry, Chin. J. Nat. Med. 147 (2016) 147–160. https://doi.org/10.3724/SP.J.1009.2016.00147.

[294] H. Li, V. Subbiah, C.J. Barrow, F.R. Dunshea, H.A.R. Suleria, Phenolic profiling of five different australian grown apples, Appl. Sci. 11 (2021) 1–21. https://doi.org/10.3390/app11052421.

[295] H. Ben Salah, S. Smaoui, R. Abdennabi, N. Allouche, LC-ESI-MS/MS Phenolic Profile of Volutaria lippii (L.) Cass. Extracts and Evaluation of Their in Vitro Antioxidant, Antiacetylcholinesterase, Antidiabetic, and Antibacterial Activities, Evidence-Based Complement. Altern. Med. 2019 (2019). https://doi.org/10.1155/2019/9814537.

[296] R.H. Mekky, E. Abdel-sattar, A. Segura-carretero, M. del M. Contreras, Metabolic Profiling of the Oil of Sesame of the Egyptian Cultivar 'Giza 32' Employing LC-MS and Tandem MS-Based Untargeted Method, Foods. 10 (2021) 1–21.

[297] F. Buiarelli, F. Coccioli, M. Merolle, R. Jasionowska, A. Terracciano, Identification of hydroxycinnamic acid-tartaric acid esters in wine by HPLC-tandem mass spectrometry, Food Chem. 123 (2010) 827–833. https://doi.org/10.1016/j.foodchem.2010.05.017.

[298] M. da Silva Mathias, R. Rodrigues de Oliveira, Differentiation of the phenolic chemical profiles of Cecropia pachystachya and Cecropia hololeuca, Phytochem. Anal. 30 (2019) 73–82. https://doi.org/10.1002/pca.2791.

[299] R. Csepregi, V. Temesfői, S. Das, Á. Alberti, C.A. Tóth, R. Herczeg, N. Papp, T. Kőszegi, Cytotoxic, antimicrobial, antioxidant properties and effects on cell migration of phenolic compounds of selected transylvanian medicinal plants, Antioxidants. 9 (2020) 1–29. https://doi.org/10.3390/antiox9020166.

[300] R.A. Veloz-García, R. Marín-Martínez, R. Veloz-Rodríguez, C.I. Muñoz-Sánchez, L. Guevara-Olvera, R. Miranda-López, M.M. González-Chavira, I. Torres-Pacheco, S.H. Guzmán-Maldonado, A. Cardador-Martínez, G. Loarca-Piña, R.G. Guevara-González, Antimutagenic and antioxidant activities of cascalote (Caesalpinia cacalaco) phenolics, J. Sci. Food Agric. 84 (2004) 1632–1638. https://doi.org/10.1002/jsfa.1852.

[301] A.A. Feregrino-Pérez, I. Torres-Pacheco, M. Vargas-Hernández, P. V. Munguía-Fragozo, G.F. Loarca-Piña, S.O. Mendoza-Díaz, R. V. Ocampo-Velázquez, E. Rico-García, R.G. Guevara-Gónzalez, Antioxidant and antimutagenic activities of Acacia pennatula pods, J. Sci. Ind. Res. (India). 70 (2011) 859–864.

[302] K. Karakoca, M. Asan-Ozusaglam, Y.S. Cakmak, M. Teksen, Phenolic compounds, biological and antioxidant activities of Onobrychis armena Boiss. & Hut flower and root extracts, Chiang Mai J.Sci. 42 (2015) 376–392.

[303] O. Kenny, T.J. Smyth, C.M. Hewage, N.P. Brunton, Antioxidant properties and quantitative UPLC-MS analysis of phenolic compounds from extracts of fenugreek (Trigonella foenum-graecum) seeds and bitter melon (Momordica charantia) fruit, Food Chem. 141 (2013) 4295–4302. https://doi.org/10.1016/j.foodchem.2013.07.016.

[304] O. Fong Lores, C. Berenguer Rivas, J. de la Vega Acosta, N. Wawoe Díaz, E. Puente Zapata,

Potencial antioxidante de un extracto acuoso de hojas del NIM (Azadirachta Indica A. Juss) TT - Antioxidant potential of an aqueous leaf extract of Neem (Azadirachta Indica A. Juss), Rev. Cuba. Plantas Med. 19 (2014) 205–207. http://scielo.sld.cu/scielo.php?script=sci_arttext&pid=S1028-47962014000200009&lng=es.%0Ahttp://scielo.sld.cu/scielo.php?script=sci_arttext&pid=S1028-47962014000200009.

[305] P. Sithisarn, R. Supabphol, W. Gritsanapan, Antioxidant activity of Siamese neem tree (VP1209), J. Ethnopharmacol. 99 (2005) 109–112. https://doi.org/10.1016/j.jep.2005.02.008.

[306] A. Ácsová, S. Martiniaková, J. Hojerová, Selectedin vitro methods to determine antioxidant activity of hydrophilic/lipophilic substances, Acta Chim. Slovaca. 12 (2020) 200–211. https://doi.org/10.2478/acs-2019-0028.

[307] S. Ou, K.C. Kwok, Ferulic acid: Pharmaceutical functions, preparation and applications in foods, J. Sci. Food Agric. 84 (2004) 1261–1269. https://doi.org/10.1002/jsfa.1873.

[308] N.A.M. Azman, S. Peiró, L. Fajarí, L. Julià, M.P. Almajano, Radical scavenging of white tea and its flavonoid constituents by electron paramagnetic resonance (EPR) spectroscopy, J. Agric. Food Chem. 62 (2014) 5743–5748. https://doi.org/10.1021/jf501707p.

[309] J. Tippel, K. Gies, B. Harbaum-Piayda, A. Steffen-Heins, S. Drusch, Composition of Quillaja saponin extract affects lipid oxidation in oil-in-water emulsions, Food Chem. 221 (2017) 386–394. https://doi.org/10.1016/j.foodchem.2016.10.055.

[310] S. Kaviarasan, G.H. Naik, R. Gangabhagirathi, C. V. Anuradha, K.I. Priyadarsini, In vitro studies on antiradical and antioxidant activities of fenugreek (Trigonella foenum graecum) seeds, Food Chem. 103 (2007) 31–37. https://doi.org/10.1016/j.foodchem.2006.05.064.

[311] M. Gallego, M. Skowyra, M. Gordon, N. Azman, M. Almajano, Effect of Leaves of Caesalpinia decapetala on Oxidative Stability of Oil-in-Water Emulsions., Antioxidants. 6 (2017) 19. https://doi.org/10.3390/antiox6010019.

[312] F. Riazi, F. Zeynali, E. Hoseini, H. Behmadi, S. Savadkoohi, Oxidation phenomena and color properties of grape pomace on nitrite-reduced meat emulsion systems, Meat Sci. 121 (2016) 350–358. https://doi.org/10.1016/j.meatsci.2016.07.008.

[313] A.M. Pisoschi, A. Pop, The role of antioxidants in the chemistry of oxidative stress: A review, Eur. J. Med. Chem. 97 (2015) 55–74. https://doi.org/10.1016/j.ejmech.2015.04.040.

[314] G. Gallego, M. Hakkarainen, M.P. Almajano, Stability of O/W emulsions packed with PLA film with incorporated rosemary and thyme, Eur. Food Res. Technol. 243 (2017) 1249–1259. https://doi.org/10.1007/s00217-016-2829-3.

[315] S. Martillanes, J. Rocha-Pimienta, M.V. Gil, M.C. Ayuso-Yuste, J. Delgado-Adámez, Antioxidant and antimicrobial evaluation of rice bran (Oryza sativa L.) extracts in a mayonnaise-type emulsion, Food Chem. 308 (2020) 125633. https://doi.org/10.1016/j.foodchem.2019.125633.

[316] N. Azman, F. Segovia, X. Martínez-Farré, E. Gil, M. Almajano, Screening of Antioxidant Activity of Gentian Lutea Root and Its Application in Oil-in-Water Emulsions, Antioxidants. 3 (2014) 455–471. https://doi.org/10.3390/antiox3020455.

[317] S. Fruehwirth, S. Egger, D. Kurzbach, J. Windisch, F. Jirsa, T. Flecker, M. Ressler, A.T. Reiner, N. Firat, M. Pignitter, Ingredient-dependent extent of lipid oxidation in margarine, Antioxidants. 10 (2021) 1–17. https://doi.org/10.3390/antiox10010105.

[318] M. Skowyra, V. Falguera, N.A.M. Azman, F. Segovia, M.P. Almajano, The effect of perilla frutescens extract on the oxidative stability of model food emulsions, Antioxidants. 3 (2014) 38–54. https://doi.org/10.3390/antiox3010038.

[319] Y.E. Sun, W.D. Wang, H.W. Chen, C. Li, Autoxidation of unsaturated lipids in food emulsion, Crit. Rev. Food Sci. Nutr. 51 (2011) 453–466. https://doi.org/10.1080/10408391003672086.

[320] L. Zhou, R.J. Elias, Antioxidant and pro-oxidant activity of (-)-epigallocatechin-3-gallate in food emulsions: Influence of pH and phenolic concentration, Food Chem. 138 (2013) 1503–1509. https://doi.org/10.1016/j.foodchem.2012.09.132.

[321] A.D.M. Sørensen, A.M. Haahr, E.M. Becker, L.H. Skibsted, B. Bergenståhl, L. Nilsson, C. Jacobsen, Interactions between iron, phenolic compounds, emulsifiers, and pH in omega-3-enriched oil-in-water emulsions, J. Agric. Food Chem. 56 (2008) 1740–1750. https://doi.org/10.1021/jf072946z.

[322] E.N. Frankel, S.W. Huang, R. Aeschbach, E. Prior, Antioxidant Activity of a Rosemary Extract and Its Constituents, Carnosic Acid, Carnosol, and Rosmarinic Acid, in Bulk Oil and Oil-in-Water Emulsion, J. Agric. Food Chem. 44 (1996) 131–135. https://doi.org/10.1021/jf950374p.

[323] W. Bo, T. Haiyan, X. Dong, Stabilizing the Oil-in-Water Emulsions Using the Mixtures of Dendrobium Officinale Polysaccharides and Gum Arabic or Propylene Glycol Alginate, Molecules. 25 (2020) 759.

[324] E.A. Decker, K. Warner, M.P. Richards, F. Shahidi, Measuring antioxidant effectiveness in food, J. Agric. Food Chem. 53 (2005) 4303–4310. https://doi.org/10.1021/jf058012x.

[325] A. Altunkaya, R. V. Hedegaard, J. Harholt, L. Brimer, V. Gökmen, L.H. Skibsted, Oxidative stability and chemical safety of mayonnaise enriched with grape seed extract, Food Funct. 4 (2013) 1647–1653. https://doi.org/10.1039/c3fo60204d.

[326] S. El-Guendouz, S. Aazza, S.A. Dandlen, N. Majdoub, B. Lyoussi, S. Raposo, M.D. Antunes, V. Gomes, M.G. Miguel, Antioxidant activity of thyme waste extract in O/W emulsions, Antioxidants. 8 (2019) 1–14. https://doi.org/10.3390/antiox8080243.

[327] C. Poyato, I. Navarro-Blasco, M.I. Calvo, R.Y. Cavero, I. Astiasarán, D. Ansorena, Oxidative stability of O/W and W/O/W emulsions: Effect of lipid composition and antioxidant polarity, Food Res. Int. 51 (2013) 132–140. https://doi.org/10.1016/j.foodres.2012.11.032.

[328] M.R. Loizzo, A. Pugliese, M. Bonesi, F. Menichini, R. Tundis, Evaluation of chemical profile and antioxidant activity of twenty cultivars from Capsicum annuum, Capsicum baccatum, Capsicum chacoense and Capsicum chinense: A comparison between fresh and processed peppers, LWT - Food Sci. Technol. 64 (2015) 623–631. https://doi.org/10.1016/j.lwt.2015.06.042.

[329] C. Robert H, T. Patrick A, The antimicrobial properties of chile peppers (Capsicum species) and their uses in Mayan medicine, J. Ethnopharmacol. 52 (1996) 61–70. https://doi.org/10.1055/s-0028-1128744.

[330] V.D. Kappel, G.M. Costa, G. Scola, F.A. Silva, M.F. Landell, P. Valente, D.G. Souza, D.C. Vanz, F.H. Reginatto, J.C.F. Moreira, Phenolic content and antioxidant and antimicrobial properties of fruits of Capsicum baccatum L. var. pendulum at different maturity stages, J. Med. Food. 11 (2008) 267–274. https://doi.org/10.1089/jmf.2007.626.

[331] L. Martínez, I. Cilla, J. A Beltrán, P. Roncalés, Antioxidant effect of rosemary, borage, green tea, pu-erh tea and ascorbic acid on fresh pork sausages packaged in a modified atmosphere: Influence of the presence of sodium chloride, J. Sci. Food Agric. 86 (2007) 1298–1307. https://doi.org/10.1002/jsfa.

[332] J. Villasante, M. Ouerfelli, A. Bobet, I. Metón, M.P. Almajano, The Effects of Pecan Shell, Roselle Flower and Red Pepper on the Quality of Beef Patties during Chilled Storage, Foods. 9 (2020) 1–17.

[333] T.A. Hashimoto, F. Caporaso, C. Toto, L. Were, Antioxidant capacity and sensory impact of

coffee added to ground pork, Eur. Food Res. Technol. 245 (2019) 977–986. https://doi.org/10.1007/s00217-018-3200-7.

[334] J.C. López-Romero, J.F. Ayala-Zavala, E.A. Peña-Ramos, J. Hernández, H. González-Ríos, Antioxidant and antimicrobial activity of Agave angustifolia extract on overall quality and shelf life of pork patties stored under refrigeration, J. Food Sci. Technol. 55 (2018) 4413–4423. https://doi.org/10.1007/s13197-018-3351-3.

[335] G. Manessis, A.I. Kalogianni, T. Lazou, M. Moschovas, I. Bossis, A.I. Gelasakis, Plant-derived natural antioxidants in meat and meat products, Antioxidants. 9 (2020) 1–30. https://doi.org/10.3390/antiox9121215.

[336] E. Muela, C. Sañudo, M.M. Campo, I. Medel, J.A. Beltrán, Effect of freezing method and frozen storage duration on instrumental quality of lamb throughout display, Meat Sci. 84 (2010) 662–669. https://doi.org/10.1016/j.meatsci.2009.10.028.

[337] E. Borch, M.L. Kant-Muermans, Y. Blixt, Bacterial spoilage of meat and cured meat products, Int. J. Food Microbiol. 33 (1996) 103–120. https://doi.org/10.1016/0168-1605(96)01135-X.

[338] M. Chaijan, S. Benjakul, W. Visessanguan, C. Faustman, Changes of pigments and color in sardine (Sardinella gibbosa) and mackerel (Rastrelliger kanagurta) muscle during iced storage, Food Chem. 93 (2005) 607–617. https://doi.org/10.1016/j.foodchem.2004.10.035.

[339] M. Ouerfelli, L.B. Ben Kâab, M.P. Almajano, Radical scavenging and antioxidant activity of anthyllis vulneraria leaves and flowers, Molecules. 23 (2018) 1–17. https://doi.org/10.3390/molecules23071657.

[340] M.E.A. do Prado, V.A.V. Queiroz, V.T. da V. Correia, E.O. Neves, E.F.S. Roncheti, A.C.A. Gonçalves, C.B. de Menezes, F.C.E. de Oliveira, Physicochemical and sensorial characteristics of beef burgers with added tannin and tannin-free whole sorghum flours as isolated soy protein replacer, Meat Sci. 150 (2019) 93–100. https://doi.org/10.1016/j.meatsci.2018.12.006.

[341] O.K. Esmer, R. Irkin, N. Degirmencioglu, A. Degirmencioglu, The effects of modified atmosphere gas composition on microbiological criteria, color and oxidation values of minced beef meat, Meat Sci. 88 (2011) 221–226. https://doi.org/10.1016/j.meatsci.2010.12.021.

[342] R.A. Mancini, M.C. Hunt, Current research in meat color, Meat Sci. 71 (2005) 100–121. https://doi.org/10.1016/j.meatsci.2005.03.003.

[343] C. Papuc, G. V. Goran, C.N. Predescu, V. Nicorescu, G. Stefan, Plant Polyphenols as Antioxidant and Antibacterial Agents for Shelf-Life Extension of Meat and Meat Products: Classification, Structures, Sources, and Action Mechanisms, Compr. Rev. Food Sci. Food Saf. 16 (2017) 1243–1268. https://doi.org/10.1111/1541-4337.12298.

[344] M.A. Zahid, J.K. Seo, J.Y. Park, J.Y. Jeong, S.K. Jin, T.S. Park, H.S. Yang, The effects of natural antioxidants on protein oxidation, lipid oxidation, color, and sensory attributes of beef patties during cold storage at 4℃, Korean J. Food Sci. Anim. Resour. 38 (2018) 1029–1042. https://doi.org/10.5851/kosfa.2018.e36.

[345] R. Domínguez, L. Purriños, C. Pérez-Santaescolástica, M. Pateiro, F.J. Barba, I. Tomasevic, P.C.B. Campagnol, J.M. Lorenzo, Characterization of Volatile Compounds of Dry-Cured Meat Products Using HS-SPME-GC/MS Technique, Food Anal. Methods. (2019). https://doi.org/10.1007/s12161-019-01491-x.

[346] T. Juntachote, E. Berghofer, S. Siebenhandl, F. Bauer, The antioxidative properties of Holy basil and Galangal in cooked ground pork, Meat Sci. 72 (2006) 446–456. https://doi.org/10.1016/j.meatsci.2005.08.009.

[347] J. García-Lomillo, M.L. Gonzalez-SanJose, R. Del Pino-García, M. Ortega-Heras, P. Muñiz-Rodríguez, Antioxidant effect of seasonings derived from wine pomace on lipid oxidation in

refrigerated and frozen beef patties, LWT - Food Sci. Technol. 77 (2017) 85–91. https://doi.org/10.1016/j.lwt.2016.11.038.

[348] S. Koona, S. Budida, Antibacterial Potential of the Extracts of the Leaves of Azadirachta indica Linn., Not. Sci. Biol. 3 (2011) 65–69. https://doi.org/10.15835/nsb315470.

[349] C.E. Realini, M.D. Guàrdia, I. Díaz, J.A. García-Regueiro, J. Arnau, Effects of acerola fruit extract on sensory and shelf-life of salted beef patties from grinds differing in fatty acid composition, Meat Sci. 99 (2014) 18–24. https://doi.org/10.1016/j.meatsci.2014.08.008.

[350] A. Aksoy, Sezer, D.B. Aydin, A. Güven, The effect of some natural antimicrobial substances on the shelf life of beef, Isr. J. Vet. Med. 69 (2014) 114–122.

[351] C.E. Maddox, L.M. Laur, L. Tian, Antibacterial activity of phenolic compounds against the phytopathogen Xylella fastidiosa, Curr. Microbiol. 60 (2010) 53–58. https://doi.org/10.1007/s00284-009-9501-0.

[352] R.R. Anjarasoa, J.R. Lovarintsoa, R.V. Eliane, R.R. Hanitra, A.D.R. Danielle, L.J. Victor, Antimicrobial activity of the extracts of Albizia masikororum R.Vig., a Fabaceae from Madagascar, African J. Microbiol. Res. 12 (2018) 464–469. https://doi.org/10.5897/ajmr2018.8864.

[353] K.S. Mistry, Z. Sanghvi, G. Parmar, S. Shah, The antimicrobial activity of azadirachta indica, mimusops elengi, tinospora cardifolia, ocimum sanctum and 2% chlorhexidine gluconate on common endodontic pathogens: An in vitro study, Eur. J. Dent. 8 (2014) 172–177. https://doi.org/10.4103/1305-7456.130591.

[354] M.P. Chaturvedi, A. Bag, V. Rawat, N.S. Jyala, V. Satyavali, P.K. Jha, Antibacterial activities of neem extracts on diabetic samples Antibacterial Antibacterial Effects of Azadirachta indica Leaf and Bark Extracts in Clinical Isolates of Diabetic Patients, Njirm. 2 (2011) 2230–9969.

[355] U.C. Chibuzo, Antimicrobial Activity of Azadirachta indica (Neem) Leaf Extract on Some Bacteria, Int. J. Curr. Microbiol. Appl. Sci. 8 (2019) 431–437. https://doi.org/10.20546/ijcmas.2019.807.053.

[356] M. Ali, Antityphoid Activity and Phytochemical Screening of Azadirachta Indica Leaf Extracts, Sci. J. Res. Rev. 2 (2020) 0–4. https://doi.org/10.33552/sjrr.2020.02.000541.

[357] K.O. Rayanil, P. Bunchornmaspan, P. Tuntiwachwuttikul, A new phenolic compound with anticancer activity from the wood of Millettia leucantha, Arch. Pharm. Res. 34 (2011) 881–886. https://doi.org/10.1007/s12272-011-0603-4.

[358] R.S. Kumar, B. Rajkapoor, P. Perumal, In vitro and in vivo anticancer activity of Indigofera cassioides Rottl. Ex. DC., Asian Pac. J. Trop. Med. 4 (2011) 379–385. https://doi.org/10.1016/S1995-7645(11)60108-9.

[359] R. Sebastian, B. Jaykar, V. Gomathi, Current status of anticancer research in fabaceae family, 9 (2020) 52–60.

[360] R.B. de L. Souza, Y.M. do Nascimento, R.G. Gouveia, A.L. Souto, M.V. Sobral, V.C.O. Costa, J.I.M. de Melo, M.S. Silva, J.F. Tavares, Dereplication-guided isolation of a new flavonoid triglycoside from Macroptilium martii and its cytotoxicity evaluation, Phytochem. Lett. 39 (2020) 144–150. https://doi.org/10.1016/j.phytol.2020.08.001.

[361] M.A. Salem, M.M. Farid, M. El-Shabrawy, R. Mohammed, S.R. Hussein, M.M. Marzouk, Spectrometric analysis, chemical constituents and cytotoxic evaluation of Astragalus sieberi DC. (Fabaceae), Sci. African. 7 (2020) e00221. https://doi.org/10.1016/j.sciaf.2019.e00221.

[362] A.M.A. Silva, H.C. da Silva, A.O. Monteiro, T.L.G. Lemos, S.M. de Souza, H. V. Santos, P.B. Alves, N.R. Romero, G.M.P. Santiago, Chemical composition, larvicidal and cytotoxic activities

of the leaf essential oil of Bauhinia cheilantha (Bong.) Steud, South African J. Bot. 131 (2020) 369–373. https://doi.org/10.1016/j.sajb.2020.03.011.

[363] D.L. Ribeiro, H.L. Cilião, A.F.L. Specian, J.M. Serpeloni, M.T. De Oliveira, E.A. Varanda, W. Vilegas, L.L. Saldanha, W. Martínez-López, A.L. Dokkedal, I.M.S. Cólus, Phytochemical study and evaluation of cytotoxicity, mutagenicity, cell cycle kinetics and gene expression of Bauhinia holophylla (Bong.) Steud. in HepG2 cells in vitro, Cytotechnology. 70 (2018) 713–728. https://doi.org/10.1007/s10616-017-0173-5.

[364] M.A. Moga, A. Bălan, C.V. Anastasiu, O.G. Dimienescu, C.D. Neculoiu, C. Gavriș, An overview on the anticancer activity of azadirachta indica (Neem) in gynecological cancers, Int. J. Mol. Sci. 19 (2018). https://doi.org/10.3390/ijms19123898.

[365] D.L. Braga, S.T.S. Mota, M.A.P. Zóia, P.M.A.P. Lima, P.C. Orsolin, L. Vecchi, J.C. Nepomuceno, C.R. Fürstenau, Y.C.P. Maia, L.R. Goulart, T.G. Araújo, Ethanolic extracts from azadirachta indica leaves modulate transcriptional levels of hormone receptor variant in breast cancer cell lines, Int. J. Mol. Sci. 19 (2018). https://doi.org/10.3390/ijms19071879.

[366] C. Sharma, A.J. Vas, P. Goala, T.M. Gheewala, T.A. Rizvi, A. Hussain, Ethanolic neem (Azadirachta indica) leaf extract prevents growth of MCF-7 and HeLa cells and potentiates the therapeutic index of cisplatin, J. Oncol. 2014 (2014) 17–20. https://doi.org/10.1155/2014/321754.

[367] P. Elumalai, D.N. Gunadharini, K. Senthilkumar, S. Banudevi, R. Arunkumar, C.S. Benson, G. Sharmila, J. Arunakaran, Ethanolic neem (Azadirachta indica A. Juss) leaf extract induces apoptosis and inhibits the IGF signaling pathway in breast cancer cell lines, Biomed. Prev. Nutr. 2 (2012) 59–68. https://doi.org/10.1016/j.bionut.2011.12.008.

[368] F. Ricci, V. Berardi, G. Risuleo, Differential cytotoxicity of MEX: A component of neem oil whose action is exerted at the cell membrane level, Molecules. 14 (2009) 122–132. https://doi.org/10.3390/molecules14010122.

[369] K. Kavitha, R. Vidya Priyadarsini, P. Anitha, K. Ramalingam, R. Sakthivel, G. Purushothaman, A.K. Singh, D. Karunagaran, S. Nagini, Nimbolide, a neem limonoid abrogates canonical NF-κB and Wnt signaling to induce caspase-dependent apoptosis in human hepatocarcinoma (HepG2) cells, Eur. J. Pharmacol. 681 (2012) 6–14. https://doi.org/10.1016/j.ejphar.2012.01.024.

[370] A.G. Atanasov, B. Waltenberger, E.M. Pferschy-Wenzig, T. Linder, C. Wawrosch, P. Uhrin, V. Temml, L. Wang, S. Schwaiger, E.H. Heiss, J.M. Rollinger, D. Schuster, J.M. Breuss, V. Bochkov, M.D. Mihovilovic, B. Kopp, R. Bauer, V.M. Dirsch, H. Stuppner, Discovery and resupply of pharmacologically active plant-derived natural products: A review, Biotechnol. Adv. 33 (2015) 1582–1614. https://doi.org/10.1016/j.biotechadv.2015.08.001.

[371] T. Wang, R. Jónsdóttir, G. Ólafsdóttir, Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds, Food Chem. 116 (2009) 240–248. https://doi.org/10.1016/j.foodchem.2009.02.041.

[372] A.G. Suganda, M. Amoros, L. Girre, B. Fauconnier, Effets inhibiteurs de quelques extraits bruts et semi purifiés de plante indigènes Françaises sur la multiplication de l'herpes virus humain 1 et du poliovirus humain 2 en culture cellulaire, J. Nat. Prod. 46 (1983) 626–32. http://www.ncbi.nlm.nih.gov/pubmed/6317803.

[373] J. Alzeer, K. Abou Hadeed, Ethanol and its Halal status in food industries, Trends Food Sci. Technol. 58 (2016) 14–20. https://doi.org/10.1016/j.tifs.2016.10.018.

[374] M.N. Safdar, T. Kausar, M. Nadeem, Comparison of Ultrasound and Maceration Techniques for the Extraction of Polyphenols from the Mango Peel, J. Food Process. Preserv. 41 (2017). https://doi.org/10.1111/jfpp.13028.

[375] J. Deng, Z. Xu, C. Xiang, J. Liu, L. Zhou, T. Li, Z. Yang, C. Ding, Comparative evaluation of maceration and ultrasonic-assisted extraction of phenolic compounds from fresh olives, Ultrason. Sonochem. 37 (2017) 328–334. https://doi.org/10.1016/j.ultsonch.2017.01.023.

[376] J.F. Osorio-Tobón, Recent advances and comparisons of conventional and alternative extraction techniques of phenolic compounds, J. Food Sci. Technol. 57 (2020) 4299–4315. https://doi.org/10.1007/s13197-020-04433-2.

[377] K.Y. Khaw, M.O. Parat, P.N. Shaw, J.R. Falconer, Solvent supercritical fluid technologies to extract bioactive compounds from natural sources: A review, Molecules. 22 (2017). https://doi.org/10.3390/molecules22071186.

[378] R. Ksouri, W. Megdiche, H. Falleh, N. Trabelsi, M. Boulaaba, A. Smaoui, C. Abdelly, Influence of biological, environmental and technical factors on phenolic content and antioxidant activities of Tunisian halophytes, Comptes Rendus - Biol. 331 (2008) 865–873. https://doi.org/10.1016/j.crvi.2008.07.024.

[379] B.L. Sampaio, M.T.F. Bara, P.H. Ferri, S. da Costa Santos, J.R. de Paula, Influence of environmental factors on the concentration of phenolic compounds in leaves of Lafoensia pacari, Brazilian J. Pharmacogn. 21 (2011) 1127–1137. https://doi.org/10.1590/S0102-695X2011005000177.

[380] W. Liu, D. Yin, N. Li, X. Hou, D. Wang, D. Li, J. Liu, Influence of environmental factors on the active substance production and antioxidant activity in Potentilla fruticosa L. and its quality assessment, Sci. Rep. 6 (2016) 1–18. https://doi.org/10.1038/srep28591.

[381] J. Villasante, M. Girbal, I. Metón, M.P. Almajano, Effects of pecan nut (Carya illinoiensis) and roselle flower (Hibiscus sabdariffa) as antioxidant and antimicrobial agents for sardines (Sardina pilchardus), Molecules. 24 (2019). https://doi.org/10.3390/molecules24010085.

[382] U. Cornelli, Antioxidant use in nutraceuticals, Clin. Dermatol. 27 (2009) 175–194. https://doi.org/10.1016/j.clindermatol.2008.01.010.

[383] R. Lahue, Spectrophotometry and Fluorometry, in: Methods Neurobiol., 1981: pp. 339–423. https://doi.org/10.1007/978-1-4684-3806-2_6.

[384] R. Apak, M. Özyürek, K. Güçlü, E. Çapanoɨlu, Antioxidant activity/capacity measurement. 1. Classification, physicochemical principles, mechanisms, and electron transfer (ET)-based assays, J. Agric. Food Chem. 64 (2016) 997–1027. https://doi.org/10.1021/acs.jafc.5b04739.

[385] H. Kameya, J. Watanabe, Y. Takano-Ishikawa, S. Todoriki, Comparison of scavenging capacities of vegetables by ORAC and EPR, Food Chem. 145 (2014) 866–873. https://doi.org/10.1016/j.foodchem.2013.09.015.

[386] J. Dai, R.J. Mumper, Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties, Molecules. 15 (2010) 7313–7352. https://doi.org/10.3390/molecules15107313.

[387] F. Ververidis, E. Trantas, C. Douglas, G. Vollmer, G. Kretzschmar, N. Panopoulos, Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part II: Reconstruction of multienzyme pathways in plants and microbes, Biotechnol. J. 2 (2007) 1235–1249. https://doi.org/10.1002/biot.200700184.

[388] N. Kumar, N. Goel, Phenolic acids : Natural versatile molecules with promising therapeutic applications, Biotechnol. Reports. 24 (2019) e00370. https://doi.org/10.1016/j.btre.2019.e00370.

[389] M. Sova, Antioxidant and Antimicrobial Activities of Cinnamic Acid Derivatives, Mini-Reviews Med. Chem. 12 (2012) 749–767. https://doi.org/10.2174/138955712801264792.

[390] B. Velika, I. Kron, Antioxidant properties of benzoic acid derivatives against Superoxide radical, Free Radicals Antioxidants. 2 (2012) 62–67. https://doi.org/10.5530/ax.2012.4.11.

[391] E.S. Park, W.S. Moon, M.J. Song, M.N. Kim, K.H. Chung, J.S. Yoon, Antimicrobial activity of phenol and benzoic acid derivatives, Int. Biodeterior. Biodegrad. 47 (2001) 209–214. https://doi.org/10.1016/S0964-8305(01)00058-0.

[392] R. van Lith, G.A. Ameer, Antioxidant Polymers as Biomaterial, Oxidative Stress Biomater. (2016) 251–296. https://doi.org/10.1016/B978-0-12-803269-5.00010-3.

[393] K.-J. Yun, D.-J. Koh, S.-H. Kim, S.J. Park, J.H. Ryu, D.-G. Kim, K.-T. Lee, J.-Y. Lee, Anti-inflammatory effects of sinapic acid through the suppression of inducible nitric oxide synthase , cyclooxygase-2 , and proinflammatory cytokines expressions via nuclear factor- K B inactivation, J. Agric. Food Chem. 56 (2008) 10265–10272.

[394] E.A. Hudson, P.A. Dinh, T. Kokubun, M.S.J. Simmonds, A. Gescher, Characterization of Potentially Chemopreventive Phenols in Extracts of Brown Rice That Inhibit the Growth of Human Breast and Colon Cancer Cells 1, 9 (2000) 1163–1170.

[395] G. Kanchana, W.J. Shyni, M. Rajadurai, R. Periasamy, Evaluation of Antihyperglycemic Effect of Sinapic Acid in Normal and Streptozotocin-Induced Diabetes in Albino Rats, 5 (2011) 33–39.

[396] X.S. Un, H.I. To, T.M. Asuoka, C.K. Amei, T.H. Atano, Effect of Polygala tenuifolia Root Extract on Scopolamine-Induced Impairment of Rat Spatial Cognition in an Eight-Arm Radial Maze Task, 30 (2007) 1727–1731.

[397] S. Mathew, T.E. Abraham, Z.A. Zakaria, Reactivity of phenolic compounds towards free radicals under in vitro conditions, J. Food Sci. Technol. 52 (2015) 5790–5798. https://doi.org/10.1007/s13197-014-1704-0.

[398] M. Naveed, V. Hejazi, M. Abbas, A.A. Kamboh, G.J. Khan, M. Shumzaid, F. Ahmad, D. Babazadeh, X. FangFang, F. Modarresi-Ghazani, L. WenHua, Z. XiaoHui, Chlorogenic acid (CGA): A pharmacological review and call for further research, Biomed. Pharmacother. 97 (2018) 67–74. https://doi.org/10.1016/j.biopha.2017.10.064.

[399] A. Victor, A. David, R. Arulmoli, S. Parasuraman, Overviews of Biological Importance of Quercetin : A Bioactive Flavonoid, Pharmacogn. Rev. 10 (2016) 89. https://doi.org/10.4103/0973-7847.194044.

[400] M. Alessandro, B. Olga, R. Daniele, B. Tatiana, S. Gianni, T. Massimo, D.R. Carmela, Research Progress in the Modification of Quercetin Leading to Anticancer Agents, Molecules. 22 (2017) 2–27. https://doi.org/10.3390/molecules22081270.

[401] S. Deepak Kumar, S. Ruchi Badoni, C. Sandra, V. Alvaro, Myricetin: A Dietary Molecule with Diverse Biological Activities, Nutrients. 8 (2016) 1–31. https://doi.org/10.3390/nu8020090.

[402] J.I. Dower, J.M. Geleijnse, P.C.H. Hollman, S.S. Soedamah-Muthu, D. Kromhout, Dietary epicatechin intake and 25-y risk of cardiovascular mortality: The Zutphen Elderly Study, Am. J. Clin. Nutr. 104 (2016) 58–64. https://doi.org/10.3945/ajcn.115.128819.

[403] J.A. Gerrard, M. Lasse, J. Cottam, J.P. Healy, S.E. Fayle, I. Rasiah, P.K. Brown, S.M. BinYasir, K.H. Sutton, N.G. Larsen, Aspects of physical and chemical alterations to proteins during food processing - Some implications for nutrition, Br. J. Nutr. 108 (2012) 288–297. https://doi.org/10.1017/S000711451200236X.

[404] K. Kawai, T. Hagiwara, Control of physical changes in food products, Adv. Exp. Med. Biol. 1081 (2018) 385–399. https://doi.org/10.1007/978-981-13-1244-1_21.

[405] C. Papuc, G. V. Goran, C.N. Predescu, V. Nicorescu, Mechanisms of Oxidative Processes in Meat and Toxicity Induced by Postprandial Degradation Products: A Review, Compr. Rev. Food Sci. Food Saf. 16 (2017) 96–123. https://doi.org/10.1111/1541-4337.12241.

[406] C. Berset, Les polyphénols en agro-alimentaire, in: P.S.& V. Cheynier (Ed.), Antioxyd.

Phénoliques Struct. Propriétés, Sources Végétales, Lavoisier, Paris, 2006: pp. 265–294.

[407] J. Cillard, P. Cillard, Mécanismes de la peroxydation lipidique et des anti-oxydations, Oléagineux, Corps Gras, Lipides. 13 (2006) 24–29. https://doi.org/10.1051/ocl.2006.6666.

[408] E. Choe, D.B. Min, Mechanisms of Antioxidants in the Oxidation of Foods, Compr. Rev. Food Sci. Food Saf. 8 (2009) 345–358. https://doi.org/10.1111/j.1541-4337.2009.00085.x.

[409] F. Tian, E.A. Decker, J.M. Goddard, Controlling lipid oxidation of food by active packaging technologies, Food Funct. 4 (2013) 669–680. https://doi.org/10.1039/c3fo30360h.

[410] WHO, Cancérogénicité de la consommation de viande rouge et de viande transformée, Who. (2016) 1–8. http://www.who.int/features/qa/cancer-red-meat/fr/.

[411] A. Ghasemzadeh, H.Z.E. Jaafar, Profiling of phenolic compounds and their antioxidant and anticancer activities in pandan (Pandanus amaryllifolius Roxb .) extracts from different locations of Malaysia, BMC Complement. Altern. Med. 13 (2013) 1–9.

[412] R. Kurata, M. Adachi, O. YAmakawa, M. Yoshimoto, Growth Suppression of Human Cancer Cells by Polyphenolics from Sweetpotato (Ipomoea batatas L .) Leaves, J. Agric. Food Chem. 55 (2007) 185–190.

[413] X. Zhang, D. Lin, R. Jiang, H. Li, J. Wan, H. Li, Ferulic acid exerts antitumor activity and inhibits metastasis in breast cancer cells by regulating epithelial to mesenchymal transition, Oncol. Rep. 36 (2016) 271–278. https://doi.org/10.3892/or.2016.4804.

[414] J. Gao, H. Yu, W. Guo, Y. Kong, lina Gu, Q. Li, S. Yang, Y. Zhang, Y. Wang, The anticancer effects of ferulic acid is associated with induction of cell cycle arrest and autophagy in cervical cancer cells, Cancer Cell Int. 18 (2018) 1–9. https://doi.org/10.1186/s12935-018-0595-y.

[415] N. Liang, D.D. Kitts, Role of chlorogenic acids in controlling oxidative and inflammatory stress conditions, Nutrients. 8 (2015) 1–20. https://doi.org/10.3390/nu8010016.

[416] W. Lim, G. Song, Inhibitory effects of delphinidin on the proliferation of ovarian cancer cells via PI3K/AKT and ERK 1/2 MAPK signal transduction, Oncol. Lett. 14 (2017) 810–818. https://doi.org/10.3892/ol.2017.6232.

[417] M. Jiang, M. Zhu, L. Wang, S. Yu, Anti-tumor effects and associated molecular mechanisms of myricetin, Biomed. Pharmacother. 120 (2019) 109506. https://doi.org/10.1016/j.biopha.2019.109506.

[418] A.A. Ruiz, Phenolic Compounds as Modulators of Leptin Signalling Pathway in Peripheral Tissues, Universitat Rovira i Virgili, Barcelona, 2018.

[419] D. Hogan, R. Kolter, Why are bacteria refractory to antimicrobials?, Contrib. to Sci. 5 (2009) 85–90. https://doi.org/10.2436/cs.v0i0.404.

[420] M.A. Pfaller, R.N. Jones, S.A. Messer, M.B. Edmond, R.P. Wenzel, National surveillance of nosocomial blood stream infection due to species of Candida other than Candida albicans: Frequency of occurrence and antifungal susceptibility in the SCOPE program, Diagn. Microbiol. Infect. Dis. 30 (1998) 121–129. https://doi.org/10.1016/S0732-8893(97)00192-2.

[421] Y.-C. Chen, Stable susceptibility of Candida blood isolates to fluconazole despite increasing use during the past 10 years, J. Antimicrob. Chemother. 52 (2003) 71–77. https://doi.org/10.1093/jac/dkg275.

[422] O. Adejare, O. Oduyebo, R. Oladele, F. Nwaokorie, F. Ogunsola, In-vitro antifungal effect of *Garcinia kola* and garlic (*Alliums sativum*) on vaginal isolates of *candida*, African J. Clin. Exp. Microbiol. 14 (2013) 140–145. https://doi.org/10.4314/ajcem.v14i3.4.

[423] P. Saillard, M. Rocca, M. Tavaud, Chimie du vieillissement, un mécanisme intracorporel, TPE.

Le Vieil. (2020) 1–6.

[424] A. Favier, Le stress oxydant: Intérêt conceptuel et expérimental dans la compréhension des mécanismes des maladies et potentiel thérapeutique, Actual. Chim. (2003) 108–115.

[425] J. Pincemail, K. Bonjean, K. Cayeux, J.O. Defraigne, Mécanismes physiologiques de la défense antioxydante, Nutr. Clin. Metab. 16 (2002) 233–239. https://doi.org/10.1016/S0985-0562(02)00166-8.

[426] R. De Guzman, H. Tang, S. Salley, K.Y.S. Ng, Synergistic effects of antioxidants on the oxidative stability of soybean oil- and poultry fat-based biodiesel, JAOCS, J. Am. Oil Chem. Soc. 86 (2009) 459–467. https://doi.org/10.1007/s11746-009-1373-8.

[427] I. Gülçin, R. Elias, A. Gepdiremen, K. Taoubi, E. Köksal, Antioxidant secoiridoids from fringe tree (Chionanthus virginicus L.), Wood Sci. Technol. 43 (2009) 195–212. https://doi.org/10.1007/s00226-008-0234-1.

[428] M. Carocho, I.C.F.R. Ferreira, A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives, Food Chem. Toxicol. 51 (2013) 15–25. https://doi.org/10.1016/j.fct.2012.09.021.

[429] T. Ozcan, A. Akpinar-Bayizit, L. Yilmaz-Ersan, B. Delikanli, Phenolics in Human Health, Int. J. Chem. Eng. Appl. 5 (2014) 393–396. https://doi.org/10.7763/ijcea.2014.v5.416.

[430] P.G. Anantharaju, P.C. Gowda, M.G. Vimalambike, S. V. Madhunapantula, An overview on the role of dietary phenolics for the treatment of cancers, Nutr. J. 15 (2016) 1–16. https://doi.org/10.1186/s12937-016-0217-2.

[431] C. Miura, H. Matsunaga, J. Haginaka, Molecularly imprinted polymer for caffeic acid by precipitation polymerization and its application to extraction of caffeic acid and chlorogenic acid from Eucommia ulmodies leaves, J. Pharm. Biomed. Anal. 127 (2016) 32–38. https://doi.org/10.1016/j.jpba.2015.12.052.

[432] S. Pal, C. Saha, A review on structure-affinity relationship of dietary flavonoids with serum albumins, J. Biomol. Struct. Dyn. 32 (2014) 1132–1147. https://doi.org/10.1080/07391102.2013.811700.

[433] S. Jeuge, M. Carlier, J. Vendeuvre, G. Nassy, L ’ oxydation des produits carnés : Méthodes de mesure et moyens de maîtrise, (2012).

[434] T.J. Nielsen, Limonene and Myrcene Sorption into Refillable Polyethylene Terephtalate Bottles, and Washing Effects on Removal of Sorbed Compounds, J. Food Sci. 59 (1994) 227–230. https://doi.org/10.1111/j.1365-2621.1994.tb06936.x.

[435] M. Aamir, M. Ovissipour, S.S. Sablani, B. Rasco, Predicting the quality of pasteurized vegetables using kinetic models: A review, Int. J. Food Sci. 2013 (2013) 29. https://doi.org/10.1155/2013/271271.

[436] S. Dal, S. Sigrist, The Protective Effect of Antioxidants Consumption on Diabetes and Vascular Complications, Diseases. 4 (2016) 24. https://doi.org/10.3390/diseases4030024.

[437] Z. Singh, R. Singh, Recent approaches in use of graphene derivatives in anticancer drug delivery systems, J. Drug Des. Res. 4 (2017) 1041–1044.

[438] R. Vardanyan, V. Hruby, Antineoplastic, in: Synth. Best-Seller Drugs, 2016: p. 868.

[439] S. Zhai, A.M. Senderowicz, E.A. Sausville, W.D. Figg, Flavopiridol, a novel cyclin-dependent kinase inhibitor, in clinical development, Ann. Pharmacother. 36 (2002) 905–911. https://doi.org/10.1345/aph.1A162.

[440] M.S.S. Cunha-Filho, B. Dacunha-Marinho, J.J. Torres-Labandeira, R. Martínez-Pancheco, M.

Landín, Characterization of β-lapachone and methylated β-cyclodextrin solid-state systems, AAPS PharmSciTech. 8 (2007). https://doi.org/10.1208/pt0803060.

[441] L. Cao, L. Zhang, X. Zhao, Y. Zhang, A hybrid chalcone combining the trimethoxyphenyl and isatinyl groups targets multiple oncogenic proteins and pathways in hepatocellular carcinoma cells, PLoS One. 11 (2016) 1–23. https://doi.org/10.1371/journal.pone.0161025.

[442] E. García, I. Fernández, A. Fuentes, Determinación de polifenoles totales por el método de Folin-Ciocalteu, Univ. Politec. Val. Dep. Tecnol. Aliment. (2015) 1–9. https://riunet.upv.es/bitstream/handle/10251/52056/Garcia Mart?nez et al.pdf?sequence=1.

[443] S. Sepahpour, J. Selamat, M.Y.A. Manap, A. Khatib, A.F.A. Razis, Comparative analysis of chemical composition, antioxidant activity and quantitative characterization of some phenolic compounds in selected herbs and spices in different solvent extraction systems, Molecules. 23 (2018) 1–17. https://doi.org/10.3390/molecules23020402.

[444] P. Schofield, D.M. Mbugua, A.N. Pell, Analysis of condensed tannins: A review, Anim. Feed Sci. Technol. 91 (2001) 21–40. https://doi.org/10.1016/S0377-8401(01)00228-0.

[445] C. Proestos, M. Komaitis, Antioxidant capacity of hops, Beer Heal. Dis. Prev. (2008) 467–474. https://doi.org/10.1016/b978-0-12-373891-2.00045-6.

[446] Wang and Zhong, Evaluation Technologies for Food Quality, 2019.

[447] A.M. Pisoschi, M.C. Cheregi, A.F. Danet, Total antioxidant capacity of some commercial fruit juices: Electrochemical and spectrophotometrical approaches, Molecules. 14 (2009) 480–493. https://doi.org/10.3390/molecules14010480.

[448] T. Weitner, S. Inić, J. Jablan, M. Gabričević, A.M. Domijan, Spectrophotometric determination of malondialdehyde in urine suitable for epidemiological studies, Croat. Chem. Acta. 89 (2016) 133–139. https://doi.org/10.5562/cca2902.

[449] K.H. Bak, T. Bolumar, A.H. Karlsson, G. Lindahl, V. Orlien, Effect of high pressure treatment on the color of fresh and processed meats: A review, Crit. Rev. Food Sci. Nutr. 59 (2019) 228–252. https://doi.org/10.1080/10408398.2017.1363712.

Websites

https://www.sigmaaldrich.com/ (Sigma Aldrich)

https://www.nih.gov/ (National Institute of Health)

9 Publications & Communications

9 Publications and communications

Published articles

Manel Ouerfelli, Leila Bettaieb Ben Kaâb, María Pilar Almajano. Radical scavenging and antioxidant activity of *Anthyllis Vulneraria* leaves and flowers. *Molecules*, 23(7) (July 2018) 1657. https://doi.org/10.3390/molecules23071657 (**Impact Factor: 4.411 (2020), JCR category rank: Q1**). **Annex 1**

Manel Ouerfelli, Juliana Villasante, Leila Bettaieb Ben Kaâb, María Pilar Almajano. Effect of Neem (*Azadirachta indica* L.) on lipid oxidation in raw chilled beef patties. *Antioxidants*, 8(8) (August 2019) 305. https://doi.org/10.3390/antiox8080305 (**Impact Factor: 6.312 (2020), JCR category rank: Q1**). **Annex 2**

Manel Ouerfelli, Nesserine Majdoub, Jihen Arroussi, María Pilar Almajano, Leila Bettaieb Ben Kaâb. Phytochemical screening and evaluation of the antioxidant and anti-bacterial activity of Woundwort (*Anthyllis vulneraria* L.) from Tunisia. *Brazilian Journal of Botany* (August 2021). https://doi.org/10.1007/s40415-021-00736-6 (**Impact Factor: 1.296 (2020), JCR category rank: Q2**). **Annex 3**

Technical article

Manel Ouerfelli and María Pilar Almajano. Estabilidad oxidativa de emulsiones alimentarias de aceite en agua. Tecnifood. La revista de la tecnología alimentaria Feb. 2021. https://techpress.es/estabilidad-oxidativa-de-emulsiones-alimentarias-de-aceite-en-agua/#. **Annex 4**

Submitted papers (Under Review)

Manel Ouerfelli, Isidoro Metón, Leila Bettaieb Ben Kaâb and María Pilar Almajano. Phenolic profile, antibacterial and antiproliferative activity of *Azadirachta indica* L. leaf extract and its effect on oxidative stability of oil-in-water emulsion. Submitted to "*Plant biosystems"*. (**Impact Factor: 2.838 (2020), JCR category rank: Q2**).

Manel Ouerfelli, Isidoro Metón, Leila Bettaieb Ben Kaâb and María Pilar Almajano. HPLC-MS profile, antiproliferative and antiradical activity of *Anthyllis vulneraria* extracts. Submitted to "*Physiology and Molecular Biology of Plants*". (**Impact Factor: 2.391 (2020), JCR category rank: Q1).**

Other publications

Nawel Jemil, **Manel Ouerfelli**, María Pilar Almajano, Jihene Elloumi-mseddi, Moncef Nasri and Noomen Hmidet (2020). The conservative effects of lipopeptides from Bacillus methylotrophicus DCS1 on sun fl ower oil-in-water emulsion and raw beef patties quality. Food Chemistry, 303 (August 2019),https://doi.org/10.1016/j.foodchem.2019.125364

(**Impact Factor: 7.514 (2020), JCR category rank: Q1**).

Noomen Hmidet, Nawel Jemil, **Manel Ouerfelli**, María Pilar Almajano and Moncef Nasri. (2020). Antioxidant properties of Enterobacter cloacae C3 lipopeptides in vitro and in model food emulsion. Journal of Food Processing and Preservation, 44(2), 1–12. https://doi.org/10.1111/jfpp.14337 (**Impact Factor: 2.190 (2020), JCR category rank: Q2**).

Juliana Villasante, **Manel Ouerfelli**, Ares Bobet, Isidoro Metón and María Pilar Almajano. (2020). The effects of pecan shell, roselle flower and red pepper on the quality of beef patties during chilled storage. *Foods*, *9*(11), 1–17. https://doi.org/10.3390/foods9111692 (**Impact Factor: 4.350 (2020), JCR category rank: Q1**).

Poster and oral communications

Manel Ouerfelli, María Pilar Almajano, Leila Bettaieb Ben Kaâb. *Anthyllis vulneraria*: Additif alimentaire naturel antioxydant. 30th International Congress of the Tunisian Society of Biotechnological Sciences, Tunisian Association of Biological Sciences (ATSB), Sousse, Tunisia 25-28 March 2019.

Manel Ouerfelli, María Pilar Almajano, Leila Bettaieb Ben Kaâb. Effet des plantes sur l'oxydation lipidique et la qualité organoleptique des produits carnés. Scientific Days MR1-BOPE "Cell Physiology and Plant Productivity", Faculty of Sciences of Tunis (FST), Tunisia 26 February 2020.

10 Annexes

10 Annexes

Annex 1

molecules

MDPI

Article

Radical Scavenging and Antioxidant Activity of *Anthyllis Vulneraria* Leaves and Flowers

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Abstract: The main targets of this work were to determine the phenolic content of *Anthyllis vulneraria* (*A. vulneraria*) leaves and flowers and to evaluate their antioxidant activity. Total polyphenols and flavonoid content (TPC and TFC, respectively) were determined. Antioxidant capacity was evaluated by the Ferric Reducing Antioxidant Power (FRAP), the Oxygen Radical Absorbance Capacity (ORAC), the Trolox Equivalent Antioxidant Capacity (TEAC) and the diphenyl picrylhydrazyl (DPPH) assays, and by the analysis of primary and secondary oxidation products in oil-in-water emulsions and in raw beef patties during storage. The results revealed that the flowers of the *A. vulneraria* contained the highest content of total polyphenols and flavonoids and extracts from these tissues exhibited the strongest antioxidant activity, as they were more effective at retarding lipid oxidation in oil-in-water emulsions and raw beef patties than extracts from the leaves which had a potent antioxidant effect only at the beginning of the oxidation process. The results of this study allowed us to obtain a deep knowledge about the properties of *A. vulneraria* and confirmed the possibility of using its biologically active extracts in the food, cosmetic and pharmaceutical industries.

Keywords: *A. vulneraria*; phenolic compounds; antioxidant activity; lipid oxidation; emulsion; beef patties

1. Introduction

In recent years, the search for novel natural and functional extracts from medicinal plants has attracted a growing amount of interest because of the rich content of bioactive molecules such as phenolic compounds, vitamins and proteins [1] present in the different parts of the plants [2]. These bioactive compounds are gaining a very important role in various industrial fields such as the pharmaceutical, cosmetics and food industries thanks to their antioxidant, antimicrobial and anticancer properties [3] allowing them to retard the development of several fatal diseases caused by Reactive Oxygen Species (ROS) [4].

Phenolic compounds are secondary metabolites occurring with an unequal qualitative and quantitative distribution in plants. They include an important variety of compounds such as phenolic acids, flavanols, anthocyanins, stilbenes, etc., that vary in their basic structure but possess an aromatic ring bearing one or more hydroxyl groups [5]. These compounds play a crucial antioxidant role through different mechanisms of action, by scavenging free radicals, quenching ROS, inhibiting oxidative enzymes and chelating transition metals [6,7]. This is one of the reasons why many studies have focused on obtaining natural antioxidants from plants as alternatives to synthetic antioxidants,

such as butylated hydroxytoluene (BHT) and butylated hydroxy-anisole (BHA), which may present harmful effects on human health in the long term [8]. Emulsions and raw meat are good models to study the antioxidant effects of herbs [9].

The benefits of phenolic compounds are not limited to their antioxidant property, but they have also been reported to protect the human organism from several chronic and degenerative health disorders and diseases due to their anticarcinogenic, antimutagenic, and antimicrobial properties that have been attributed to their antioxidant activity [10,11]. Despite advanced research and the discovery of treatments, many infectious and cancerous diseases remain a global problem causing deaths worldwide. Therefore, numerous studies have been conducted to identify new antimicrobial and anticancer agents from herbs [12,13].

Among the wild plant families most exploited for their secondary metabolites, the Fabaceae family, with more than 18,000 identified species, is considered as an excellent source of proteins, dietary fibers and various phytochemicals with important health benefits, such as anticancer, antimicrobial, and anti-obesity effects [14,15].

Anthyllis vulneraria L. (*Anthyllidis flos, herba; Papilionaceae*) [16], is a Mediterranean medicinal plant that belongs to the Fabaceae family and is common in the pastures of mountainous regions in Europe, North Africa, South Africa and Southeast Asia [17]. Ethanolic extracts of *A. vulneraria* have been used in traditional medicine to inhibit the multiplication of human herpesvirus 1 and poliovirus 2 in cell culture [18]. Its flowers were used to treat wounds, high blood pressure, heart failure, portal hypertension, vomiting, inflammation, acne and disturbances of metabolism, and to purify the body by promoting the elimination of toxins. They were also used to heal mouth and throat pain and to enhance hair growth [16,19]. Moreover, its leaves contain several bioactive substances such as phenolic acids, flavonoids, carotenoids, tannins and saponins [20].

The main targets of this study were to determine the total polyphenol and flavonoid contents of the leaf and flower extracts from *A. vulneraria* collected from the northeast of Tunisia, to evaluate their antioxidant activity by the ORAC, FRAP, TEAC and DPPH assays and to assess their usefulness in oil-in-water emulsions and in raw beef patties.

2. Results and Discussion

2.1. Total Polyphenol and Flavonoidcontents

The TPC and the TFC of the *A. vulneraria* leaf and flower extracts in 50% aqueous ethanol were determined and the results obtained with significant differences between the samples ($p < 0.05$) are shown in Table 1.

Table 1. TPC (Total Polyphenol Content) and TFC (Total Flavonoid Content) results of the leaf and flower extracts of *A. vulneraria* in 50% aqueous ethanol.

Samples	TPC (mg GAE/g Dry Plant)	TFC (mg QE/g Dry Plant)
Leaf	82.86 ± 1.22^{b}	20.14 ± 0.18^{b}
Flower	134.31 ± 1.64^{a}	33.58 ± 4.07^{a}

Results represent the mean of three replicates ($n = 3$) and are expressed as mean value ± SD; different letters indicate significant differences in each column at $p < 0.05$. GAE (Gallic Acid Equivalent), QE (Quercetin Equivalent).

The flower extract of *A. vulneraria* contained 62% higher total polyphenol content and 67% higher flavonoid content than the leaf extract.

Several authors have determined the contents of total polyphenols and flavonoids in *A. vulneraria* from different tissues. Godevac et al. [19] studied the chemical composition of *A. vulneraria* and reported similar total polyphenols content of 108.1 and 79.34 mg GAE/g dry plant in the flower extracts of *A. vulneraria* from Montenegro and Serbia, respectively. However, Tusevski et al. [21] found that the extracts of the aerial part of *A. vulneraria* from Macedonia contained lower contents of total

polyphenols and flavonoids than those obtained in this study with values 12.02 mg GAE/g DW and 2.22 mg CE/g DW, respectively.

Many other medicinal plants belonging to the Fabaceae family, including *Albizia julibrissin*, *Desmodium caudatum*, *Lespedeza bicolor*, *L. cuneata*, *L. maximowiczii*, *Pueraria lobata*, *Robinia pseudoacacia*, *Sophora flavescens*, *S. japonica* and *Erythrina stricta Roxb* contain high contents of phenolic compounds [22]. Skowyra and Gallego [23] reported high contents of total polyphenols and flavonoids in *Caesalpinia spinosa* pods estimated at 460.2 mg GAE/g dry plant and 2.93 mg CE/g dry plant, respectively. Gallego et al. [2] determined the phenolic compounds in the leaf extracts from Tara (*Caesalpinia spinosa*) and Mysore thorn (*Caesalpinia decapetala*) and found that the *C. spinosa* leaf extract extracted with 50% aqueous ethanol contained higher contents of total polyphenols compared with our results, while *C. decapetala* containeda lower polyphenol content of 63.8 mg GAE/g dry plant.

Differences in the distribution of the polyphenols and flavonoids arise from various factors that can be biological e.g., the part analyzed and the vegetative stage of the plant [24] and technical such as the extraction method, the solvents and their concentrations [25,26] and there are also differences in the structure and properties of the phenolic compounds present in the different samples analyzed [27].

Recent studies confirmed the key role that the polarity of the extraction solvent plays in extracting phenolic compounds from plant materials. Cheung et al. [28] and Ye et al. [29] found that aqueous alcohols are more effective solvents for extraction of phenolics from the florets of Sunflower and Moringa. Moreover, ethanol is preferred to other solvents and wildly used as a solvent to extract phenolics from plants because it is considered as a GRAS solvent (Generally Recognized As Safe) that can be used safely for food and industrial products without fear of toxicity [30,31].

2.2. *Antioxidant Activity*

2.2.1. Free Radical Scavenging Activity

The results of the antioxidant activity of the *A. vulneraria* leaf and flower extracts are presented in Table 2.

Table 2. Antioxidant activity of *A. vulneraria* leaf and flower extracts extracted with 50% aqueous ethanol.

Samples	FRAP (mM Trolox/g Dry Plant)	ORAC (mM Trolox/g Dry Plant)	TEAC (mM Trolox/g Dry Plant)	DPPH (mM Trolox/g Dry Plant)
Leaves	1.90 ± 0.01^{b}	0.98 ± 0.003^{b}	0.82 ± 0.002^{b}	0.33 ± 1.08^{b}
Flowers	3.30 ± 0.01^{a}	1.64 ± 0.07^{a}	1.42 ± 0.01^{a}	0.64 ± 0.96^{a}

Results represent the mean of three replicates ($n = 3$) and are expressed as mean value ± SD; different letters indicate significant differences in each column at $p < 0.05$.

Significant differences in the antioxidant activity determined by the different methods were found ($p < 0.05$). The flower extract showed the best antioxidant activity with 74%, 67%, 73% and 94% higher values than the leaf extract determined by the FRAP, ORAC, TEAC and DPPH assays, respectively. In previous studies, the antioxidant activity of *Anthyllis* extracts has been determined using the TEAC method. Godevac et al. [19] obtained lower TEAC values of 0.448 mM Trolox/g dry plant in the flower extract of *A. vulneraria* from Serbia and 0.909 mM Trolox/g dry plant in the flowers extract of *A. aurea*. High antioxidant activity has also been found in different extracts from members of the *Fabaceae* family, for instance the extracts from the pods of *Caesalpinia cacalaco* [32], *Acacia pennatula* [33], extracts from the flowers and roots of *Onobrychis armena* [34] and extracts from the seeds of *Trigonella foenum-graecum* [35].

2.2.2. Effects of the *A. vulneraria* Extracts on the Oxidative Stability of Emulsions

Several studies relevant to the food industry have investigated the antioxidant effects of extracts containing phenolic compounds on lipid oxidation in food systems. In the present study, the effect of the *A. vulneraria* leaf and flower extracts, extracted with 50% aqueous ethanol, on lipid oxidation in oil-in-water emulsion was investigated during 30 days of storage at 33 ± 1 °C. The evolution of

lipid oxidation in the emulsion samples was determined by monitoring the changes in the peroxide values (PV), the pH and the Thiobarbituric acid reactive substances (TBARS) values of the emulsions during the storage time. The evolution of primary oxidation (PV) over the storage time is illustrated in Figure 1.

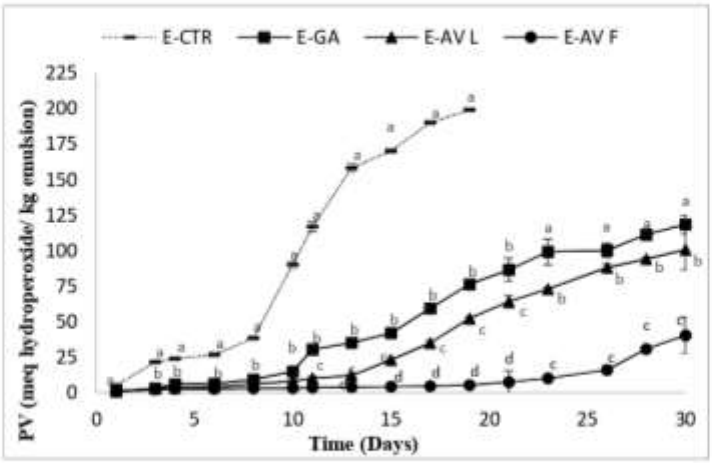

Figure 1. Peroxide values (PV) of emulsions containing *A. vulneraria* leaf and flower extracts during storage. E-CTR (Control emulsion sample), E-GA (Galic acid emulsion sample), E-AVL (*A. vulneraria* leaf emulsion sample), and E-AVF (*A. vulneraria* flower emulsion sample). Error bars represent the standard deviation ($n = 3$) and different letters in the same day indicate significant difference between samples at $p < 0.05$.

To ensure a good quality of consumable products containing fats, the maximum peroxide value was estimated by the Codex Alimentarius to be 10 meqhydroperoxide/kg of oil [26]. Considering this value as a measure of emulsion stability, the formation of hydroperoxides was significantly swiftest in the E-CTR sample, which showed an increase of the oxidation values from the first day of storage. The E-GA and the E-AVL remained stable against lipid oxidation for 8 to 11 days respectively, and then exhibited a faster oxidation, whereas the E-AVF sample was stable against lipid oxidation until 23 days. After 19 days of storage, the PV of the E-CTR sample reached a maximum of hydroperoxide content with 199.37 meq hydroperoxide/kg emulsions. At the end of the storage period (30 days), the E-GA and E-AVL samples had also deteriorated with maximum peroxide values of 188.47 and 100.78 meq hydroperoxide/kg emulsion, respectively. The order of the oxidation stability of the different emulsions was consistent with the order of the phenolic contents and the antioxidant assay values. The E-AVF sample presented the best protective effect against the formation of the primary oxidation products, followed by the E-AVL then the E-GA.

The *A. vulneraria* extracts were more effective at retarding lipid oxidation compared with several previous research reports of the antioxidant effect of herbs on protecting emulsions from lipid oxidation. Gallego et al. [2] determined the peroxide values of emulsions containing 10% of purified sunflower oil containing extracts from *Caesalpinia spinose* (*C. spinose*) and *Caesalpinia decapetala* (*C. decapetala*) at a concentration of 0.5% during 33 days of storage at 33 °C. These authors reported that their extracts reached the limit allowed for products containing edible fats (10 meg/kg oil) after 18 days of storage and were successful in slowing down lipid oxidation during the whole storage period with peroxide values of 6.7 and 18.2 meg hydroperoxides/kg emulsion, respectively. In another study conducted by Skowyra et al. [36], the *Artemisia annua* extract was effective also in slowing down the formation of hydroperoxides and reached 10 meg/kg oil after 28 days. An amount of 0.5% w/w of *Gentiana lutea* extract was able to retard lipid oxidation throughout storage with samples reaching the limit for fat

products after 10 days [37]. An extract from Pineapple Waste was effective at retarding lipid oxidation according to the study of Segovia and Almajano [38].

The oxidation of lipids causes qualitative and nutritional alterations such as rancidity, loss of vitamins and even toxicity due to lipid peroxidation products like peroxides and aldehydes. The hydroperoxides are the main primary products generated by lipid oxidation. However, their instability causes their easy decomposition into secondary compounds, resulting in the appearance of aldehydes and acidic oxidation products [39]. For this reason, the pH of the different samples was also determined in our study as an indicator of emulsion lipid oxidation. The evolution of the peroxide values (Figure 1) and the pH values (Figure 2) were inversely proportional.

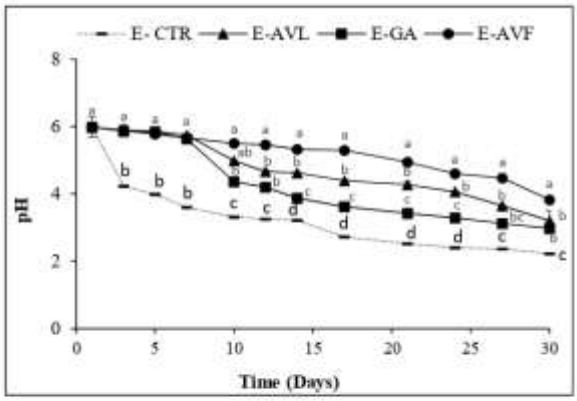

Figure 2. pH evolution during 30 days of study. E-CTR (Control emulsion sample), E-AVL (*A. vulneraria* leaf emulsion sample), E-GA (Galic acid emulsion sample), E-AVF (*A. vulneraria* flower emulsion sample). Error bars represent the standard deviation ($n = 3$) and different letters in the same day indicate significant difference between samples at $p < 0.05$.

All the samples started with an initial average pH value of 5.98. The first decrease of the pH values was observed in the E-CTR from 5.98 to 3.89 after only three days of storage. The E-AVF was the only sample that remained stable at around 5 for more than 17 days before decreasing to 3.82 at the end of the storage time. The E-GA had a similar behavior to the E-AVL. Their pH remained stable at around 5.51 for 8 days, then decreased to a pH value of 2.97 and 3.20, respectively, at the end of the storage time. The change and fall of the pH during lipid oxidation is due to the decrease in the efficacy of the phenolic compounds present in the extracts to prevent the formation of oxidation products including hydroperoxides and their acidic degradation products [40]. Decker et al. [41] showed that pH also affects the rate of oxidation by different mechanisms. For example, pH can affect the redox state of metals and the activity, solubility, stability, and chelation capacity of antioxidants, and it can influence the distribution of antioxidants between the aqueous and lipid phases.

The degradation of hydroperoxides into second compounds leads to the formation of aldehydes. Malondialdehyde (MDA) is one of the main secondary products responsible for the bad flavor, the rancid odor and undesirable taste of oxidized fats [42]. In the present study, this compound was monitored by the measurement of the TBARS values (Figure 3).

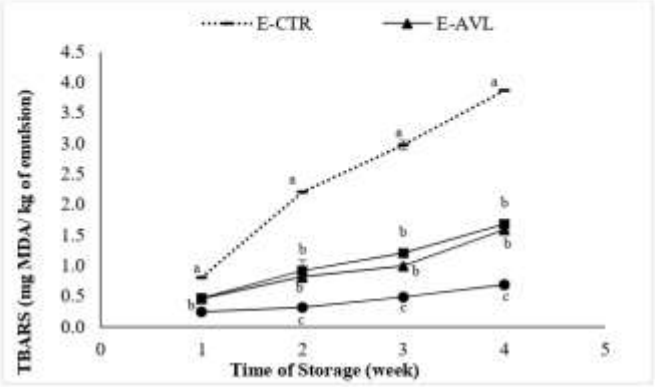

Figure 3. Changes in Thiobarbituric Acid Reactive Substance (TBARS) values of the emulsion containing *A. vulneraria* leaf and flower extracts during storage. E-CTR (Control emulsion sample), E-AVL (*A. vulneraria* leaf emulsion sample), E-GA (Galic acid emulsion sample), E-AVF (*A. vulneraria* flower emulsion sample). Error bars represent standard deviation ($n = 3$) and different letters in the same day indicate significant difference between samples at $p < 0.05$.

2.2.3. Effects of Powdered *A. vulneraria* on Raw Meat Oxidative Stability

Lipid Oxidation and Changes in pH Values

The effect of powdered *A. vulneraria* leaves and flowers on the lipid oxidation of raw beef patties during 11 days of storage (DOS) at 4 ± 1 °C was evaluated and the results are shown in Figure 4.

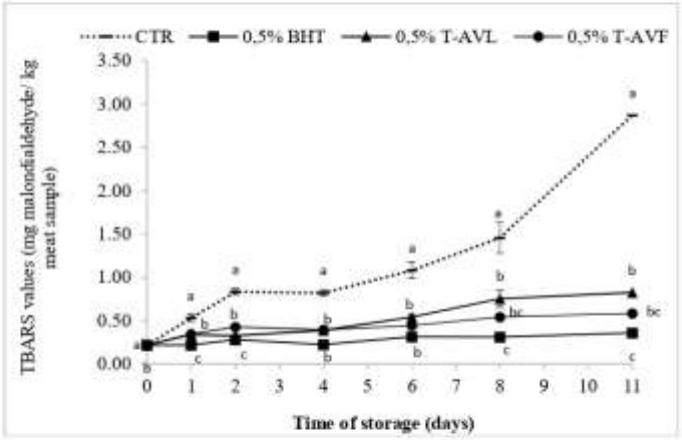

Figure 4. Thiobarbituric Acid Reactive Substance (TBARS) values of raw beef patties formulated with powdered leaves and flowers of *A. vulneraria* during storage at 4 ± 1 °C. CTR (Control sample without antioxidant), 0.5% BHT (treatment with 0.5% synthetic antioxidant (Butylated hydroxytoluene)), 0.5% T-AVL (treatment with 0.5% *A. vulneraria* leaf), 0.5% T-AVF (treatment with 0.5% *A. vulneraria* flower). Results represent the mean of three replicates ($n = 3$) and are expressed as mean value ± SD; different letters in the same day indicate significant difference between samples at $p < 0.05$.

The TBARS values increased continually ($p < 0.05$) despite the low temperature of storage from 0.22 ± 0.01 mg MDA/kg ground beef sample (initial value) to 2.87 ± 0.18, 0.36 ± 0.02, 0.83 ± 0.10 and 0.59 ± 0.03 in the control sample, 0.5% BHT, 0.5% T-AVL and 0.5% T-AVF, respectively. According to Leygonie et al. [43], storage time and temperature have a significant influence on lipid oxidation in raw beef patties. Cool temperature storage is not completely effective in preventing meat from deterioration and the freezing and thawing of raw meat is an incentive to accelerate lipid oxidation, and thus the formation of secondary products.

The results obtained showed that natural antioxidants have a significant influence ($p < 0.05$) on the development of lipid oxidation. The TBARS values of all the treated samples including 0.5% BHT, 0.5% T-AVL and 0.5% T-AVF, were significantly ($p < 0.05$) lower than for the CTR sample. Leaf powder contributed stronger lipid stability than flower powder during storage as shown by the lower TBARS values. This can be explained by the higher content of natural polyphenol, which are responsible for strong antioxidant activity, due to their ability to neutralize and eliminate free radicals [44]. The effectiveness of natural antioxidants for limiting lipid oxidation and extending the shelf-life of meat products was demonstrated in a previous study [45], which showed that Tara pod powder, when battered with pork meat, had high antioxidant activity and retarded lipid oxidation during chilled storage. The results obtained are consistent with those reported by Azman et al. [46] who found that 0.1% *Convolvulus arvensis* samples were more stable ($p < 0.05$) than samples treated with 0.1% BHT. It was also reported that a by-product, avocado waste, was also efficient in inhibiting rancidity deterioration of beef and pig meat [43,44].

Changes in pH values during storage of raw beef patties formulated with powdered leaves and flowers of *A. vulneraria* are shown in Table 3.

Table 3. The pH values of raw beef patties formulated with powdered leaves and flowers of *A. vulneraria* during storage at 4 ± 1 °C CTR (Control sample without antioxidant), BHT (treatment with synthetic antioxidant (Butylated hydroxytoluene)), T-AVL (treatment with *A. vulneraria* leaf), T-AVF (treatment with *A. vulneraria* flower).

Days of Storage	1	2	4	6	8	11
CTR	5.86 ± 0.03^{a}	5.89 ± 0.02^{a}	5.92 ± 0.01^{a}	5.97 ± 0.02^{a}	6.01 ± 0.02^{a}	6.19 ± 0.03^{a}
BHT	5.69 ± 0.01^{c}	5.70 ± 0.11^{b}	5.72 ± 0.02^{c}	5.74 ± 0.01^{b}	5.77 ± 0.01^{c}	5.78 ± 0.02^{c}
T-AVL	5.76 ± 0.01^{b}	5.78 ± 0.03^{ab}	5.82 ± 0.03^{b}	5.87 ± 0.1^{ab}	5.93 ± 0.1^{ab}	6.01 ± 0.05^{b}
T-AVF	5.71 ± 0.01^{c}	5.74 ± 0.02^{b}	5.79 ± 0.05^{bc}	5.81 ± 0.03^{ab}	5.84 ± 0.03^{bc}	5.86 ± 0.02^{c}

Results represent the mean of three replicates ($n = 3$) and are expressed as mean value ± SD, different letters in the same day indicate significant difference between samples at $p < 0.05$.

The pH values were significantly different between the different samples ($p < 0.05$) but not in the same sample during 11 days and increased continually with storage time from 5.86 ± 0.03; 5.69 ± 0.01; 5.76 ± 0.01 and 5.71 ± 0.01 on the first day to 6.19 ± 0.03; 5.78 ± 0.02; 6.01 ± 0.05 and 5.86 ± 0.02 on day 11 (last day) for the CTR, 0.5% BHT, 0.5% T-AVL and 0.5% T-AVF, respectively. After 11 days of refrigerated storage, the control had the highest pH values (6.19 ± 0.03), whereas the 0.5% BHT sample had the lowest pH value (5.78 ± 0.02). T-AVF had lower pH values compared with the control and T-AVL. Muela et al. [47] argued that degradation of proteins in muscle tissues, due to microorganisms, results in the production and accumulation of ammonia, amines and other basic substances that are responsible for the increase in the pH of the meat and its products.

Color Changes

The color of meat is a good sign of its quality and freshness, which are considered amongst the most influential factors that affect consumer acceptance and purchasing decisions [48]. The effect of the addition of powdered *A. vulneraria* leaves and flowers to raw beef patties on their surface color is shown in Table 4.

Table 4. Color characteristics of raw beef patties formulated with powdered leaves and flowers of *A. vulneraria* during storage at 4 ± 1 °C CTR (Control sample without antioxidant), BHT (treatment with synthetic antioxidant (Butylated hydroxytoluene)), T-AVL (treatment with *A. vulneraria* leaf), T-AVF (treatment with 0.5% *A. vulneraria* flower).

Trait	Days	CTR	BHT	T-AVL	T-AVF
Redness (a*)	1	36.67 ± 2.37 ^{a}	52.32 ± 0.98 ^{b}	43.75 ± 4.28 ^{ab}	49.46 ± 0.37 ^{ab}
	2	33.80 ± 0.37 ^{a}	49.26 ± 2.56 ^{b}	39.96 ± 1.23 ^{ab}	48.01 ± 0.05 ^{ab}
	4	27.95 ± 0.71 ^{a}	48.91 ± 1.63 ^{b}	38.21 ± 0.95 ^{ab}	43.07 ± 0.03 ^{ab}
	6	27.76 ± 0.84 ^{a}	48.77 ± 0.69 ^{b}	38.02 ± 0.47 ^{ab}	41.80 ± 1.85 ^{ab}
	8	25.14 ± 0.12 ^{a}	43.68 ± 0.81 ^{b}	34.09 ± 0.83 ^{ab}	31.22 ± 0.18 ^{ab}
	11	24.89 ± 1.23 ^{a}	37.31 ± 1.09 ^{b}	26.84 ± 1.46 ^{ab}	25.74 ± 1.03 ^{ab}
Yellowness (b*)	1	10.30 ± 0.17 ^{a}	15.35 ± 0.02 ^{b}	10.45 ± 1.24 ^{a}	14.34 ± 0.57 ^{b}
	2	10.28 ± 1.92 ^{a}	14.63 ± 1.19 ^{b}	9.93 ± 2.51 ^{a}	13.32 ± 0.11 ^{b}
	4	9.58 ± 1.93 ^{a}	13.43 ± 0.41 ^{b}	9.88 ± 0.15 ^{a}	13.29 ± 0.04 ^{b}
	6	9.46 ± 0.55 ^{a}	13.35 ± 0.10 ^{b}	8.78 ± 0.04 ^{a}	12.11 ± 0.24 ^{b}
	8	5.63 ± 0.07 ^{a}	11.79 ± 1.24 ^{b}	8.10 ± 1.00 ^{a}	10.79 ± 0.53 ^{b}
	11	5.17 ± 1.03 ^{a}	11.13 ± 0.46 ^{b}	5.52 ± 0.95 ^{a}	9.92 ± 0.14 ^{b}
Lightness (L*)	1	56.18 ± 0.14 ^{a}	70.80 ± 2.54 ^{ab}	68.36 ± 2.09 ^{ab}	70.99 ± 2.19 ^{b}
	2	55.42 ± 3.07 ^{a}	61.86 ± 1.62 ^{ab}	65.07 ± 2.12 ^{ab}	65.09 ± 4.12 ^{b}
	4	55.30 ± 0.25 ^{a}	60.61 ± 0.33 ^{ab}	61.52 ± 1.80 ^{ab}	63.70 ± 3.63 ^{b}
	6	55.04 ± 1.60 ^{a}	56.78 ± 2.78 ^{ab}	58.64 ± 1.97 ^{ab}	62.18 ± 0.40 ^{b}
	8	52.59 ± 0.50 ^{a}	56.65 ± 1.80 ^{ab}	55.54 ± 1.72 ^{ab}	59.53 ± 0.23 ^{b}
	11	43.63 ± 2.98 ^{a}	49.39 ± 0.04 ^{ab}	49.74 ± 0.21 ^{ab}	55.61 ± 4.65 ^{b}

Results represent the mean of three replicates ($n = 3$) and are expressed as mean value ± SD; different letters in the same day indicate significant difference between samples at $p < 0.05$.

The most important color parameter to evaluate the consumer preference of meat is the Redness (a*) value since its decrease alienates consumers [49]. In this study, Redness (a*) values decreased in all the samples of patties during the period of storage at 4 ± 1°C. Compared with 0.5% T-AVL, Redness (a*) values of 0.5% T-AVF sample were significantly higher ($p < 0.05$) but slightly lower than the 0.5% BHT sample. Redness (a*) values of the 0.5% T-AVF sample decreased from 49.46 ± 0.37 in the first day of analysis to 25.74 ± 1.03 in day 11. The lowest values of Redness were recorded in the control sample, which were considerably ($p < 0.05$) decreased during chilled storage from 36.67 ± 2.37 in the first day to 24.89 ± 1.23 in the last day. A similar trend for fresh beef patties containing olive cake powder during storage at 4 °C (±1) was reported [50]. According to Mancini and Hunt [51], the reduction of Redness (a*) values during storage is probably due to the oxidation of myoglobin and the formation of metmyoglobin.

Besides Redness (a*) values, powdered *A. vulneraria* leaves and flowers enhanced the Yellowness (b*) values of raw beef patties. The Yellowness (b*) values of non-treated (CTR) and treated (0.5% BHT, 0.5% T-AVL and 0.5% T-AVF) samples decreased significantly ($p < 0.05$) during refrigerated storage. The lowest Yellowness (b*) values were observed in the stored control samples and values increased with the addition of *A. vulneraria* leaf and flower powder. Higher values were recorded in 0.5% T-AVF samples than in 0.5% T-AVL samples and were close to the values recorded in 0.5% BHT samples. An increase in Yellowness (b*) values was observed also in raw ground pork patties containing *Moringa oleifera* leaves [52]. Eventually, as shown in Table 4, the Lightness (L*) values of each sample decreased significantly over time of storage.

The mean Lightness (L*) values of the control sample were lower than those of the treated samples. The addition of powdered *A. vulneraria* leaves and flowers had a significant effect when compared with the control. In previous studies conducted by Esmer et al. [53] and Gallego et al. [49], slight changes in Lightless (L*) values in meat during chilled storage were reported:

Antioxidant Capacity Determined by the FRAP Assay

The results of the antioxidant capacity assay determined by the FRAP-water and FRAP-lipid assays on day 0 (initial day) and day 11 (last day) are summarized in Figures 5a and 5b, respectively.

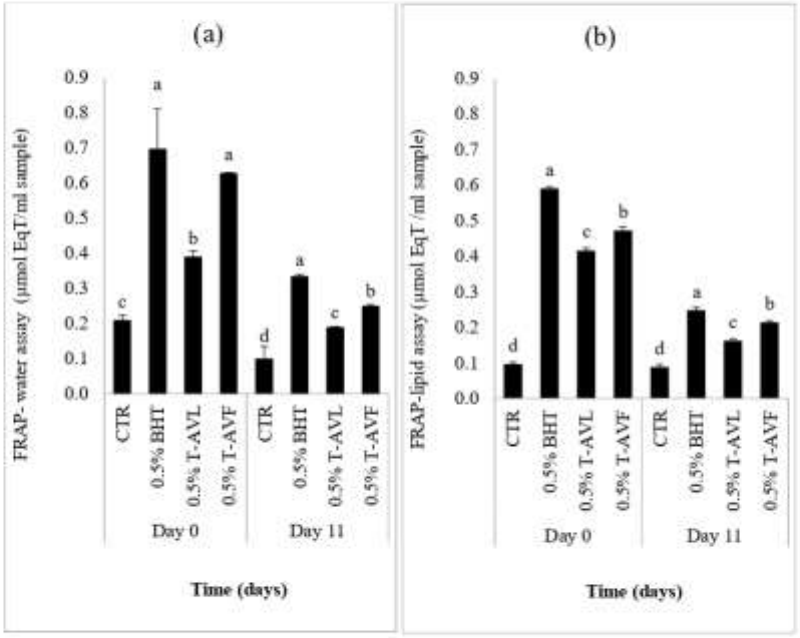

Figure 5. Antioxidant Capacity. (AOC) measurements by FRAP water (**a**) and FRAP lipid (**b**) assays of each beef patty sample formulated with powdered leaf and flower of *A. vulneraria*. CTR (Control sample without antioxidant), 0.5% BHT (treatment with 0.5% synthetic antioxidant (Butylated hydroxytoluene)), 0.5% T-AVL (treatment with 0.5% *A. vulneraria* leaf), 0.5% T-AVF (treatment with 0.5% *A. vulneraria* flower). Results represent the mean of three replicates and are expressed as mean value ± SD; different letters in the same day indicate significant difference between samples at $p < 0.05$.

Each sample was tested by the hydrophilic and lipophilic antioxidant activity assays. The significantly highest values ($p < 0.05$) of the hydrophilic antioxidant assay were recorded in the 0.5% BHT sample (0.34 ± 0.01), followed by the 0.5% T-AVF (0.25 ± 0.01) and then 0.5% T-AVL samples (0.19 ± 0.02), while the CTR had the lowest antioxidant activity (0.10 ± 0.01) µmol eq Trolox/mL sample (Figure 5a). For the hydrophilic antioxidant assay, the significantly highest values ($p < 0.05$) of the lipophilic antioxidant assay were recorded in the 0.5% BHT sample (0.25 ± 0.02), followed by the 0.5% T-AVF (0.21 ± 0.02) then 0.5% T-AVL samples (0.16 ± 0.05) while the control represented the lowest antioxidant activity (0.09 ± 0.01) µmol eq Trolox/mL sample (Figure 5b). All the samples, including the control, had higher hydrophilic FRAP values than lipophilic ones with no significant difference between the same samples in each FRAP assay. The results obtained in this study were higher than those of the *Caesalpinia decapetala* extract showing that the *C. decapetala* can be a good source of natural antioxidant since it had higher antioxidant capacity determined with hydrophilic and lipophilic FRAP assays and that hydrophilic FRAP values are higher than lipophilic values [49]. Generally, antioxidants can be classified into two different groups, namely lipophilic antioxidants (tocopherols and carotenoids) and hydrophilic antioxidants (ascorbic acid and the majority of phenolic

compounds), which contribute to a high antioxidant capacity, enabling the meat products treated with natural antioxidants to be protected against oxidation.

Sensory Characteristics

In the present study, a triangle test was used to know if the cooked beef patties formulated with powdered leaves (T-AVL) and flowers (T-AVF) of *A. vulneraria* were identical to the control beef patties (without natural or synthetic antioxidants). Results presented in Table 5 show the sensory evaluation of the beef patties samples.

Table 5. Sensorial analysis of beef patties formulated with powdered leaves and flowers of *A. vulneraria* T-AVL (treatment with *A. vulneraria* leaf), T-AVF (treatment with *A. vulneraria* flower).

Meat Samples	Number of Assessors			Number of Smokers/Non-Smokers		Odd Samples Identified		Level of Significance
	Male	Female	Total	Smoker	Non-Smoker	Correct (+)	Incorrect (-)	
T-AVL	18	7	25	1	24	23	2	0.1%
T-AVF	18	12	30	3	27	23	7	0.1%

In fact, 23 assessors from 25 agreed that T-AVL samples had a different taste compared to the control samples. Furthermore, 23 assessors from 30 agreed also that T-AVF samples had a different taste than the control samples. According to the table used to interpret the triangular test results, if the number of assessors is 23 and 30 and the number of correct answers is 15 and 19 respectively, the level of significance is equal to 0.1%. Thus, we conclude from this result that there is a significant difference between T-AVL/T-AVF samples and the control samples. In other words, the reformulated beef patties are significantly different from the original ones.

3. Materials and Methods

3.1. Reagents and Chemicals

Alumina; aluminum chloride ($AlCl_3$), ethanol (EtOH), iron(II) chloride ($FeCl_2$), ferric chloride hexahydrate ($Cl_3Fe{\cdot}6\ H_2O$), gallic acid (GA), methanol (MeOH), potassium persulfate ($K_2S_2O_8$), sodium carbonate (Na_2CO_3), Trolox, tween, 2-thiobarbituric acids (TBA), dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), 2,4,6-tripyridyl-s-triazine (TPTZ), 2,2′-azo-bis-2-amidinopropane hydrochloride (AAPH); 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain).

Acetic acid, acetone, ammonium thiocyanate (NH_4SCN), fluorescein, Folin–Ciocalteu reagent, hydrochloric acid (HCl), phosphate buffered saline (PBS), quercetin, trichloroacetic acid (TCA) were acquired from Panreac Química S.L.U (Barcelona, Spain).

3.2. Spectrophotometric Measurements

Spectrophotometric measurements were performed using FLUOstar® Omega (Ortenberg, Germany), a multimode micro-plate reader with five detection modes using an ultra-fast UV/Vis spectrometer. The spectrophotometer was purchased from BIOGEN Cientifica, S.L. (Madrid, Spain).

3.3. Determination of Phenolic Compounds and Free Radical Scavenging Capacity

3.3.1. *A. vulneraria* Samples and Extraction Procedure

The *A. vulneraria* was harvested at the beginning of April, corresponding to the flowering period, from the mountains of Zaghouan situated in the northeast of Tunisia characterized by a semi-arid climate. The leaves and flowers were separated and dried in air during two weeks, until the achievement of constant weight, and then were ground using a blender. The homogenous powder obtained was stored in amber glass bottles at room temperature for later use. The different extracts

were prepared by mixing and stirring 0.25 g of each vegetable matter (leaves and flowers) in 5 mL of 50% ethanol: water (*v*/*v*). The mixtures obtained were centrifuged at 3723 g (Orto Alresa Mod. Consul, Ortoalresa, Ajalvir, Madrid, Spain) for 10 min and then each supernatant was filtered using Whatman filters N°4 and stored at 4 °C.

For all parameters studied below, samples were analyzed in triplicate.

3.3.2. Total Polyphenol Content (TPC)

TPC was determined using the method based on the Folin–Ciocalteu reagent [38]. Briefly, 20 µL of each diluted sample (1:10, *v*:*v*) were mixed with 80 µL of Folin–Ciocalteu reagent (2N), 80 µL of Na_2CO_3 20%. After mixing for 2 min and incubation at room temperature for 1 h in darkness, the absorbance was measured at 765 nm against a blank, where extracts were replaced by Milli-Q water. The absorbance was measured at 765 nm and Gallic acid (100–1700 µM, R^2 = 0.992) was used as the standard for the calibration. The results were expressed as mg GAE/g dry plant.

3.3.3. Total Flavonoid Content (TFC)

TFC was determined following the method of Pękal and Pyrzynska [54] with some modifications. An aliquot of 150 µL of each sample was allowed to react with 50 µL of $AlCl_3$ (20 mg/mL in acetic acid 5% prepared with MeOH 3:1 ratio). After 30 min of incubation in darkness, the absorbance was measured at 405 nm and the measurements were compared to a calibration curve prepared with Quercetin (50–500 µM, R^2 = 0.998). Results are expressed as mg QE/g dry plant.

3.3.4. Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP method was carried out as described by Skowyra et al. [36]. In short, a suitable dilution of the *A. vulneraria* extracts was allowed to react with the FRAP reagent and incubated at 37 °C. FRAP reagent was prepared with acetate buffer (300 mM; pH 3.6), 2.4.6. Tri-Pyridyl-5-Triazine (10 mM in HCl, 40 mM) and $FeCl_3$ (20 Mm), which were all mixed in the ratio 10:1:1 (*v*/*v*/*v*), respectively. The absorbance was recorded at 593 nm and the FRAP value was determined from a calibration with Trolox (3–20 µM, R2 = 0.989) and expressed as mM Trolox/g dry plant.

3.3.5. Oxygen Radical Absorbance Capacity (ORAC) Assay

ORAC assay was carried out at 37 °C [38]. Shortly, 40 µL of each diluted sample (1:100; *v*:*v*) was mixed with 120 µL of Fluorescein (0.01 mM) and agitated for 2 min.. An initial reading of 2 min at an excitation wavelength of 485 nm was taken, and then 40 µL AAPH (0.3 M) was added to the mixture and the measurement was continued for 3 h at a wavelength of 535 nm. The decrease in fluorescence over time was quantified as area according to Equation (1):

$$AUC = \frac{\left(0.5 + \sum_{t_c}^{N_c} f_n\right)}{f_i} \quad (1)$$

where AUC represents the area under the sample curve in the well, f_i represent the fluorescence reading at the initiation of the reaction, f_n represent la the last measurement, N_c represent the number of cycles and t_c represent the time of each cycle (2 min).

To calculate the ORAC value, a calibration curve was prepared using Trolox at different concentrations ranging from 0.5 to 14.78 mg Trolox/L. Equation (2) below is specific for determining the decrease in fluorescence at the sample level:

$$\text{Decrease in fluorescence} = AUC - AUC_{Bl} \quad (2)$$

where AUC_{Bl} expresses the area under the blank curve. ORAC values were expressed in mM Trolox/g dry plant mass.

3.3.6. Trolox Equivalent Antioxidant Capacity (TEAC) Assay

TEAC assay was performed according to the procedure followed by Gallego et al. [26]. ABTS radical solution was prepared with ABTS radical cation (7mM) and $K_2S_2O_3$ (24.24 mM) and diluted with 10 mM PBS (pH 7.4) to be adjusted to an absorbance of 0.72 then incubated at 30 °C. To perform the assay, 200 µL of the ABTS radical solution was mixed with 20 µL of each sample. The absorbance was measured at 734 nm for 20 min. The final results were taken after 5 min of the absorbance reading. The TEAC values were determined from a Trolox calibration curve with final concentrations ranging from 1 to 10 µM and $R^2 = 0.998$. The results are expressed as mM Trolox/g dry plant.

3.3.7. Diphenyl Picrylhydrazyl (DPPH) Assay

The ability of the *A. vulneraria* extracts to scavenge DPPH radicals was assessed by the method of Shalaby and Shanab [55]. An aliquot of each extract was added to 5.07 mM DPPH methanolic solution (concentration of 10%; *v*/*v* of sample and 90%; *v*/*v* of radicals). Then, the mixtures were left in the darkness at 37 °C. The model that explains the activity of a compound as antiradical is illustrated by Equation (3):

$$\text{DPPH.} + (\text{AH})n \rightarrow \text{DPPH-H} + (\text{A.})n \quad (3)$$

where AH is an antioxidant that acts as an anti-radical donor of hydrogen atoms, resulting in relatively stable molecular structures and relocation that stops the chain reaction. The newly formed radical (A.) may interact with other molecules to form stable radicals (DPPH-A, A-A). The absorbance was recorded at 517 nm every 15 min during 75 min in total. The results are expressed as mM Trolox/g dry plant.

3.4. Evaluation of the Antioxidant Effect of the A. vulneraria Extracts in an Oil-in-Water Emulsion System

3.4.1. Preparation of Emulsion

Alumina was activated in the oven at 200 °C for 24 h, and then cooled in a desiccator until reaching room temperature. Sunflower oil was purchased from a local market and purified two times through alumina in absolute darkness to exclude tocopherols then stored at −80 °C. To prepare the oil-in-water emulsion, 10 % of the purified sunflower oil was added drop by drop to an aqueous mixture containing 1% of Tween-20 and Milli-Q water (Barcelona, Spain) and cooled in an ice bath while sonicating for 10 min.

3.4.2. Conditioning of the *A. vulneraria* Extracts in Emulsions

The initial emulsion obtained was divided into vials to have at the end 4 samples (each sample was prepared in triplicate) including: a control (emulsion without antioxidant, E-CTR), emulsion containing Gallic acid (E-GA), emulsion containing the *A. vulneraria* leaf extract (E-AVL) and emulsion containing the *A. vulneraria* flower extract (E-AVF). All of the emulsion samples were stored in the oven and allowed to oxidize for 30 days at 35 ± 1 °C in darkness and under constant slow agitation.

3.4.3. Peroxide Value (PV) and pH Measurement

The PV was determined by the ferric thiocyanate method. $FeCl_2$ solution (2mM) (made with HCl (1M) and $FeCl_2$) and NH_4SCN solution (2mM) was prepared as the reagent. The assay was performed by diluting a drop of emulsion in the range of 0.007 to 0.0130 g in 1 mL of EtOH 96%. An aliquot from this solution was mixed with 3 mL of EtOH in plastic cuvette and then 75 µL of each reagent was added to the mixture. The absorbance was measured at 500 nm and the results are expressed as meq hydro peroxide/kg emulsion.

The pH was measured in triplicate each 2 day (pH-meter GLP21, Criston Instruments, Barcelona, Spain) to determine its correlation with the peroxide values.

3.4.4. Thiobarbituric Acid Reactive Substances (TBARS) Assay

The TBARS assay was determined following the method described in the study of Maqsood and Benjakul [5]. In brief, 0.3 g of each emulsion sample was mixed with 3 mL of TBARS reagent (15% TCA, 0.375% TBA and HCl 2.1%). The samples were mixed in an ultrasonic bath (Prolabo brand equipment, Lutterworth, UK) for 10 min and then placed in a hot water bath at 95 °C for 10 min. After cooling, samples were centrifuged and the absorbance of each supernatant was measured at 531 nm. Results are expressed as mg MDA/kg of emulsion sample.

3.5. Evaluation of the Antioxidant Effect of the Powdered A. vulneraria on Raw Ground Meat Quality

3.5.1. Treatment, Preparation and Storage Conditions of the Raw Beef Patties

Three different pieces of ground meat (each piece weighed 300 g), taken from the round part of 3 different cuts, were purchased fresh on 3 different days. Each piece was mixed with salt (1.5% *w*/*w*) and divided into 4 different parts, and then each part was mixed, to homogeneity, with different compounds to finally obtain 4 different treated beef samples: control (without antioxidant), 0.5% T-BHT (treatment with 0.5% synthetic antioxidant (BHT)), 0.5% T-AVL (treatment with 0.5% *A. vulneraria* leaves) and 0.5% T-AVF (treatment with 0.5% *A. vulneraria* flowers). After 3 min of kneading, each blend was flattened and formed into patties of 3–4 g of weight, 4 cm of diameter and 0.5 cm in thickness, using a round cutter. Then, beef patty samples were placed in plastic trays and covered with film and stored in the refrigerator at 4 ± 1 °C for 11 days. The lipid oxidation inhibition and the quality of the beef patties were monitored every 2 days during 11 days. The microbiological analyses were done every 5 days.

3.5.2. Thiobarbituric Acid Reactive Substance Values (TBARS) and pH Measurement

The determination of TBARS in raw beef patties was assessed according to Skowyra et al. [45]. The absorbance of each sample was measured at 531 nm and the results were expressed as mg MDA/kg meat sample. The pH measurement of the beef patties was determined electrometrically every two days using an Orion 3-Star pH Benchtop Meter (Thermo Fisher Scientific, Waltham, MA, USA).

3.5.3. Color Stability Evaluation

The effect of powdered *A. vulneraria* leaves and flowers on the color stability of raw beef patties was evaluated using a reflectance colourimeter Minolta CR-400 (Konica Minolta, Tokyo, Japan) and it was expressed against the scale of L* (lightness), a* (redness) and b* (yellowness) in the CIELab colour space system. Before each measuring session (light source of D65 and 10° standard observer), the instrument was calibrated (white reference: $Y = 93.8$; $x = 0.315$; $y = 0.332$). Three measurements from three different locations on the raw beef patties surface were taken on each day of analysis. Fat zones were avoided in order to obtain correct measurements.

3.5.4. Antioxidant Capacity Measurement (AOC)

AOC of meat samples was determined by the hydrophilic and lipophilic FRAP assays. The preparation of the samples was conducted as described by Gallego et al. [49] using hydrophilic (distilled water) and lipophilic (acetone, ethanol and distilled water (5:4:1; *v*/*v*/*v*)) solvents, used for the extraction of hydrophilic and lipophilic antioxidants, respectively. The different extracts were used to perform the FRAP assay as described previously. The results were expressed as µmol eq Trolox/mL.

3.5.5. Sensory Analysis

The sensory characteristics of beef pattie samples were evaluated by the discriminative triangle test. A non-trained panel composed of 31 students between 18 and 22 years old were selected from the School of Industrial Engineering of Barcelona (ETSEIB). The different beef pattie samples (Control,

T-AVL and T-AVF) were cooked in a Hamburger Grill (Tristar, GR-2843, Barcelona, Spain) at full power for 3 min and presented directly to the panelists. In the triangle test, each panelist chose the odd one of the three samples (two similar samples and a different one). Water, apples and biscuits were provided for cleaning the palate after tasting each sample.4.7. Data analysis

Analysis was carried out in triplicate ($n = 3$) and standard deviations (SD) were calculated. All the data were analyzed by the MINITAB software program (Version 18, München, Germany) using Tukey's test. The significance of differences ($p < 0.05$) between mean values was determined by the one-way analysis of variance (ANOVA).

4. Conclusions

The results obtained in this study showed that the leaves and flowers of *A. vulneraria* are rich in phenolic compounds and have significant antioxidant activity with significant differences between the two parts, and the flowers presented the best results. The antioxidant effect of the *A. vulneraria* leaves and flowers in a model food emulsion was investigated for the first time in this study. The flowers had a more notable effect on oxidation in the oil-in-water emulsion system than the leaves, as they had the most effective antioxidant effect against lipid oxidation in raw beef patties during refrigerated storage. In conclusion, the use of the *A. vulneraria* as a natural additive in food, cosmetic and pharmaceutical products is an effective strategy to improve their nutritional and medical value while ensuring consumer safety.

Author Contributions: Maria Pilar Almajano and Leila Bettaieb Ben Kâab conceived the idea; Maria Pilar Almajano designed the experiments; Manel Ouerfelli performed the experiments; Manel Ouerfelli and Maria Pilar Almajano analyzed the data; Maria Pilar Almajano contributed reagents/materials/analysis tools; and Manel Ouerfelli wrote the paper. All the authors read and approved the final paper.

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References

1. Ouerghemmi, I.; Bettaieb Rebey, I.; Rahali, F.Z.; Bourgou, S.; Pistelli, L.; Ksouri, R.; Marzouk, B.; Saidani Tounsi, M. Antioxidant and antimicrobial phenolic compounds from extracts of cultivated and wild-grown Tunisian *Ruta chalepensis*. *J. Food Drug Anal.* **2017**, *25*, 350–359. [CrossRef] [PubMed]
2. Gallego, M.G.; Rodriguez, T.; Rodriguez, I.; Almajano, M.P. Analytical Characterization of Polyphenols from Tara and *Caesalpinia decarpetala* as Stabilizers of O/W Emulsions. *J. Food Sci.* **2016**, *81*, C2676–C2685. [CrossRef] [PubMed]
3. Kchaou, W.; Abbès, F.; Mansour, R.B.; Blecker, C.; Attia, H.; Besbes, S. Phenolic profile, antibacterial and cytotoxic properties of second grade date extract from Tunisian cultivars (*Phoenix dactylifera* L.). *Food Chem.* **2016**, *194*, 1048–1055. [CrossRef] [PubMed]
4. Zilani, M.N.H.; Sultana, T.; Asabur Rahman, S.M.; Anisuzzman, M.; Islam, M.A.; Shilpi, J.A.; Hossain, M.G. Chemical composition and pharmacological activities of *Pisum sativum*. *BMC Complement. Altern. Med.* **2017**, *17*, 1–9. [CrossRef] [PubMed]
5. Maqsood, S.; Benjakul, S. Comparative studies of four different phenolic compounds on in vitro antioxidative activity and the preventive effect on lipid oxidation of fish oil emulsion and fish mince. *Food Chem.* **2010**, *119*, 123–132. [CrossRef]
6. Pisoschi, A.M.; Pop, A. The role of antioxidants in the chemistry of oxidative stress: A review. *Eur. J. Med. Chem.* **2015**, *97*, 55–74. [CrossRef] [PubMed]
7. Gallego, G.; Hakkarainen, M.; Almajano, M.P. Stability of O/W emulsions packed with PLA film with incorporated rosemary and thyme. *Eur. Food Res. Technol.* **2016**, 1–11. [CrossRef]

8. Lin, M.-J.; Chang, S.-C.; Jea, Y.-S.; Liao, J.-W.; Fan, Y.-K.; Lee, T.-T. In vitro antioxidant capability and performance assessment of White *Roman goose* supplemented with dried *Toona sinensis*. *J. Appl. Anim. Res.* **2016**, *44*, 395–402. [CrossRef]
9. Wardhani, D.H.; Fuciños, P.; Vázquez, J.A.; Pandiella, S.S. Inhibition kinetics of lipid oxidation of model foods by using antioxidant extract of fermented soybeans. *Food Chem.* **2013**, *139*, 837–844. [CrossRef] [PubMed]
10. Balasundram, N.; Sundram, K.; Samman, S. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chem.* **2006**, *99*, 191–203. [CrossRef]
11. Wang, Y.C.; Chuang, Y.C.; Ku, Y.H. Quantitation of bioactive compounds in citrus fruits cultivated in Taiwan. *Food Chem.* **2007**, *102*, 1163–1171. [CrossRef]
12. Palici, I.F.; Liktor-Busa, E.; Zupkó, I.; Touzard, B.; Chaieb, M.; Urbán, E.; Hohmann, J. Study of in vitro antimicrobial and antiproliferative activities of selected Saharan plants. *Acta Biol. Hung.* **2015**, *66*, 385–394. [CrossRef] [PubMed]
13. Mhalla, D.; Bouaziz, A.; Ennouri, K.; Chawech, R.; Smaoui, S.; Jarraya, R.; Tounsi, S.; Trigui, M. Antimicrobial activity and bioguided fractionation of Rumex tingitanus extracts for meat preservation. *Meat Sci.* **2017**, *125*, 22–29. [CrossRef] [PubMed]
14. Gepts, P.; Beavis, W.D.; Brummer, E.C.; Shoemaker, R.C.; Stalker, H.T.; Weeden, N.F.; Young, N.D. Legumes as a model plant family. Genomics for food and feed report of the Cross-Legume Advances Through Genomics Conference. *Plant Physiol.* **2005**, *137*, 1228–1235. [CrossRef] [PubMed]
15. Graham, P.H.; Vance, C.P. Update on Legume Utilization Legumes: Importance and Constraints to Greater Use. *Plant Physiol.* **2014**, *131*, 872–877. [CrossRef] [PubMed]
16. Nartowska, J.; Wawer, I.; Strzelecka, H. Triterpenoid sapogenin from *Anthyllis vulneraria L.*. *Acta Pol. Pharm. Drug Res.* **2001**, *58*, 289–291.
17. Halabalaki, M.; Urbain, A.; Paschali, A.; Mitakou, S.; Tillequin, F.; Skaltsounis, A.L. Quercetin and kaempferol 3-*O*-[α-L-rhamnopyranosyl-(→2)-α-L-arabinopyranoside]-7-*O*-α-L-rhamnopyranosides from *Anthyllis hermanniae*: Structure determination and conformational studies. *J. Nat. Prod.* **2011**, *74*, 1939–1945. [CrossRef] [PubMed]
18. Suganda, A.G.; Amoros, M.; Girre, L.; Fauconnier, B. Purifies De Plantes Indigenes Francaises Sur La Multiplication De L ' Herpesvirus Humain 1 Et Du. *J. Nat. Prod.* **1983**, *4*, 626–632. [CrossRef]
19. Godevac, D.; Zdunić, G.; Šavikin, K.; Vajs, V.; Menković, N. Antioxidant activity of nine Fabaceae species growing in Serbia and Montenegro. *Fitoterapia* **2008**, *79*, 185–187. [CrossRef] [PubMed]
20. Csepregi, R.; Bencsik, T.; Papp, N. Examination of secondary metabolites and antioxidant capacity of *Anthyllis vulneraria*, *Fuchsia* sp., *Galium mollugo* and *Veronica beccabunga*. *Acta Biol. Hung.* **2016**, *67*, 442–446. [CrossRef] [PubMed]
21. Tusevski, O.; Kostovska, A.; Iloska, A.; Trajkovska, L.; Simic, S.G. Phenolic production and antioxidant properties of some Macedonian medicinal plants. *Cent. Eur. J. Biol.* **2014**, *9*, 888–900. [CrossRef]
22. Son, S.Y.; Kim, N.K.; Lee, S.; Singh, D.; Kim, G.R.; Lee, J.S.; Yang, H.S.; Yeo, J.; Lee, S.; Lee, C.H. Metabolite fingerprinting, pathway analyses, and bioactivity correlations for plant species belonging to the Cornaceae, Fabaceae, and Rosaceae families. *Plant Cell Rep.* **2016**, *35*, 1917–1931. [CrossRef] [PubMed]
23. Skowyra, M.; Gallego, G. Antioxidant properties of aqueous and ethanolic extracts of tara (*Caesalpinia spinosa*) pods in vitro and in model food emulsions. *J. Sci Food Agric.* **2013**, *94*, 911–918. [CrossRef] [PubMed]
24. Ksouri, R.; Megdiche, W.; Falleh, H.; Trabelsi, N.; Boulaaba, M.; Smaoui, A.; Abdelly, C. Influence of biological, environmental and technical factors on phenolic content and antioxidant activities of Tunisian halophytes. *Comptes Rendus Biologies* **2008**, *331*, 865–873. [CrossRef] [PubMed]
25. Santas, J.; Carbó, R.; Gordon, M.H.; Almajano, M.P. Comparison of the antioxidant activity of two Spanish onion varieties. *Food Chem.* **2008**, *107*, 1210–1216. [CrossRef]
26. Gallego, M.G.; Gordon, M.H.; Segovia, F.J.; Skowyra, M.; Almajano, M.P. Antioxidant properties of three aromatic herbs (rosemary, thyme and lavender) in oil-in-water emulsions. *J. Am. Oil Chem. Soc.* **2013**, *90*, 1559–1568. [CrossRef]
27. Pahua-Ramos, M.E.; Ortiz-Moreno, A.; Chamorro-Cevallos, G.; Hernández-Navarro, M.D.; Garduño-Siciliano, L.; Necoechea-Mondragón, H.; Hernández-Ortega, M. Hypolipidemic Effect of Avocado (*Persea americana Mill*) Seed in a Hypercholesterolemic Mouse Model. *Plant Foods Hum. Nutr.* **2012**, *67*, 10–16. [CrossRef] [PubMed]

28. Cheung, L.M.; Cheung, P.C.K.; Ooi, V.E.C. Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chem.* **2003**, *81*, 249–255. [CrossRef]
29. Ye, F.; Liang, Q.; Li, H.; Zhao, G. Solvent effects on phenolic content, composition, and antioxidant activity of extracts from florets of sunflower (*Helianthus annuus L.*). *Ind. Crops Prod.* **2015**, *76*, 574–581. [CrossRef]
30. Saha, J.; Debnath, M.; Saha, A.; Ghosh, T.; Sarkar, P.K. Response surface optimisation of extraction of antioxidants from strawberry fruit, and lipid peroxidation inhibitory potential of the fruit extract in cooked chicken patties. *J. Sci. Food Agric.* **2011**, *91*, 1759–1765. [CrossRef] [PubMed]
31. Fernández-Agulló, A.; Pereira, E.; Freire, M.S.; Valentão, P.; Andrade, P.B.; González-álvarez, J.; Pereira, J.A. Influence of solvent on the antioxidant and antimicrobial properties of walnut (*Juglans regia* L.) green husk extracts. *Ind. Crops Prod.* **2013**, *42*, 126–132. [CrossRef]
32. Veloz-García, R.A.; Marín-Martínez, R.; Veloz-Rodríguez, R.; Muñoz-Sánchez, C.I.; Guevara-Olvera, L.; Miranda-López, R.; González-Chavira, M.M.; Torres-Pacheco, I.; Guzmán-Maldonado, S.H.; Cardador-Martínez, A.; et al. Antimutagenic and antioxidant activities of cascalote (*Caesalpinia cacalaco*) phenolics. *J. Sci. Food Agric.* **2004**, *84*, 1632–1638. [CrossRef]
33. Feregrino-Pérez, A.A.; Torres-Pacheco, I.; Vargas-Hernández, M.; Munguía-Fragozo, P.V.; Loarca-Piña, G.F.; Mendoza-Díaz, S.O.; Ocampo-Velázquez, R.V.; Rico-García, E.; Guevara-Gónzalez, R.G. Antioxidant and antimutagenic activities of *Acacia pennatula* pods. *J. Sci. Ind. Res.* **2011**, *70*, 859–864.
34. Karakoca, K.; Asan-Ozusaglam, M.; Cakmak, Y.S.; Teksen, M. Phenolic Compounds, biological and antioxidant activities of *onobrychis armena Boiss. & Huet* flower and root extracts. *Chiang Mai J. Sci.* **2015**, *42*, 376–392.
35. Kenny, O.; Smyth, T.J.; Hewage, C.M.; Brunton, N.P. Antioxidant properties and quantitative UPLC-MS analysis of phenolic compounds from extracts of fenugreek (*Trigonella foenum-graecum*) seeds and bitter melon (*Momordica charantia*) fruit. *Food Chem.* **2013**, *141*, 4295–4302. [CrossRef] [PubMed]
36. Skowyra, M.; Gallego, M.; Segovia, F.; Almajano, M. Antioxidant Properties of *Artemisia annua* Extracts in Model Food Emulsions. *Antioxidants* **2014**, *3*, 116–128. [CrossRef] [PubMed]
37. Azman, N.; Segovia, F.; Martínez-Farré, X.; Gil, E.; Almajano, M. Screening of Antioxidant Activity of *Gentian Lutea* Root and Its Application in Oil-in-Water Emulsions. *Antioxidants* **2014**, *3*, 455–471. [CrossRef] [PubMed]
38. Segovia Gómez, F.; Almajano Pablos, M.P. Pineapple Waste Extract for Preventing Oxidation in Model Food Systems. *J. Food Sci.* **2016**, *81*, C1622-C1628. [CrossRef] [PubMed]
39. Davey, M.W.; Stals, E.; Panis, B.; Keulemans, J.; Swennen, R.L. High-throughput determination of malondialdehyde in plant tissues. *Anal. Biochem.* **2005**, *347*, 201–207. [CrossRef] [PubMed]
40. Frankel, E.N.; Huang, S.W.; Aeschbach, R.; Prior, E. Antioxidant Activity of a Rosemary Extract and Its Constituents, Carnosic Acid, Carnosol, and Rosmarinic Acid, in Bulk Oil and Oil-in-Water Emulsion. *J. Agric. Food Chem.* **1996**, *44*, 131–135. [CrossRef]
41. Decker, E.A.; Warner, K.; Richards, M.P.; Shahidi, F. Measuring antioxidant effectiveness in food. *J. Agric. Food Chem.* **2005**, *53*, 4303–4310. [CrossRef] [PubMed]
42. Sun, Y.E.; Wang, W.D.; Chen, H.W.; Li, C. Autoxidation of unsaturated lipids in food emulsion. *Crit. Rev. Food Sci. Nutr.* **2011**, *51*, 453–466. [CrossRef] [PubMed]
43. Leygonie, C.; Britz, T.J.; Hoffman, L.C. Impact of freezing and thawing on the quality of meat: Review. *Meat Sci.* **2012**, *91*, 93–98. [CrossRef] [PubMed]
44. Riazi, F.; Zeynali, F.; Hoseini, E.; Behmadi, H.; Savadkoohi, S. Oxidation phenomena and color properties of grape pomace on nitrite-reduced meat emulsion systems. *Meat Sci.* **2016**, *121*, 350–358. [CrossRef] [PubMed]
45. .Skowyra, M.; Janiewicz, U.; Salejda, A.M.; Krasnowska, G.; Almajano, M.P. Effect of tara (*Caesalpinia spinosa*) pod powder on the oxidation and colour stability of pork meat batter during chilled storage. *Food Technol. Biotechnol.* **2015**, *53*, 419–427. [CrossRef] [PubMed]
46. Azman, A.; Gallego, M.; Julià, L.; Fajari, L.; Almajano, M.P. The effect of *convolvulus arvensis* dried extract as a potential antioxidant in food models. *Antioxidants* **2015**, *4*, 170–184. [CrossRef] [PubMed]
47. Muela, E.; Sañudo, C.; Campo, M.M.; Medel, I.; Beltrán, J.A. Effect of freezing method and frozen storage duration on instrumental quality of lamb throughout display. *Meat Sci.* **2010**, *84*, 662–669. [CrossRef] [PubMed]
48. Li, M.; Li, X.; Xin, J.; Li, Z.; Li, G.; Zhang, Y.; Du, M.; Shen, Q.W.; Zhang, D. Effects of protein phosphorylation on color stability of ground meat. *Food Chem.* **2017**, *219*, 304–310. [CrossRef] [PubMed]

49. Gallego, M.G.; Gordon, M.H.; Segovia, F.J.; Almajano, M.P. Caesalpinia decapetala Extracts as Inhibitors of Lipid Oxidation in Beef Patties. *Molecules* **2015**, *20*, 13913–13926. [CrossRef] [PubMed]
50. Hawashin, M.D.; Al-Juhaimi, F.; Ahmed, I.A.M.; Ghafoor, K.; Babiker, E.E. Physicochemical, microbiological and sensory evaluation of beef patties incorporated with destoned olive cake powder. *Meat Sci.* **2016**, *122*, 32–39. [CrossRef] [PubMed]
51. Mancini, R.A.; Hunt, M.C. Current research in meat color. *Meat Sci.* **2005**, *71*, 100–121. [CrossRef] [PubMed]
52. Muthukumar, M.; Naveena, B.M.; Vaithiyanathan, S.; Sen, A.R.; Sureshkumar, K. Effect of incorporation of Moringa oleifera leaves extract on quality of ground pork patties. *J. Food Sci. Technol.* **2012**, *51*, 3172–3180. [CrossRef] [PubMed]
53. Esmer, O.K.; Irkin, R.; Degirmencioglu, N.; Degirmencioglu, A. The effects of modified atmosphere gas composition on microbiological criteria, color and oxidation values of minced beef meat. *Meat Sci.* **2011**, *88*, 221–226. [CrossRef] [PubMed]
54. Pękal, A.; Pyrzynska, K. Evaluation of Aluminium Complexation Reaction for Flavonoid Content Assay. *Food Anal. Methods.* **2014**, *7*, 1776–1782. [CrossRef]
55. Shalaby, E.A.; Shanab, S.M.M. Comparison of DPPH and ABTS assays for determining antioxidant potential of water and methanol extracts of *Spirulina platensis*. *Indian J. Geomarine Sci.* **2013**, *42*, 556–564.

Sample Availability: Samples of the *A. vulneraria* are available from the authors.

Annex 2

antioxidants

MDPI

Article

Effect of Neem (*Azadirachta indica* L.) on Lipid Oxidation in Raw Chilled Beef Patties

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Abstract: The aim of this study was to determine the total polyphenol content, radical scavenging and antimicrobial activities of *Azadirachta indica* (*A. indica*) and to evaluate their effect on shelf-life stability of raw beef patties during refrigerated storage at 4 ± 1 °C. During 11 days of storage, the antioxidant effect of *A. indica* on ground beef meat was investigated by the determination of lipid oxidation, pH, anti-radical activity, color, hexanal content, and microbial growth. The results obtained showed that fresh *A. indica* leaves and synthetic conservative behaved in the same way and retarded the lipid oxidation of chilled beef patties while increasing their pH (5.40 and 5.45, respectively). It can also be said that *A. indica* limited the loss of color, reduced the metmyoglobin formation (36.70%) and had a significant effect on bacterial growth and hexanal content. In addition, the results obtained through anti-radical and antimicrobial properties showed proportional values of total polyphenol content and radical scavenging activity of leaf extracts as they showed their antimicrobial effect against some bacteria such as *Staphylococcus aureus* and *Micrococcus luteus*, among others. These results support the involvement of *A. indica* in the food industry as a natural antioxidant that could replace synthetic ones.

Keywords: *Azadirachta indica*; antimicrobial activity; antioxidants; lipid oxidation; beef meat

1. Introduction

For decades, red meat from beef, sheep and pork has played an important role in the human diet [1] due to its high quality proteins, minerals, vitamins and many other essential nutrients indispensable for human health [2]. However, meat and meat products are susceptible to two main factors that lead to their deterioration, which are microbial growth and rapid lipid oxidation caused by the high concentrations of moisture, unsaturated lipids, hemo-pigments and other different oxidizing agents present in the muscle tissues [3].

Microbial growth and rapid lipid oxidation are generally accompanied by the formation of toxic compounds, texture deterioration, color and nutrient loss, accumulation of harmful compounds and shelf-life lessening, which decrease the nutritional quality of the meat products [4].

Therefore, different methods were developed to preserve meat and meat products from deterioration [5], like adding exogenous antioxidants considered beneficial for the meat quality. For instance, synthetic antioxidants such as the butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were widely added in meat products to prevent and reduce lipid oxidation [6]. However, their application in meat products, has recently being questioned, mainly by consumers, due to their possible side effects [5,7]. For this reason, consumers tend to prefer meat products that contain the minimum quantities of synthetic antioxidants and prefer products with natural

preservatives from plants with high antioxidant and antibacterial properties to reduce the risk of lipid oxidation in the meat and at the same time to ensure it is a healthy product for the consumer [8].

Several studies demonstrated the efficiency of plant materials rich in phenolic compounds as good natural antioxidants in meat and meat products. As examples, pomegranate peel [9], leaf extracts from *Rumex tingitanus* [10], Lucerne (alfalfa) [11], *Urtica dioica* and *Hibiscus sabdariffa* [12] can be cited. Moreover, plants represent good opportunities to control microorganisms in food as an alternative to synthetic preservatives [13]. For instance, *Azadirachta indica* A. Juss., commonly denominated as the Neem tree, which belongs to the Meliaceae family, is one of the most useful plants in traditional medicine in the Indian culture thanks to its various therapeutic benefits [14,15]. Neem is considered one of the most important plants worldwide, due to its diverse therapeutic applications and the variety of bioactive constituents present in its different parts [16]. The documented medicinal virtues of the Neem showed that its different parts (leaves, flowers, seeds, fruits, roots and bark) have been used to treat several human ailments such as inflammation, diarrhea, bacterial infection, constipation and even cancer [17].

Several reports suggested the importance of the Neem as an edible plant rich in bioactive molecules and pharmacological properties [18]. However, despite the diverse investigations done to demonstrate the biological and therapeutic benefits of Neem's different parts, its effect on meat products remains unclear given the poorness of research regarding the use of Neem. For these reasons, the main target of this study is to determine phenolic compounds and antimicrobial activities of *A. indica* leaf extracts and evaluate the antioxidant property of its dry leaves on lipid oxidation and shelf life of raw beef patties during refrigerated storage at 4 ± 1 °C.

2. Materials and Methods

2.1. Natural Products

The leaves of *A. indica* were collected from Punjab in the north of India; while *C. baccatum* fruits were purchased from a local market (Mercadona, Barcelona, Spain).

2.2. Microbial Strains

Six different microbial strains from the collection ATCC were obtained from the Universitat de Barcelona (UB). *Staphylococcus aureus* (*S. aureus*, ATCC 25423), *Micrococcus luteus* (*M. luteus*, ATCC 4698), *Listeria* (ATCC 15313) and *Bacillus cereus* (*B. cereus*, ATCC 11778) are Gram-positive (Gram+) bacteria, while *Escherichia coli* (*E. coli*, ATCC 25022) and *Salmonella paratyphi A* (*S. paratyphi*, ATCC 9150) are Gram-negative (Gram−).

2.3. Meat

Fresh ground beef meat was purchased from "Carns Blai" butchery (Barcelona, Spain) and brought to the laboratory under refrigeration (4 ± 1 °C).

2.4. Chemicals and Products

Ethanol (EtOH), methanol (MeOH), gallic acid (GA), trolox, 2-thiobarbituric acids (TBA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), hexanal and peptone water were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain). Acetone (Ac), Folin–Ciocalteu reagent and phosphate buffered saline (PBS) were acquired from Panreac Química S.L.U (Barcelona, Spain). Miller Hinton agar, tryptone glucose yeast agar and Penicillin-Streptomycin (Pen-Strep) were bought from Thermo-Fisher Scientific (Barcelona, Spain).

The synthetic preservative used as a positive control in the determination of the antioxidant effect of powdered *A. indica* dry leaves on beef meat quality is "Conservative CAMPA N°3 (A), code 403600", elaborated by "La Campana" for burger meat and composed of: dextrose, preservatives:

(sulfur dioxide 5.7%), E-224 (sulfite) and antioxidants (E-301 and E-331 iii). The synthetic preservative was purchased from la CAMPANA (Barcelona, Spain).

2.5. Instrumentation

All absorbance analyses were performed on multimode micro-plate reader FLUOstar® Omega (Ortenberg, Germany) equipped with five detection modes using an ultra-fast UV/Vis.

2.6. Total Polyphenol Content and Radical Scavenging Activity of A. indica and C. baccatum Extracts

2.6.1. Extracts Preparation

Dry leaves of *A. indica* and *C. baccatum* were ground with liquid nitrogen until fine and homogeneous powders were obtained. The extraction was carried out according to the method of Slatnar et al. [19] with few adaptations. An amount of 1 g from each powdered sample was extracted with 10 mL of 50% aqueous EtOH (v/v) at 4 ± 1 °C under sonication for 1 h. Then, the extracts were centrifuged at 16,800× *g* for 10 min at 4 °C (Orto Alresa Mod. Consul, Ortoalresa, Ajalvir Madrid, Spain). The supernatants obtained were filtered through Whatman filters N°1 and lyophilized for 2 days (Unicryo MC2L, UniEquip Laborgerätebau & Vertr. GmbH, Martinsried, Munich, Germany). The final dry extracts obtained were dissolved in 5 mL of different solvents with increased polarity (80% EtOH, 80% MeOH and deionized water) then stored at 4 °C after being filtered through 0.45 µm pore filters to ensure their purity. All the extracts were used to determine their total polyphenols content and radical scavenging activity, following DPPH assay, and 80% MeOH samples were used to assess their antimicrobial activity.

For all parameters studied below, samples were analyzed in triplicate.

2.6.2. Total Polyphenols Content (TPC)

TPC was determined following the Folin–Ciocalteu phenol reagent method as described by Villasante et al. [20]. The absorbance was measured at 765 nm and measurements were based on a calibration curve made with Gallic Acid (100–1700 µM, $R^2 = 0.992$). The results are expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g DW).

2.6.3. Radical Scavenging Activity (RSA): DPPH Assay

The ability of *A. indica* and *C. baccatum* extracts to scavenge DPPH radicals was assessed following the method adapted by Maqsood and Benjakul [21]. The results are expressed as milli-mole of Trolox equivalents per gram of dry weight (mM TE/g of DW).

2.7. Antibacterial Activity of A. indica and C. baccatum Extracts, by Agar Disk-Diffusion Method (Inhibitory Zone Assay)

Inhibitory zone assay was assessed following the method of Balouiri et al. [22] using sterile disks (inner diameter 6 mm). The 80% aqueous MeOH extract of *A. indica* and *C. baccatum* were sterilized by filtration through 0.22 µm milli-pore filters. Mueller–Hinton agar was solidified in different culture dishes and each dish was inoculated with 100 µL of a different bacterial suspension. Then, sterile disks impregnated with 100 µL of each extract were put in each dish. Disks soaked in penicillin (100 µg/mL) were used as positive control, while disks soaked in 80% MeOH were used as negative control. All the culture plates were incubated at 35 ± 2 °C for 18 h. The antibacterial activity of the different samples was determined by measuring the diameter of inhibition zones against the tested microorganisms.

2.8. Antioxidant Effect of Powdered A. indica Dry Leaves on Beef Meat Quality

2.8.1. Preparation and Storage Conditions of Raw Beef Patties

Three minced meat pieces of three different cuts taken from the round part of three different beef were bought fresh on three different days. The meat mixture was done by mincing the meat twice and passing it through a 4 mm diameter hole, so that the fat was homogeneously distributed. Each piece was well mixed with salt (1.5%, w/w) and divided into five different parts, one part was considered as a control and each one of the remaining four parts was mixed with a different compound to finally obtain five different beef meat parts: CTR (CTR, meat sample with no antioxidant), S.C (meat samples formulated with 0.7% (w/w) synthetic conservative), *C.B* (meat samples formulated with 0.7% (w/w) powdered *C. baccatum* fruits), *A.I* (meat sample formulated with 0.7% (w/w) *A. indica* dry powdered leaves) and *A.I + C.B* (meat samples formulated with 0.7% (w/w) fresh powdered *A. indica* leaves combined with 0.7% (w/w) powdered *C. baccatum* fruits).

Various patties (3 to 4 g in weight, 4 cm in diameter and 0.5 cm in thickness) were subsequently formed using a round cutter, then placed in plastic trays after being covered with plastic films and kept in the refrigerator at 4 ± 1 °C. During 11 days of chilled storage, the lipid oxidation delay and metmyoglobin (MetMb) content were measured every 2 days while the quality of beef patties (pH and color variations) was monitored daily. The microbiological analysis, the anti-radical activity determined by the hydrophilic and lipophilic DPPH assays, and the determination of hexanal content were carried out every 5 days.

2.8.2. Lipid Oxidation and pH Value Evolution

Evolution of lipid oxidation in raw beef patties was assessed by thiobarbituric acid reactive substance (TBARS) assay as described by Fan et al. [23]. The absorbance was measured at 531 nm and the results are expressed as milligrams of malondialdehyde (MDA) per kilogram of meat sample (mg MDA/kg meat sample).

The pH measurement of beef samples was determined using an Orion 3-Star pH Benchtop Meter (Thermo Fisher Scientific, Waltham, MA, USA).

2.8.3. Antioxidant Capacity (AOC)

AOC of meat samples was determined by hydrophilic and lipophilic DPPH assays (H-DPPH and L-DPPH, respectively). As described by Gallego et al. [24], the first extract solvent was Milli-Q water (Simplicity®, C9210, Merck KGaA, Darmstadt, Germany) and the second one was composed of Ac, EtOH and Milli-Q water (5:4:1; v/v/v) to extract hydrophilic and lipophilic anti-radicals, respectively. The extracts obtained were used to perform DPPH assays as described previously. The results were expressed as micromole of Trolox equivalents per milliliter of extract (µmol TE/mL).

2.8.4. Color Fading Measurement

Color fading of the different treated meat samples was measured in triplicate at three different locations on the surface of the raw beef patties while avoiding the fatty zones in order to obtain correct measurements [25]. Color stability of raw beef patties was evaluated using a reflectance colorimeter Minolta CR-400 (Konica Minolta, Tokyo, Japan) and was expressed against the scale of Lightness (L*), Redness (a*) and Yellowness (b*) in the CIELab color space system.

2.8.5. Metmyoglobin (MetMb) Reducing Activity

According to Aini et al. [26], an amount of 5 g from each beef meat sample was homogenized with 25 mL of PBS (0.04M, pH = 6.8) for 30 sec using Ultra Turrax (IKA, Model T18 Basic, Germany). The homogenized mixture was stored for 1 h at 4 °C and centrifuged at 1500× *g* for 20 min at 4 °C.

The absorbance of the supernatant was read at 572, 565, 545 and 525 nm. The percentage of MetMb (%) was determined using the Krzywicki equation (1) described below:

$$\text{MetMb (\%)} = [2.514 \times (A_{572}/A_{525}) + 0.777 \times (A_{565}/A_{525}) + 0.8 \times (A_{545}/A_{525}) + 1.098] \quad (1)$$

where A_{572} is absorbance at a wavelength of 572 nm, A_{525} is absorbance at a wavelength of 525 nm, A_{565} is absorbance at a wavelength of 565 nm and A_{545} is absorbance at a wavelength of 545 nm.

2.8.6. Hexanal Content Determination by HS-GC/MS

The different meat samples were prepared by mixing 500 mg of each treated beef sample with 1.5 mL of Milli-Q water in a headspace vial. Then, each vial was sealed air-tight with a PTFE septum. Hexanal was used as standard for the calibration at different concentrations ranging from 0.005 to 0.250 ppm. To determine the hexanal content, vials were incubated at 80 °C for 30 min. The analysis was performed using HS-GC/MS by injecting 1 mL of vapor phase through a syringe kept at 85 °C. Equipment used consisted of a Trace GC gas chromatograph with a Head Space Tri-plus auto-sampler coupled to a DSQII mass spectrometer (Thermo Fisher Scientific, Austin, Texas, USA) with TRB-624 (60 m × 0.32 mm × 1.8 mm) column, 1.8 mL/min helium flow. The injector temperature was 220 °C with split mode injection (split flow 20 mL/min). Temperature program was 60 °C held for 2 min and then raised to 220 °C at the rate of 8 °C /min (5 min). Interface temperature was 260 °C and ionization source temperature 230 °C [27]. Results are expressed in milligram of hexanal per gram of meat sample (mg hexanal/g meat sample).

2.8.7. Microbial Analysis

The presence of colony-forming units was determined according to the method described by Hawashin et al. [28]. Shortly, 10 g of each beef patty were weighted into a stomacher bag (Stomacher® Lab system, Seward) and homogenized with 100 mL of 0.1% sterile peptone water, then well mixed using a stomacher (Stomacher® 80 Lab Blender, Galileo Equipos, Madrid, Spain) for 2 to 3 min. Different dilutions were prepared and 100 µL of each dilution were transferred to a standard agar coated plate (with Tryptone Glucose Yeast Agar, TGYA). The plates were incubated at 37 °C for 48 h. The microbial colonies were observed on the initial, fifth and last days of analysis.

2.8.8. Sensory Analysis

A panel composed of seven semi-trained judges, familiar with quality of meat and meat products, evaluated the cooked beef patties on day 1. The subjects were gender balanced (four females and three males) between the age of 20 and 28 years old. Each judge was instructed to taste each sample and grade them from 1 (least preferred) to 10 (most preferred) [29]. Control beef patties (without antioxidant) and beef patties formulated with synthetic conservative, *A. indica* leaves and *C. baccatum* fruits were cooked in a Hamburger Grill (Tristar, GR-2843, Barcelona, Spain) at full power for 5 min then coded and presented directly to the panelists. Water, apples and biscuits were provided to clean the palate after tasting each sample. Results were analyzed using the tables developed by Basker [30].

2.8.9. Statistical Analysis

Data analysis was carried out in triplicate ($n = 3$) and standard deviations (SD) were calculated by the MINITAB software program (Version 17, München, Germany). Tukey's test was used to calculate the significant differences ($p < 0.05$) between mean values.

3. Results and Discussion

3.1. Total Polyphenol Content (TPC) and Radical Scavenging Activity (RSA)

TPC and RSA of *A. indica* and *C. baccatum* extracts were determined and the results obtained are presented in Table 1.

Table 1. Total polyphenol content (TPC) and radical scavenging activity (RSA) of *A. indica* leaves and *C. baccatum* fruits aqueous extracts.

Samples	Extract Solvents	TPC (mg GAE/g DW)	RSA: DPPH Assay (mM TE/g DW)
A. indica	80% EtOH	47.47 ± 0.03 ^{c}	0.37 ± 0.013 ^{b}
	80% MeOH	107.41 ± 0.03 ^{b}	0.72 ± 0.004 ^{a}
	Deionized water	20.93 ± 0.08 ^{a}	0.27 ± 0.002 ^{c}
C. baccatum	80% EtOH	34.78 ± 0.03 ^{a}	0.29 ± 0.002 ^{b}
	80% MeOH	53.91 ± 0.02 ^{b}	0.42 ± 0.001 ^{a}
	Deionized water	20.23 ± 0.01 ^{c}	0.17 ± 0.004 ^{c}

Results represent the mean of three replicates (n = 3) and are expressed as mean value ± SD. For each sample different letters in the same column indicate significant differences in each column at $p < 0.05$. DPPH (Diphenyl-1-picrylhydrazyl), MeOH (methanol), GAE (gallic acid equivalent) and TE (Trolox equivalent).

Depending on the extraction solvent polarity, TPC results showed significant differences ($p < 0.05$) between samples. The leaves of *A. indica* extracted in 80% MeOH presented higher content of total polyphenols with 107.41 ± 0.03 mg GAE/g DW than extracts prepared in 80% EtOH and deionized water which contained lower TPC estimated at 47.47 ± 0.03 and 20.93 ± 0.08 mg GAE/g DW, respectively. TPC was also highest in *C. baccatum* fruits extracted in 80% MeOH with 53.91 ± 0.02 mg GAE/g DW followed by 80% EtOH extract with 34.78 ± 0.03 mg GAE/g DW and deionized water extract which contained the lowest content determined at 20.23 ± 0.01 mg GAE/g LP. Furthermore, the data obtained from the determination of *A. indica* leaf and *C. baccatum* fruit extract ability to scavenge DPPH radicals also presented significant differences ($p < 0.05$) between samples (Table 1). RSA was significantly higher ($p < 0.05$) in *A. indica* leaves and *C. baccatum* fruits extracted in 80% MeOH (0.72 ± 0.004 and 0.42 ± 0.001 mM TE/g LP, respectively) than 80% EtOH and deionized water extracts.

From the point of view of the samples investigated, *A. indica* leaves 80% MeOH, 80% EtOH and deionized water extracts presented, respectively, 27, 50 and 3% higher TPC and 22, 42 and 37% stronger RSA than *C. baccatum* extracts.

Variations in polyphenol contents and anti-radical activity were observed in different studies. For example, Ghimeray et al. [31] reported similar results for TPC expressed in tannic acid equivalents in *A. indica* leaves extracted in MeOH and water. However, RSA was higher in the present study. Datta et al. [32] reported lower TPC in *A. indica* leaves extracted with water than those obtained in this study. In addition, Sora et al. [33] made a comparative study of the phenolic content and antioxidant activity of *C. baccatum* ethanol extract and found TPC and RSA 5 and 7 times, respectively, higher than those found in the present study.

These variations in TPC could be explained by the type of extraction solvent used. MeOH and EtOH extracted polyphenols better in comparison with deionized water. This can be explained by the fact that the extraction yield of polyphenols is higher with solvents that have lower polarities than water [34]. The degradation of phenolic compounds by enzymes called polyphenol oxidases can also be an origin of the low TPC in the samples extracted with deionized water since these enzymes are active in water whereas they are inactive in alcoholic solutions [35]. This great variability found in literature values could also be related to the origin of each plant studied [20]. Moreover, the results collected by the TPC determination and the measurement of the anti-radical potential of *A. indica* and *C. baccatum* extracts to scavenge DPPH radicals revealed that the variation of the anti-radical activity observed in the different extracts of the two plants investigated depends on the TPC. In fact, the values obtained vary proportionally; the higher the polyphenols content, the stronger the antioxidant activity,

which shows that the antioxidant activity of the various extracts may be due to their TPC. This agrees with the result found by Gallego et al. [36] and Kaviarasan et al. [37] who demonstrated that phenolic compounds are indeed responsible for the antioxidant activity of plant extracts [38].

3.2. Screening of Antibacterial Activity

The results obtained (Table 2) showed that *A. indica* and *C. baccatum* 80% MeOH extracts had antimicrobial activity against the majority of bacterial strains tested. The best antimicrobial activity of *A. indica* extract was obtained in an *E. coli* strain with an inhibition zone estimated at 21 mm, followed by *S. aureus*, *M. luteus and S. paratyphi* strains with inhibition zones estimated at 19, 12 and 10 mm, respectively. However, *A. indica* extract had no activity on *Listeria* and *B. cereus* strains which had inhibition diameters of 15 and 10 mm, respectively, in the presence of penicillin. In addition, inhibition halos were observed in the presence of the *C. baccatum* extract. The strongest antimicrobial activity of *C. baccatum* extract was recorded in *S. aureus* strain with an inhibition zone estimated at 22 mm, followed by *M. luteus* and *Listeria* strains with inhibition zones estimated both at 10 mm. However, *C. baccatum* extract had no activity on *B. cereus*, *S. paratyphi* and *E. coli* strains which had diameters of 10, 30 and 27 mm, respectively, in the presence of penicillin.

Table 2. Antibacterial activity of *A. indica* and *C. baccatum* extracts determined by inhibitory zone assay.

			Inhibitory Zone (mm)			
	Microorganisms	**Strains**	***A. indica***	***C. baccatum***	***penicillin***	**80% MeOH**
Gram+	*S. aureus*	ATCC 25423	19	22	24	-
	M. luteus	ATCC 4698	12	10	17	-
	Listeria	ATCC 15313	-	10	15	-
	B. cereus	ATCC 11778	-	-	10	-
Gram−	*S. paratyphi*	ATCC 9150	10	-	30	-
	E. coli	ATCC 25022	21	-	27	-

A. indica 80% MeOH extract. *C. baccatum* 80% MeOH extract. Penicillin is used as positive control. Sterile 80% MeOH is used as negative control. Gram+: Gram positive bacteria. Gram−: Gram negative bacteria. No inhibition zone is indicated by (−).

The analysis of the results in Table 2 showed that 80% MeOH *A. indica* extract had antibacterial activity on both Gram-positive and Gram-negative bacteria, while *C. baccatum* extract had only an effect on Gram-positive bacteria. On one hand, this antibacterial activity is conferred by the presence of capsaicin and dihydrocapsaicin in extracts in addition to phenolic compounds whose antibacterial properties have been demonstrated in many researches [39,40]. On the other hand, the absence of antimicrobial activity in *A. indica* and *C. baccatum* against some bacterial strains could be explained by the fact that these strains developed resistance mechanisms. Many studies have shown that phenotypic variability may be a strategy put in place by certain microorganisms to resist certain compounds [41,42]. It is also possible that the solvent used during the extraction may not have been able to retain the desired molecules because of its polarity or concentration. Several investigations suggested that the antimicrobial activity of herbal extracts required high concentrations [43]. This probably suggests that the extract concentration used in the present study was lower than expected and this could explain the lack of activity.

In conclusion, different factors may be at the origin of the presence or absence of the extracts' antibacterial activity. It all depends on the type of bacterial strain and its resistance, the polarity of the extraction solvent, the concentration of the extract and the composition and activity of the plant investigated.

3.3. Effect of Powdered A. indica Leaves on Beef Meat Quality During Refrigerated Storage

3.3.1. Lipid Oxidation and pH Variation

The direct incorporation of *A. indica* dry leaves in chilled ground raw beef meat has been carried out in order to evaluate their efficacy against the formation of malonaldehyde, aldehyde compounds, and ketones resulting from lipid oxidation. This evaluation was determined by the TBARS assay and the results obtained are illustrated in Figure 1.

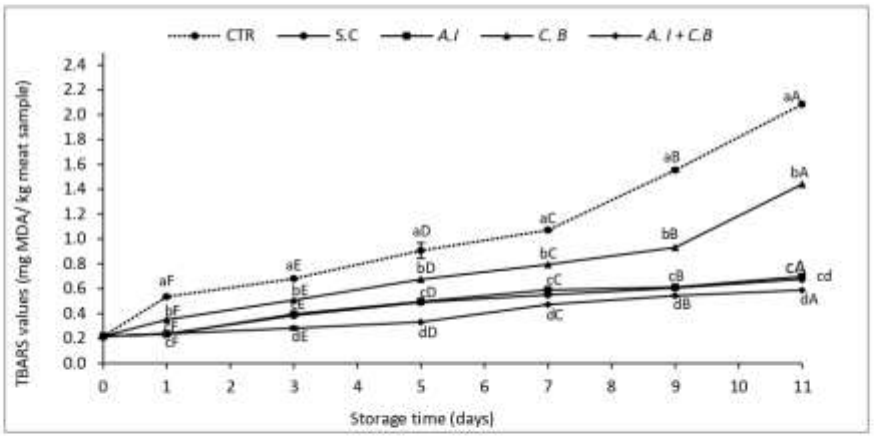

Figure 1. Thiobarbituric acid reactive substance (TBARS) values of raw beef patties during storage at 4 ± 1 °C. CTR (Control sample without antioxidant), S.C (treatment with 0.7% (w/w) synthetic conservative), *A.I* (treatment with 0.7% (w/w) powdered *A. indica* leaves), *C.B* (treatment with 0.7% (w/w) powdered *C. baccatum* fruits) and *A.I* + *C.B* (treatment with 0.7% (w/w) powdered *A. indica* leaves and 0.7% (w/w) powdered *C. baccatum* fruits). Results represent the mean of three replicates ($n = 3$) and are expressed as mean value ± SD; different letters in the same day indicate significant difference between samples at $p < 0.05$, different capital letters indicate significant difference between storage days at $p < 0.05$ for the same sample.

The results showed that the secondary oxidation of beef samples significantly increased during refrigerated storage ($p < 0.05$). CTR beef sample had the highest TBARS value estimated at 2.08 mg MDA/kg meat compared with *A.I* + *C.B* (0.7%, w/w) which presented a synergistic antioxidant effect in raw beef patties and produced a combined inhibitory effect of MDA formation greater than the rest of formulated meat samples and reached a value of 0.59 mg MDA /kg meat by the end of the storage period. Secondary oxidation of *A.I*, S.C and *C.B* meat samples also increased progressively with storage time. The *A.I* meat sample showed an effective antioxidant effect against lipid degradation almost similar to the S.C sample and reached 0.68 and 0.70 mg MDA/kg meat, respectively, while *C.B* beef samples presented higher TBARS values estimated at 1.44 mg MDA/kg meat. TBARS values recorded in *A.I* and *A.I* + *C.B* beef patties were considered to be a good sign of their efficiency against lipid oxidation in beef patties since they didn't exceed 1.5 mg MDA/kg meat. This value is considered as an indicator of lipid degradation in meat as reported by Martínez et al. [44].

The pH of beef patties throughout chilled storage period was also measured to determine its correlation with TBARS values. The results obtained are presented in Figure 2.

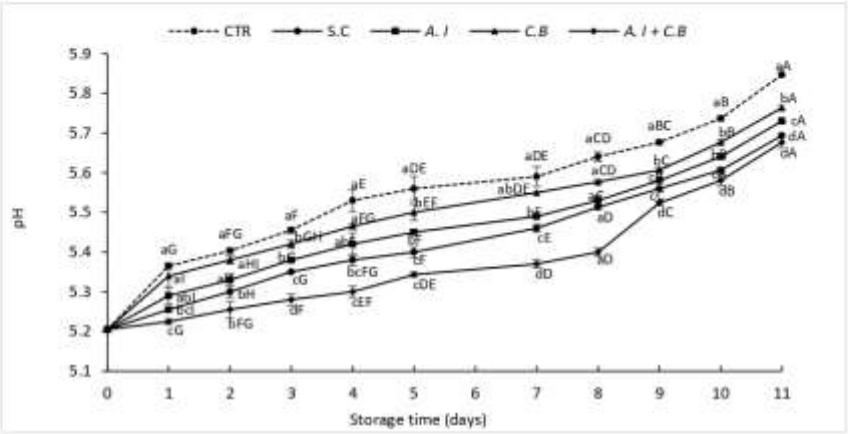

Figure 2. Evolution of pH values of raw beef patties during storage at 4 ± 1 °C. CTR (Control sample without antioxidant), S.C (treatment with 0.7% (w/w) synthetic conservative), *A.I* (treatment with 0.7% (w/w) powdered *A. indica* leaves), *C.B* (treatment with 0.7% (w/w) powdered *C. baccatum* fruits) and *A.I* + *C.B* (treatment with 0.7% (w/w) powdered *A. indica* leaves and 0.7% (w/w) powdered *C. baccatum* fruits). Results represent the mean of three replicates ($n = 3$) and are expressed as mean value ± SD; different letters in the same day indicate significant difference between samples at $p < 0.05$, different capital letters indicate significant difference between storage days at $p < 0.05$ for the same sample.

At every storage day, the pH values were significantly different between meat samples ($p < 0.05$) and increased steadily over storage time. The highest pH values were recorded in the CTR meat sample which increased from 5.21 at day 1 to 5.85 at day 11 followed by the pH of *C.B* beef patties presenting pH values ranging from 5.21 to 5.76 at day 1 and day 11, respectively. The S.C, *A.I* and *A.I* + *C.B* kept their pH relatively low, especially *A.I* + *C.B* beef meat which presented the best pH values ranging from 5.21 in the initial day to 5.37 in the final day 11 of chilled storage.

Our findings about the positive impact of edible plants rich in phenolic compounds on the lipid oxidation process in meat are in agreement with other studies. Özer et al. [45] assessed the effects of quinoa flour on lipid oxidation in raw beef burger during long term frozen storage and found that the addition of quinoa significantly decreased TBARS values for raw burger compared to control group during storage. Abdelhakam et al. [46], also studied the quality characteristics of beef hamburgers enriched with red grape pomace powder during freezing storage and found similar results. The antioxidant effect of roasted coffees added to refrigerated ground pork over 21 days was determined by Hashimoto et al. [47] who found TBARS values in treated meat samples lower than those of control. Fan et al. [23] investigated the effects of *Portulaca oleracea* L. on lipid oxidation of pork meat during refrigerated storage and obtained results supporting the hypothesis that the addition of natural products enriched in polyphenols extends the shelf life of fresh meat and delays lipid oxidation.

The antioxidant effect of *A. indica* dry leaves and *C. baccatum* fruits on oxidative stability could possibly be associated with their wealth of phenolic compounds which present strong antioxidant activity allowing them to scavenge hydroperoxides, whose decomposition results in secondary oxidation products responsible for the deterioration of meat quality [48].

Moreover, the variation in pH values in beef meat during chilled storage can be influenced by different factors. Many studies have addressed the decrease in acidity on microorganisms and enzymes that degrade meat proteins and produce ammonia, amines and other toxic compounds. This conducts to high pH values. These compounds are formed rapidly when meat starts to decompose [49].

3.3.2. AOC Assay

The results of the anti-radical capacity assay determined by the H-DPPH and L-DPPH assays in the initial and last day of refrigerated storage are summarized in Figure 3a,b, respectively.

The results obtained showed that AOC values determined by the H-DPPH assay (Figure 3a) are lower than those obtained by L-DPPH assay (Figure 3b). According to the H-DPPH assay, the result differences obtained are significant ($p < 0.05$) between samples on each day of analysis. The different samples exhibited stronger anti-radical activity at day 1 than day 11. The highest value was recorded in the meat sample formulated with *A.I* + *C.B* ranging from 0.08 to 0.07 µmol TE/mL, followed by the S.C *and A.I* beef samples which had almost the same anti-radical activity ranging from 0.07 to 0.05 µmol TE/mL and had no significant difference at day 1. CTR and *C.B* meat samples presented the lowest AOC values estimated at 0.05 and 0.03 µmol TE/mL at day 1 and 0.05 and 0.04 µmol TE/mL at day 11, respectively.

AOC values determined by L-DPPH assay (Figure 3b) also presented significant differences between samples ($p < 0.05$). CTR and *C.B* beef meat had the lowest anti-radical activities estimated at 0.27 and 0.25 µmol TE/mL in day 1 and 0.16 and 0.22 µmol TE/mL at day 11. The significantly highest values were recorded in *A.I* + *C.B* sample (0.70 and 0.53 µmol TE/mL at day 1 and 11, respectively), followed by S.C and *A.I* beef samples (with no significant difference at day 1) ranging from 0.44 to 0.30 µmol TE/mL at day 1 and 0.43 to 0.28 µmol TE/mL at day 11, respectively.

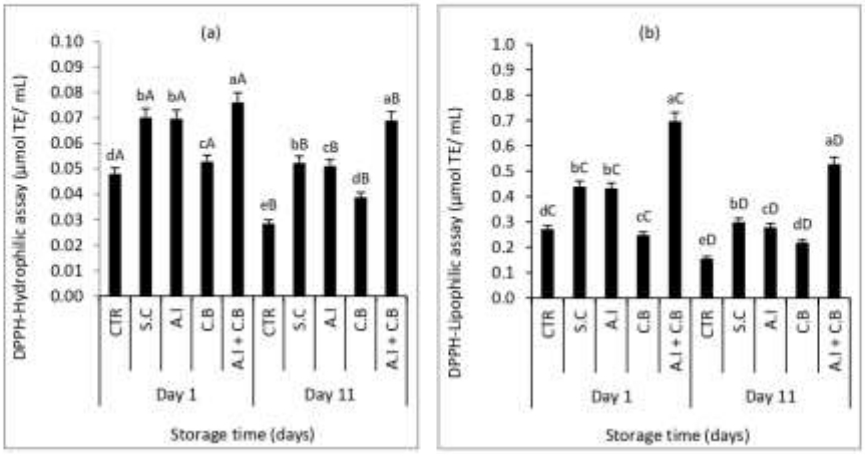

Figure 3. Antioxidant capacity (AOC) measurements by DPPH-Hydrophilic (**a**) and DPPH-Lipophilic (**b**) assays of raw beef patties during storage at 4 ± 1 °C. CTR (Control sample without antioxidant), S.C (treatment with 0.7% (w/w) synthetic conservative), *A.I* (treatment with 0.7% (w/w) powdered *A. indica* leaves), *C.B* (treatment with 0.7% (w/w) powdered *C. baccatum* fruits) and *A.I* + *C.B* (treatment with 0.7% (w/w) powdered *A. indica* leaves and 0.7% (w/w) powdered *C. baccatum* fruits). Results represent the mean of three replicates ($n = 3$) and are expressed as mean value ± SD; different letters in the same day indicate significant differences between samples at $p < 0.05$, different capital letters indicate significant differences between storage days at $p < 0.05$ for the same sample.

Lipophilic antioxidants, such as tocopherols and carotenoids, and hydrophilic antioxidants like ascorbic acid and the majority of phenolic compounds are two different groups of antioxidants which contribute to a high antioxidant capacity, protecting the meat products treated with natural antioxidants to be against oxidation [50]. Several studies demonstrated that these antioxidants can improve the nutrition value of meat. For instance, Gallego et al. [24] and Ouerfelli et al. [50] achieved similar results with differences in the values obtained and found that the *Caesalpinia decapetala* and *Anthyllis vulneraria*,

respectively, can be good sources of natural antioxidants since they had higher antioxidant capacity determined with hydrophilic and lipophilic FRAP assays as they noticed that hydrophilic FRAP values are higher than lipophilic ones.

3.3.3. Color Fading and MetMb Reducing Activity

Color changes measured on the surface of the different beef patties during 11 days of refrigerated storage are illustrated in Table 3.

Table 3. Color changes (a*, b*, L*) in treated raw beef patties during refrigerated storage at 4 ± 1 °C.

Trait	Day	CTR	S.C	*A.I*	*C.B*	*A.I* + *C.B*
Redness (a*)	1	39.27 ± 2.37^{aA}	50.12 ± 0.77^{bA}	42.65 ± 2.16^{cA}	48.36 ± 0.37^{dA}	45.19 ± 1.26^{cA}
	2	33.32 ± 0.37^{aB}	49.14 ± 1.32^{bB}	39.43 ± 0.96^{cB}	47.02 ± 0.05^{bB}	38.77 ± 2.14^{cB}
	3	30.15 ± 0.26^{aC}	48.89 ± 1.52^{bB}	38.73 ± 1.03^{cC}	45.07 ± 0.33^{dC}	38.23 ± 2.45^{cB}
	4	27.65 ± 0.71^{aD}	48.62 ± 0.51^{bB}	38.31 ± 0.65^{cC}	43.47 ± 0.23^{dD}	37.45 ± 1.45^{cC}
	5	27.12 ± 0.31^{aD}	45.56 ± 0.21^{bC}	38.04 ± 0.27^{cC}	42.11 ± 0.15^{bE}	37.01 ± 1.32^{cC}
	7	25.88 ± 0.17^{aE}	41.96 ± 0.23^{bD}	36.01 ± 0.25^{cD}	37.85 ± 1.22^{cF}	36.04 ± 1.23^{cD}
	8	25.24 ± 0.12^{aE}	39.78 ± 1.02^{bE}	34.29 ± 0.63^{cE}	31.11 ± 0.47^{dG}	35.77 ± 1.09^{cE}
	9	$23.99 \pm 0,13^{aF}$	39.02 ± 1.01^{bE}	31.12 ± 0.16^{cF}	29.63 ± 0.23^{dH}	35.19 ± 1.47^{dE}
	10	23.06 ± 0.19^{aF}	38.45 ± 0.98^{bF}	28.29 ± 0.77^{cG}	27.03 ± 0.11^{cI}	30.44 ± 2.52^{dF}
	11	22.89 ± 1.23^{aG}	37.21 ± 1.00^{bG}	26.64 ± 1.26^{cH}	25.24 ± 1.14^{cJ}	30.01 ± 0.99^{dF}
Yellowness (b*)	1	11.28 ± 0.11^{aA}	15.25 ± 0.11^{bA}	14.14 ± 0.67^{bA}	10.55 ± 1.21^{dA}	12.25 ± 1.01^{cA}
	2	10.56 ± 1.32^{aB}	14.43 ± 1.14^{bB}	13.49 ± 1.26^{bB}	9.83 ± 2.01^{dB}	12.04 ± 1.61^{cA}
	3	9.89 ± 1.22^{aC}	13.98 ± 1.22^{bC}	13.32 ± 1.25^{bB}	9.78 ± 0.55^{aB}	11.78 ± 1.44^{cB}
	4	9.54 ± 1.03^{aC}	13.43 ± 0.11^{bC}	12.87 ± 0.42^{cC}	8.66 ± 1.06^{dC}	11.59 ± 1.25^{dB}
	5	9.43 ± 0.15^{aC}	13.25 ± 0.25^{bC}	12.44 ± 0.17^{cC}	8.51 ± 0.09^{dC}	11.23 ± 1.09^{cB}
	7	7.45 ± 0.18^{aD}	12.12 ± 0.13^{bD}	11.01 ± 0.44^{cD}	7.88 ± 0.15^{aD}	10.76 ± 1.21^{dC}
	8	5.33 ± 0.07^{aE}	11.69 ± 1.09^{bE}	10.82 ± 0.73^{cE}	7.06 ± 0.45^{dD}	10.66 ± 1.56^{cC}
	9	5.24 ± 0.04^{aE}	11.23 ± 0.33^{bE}	10.12 ± 0.01^{cE}	6.59 ± 0.16^{dE}	10.09 ± 1.33^{cC}
	10	5.17 ± 1.22^{aE}	10.76 ± 0.26^{bF}	9.72 ± 0.59^{cF}	6.13 ± 0.19^{dE}	9.44 ± 2.47^{cD}
	11	5.02 ± 1.18^{aE}	10.27 ± 0.11^{bF}	9.42 ± 0.19^{cF}	5.32 ± 1.69^{aF}	9.21 ± 2.89^{cD}
Lightness (L*)	1	57.37 ± 0.74^{aA}	$70,79 \pm 2.03^{bA}$	$68,70 \pm 2.24^{cA}$	65.16 ± 2.00^{dA}	66.15 ± 2.20^{dA}
	2	56.32 ± 2.17^{aB}	67.09 ± 3.02^{bB}	$66.46 \pm 1,42^{cB}$	65.17 ± 2.02^{dA}	66.01 ± 2.09^{cA}
	3	56.01 ± 1.85^{aB}	65.33 ± 1.23^{bC}	64.09 ± 1.74^{cC}	63.46 ± 1.46^{dB}	65.19 ± 2.33^{bB}
	4	55.84 ± 1.40^{aC}	64.70 ± 3.53^{bC}	$62,61 \pm 0,33^{bD}$	61.43 ± 1.52^{cC}	61.23 ± 2.40^{cC}
	5	54.98 ± 1.78^{aD}	63.12 ± 2.10^{bD}	58.12 ± 0.78^{cE}	59.87 ± 1.96^{cD}	54.54 ± 2.10^{aD}
	7	53.13 ± 1.45^{aE}	60.15 ± 1.64^{bE}	56.45 ± 1.77^{cF}	56.16 ± 1.69^{cE}	52.33 ± 2.44^{dE}
	8	52.41 ± 0.50^{aF}	59.23 ± 0.23^{bF}	54.15 ± 1.26^{cG}	55.14 ± 1.62^{cF}	51.46 ± 2.30^{dF}
	9	$51. 26 \pm 1.45^{aG}$	56.54 ± 0.19^{bG}	$52. 49 \pm 1.65^{cH}$	53.75 ± 1.36^{dG}	50.73 ± 2.96^{eG}
	10	47.46 ± 1.85^{aH}	54.16 ± 0.15^{bH}	50.46 ± 1.87^{cI}	51.36 ± 1.64^{dH}	49.16 ± 2.23^{eH}
	11	43.23 ± 2.96^{aI}	54.41 ± 2.65^{bF}	49.22 ± 0.04^{cJ}	48.04 ± 0.21^{cJ}	47.36 ± 2.08^{dI}

Results represent the mean of three replicates ($n = 3$) and are expressed as mean value ± SD. CTR (Control sample without antioxidant), S.C (treatment with 0.7% (w/w) synthetic conservative), *A.I* (treatment with 0.7% (w/w) powdered *A. indica* leaves), *C.B* (treatment with 0.7% (w/w) powdered *C. baccatum* fruits) and *A.I* + *C.B* (treatment with 0.7% (w/w) powdered *A. indica* leaves and 0.7% (w/w) powdered *C. baccatum* fruits). Different letters in the same day indicate significant differences between samples at $p < 0.05$, different capital letters indicate significant differences between storage days at $p < 0.05$ for the same sample.

The red color of meat is one of the most important factors that determines the purchase decision of consumers. The results presented in the Table 3 showed Lightness (L*) values of the different treated beef patties which decreased significantly ($p < 0.05$) over storage time.

The results showed also that the beef patties formulated with synthetic conservative had the highest Redness (a*) values ranging from 50.12 at day 1 to 37.21 at day 11, followed by the sample containing *A.I* + *C.B*, while CTR and *C.B* meat samples presented the lowest values during storage period, because it is meat that has a higher proportion of metmyoglobin, a color with a tendency to brown. On the other hand CTR and *C.B* meat samples presented the lowest values during storage period. *A.I* beef samples showed better red color values than the control samples despite the dark color that the powdered leaves attributed to the beef patties.

In addition to Redness (a*), synthetic conservative enhanced the Yellowness (b*) of raw beef patties during storage period and presented lower values ranging from 15.25 at day 1 to 10.27 at

day 11, compared to the Yellowness (b*) values of the CTR and *C. B* meat samples which decreased significantly ($p < 0.05$) during refrigerated storage and presented the lowest Yellowness (b*) values.

Figure 4 presented the changes in the MetMb percentage in beef patties treated during refrigerated storage.

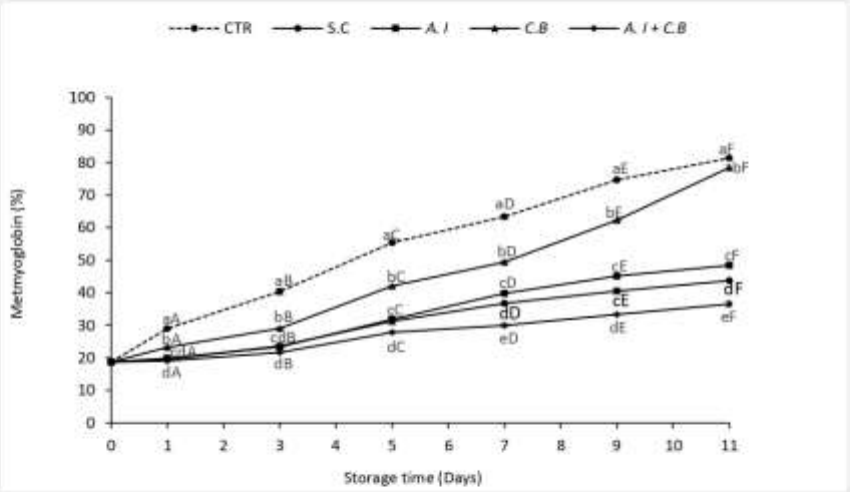

Figure 4. Effects of powdered *A. indica* leaves added at 0.7% (w/w) on MetMb changes in beef patties during 11 days of refrigerated storage at 4 ± 1 °C. CTR (Control sample without antioxidant), S.C (treatment with 0.7% (w/w) synthetic conservative), *A.I* (treatment with 0.7% (w/w) powdered *A. indica* leaves), *C.B* (treatment with 0.7% (w/w) powdered *C. baccatum* fruits) and *A.I + C.B* (treatment with 0.7% (w/w) powdered *A. indica* leaves and 0.7% (w/w) powdered *C. baccatum* fruits). Results represent the mean of three replicates ($n = 3$) and are expressed as mean value ± SD; different letters in the same day indicate significant differences between samples at $p < 0.05$, different capital letters indicate significant differences between storage days at $p < 0.05$ for the same sample.

The MetMb increased ($p < 0.05$) progressively in all the beef samples as the treatment time was further prolonged. The CTR sample presented the highest percentage estimated from 18.73% at the initial day of storage to 81.39% at the end of the storage period, while *A.I + C.B* beef patties presented the lowest percentage that did not exceed 36.55% after 11 days of chilled storage. Beef samples treated with S.C and *A.I* exhibited almost the same MetMb percentage recorded at 43.69% and 48.42%, respectively, at the end of storage time. However, *C.B* beef samples increased gradually and had higher MetMb percentage ranging from 18.73% to 78.47%.

Different studies reported similar results and suggested that free radicals produced during lipid oxidation may damage the structure of muscle fibers and reduce pigmentation [23,50,51].

Redness (a*) is the most important color parameter of meat and meat products [24]. The fading of red color of beef patties during storage can be explained by the oxidation of myoglobin over time when meat decomposes and MetMb starts to be formed [52]. In addition, the main cause of the color change in meat is the oxidation of myoglobin from Fe(II) of myoglobin to Fe(III) giving met-myoglobin (MetMb) [40].

To conclude, the measurement of the beef patties color did not show results in agreement with those determined by TBARS, pH and AOC assays. This may be due to the color change that occurred when the beef patties were mixed with the powdered plants, especially *A. indica* whose leaves darkened the beef patties, hence the red color of raw meat had been masked. However, the determination of MetMb percentage in beef patties was effective to support the color measurements results obtained.

3.3.4. Hexanal Content

The hexanal content of meat samples stored at 4 ± 1 °C was determined at day 1, 5 and 11 and the results obtained are represented in Figure 5.

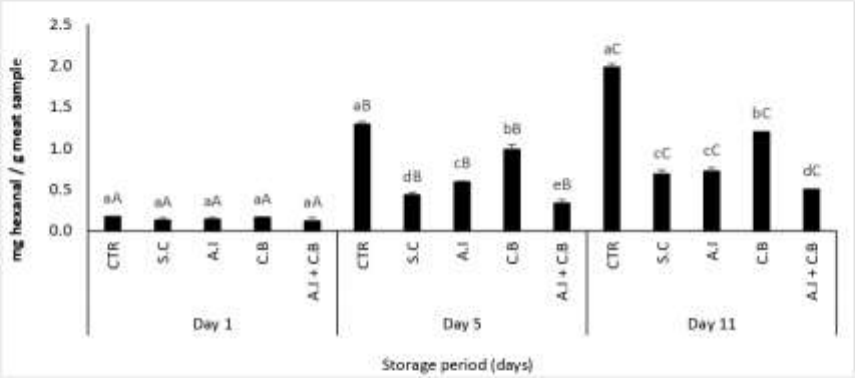

Figure 5. Hexanal content in beef patties during 11 days of refrigerated storage at 4 ± 1 °C. CTR (Control sample without antioxidant), S.C (treatment with 0.7% (w/w) synthetic conservative), *A.I* (treatment with 0.7% (w/w) powdered *A. indica* leaves), *C.B* (treatment with 0.7% (w/w) powdered *C. baccatum* fruits) and *A.I* + *C.B* (treatment with 0.7% (w/w) powdered *A. indica* leaves and 0.7% (w/w) powdered *C. baccatum* fruits). Results represent the mean of three replicates ($n = 3$) and are expressed as mean value ± SD; different letters in the same day indicate significant difference between samples at $p < 0.05$, different capital letters indicate significant difference between storage days at $p < 0.05$ for the same sample.

The hexanal content of meat samples increased significantly over storage time. The first day of storage there were no significant differences observed between S.C and *A.I* beef meat samples, whereas CTR, *C.B* and *A.I* + *C.B* samples showed significant differences. After 5 days of chilled storage, the content of hexanal increased significantly in all the samples. The highest content of hexanal was observed in CTR and *C.B* samples with 1.29 and 0.99 mg hexanal/g meat sample, respectively, while the lowest hexanal content was observed in *A.I* + *C.B* sample with 0.34 mg hexanal/g meat sample. The S.C and *A.I* meat samples exhibited almost the same effect and presented hexanal contents estimated at 0.436 and 0.594 mg hexanal/g meat sample, respectively. At the end of the storage period, CTR presented the highest content of hexanal at 1.98 mg hexanal/g meat sample while the *A.I* + *C.B* sample presented the lowest hexanal content estimated at 0.50 mg hexanal/g meat sample.

Just like color, aroma is an important criterion that influences the decision of customers to buy meat and meat products [53]. Oxidation reactions cause the creation of volatile compounds. The analysis of these volatile compounds is a good indicator of the oxidation state of the meat products [54]. The main compounds sought is hexanal, which is predominant in the volatile fractions of meat products [24].

Similar observations have also been made by Juntachote et al. [55] about holy basil and galangal in pork meat. Gallego et al. [24] also reported similar results about *Caesalpinia decapetala* showing that natural antioxidants exhibited better antioxidant effect than that shown by synthetic conservatives, when assessed by hexanal formation.

3.3.5. Antimicrobial Analysis

Presence of colony-forming units evaluated in the control and treated beef samples at the first; fifth and last day of incubation is presented in Table 4.

Table 4. Effect of *A. indica* and *C. baccatum* on microbial quality of raw beef meat during refrigerated (4 ± 1 °C) storage.

Aerobic Mesophilic Bacteria Presence	Refrigerated Storage Period (Days)		
	0	5	11
CTR	−	+	+
S.C	−	−	−
A.I	−	−	−
C.B	−	−	+
A.I + C.B	−	−	−

(−) indicates less of 10^4 CFU/g sample of aerobic mesophilic bacteria in the meat sample; (+) indicates a number of aerobic mesophilic bacteria between 10^4 and 10^5 CFU/g.

The number of mesophilic bacteria present in all samples at the first day of incubation was less than 10^4 CFU/g sample. After 5 days of incubation, number of mesophilic bacteria present in CTR beef meat increased to 4.2×10^4 CFU/g, while the rest of samples kept their effective antibacterial properties. At the last day of the experiment, the antimicrobial activity of *C. baccatum* meat sample became weak, hence the increase in the number of bacteria. Our findings are consistent with previous reports in which bioactive compounds from plants were successfully used to disinfect meat samples [27,46,56].

3.3.6. Sensory Analysis

In order to know the total acceptability of the different meat samples, a grade sensorial analysis was made. The results are shown in Figure 6. It should be noted that there are no major differences between the samples, except for the one that incorporates *C. baccatum* fruits that is perceived as extremely spicy. In this sense it depends on the taste and what the consumer is looking for in the hamburger. Its incorporation may be positive, but not always.

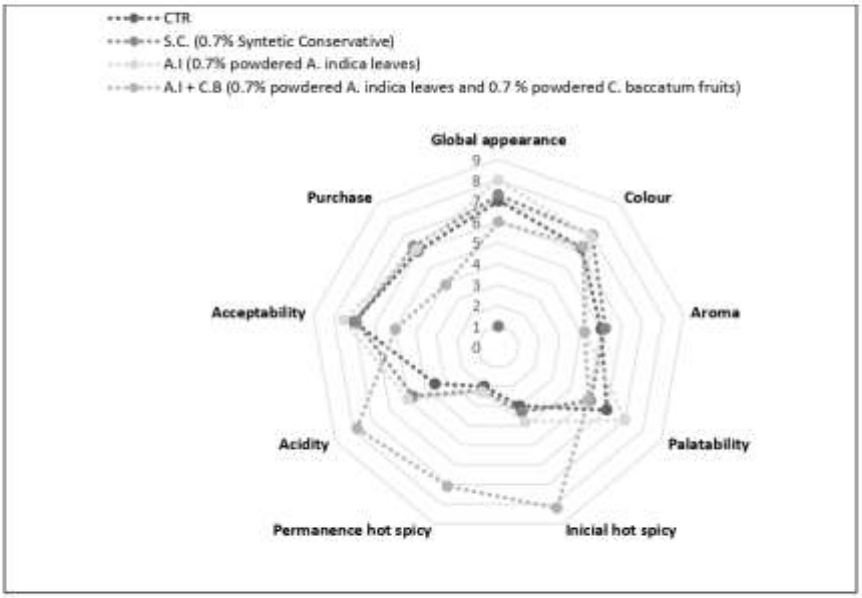

Figure 6. Sensorial analysis of CTR (Control sample without antioxidant), S.C (treatment with 0.7% (w/w) synthetic conservative), *A.I* (treatment with 0.7% (w/w) powdered *A. indica* leaves), and *A.I + C.B* (treatment with 0.7% (w/w) powdered *A. indica* leaves and 0.7% (w/w) powdered *C. baccatum* fruits).

4. Conclusions

Antioxidant effect of *A. indica* powdered leaves added directly to raw beef patties during refrigerated storage at 4 ± 1 °C was investigated in this study. The results obtained from the analysis of lipid oxidation, changes in pH and color, microbial growth, MetMb formation, hexanal content and antioxidant capacity proved that *A. indica* contains natural antioxidants that might substitute synthetic ones since they presented similar protective effect against deterioration.

To conclude, the use of *A. indica* as a natural antioxidant in beef meat products might be a good strategy to improve the nutritional value of meat products while ensuring consumers' safety.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Jiang, J.; Xiong, Y.L. Natural antioxidants as food and feed additives to promote health benefits and quality of meat products: A review. *Meat Sci.* **2016**, *120*, 107–117. [CrossRef] [PubMed]
2. Cao, Y.; Gu, W.; Zhang, J.; Chu, Y.; Ye, X.; Hu, Y.; Chen, J. Effects of chitosan, aqueous extract of ginger, onion and garlic on quality and shelf life of stewed-pork during refrigerated storage. *Food Chem.* **2013**, *141*, 1655–1660. [CrossRef] [PubMed]
3. Falowo, A.B.; Fayemi, P.O.; Muchenje, V. Natural antioxidants against lipid-protein oxidative deterioration in meat and meat products: A review. *Food Res. Int.* **2014**, *64*, 171–181. [CrossRef] [PubMed]
4. Shah, M.A.; Bosco, S.J.D.; Mir, S.A. Plant extracts as natural antioxidants in meat and meat products. *Meat Sci.* **2014**, *98*, 21–33. [CrossRef] [PubMed]
5. Duman, M.; Özpolat, E. Effects of water extract of propolis on fresh shibuta (*Barbus grypus*) fillets during chilled storage. *Food Chem.* **2015**, *189*, 80–85. [CrossRef]
6. Aziz, M.; Karboune, S. Natural antimicrobial/antioxidant agents in meat and poultry products as well as fruits and vegetables: A review. *Crit. Rev. Food Sci. Nutr.* **2018**, *58*, 486–511. [CrossRef]
7. Lin, M.J.; Chang, S.C.; Jea, Y.S.; Liao, J.W.; Fan, Y.K.; Lee, T.T. In vitro antioxidant capability and performance assessment of White Roman goose supplemented with dried *Toona sinensis*. *J. Appl. Anim.Res.* **2016**, *44*, 395–402. [CrossRef]
8. Gupta, S.; Abu-Ghannam, N. Recent developments in the application of seaweeds or seaweed extracts as a means for enhancing the safety and quality attributes of foods. *Innov. Food Sci. Emerg. Technol.* **2011**, *12*, 600–609. [CrossRef]
9. Turgut, S.S.; Soyer, A.; Işıkçı, F. Effect of pomegranate peel extract on lipid and protein oxidation in beef meatballs during refrigerated storage. *Meat Sci.* **2016**, *116*, 126–132. [CrossRef]
10. Mhalla, D.; Bouaziz, A.; Ennouri, K.; Chawech, R.; Smaoui, S.; Jarraya, R.; Tounsi, S.; Trigui, M. Antimicrobial activity and bioguided fractionation of *Rumex tingitanus* extracts for meat preservation. *Meat Sci.* **2017**, *125*, 22–29. [CrossRef]
11. Baldi, G.; Chauhan, S.S.; Linden, N.; Dunshea, F.R.; Hopkins, D.L.; Sgoifo Rossi, C.A.; Dell'Orto, V.; Ponnampalam, E.N. Comparison of a grain-based diet supplemented with synthetic vitamin E versus a lucerne (alfalfa) hay-based diet fed to lambs in terms of carcass traits, muscle vitamin E, fatty acid content, lipid oxidation, and retail colour of meat. *Meat Sci.* **2019**, *148*, 105–112. [CrossRef]
12. Seçil Karabacak, H.; Bozkurt, S. Effects of *Urtica dioica* and *Hibiscus sabdariffa* on the quality and safety of sucuk (Turkish dry-fermented sausage). *Meat Sci.* **2008**, *78*, 288–296. [CrossRef]

13. Del Serrone, P.; Toniolo, C.; Nicoletti, M. Neem (*Azadirachta indica* A. Juss) Oil: A natural preservative to control meat spoilage. *Foods* **2015**, *4*, 3–14. [CrossRef]
14. Sharma, O.P.; Bhat, T.K. DPPH antioxidant assay revisited. *Food Chem.* **2009**, *113*, 1202–1205. [CrossRef]
15. Kumar, V.S.; Navaratnam, V. Neem (*Azadirachta indica*): Prehistory to contemporary medicinal uses to humankind. *Asian Pac. J. Trop. Biomed.* **2013**, *3*, 505–514. [CrossRef]
16. Fernandes, S.R.; Barreiros, L.; Oliveira, R.F.; Cruz, A.; Prudêncio, C.; Oliveira, A.I.; Pinho, C.; Santos, N.; Morgado, J. Chemistry, bioactivities, extraction and analysis of azadirachtin: State-of-the-art. *Fitoterapia* **2019**, *134*, 141–150. [CrossRef]
17. Duangjai, A.; Nuengchamnong, N.; Lee, L.H.; Goh, B.H.; Saokaew, S.; Suphrom, N. Characterisation of an extract and fractions of *Azadirachta indica* flower on cholesterol lowering property and intestinal motility. *Nat. Prod. Res.* **2017**, *6419*, 1–4. [CrossRef]
18. Al Akeel, R.; Mateen, A.; Janardhan, K.; Gupta, V.C. Analysis of anti-bacterial and anti oxidative activity of *Azadirachta indica* bark using various solvents extracts. *Saudi J. Biol. Sci.* **2017**, *24*, 11–14. [CrossRef]
19. Slatnar, A.; Mikulic-Petkovsek, M.; Stampar, F.; Veberic, R.; Solar, A. Identification and quantification of phenolic compounds in kernels, oil and bagasse pellets of common walnut (*Juglans regia* L.). *Food Res. Int.* **2015**, *67*, 255–263. [CrossRef]
20. Villasante, J.; Girbal, M.; Metón, I.; Almajano, M.P. Effects of pecan nut (*Carya illinoiensis*) and roselle flower (*Hibiscus sabdariffa*) as antioxidant and antimicrobial agents for sardines (*Sardina pilchardus*). *Molecules* **2019**, *24*, 85. [CrossRef]
21. Maqsood, S.; Benjakul, S. Comparative studies of four different phenolic compounds on in vitro antioxidative activity and the preventive effect on lipid oxidation of fish oil emulsion and fish mince. *Food Chem.* **2010**, *119*, 123–132. [CrossRef]
22. Balouiri, M.; Sadiki, M.; Ibnsouda, S.K. Methods for in vitro evaluating antimicrobial activity: A review. *J. Pharm. Anal.* **2016**, *6*, 71–79. [CrossRef]
23. Fan, X.J.; Liu, S.Z.; Li, H.H.; He, J.; Feng, J.T.; Zhang, X.; Yan, H. Effects of *Portulaca oleracea* L. extract on lipid oxidation and color of pork meat during refrigerated storage. *Meat Sci.* **2019**, *147*, 82–90. [CrossRef]
24. Gallego, M.G.; Gordon, M.H.; Segovia, F.J.; Almajano, M.P. *Caesalpinia decapetala* extracts as inhibitors of lipid oxidation in beef patties. *Molecules* **2015**, *20*, 13913–13926. [CrossRef]
25. Wrona, M.; Nerín, C.; Alfonso, M.J.; Caballero, M.Á. Antioxidant packaging with encapsulated green tea for fresh minced meat. *Innov. Food Sci. Emerg. Technol.* **2017**, *41*, 307–313. [CrossRef]
26. Aini, N.; Azman, M.; Gallego, M.G.; Juliá, L.; Fajari, L.; Apilar Almajano, M.; Miguel, M.G.; Rocha, J.O.; Abourashed, E.A. The effect of *Convolvulus arvensis* dried extract as a potential antioxidant in food models. *Antioxidants* **2015**, *4*, 170–184. [CrossRef]
27. Sirocchi, V.; Devlieghere, F.; Peelman, N.; Sagratini, G.; Maggi, F.; Vittori, S.; Ragaert, P. Effect of *Rosmarinus officinalis* L. essential oil combined with different packaging conditions to extend the shelf life of refrigerated beef meat. *Food Chem.* **2016**. [CrossRef]
28. Hawashin, M.D.; Al-Juhaimi, F.; Ahmed, I.A.M.; Ghafoor, K.; Babiker, E.E. Physicochemical, microbiological and sensory evaluation of beef patties incorporated with destoned olive cake powder. *Meat Sci.* **2016**, *122*, 32–39. [CrossRef]
29. Saha, J.; Debnath, M.; Saha, A.; Ghosh, T.; Sarkar, P.K. Response surface optimisation of extraction of antioxidants from strawberry fruit, and lipid peroxidation inhibitory potential of the fruit extract in cooked chicken patties. *J. Sci. Food Agric.* **2011**, *91*, 1759–1765. [CrossRef]
30. Christensen, Z.T.; Ogden, L.V.; Dunn, M.L.; Eggett, D.L. Multiple comparison procedures for analysis of ranked data. *J. Food Sci.* **2006**, *71*. [CrossRef]
31. Ghimeray, A.K.; Jin, C.; Ghimire, B.K.; Cho, D.H. Antioxidant activity and quantitative estimation of azadirachtin and nimbin in *Azadirachta Indica* A. Juss grown in foothills of Nepal. *J. Biotechnol.* **2009**, *8*, 3084–3091.
32. Datta, A.; Grün, I.U.; Kwasniewski, M.T.; Fernando, L.N. Comparison of two adsorbent based de-bittering procedures for neem (*Azadirachta indica* A. Juss) tea—Effect on polyphenols, anti-oxidant capacity, color and volatile profile. *Plant Foods for Hum. Nutr.* **2017**, *72*, 88–95. [CrossRef]

33. Sora, G.T.S.; Haminiuk, C.W.I.; da Silva, M.V.; Zielinski, A.A.F.; Gonçalves, G.A.; Bracht, A.; Peralta, R.M. A comparative study of the capsaicinoid and phenolic contents and in vitro antioxidant activities of the peppers of the genus *Capsicum*: An application of chemometrics. *J. Food Sci. Technol.* **2015**, *52*, 8086–8094. [CrossRef]
34. Dhanani, T.; Shah, S.; Gajbhiye, N.A.; Kumar, S. Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of *Withania somnifera*. *Arab. J. Chem.* **2017**, *10*, S1193–S1199. [CrossRef]
35. Lubomirova Christova-Bagdassarian, V.; Stefanova Atanassova, M.; Hristova, V.K.; Ayaz Ahmad, M. Soild-liquid extraction kinetics of total phenolic, total flavonoid, rutin and tannin contents in 50% ethanol extract of *Cotinus coggygria*. *Int. J. Sci. Eng. Res.* **2016**, *7*, 1466–1472.
36. Gallego, M.; Skowyra, M.; Gordon, M.; Azman, N.; Almajano, M. Effect of leaves of *Caesalpinia decapetala* on oxidative stability of oil-in-water emulsions. *Antioxidants* **2017**, *6*, 19. [CrossRef]
37. Kaviarasan, S.; Naik, G.H.; Gangabhagirathi, R.; Anuradha, C.V.; Priyadarsini, K.I. In vitro studies on antiradical and antioxidant activities of fenugreek (*Trigonella foenum graecum*) seeds. *Food Chem.* **2007**, *103*, 31–37. [CrossRef]
38. Riazi, F.; Zeynali, F.; Hoseini, E.; Behmadi, H.; Savadkoohi, S. Oxidation phenomena and color properties of grape pomace on nitrite-reduced meat emulsion systems. *Meat Sci.* **2016**, *121*, 350–358. [CrossRef]
39. Cui, Y.; Oh, Y.J.; Lim, J.; Youn, M.; Lee, I.; Pak, H.K.; Park, W.; Jo, W.; Park, S. AFM study of the differential inhibitory effects of the green tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) against Gram-positive and Gram-negative bacteria. *Food Microbiol.* **2012**, *29*, 80–87. [CrossRef]
40. Papuc, C.; Goran, G.V.; Predescu, C.N.; Nicorescu, V.; Stefan, G. Plant polyphenols as antioxidant and antibacterial agents for shelf-life extension of meat and meat products: Classification, structures, sources, and action mechanisms. *Compr. Rev. Food Sci. Food Saf.* **2017**, *16*, 1243–1268. [CrossRef]
41. Pfaller, M.A.; Jones, R.N.; Messer, S.A.; Edmond, M.B.; Wenzel, R.P. National surveillance of nosocomial blood stream infection due to species of Candida other than *Candida albicans*: Frequency of occurrence and antifungal susceptibility in the SCOPE program. *Diagn. Microbiol. Infect. Dis.* **1998**, *30*, 121–129. [CrossRef]
42. Chen, Y.-C. Stable susceptibility of *Candida* blood isolates to fluconazole despite increasing use during the past 10 years. *J. Antimicrob. Chemother.* **2003**, *52*, 71–77. [CrossRef]
43. Adejare, O.; Oduyebo, O.; Oladele, R.; Nwaokorie, E.; Ogunsola, F. In-vitro antifungal effect of *Garcinia kola* and garlic (*Alliums sativum*) on vaginal isolates of *candida*. *Afr. J. Clin. Exp. Microbiol.* **2013**, *14*, 140–145. [CrossRef]
44. Irene Cilla, L.M.; Beltrán, J.A.; Roncalés, P. Antioxidant effect of rosemary, borage, green tea, pu-erh tea and ascorbic acid on fresh pork sausages packaged in a modified atmosphere: Influence of the presence of sodium chloride. *J. Sci. Food Agric.* **2007**, *86*, 1298–1307. [CrossRef]
45. Özer, C.O.; Seçen, S.M. Effects of quinoa flour on lipid and protein oxidation in raw and cooked beef burger during long term frozen storage. *Food Sci. Technol.* **2018**, *2061*, 1–7. [CrossRef]
46. Abdelhakam, O.S.; Elsebaie, E.M.; Ghazi, A.K.; Gouda, M.S. Quality characteristics of beef hamburger enriched with red grape pomace powder during freezing storage. *Slov. Vet. Res.* **2019**, *56*, 333–340. [CrossRef]
47. Hashimoto, T.A.; Caporaso, F.; Toto, C.; Were, L. Antioxidant capacity and sensory impact of coffee added to ground pork. *Eur. Food Res. Technol.* **2019**, *245*, 977–986. [CrossRef]
48. Gallego, M.G.; Gordon, M.H.; Segovia, F.J.; Skowyra, M.; Almajano, M.P. Antioxidant properties of three aromatic herbs (rosemary, thyme and lavender) in oil-in-water emulsions. *J. Am. Oil Chem. Soc.* **2013**, *90*, 1559–1568. [CrossRef]
49. Muela, E.; Sañudo, C.; Campo, M.M.; Medel, I.; Beltrán, J.A. Effect of freezing method and frozen storage duration on instrumental quality of lamb throughout display. *Meat Sci.* **2010**, *84*, 662–669. [CrossRef]
50. Ouerfelli, M.; Bettaieb Ben Kâab, L.; Almajano, M.P. Radical scavenging and antioxidant activity of *Anthyllis vulneraria* leaves and flowers. *Molecules* **2018**, *23*, 1657. [CrossRef]
51. De Oliveira Ferreira, N.S.; Rosset, M.; Lima, G.; Stuelp Campelo, P.M.; de Macedo, R.E.F. Effect of adding *Brosimum gaudichaudii* and *Pyrostegia venusta* hydroalcoholic extracts on the oxidative stability of beef burgers. *LWT* **2019**, *108*, 145–152. [CrossRef]
52. Mancini, R.A.; Hunt, M.C. Current research in meat color. *Meat Sci.* **2005**, *71*, 100–121. [CrossRef]
53. Domínguez, R.; Purriños, L.; Pérez-Santaescolástica, C.; Pateiro, M.; Barba, F.J.; Tomasevic, I.; Campagnol, P.C.B.; Lorenzo, J.M. Characterization of volatile compounds of dry-cured meat products using HS-SPME-GC/MS technique. *Food Anal. Methods* **2019**. [CrossRef]

54. Bosse Née Danz, R.; Wirth, M.; Konstanz, A.; Becker, T.; Weiss, J.; Gibis, M. Determination of volatile marker compounds in raw ham using headspace-trap gas chromatography. *Food Chem.* **2017**, *219*, 249–259. [CrossRef]
55. Juntachote, T.; Berghofer, E.; Siebenhandl, S.; Bauer, F. The antioxidative properties of Holy basil and Galangal in cooked ground pork. *Meat Sci.* **2006**, *72*, 446–456. [CrossRef]
56. Realini, C.E.; Guàrdia, M.D.; Díaz, I.; Garcia-Regueiro, J.A.; Arnau, J. Effects of acerola fruit extract on sensory and shelf-life of salted beef patties from grinds differing in fatty acid composition. *Meat Sci.* **2014**, *99*, 18–24. [CrossRef]

Annex 3

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BIOCHEMISTRY & PHYSIOLOGY - ORIGINAL ARTICLE

Phytochemical screening and evaluation of the antioxidant and anti-bacterial activity of Woundwort (*Anthyllis vulneraria* L.)

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Abstract
For millennia, medicinal plants have been used to prevent and cure diseases. Up to now there is a growing interest in their use in several areas as conducted for *Anthyllis* in our investigation. Actually the present research aims to investigate the biochemical characterisation of a medicinal plant collected from Tunisia named Woundwort [*Anthyllis vulneraria* L. (Jones and Turrill 1933)] by determining the mineral content, phenolic compound contents and biological activities of its leaf and flower extracts. On the one hand, the results obtained showed that *A. vulneraria* accumulated minerals at different amounts with significant differences between leaves and flowers extracts. On the other hand, the data revealed that the hydroethanolic flower extract contained the highest content of total polyphenols, flavonoids and condensed tannin, as it exhibited the strongest antioxidant activity. The flower extract also showed better antibacterial effect than leaf extract. These results support the exploitation of active compounds extracted from the leaves and especially the flowers of *A. vulneraria*, which can provide new alternatives to the use of certain drugs, additives, among others, as they can be used as structure–activity models for the development of new products.

Keywords Biological activity · Medicinal plant · Minerals · Phenolic compounds

Abbreviations

$AlCl_3$	Aluminium chloride
ATCC	American Type Culture Collection
EtOH	Ethanol
$FeCl_2$	Iron(II) chloride
H_2O_2	Hydrogen peroxide
MeOH	Methanol
UV-B	Ultraviolet (280–315 nm)

1 Introduction

Anthyllis vulneraria [*A. vulneraria* (Jones and Turrill 1933)], commonly named "Woundwort" is a mediterranean medicinal plant that belongs to the Fabaceae family (Nartowska et al. 2001). The term "*Anthyllis*" comes from the Latin words "*Anthos*" and "*ioulos*", which mean "flower" and "downy", respectively (as are the undersides of leaf), while "*vulneraria*" in Latin is "*vulnus*", which means "injury" referring generally to wounds healing (Halabalaki et al. 2011). As its name suggests, *A. vulneraria* is a popular remedy for burns and skin rashes. In the traditional medicine, *A. vulneraria* flowers were used to heal wounds, low the high blood pressure, treat inflammation, vomiting, acne and purify the body by promoting the elimination of toxins (Nartowska et al. 2001). They were also used to heal mouth and throat pain, to limit hair loss and promote hair growth (Menković et al. 2011). In recent years, *A. vulneraria* has attracted the attention of researchers to quantify its phenolic compounds and prove its potential as a source of bioactive molecules with effective biological properties. Different environmental factors, including biotic (microbial invasion, insect pests and herbivores) and abiotic (cold and drought) factors stimulate the production of a wide variety

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of bioactive substances in plants to protect themselves (Yang et al. 2018) and which also play a crucial role in preventing and curing human diseases (Sales de Oliveira et al. 2020).

In response to these external changes, plants vary their chemical composition, which explains the qualitative and quantitative differences in their metabolites during a given season and, consequently, the variation in their pharmacological property (Yang et al. 2018). Antioxidant components, such as phenolic compounds are generally received by the human organism as food, drugs and supplements (Lushchak 2014). The antioxidant activity is one of the most important biological properties that characterises bioactive substances produced by plants (Stagos 2020). Plants rich in phenolic compounds have an important pharmacological potential because of their antioxidant activity that allows them to adsorb, neutralise and eliminate free radicals (Stagos 2020) and thus, to defend against some cancer, infections, inflammation, microorganisms and cardiovascular disorders (Pandey and Rizvi 2009; Ncube et al. 2012). Plants also have long been known by their antimicrobial property. For this aim, many scientists looking for new antimicrobial agents are paying particular attention to the plant kingdom (Gorlenko et al. 2020).

The main target of the present study was to quantify mineral content in powdered *A. vulneraria* leaves and flowers and to determine the phenolic compound contents, antioxidant activity and antibacterial property of the leaf and flower ethanol extracts. Hence, in addition to previous findings, this investigation gives further information about the nutritive value of *A. vulneraria* as dietary supplements rich in natural antioxidants and could be also a supportive data for using the specie as a new industrial crop in cosmetic, pharmaceutical and food industries.

2 Material and methods

Chemicals and reagents – Catechin, EtOH, $FeCl_2$, ferrozine, gallic acid, vanillin, ascorbic acid and quercetin were purchased from Sigma-Aldrich Chimie (*S.Q.F, France*). $AlCl_3$, H_2O_2, phosphate buffer, nitric acid and perchloric acid were acquired from Loba Chemie Pvt. Ltd (*Mumbai, India*). Mueller Hinton agar and Penicillin-Streptomycin (Pen-Strep) were bought from Thermo-Fisher Scientific (*Barcelona, Spain*).

Plant sampling – *Anthyllis vulneraria* was collected at the flowering stage (beginning of April) from Zaghouan located in the North of the Tunisian ridge (latitude 36°, 24 min, 10 s North; longitude 10°, 08 min, 34 s East). Once brought back to the laboratory, *A. vulneraria* leaves and flowers were allowed to dry in the shade at room temperature. The weight was measured every 2 days until a constant weight (dry weight) was obtained. Then, dry samples were ground into a fine powder and stored in amber glass bottles for further analysis.

Determination of mineral content – Mineral content was determined in powdered *A. vulneraria* leaves and flowers by hot mineralisation with nitric and perchloric acids (Larsson et al. 1998; Rezgui et al. 2017). After the complete evaporation of the mixture and obtaining a white haze dry residue, 20 mL of nitric acid (N7) were added. The determination of iron (Fe), copper (Cu) and zinc (Zn) contents was carried out using an atomic absorption spectrophotometer (*Varian SpectrAA 220FS, Canada*), whereas potassium (K) and calcium (Ca) contents were determined using a photoelectric flame photometer (*Model 410 Flame Photometer Range, Cambridge, United Kingdom*). The results obtained are expressed as microgram per gram of Dry Weight ($\mu g\ g^{-1}$ DW).

Phenolic compound contents in *A. vulneraria* leaf and flower extracts –

Extracts preparation. The preparation of *A. vulneraria* leaf and flower extracts was assessed using two different methods, including cold maceration extraction and ultrasound assisted extraction.

To perform the cold maceration extraction, 1 g of each dry sample was mixed with 10 mL of different concentrations of EtOH (absolute, 75% and 50%) and extracted during 24 h at 4 °C under stirring using a multi-position magnetic stirrer (*Ovan, MM90E, Barcelona, Spain*). Then, the extracts were centrifuged (*Orto Alresa Mod. Consul, Ajlvir, Madrid, Spain*) at 1500×*g* for 10 min. The different supernatants were concentrated using a sample concentrator (*Techne FSC496D sample concentrator, Madrid, Spain*) under a jet of moderate nitrogen gas, then lyophilised using a freeze dryer (*Unicryo MC2L, UniEquip Laborgerätebau & Vertr. GmbH, Munich, Germany*) for 2 days.

The ultrasound assisted extraction method also was carried out using different concentrations of EtOH. Shortly, 1 g from each powdered leaf and flower was mixed with 10 mL of EtOH at different concentrations (absolute, 75% and 50%). The different mixtures were stirred using a vortex (*FALC Instruments, A121498, Italy*) for 1 min and extracted in an ultrasonic bath (*COXO Medical Instrument CO., LTD, DB4820, Medical World Company*) for 15 min at 25 °C at a frequency of 40 kHz and a power of 100 W. Then, the extracts were filtered with filter paper (Wattman no. 4) and the supernatants obtained were concentrated then lyophilised for 2 days.

Anthyllis vulneraria leaf and flower freeze-dried extracts were dissolved in absolute EtOH, 75%-aqueous EtOH and 50%-aqueous EtOH and stored in darkness at 4 °C until their use.

Extraction yield. The final dry weight of each lyophilised extract was used to calculate the extraction yield according to the following formula:

$$EY\,(\%) = \frac{W_1}{W_2} \times 100$$

where W_1 represents the weight of the dry extract after the lyophilisation and W_2 represents the weight of the dry ground plant material.

Total polyphenol content. Total polyphenol content (TPC) in the leaf and flower extracts of *A. vulneraria* was determined following the method described by Singleton et al. (1965) and reported by Segovia Gómez and Almajano Pablos (2016). The absorbance reading was recorded at 765 nm using a spectrophotometer (*MAPADA spectrophotometer, UV-1600. Shanghai Mapada Instruments co., Ltd*). The calibration curve was prepared with gallic acid at different concentrations ranging from 100 to 1700 µM ($R^2 = 0.992$). The results of TPC are expressed as milligram of Gallic Acid Equivalent per gram of Dry Weight (mg GAE g^{-1} DW).

Total flavonoid content. Total flavonoid content (TFC) was determined using the $AlCl_3$ colorimetric method as described by Skowyra et al. (2014). The absorbance was measured at 405 nm, and the calibration curve was prepared with quercetin at increasing concentrations from 50 to 500 µM, ($R^2 = 0.998$). Results are expressed as milligram of Quercetin Equivalent per gram of Dry Weight (mg QE g^{-1} DW).

Condensed tannin content. Condensed tannin content (CTC) was determined by the vanillin method described by Julkunen-Tiitto (1985). The absorbance was measured at 550 nm, and the calibration curve was prepared with catechin at different concentrations ranging from zero to 1000 µg mL^{-1}, ($R^2 = 0.997$). Results are expressed as milligram of Catechin Equivalent per gram of Dry Weight (mg CE g^{-1} DW).

Antioxidant activity of *A. vulneraria* leaf and flower extracts –

Total antioxidant capacity by phosphor-molybdenum method. Total antioxidant capacity (TAC) of *A. vulneraria* leaf and flower extracts was determined as reported by Zengin et al. (2015). The absorbance of the mixtures obtained was measured at 695 nm. The calibration curve was prepared with ascorbic acid at different concentrations ranging from zero to 100 µg mL^{-1}, ($R^2 = 0.998$) and the TAC results are expressed as milligram of Ascorbic Acid Equivalent per gram of Dry Weight (mg AAE g^{-1} DW).

Ferrous ion chelating assay. Ferrous ion chelating (FIC) assay was assessed following the method of Dinis et al. (1994). Shortly, 100 µL of each diluted extract were mixed with 0.2 mL of 2 mM $FeCl_2$. The reaction was initiated by the addition of 0.4 mL of 5 mM ferrozine and the mixtures were adjusted to 4 mL with EtOH. After shaking, the mixtures were incubated in the dark at room temperature for 10 min. The absorbance of the extracts was measured at 562 nm and the percentage of Fe^{2+} chelating effect (FIC effect (%)) was calculated as follows:

$$\text{FIC effect}\,(\%) = \left(1 - \frac{As}{Ac}\right) \times 100$$

where Ac is the absorbance of the blank (containing $FeCl_2$—ferrozine complex) and As is the absorbance of the extract.

Hydrogen peroxide scavenging assay. Hydrogen peroxide scavenging (HPS) assay was determined as described by Ruch et al. (1989). A solution of 43 mM H_2O_2 was prepared with phosphate buffer (0.1 M, pH = 7.4), then 100 µL from each extract were mixed with 0.6 mL of H_2O_2 solution. The percentage of H_2O_2 scavenging effect (HPS effect (%)) was calculated using the following formula:

$$\text{HPS effect}\,(\%) = \left(1 - \frac{As}{Ac}\right) \times 100$$

where Ac is the absorbance of the blank (containing sodium phosphate buffer without H_2O_2) and As is the absorbance of the extract.

Determination of antibacterial activity of *A. vulneraria* leaf and flower extracts –

Bacterial strains tested. Six different microbial strains, causing infective and toxic food poisoning, were provided by the "Departament de Biologia, Sanitat i Medi Ambient" of the Universitat de Barcelona to be tested; including *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 15,313), *Micrococcus luteus* (ATCC 4698), *Escherichia coli* (ATCC 25922) and *Salmonella paratyphi* (ATCC 9150).

Inhibitory zone assay (disc diffusion method). To perform the disc diffusion method, 15 mL of Mueller Hinton agar was inoculated with 200 µL of bacterial suspension (0.4×10^4 CFU mL^{-1}) then kept in culture dishes until solidification. Oxford discs were placed in the inoculated plates and impregnated with each extract (100 µg mL^{-1}), and then the plates were incubated at 30 °C. The penicillin (100 µg mL^{-1}) was used as a positive control, while the sterile EtOH 50% was used as a negative control (Fan et al. 2019). The plates were read from the back against a dark

background. The diameter of the zone inhibition measured in millimetre (mm) correlates to the sensitivity of the strain to the extract.

Minimum inhibitory zones (MIC). The minimum inhibitory concentration (MIC) of the extracts (50%-aqueous EtOH) was determined using the broth dilution method reported by Manandhar et al. (2019) with some modifications. Two-fold serial dilutions of the antibiotic (penicillin) and the different extracts were prepared (0.062, 0.125, 0.25, 0.5 and 1 mg mL^{-1}), then 0.2 mL of the different bacterial suspensions were added to each test tube except the negative control (CTR-) and tubes were incubated for 24 h at 37 °C. The MIC was calculated following the formula described below:

$$\mathrm{MIC}\left(\mathrm{mg\,mL^{-1}}\right) = \frac{\mathrm{Lc} + \mathrm{Hc}}{2}$$

where Lc represents the extract's lowest concentration inhibiting the growth of microbial strains and Hc represents the extract's highest concentration allowing the growth of microbial strains.

Statistical analysis – For all the parameters studied below, extracts were analysed in triplicate ($n = 3$). Statistical analysis was performed using the one-way analysis of variance (ANOVA) in Minitab software (*Version 18, München, Germany*), where Tukey test was used at a significance level of $p < 0.05$.

3 Results

Mineral content – Minerals content was determined in powdered *A. vulneraria* leaves and flowers and the results obtained are presented in Table 1.

The results obtained showed that powdered *A. vulneraria* leaves and flowers accumulated important minerals contents with significant differences among extracts ($p < 0.05$). Flowers contained higher contents of Ca, Cu and Zn than leaves extract with significant differences estimated at 17.5, 37.13 and 21.79%, respectively. Additionally, Fe content in flowers was two folds higher than Fe content determined in leaves extract. Contrariwise, *A. vulneraria* leaves contained the highest K content estimated at 12,320 µg mg^{-1} DW, while flowers contained only 9462 µg mg^{-1} DW.

There are various reports in literature about minerals amounts analyses in different species, meanwhile there is no data concerning the mineral composition of Tunisian *A. vulneraria*, which prompt us to conduct these analyses. For instance, Butkut et al. (2018) determined the mineral composition of two *Astragalus* species from the Fabaceae family, including *Astragalus glycyphyllos* and *Astragalus cicer*, and reported that K, Ca, Zn and Fe contents in the leaves of *Astragalus glycyphyllos* were lower than contents in leaves of *A. vulneraria* with values estimated at 89.7, 22.2, 27 and 226.6 µg mg^{-1} DW, respectively, and were lower in flowers with contents equal to 78.8, 29.4, 47.6 and 141 µg mg^{-1} DW, respectively. Mineral contents were also lower in *Astragalus cicer* leaves and flowers compared with mineral contents in *A. vulneraria* leaves and flowers.

Phenolic compound content in *A. vulneraria* leaf and flower extracts –

Extraction yield of extracts. The extraction solvent and method efficiency to extract phenolic compounds from *A. vulneraria* leaf and flower extracts was determined and the results are represented in Table 2.

Extraction yield values were significantly different ($p < 0.05$) depending on the extraction method and the solvent concentration used for the extraction. From the perspective of the extraction method, the highest extraction yields were found in the leaf and flower extracts extracted using ultrasound assisted extraction method, which were 1.5-, 2.4- and 2.5-folds higher in leaf extract and 1.5, 1.8- and 1.4- in flower extract compared with leaf and flower extracts extracted using cold maceration method when extracted with absolute EtOH, 75% and 50%-aqueous EtOH, respectively.

The results depicted in Table 2 also revealed a significant influence of the extraction power of the solvent on the yield. Hydroethanolic solvent was the most suitable for better extraction of phytochemical components and flower extract presented the highest extraction yield. The leaves and flowers extracted with 50%-aqueous EtOH showed better extraction yield estimated at 58.15% and 66.19% when extracted by ultrasound assisted extraction method and 22.72% and 46.14% when extracted by cold maceration

Table 1 Minerals content in powdered *A. vulneraria* leaves and flowers

	Minerals content (µg mg^{-1} DW)				
	K	Ca	Cu	Fe	Zn
Leaves	$12{,}320 \pm 35.2^{b}$	$16{,}320 \pm 36.9^{a}$	51.82 ± 1^{a}	792.27 ± 3.1^{a}	155.81 ± 1.3^{a}
Flowers	9462 ± 23.2^{a}	$19{,}190 \pm 11.3^{b}$	71.06 ± 0.6^{b}	1546.66 ± 26.1^{b}	199.24 ± 3^{b}

Results represent the mean of three replicates ($n = 3$) and are expressed as mean value ± SD. Different letters in each column indicate significant differences between extracts at $p < 0.05$

Table 2 Extraction yield of *A. vulneraria* leaf and flower extracts

Extract	Extraction yield (%)					
	Maceration extraction			Ultrasound assisted extraction		
	EtOH	75% EtOH	50% EtOH	EtOH	75% EtOH	50% EtOH
Leaf	8.61^{b}	14.21^{b}	22.72^{b}	12.72^{b}	34.41^{b}	58.15^{b}
Flower	16.48^{a}	27.12^{a}	46.14^{a}	24.98^{a}	49.26^{a}	66.19^{a}

Results represent the mean of three replicates ($n=3$) and are expressed as mean value ± SD. Different lowercase letters indicate significant differences between extracts at $p<0.05$

Table 3 Phenolic compounds contents of *A. vulneraria* leaf and flower extracts

50%-EtOH extract	TPC (mg GAE g^{-1} DW)	TFC (mg QE g^{-1} DW)	TCTC (mg CE g^{-1} DW)
Leaf	93.27 ± 0.21^{b}	37.88 ± 0.18^{b}	22.72 ± 0.11^{b}
Flower	147.77 ± 0.11^{a}	48.83 ± 0.37^{a}	24.24 ± 0.14^{a}

Results represent the mean of three replicates ($n=3$) and are expressed as mean value ± SD. Different lowercase letters indicate significant differences between extracts at $p<0.05$

method, respectively, followed by the leaves and flowers extracted with 75%-aqueous EtOH. Extracts prepared with absolute EtOH presented the lowest extraction yield values.

Since it showed the best extraction yield, EtOH 50%-aqueous extracts obtained by ultrasound assisted extraction were used to determine the phenolic compound contents, antioxidant activity and antibacterial properties of *A. vulneraria* leaves and flowers.

Spectrophotometric determination of phenolic compounds. The different phenolic compounds contents determined spectrophotometrically are represented in Table 3.

The phenolic compound contents found in *A. vulneraria* leaf and flower extracts varied among extracts with significant differences at $p<0.05$. The highest phenolic compound contents were found in the flower extract and were estimated at 147.77 mg GAE g^{-1} DW for TPC, 48.83 mg QE g^{-1} DW for TFC and 24.24 mg CE g^{-1} DW for TCTC, while the leaf extract contained TPC and TFC 1.6- and 1.3-folds, respectively, lower and a TCTC broadly similar to that determined in the flower extract.

Quantitative and qualitative variation of phenolic compound contents in *A. vulneraria* extracts was observed in several recent studies. For example, Moradi et al. (2018) determined TPC and TFC in *A. vulneraria* leaves collected from the south west city of Iran and extracted in EtOH. The results obtained were two folds higher than contents found in the present study. Csepregi et al. (2016) measured also TPC, TFC and CTC in *A. vulneraria* extract and found contents very lower than contents found in our study estimated at 6.8, 1.7 and 4.5 g GAE g^{-1} DW, respectively. Such trend has been previously studied on Fabaceae family plants. For instance, Al-Dabbagh et al. (2018) determined TPC and TFC in the leaves extract of *Trigonella foenum-graecum* and *Cassia acutifolia* extracted with 70% EtOH and found

Table 4 Antioxidant activity of *A. vulneraria* leaf and flower extracts

	Leaf	Flower
TAC (mg AAE g^{-1} DW)	219.7 ± 0.02^{b}	293.3 ± 0.01^{a}
FIC (%)	48 ± 0.03^{b}	65 ± 0.06^{a}
HPS (%)	27 ± 0.02^{b}	38 ± 0.08^{a}

Results represent the mean of three replicates ($n=3$) and are expressed as mean value ± SD. Different lowercase letters indicate significant differences between extracts at $p<0.05$

TPC lower than *A. vulneraria* estimated at 9.7 and 10.5 mg GAE g^{-1} DW, respectively, and TFC almost similar to TFC found in our study estimated at 14.6 and 20.8 mg QE g^{-1}, respectively. Chen et al. (2018) quantified also TPC and TFC in the flowers of *Pueraria lobate* and *Sophora japonica* and reported TPC of 23.99 and 81.17 mg GAE g^{-1} DW, respectively, and TFC estimated at 14.59 and 42.88 mg RE g^{-1} DW, respectively.

Antioxidant activity – The evaluation of the antioxidant activity of *A. vulneraria* leaf and flower extracts was carried out in vitro by different analytical methods and the results are in Table 4.

Radical scavenging activity was significantly higher in *A. vulneraria* flower extract than in leaf extract ($p<0.05$). On the one hand, the flower extract showed a significantly higher TAC ($p<0.05$) compared to the leaf extract estimated at 293.3 mg AAE g^{-1} DW. Furthermore, the flower extract of *A. vulneraria* exhibited as well FIC and HPS activities 1.3- and 1.4-folds higher than the leaf extract, respectively.

Antioxidant activity of medicinal plants belonging to different families was determined in previous studies. For example, antioxidant activity of medicinal plants belonging to different families was determined in previous studies. For

example, Sharma and Vig (2014) found better TAC values in *Parkinsonia aculeate* extract than TAC obtained in the present study estimated at 360 mg g^{-1} of extract. Osman et al. (2018) also determined TAC in *Dialium indum* extract and found values ranging between 104.52 and 1515.79 µmol TE/g Dry Extract.

Based on Tables 3 and 4 results, it can be suggested that antioxidant activities of extracts are linked to their polyphenol contents, which can support the hypothesis of a positive correlation between the effectiveness of extracts antioxidant capacities and their phenolic amounts as submitted by supportive studies of Pisoschi and Pop (2015) and Gabriela et al. (2016).

Antibacterial activity screening –

Inhibitory zone. The sensitivity of bacterial strains against *A. vulneraria* leaf and flower extracts was determined after 48 h of incubation at 37 °C. The area developed around the discs treated with extracts was measured and the results obtained are represented in Table 5.

According to the results obtained, the leaf and flower extracts of *A. vulneraria* (100 µg mL^{-1}) showed a significant inhibitory effect against the bacteria growth ($p < 0.05$). The flower extract showed better antibacterial activity than the leaf extract. The best antibacterial activity of the flower extract was against *S. aureus* strain with an inhibition zone estimated at 18 mm, while the best antibacterial activity of the leaf extract was observed against *M. luteus* strain with an inhibitory zone of 12 mm. However, the leaf and flower extracts did not show any antimicrobial activity against *S. paratyphi* and *E. coli* strains, respectively, as compared with the penicillin (100 µg mL^{-1}), which showed inhibition zones of 30 and 27 mm, respectively.

Table 5 Diameters of the inhibitory zones (mm) developed around discs treated with *A. vulneraria* leaf and flower extracts (100 µg mL^{-1})

	Inhibitory zone (mm)				
	Microorganism	50% EtOH	Penicillin	Leaf	Flower
Gram +	*S. aureus*	NS	24 ± 1.8^{c}	10 ± 0.6^{a}	18 ± 1.4^{b}
	M. luteus	NS	19 ± 1.2^{c}	12 ± 0.2^{a}	15 ± 0.5^{b}
	L. monocytogenes	NS	15 ± 0.9^{b}	10 ± 0.3^{a}	11 ± 0.6^{a}
	B. cereus	NS	10 ± 0.3^{a}	9 ± 0.2^{a}	14 ± 0.8^{b}
Gram −	*S. paratyphi*	NS	30 ± 1.6^{b}	NS	4 ± 1.5^{a}
	E. coli	NS	27 ± 1.8^{b}	5 ± 1^{a}	NS

Results are means of three different experiments ($n = 3$). Means in the same row with different letters are significantly different ($p < 0.05$). Penicillin (100 µg mL^{-1}) was used as a positive control. Sterile 50%-aqueous EtOH was used as negative control. No inhibition zone is indicated by NS (Not Sensitive)

Minimum inhibitory concentration. The minimum inhibitory concentration (MIC) assay was assessed for only the bacterial strains that showed a sensitivity to *A. vulneraria* leaf and flower extracts (100 µg mL^{-1}) in the disc diffusion method previously performed and the results obtained are in Table 6.

Table 6 shows the turbidity of the penicillin, *A. vulneraria* leaf and flower inoculations, and positive (CTR^{+}) and negative (CTR^{-}) controls after 24 h of incubation. The CTR^{+} containing the broth nutrient, bacterial culture and antibiotic or *A. vulneraria* extracts showed turbidity (bacterial growth) after the 24 h of incubation and was used to test the growing ability of the medium, while the CTR^{-} containing only the broth nutrient and the antibiotic or *A. vulneraria* extract did not show turbidity (no bacterial growth) after the 24 h of incubation and was used to test the sterility of the medium and equipment.

Bacterial growth (indicated by the presence of turbidity in the inoculum) was observed for all the bacterial strains tested at the concentrations of penicillin 0.062 and 0.125 mg mL^{-1} and only for *L. monocytogenes* and *E. coli* at the penicillin concentration 0.25 mg mL^{-1}. In one hand, the penicillin at 0.5 and 1 mg mL^{-1} inhibited totally the growth of all the bacterial strains. Likewise, *A. vulneraria* leaf extract at 0.062, 0.125 and 0.25 mg mL^{-1} was not enough to inhibit

Table 6 Turbidity of the Penicillin, *A. vulneraria* leaf and flower inoculations after 24 h of incubation at 37 °C

Cc (mg mL^{-1})	Penicillin					*A. vulneraria* leaf extract					*A. vulneraria* flower extract					CTR +	CTR −
	0.062	0.125	0.25	0.5	1	0.062	0.125	0.25	0.5	1	0.062	0.125	0.25	0.5	1		
S. aureus	+	+	−	−	−	+	+	+	−	−	+	+	−	−	−	+	−
M. luteus	+	+	−	−	−	+	+	−	−	−	+	+	−	−	−	+	−
L. monocytogenes	+	+	+	−	−	+	+	+	−	−	+	+	−	−	−	+	−
B. cereus	+	+	−	−	−	+	+	+	−	−	+	+	−	−	−	+	−
S. paratyphi	+	+	−	−	−	NT		+	+	−	−	+	−				
E. coli	+	+	+	−	−	+	+	+	+	+	NT	+	−				

(+) Turbidity indicating bacterial growth, (−) no turbidity indicating no bacterial growth and NT indicates microbial strain not tested

the growth of all the bacterial strains, except for *M. luteus*, which showed resistant at 0.25 mg mL^{-1}. No bacterial growth was observed at 0.5 and 1 mg mL^{-1} of *A. vulneraria* leaf extract, except for *E. coli*. In the other hand, *A. vulneraria* flower extract showed better antimicrobial activity than the penicillin and *A. vulneraria* leaves extract by inhibiting the growth of all the bacterial strains without exception at 0.25 mg mL^{-1}.

Based on results of Table 6, MIC of the different extracts tested against the different bacterial strains were calculated and the results obtained are represented in Table 7.

Anthyllis vulneraria flower extract was considered to have better antibacterial activity than penicillin and leaf extract. The flower extract of *A. vulneraria* had the lowest MIC values equal to 0.625 mg mL^{-1} to inhibit the growth of all the bacterial strains tested compared with the penicillin that showed similar results except for *L. monocytogenes* and *E. coli*, which showed sensitivity at a MIC value of 0.75 mg mL^{-1}. The leaves of *A. vulneraria* presented the lowest antimicrobial effect with MIC values higher than those recorded in the penicillin and *A. vulneraria* flowers inoculums estimated at 0.75 mg mL^{-1} against *S. aureus, L. monocytogenes* and *B. cereus* strains, except for *M. luteus* strain (0.625 mg mL^{-1}) and *E. coli* where MIC value was higher than the highest concentration of the penicillin used ($\geq$ 1 mg mL^{-1}).

4 Discussion

Plants frequently accumulate metabolites, mainly phenolic compounds, which represent an important source of molecules that can be used by humans for different purposes. These phenolic compounds, such as phenolic acids, flavonoids, stilbenes and lignans, etc., correspond to a very wide range of chemical structures and are characterised by an unequal qualitative and quantitative distribution. After several years of research, different studies have shown the biological virtues of these compounds in different fields (Lin et al. 2016; Cosme et al. 2020). The main property of these compounds is their antioxidant activity, which consists in neutralising free radicals that are harmful for living organisms. Beyond the antioxidant property, phenolic compounds are characterised by their powerful antimicrobial property as well as many other biological activities (Wang et al. 2009). In the present work, *A. vulneraria* was studied since it was widely used in folk medicine for centuries owing to its varied chemical composition and active ingredients. Several previous investigations have focused on the biological properties of *A. vulneraria* have demonstrated its efficiency to prevent and treat different diseases. For instance, the consumption of *A. vulneraria* aerial part as an infusion has been shown to treat vomiting, diabetes, and stomach disorder (Csepregi et al. 2020). The aerial part also have been used to treat wounds and swelling by direct application (Godevac et al. 2008). Moreover, *A. vulneraria* leaves have been proven to be effective in inhibiting human herpes-virus and poliovirus (Suganda et al. 1983). The flowers as well have a potent therapeutic efficacy against wounds, high blood pressure, heart failure, portal hypertension, vomiting, inflammation, acne and throat pain (Nartowska et al. 2001). The objectives of the present research work were to quantify the mineral content in *A. vulneraria* leaves and flowers, determine their phenolic compound contents and evaluate their antioxidant and antibacterial properties.

Essential minerals, like K and Ca, and trace elements, like Fe, Zn, and Cu, are important for plants metabolic processes, such as chlorophyll synthesis, respiration, as well as protein structure and function (Majdoub et al. 2017) and the consumption of plants rich in minerals is associated with numerous health benefits (Ryan-harshman and Aldoori 2005). Plants absorb minerals from the soil with their roots in varying amounts. This quantitative and qualitative absorption of minerals is influenced by environmental factors, such as the nature of the soil, aeration and temperature (Pallardy 2007). Each mineral nutrient plays a specific role in the development of the plant, for this reason some plants adopt different strategies to avoid the consequences related to the deficiency or excess of minerals and to cope with toxic heavy metals leading to serious physiological disorders (Rouached and Tran 2015). Several researchers showed that some Fabaceae species can tolerate high concentrations of heavy metals in the soil, such as Cu and Zn (Sujkowska-Rybkowska et al. 2020). In addition, owing to their ability to establish symbiotic association with nitrogen-fixing bacteria (nodulated rhizobia), Fabaceae species are capable to grow in metal contaminated sites (Ma et al. 2011; Karthik et al. 2017). Furthermore, Neubauer et al. (2000) reported that the root

Table 7 MIC values of the penicillin and *A. vulneraria* extracts against the different bacterial strains

	MIC (mg mL^{-1})		
	Penicillin	Leaf	Flower
S. aureus	0.625	0.75	0.625
M. luteus	0.625	0.625	0.625
L. monocytogenes	0.75	0.75	0.625
B. cereus	0.625	0.75	0.625
S. panatyphi	0.625	NT	0.625
E. coli	0.75	*	NT

*MIC is higher than the highest concentration of the antibiotic/extract in the first tube, NT indicates microbial strain not tested

exudates resulting from the rhizobium-legume symbiosis in Fabaceae plants can also immobilise metal ions and reduce their harmful effects on plants when they are present in very high contents. Seeing their high adaptability to sites contaminated with heavy metals, Fabaceae species like *A. vulneraria* are applied in re-vegetation and phyto-stabilisation of mine tailings in Europe and northern Africa (Mohamad et al. 2017; Fagorzi et al. 2018; Sujkowska-Rybkowska and Ważny 2018), which can explain the high levels of Cu and Zn found in the leaves and flowers of *A. vulneraria*. The richness of medicinal and edible plants in minerals confers them interesting biological properties provided when they are consumed as food or administered as drugs (Karppanen 1991). For instance, trace elements, such as Cu, Manganese (Mn), Selenium (Se), Fe and Zn, are indispensable co-factors for metabolic reactions of antioxidant enzymes like SOD, CAT and GPx to protect the human body from radicals (Leung 1998). Moreover, several researches demonstrated the anti-inflammatory property of the Zn and its ability to decrease oxidative stress biomarkers (Prasad 2014). An investigation carried out by Roughead et al. (1999) confirmed as well the antioxidant activity of Fe and its capacity to decrease risk of heart disease and cancer.

The phenolic content of the leaf and flower extracts of *A. vulneraria* was determined as well. EtOH solvent was chosen to extract phenolic compounds since it is considered one of the most powerful pure natural solvents that does not present a danger for human consumption. In addition, EtOH is labelled by the *Food and Drug Administration* (FDA) as *Generally Recognised As Safe* (GRAS) food substance products (Alzeer and Abou Hadeed 2016), hence the possibility of its safe use in pharmaceutical, food and cosmetic products without fear of intoxication. The results obtained showed that, for *A. vulneraria*, the best extraction yield value and the highest phenolics contents were observed in the flowers extracted with 50%-aqueous EtOH by ultrasound assisted extraction method. Being such an efficient and widely used extraction method on the industrial scale, the ultrasound assisted extraction method saves time as the chemical compounds, such as carotenoids and polyphenols, etc., diffuse more rapidly into the extraction medium, while producing high quality extracts that can be used for foods, supplements and pharmaceutical products (Safdar et al. 2017; Deng et al. 2017; Osorio-Tobón 2020). Moreover, Do et al. (2014) showed that increasing the water concentration in EtOH solvent improves the extraction efficiency and facilitates the extraction of chemical compounds that are soluble in both inorganic and organic solvents (Khaw et al. 2017) and thus, the extraction of the maximum contents of phenolic compounds. The results obtained also showed a significant difference ($p < 0.05$) in phenolics contents among the different extracts. The variability in phenolic compound contents observed in the different plant's parts may be due to biological (vegetative stage), environmental (climate) and technical (methods and extraction solvents polarity) factors (Ksouri et al. 2008; Sampaio et al. 2011; Liu et al. 2016). In our investigation, the estimated phenolic compounds varied significantly with respect to the used plant part and the extraction method and solvent, which is in line with the findings of Zengin et al. (2015) and Villasante et al. (2019). Researches about phenolic compounds, in particular flavonoids, seem to be very advanced by reason of their various physiological properties, such as anti-allergic, anti-inflammatory, antimicrobial, antiviral, antibacterial, anti-carcinogenic, antithrombotic, cardio-protective and vasodilator activities (Generali et al. 2019). The beneficial effects of polyphenols are of a particular interest in pharmaceutical, cosmetic and food industry. According to several researches that studied the positive impact of polyphenol consumption on health and prevention of diseases, manufacturers are now marketing polyphenol-enriched foods and dietary supplements (Martin and Apple 2009). In addition, their antioxidant activity ensures better preservation of cosmetic and food products by preventing lipid peroxidation (Chang and Kim 2018). In cosmetic industry, phenolic compounds are added in cosmetic products owing to their well-recognised properties, such as antioxidant, anti-inflammatory, antimicrobial, antimutagens and anti-aging activities, as well as their emollients, humectant, wound healing, protective agents against UV-B damage, and reducing skin discoloration effects (Halla et al. 2018).

Over the few last decades, several different analytical methods have been developed and improved in order to measure the antioxidant activity of plant's extracts (Cornelli 2009). In the present work, the antioxidant activity of *A. vulneraria* extracts was assessed by different analytical methods, including TAC, FIC and HPS assays and the results obtained showed as well a significant difference ($p < 0.05$) in antiradical assays among the different extracts. The results followed the same trend as the phenolics contents and showed that, for *A. vulneraria*, the strongest radical scavenging activities were exhibited by the flowers extracted with 50%-aqueous EtOH by ultrasound assisted extraction method. The significant difference observed among the values obtained by the different antiradical methods can be explained by the type of the methods and their chemical backgrounds (Lahue 1981; Apak et al. 2016).

In addition to their antioxidant activity, *A. vulneraria* leaf and flower extracts showed potent antibacterial activity. Foodborne infections caused by bacteria or their toxins, viruses or parasites or unconventional agents are considered one of the serious problems that threaten food industries and consumer's health. A strong correlation between phenolic compounds and antibacterial activity has been found to be

significant in several recent studies (Maddox et al. 2010). In this research work, the antimicrobial analysis revealed that *A. vulneraria* leaf extract inhibited the growth of *Staphylococcus aureus*, *Micrococcus luteus*, *Listeria monocytogenes*, *Bacillus cereus* and *Escherichia coli*, but it did not show any antimicrobial activity against *Salmonella paratyphi* strain, while flower extract had an antimicrobial activity against all the bacterial strains tested except *Escherichia coli* strain. Previous studies showed that the antibacterial activity of plant's extracts depended mainly on their richness on polyphenol contents (Maddox et al. 2010). Polyphenols are endowed with significant antimicrobial activity. Their activity is probably due to their ability to complex with extracellular proteins and makes complexes with the bacterial cell membrane. One of the most important functions of phenolic compounds, mainly flavonoids, is their role in protective effect against microbial invasion. This involves their accumulation as phytoalexins in response to microbial attack. Due to their ability to inhibit photogenic spore germination in plants, they have also been proposed for use against fungal pathogens in humans (Cho and Lee 2015). Several studies reported the regular presence of antimicrobial activity in flavonoids. The majority of flavonoids, recognised as antifungal constituents, are isoflavonoids, flavones and flavanones (Qiu et al. 2014). Additionally, the absence of antimicrobial activity in leaves extract against *S. paratyphi* strain and in flowers extract against *E. coli* strain could be explained by the fact that those strains developed resistance mechanisms or the concentration of *A. vulneraria* extracts are not high enough to inhibit bacterial growth.

To summarise, the results obtained in the present study suggest that the richness of *A. vulneraria* in minerals and phenolic compounds and their unequal distribution in its leaf and flower extracts confer it antioxidant and antibacterial activity and supported its involvement in pharmaceutical, cosmetic and food products to enhance their quality while insuring consumer health.

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Author's contributions MO contributed to the study conception, design, conceptualisation, methodology, software, investigation and data curation. The first draft of the manuscript was written by MO. Material preparation, data collection and analysis were performed by MO, NM, JA and MPA. LBK and MPA supervised and validated the work. All authors commented on previous versions of the manuscript, and read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

Al-Dabbagh B, Elhaty IA, Al Hrout A et al (2018) Antioxidant and anticancer activities of *Trigonella foenum-graecum*, *Cassia acutifolia* and *Rhazya stricta*. BMC Complement Altern Med 18:1–12. https://doi.org/10.1186/s12906-018-2285-7

Alzeer J, Abou Hadeed K (2016) Ethanol and its Halal status in food industries. Trends Food Sci Technol 58:14–20. https://doi.org/10.1016/j.tifs.2016.10.018

Apak R, Özyürek M, Güçlü K, Çapanoğlu E (2016) Antioxidant activity/capacity measurement. 1. Classification, physicochemical principles, mechanisms, and electron transfer (ET)-based assays. J Agric Food Chem 64:997–1027. https://doi.org/10.1021/acs.jafc.5b04739

Batkut B, Dagilyt A, Benetis R et al (2018) Mineral and phytochemical profiles and antioxidant activity of herbal material from two temperate *Astragalus* species. BioMed Res Int. https://doi.org/10.1155/2018/6318630

Chang E, Kim CY (2018) Lipid peroxidation and antioxidant activities of the aqueous rhizome extract of *Rheum officinale* Baillon. J Food Qual. https://doi.org/10.1155/2018/5258276

Chen GL, Chen SG, Xiao Y, Fu NL (2018) Antioxidant capacities and total phenolic contents of 30 flowers. Ind Crops Prod 111:430–445. https://doi.org/10.1016/j.indcrop.2017.10.051

Cho MH, Lee SW (2015) Phenolic phytoalexins in rice: biological functions and biosynthesis. Int J Mol Sci 16:29120–29133. https://doi.org/10.3390/ijms161226152

Cornelli U (2009) Antioxidant use in nutraceuticals. Clin Dermatol 27:175–194. https://doi.org/10.1016/j.clindermatol.2008.01.010

Cosme P, Rodríguez AB, Espino J, Garrido M (2020) Plant phenolics: bioavailability as a key determinant of their potential health-promoting applications. Antioxidants 9:1–20. https://doi.org/10.3390/antiox9121263

Csepregi R, Bencsik T, Papp N (2016) Examination of secondary metabolites and antioxidant capacity of *Anthyllis vulneraria*, *Fuchsia* sp., *Galium mollugo* and *Veronica beccabunga*. Acta Biol Hung 67:442–446. https://doi.org/10.1556/018.67.2016.4.10

Csepregi R, Temesfői V, Das S et al (2020) Cytotoxic, antimicrobial, antioxidant properties and effects on cell migration of phenolic compounds of selected transylvanian medicinal plants. Antioxidants 9:1–29. https://doi.org/10.3390/antiox9020166

Deng J, Xu Z, Xiang C et al (2017) Comparative evaluation of maceration and ultrasonic-assisted extraction of phenolic compounds from fresh olives. Ultrason Sonochem 37:328–334. https://doi.org/10.1016/j.ultsonch.2017.01.023

Dinis T, Victor M, Leonor A (1994) Action of phenolic derivatives (Acetaminophen). Arch Biochem Biophys 315:161–169

Do QD, Angkawijaya AE, Tran-Nguyen PL et al (2014) Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. J Food Drug Anal 22:296–302. https://doi.org/10.1016/j.jfda.2013.11.001

Fagorzi C, Checcucci A, Dicenzo GC et al (2018) Harnessing rhizobia to improve heavy-metal phytoremediation by legumes. Genes (basel). https://doi.org/10.3390/genes9110542

Fan XJ, Liu SZ, Li HH et al (2019) Effects of *Portulaca oleracea* L. extract on lipid oxidation and color of pork meat during refrigerated storage. Meat Sci 147:82–90. https://doi.org/10.1016/j.meatsci.2018.08.022

Gabriela G, Minna H, María Pilar A (2016) Stability of O/W emulsions packed with PLA film with incorporated rosemary and thyme. Eur Food Res Technol. https://doi.org/10.1007/s00217-016-2829-3

Generali IM, Skroza D, Ljubenkov I et al (2019) Antioxidant and antimicrobial potential of phenolic metabolites from traditionally used mediterranean. Foods 8:16

Godevac D, Zdunić G, Šavikin K et al (2008) Antioxidant activity of nine Fabaceae species growing in Serbia and Montenegro. Fitoterapia 79:185–187. https://doi.org/10.1016/j.fitote.2007.10.001

Gorlenko CL, Kiselev HY, Budanova EV et al (2020) Plant secondary metabolites in the battle of drugs and drug-resistant bacteria: new heroes or worse clones of antibiotics? Antibiotics. https://doi.org/10.3390/antibiotics9040170

Halabalaki M, Urbain A, Paschali A et al (2011) Quercetin and kaempferol 3-O-[α-L-rhamnopyranosyl-(→2)-α-L-arabinopyranoside]-7-O-α-L-rhamnopyranosides from *Anthyllis hermanniae*: structure determination and conformational studies. J Nat Prod 74:1939–1945. https://doi.org/10.1021/np200444n

Halla N, Fernandes IP, Heleno SA et al (2018) Cosmetics preservation: a review on present strategies. Molecules 23:1–41. https://doi.org/10.3390/molecules23071571

Jones EM, Turrill W (1933) Studies in variation of *Anthyllis vulneraria*. J Genet 27:261–285

Julkunen-Tiitto R (1985) Phenolic constituents in the leaves of northern Willows: methods for the analysis of certain phenolics. J Agric Food Chem 33:448

Karppanen H (1991) Minerals and blood pressure. Ann Med 23:299–305. https://doi.org/10.3109/07853899109148064

Karthik C, Elangovan N, Kumar TS et al (2017) Characterization of multifarious plant growth promoting traits of rhizobacterial strain AR6 under Chromium(VI) stress. Microbiol Res 204:65–71. https://doi.org/10.1016/j.micres.2017.07.008

Khaw KY, Parat MO, Shaw PN, Falconer JR (2017) Solvent supercritical fluid technologies to extract bioactive compounds from natural sources: a review. Molecules. https://doi.org/10.3390/molecules22071186

Ksouri R, Megdiche W, Falleh H et al (2008) Influence of biological, environmental and technical factors on phenolic content and antioxidant activities of Tunisian halophytes. Comptes Rendus Biol 331:865–873. https://doi.org/10.1016/j.crvi.2008.07.024

Lahue R (1981) Spectrophotometry and fluorometry. In: Lahue R (ed) Methods in neurobiology. Springer US, Boston, pp 339–423

Larsson EH, Bornman JF, Asp H (1998) Influence of UV-B radiation and Cd^{2+}on chlorophyll fluorescence, growth and nutrient content in *Brassica napus*. J Exp Bot 49:1031–1039. https://doi.org/10.1093/jxb/49.323.1031

Leung FY (1998) Trace elements that act as antioxidants in parenteral micronutrition. J Nutr Biochem 9:304–307. https://doi.org/10.1016/S0955-2863(98)00018-7

Lin D, Xiao M, Zhao J et al (2016) An overview of plant phenolic compounds and their importance in human nutrition and management of type 2 diabetes. Molecules. https://doi.org/10.3390/molecules21101374

Liu W, Yin D, Li N et al (2016) Influence of environmental factors on the active substance production and antioxidant activity in *Potentilla fruticosa* L. and its quality assessment. Sci Rep 6:1–18. https://doi.org/10.1038/srep28591

Lushchak VI (2014) Free radicals, reactive oxygen species, oxidative stress and its classification. Chem Biol Interact 224:164–175. https://doi.org/10.1016/j.cbi.2014.10.016

Ma Y, Prasad MNV, Rajkumar M, Freitas H (2011) Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. Biotechnol Adv 29:248–258. https://doi.org/10.1016/j.biotechadv.2010.12.001

Maddox CE, Laur LM, Tian L (2010) Antibacterial activity of phenolic compounds against the phytopathogen Xylella fastidiosa. Curr Microbiol 60:53–58. https://doi.org/10.1007/s00284-009-9501-0

Majdoub N, el-Guendouz S, Rezgui M et al (2017) Growth, photosynthetic pigments, phenolic content and biological activities of *Foeniculum vulgare* Mill., *Anethum graveolens* L and *Pimpinella anisum* L. (Apiaceae) in response to zinc. Ind Crops Prod 109:627–636. https://doi.org/10.1016/j.indcrop.2017.09.012

Manandhar S, Luitel S, Dahal RK (2019) In vitro antimicrobial activity of some medicinal plants against human pathogenic bacteria. J Trop Med. https://doi.org/10.1155/2019/1895340

Martin KR, Apple CL (2009) Polyphenols as dietary supplements: a double-edged sword. Nutr Diet Suppl. https://doi.org/10.2147/nds.s6422

Menković N, Šavikin K, Tasić S et al (2011) Ethnobotanical study on traditional uses of wild medicinal plants in Prokletije Mountains (Montenegro). J Ethnopharmacol 133:97–107. https://doi.org/10.1016/j.jep.2010.09.008

Mohamad R, Maynaud G, Le Quéré A et al (2017) Ancient heavy metal contamination in soils as a driver of tolerant *Anthyllis vulneraria* rhizobial communities. Appl Environ Microbiol 83:1–13. https://doi.org/10.1128/AEM.01735-16

Moradi MT, Karimi A, Alidadi S, Hashemi L (2018) *In vitro* anti-herpes simplex virus activity, antioxidant potential and total phenolic compounds of selected iranian medicinal plant extracts. Indian J Tradit Knowl 17:255–262

Nartowska J, Wawer I, Strzelecka H (2001) Triterpenoid sapogenin from *Anthyllis vulneraria* L. Acta Pol Pharm - Drug Res 58:289–291

Ncube B, Finnie JF, Van Staden J (2012) Quality from the field: the impact of environmental factors as quality determinants in medicinal plants. S Afr J Bot 82:11–20. https://doi.org/10.1016/j.sajb.2012.05.009

Neubauer U, Nowack B, Furrer G, Schulin R (2000) Heavy metal sorption on clay minerals affected by the siderophore desferrioxamine B. Environ Sci Technol 34:2749–2755. https://doi.org/10.1021/es990495w

Osman MF, Hassan NM, Khatib A, Tolos SM (2018) Antioxidant activities of *Dialium indum* L. Fruit and gas chromatography–mass spectrometry (GC–MS) of the active fractions. Antioxidants 7:154. https://doi.org/10.3390/antiox7110154

Osorio-Tobón JF (2020) Recent advances and comparisons of conventional and alternative extraction techniques of phenolic compounds. J Food Sci Technol 57:4289–4315. https://doi.org/10.1007/s13197-020-04433-2

Pallardy SG (2007) Nutrients and effects. Physiol Woody Plants. https://doi.org/10.1016/B978-0-12-088765-1.50011-7

Pandey KB, Rizvi SI (2009) Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev 2:270–278. https://doi.org/10.4161/oxim.2.5.9498

Pisoschi AM, Pop A (2015) The role of antioxidants in the chemistry of oxidative stress: a review. Eur J Med Chem 97:55–74. https://doi.org/10.1016/j.ejmech.2015.04.040

Prasad AS (2014) Zinc is an antioxidant and anti-inflammatory agent: its role in human health. Front Nutr 1:1–10. https://doi.org/10.3389/fnut.2014.00014

Qiu GH, Xie X, Xu F et al (2014) Distinctive pharmacological differences between liver cancer cell lines HepG2 and Hep3B. Cytotechnology 67:1–12. https://doi.org/10.1007/s10616-014-9761-9

Rezgui M, Majdoub N, Ben-Kaab S et al (2017) How salt stress represses the biosynthesis of marrubiin and disturbs the antioxidant activity of *Marrubium vulgare* L. Polish J Environ Stud 26:267–277. https://doi.org/10.15244/pjoes/64792

Rouached H, Tran LSP (2015) Regulation of plant mineral nutrition: transport, sensing and signaling. Int J Mol Sci 16:29717–29719. https://doi.org/10.3390/ijms161226198

Roughead ZK, Johnson LK, Hunt JR (1999) Dietary copper primarily affects antioxidant capacity and dietary iron mainly affects iron status in a surface response study of female rats fed varying concentrations of iron, zinc and copper. J Nutr 129:1368–1376. https://doi.org/10.1093/jn/129.7.1368

Ruch RJ, Cheng S, jun, Klaunig JE, (1989) Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from chinese green tea. Carcinogenesis 10:1003–1008. https://doi.org/10.1093/carcin/10.6.1003

Ryan-harshman M, Aldoori W (2005) Food for thought health benefits of selected minerals. Can Fam Physician 51:673–675

Safdar MN, Kausar T, Nadeem M (2017) Comparison of ultrasound and maceration techniques for the extraction of polyphenols from the mango peel. J Food Process Preserv. https://doi.org/10.1111/jfpp.13028

Sales de Oliveira V, Augusta IM, da Conceicao Braz MV et al (2020) Aroeira fruit (*Schinus terebinthifolius Raddi*) as a natural antioxidant: chemical constituents, bioactive compounds and *in vitro* and *in vivo* antioxidant capacity. Food Chem 315:126274. https://doi.org/10.1016/j.foodchem.2020.126274

Sampaio BL, Bara MTF, Ferri PH et al (2011) Influence of environmental factors on the concentration of phenolic compounds in leaves of *Lafoensia pacari*. Braz J Pharmacogn 21:1127–1137. https://doi.org/10.1590/S0102-695X2011005000177

Segovia Gómez F, Almajano Pablos MP (2016) Pineapple waste extract for preventing oxidation in model food systems. J Food Sci 81:C1622–C1628. https://doi.org/10.1111/1750-3841.13341

Sharma S, Vig AP (2014) Preliminary phytochemical screening and *in vitro* antioxidant activities of *Parkinsonia aculeata* linn. Biomed Res Int. https://doi.org/10.1155/2014/756184

Singleton VL, Rossi JA Jr (1965) Colorimetry of total phenolics with acid reagents. Am J Enol Vitic 16:144–158

Skowyra M, Gallego MG, Segovia F, Almajano MP (2014) Antioxidant properties of *Artemisia annua* extracts in model food emulsions. Antioxidants 3:116–128. https://doi.org/10.3390/antiox3010116

Stagos D (2020) Antioxidant activity of polyphenolic plant extracts. Antioxidants. https://doi.org/10.3390/antiox9010019

Suganda AG, Amoros M, Girre L, Fauconnier B (1983) Effets inhibiteurs de quelques extraits bruts et semi purifies de plantes indigenes francaises sur la multiplication de l'herpesvirus humain 1 et du polioviras humain 2 en culture cellulaire. J Nat Prod 46:626–632

Sujkowska-Rybkowska M, Ważny R (2018) Metal resistant rhizobia and ultrastructure of *Anthyllis vulneraria* nodules from zinc and lead contaminated tailing in Poland. Int J Phytoremediation 20:709–720. https://doi.org/10.1080/15226514.2017.1413336

Sujkowska-Rybkowska M, Banasiewicz J, Rekosz-Burlaga H, Stępkowski T (2020) *Anthyllis vulneraria* and *Lotus corniculatus* on calamine heaps form nodules with *Bradyrhizobium liaoningense*-related strains harboring novel in Europe symbiotic nifD haplotypes. Appl Soil Ecol. https://doi.org/10.1016/j.apsoil.2020.103539

Villasante J, Girbal M, Metón I, Almajano MP (2019) Effects of pecan nut (*Carya illinoiensis*) and roselle flower (*Hibiscus sabdariffa*) as antioxidant and antimicrobial agents for sardines (*Sardina pilchardus*). Molecules. https://doi.org/10.3390/molecules24010085

Wang T, Jónsdóttir R, Ólafsdóttir G (2009) Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. Food Chem 116:240–248. https://doi.org/10.1016/j.foodchem.2009.02.041

Yang L, Wen KS, Ruan X et al (2018) Response of plant secondary metabolites to environmental factors. Molecules 23:1–26. https://doi.org/10.3390/molecules23040762

Zengin G, Uysal S, Ceylan R, Aktumsek A (2015) Phenolic constituent, antioxidative and tyrosinase inhibitory activity of *Ornithogalum narbonense* L. from Turkey: a phytochemical study. Ind Crops Prod 70:1–6. https://doi.org/10.1016/j.indcrop.2015.03.012

Annex 4

Manel Ouerfelli; María Pilar Almajano *Estabilidad oxidativa de emulsions alimentarias de aceite en agua* "Tecifood", Febrer 2021, p. 196-198

ATTENTION¡¡

Pages 246 to 248 of the thesis, containing the article mentioned above

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