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Title

Study of the influence of the geographical variation and the mode of preparation on some pharmacological properties of Juniper *Juniperus oxycedrus* in Algeria

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Introduction

Introduction

Medicinal plants have played a crucial role in medicine and pharmacology for many years. It is estimated that approximately 80% of the global population relies on botanical preparations as medicines to address their health needs (Ogbera et al. 2010). Currently, a significant focus of research lies in studying bioactive molecules of plant origin, such as vitamins, carotenoids, and polyphenols, intending to replace synthetic alternatives due to potential associated toxicological risks (Athamena et al. 2010). Secondary metabolites, being natural compounds widely distributed in the plant kingdom, have gained increasing importance due to their beneficial effects on health (Koechlin-ramonatxo et al. 2006).

Throughout centuries, various species of juniper have been used in traditional medicine as diuretics, remedies for indigestion, and as a source of tar (Medini et al. 2009). *Juniperus oxycedrus* L. is a plant that has caught the interest of the pharmaceutical and food industries in recent years due to its potential as a source of secondary metabolites with various pharmacological effects. Phytochemical research conducted on Juniperus has demonstrated a diverse array of these compounds, such as terpenoids, flavonoids, and alkaloids, that may have potential therapeutic benefits (El-Tantawy et al. 2019; Goudali et al. 2020; Hazzit et al. 2018). Further studies have explored the potential health benefits of *J. oxycedrus*, including its antioxidant, anti-inflammatory, and antiproliferative properties, among others (Goudali et al. 2020; Tavakoli et al. 2017). As such, it can be considered a promising candidate for the development of novel drugs and functional foods.

In this context, the main objective of this work is the valorization of the natural biological resources, mainly medicinal plants of Algeria, and to collect the maximum of information in order to obtain a better knowledge on the medicinal flora. The specific aim of the present study is to evaluate some pharmacological properties of infusion and maceration extracts of needles of *J. oxycedrus* belonging from two contrasting regions namely, Harmla and Ouled Boughedou (Tiaret, Algeria). Therefore, secondary metabolites have been quantified and the antioxidant, anti-inflammatory, hemolytic, and anti-hemolytic activities have been evaluated.

Literature review

Literature review

The use of traditional medicine has been documented in various parts of the world and dates back to ancient times (WHO 2021). Traditional medicines encompass a range of methodologies and approaches, including herbal remedies, massage, acupuncture, and dietary interventions, among others (Bodeker 2015). Despite some challenges in their scientific validation and efficacy, traditional practices continue to play an important role in preserving health and treating a wide range of conditions in many communities globally (NCCIH 2021). It is deeply rooted in the cultural beliefs and experiences of diverse populations and is often closely tied to spiritual and ritual practices (WHO 2019).

1. Description of *Juniperus oxycedrus* L.

Juniperus oxycedrus L. is a species of evergreen shrub or small tree belonging to the family Cupressaceae. It is commonly known as the prickly juniper, sharp cedar, or cade juniper. It can grow up to 10 meters tall with a dense, conical, or columnar crown, and a reddish-brown bark with flaky plates (Pardo-de-Santayana et al. 2007).

The leaves of this plant are needle-like and arranged in whorls of three, and they are about 4-12 mm long and sharp-pointed. The male and female cones are usually produced on separate trees. The male cone is small, round, and yellow, while the female cone is larger, bluish-green, and rounded to oblong in shape. The fruit is a small, dark blue-purple fleshy berry that contains one to three seeds (Arli et al. 2016).

Figure 1. *Juniperus oxycedrus* L. 1753

J. oxycedrus L. is characterized by its strong and spicy fragrance due to the presence of essential oils. This plant has diverse uses in traditional medicine, as a flavoring agent, and in various industries for its wood and oil. In particular, the oil extracted from cedarwood has been used in perfumery, aromatherapy, and as a natural insect repellent (Demirci et al. 2017).

2. Taxonomy

According to the APG (Angiosperm Phylogeny Group 2016), the juniper plant, scientifically known as *Juniperus oxycedrus*, belongs to the Clade Gymnospermae within the Pinidae subclass. It is classified under the Cupressales order and the Cupressaceae family. The genus name for this plant is Juniperus, while the specific species is *Juniperus oxycedrus*. It has a subspecies known as *Juniperus oxycedrus* subsp. *Oxycedrus*. In the Arabic language, it is referred to as aaraar or taga (العرعر، التق), while in Berber, it is called taqqa, taka, tiqqi, or teki. In English, it is commonly known as prickly cedar, medlar tree, juniper bush, juniper bark, juniper berry, or sharp cedar. In French, popular names for this plant include cade, genévrier oxycèdre, oxycèdre, or cadier.

3. Geographical distribution

J. oxycedrus is distributed throughout the Mediterranean region in Europe, Asia, and North Africa. It is found on hills, slopes, and mountains, with a preference for calcareous soils, at altitudes of up to 2000 m. The species' range extends from Portugal in the west to Cyprus in the east and from Turkey in the north to Morocco in the south. In addition, *J. oxycedrus* is also found in the Canary Islands, Madeira, Azores, and Cape Verde. The species is a vital source of food for wildlife, and its wood and oil extracts are used in various industries. However, several factors, including habitat fragmentation, fire, disease, and overgrazing, have caused a decline in the species populations. Therefore, conservation efforts are needed to preserve this important species (Atici et al. 2010).

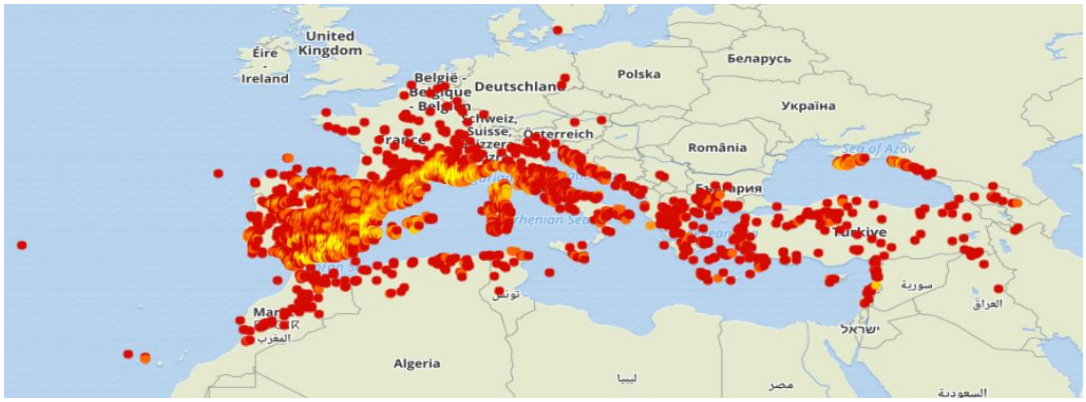


Figure 2. Global Presence of *Juniperus oxycedrus* L. (Accessed via Global Biodiversity Information Facility "GBIF" 2023)

In Algeria, *J. oxycedrus* occupies several bioclimatic zones, including the humid and the hyperarid regions. According to the Flora of Algeria, this species is found in the mountainous areas of the country, including the Atlas Mountains, the Aurès Mountains, and the Kabylie Mountains. The presence of *Juniperus oxycedrus* L. in the semi-arid and sub-humid climates of the Northeastern High Plateaus in Algeria (Kacem et al. 2010).

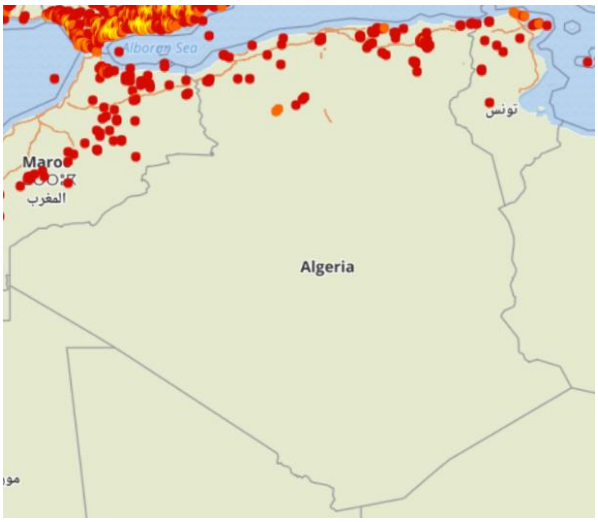


Figure 3. Presence of *Juniperus oxycedrus* L. in Algeria (Accessed via Global Biodiversity Information Facility "GBIF" 2023)

4. Chemotaxonomy

After the identification of *Juniperus oxycedrus* L. essential oil, 42 compounds were founded. The chemical composition of the plant oil was dominated by monoterpene hydrocarbons, oxygenated sesquiterpenes, and sesquiterpene hydrocarbons. While the monoterpene hydrocarbons and diterpenes were present in low percentages. Like the essential oil from other Juniperus species, the major constituents of the oil were α-pinene, manoyl oxide, Z-caryophyllene, δ-3-carene, geranyl acetone, and caryophyllene oxide. There may be differences in the oil composition and yield may be due to several factors such as time of collection, and geographic and climatic conditions (Amri Ismail et al. 2013)

5. Phytochemistry

According to Negreche et al. (2019), the phytochemistry analysis of *Juniperus oxycedrus* reveals several findings. The plant exhibits a significant presence of tannins in large quantities, along with a moderate quantity of terpenes. However, it shows low levels of alkaloids and free flavonoids. Notably, Anthocyanin and Saponosides are found to be absent in this plant species.

6. Toxicity

The primary source of toxicity associated with *Juniperus oxycedrus* is its essential oil, known as cade oil. Cade oil contains various compounds, including thujone and cadinene, which can be toxic when ingested or used improperly. Thujone, a neurotoxin found in high concentrations in *Juniperus oxycedrus* oil (Adams 2012), can have adverse effects on the central nervous system. Ingesting significant amounts of the oil or its products can lead to symptoms such as abdominal pain, nausea, vomiting, diarrhea, dizziness, confusion, convulsions, and potential organ damage. It is important to note that these toxic effects are generally observed with high doses or prolonged exposure. Additionally, direct and undiluted contact with the skin can cause irritation, redness, and allergic reactions in some individuals (Duke 2002). To ensure safety, it is advisable to consult with healthcare professionals or trained aromatherapists before using any products derived from Juniperus oxycedrus, especially if you have specific health conditions or concerns (Blumenthal 2000).

7. Secondary metabolites

Secondary metabolites are diverse plant chemicals that are not essential for growth but play important ecological roles as defenses against herbivores, pathogens, or competitors. These metabolites can vary in distribution, chemical class, and function. They often have complex structures and can accumulate in specific tissues or organs, making them valuable sources of bioactive for various applications, such as drugs, fragrances, or food additives (Macheix et al. 2005).

7.1. Polyphenols

Phenolic compounds, which are secondary metabolites in plants, share a common characteristic of containing one or more benzene rings with one or more hydroxyl groups attached to them. The structure of natural phenolic compounds varies across a wide range, from simple molecules such as simple phenolic acids to highly polymerized molecules like condensed tannins (Urquiaga et Leighton 2000). These compounds serve as the foundation for the active principles found in medicinal plants, albeit their production can be challenging. In humans, these trace compounds play a significant role by directly influencing the nutritional quality of fruits and vegetables and their impact on consumer health. They exhibit various effects, including antioxidant properties and a protective role against certain types of cancers, among others. (Macheix et al. 2005).

Phenolic compounds are characterized by the presence of both a phenol function and an acidic group, and they can be divided into two subclasses: hydroxycinnamic acids and

hydroxybenzoic acids. These compounds possess intriguing biological properties, including anti-inflammatory, antiseptic, immunostimulatory (Hennebelle et al. 2004), and antioxidant effects (Bossokpi 2002). Among them, caffeic acid has received extensive pharmacological attention and has demonstrated remarkable efficacy against viruses, bacteria, and fungi (Cowan 1999). Gallic acid, on the other hand, has shown the potential in reducing the viability of lung cancer cells in vitro in mice. Furthermore, combining gallic acid with anti-cancer medications like cisplatin holds promise as a potentially effective treatment for this type of cancer (Rangkadilok et al. 2007).

7.2. Flavonoids

The group of phenolic compounds is highly representative and encompasses a wide range of chemical structures with distinct characteristics. These molecules are abundantly present in plant fruits, leaves, and seeds (Tsimogiannins 2006), and are commonly recognized as nearly universal plant pigments that play crucial roles in photosynthesis (Mukohata et al. 1978), gene regulation, and growth metabolism (Havsteen 2002). Currently, there is knowledge of approximately 4000 flavonoid compounds (Edenharder 2003), all sharing a fundamental structure composed of fifteen carbon atoms arranged in a phenyl-2-benzopyran configuration of C_6-C_3-C_6 (Yao et al. 2004).

7.3. Tannins

The term "tannins" refers to a group of polymeric phenolic substances with a molecular mass ranging from 500 to 3000. These substances demonstrate typical phenol reactions and have a unique ability to precipitate alkaloids, gelatin, and other proteins (Haslam 1996; Cowan 1999). Tannins are commonly found in various plant parts such as bark, wood, leaves, fruits, and roots, and are known for their astringent taste (Scalbert 1991). There are two distinct types of tannins, namely hydrolyzable tannins and condensed tannins, which can be distinguished based on their structure and biogenetic origin.

Plants rich in tannins have been utilized for different purposes, including the treatment of colds, sore throats, excessive secretions, internal or external infections, wounds, cuts, and burns (Bruneton 1999). Moreover, tannins exhibit remarkable antibacterial properties (Mahamat et al. 1995), antiviral effects (Nonaka et al. 1990), and anti-inflammatory properties (Mota al. 1985).

7.4. Alkaloids

An alkaloid, primarily derived from natural sources such as plants, is an organic compound that contains nitrogen and exhibits significant pharmacological properties (Padrimi et al. 2003).

Alcohols are present in all parts of a plant, with varying concentrations in leaves, seeds, bark, or rhizomes (Verdrager 1978). Alcohols tend to retain their efficacy when plants are dried and can contribute to the toxic effects of certain medications. Although only a small number of alkaloids have an impact on the heart, some are employed to regulate blood pressure due to their physiological effects on the central nervous system, which influences circulation and respiration. Depending on their properties, alkaloids can act as depressants or stimulants on the nervous system, and they may also possess anti-spasmodic, mydriatic, and narcotic properties. Furthermore, they find use as appetite stimulants, (Schauenberg 2005). Additionally, also their applications as analgesics, antitussives, and laxatives (Grunwald 2006).

7.5. Terpenoids

Terpenes, which are organic compounds found in plants, form a vast and diverse group comprising approximately 15,000 identified molecular structures. Their exceptional diversity arises from the numerous building blocks they consist of and the various ways in which they are assembled. Terpenes can be categorized into specific groups based on the number of carbon atoms they possess monoterpenes (10 carbon atoms), sesquiterpenes (15 carbon atoms), diterpenes (20 carbon atoms), triterpenes (30 carbon atoms), and tetraterpenes (40 carbon atoms) (Belbache 2008).

8. Traditional medicine and local knowledge

This plant has a long history of use in traditional medicine for treating various ailments including hyperglycemia, obesity, tuberculosis, bronchitis, and pneumonia (Swanston-flatt et al. 1990; Sanchez et al. 1994). It is prepared as a decoction to address gastric disorders and is also utilized as an oral analgesic (Fernandez et al. 1996). Furthermore, this plant genus was considered a panacea, and its fumigations were recognized for their disinfectant properties, commonly employed in the streets to combat outbreaks of diseases such as plague and cholera. "Vin de genièvre" (juniper wine) was believed to possess diuretic properties. In the field of human dermatology, it is used as an antiseptic and parasiticide, applied in the form of an ointment to treat chronic eczema and various skin conditions, including scabies. Presently, the essential oil derived from this plant is also recommended for addressing scalp conditions and as a vermifuge for domestic animals (Becker et al. 1982; Bouhlal et al. 1988; Tavares et al. 2012).

9. Biological activities

9.1. Antioxidant activity

Protection against deleterious effects induced by reactive oxygen species (ROS) is attained through various mechanisms, including non-enzymatic proteins, enzymes such as superoxide dismutases (SODs) and glutathione peroxidases (GPx), as well as dietary antioxidants such as carotenoids, tocopherols (vitamin E), ascorbic acid (vitamin C), and polyphenols (Ferreira et al. 2019; Goyal et al. 2020). Non-enzymatic proteins such as transferrin, ceruloplasmin, and ferritin function as ROS scavengers. Enzymes like SODs, GPx, and catalase (CAT) play a crucial role in mitigating oxidative stress in living organisms (Valko et al. 2005). Antioxidants such as arotenoids (e.g., beta-carotene, lycopene, and lutein), tocopherols (vitamin E), ascorbic acid (vitamin C), and polyphenols (e.g., resveratrol, quercetin, and catechins) are essential antioxidants for humans as they play a crucial role in the scavenging of ROS and reducing oxidative stress-related damage (Kapoor et al. 2018; Rothwell et al. 2019). Therefore, by consuming a balanced diet enriched with an adequate amount of these essential antioxidants, we can combat the adverse effects of oxidative stress on our bodies.

The use of antioxidants is widespread in primary and secondary prevention. Commonly recognized antioxidants, such as β-carotene, ascorbic acid, anthocyanins, polyphenols, and flavonoids, are widely used in this regard (Bjelakoic et al. 2007). Flavonoids can trap free radicals generated by our body in response to environmental aggressions, which promote cellular aging (Karbin et al. 2015).

9.2. Anti-inflammatory activity

Anti-inflammatory substances are diverse compounds that aim to reduce prolonged inflammatory responses. They can be categorized into three classes: steroidal anti-inflammatories, non-steroidal anti-inflammatories, and plant-derived anti-inflammatories (Mishra and Palanivelu 2008).

9.2.1. Steroidal anti-inflammatories

Steroidal anti-inflammatories (SAIs) form a large group of medications derived from cortisol; the primary glucocorticoid hormone produced by the adrenal glands. Glucocorticoids are derived from cholesterol under the influence of the adrenocorticotropic hormone (ACTH) secreted by the pituitary gland. SAIs have various biological effects, particularly on inflammation. They can suppress the expression of pro-inflammatory genes, induce the expression of anti-inflammatory genes, or inhibit the production of prostaglandins (Weill et al. 2003).

9.2.2. Non-steroidal anti-inflammatories

Non-steroidal anti-inflammatories (NSAIDs) work by preventing the formation of prostaglandins, which are responsible for triggering inflammation. They are effective in alleviating pain and reducing fever. However, their anti-inflammatory properties are most prominent at higher doses, making NSAIDs one of the most widely used therapeutic classes globally. They exhibit strong binding affinity to plasma proteins and distribute widely in various tissues. The primary role of NSAIDs is to inhibit cyclooxygenase, an enzyme involved in the production of prostaglandins from arachidonic acid. (Krzesinski et al. 2002).

The use of NSAIDs and SAIs in medical prescriptions is increasingly prevalent due to their effectiveness in managing pain. Nevertheless, this therapeutic class can lead to various side effects. Hence, it is advantageous to explore traditional medicine based on medicinal plants to harness the potential of bioactive compounds, particularly phenolic compounds known for their anti-inflammatory effects. The subsequent table illustrates the anti-inflammatory effects of selected medicinal plants (Barnes 1998).

9.2.3. Hemolysis

Hemolysis is an irreversible physiological process that results in the rupture of red blood cell membranes, leading to the release of intra-erythrocytic components into the plasma, particularly hemoglobin. This phenomenon can be visually identified by a pink-to-red coloration in the sample after centrifugation or by measuring the optical density of the supernatant (hemoglobin) using spectrophotometry (Mezzou et al. 2006). Hemolysis is characterized by increased levels of hemoglobin, lactate dehydrogenase (LDH), phosphate, and creatine kinase (CK) in the serum. (Ali et al. 2014). It is also associated with decreased levels of haptoglobin and glycosylated hemoglobin. The liberated hemoglobin during hemolysis is either broken down into unconjugated bilirubin or forms a complex with haptoglobin, which is rapidly cleared by the liver (Marchand et al. 1980).

9.2.4. Anti-hemolytic activity

Anti-hemolytic activity is a term used to describe the capacity of a substance to protect red blood cells from hemolysis under stressful conditions. Hemolysis is a common problem encountered in various pathological and clinical situations such as sepsis, inflammation, renal dysfunction, and oxidant stress. The present literature offers a vast array of natural and synthetic agents that could protect against hemolysis through their antioxidant, anti-inflammatory, anti-apoptotic, and membrane-stabilizing activities. For example, Cicek et al. (2015) demonstrated the anti-hemolytic activity of natural and synthetic antioxidants attributed to their antioxidant

properties. Moreover, Frijhoff et al. (2015) highlighted the importance of oxidative stress biomarkers in various clinical conditions. Anti-apoptotic activity of certain substances was also investigated for their potential in protecting erythrocytes against hemolysis. Ghaffarloo et al. (2014) reported that quercetin prevented hemolysis of erythrocytes and modulated the apoptotic pathway *in vitro*. Furthermore, Memariani et al. (2012) studied the erythrocyte membrane stabilizing properties of plant extracts *in vitro*. Finally, the deficiency or imbalance of certain ions, such as magnesium and calcium, in the red blood cells may lead to hemolysis. Ozcan and Halmagyi (2010) reported on the calcium and magnesium contents of various foods and herbal infusions and their impact on erythrocyte stability. Overall, anti-hemolytic substances play a vital role in preventing hemolysis and reducing the risk of clinical complications.

Material and methods

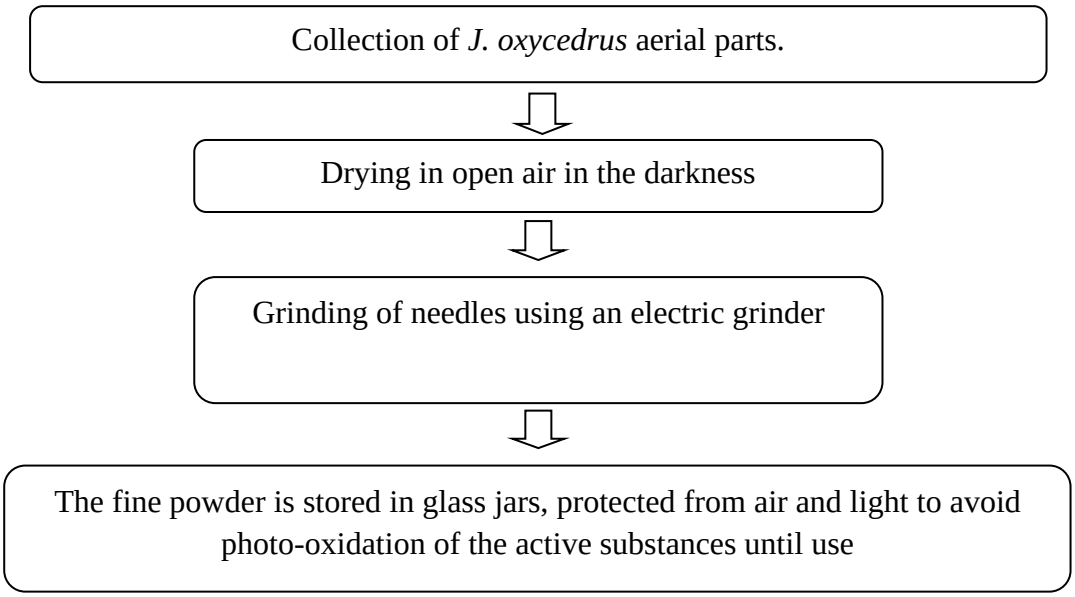
Material and methods

1. Plant material

The medicinal plant *Juniperus oxycedrus* L. was harvested from two different regions of contrasting environmental conditions in Tiaret (Algeria) namely Harmla (from the southern arid part) and Ouled Boughedou (from the northern mountainous part) during January 2023.

2. Preparation of plant powder

After the drying of aerial plant parts for one week in the darkness at room temperature, the samples were grinded using an electric grinder to obtain a fine powder. The obtained powders were put in closed bottles, labeled, and kept at room temperature until the moment of extraction (Fig. 4).



FiguVVre 3. Preparation of the plant powder.

Figure 04. Preparation and conservation methods of the plant powder of Juniper.

3. Preparation of herbal teas

Herbal teas of *J. oxycedrus* were prepared according to the traditional use reported by the local population, hence, infusion and maceration were prepared and tested throughout this study.

3.1. Infusion

The infusion method involves submerging the plant parts in boiling water, which is particularly effective for delicate plant parts and those containing abundant essential oils such as *J. oxycedrus*. The duration of the infusion process varies depending on the specific plant, ranging from a few minutes to an hour. In our case, 5 g of the powder of the aerial parts (mainly needles) have been mixed with 100 mL of distilled water and stirred for 15 minutes in the darkness at ambient temperature.

3.2. Maceration

Maceration (solid-liquid extraction method) is the process of contacting the grinded powder with water as a solvent to extract the active ingredients. The extraction was carried out at room temperature by introducing 5 g of powder into 100 mL of distilled water in a shaded beaker protected with aluminum foil to avoid any degradation of the molecules by light and were kept macerated for 48 hours at room temperature. The duration of maceration may vary depending on the inherent characteristics of the plant, but it is important to avoid excessively prolonged maceration with water to prevent any potential risks of fermentation.

4. Evaluation of the biological activities of herbal teas

4.1. Hemolytic activity

The hemolytic activity was determined according to the method described by Haddouchi et al. (2016). Briefly, 1 mL of red blood cell suspension was mixed with 1 mL of extracts at different concentrations prepared in PBS (0.625, 1.25, 2.5, 5 mg/mL). The mixture was then incubated at 37°C for 30 minutes, followed by centrifugation at 1500 rpm for 10 minutes. The resulting supernatant was measured at a wavelength of 540 nm. Distilled water and PBS were used under the same conditions for the positive and negative controls. Each experiment was replicated three times. The percentage of hemolysis was calculated according to the following equation:

$$\text{Hemolysis (\%)} = (AT - AN / AC - AN) \times 100$$

With **AT:** absorbance of the tested extract. **AN:** absorbance of the negative control (PBS).

AC: absorbance of the positive control (distilled water).

Figure 05. Hemolytic activity samples before incubation.

4.2. Anti-hemolytic activity

The anti-hemolytic activity was assessed using a total of 10 milliliters of blood collected from healthy individuals with blood group O positive. The blood was collected in tubes containing vials with 10% EDTA and then centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded, and the resulting pellet was washed three times with 0.2M PBS (pH 7.4). After each wash, centrifugation was performed at 3000 rpm for 10 minutes, followed by resuspension of the pellet in a saline solution of 0.9% NaCl. To initiate the assay, 0.8 mL of various extracts (0.25, 0.50, 0.75, 1, 1.25, 2.5, and 5 mg/mL in PBS) were mixed with 0.8 mL of an erythrocyte suspension and incubated at 37°C for 5 minutes. Subsequently, 0.4 mL of H_2O_2 solution (0.82 M in PBS) was added to induce oxidative degeneration of lipid membranes. After incubating the samples for 3 hours at a temperature of 37°C and a speed of 120 rpm, the samples were centrifuged at 3000 rpm for 10 minutes, and the supernatant was measured using a UV-Vis spectrophotometer at 540 nm. Following that, 0.2 mL of H_2O_2 solution (0.82 M in PBS) was added to induce oxidative denaturation of lipid membranes. The samples were incubated for an additional 3 hours at 37°C and 120 rpm. After incubation, the samples were centrifuged at 3000 rpm for 10 minutes, and the supernatant was measured using a UV-Vis spectrophotometer at 540 nm.

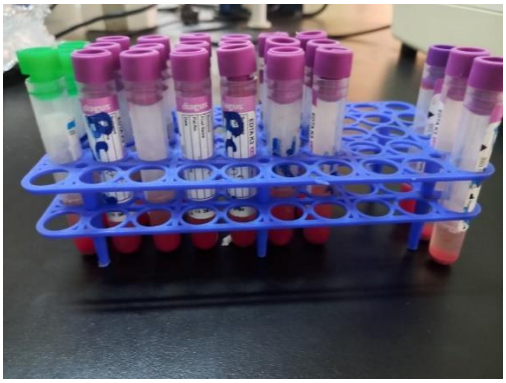


Figure 06. Anti-hemolytic activity samples after incubation.

4.3. *In vitro* anti-inflammatory activity

The anti-inflammatory activity was determined *in vitro* by the thermal denaturation of BSA (Kandikattu et al. 2013). A volume of 1 mL of each extract was mixed with 1 mL of Bovine Serum Albumin solution (0.2%) prepared in Tris-HCl buffer (50 mM, pH 6.6). The mixture was allowed to stand for 15 min at 37° and then heated in bath water at 72 °C for 5 min. The absorbance was recorded at 660 nm using a UV-visible spectrophotometer after cooling to room temperature. The experiment was performed in triplicate.

Diclofenac sodium was used as a standard. The protective effect of samples against the denaturation of BSA was presented as inhibition percentages calculated using the formula:

$$\text{Inhibition (\%)} = [(\text{Abs control} - \text{Abs sample}) / \text{Abs control}] \times 100$$

4.4. Antioxidant activity using DPPH assay

The measurement of free radical scavenging activity involved assessing the reduction in absorbance of a methanol solution containing DPPH. Specifically, 200 µL of the sample was combined with 1 mL of a methanol solution containing DPPH (20 mg/L). After 30 minutes, the absorbance was determined at 517 nm. The DPPH free radical scavenging activity was determined by referencing a calibration curve, and the outcomes were expressed as milligrams of ascorbic acid equivalent (AAEq) per gram of extract (Kumar et al. 2007).

The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{IC (\%)} = [(\text{Abs Control} - \text{Abs Sample})]/ (\text{Abs Control})] \times 100$$

Where;

Abs Control is the absorbance of DPPH radical methanol,

Abs Sample is the absorbance of DPPH radical + sample extract/standard.

5. Secondary metabolites

5.1. Polyphenols

The total phenolic content was determined by the Folin-Ciocalteu method as described by Singleton et al. (1999). The aqueous extract (1 mL) was mixed with 5 mL of Folin-Ciocalteu reagent previously diluted 10-fold with distilled water. After 1 min, 15 mL of Na_2CO_3 (20%) was added to the mixture, and the volume was adjusted to 10 mL with distilled water. The solution was kept for 2 hours of incubation in the dark at room temperature. The absorbance was then measured at 760 nm. The total polyphenol content is calculated from the calibration curve and the results are expressed as mg gallic acid equivalent (GAE)/g dry weight of the extracts.

5.2. Flavonoid

Total flavonoid content was determined following the method described by Acharya et al. (2015). Briefly, 1 mL of the aqueous extract was added to 1 mL of AlCl3 (2%). After 15 min, the absorbance was read at 430 nm. Total flavonoid content is calculated from the calibration curve and the results are expressed as mg quercetin equivalent (QE) /g dry weight of the extracts.

5.3. Tannins

The quantification of tannins was carried out according to the method of Ouerghemmi et al. (2017). An aliquot (50 mL) of extracts was mixed with 3 ml of 4% methanol vanillin solution and 1.5 mL of H_2SO_4. After 15 min, the absorbance was measured at 500 nm. Tannin contents were expressed as mg cyanidin equivalents (CE)/ g dry weight through the calibration curve.

Results

Results

The biological activities and secondary metabolites content of needles of *Juniperus oxycedrus* collected from two different regions in Tiaret (Algeria) namely Harmla and Ouled Boughedou have been investigated throughout this study.

1. Hemolytic activity

Overall, infusion of *J. oxycedrus* has a high hemolytic effect in comparison to maceration. In addition, the highest hemolytic effect was observed in samples treated with the infusion of Harmla provenance. On the opposite, the highest hemolytic effect was observed in samples treated with maceration of Ouled Boughedou provenance.

The effect of both methods of extraction acts in a dose-dependent manner; the increase of the concentration of the extract in the medium leads to an increase in hemolytic activity.

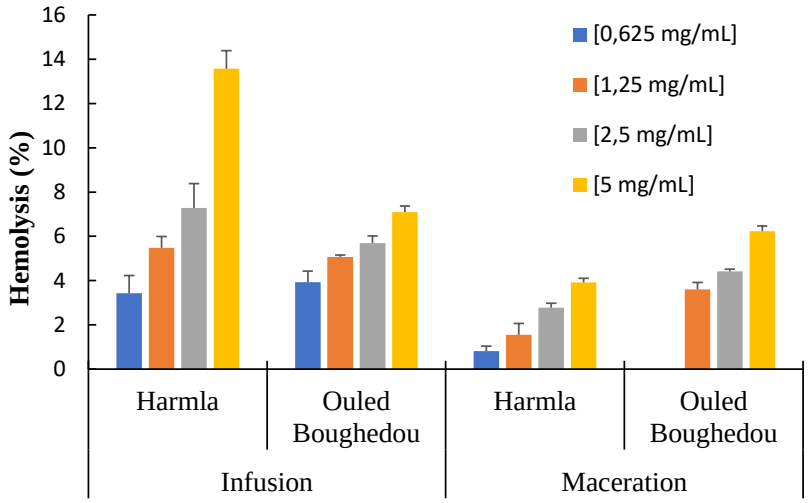


Figure 7. Evolution of the hemolysis rate as a function of the concentration of plant extracts.

When samples were subjected to 0.625 mg/mL, hemolysis was very low for both populations, recording 3.4% for Harmla and 3.9% for Ouled Boughedou. However, when the concentration of the extract rises to 5 mg/mL, the infusion from both populations induced a high hemolytic effect of 13.6% for Harmla and 7.1% for Ouled Boughedou (Fig. 7).

2. Anti-hemolytic activity

The antihemolytic effects of the infusion were significantly higher than that of maceration for both provenances. In addition, the extract of the population Ouled Boughedou exhibits a higher antihemolytic effect than the extract of Harmla for both extraction methods.

By the same, the antihemolytic activity of both extracts increases when the concentrations of the plant extract increase in the reactional medium.

In general, the extract of Harmla manifested higher antihemolytic power under the concentrations 0.25 mg/mL to 1.25 mg/mL for both provenances and extraction methods. However, when the concentration of the extracts exceeds 2.5 mg/mL, Ouled Boughedou's provenance revealed higher antihemolytic potential.

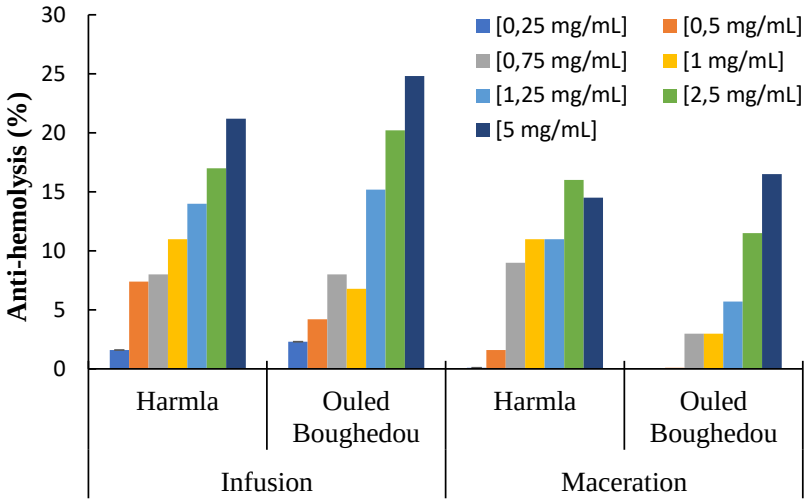


Figure 8. Evolution of anti-hemolytic activity as a function of the concentration of extracts.

For a concentration of 0.25 mg/mL, Harmla exhibited an anti-hemolytic activity of 1.6%, while Ouled Boughedou showed a slightly higher activity of 2.3%. Both provenances displayed low anti-hemolytic activity under this concentration. When the concentration doubled to 0.5 mg/mL, Ouled Boughedou extract demonstrated higher antihemolytic activity than Harmla extract, with 7.4% and 4.2% respectively. When the concentration of the extracts passes to 0.75 mg/mL, both provenances exhibited comparable activity around 8%.

Similar results have been observed under 1 mg/mL, 1.25 mg/mL, and 2.5 mg/mL. regarding the concentration of 5 mg/mL, both provenances exhibited higher antihemolytic power of 14.5% and 24.8% in Harmla and Ouled Boughedou respectively (Fig. 8).

3. Anti-inflammatory activity

The anti-inflammatory activity was higher in the maceration extract. It demonstrated a concomitant increase when the concentration of the extract increased in the reactional medium.

Under the concentration of 0.625 mg/mL, Harmla provenance exhibited respective anti-inflammatory rates of 78% and 86% in the infusion and maceration extracts, while Ouled Boughedou showed only 66% and 78% (Fig. 9).

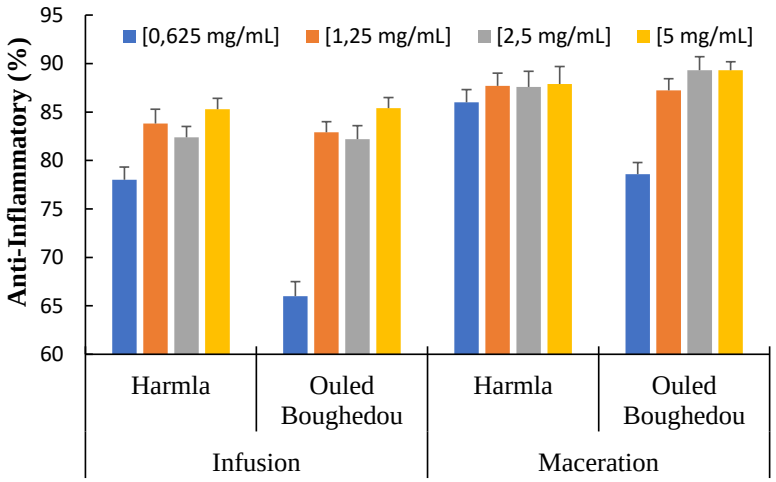


Figure 09. Variation of anti-inflammatory activity between infusion and maceration of two origins of *Juniperus oxycedrus* L.

The anti-inflammatory rate was around 83% for both provenances in the infusion extract at the concentration of 1.25 mg/mL and 87% in the maceration extract. When the concentration of the extract rises to 2.5 mg/mL in the infusion, both provenances showed high anti-inflammatory activity around 82.2% while it increases to 89% in the maceration extract. At the concentration of 5 mg/mL, both provenances showed 85% anti-inflammatory activity in the infusion extract and 88% in the maceration extract (Fig. 9).

4. Antioxidant activity

For both types of extraction, Harmla provenance exhibited higher DPPH IC_{50} than Ouled Boughedou provenance. It was around 1.14 mg/mL in the infusion extract for Harmla against 0.672 mg/mL for Ouled Boughedou.

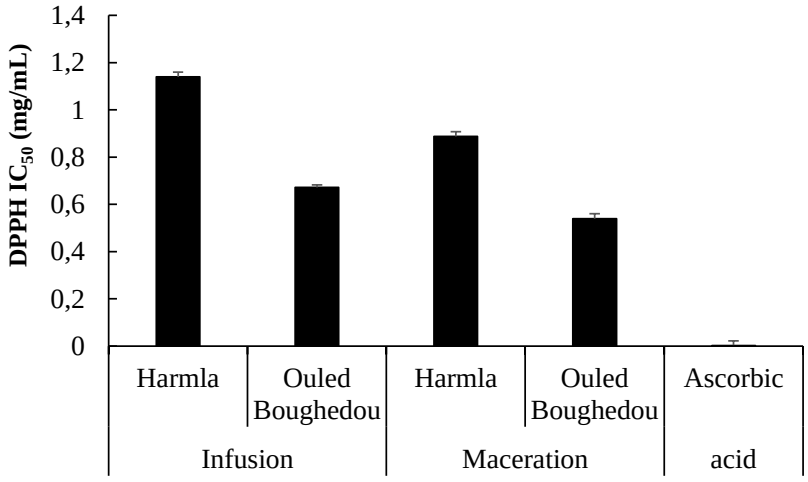


Figure 10. Variation of antioxidant activity (DPPH IC50) between infusion and maceration of two origins of *Juniperus oxycedrus* L.

For maceration, Harmla's provenance showed DPPH IC_{50} of 0.88 mg/mL while Ouled Boughedou's provenance showed 0.54 mg/mL (Fig. 10). The obtained values remain higher when compared with ascorbic acid which exhibited a DPPH IC_{50} of 0.0025 mg/mL.

5. Phytochemical characterization

5.1. Phenolic compounds

Polyphenol contents are higher in infusion extract compared to maceration extract. The highest content is recorded in the infusion of the provenance Harmla (261±18 µg eAG/mg extract), which was slightly higher than the same extract of Ouled Boughedou provenance (257±4.7 µg eAG/mg extract).

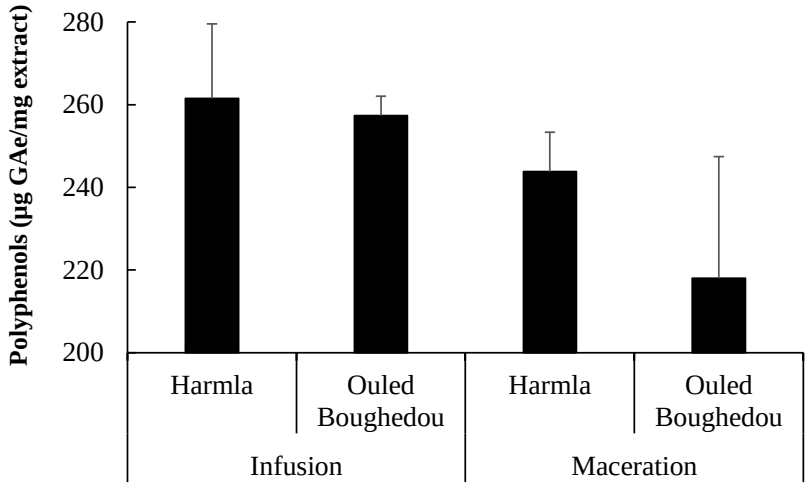


Figure 11. Variation of polyphenol contents between infusion and maceration of two origins of *Juniperus oxycedrus L.*

The same observation has been made in the maceration extract; Harmla provenance exhibited 243.8±9.5 µg eAG/mg extract while Ouled Boughedou provenance exhibited 217.9±29.5 µg eAG/mg extract (Fig. 11).

5.2. Flavonoids

The flavonoids content in the infusion extract was higher in Harmla provenance which exhibited high flavonoids content of 34.92 µg Qe/mg extract, which is significantly higher than that of Ouled Boughedou with an average content of 16.25 µg Ce/mg extract.

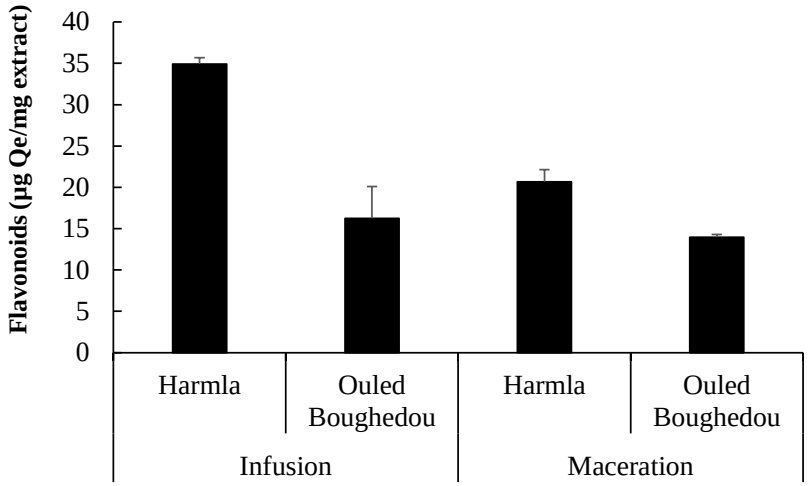


Figure 12. Variation of flavonoid contents between infusion and maceration of two origins of *Juniperus oxycedrus* L.

By the same, Harmla provenance demonstrated high flavonoids content of 20.67 µg Ce/mg extract in the maceration extract, while Ouled Boughedou provenance showed a low content of 13.96 µg Ce/mg extract (Fig. 12).

5.3. Tannins

In general, the content of tannins was higher in the maceration extract in comparison to the infusion. In infusion extract, Harmla provenance exhibited a higher tannins content of 108.46 µg Ce/mg extract, than Ouled Boughedou provenance (69.89 µg Ce/mg extract).

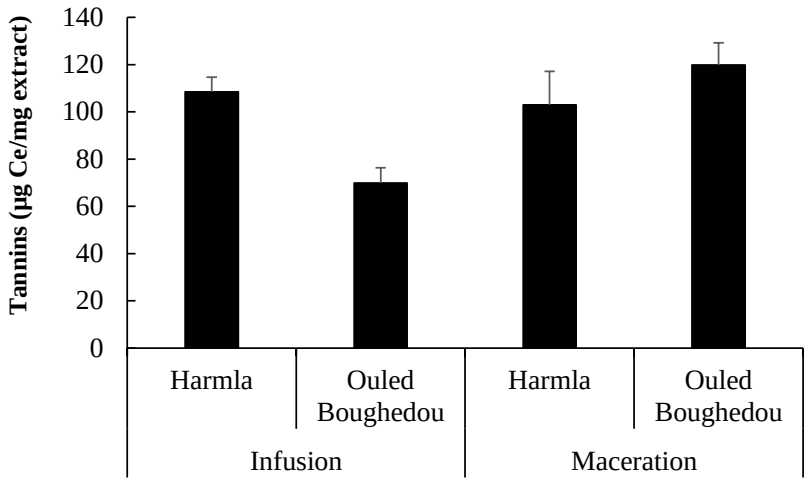


Figure 13. Variation of tannin content between infusion and maceration of two origins of *Juniperus oxycedrus* L.

For maceration, Ouled Boughedou provenance exhibited higher content with an average of 119.89 µg Ce/mg extract than Harmla provenance (102.90 µg Ce/mg extract) (Fig. 13).

Discussion

Discussion

Medicinal plants have been integrated in healthcare for centuries, with a significant proportion of the global population relying on botanical preparations for their health needs (Ghorbani 2017). The increasing interest in natural compounds has led to a focus on studying the therapeutic potential of plant-derived molecules (Pandey et al. 2009). *Juniperus oxycedrus*, a species of juniper with a rich traditional medicinal history, has gained recent attention in the pharmaceutical and food industries (Rigling et al. 2020). This study aims to quantify the secondary metabolites and evaluate the antioxidant, anti-inflammatory, hemolytic, and anti-hemolytic activities of infusion and maceration extracts of *J. oxycedrus* belonging to two different environmental conditions. The findings will contribute to understanding the pharmacological effects and potential health benefits of this medicinal plant as well as the influence of geographical variability on the phytochemical profile and the biological potential of its active compounds.

The analysis of hemolytic activity demonstrates that both infusion and maceration induce hemolytic rate inferior to 15% which demonstrate that they are not toxic for human consumption. The hemolytic activity of the aqueous, methanolic, hexane and dichloromethane extract of the root bark of *J. oxycedrus* was assessed by Chaouche et al. (2014). They reported its hemolytic activity and the values of the aqueous extract varied between 2 and 3.5%.

The infusion extract shows high hemolytic activity than maceration. Gul et al. (2015) showed that the hemolytic activity of plant extracts can be influenced by various factors such as extraction method, and concentration of the plant extract. Simões et al. (2020) investigated the hemolytic potential of juniper infusion and maceration. The results revealed that juniper infusion exhibited a significantly higher hemolytic activity compared to maceration. At the same time, hemolysis rate vary depending on the geographical origin. Shafique et al. (2018) showed that the hemolytic activity of plant extracts can be affected by geographical variation.

The anti-hemolytic rate is relatively high which means that this plant can be used to protect efficiently erythrocytes from hemolysis. Plant extracts prevent hemolysis by scavenging the reactive oxygen species that cause oxidative stress leading to the destruction of erythrocytes. Plant polyphenols, flavonoids, and tannins have been identified as potent antioxidants that can protect erythrocytes from hemolysis. These compounds act by inhibiting lipid peroxidation, preserving the integrity of erythrocyte membranes, and chelating free iron ions in erythrocytes.

Several studies have identified plant extracts such as flavonoids from *Moringa oleifera* and tannins from *Terminalia arjuna* that have been shown to protect erythrocytes from hemolysis (Ōkubo et al. 2018; Chidume et al. 2019).

The infusion extracts show high anti-hemolytic activity than maceration. The efficacy of plant extracts in terms of their anti-hemolytic activity varies based on different extraction methods such as infusion, maceration, and solvent extraction (Giroux et al. 2013; Khatami et al. 2016). Several studies suggest that anti-hemolytic activity in plants can vary depending on biogeographical factors such as altitude, soil composition, and climate conditions (Laulhere et al. 2019; Scartezzini et al. 2005). Djilani and Dicko (2012) compared the anti-hemolytic activity of juniper extract obtained through infusion and maceration. They found that the infusion method produced higher level of anti-hemolytic activity than the maceration method.

Our plant extracts have exhibited remarkable potential in mitigating inflammatory diseases which means that these extracts constitute a potential source and alternative of modern medicine for the treatment of inflammatory diseases.

Various studies report that biomolecules such as curcumin, resveratrol, quercetin, and epigallocatechin-3-gallate (EGCG) suppress the production of inflammatory cytokines and modulate immune responses in a variety of human diseases (Sharma et al. 2018; Han et al. 2019; Leite et al. 2019). For instance, curcumin extracted from turmeric has been shown to reduce oxidative stress, inflammation, and apoptosis in various inflammatory diseases, including arthritis and ulcerative colitis (Umar et al. 2013; Kuptniratsaikul et al. 2014). Additionally, EGCG extracted from green tea exhibits potent anti-inflammatory effects by suppressing the production of pro-inflammatory cytokines and chemokines, and inhibiting inflammatory signaling pathways (Yang et al. 2021).

The anti-inflammatory potential was high in maceration extracts than in infusion. The anti-inflammatory potential of plant extracts is known to be affected by various factors, including extraction methods. Studies have reported that solvent extraction is one of the most effective methods in extracting bioactive compounds with anti-inflammatory properties from different plants (Kasote et al. 2013; Bouyahya et al. 2020). In addition, the anti-inflammatory activity varies depending on the biogeography and the different concentrations. The bioactive compounds present in plant extracts, including those with anti-inflammatory properties, can vary depending on the biogeography and environmental factors. Studies have reported the influence of biogeographical origin on the phytochemical composition and anti-inflammatory activity of plant extracts. For instance, Almaraz-Abarca et al. (2019) evaluated the anti-inflammatory and antioxidant potential of *Rhizophora mangle* extracts collected from different biogeographical

locations. Results showed that the anti-inflammatory activity varied significantly between extracts from different regions and different concentrations.

Tadesse et al. (2018) found that both extraction methods (infusion and maceration) were useful in producing extracts with anti-inflammatory activity, which was attributed to the high concentration of flavonoids and other compounds. The anti-inflammatory activity of *J. oxycèdrus* is also significant; it is around 95%. Kupeli Akkol et al. (2009) recorded an anti-inflammatory activity of 28.9% of the methanolic extract of the leaves and fruits of *J. oxycedrus*.

The extracts from Harmla region show high antioxidant activity. Previous research has demonstrated that the antioxidant activity of plants can be significantly influenced by the ecological conditions during their growth and development (Rauf et al. 2020). Plants exposed to high levels of environmental stress, such as drought, salinity, or heavy metals, may produce higher levels of antioxidant activity as a defense mechanism against cellular damage caused by the stressor. The increased production of antioxidants in response to stress is an adaptive response to protect the plant from oxidative damage (Gill et al. 2019).

The antioxidant activity varies between different extracts; the infusion extracts have the higher amounts. Sauvain et al. (1997) compared the antioxidant activity of various medicinal plants using infusion and maceration methods. The results revealed that the infusion method produced extracts with higher antioxidant activity levels compared to the maceration method. The infusion method involves the steeping of plant material in hot water, which can facilitate the release of bioactive compounds, leading to higher antioxidant activity. On the other hand, maceration involves soaking the plant material in a solvent for a prolonged period, resulting in a slower extraction process, which may lead to lower antioxidant activity levels (Ciriminna et al. 2016).

According to a study conducted by Canadanovic-Brunet et al. (2005), the infusion method yielded higher antioxidant activity compared to maceration. The results of another study by Baydar et al. (2005) also showed that the infusion of juniper berries has a higher antioxidant activity than maceration. Chaouche et al. (2013) reported that the antioxidant activity of the hydro-methanolic extract of the root and needle barks of *J. oxycedrus* is remarkable (0.02 mg/mL). The extracts from Harmla region show high phenolic content. Phenolic content, including flavonoids and phenolic acids, possesses antioxidant properties and plays a crucial role in protecting plants from oxidative damage induced by stress (Fini et al. 2011). The increase in phenolic content in infusion extracts can be attributed to the thermal degradation of cellular components, leading to the release of phenolic content during the extraction process. Moreover, prolonged incubation time enables the solvent to penetrate deeper into the plant material and extract more phenolic compounds (Wu et al. 2014). Miguel et al. (2005) compared the phenolic

content of extracts obtained from medicinal plants using maceration and infusion methods. The results showed that the infusion method produced extracts with higher phenolic content than the maceration method. According to Lachowicz et al. (2008), the phenolic content of leaves from *Juniperus communis* can be enhanced through both infusion and maceration methods. Both methods resulted in high levels of phenolic compounds, with the infusion method producing slightly higher levels than the maceration method.

Kuhnle et al. (2018) showed that the levels of flavonoids in plants are significantly influenced by their ecological conditions during development. Additionally, plants exposed to high levels of stress, such as from drought or salinity, also produced higher levels of flavonoids. These findings demonstrate the adaptability of plants to their environment and the role of flavonoids in plant resilience. Our results also show that the total flavonoid contents vary between different extracts; the infusion extracts have higher amounts. Kulisic et al. (2004) showed that the flavonoid content in plant extracts can vary depending on the extraction method used. Azmir et al. (2013) demonstrated that the flavonoid content significantly increased with higher temperatures and longer incubation time during the extraction process. Cemali et al. (2018) reported that juniper tea is a good source of flavonoids, which have potential health benefits for preventing chronic diseases. However, Kowalska et al. (2012) demonstrated that macerations of leaves had the highest flavonoid content overall.

Quideau et al. (2011) found that the choice between infusion and maceration as the extraction method can significantly affect the tannins content of plant extracts. Infusions generally led to lower tannin content than macerations, which were more effective at extracting tannins over a longer period. Furthermore, it is evident that the levels of tannins differ based on the biogeography and varying concentrations. Smeriglio et al. (2019) found that the tannin content of Juniper leaves varied depending on the location of the plant. Lachowicz et al. (2008) demonstrated that both infusion and maceration methods can effectively extract tannins from the leaves of the Juniper. The researchers found that the maceration method yielded higher levels of tannins than the infusion method.

The study conducted on *Juniperus oxycedrus* in Algeria revealed that the plant extracts have remarkable biological properties. The extracts displayed low hemolytic activity but high anti-hemolytic activity accompanied by significant anti-inflammatory and antioxidant activities, with high levels of phenolic compounds, flavonoids, and tannins. Interestingly, the chemical composition of flavonoids and tannins varied depending on the geographic location of the plant, indicating its potential use in various applications. The findings highlight Juniperus oxycedrus as a promising source of bioactive compounds for therapeutic and industrial purposes, although further studies are necessary to fully characterize its potential.

Conclusion

Conclusion

Currently, medicinal plants continue to serve as the primary storehouse for novel pharmaceuticals. They are recognized as a vital resource of fundamental ingredients that aid in the exploration of new compounds crucial for the advancement of future medications. Consequently, extensive research is dedicated to studying secondary metabolites and their influence on human and animal health. These molecules are extensively utilized in therapeutic applications. In light of this, our investigation centres on examining the phytochemical and biological aspects of *Juniperus oxycedrus* infusion and maceration extract.

The present study revealed that the plant possesses interesting pharmacological properties, including high antioxidant and anti-inflammatory activities, as well as significant levels of phenolic compounds, flavonoids, and tannins that vary depending on the geographic location of the plant. Although some extracts showed antihaemolytic activity with therapeutic potential.

The findings suggest that *Juniperus oxycedrus* can be used as a promising source of bioactive compounds for therapeutic and industrial purposes. This study provides valuable insights into the pharmacological efficacy of the studied medicinal plant and its potential application in human and animal health, but further studies are necessary to fully characterize its potential.

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