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Phytochemical investigation of *Centaurea calcitrapa* leaves and thorns extracts and their interactions with a biomembrane model.

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AAPH: 2,2'-Azobis(2-amidino-propane) dihydrochloride.

ANOVA: Analysis of variance

***C. calcitrapa* :** *Centaurea calcitrapa*

DPPH: 2,2-diphenyl-1-picrylhydrazyl

Eq.AG: Gallic acid equivalent
Eq.AT: Tannic acid equivalent

ES: Erythrocyte suspension

EtOH : Ethanol

F.C.: Folin Ciocalteu

H_2O_2: Hydrogen peroxide

Na_2CO_3: Sodium carbonate

PBS: phosphate-buffered saline

QE: quercetin equivalent
RBC: Red blood cell.

RNS: Reactive nitrogen species

ROS: reactive oxygen species

SD: Standard deviation.

STLs: sesquiterpene lactones

TCTC: Total condensed tannins content
TFC: Total flavonoid content
THTC: Total hydrolized tannins content
TPC: Total phenol content

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Introduction

Medicinal plants have been extensively recognized as a source of bioactive compounds with pharmacological applications. Among these compounds, phenolic compounds have attracted increasing attention because of their diverse biological activities such as antioxidant, anti-inflammatory, antimicrobial and anticancer properties. In recent years the interaction of these natural compounds with cellular membranes has become a subject of growing scientific interest in order to understand their mechanisms of action at molecular level **(Dimkić *et al.*, 2020)**.

Centaurea calcitrapa, commonly known as purple starthistle, is a member of Asteraceae family and has been traditionally used in medicine for various ailments. It is known for its phytochemical composition, particularly its rich contents of secondary metabolites such as flavonoids, tannins, and phenolic acids which are believed to contribute to medicinal properties **(Dimkić *et al.*, 2020)**. However, despite its traditional use and known phytochemical richness, the specific interactions between *Centaurea calcitrapa* phenolic compounds and biological membranes remain poorly understood.

Biomembranes are fundamental to cellular integrity for they form cells and enable separation between the inside and outside of an organism, controlling by means of their selective permeability which substances enter and leave. Biomembranes are composed of phospholipids, proteins and sugars. They play a role in transport, signal traduction and enzymatic activity **(Watson, 2015)**. Understanding the interactions between plant phenolic compounds and biomembranes is crucial for assessing their pharmacokinetics, bioavailability, and therapeutic potential. These interactions are explored using erythrocyte model. Research indicates that phenolic compounds can modulate membrane fluidity, permeability, and stability, thereby influencing cellular functions and drug absorption **(Hossain *et al.*, 2021)**.

The aim of the study is to investigate the phytochemical composition of *Centaurea calcitrapa*, leaves and thorns and their interactions with biomembrane model to understand their potential biological activities, antioxidant and membrane modulating properties.

Bibliographic review

I.1. *Centaurea calcitrapa*

I.1.1. Description

Centaurea calcitrapa, commonly known as purple starthistle **(Figure 01)**, is a biennial herbaceous plant that can grow up to 60 cm in height. Its flowers are arranged lateraly and at the top of the shoot with many bright purple-red tubular flowers. Involucre is ovoid, consisting of many phyllaries of which external are with long, yellow, terminal spine distinctive for this species. It grows along roads, in waste places, between the rails and prefer rocky place, arable land, sunny and warm slopes **(Dimkić *et al.,* 2020)**. The hierarchical classification (Taxonomy) of *Centaurea calcitrapa* is reported in **table I.**

Figure 01: *Centaurea calcitrapa* L. (Village Sakule, Opovo municipality, the South Banat District, Vojvodina province, Serbia) **(Dimkić *et al.*, 2020)**

Table I. The hierarchical classification (Taxonomy) of *Centaurea calcitrapa* L.1753. according to the national inventory of natural heritage **(INPN, 2003)**.

Domain	Botanical
Kingdom	Plant
Class	Dicotyledon
Order	Astral
Family	Asteraceae
Genus	Centaurea L.
Species	*Centaurea calcitrapa* L.

Members of the Centaurea genus are distributed all around the world, particularly in the Mediterranean region and Western Asia **(Dimkić *et al.,* 2020)**. *Centaurea calcitrapa* is widespread in the western and southern parts of Central Europe, North Africa, and Western Asia, extending to

Northwestern India. It thrives along roadsides, in waste places, between railway tracks, and prefers rocky areas, arable land, and sunny, warm slopes. In Southern Italy (Vulture area), young shoots of *C. calcitrapa* are consumed, either boiled or fried, often mixed with other wild herbaceous plants **(Pieroni *et al*., 2002)**.

In Algeria, 45 species are present, including 7 localized in the Sahara. This review compiled reports on the chemical composition and biological activities of 26 selected Centaurea species and related genera found in the country until early 2018.Numerous phytochemical studies on Algerian *Centaurea* species have revealed their abundance in bioactive secondary metabolites, particularly flavonoids and sesquiterpene lactones. Pharmacological research has demonstrated that various crude extracts and isolated compounds from these species exhibit significant biological activities, including cytotoxic, antimicrobial, antioxidant, and antiplasmodial effects **(Ayad & Akkal, 2019).**

I.1.3. Use in Medicine

Traditional knowledge as revealed by ethnobotanical and ethnopharmacological studies highlights extensive use of various Centaurea species in folk medicine for the treatment of a wide range of diseases **(Khammar & Djeddi, 2012)**. The plants have been utilized from ancient times due to their pharmacological activities like antidiarrheal, anti-inflammatory, antipyretic, antirheumatic, antibacterial, and cytotoxicity. In a few regions, leaves or other plant parts like Centaurea triumfettii All., C. urvillei DC. spp. stepposa Wagenitz, C. pullata L., and C. calcitrapa L. have either been eaten raw or treated, and utilized even in the preparation of traditional tonics and beverages **(Khammar & Djeddi, 2012)**. Specifically, C. calcitrapa has been employed in traditional treatments for ophthalmia, fever, jaundice, gastrointestinal disorders, as well as various skin disorders **(Dimkić *et al.*, 2020)**.

Phytochemical investigations of the Centaurea genus have revealed a wide range of naturally occurring active compounds. The most prominent among them are sesquiterpene lactones, phenolic acids, flavonoids, and steroids of various kinds, which are accountable for the pharmacological activity of the plant **(Dob *et al.*, 2009; Mekky *et al.*, 2024)**. These bioactive constituents are largely responsible for the biological activities attributed to the genus, delineating its therapeutic significance within the Asteraceae plant family **(Dumlu & Gürkan, 2006)**. More specifically, studies on Centaurea calcitrapa have reported the presence of sterols, triterpenes,

sesquiterpene lactones, lignans, flavonoids, and bisabolenes, warranting its use in traditional and modern pharmacology **(Al-Easa & Rizk, 1992)**.

Experimental evidence also bears witness to the therapeutic potential of C. calcitrapa, with different extracts possessing notable biological activities. Both water and methanol (MeOH) extracts have been shown to have good antioxidant activity. Notably, the MeOH extract has been shown to be cytotoxic on certain cancer cell lines such as HeLa (cervical carcinoma) and Vero (kidney epithelial cells of African green monkeys) **(Erol-Dayi *et al.*, 2011)**. In addition, antimicrobial assays revealed the activity of the MeOH extract against a series of bacterial pathogens, including Bacillus, Pseudomonas, Staphylococcus, Streptococcus, Salmonella, Enterobacter, Enterococcus, Acinetobacter, and Escherichia species **(Dimkić *et al.*, 2020)**.

The biological activities of *Centaurea calcitrapa* are closely linked to its diverse array of plant metabolites. Phytochemical analyses have identified a variety of compounds, including phenolic acids, flavonoids, and sesquiterpene lactones, which are believed to contribute to the plant's antioxidant, cytotoxic, and antibacterial properties **(Dimkić *et al.*, 2020)**. For instance, in the same study, (Dimkić et al., 2020) identified 55 phenolic compounds in *C. calcitrapa* extracts, comprising 30 phenolic acids and 25 flavonoid derivatives. These metabolites are known for their bioactive potential, supporting the plant’s traditional medicinal uses.

Additionally, research by **Mekky *et al.*, (2024)** investigated the phytochemical profile of *C. calcitrapa*'s aerial flowering parts and found high concentrations of total phenolic acids and tannins. The methanol extract exhibited substantial antibacterial effects against human pathogens and demonstrated strong antioxidant properties, further reinforcing the link between its bioactive compounds and pharmacological activities. These findings highlight the significance of plant metabolites in the biological potential of *C. calcitrapa*, providing a foundation for further studies on its medicinal applications.

I.2. Plant metabolites

Plant metabolites are chemical substances isolated from plants and they are synthesized through primary and secondary metabolism pathways. Metabolism refers to the sum of all chemical reactions that take place within the plant to produce energy for processes like growth, development, environmental adaptation and synthesizing new organic materials. Plant Metabolites

are divided into primary metabolites (PMs) and secondary metabolites (SMs) **(Elshafie *et al.*, 2023).**

I.2.1. Primary metabolites

Primary metabolites are biochemical compounds produced during metabolic processes that are crucial for the growth, development, and survival of organisms. These include carbohydrates, amino acids, fatty acids, and nucleotides, which serve as the building blocks for more complex molecules like proteins, lipids, and nucleic acids **(Butnariu & Bocso, 2022)**. Primary metabolites are involved in fundamental metabolic processes such as photosynthesis, nutrient assimilation, and respiration, and have an essential role in plant growth, development, and stress adaptation **(Krstić *et al.*, 2022)**.

I.2.2. Secondary metabolites

Plant secondary metabolites are group of molecules that participate to the adaptation of plants to their environment, but are not part of the main pathways biochemical mechanisms of cell growth and reproduction. Research in secondary metabolites has increased significantly in the last few years, due to their antioxidant, antiviral, antibacterial and anticancer effects. They effectively participate in the tolerance of plants to various stresses like pathogens attack, drought and UV light. These molecules often constitute the basis of the active principles of medicinal plants **(Ayad, 2013).**They are divided into three classes which are terpenoids, phenolic compounds and nitrogen containing compounds **(Elshafie *et al.*, 2023)**.

I.2.1.1. Secondary metabolites of *Centaurea calcitrapa*

The major secondary metabolites identified in *Centaurea calcitrapa* are sesquiterpene lactones (STLs) and flavonoid (Formisano et al., 2006). (Bruno et al., 2013) conducted a review that documented nine distinct STLs, while (Formisano et al., 2006) compiled a list of twelve flavonoids. Additionally, **Kitouni *et al.* (2015)** identified eupatorin, cirsiliol, and jaceosidin as flavonoids present in *Centaurea calcitrapa* from Algeria. Similarly, **(Dob *et al.*, 2009; Marco *et al.*, 1992)** reported the presence of lignans, a type of phenolic compound, in this plant. These findings are presented in **Table II.**

Table II: Main secondary metabolites found in *C. calcitrapa* (**Marco et al., 1992; Formisano et al., 2006; Bruno et al., 2013; Kitouni et al., 2015**).

Sesquiterpenes	
Germacanes	Cnicin, Cnicin4'-O-acetyl, Salonitenolide,11β,13-dihydrosalonitenolide , 11α,13-dihydrosalonitenolide.
Elemanes	8α-(3',4'-dihydroxy2'methylenebutanoyloxy)-dehydromelitensin; isocnicin and Melitensin.
Bisabolanes	4,9-Dioxo-bisabol-2,7(14),10-triene, 4,9-Dioxo-bisabol-2,7E,10-triene.
Flavonoids	
Flavones	Apigenin, Luteolin, Eupatilin, Cirsiliol, Eupatorin and Jaceosidin
Flavonols	Kaempferol, Quercetin
Flavanones	Naringenin
Flavone Glycosides	Cosmosiin, Apigenin-7-O-b-d-galacturonic acid methyl ester, Scutellarin
Flavonol Glycosides	Rutin, Chrysanthemin, Cyanin
Other Secondary Metabolites	
Lignans	Arctigenin, Pinoresinol and 7′(*S*)-hydroxyarctigenin

I.3. Oxidative stress and antioxidant activity

I.3.1. Oxidative stress

Oxidative stress refers to an imbalance between harmful molecules called free radicals and reactive oxygen species (ROS), and the body's natural protective systems. This imbalance results in the body producing more harmful oxidants than it can neutralize. When this balance is disrupted, it can damage important cell parts like proteins, DNA, and cell membranes, which may eventually lead to cell death. Oxidative stress is also linked to many biological processes and diseases, including aging, DNA harm, cell growth issues, and reduced cell survival (**Sharma, 2014**).

I.3.2. Free Radicals and reactive oxygen and nitrogen species

Free radicals are molecules that contain an unpaired electron and are highly reactive. Free radicals can initiate chain reactions in which cellular components such as lipids, proteins, and nucleic acids are damaged **(Halliwell, 2007)**. The most prevalent free radicals in biological systems are ROS, such as the superoxide anion (O_2^-) and the hydroxyl radical (OH·) **(Birben *et al.*, 2012)**.

Overproduction of ROS causes oxidative stress, which is involved in various pathological disorders such as inflammation, neurodegenerative diseases, cardiovascular disease, and cancer **(Halliwell & Gutteridge, 2015)**. The reaction of ROS with cellular biomembranes can cause lipid peroxidation, which results in membrane dysfunction and instability **(Zhou *et al.*, 2020)**.

Table III : Reactive oxygen and nitrogen species **(Phaniendra *et al.,* 2014).**

Reactive oxygen species(ROS)	Reactive nitrogen species(RNS)
Radicals	**Radicals**
Superoxide ($O^•$) Hydroxyl ($OH^•$) Alkoxyl radical ($RO^•$) Peroxyl Radical ($ROO^•$)	Nitric oxide ($NO^•$) Nitrogen dioxide ($NO_2^•$)
Non radicals	**Non radicals**
Hydrogen peroxide (H_2O_2) Singlet oxygen ($1O_2$) Ozone (O_3) Organic peroxide (ROOH) Hypochlorous acid (HOCl) Hypobromous acid (HOBr)	Peroxynitrite (ONOO-) Nitrosyl cation (NO) Nitroxyl anion (NO-) Dinitrogen trioxide (N_2O_3) Dinitrogen tetraoxide (N_2O_3) Nitrous acid (HNO_2) Nitryl chloride (NO_2Cl) Peroxynitrous acid (ONOOH)

I.3.3. Sources of free radicals

Free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), are produced both endogenously and exogenously within biological systems. Endogenously, free radicals are generated primarily as by-products of normal cellular metabolism, particularly in mitochondria during oxidative phosphorylation, peroxisomes, the endoplasmic reticulum, and phagocytic cells involved in immune processes. Specifically, the superoxide anion radical (O2•–) is enzymatically formed by a variety of enzymes such as xanthine oxidase, lipoxygenase, cyclooxygenase, and NADPH oxidase and non-enzymatically via electron transfer reactions to

molecular oxygen. Another protonated superoxide derivative, the hydroperoxyl radical (HO_2•), is formed and is also involved in initiating fatty acid peroxidation. Hydroxyl radicals (•OH) are generated primarily through Fenton and Haber–Weiss reactions between transition metal ions such as Fe^{2+} and hydrogen peroxide (H_2O_2). Nitric oxide (NO•) and nitrogen dioxide (NO_2•) are reactive nitrogen species generated by nitric oxide synthases. At physiological concentrations, these radicals are engaged in critical cellular processes; however, their excessive generation results in oxidative and nitrosative stress, causing damage to lipids, proteins, and DNA. Exogenously, free radicals are introduced or produced by environmental pollutants, cigarette smoking, alcohol consumption, heavy metal exposure, industrial pollutants, certain drugs, and ionizing radiation, [. Ultimately, cellular wellness is based upon the balance of free radical production and antioxidant defense, which sets free radical biology at a fundamental interest in disentangling aging and numerous pathophysiological illnesses **(Kohen & Nyska, 2002; Pham-Huy *et al.*, 2008)**.

I.3.4. Beneficial actifities of free radicals

Free radicals like reactive oxygen species (ROS) and reactive nitrogen species (RNS) are essential positive players in human health when maintained at low or moderate levels. They are essential signaling molecules involved in physiological processes like cellular response and immune defense. For example, superoxide anion radicals produced by phagocytes are involved in the elimination of intruding pathogens, while nitric oxide controls blood flow, thrombosis, and neural transmission and helps eliminate intracellular pathogens and tumors. Free radicals are also implicated in causing mitogenic effects to promote cell multiplication needed in tissue repair and regeneration. Whereas highly reactive molecules like hydroxyl radicals are toxic in excess, controlled levels of free radicals are essential to normal cellular functions and immune ability **(Pham-Huy *et al.*, 2008).**

I.3.5. Destructive activities of free radicals

When excessively produced, free radicals and oxidants induce oxidative stress, a pathological condition that can cause extensive damage to cellular structures like proteins, lipids, lipoproteins, and DNA. The imbalance arises when the rate of production of reactive oxygen and nitrogen species exceeds the ability of the body to detoxify them. Free radicals in excess cause lipid peroxidation, which damages cell membranes and lipoproteins and can initiate mutations and impairment in vital biomolecules. Oxidative stress has been implicated in the pathogenesis of

numerous chronic and degenerative diseases such as cancer, autoimmune diseases, aging, cataract, rheumatoid arthritis, cardiovascular disease, neurodegenerative disease, pre-eclampsia, and intra-uterine growth retardation. For example, overexpression of placental NADPH oxidase isoforms leads to enhanced production of superoxide in pre-eclampsia pathogenesis. Therefore, while free radicals are needed in small amounts, overproduction is dangerous and is the hallmark of pathogenesis in the majority of human illnesses **(Pham-Huy *et al.*, 2008)**.

I.3.6. Antioxidant

Antioxidants are compounds that prevent oxidation through the neutralization of free radicals, thus preventing damage to cells **(Lobo *et al.*, 2010)**. Antioxidants are important in the preservation of redox balance in biological systems and the prevention of oxidative stress-induced damage **(Birben *et al.*, 2012)**. Natural antioxidants from medicinal plants like *Centaurea calcitrapa* have been found to be promising in the protection of cellular components against oxidative damage, thus their importance in the prevention of diseases as well as therapeutic use **(Aboul-Soud *et al*., 2022).**

Heijnen et al. (2002) gives a general evaluation of the antioxidant activity of flavonoids quercetin and rutin. According to the study, the two flavonoids have high inhibitory activity against iron ion-dependent lipid peroxidation through the chelation of iron ions to form inactive complexes that are unable to cause lipid peroxidation. In addition, these flavonoid-iron complexes retain their free radical scavenging activities, pointing to their dual function in opposition to oxidative stress. The article upholds that rutin and quercetin suppress free radical processes at various stages, such as the generation of superoxide ions, generation of hydroxyl radicals in the Fenton reaction, and generation of lipid **(Afanas'ev *et al.*, 1989)**.

Metal chelation is one of the major antioxidant mechanisms that operates via complexation with metal ions,like iron,implicated in the formation of free radicals. Synthetic pentapeptides, quercetin, and ferulic acid were revealed by recent studies to have the capacity to chelate iron ions and, therefore, inhibit their activity in the Fenton reaction responsible for the production of extremely reactive hydroxyl radicals. By reducing the level of available free Fe^{2+}, these bioactive molecules prevent the initiation of oxidative chain reactions and protect cells from oxidative damage. This dual activity,chelating metal and scavenging free radicals, accounts for their total antioxidant

activity and highlights their therapeutic usefulness in the management of oxidative stress-related diseases

Apart from this, antioxidants are also able to modulate the activity of endogenous antioxidant enzymes like superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). Polyphenols like resveratrol have been reported to enhance the activity and expression of these enzymes, thereby enhancing the antioxidant defense of the body against oxidative stress-induced cell injury **(Sreelatha & Padma, 2015)**. Antioxidants can be divided into two general categories:

I.3.6.1. Endogenous Antioxidant

These are endogenously produced in the body and consist of enzymatic antioxidants like SOD, catalase, and glutathione peroxidase, and non-enzymatic antioxidants like glutathione, uric acid, and bilirubin **(Birben *et al.,* 2012)**. These have a significant role to play in cell protection against oxidative damage through detoxification of ROS produced during metabolism.

I.3.6.2. Exogenous Antioxidants

These exogenous antioxidants are acquired from external sources, mainly from the diet. They consist of vitamins like vitamin C and E, minerals such as selenium and zinc, and phytochemicals like flavonoids and phenolic acids in plants **(Lobo *et al.,* 2010)**. *Centaurea calcitrapa* is a good source of these exogenous antioxidants, which explains its medicinal significance.

I.4. Polyphenols and their interactions with biomembranes

Polyphenols are naturally occurring secondary metabolites of plants that contain greater than one phenolic hydroxyl group attached on aromatic rings **(Akar *et al.*, 2017).** They are synthesized through shikimate and acetate-malonate pathways **(Zagoskina *et al.,* 2023).** Polyphenols carry important roles in plant physiology as antioxidants that are implicated for protecting plant cells against oxidative stress by scavenging ROS. Antioxidant property of polyphenols averts damage to molecules by free radicals and hence is involved in plant defense mechanisms. Beyond their significance in plants, polyphenols have effects in human beings, like protection against diseases such as cardiovascular diseases, cancer, and inflammatory diseases due to their ability to neutralize oxidative stress and modulate cellular signaling pathways **(Akar *et al.*, 2017)**.

I.4.1. Classification of polyphenols

Over 8,000 polyphenolic compounds have been identified. They are characterized by structures containing one or more aromatic phenolic rings with hydroxyl groups attached. These compounds are differentiated by the number and position of hydroxyl groups and the presence of different substituents **(Prabhu *et al*., 2021).**

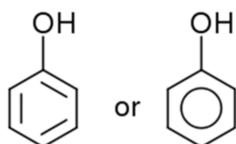

Figure 02: The structural formula of phenol **(Zagoskina *et al.*, 2023).**

Phenolic acids

Phenolic acids are phenol-derived compounds in plants, containing a benzene ring, one or more hydroxyl groups, and a carboxylic acid function. Phenolic acids are usually classified into two types, according to the carbon skeleton: hydroxybenzoic acids (C6–C1 structure) and hydroxycinnamic acids (C6–C3 structure). Their general structure of an aromatic ring with hydroxyl substitutions and a carboxylic acid group determines their chemical and biological activity, including antioxidant and antimicrobial activity **(N'Guessan *et al*., 2023).**

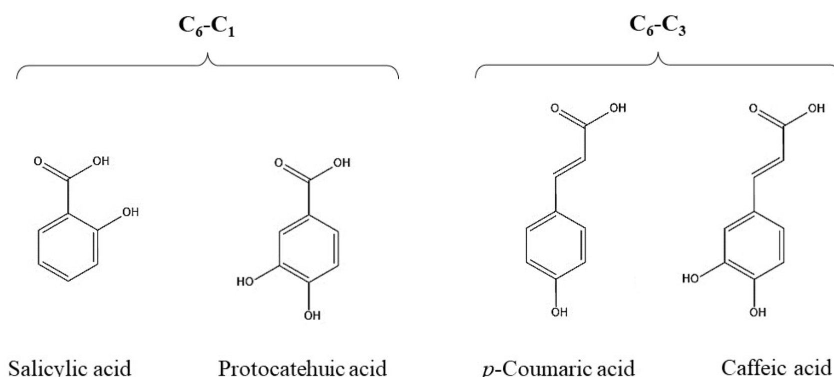

Figure 03: Structural formulas of simple polyphenols **(Zagoskina *et al.*, 2023).**

Flavonoids

Flavonoids represent one of the major classes of wood extractives phenolic compounds, which have in common a 15-carbon skeleton in the form of C6-C3-C6. The framework includes two aromatic rings (A and B rings) that are bonded together by a heterocyclic pyran ring (C ring) to form the central three-carbon bridge. The differences in the hydroxylation pattern and other substitutions, especially on the B ring, account for the wide range of flavonoids, including

anthocyanins, flavan-3-ols, flavones, flavanones, flavonols, isoflavones, neoflavonoids, and chalcones **(N'Guessan *et al*., 2023).**

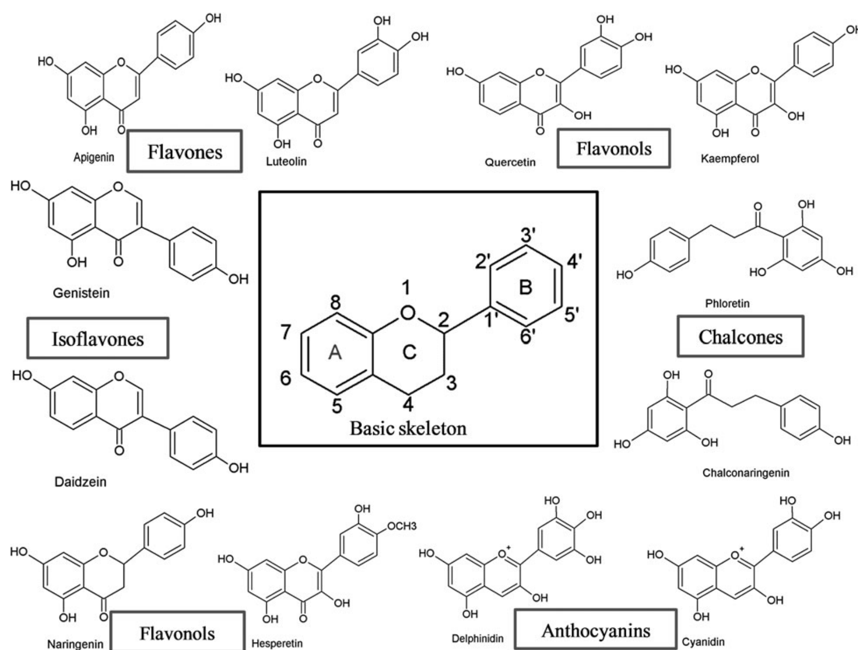

Figure 04: Basic skeleton structure of flavonoids and their classes **(Panche *et al.*, 2016).**

Tannins

Tannins are polyphenolic compounds divided into hydrolysable tannins, based on sugar esters of phenolic acids (**Figure 05**), and condensed tannins, which are polymers of flavan-3-ol units linked mainly by C4–C8 or C4–C6 bonds. These carbon–carbon linkages define the structure of condensed tannins, enabling their protein-precipitating ability and biological activities like antioxidant and antimicrobial effects **(N'Guessan et al., 2023).**

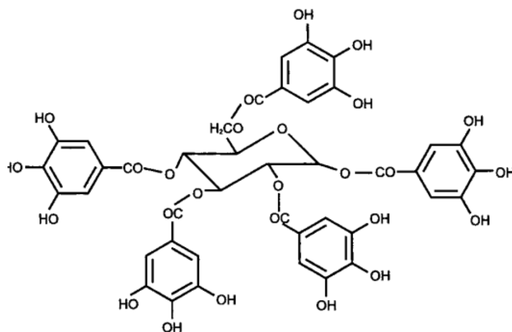

Figure 05: Structure of tannic acid **(Bravo, 2009).**

I.4.2. Biological properties of polyphenols

Natural polyphenols are a heterogenous group of plant polyphenolic compounds having more than one phenol structural unit. Natural polyphenols are generally strong antioxidants with the capacity to trap free radicals and inhibit oxidative stress by scavenging reactive oxygen species (ROS) and metal ions that facilitate ROS generation. In addition to their antioxidative potential, polyphenols also possess anti-inflammatory, anticancer, cardioprotective, anti-ageing, and antimicrobial activity, impacting different biological pathways like enzyme inhibition as well as regulation of signal transduction and cell receptors **(Li *et al.*, 2014).**

I.4.3. Interaction phenolic-lipids

Polyphenols, especially flavonoids, phenolic acids, and tannins, have been shown to exhibit strong interactions with lipid bilayers, which contribute importantly to the determination of biological activity. The type of interaction like hydrogen bonding, hydrophobic contacts is very much reliant on the chemical structure of the polyphenol and the lipid bilayer composition. It is revealed through research that polyphenols are able to cross lipid membranes, changing their structural and biophysical characteristics, such as fluidity and phase transition temperatures **(Karonen, 2022; Poklar Ulrih *et al.,* 2017)**.

One of the major determinants of polyphenol-lipid interactions is the polyphenol's hydrophobicity, as this affects the partitioning of the polyphenol into a membrane. More non-polar molecules like aglycones partition more into lipid bilayers than very hydrophilic ones **(Karonen, 2022; Poklar Ulrih *et al*., 2017).**

For instance, quercetin, a flavonoid, enters lipid bilayers mainly via a mixture of hydrogen bonding synthesis and hydrophobic interactions. Its ring system aromatic gives it the capability to stack parallel to the chains of membrane's fatty acyls, and hydroxyl groups present on its rings form hydrogen bonds to polar headgroups and lipid carbonyl groups. Such incorporation into membranes can lead to membrane fluidity, permeability, and phase behavior alterations, and thus on the membrane activity as well as on integral protein functioning. The nature of interaction is structure-dependent on the phenolic molecule and hydrophobicity reflecting the significance of molecular structure in phenolic-lipid interaction **(Karonen, 2022).**

Materials and Methods

II.1. Materials

II.1.1. Plant material

Centaurea calcitrapa was harvested at Ihaddaden (Bejaia) in May 2024 and dried in oven at **40°C** to remove all traces of moisture. The leaves and thorns of *Centaurea calcitrapa* were grinded using high performance grinder and sieved using a sieve of **1mm**, **500µm**, **125µm** of diameter to get a finer powder which was stored in dry glass jars away from light and moisture.

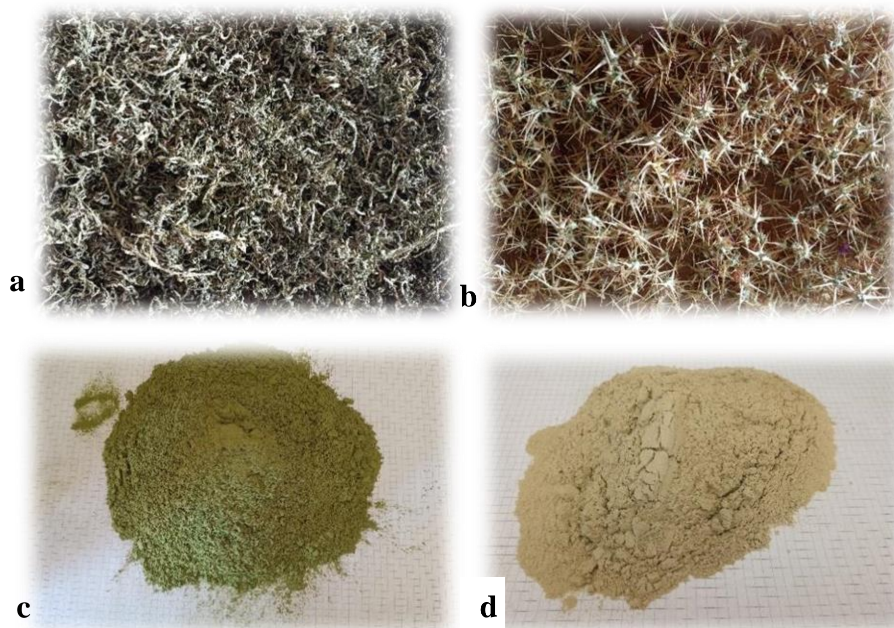

Figure 6 : *Centaurea calcitrapa* original photographs : **(a)** leaves **(b)** thorns **(c)** grinded leaves **(d)** grinded thorns.

II.1.2. Human blood samples

Human blood samples were obtained from the Lalaoui's Medical analysis Laboratory and were used to evaluate the hemolytic and anti-hemolytic effects of *Centaurea calcitrapa* extracts.

II.1.3. Chemicals and apparatus

Chemicals and apparatus used were presented in a table below:

Table IV: Chemical products and apparatus used.

Chemicals	Ethanol(96%), methanol, gallic acid, tannic acid, folin-ciocalteu reagent(1N), catechine, quercetin, aluminium chloride ($AlCl_3$), sodium carbonate (Na_2CO_3), 2,2-diphenyl-1-picrylhydrazyl(DPPH), sulfuric acid (H_2SO_4), hydrochloric acid (HCl), ferric chloride, tannic acid, anhydride acetate, iron chloride ($FeCl_3$), hydrogen peroxide(H_2O_2), Thiobarbituric acid (TBA), distilled water, Sodium azide (N_3Na).
Aparratus	High power grinder, rotary vacuum, centrifuge, stirrer, spectrophotometer, micropipette, beakers, test tubes, hemolysis tubes, heparin tubes, PH meter, Agitator, Balance, Oven, Micropipettes (50µl-100µl-1000µl), Eppendoff tubes, gloves.

II.2. Methods

II.2.1. Extraction of plant material

100g of *Centaurea calcitrapa* leaves and thorns powder was macerated in **1000ml** of hydroethanol solvent (ethanol/water, **80:20**, **4:1**, v/v). The mixture was agitated at **650 rpm** for **24 hours** at room temperature. The mixture was left to decant in a cylinder overnight then the supernatant was poured into a flask and kept at 4°C. The maceration was repeated two times to optimize metabolites extraction. Obtained supernatants were mixed then centrifuged for further purification at **3000 rpm** for **10 minutes** to get the final extract. The liquid was evaporated using a rotary vacuum evaporator **(figure 6)** at **40°C** and **130-140 rpm** until the volume was significantly reduced. The remaining extract was poured into petri dishes and completely dried in a ventilated oven at **40°C**. The extraction yield was calculated as follows:

$$\text{Yield (\%)} = (\text{Dried weight of extract}/\text{Dried weight plant material}) \times 100$$

Figure 7: Evaporating the ethanol from the extract using the rotary vacuum evaporator.

II.2.2. Phytochemical analysis

II.2.2.1. Total phenolic content

The total phenolic contents of *Centaurea calcitrapa* leaves and thorns were determined according to the protocol described by **Kahkonen *et al.* (1999).** The method is based on the Folin-Ciocalteu reagent reaction with phenolic compounds. During the process, phenols are oxidized, leading to the reduction of the initially yellow reagent and the development of a blue colored complex. The intensity of the blue color, as quantified by absorbance at **760 nm**, is proportional to the total phenol content in the extract.

To conduct the assay, **20 µl** of the methanoic extract solution (10mg/ml) was combined with **80 µl** of a **7.5%** sodium carbonate (Na_2CO_3) solution. To this, **100µl** of Folin–Ciocalteu (1N) reagent, was added. The corresponding blank was prepared by mixing **20µl** of methanol, **80µl** of sodium carbonate and **100µl** of folin-ciocalteu. The resulting solution was incubated in the dark for **30 minutes** at room temperature to ensure full reaction. The blue complex formed absorbance was measured at **760 nm** using spectrophotometer. The content of total phenolic was calculated from a calibration curve prepared with gallic acid and was expressed in terms of milligrams of gallic acid equivalents per gram of extract (mg GA Eq/gE) **(Annex 1).**

II.2.2.2. Flavonoid content

Flavonoid content was estimated by the aluminum chloride colorimetric method described by **Maksimovic et *al.* (2005)**. Flavonoid molecules chelate with aluminum ions to produce stable complexes that shift their color to yellow. The yellow color is measurable at **430 nm**. The intensity of the color indicates the concentration of flavonoids in the sample.

During this process, **120µl** of extract solution was combined with **60 µl** of aluminum chloride reagent in which **66.5 mg** of aluminum chloride ($AlCl_3$) and **200 mg** of sodium acetate were dissolved in **100 ml** of distilled water. The blank was prepared by mixing **120µl** of extract solution and **60 µl** of distilled water. The mixture was incubated for **10 minutes** at room temperature. After incubation, the absorbance was recorded at **430 nm** with the UV-visible spectrophotometer. The flavonoid content was also measured in reference to a calibration curve of a quercetin standard and

the results recorded in terms of milligrams of quercetin equivalents per gram of extract (mg Q Eq/gE) **(Annex 2).**

II.2.2.3. Tannins content

II.2.2.3.1. Condensed tannins content

The quantification of condensed tannins was performed using the vanillin assay in an acidic medium described by **Dif *et al.* (2015**). The condensed tannins with vanillin form brown-coloured complex that is measured at **500 nm**. The intensity of the color indicates the concentration of condensed tannins in the sample.

To determine the concentration of condensed tannins, **120µl** of vanillin sulfuric was combined with **60µl** of sample (catechine /extract). A control containing **120µl** vanillin sulfuric and **60µl** of methanol instead of extract served as the reference. **100 mL** of a vanillin solution, was prepared by dissolving **1g** of vanillin in **77.77 mL** of **70%** sulfuric acid and adjusting the volume to **100 mL** with distilled water. The reaction mixture was incubated for **15 seconds**, protected from light. The absorbance of the resulting brown-colored complex was measured at **500 nm** using a UV-Vis spectrophotometer. The condensed tannins content was measured in reference to a calibration curve of catechine standard and the results recorded in terms of milligrams of catechine equivalents per gram of extract (mg Q Eq/gE) **(Annex 3).**

II.2.2.3.2. Hydrolysable tannins content

The determination of hydrolyzed tannins was carried out using the ferric chloride colorimetric method described by **Dif *et al.* (2015**). The hydrolysabled tannins with ferric chloride form reddish-violet complex that is measured at **660 nm**. The intensity of the color indicates the concentration of condensed tannins in the sample.

To assess the concentration of hydrolyzed tannins, the solution of hydrochloric acid **(0.001M)** was prepared by adding **41.5 µl** of concentrated hydrochloric acid to **500ml** of distilled water. **100 ml** of the final solution was prepared by adding **162.21 mg** ferric chloride **(0.01 M)** in **100ml** of HCl **(0.001M)**. The absorbance of the reddish-violet complex formed was measured **15 seconds** after reagent addition at a wavelength of **660 nm** using a UV-Vis spectrophotometer. The hydrolysable tannins content was measured in reference to a calibration curve of gallic acid standard and the

results recorded in terms of milligrams of gallic acid equivalents per gram of extract (mg Q Eq/gE) (Annex 4).

II.2.3. Antioxidant activities

II.2.3.1. DPPH Radical Scavenging Assay

The antioxidant potential of the extracts/standard was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging assay of **Rangkadilok et al. (2007).** This method measures hydrogen-donating capacity by tracking the reduction of the purple DPPH radical to its non-radical yellow form (DPPH-H).

To determine the DPPH scavenging Assay, **180µl** of DPPH was added to **20 µl** of standard/extract. DPPH solution was prepared by combining **20 mg** of DPPH and **50 mL** of ethanol. A control containing **180µl** of DPPH and **20µl** of methanol instead of extract served as the reference. The absorbance was recorded at **515 nm** using a UV-visible spectrophotometer after incubating the mixture for 3**0 minutes** under subdued light to prevent photo-degradation. Results were quantified as **IC_{50}**, representing the extract concentration required to scavenge **50%** of DPPH radicals.

II.3. Evaluation of the hemolytic and anti-hemolytic activity of ethanoic extracts from *Centaurea calcitrapa.*

Hemolytic and anti-hemolytic tests on erythrocytes were performed according to the protocol of **Rafat** ***et al.*** **(2009)** described by **Sundaram** ***et al.*** **(2011).**

II.3.1. Preparation of erythrocytes suspensions.

The blood was collected in heparinized tubes and centrifuged at **1500 rpm** in **10 min** to remove plasma and the buffy coat. The (RBCs) red blood cells were washed three times by centrifugation **(3500 rpm/10 min)** with phosphate-buffered saline at **pH 7.4 (0.58 mM KH2PO4, 3.4 mM $NaHPO_4$, 150 mM NaCl and 1 mM Sodium azide).** The phosphate-buffered saline composed of **1.8 mM** KH_2PO_4, **10 mM** K_2HPO_4 and **50 mM** NaCl. The washed erythrocytes were re-suspended at 10% hematocrit in the same buffer.

II.3.2. Evaluation of hemolytic activity of ethanoic extracts of *Centaurea calcitrapa* on human erythrocytes.

The hemolytic activity of ethanoic extracts of *Centaurea calcitrapa* was carried out on erythrocyte suspensions at **10%** hematocrit. In hemolysis tubes, **250µl** of ethanoic extracts of *Centaurea calcitrapa* at different concentrations (**1-100 µg/ml**) were added to **125µl** of the erythrocyte suspension and then incubated in a water bath at **37°C** for **3 hours**. A negative control and a positive control were prepared by substituting the ethanoic extracts of *Centaurea calcitrapa* solution with the phosphate buffer saline solution and distilled water. After incubation, the volume of the different samples was adjusted to **2250 µl** with phosphate buffer, then directly centrifuged the tubes at **2000 rpm / 10 minutes**. The absorbance of the supernatants was recorded at **540 nm** using a UV-Visible spectrophotometer.

II.3.3. Anti-hemolytic effects of ethanoic extracts of *Centaurea calcitrapa* on human erythrocytes.

II.3.3.1. Anti-hemolytic effects of ethanoic extracts of *Centaurea calcitrapa* on H_2O_2 induced hemolysis on human erythrocytes.

Following the hemolytic tests of ethanoic extracts, concentrations that did not exhibit significant toxicity were selected for further evaluation of their anti-hemolytic effects.

It consists of adding a volume of **250 µl** of plant extract to **125 µl** of the suspension erythrocyte. After **30 min** of pre-incubation at **37 °C**, **250 µl** of **8 mM** H_2O_2 was added to the reaction medium and the mixture is re-incubated for up to **3 hours**. negative control (prepared with phosphate buffer) and two positive controls (prepared with distilled water and H_2O_2) were carried out in parallel. After incubation, the volume of all tests was adjusted to **2250 µl**. Hemolysis was measured at **540 nm** after centrifugation at **2000 rpm** for **10 min**, and the inhibition of hemolysis was calculated as follows:

% inhibition = [(% **H_2O_2 hemolysis - % extract hemolysis**) / % **H_2O_2 hemolysis] *100**

II.3.3.2. Anti-hemolytic effects of ethanoic extracts of *Centaurea calcitrapa* on AAPH induced hemolysis on human erythrocytes.

The anti-hemolytic effects of ethanoic extracts of *Centaurea calcitrapa* were evaluated against AAPH induced hemolysis, following the method of **Rafat *et al.* (2009),** with slight modifications.

125 µl of a **10%(v/v)** human erythrocyte suspension in phosphate-buffered salin was pre-incubated with varying concentrations (**50-300µg/ml**) of *Centaurea calcitrapa* extracts. Hemolysis was then induced by adding **250 µl** of AAPH solution to achieve the final concentration of **200 mM**, followed by incubation at **37°C** for **4 hours**. After incubation, all samples were adjusted to **2250 µl** with PBS and centrifuged at **2000 rpm** for **10 minutes**. The extent of hemolysis was quantified by measuring supernatant hemoglobin absorbance at **540 nm**. The inhibition percentage of hemolysis was calculated using the formula:

Hemolysis inhibition (%) $= [(A_{AAPH} - A_{Extract})/A_{AAPH}] \times 100$

Statistical analysis

Statistical analysis was performed using GraphPad Prism software where the results are expressed as ± SD. The data was subjected to one-way analysis of variation (ANOVA) and used Dunnett's Multiple Comparison Test. P<**0.05** was considered significative.

Results and Discussion

III.1. Results and interpretations

III.1.1. Extraction yield.

Centaurea calcitrapa, leaves and thorns, extracts were prepared using ethanol: water (80:20) by maceration that was repeated twice. The extraction yields were calculated and if presented in the table below:

Table V: Extraction yield from Centaurea calcitrapa (leaves and thorns)

	C.calcitrapa leaves	*C.calcitrapa* thorns
Extraction yield (%)	29.22	19.15

The extraction yield of phenolic compounds from *Centaurea calcitrapa* was higher in leaves **(29.22%)** compared thorns **(19.15%) (Table VI)**. The high yield obtained could be explained by the prolonged extraction of our plant material in the solvent by maceration that was repeated twice. The higher yield could also be due to the solvents used (ethanol-water). The lower yield in thorns observed could be caused by their lignified, rigid structure, which limits solvent accessibility and reduce extraction efficiency.

III.1.2. Determination of phenolic contents of *Centaurea calcitrapa* extracts.

The phenolic contents of *Centaurea calcitrapa* extracts, leaves and thorns, were estimated by colorimetric methods. The results are represented in (**Table VI).**

Table VI: Results of the quantification of total polyphenols, flavonoids, hydrolysable tannins and condensed tannins from hydroethanoic extracts of *Centaurea calcitrapa* plant materials (leaves and thorns).

	C.calcitrapa Leaves	*C.calcitrapa* Thorns
Total phenolic content (mg Eq. GA/mg d'extrait sec)	2415.00±564.71	752.17±53.83
Total flavonoids content (µg Eq. Q/mg d'extrait sec)	5.94±0.44	4.44±1.02
Total hydrolysable tannins content (µg Eq. GA/mg d'extrait sec)	1.13±0.692	1.03±0.71
Total condensed tannins content (µg Eq. Cat/mg d'extrait sec)	31.35±2.17	82.76±11.92

The TPC was determined by the Folin-Ciocalteu method using gallic acid as a reference and calculated using the gallic acid standard curve equation **($y = 0.0026x - 0.0929$, $R^2 = 0.9493$) (Annex 1)**, the TFC was determined with the aluminium chloride colorimetric assay using quercitin as a reference and calculated with the quercitin standard curve equation **($y=0.2477x - 0.0105$, $R^2 = 0.977$) (Annex 2)**, the THTC was assessed by the ferric chloride colorimetric method using gallic acid as a reference and calculated with gallic acid standard curve equation **($y = 0.603x - 0.4071$, $R^2 = 0.9642$) (Annex 3)** and the TCTC was determined by the vanillin assay using catechine as a reference and calculated with catechine standard curve equation **($y = 0.0092x - 0.0112$, $R^2 = 0.9912$) (Annex 4)**.

Our extract demonstrated a high TPC, TFC, THTC in leaves than in thorns. The TPC from leaves **(2415 µg/mg)** are thrice higher than in thorns **(754.17 µg/mg)**. The TFC from leaves (**5.94 µg/mg)** are slightly higher than those from thorns (**4.44 µg/mg)**. Similarly, THTC from leaves (**1,13 µg/mg)** are slightly higher than those from thorns (**1,03 µg/mg)**. However, TCTC from thorns (**82,76 µg/mg)** are twice higher than those from leaves (**31,35 µg/mg)**.

Our results of THTC in leaves are slightly higher than in thorns. The results of TCTC in thorns are higher than in leaves. Given the originality of this work, there is no comparison with the previous research. This inversion may reflect unique *C. calcitrapa* adaptation where thorns invest in condensed tannins for physical and chemical protection.

III.1.3. Antioxidant activities

III.1.3.1. DPPH scavenging activity of *Centaurea calcitrapa* of extracts (leaves and thorns).

The antioxidant activity of *C. calcitrapa* extracts was evaluated using the DPPH scavenging assay, a method for assessing free radical neutralization activity. The results are represented in **(Figure 8)**.

(a)

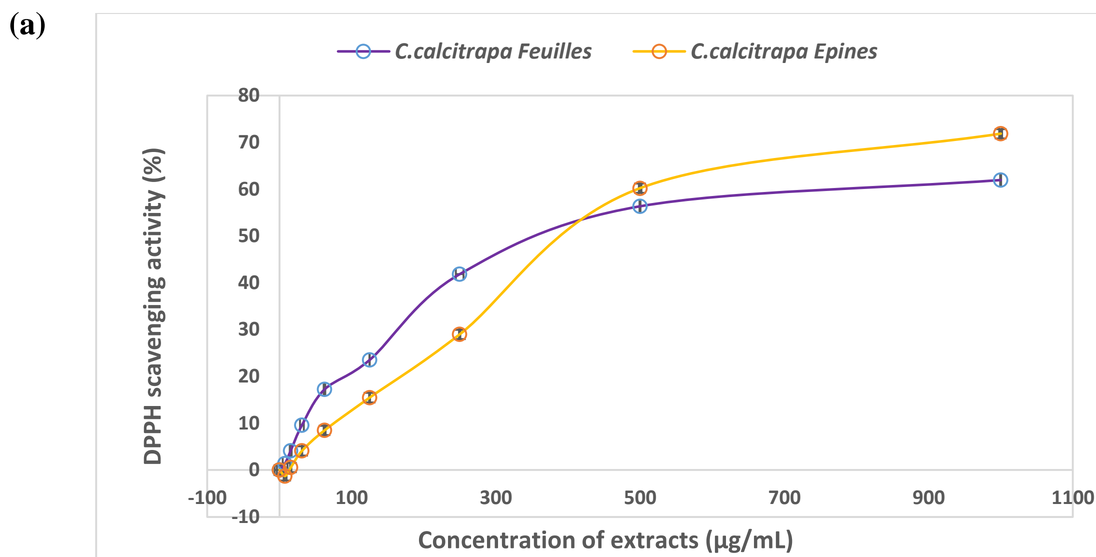

Figure 8: Results of DPPH radical scavenging activities by *Centaurea calcitrapa* extracts (leaves and thorns).

The results indicate that both leaves and thorns extracts increase in DPPH scavenging activity. Thorns extract**,** on the other hand, reached a slightly higher scavenging activity than leaves, indicating a stronger antioxidant potential**.**

According to **Dimkić et al. (2021),** phenolic-rich plant extracts show comparable radical scavenging patterns with R^2 values typically above 0.95 in DPPH assays, affirming the reliability of the linearity observed in this study.

Table VII: 50% inhibitrory Concentrations of DPPH radical by *Centaurea calcitrapa* extracts (leaves and thorns) and those of phenolic molecules tested under the same experimental conditions (**Curve in Anexe 1,2,3).**

	Hydroalcoholic extracts	
	C.calcitrapa leaves	*C.calcitrapa* thorns
	290.15	415.06
IC_{50} (µg/mL)	**Pure phenols**	
	Tannic acid	Quercetine
	6.68	13.90
	Gallic acid	Catechine
	4,21	6,24

The antioxidant potential of reference phenolic compounds, gallic acid and tannic acid was evaluated using the DPPH radical scavenging assay under the same conditions as the *Centaurea calcitrapa* extracts. The results are presented in **Figure 9**, and the corresponding IC_{50} values are summarized in **Table VIII**.

Gallic acid exhibited the strongest radical scavenging activity with an IC_{50} of 13. 9µg/mL, indicating high antioxidant potency**.**

The results also showed that *C. calcitrapa* thorns extracts had high IC_{50} (415.06 µg/ml) compared to *C. calcitrapa* leaves IC_{50} (290.15 µg/ml) .

III.1.4. Evaluation of the hemolytic and anti-hemolytic activity of ethanoic extracts from *Centaurea calcitrapa.*

III.1.4.1. Interaction of *Centaurea calcitrapa* extracts (leaves and thorns) with humain erythrocytes.

To assess the interaction of gallic acid and *Centaurea calcitrapa* extracts (leaves and thorns) on erythrocytes, various concentrations were used. The results are presented in **(Figure 11).**

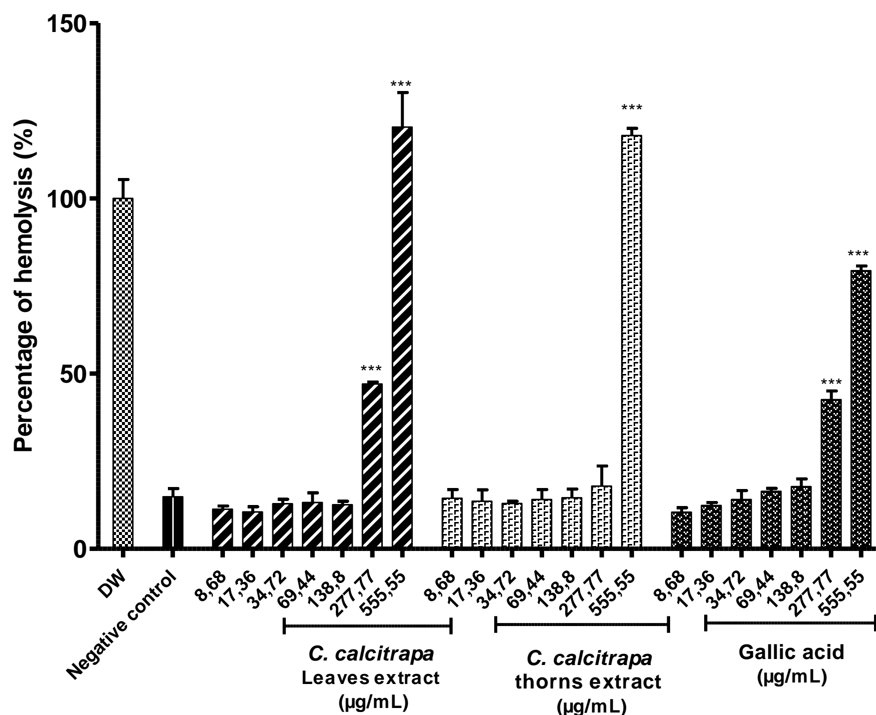

Figure 9: Results of interactions of gallic acid and *Centaurea calcitrapa* extracts (leaves and thorns) with human erythrocytes humain. Values are expressed in mean, mean ± SD (n= 3).

At lower concentrations (8.68 µg/mL and 17.36 µg/mL), the leaves extract exhibited negligible hemolytic activity, comparable to the negative controls. However, a striking dose-dependent increase in hemolysis was observed with escalating concentrations. At 34.72 µg/mL, the hemolytic effect became significant. This effect became profoundly pronounced at 69.44 µg/mL and 138.88 µg/mL, with hemolysis percentages exceeding 100% at the highest concentration. This suggests complete lysis of erythrocytes then membrane damage, indicating a strong hemolytic potential.

The thorns extract displayed a similar pattern to the leaves extract. Low concentrations (8.68 µg/mL and 17.36 µg/mL) showed minimal hemolysis. Significant and dose-dependent hemolytic activity was observed at higher concentrations, particularly from 34.72 µg/mL upwards. The highest concentrations (69.44 µg/mL and 138.88 µg/mL) also led to very high percentages of hemolysis, similar to the leaves extract, confirming that the thorn extract also possesses considerable hemolytic properties at elevated doses.

Gallic acid showed very low hemolytic activity at lower concentrations (8.68 µg/mL, 17.36 µg/mL, and 34.72 µg/mL). A significant dose-dependent increase in hemolysis was observed at 69.44 µg/mL and 138.88 µg/mL. However, the maximum hemolysis induced by gallic acid (70-80% at 138.88 µg/mL) was notably lower than that caused by the highest concentrations of either *Centaurea calcitrapa* extract. This indicates that while gallic acid can induce hemolysis, its potency is less pronounced than that of the plant extracts.

III.1.4.2. Effects of *Centaurea calcitrapa* extracts (leaves and thorns) against H_2O_2 –induced hemolysis.

Different concentrations of gallic acid and *Centaurea calcitrapa* extracts were used against H_2O_2 on human erythrocytes to evaluate their effect. The results are shown in **(Figure 12).**

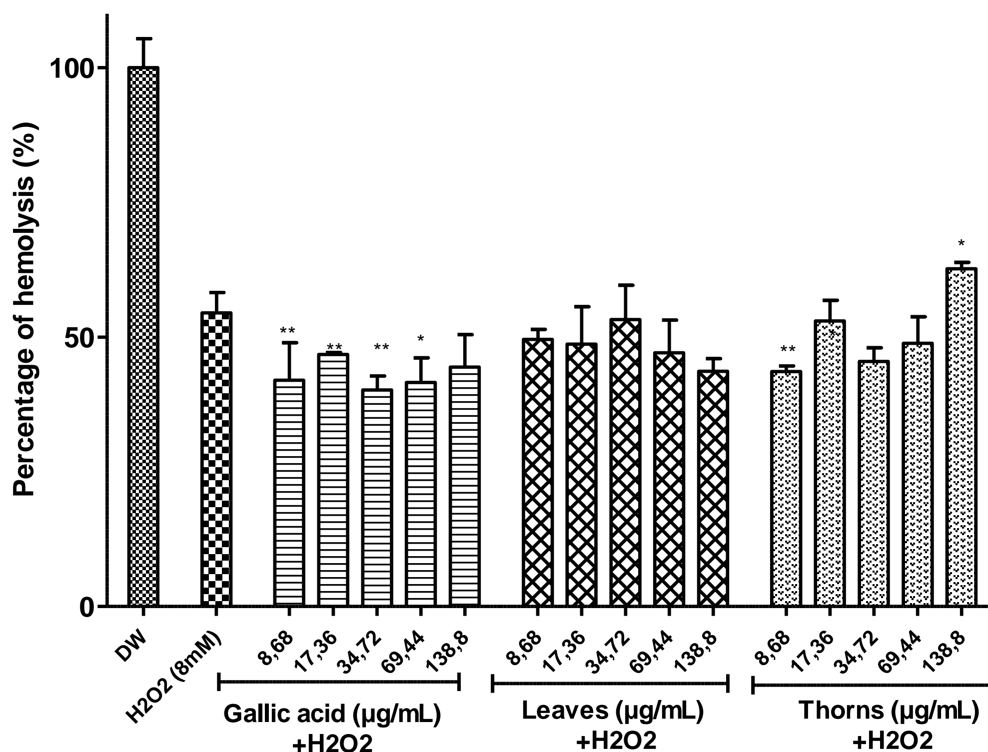

Figure 10: Results of the effets of extracts de *Centaurea calcitrapa* (leaves and thorns) and gallic acid on hemolysis induced by H_2O_2**.** Values are expressed in mean, mean ± SD (n= 3).

The results shown in **Figure 12** show different rates of activity. The positive control, H_2O_2 (8mM) caused approximately 60% hemolysis, consistent with its ability to produce peroxyl radicals. The negative control, distilled water caused 100% hemolysis. Gallic acid showed significant inhibition of hemolysis across the concentrations. Notably, *C. calcitrapa* thorn extracts overperformed the *C. calcitrapa* leaves extract in reducing hemolysis at higher concentrations.

III.1.4.3. Effects of *Centaurea calcitrapa* extracts (leaves and thorns) against AAPH-induced hemolysis.

Different concentrations of gallic acid, *Centaurea calcitrapa* (leaves and thorns) extracts were used against AAPH on human erythrocytes to evaluate their effect. The results are shown in **(Figure 13).**

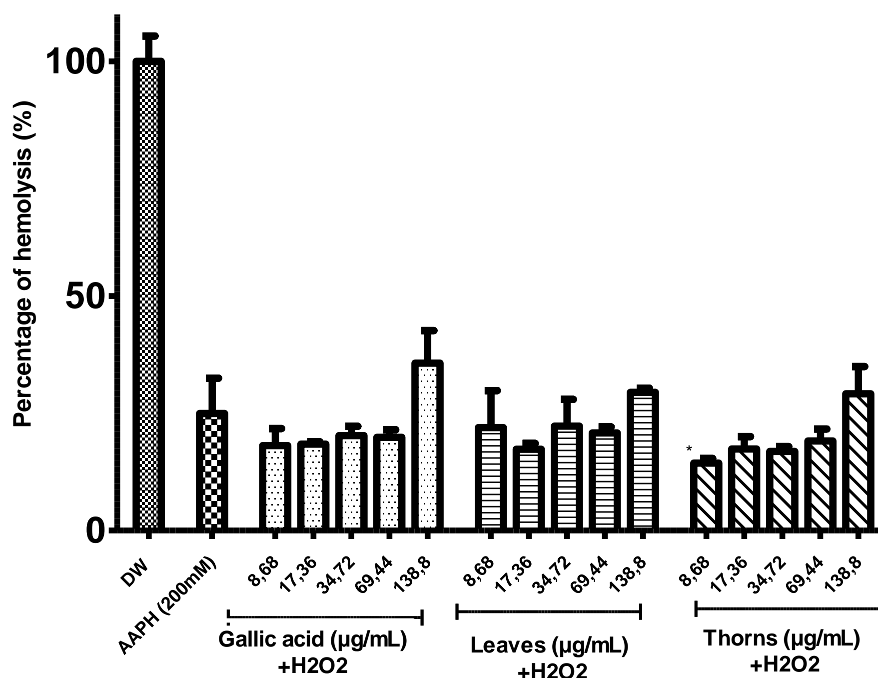

Figure 11: Results of the effets of extracts de *Centaurea calcitrapa* (leaves and thorns) on hemolysis induced by AAPH. Values are expressed in mean, mean ± SEM (n= 3).

The positive control, AAPH (200mM) showed nearly 25% hemolysis, confirming that AAPH induces significant oxidative damage to erythrocyte membranes. In addition, distilled water (DH) showed 100% hemolysis, serving as a negative control.

Gallic acid exhibited the strongest protective effect, significantly reducing hemolysis. At higher concentrations around 87.565 µg/mL, hemolysis was reduced and this confirms its potent antioxidant and membrane-protective properties.

Leaf extracts also reduced hemolysis although the protective effect was less than that of gallic acid. Moderate concentrations showed some level of protection but the effect was more variable and less significant. This suggests that leaves contain active phenolic compounds at a lower concentration than pure gallic acid.

The thorn extract demonstrated the least protective effect among the three treatments. Hemolysis remained relatively high across all tested concentrations, indicating weaker antioxidant potential compared to both gallic acid and leaf extracts.

III.2. Discussion

Several experiments have determined that the rate of secondary metabolites such as Phenolic compounds are extracted from medicinal plants, which contain a power antioxidant thanks to their hydroxyl groups, protect against the radicals responsible of various pathologies.

The preparation of leaves and thorns extracts of *C. centaurea* was done using maceration method in ethanol and water. The difference in the contents obtained can be explained by the nature of the active compounds extracted, the particle size of the plant powder, which can increase the surface area for interaction between the solvent and the powder, as well as the extraction conditions **(Bonnaillie *et al*., 2012).** It can also be suggested that the difference in contents is due to the duration of maceration

Similarity, studies have shown that extraction rates are influenced by the type of solvent. It has been proven that ethanol is the best solvent to extract the maximum number of phenolic compounds **(Cowan, 1999).** It is known for its ability to extract not only polar compounds but also non-polar compounds, unlike distilled water, which only extracts highly polar substances **(Bonnaillie *et al*., 2012).**

Phenolic compounds such as total phenols, flavonoids, condensed tannins and hydrolysable tannins form the most important group of phytochemical compounds in plants. The dosage results show that the condensed tannin content is higher in the ethanolic extract. This may be explained by the richness of the latter in hydroxyl OH groups **(Bravo, 1998),** which promotes their solubility in alcohols such as ethanol. Nevertheless, it is a class of compounds with a complex structure given their high molecular weight and the degree of their polymerization, which would limit their solubility in aqueous phases **(Mahmoudi *et al.*, 2013).**

A study conducted by **Berboucha et al. (2009)** on this plant shows a slight similarity in the rates of phenolic compounds compared with the contents obtained currently, mainly total phenols, whereas for flavonoid contents and tannins. These differences may be due to climatic factors and harvest regions. These metabolites are widely reported for their biological activities, notably antioxidants, by different protocols and experimental models.

The red blood cell remains an interesting model for cellular study because its structure is simple, and its function of transporting oxygen and hemoglobin exposes it particularly to radical attacks **(Antonelou *et al.*, 2010).** It is thus prone to oxidation due to its high content of polyunsaturated fatty acids, its oxygen-saturated molecular environment, as well as the presence of transition metals such as iron and copper **(Rocha *et al.*, 2009).**

H2O2 is one of the most potent ROS. It exerts an effect on erythrocytes, promoting their lipid peroxidation, consequently cell death **(Edwards and Fuller, 1996).** This hemolysis is happens due to the oxidation of erythrocytes by H2O2 following its diffusion across erythrocyte membranes, this phenomenon can be explained by the Fenton reaction where oxygen binds with hydrogen peroxide in the intracellular medium which promotes the oxidation of ferrous iron hemoglobin (Fe^{2+}) to ferric iron methemoglobin (Fe^{3+}), then formation of the hydroxyl radical (OH), which acts on erythrocyte membrane lipids, thus causing membrane lysis, erythrocytes then release of hemoglobin **(luchi, 2012),** expressing levels hemolysis induced by oxidative stress.

Biological activities are dose-dependent this is the same for the bioactivity and toxicity of a substance. In this study, the doses used for anti-hemolytic activities were determined after a toxicity study of the extracts studied, which consists of evaluating hemolysis induced by the extracts themselves.

In this context, hydroethanoic extracts of C. calcitrapa were tested on human erythrocytes. The results indicate that the extract at high concentration (277.77 µg/ml–555.55µg/ml) induced hemolysis.

The findings in this study indicates that both gallic acid and *C. calcitrapa* extracts have significative effect against AAPH and H_2O_2 induced hemolysis. Gallic acid, a phenolic compound, had a strong protective effect against oxidative damage in erythrocytes.

A study by **Trabsa et al. (2020)** showed that the metabolites of *Centaurea calcitrapa*, particularly its flavonoid compounds, demonstrate significant interactions with erythrocyte membranes, contributing to their protective antioxidant effects. The study highlights that the ethyl acetate

extract (EaE) contains the highest flavonoid content (50.71 ± 0.65 mg Eq / Quercetin g dry extract). In the in vitro assays, the extracts notably reduced β-carotene bleaching by at least 70%, and the ethyl acetate extract achieved an inhibition rate comparable to standard antioxidants like BHT. These findings suggest that flavonoids and phenolic compounds in the plant may incorporate into erythrocyte membranes, stabilizing lipid bilayers by scavenging free radicals and inhibiting lipid peroxidation, thus preventing oxidative damage or hemolysis. The ability of these metabolites to enhance membrane resilience aligns with their known membrane-stabilizing and antioxidant properties, thereby having therapeutic potential in protecting erythrocytes from oxidative stress-induced hemolysis **(Joujeh *et al.*, 2020)**.

Gallic acid (GA) is a polyphenol present in many plants. The study by **Suwalsky et al. (2016)** was aimed to investigate the molecular interaction of GA with the human erythrocyte membrane and to determine its antioxidant capacity. The molecular interaction with the membrane of human red cells and the antioxidant property was assayed on both human red cells and molecular models of its membrane. Observations by optical, scanning electron, and defocusing microscopy demonstrated that GA is capable to convert red cells from their normal biconcave shape to crenated echinocytes. This result indicates that GA molecules are positioned in the outer monolayer of the erythrocytes. Dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE) were selected as classes of phospholipids found in the outer and inner monolayers of the red cell membrane, respectively. X-ray diffraction and differential scanning calorimetry showed that GA was preferentially bound to DMPC bilayers. Experiments related to the antioxidant capacity of GA indicated that this compound prevent oxidative capacity on DMPE bilayers. As a conclusion, GA would be capable to block the access of oxidants into the lipid bilayer, and thus avoid their access into red cells **(Suwalsky *et al.,* 2016).**

Conclusion and Perspectives

This study investigated the interaction of phenolic compounds extracted from *Centaurea calcitrapa* with biomembranes models, specifically human erythrocytes membranes in order to evaluate their potential bioactivities. Through a series of in vitro assays, including hemolysis and membrane protection tests, the results demonstrated that *Centaurea calcitrapa* exhibits significant antioxidant and membrane stabilizing properties attributed to its rich content in phenolic compounds.

The extract showed a concentration-dependent effect on erythrocytes membranes with lower concentrations exhibiting protective effect against oxidative stress-induced hemolysis, while higher concentrations led to hemolysis. These results suggest that the phenolic compounds may play an important role in modulating membrane integrity which can be used for therapeutic purposes when carefully used.

In summary, this work contributes to the growing evidence supporting the use of plant-derived in biomedical applications. It highlights the importance of *Centaurea calcitrapa* as a potential source of bioactive molecules with antioxidant and cytoprotective properties which may be relevant in the development of natural therapies targeting oxidative stress and membrane related pathologies.

Future studies should focus on explaining the molecular mechanisms underlying these interactions and exploring the *in vivo* efficacy of these extracts. Additionally, isolating active compounds may enhance their therapeutic potential and lead to novel pharmacological developments.

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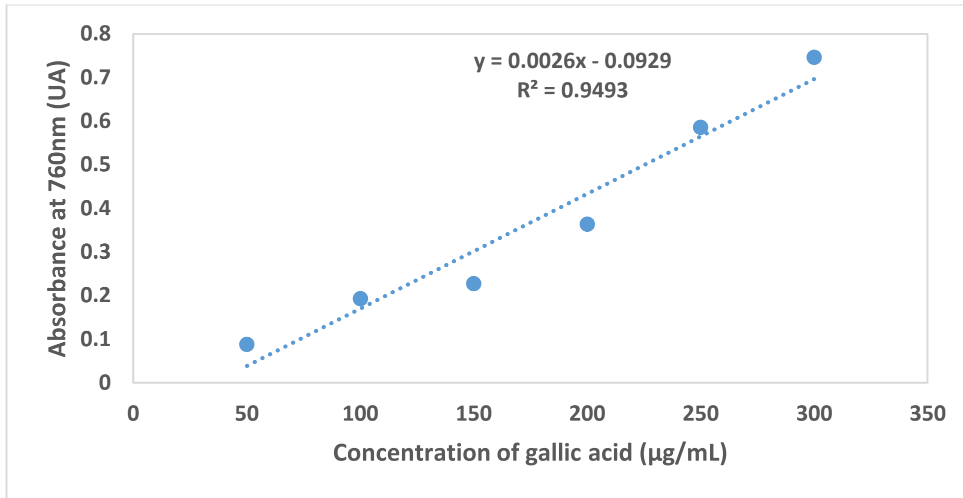
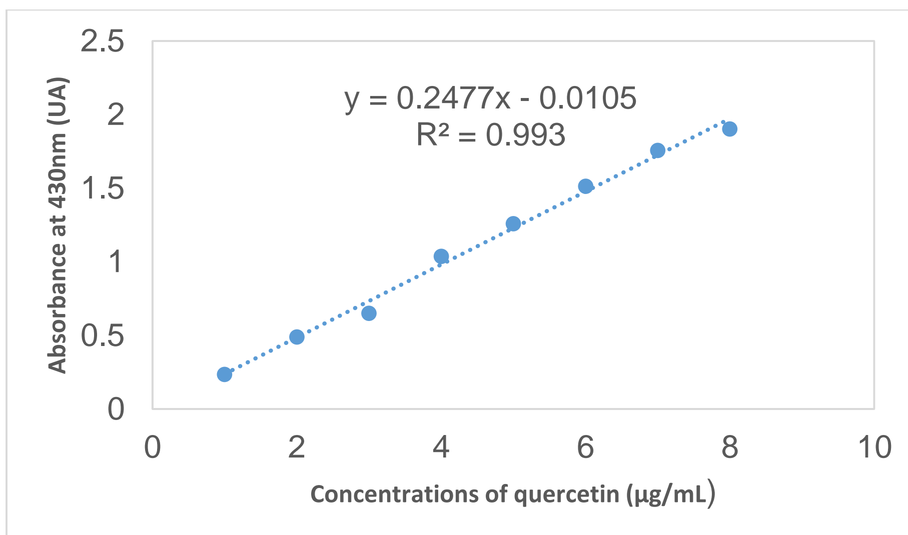
Annexes

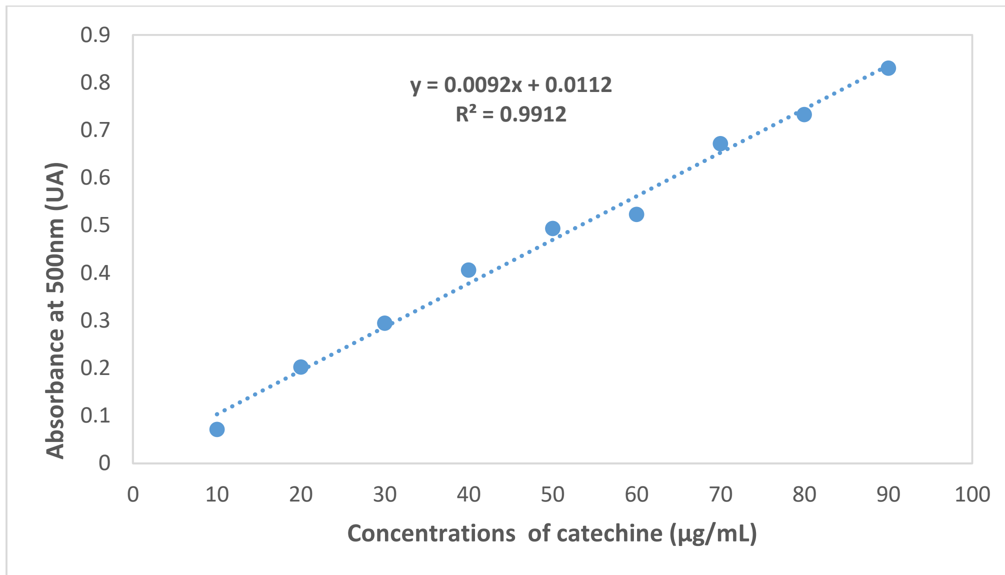
Annexe 1: Gallic acid standard curve for total polyphenol content determination.



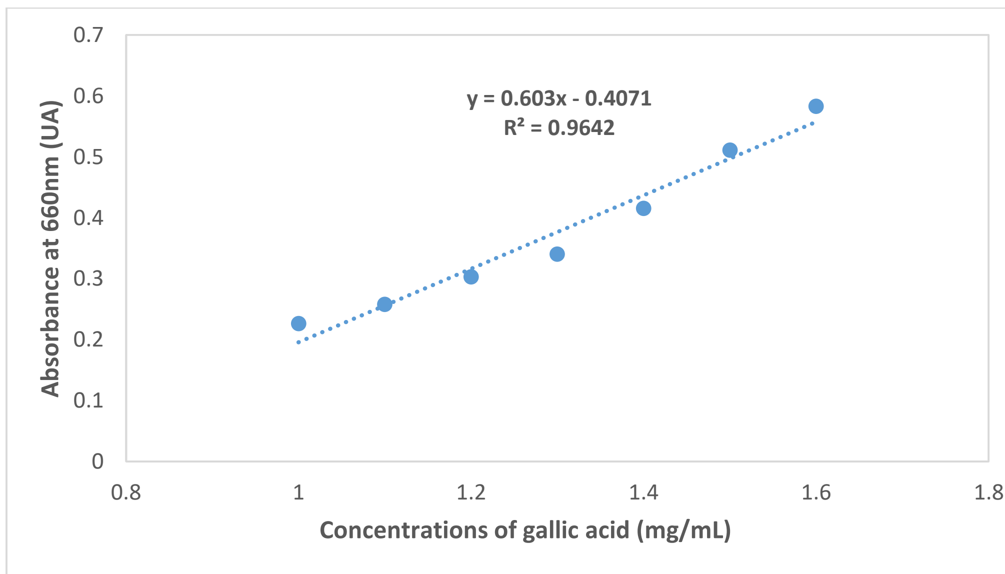
Annexe 2: Quercitin standard curve for total flavonoid content determination.



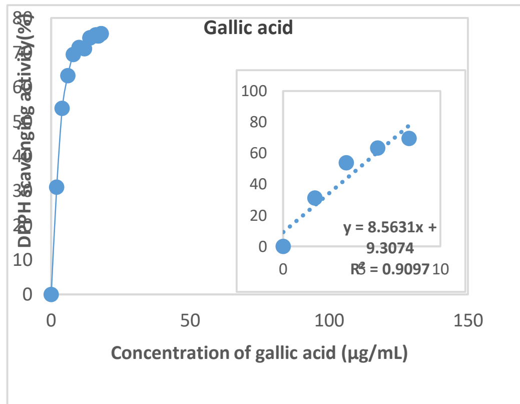
Annexe 3 : Catechine standard curve for total condensed tannins content determination.

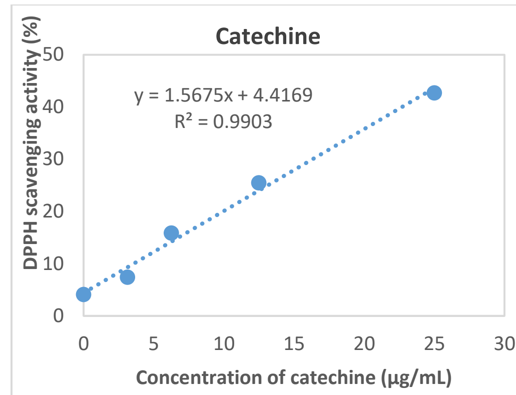


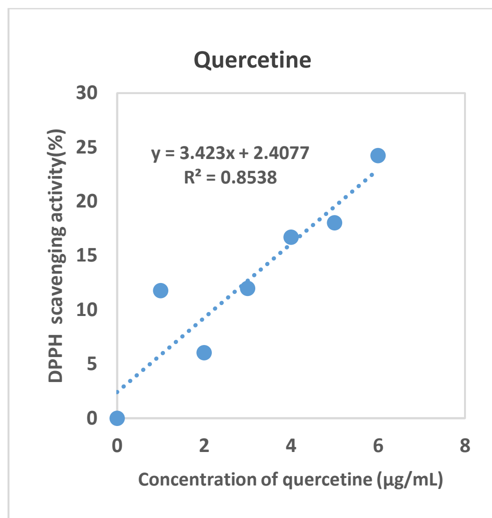
Annexe 4: Gallic acid standard curve for total hydrolysable tannins content determination.

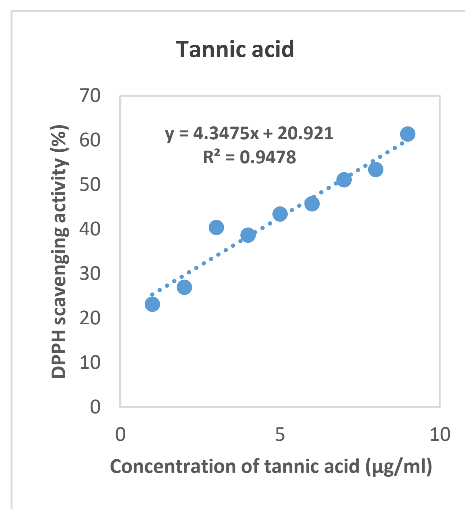


Annexe 5: DPPH radical scavenging activities by pure phenolic molecules tested under the same experimental conditions as the extracts studied.









Abstract

Centaurea calcitrapa leaves and thorns are known for their use in traditional medicine and more specifically for the treatment of pathologies related to oxidative stress. This work aims to test the protective effect of different concentrations of ethanoic extracts of these parts of the plant against hemolysis induced by H_2O_2 (**8 mM**) and AAPH (**200 mM**) carried out *in vitro* on a membrane model of human erythrocytes. Furthermore, the toxicity of these extracts was studied and their contents in total phenols, flavonoids and tannins were estimated by colorimetric assays. The results demonstrate that the ethanoic extracts have a high in total phenols followed by condensed tannins, flavonoids then hydrolysable tannins. They exhibited a toxic effect at high concentrations on the erythrocytes membranes. Additionally, the concentrations not showing hemolytic effect showed their preventive effect. In conclusion, these results strengthen the scientific basis and justify the use of *Centaurea calcitrapa* in the traditional treatment and prevention of pathologies related to oxidative stress.

Keywords: *Centaurea calcitrapa*, Secondary metabolites, Biomembranes, Interactions of phenolic compounds – biomembranes

Resumé

Les feuilles et les épines de *Centaurea calcitrapa* sont connues pour leur utilisation en médecine traditionnelle, et plus particulièrement pour le traitement des pathologies liées au stress oxydatif. Ce travail vise à tester l'effet protecteur de différentes concentrations d'extraits éthanoiques de ces parties de la plante contre l'hémolyse induite par H_2O_2 (8 mM) et AAPH (200 mM), réalisé in vitro sur un modèle membranaire d'érythrocytes humains. De plus, la toxicité de ces extraits a été étudiée et leurs teneurs en phénols totaux, flavonoïdes et tanins ont été estimées par dosages colorimétriques. Les résultats démontrent que les extraits éthanoiques sont riches en phénols totaux, puis en tanins condensés, flavonoïdes et tanins hydrolysables. Ils ont montré un effet toxique à fortes concentrations sur les membranes érythrocytaires. De plus, les concentrations ne montrant pas d'effet hémolytique ont montré leur effet préventif. En conclusion, ces résultats renforcent les bases scientifiques et justifient l'utilisation de *Centaurea calcitrapa* dans le traitement et la prévention traditionnels des pathologies liées au stress oxydatif.

Mots-clés : *Centaurea calcitrapa*, Métabolites secondaires, Biomembranes, Interactions composés phénoliques – biomembranes.

الملخص

علاج في وتحديدًا ،التقليدي الطب في باستخدامها (Centaurea calcitrapa) الكالسيترابا القنطريون نبات وأشواك أوراق تُعرف لهذه الإيثانوية المستخلصات من مختلفة لتركيزات الوقائي التأثير اختبار إلى البحث هذا يهدف .التأكسدي بالإجهاد المرتبطة الأمراض نموذج على المختبر في أُجري والذي ،(مولار ملي 200) AAPHو (مولار ملي 8) H2O2 عن الناتج الدم انحلال ضد النبات من الأجزاء الكلية الفينولات من محتواها وقُدِّر ،المستخلصات هذه سمية دُرست ،ذلك على علاوة .البشرية الحمراء الدم لكريات غشائي من عالية نسبة على تحتوي الإيثانوية المستخلصات أن النتائج أظهرت .اللون قياس فحوصات باستخدام والعفصات والفلافونويدات تركيزاتها عند سامًا تأثيرًا أظهرت وقد .المائي للتحلل القابلة العفصات ثم ،الفلافونويدات ثم ،المكثفة العفصات تليها ،الكلية الفينولات تُعزز ،الختام في .وقائيًا تأثيرًا انحلاليًا تأثيرًا تُظهر لا التي التركيزات أظهرت ،ذلك إلى بالإضافة .الحمراء الدم كريات أغشية على العالية بالإجهاد المرتبطة الأمراض من والوقاية التقليدي العلاج في كالسيترابا سنتوريا نبات استخدام وتُبرر العلمي الأساس النتائج هذه .التأكسدي

.الحيوية الأغشية مع الفينولية المركبات تفاعلات ،الحيوية الأغشية ،الثانوية المستقلبات ،كالسيترابا سنتوريا :المفتاحية الكلمات