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**Extraction, evaluation of antioxidant and
antibacterial activities of *Juniperus oxycedrus***

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ABSTRACT

The aim of this study is the determination of the phytochemical constituent and evaluation of the antioxidant and antibacterial activities of *Juniperus oxycedrus L.* leaves extracts and essential oil.

J. oxycedrus has many pharmacological and therapeutic properties including antimicrobial, and antioxidant activities. Due to this importance the leaves was analyzed for preliminary phytochemical studies. The qualitative analysis using TLC showed the presence of flavonoids in different studied extracts. DPPH free radical scavenging method was used for antioxidant activity analysis. All the extracts exhibit a good antioxidant activity; soxhlet ethanolic extract exhibited the highest radical scavenging activity with an IC₅₀ values 0.25µg/ml. The extracts were also tested for their possible *in vitro* antibacterial activity against two bacterial strains (*Escherichia coli* and *Staphylococcus aureus*) using the disc diffusion method. The results of showed that the highest activity was observed to decoction extract (diameter of zone inhibition 13 mm) against *Staphylococcus aureus* and no effect was observed on *E.coli*, while essential oil give a positive result for both strains.

Keywords: *Juniperus oxycedrus*, phenolic compounds, TLC, antioxidant activity, antibacterial activity.

ملخص

هدف هذه الدراسة هو تحديد الفحص الكيميائي النباتي. وتقييم النشاطية المضادة للأكسدة والمضادة للجراثيم لمستخلص الاوراق و الزيتالاساسي لنبتة *Juniperus oxycedrus*

لنبتة العرعر الشربيني العديد من الخصائص الدوائية والعلاجية بما في ذلك مضادات الميكروبات ، مضادات ، ومضادات الأكسدة. بسبب هذه الأهمية تم تحليل الاوراق للدراسات الكيميائية النباتية الأولية. في هذه الدراسة ، تم جمع واختبار مستخلص النبات حيث تم جمعه و اختباره للكشف عن المواد الكيميائية النباتية. كشفت التحليل الكيفي باستخدامTLCوجود فلافونويد في مختلف المستخلصات. تم استخدام طريقة كسح الجذور الحرة DPPH لتحليل نشاط مضادات الأكسدة. جميع المستخلصات أظهرت نشاط جيد مضاد للأكسدة. أظهر المستخلص الايثانولي لل Soxhlet أعلى نشاط للمسح الجذري بتركيز مثبط IC50 مقدر ب 0.25 ميكروغرام / مل. كما تم اختبار المستخلصات لنشاطها المضاد للبكتيريا في المختبر ضد سلالتين بكتيرية (*Escherichia coli* و *Staphylococcus aureus*) باستخدام طريقة انتشار القرص. وأظهرت النتائج علوانشاطية المضادة للبكتيريا أن أعلى نشاط كان يعزى إلى مستخلص الماء الكحولي مع الحد الأقصى لمنطقة تثبيط كان 13 ملم ضد *Staphylococcus aureus*. ولم يظهر اي نشاط ضد *E.coli* بينما اظهر الزيت الاساسي نشاط مضاد للبكتيريا على كل من السلالتين.

كلمات مفتاحية: مركبات الفينولية، النشاط المضاد للأكسدة، القدرة المضادة للجراثيم، *Juniperus oxycedrus*.

ABREVIATIONS

HCL: Hydrochloric

H₂SO₄: sulfuric acid

NH₄OH: Ammonia solution

TLC: Thin layer chromatography

UV: Ultraviolet

GAE: Gallic acid equivalent

DPPH: 2,2'-diphenyl-1-picrylhydrazyl

IC₅₀: Inhibitory concentration for 50% of activity

DMSO: Dimethyl sulfoxide

AI: Activity Index

InfE: Infusion extract

DecE: Decoction extract

MeEbS: Meanollic extract by soxhlet

EtEbS: Etanollic extract by soxhlet

HMeEbM: hydromethanolic extract by Maceration

HEtEbM: hydroethanol extract by Maceration

IZ: Inhibition zone

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Introduction

Introduction

During the processes of creation and expansion of the universe which evolves over the fifteen billion years, two events we concerning more directly have occurred: the organization of solar system with the creation of our planet, Earth, there are four and half billion years ago and the appearing of what is known as life, there are four billion years ago (Serge et al. 2004).

But, what is life? How we can identify it?

Religions and philosophers have suggested definitions for that question, but scientists always have a lot of difficulties to answer this question. What are the necessary sufficient conditions that qualified a system to be alive? In fact, the border is not always obvious between inorganic matter and the most primitive life forms and therefore towards chemistry that we are currently turning to find explanatory hypotheses (Christian et al. 2008)

One of the most fundamental and ancient biochemical processes of life is photosynthesis. It appeared in some primitive bacteria, about three billion years ago, and then it evolved in more evolutionary bacteria 'cyanobacteria'. Photosynthetic activity of cyanobacteria accompanied by liberation of O₂ in the environment followed by creation of atmosphere and specifically ozone layer, and then appearing of large biological richness such as animals, plants, marine organisms, microorganisms...(Serge et al. 2004).

The arrival of new component that called human being makes remarkable changes on biodiversity, this new specie found all what it needs in its environment that gives everything for surviving like oxygen, nutrition, clothes and especially medicaments.

The aim of this study was to present the phytochemical screening, and antioxidant and antibacterial potential of leaves of *juniperus oxycedrus*. The concentration of these bioactive in different extracts prepare by different extraction methods was also carried out.

Bibliographic research

1. Natural products

Natural products are chemical compounds or substances isolated from living organism. The chemistry of the natural product include their biosynthesis, extraction, identification, quantification, structural elucidation, physical and chemical properties and reaction They are produced by the pathway of primary or secondary metabolism (Nwokeji et al., 2016)

Natural products, whether collected from nature or synthesized in the laboratory, play a large and increasing role in our everyday lives. Besides the obvious role of keeping us alive (as do vitamins, hormones, etc.), natural products are used as therapeutic agents, as flavoring additives, as insecticides, for skin protection, and in many other applications. They were used ever since man learned to interact with its environment. Centuries ago, they were administered as plant or animal extracts, or in other forms, and they were used to treat diseases or as hallucinogens, etc. The effects of the extracts were known but the agent(s) responsible for the effect could not then be identified. Today, with the help of modern science and technology, chemists are able to identify the components of an extract and to assign a biological activity or other function to each component. This branch of chemistry is called "natural product isolation and structure elucidation". But the science doesn't stop there. Chemists are looking for ways to synthesize natural products in the laboratory. That way, they can obtain large supplies of rare, scarce and difficultly accessible natural products. They also can modify the structure of natural products and test the analogues for increased biological activity, decreased side effects, or metabolic stability. New chemicals can be obtained that are more powerful, more selective, or cheaper than the original natural products (Claude. 2016).

Natural products are classified according to four main areas:

- Chemical Structure; classification according to broad structural features, e.g., aliphatic, aromatic, heterocyclic, etc.;
- Physiological Activity; classification according to their role in biological systems, e.g., antibiotics, vitamins, hormones, antineoplastic, etc.;
- Taxonomy; classification according to the source of the compound. Based on plant taxonomy, e.g., alkaloids, terpenoids, etc.; and

- Biogenesis; classification according to their chemical origin or biosynthesis. Usually the compounds are named after the mechanisms by which they are produced, e.g., isoprenoids, acetogenins (polyketides), shikimic acid pathway, etc

Usually the compounds are named after the mechanisms by which they are produced, e.g., isoprenoids, acetogenins (polyketides), shikimic acid pathway, etc. (Claude. 2016).

2. Plant's secondary metabolisms

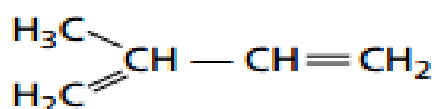
The secondary metabolites are also known as phytochemicals, natural products or plant constituents. They are organic molecules characterized by an enormous chemical diversity, every organism has its own characteristic set of secondary metabolites, some of which they may share with other related or totally unrelated organisms. For many years secondary metabolites have been considered as more or less waste products, with no apparent use for the plant. Still our knowledge about the role of the secondary metabolites is limited, but now it is generally accepted that secondary metabolism is involved in the relationship of the organism with its environment, e.g. in resistance against pests and diseases, as attractant of pollinators, or as signal compound (Verpoorte and Alfermann, 2000).

In most references, it is stated that the secondary metabolites extracted from plants are subdivided in three major classes: terpenoids, alkaloids and phenolics. Glycosides and saponins are part of them according their specific structure.

2.1. Terpenoids

Terpenoids are the largest class of all known natural products. With Over then 20,000 terpenoids have been identified, and more are being discovered continuously (Natanya, 2012). The diverse substances of this class are generally insoluble in water. They are biosynthesized from acetyl-CoA or glycolytic intermediates (Lincoln and Eduard, 2003).

The basic structural elements of terpenes are sometimes called isoprene units because terpenes can decompose at high temperatures to give isoprene:



Terpenes are classified by the number of five-carbon units they contain, although extensive metabolic modifications can sometimes make it difficult to pick out the original five-carbon residues. Ten-carbon terpenes, which contain two C₅ units, are called monoterpenes; 15 carbons terpene (three C₅ units) are sesquiterpenes; and 20-carbons terpene (four C₅ units) are diterpenes. Larger terpenes include triterpenes (30 carbons), tetraterpenes (40 carbons) and polyterpenoids ([C₅] n carbons, where n > 8) (Lincoln and Eduardo, 2003).

The terpene family includes hormones (gibberellins and abscisic acid), the carotenoid pigments (carotene and xanthophyll) which give the yellow, red and orange colour in some plants like carrot, sterols (ergosterol, sitosterol and cholesterol) and sterol derivatives (e.g., cardiac glycosides), latex (the basis for natural rubber) and many of the essential oils that give plants their distinctive odors and flavors (William and Norman, 2008).

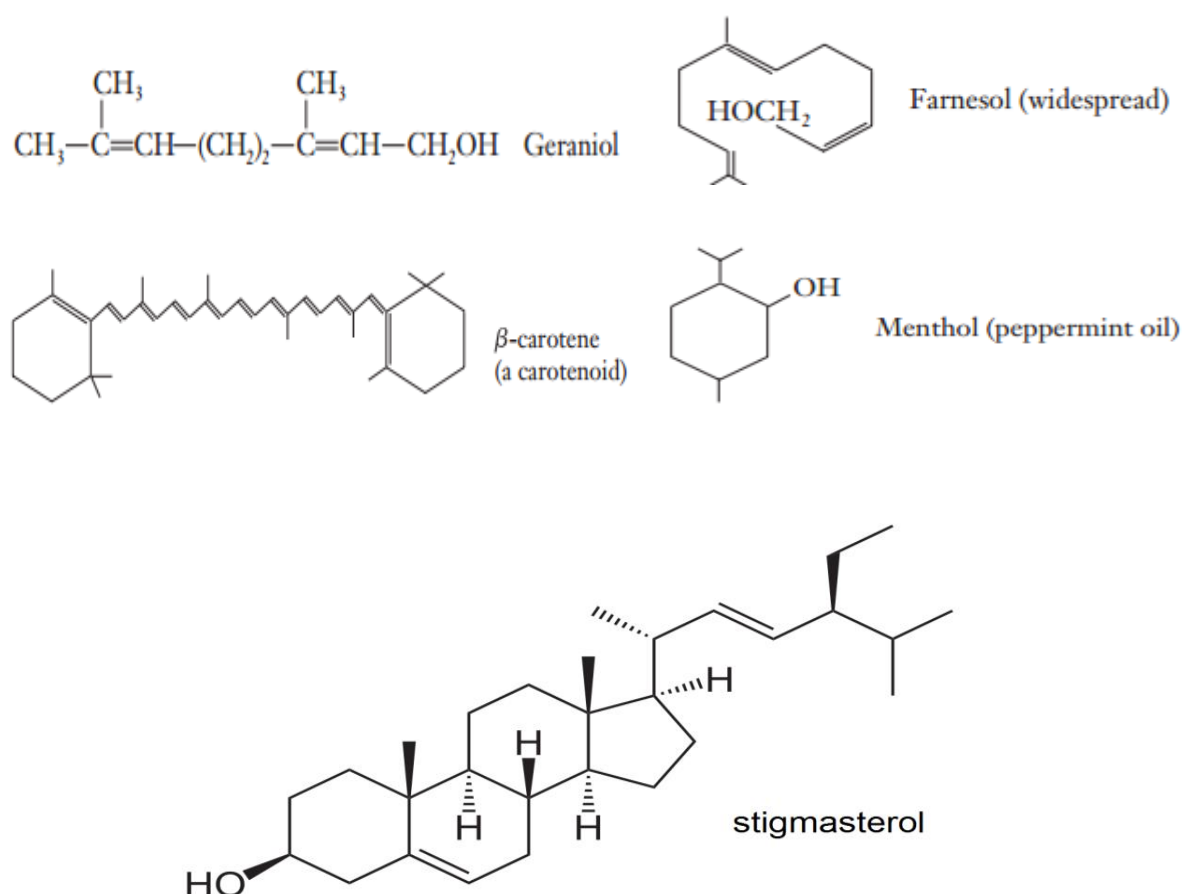


Figure 1: Example of some terpenoids (William and Norman, 2008).

The roles of plant terpenoids are incredibly diverse but are associated most often with defense and communication of sessile plants interacting with other organisms. Examples include terpenoid chemicals that form physical and chemical barriers, antibiotics, phytoalexins, repellents and antifeedants against insects and other herbivores, toxins, attractants for pollinators or fruit-dispersing animals. They are the most diverse group of plant chemicals and have been used by humans for centuries in both traditional and modern industrial applications (Natanya, 2012).

2.2. Phenolics Compounds

Phenolic compounds from plants are one of largest groups of secondary plants metabolites. They are distributed in almost all plants. They are diverse in structure, and present in common the hydroxylated aromatic rings (Justin, 2014).

They represent a diverse group of compounds having such various roles as defending plants, determining certain distinguishing features of different woods and barks (e.g., durability), establishing flower color, and contributing substantially to certain flavors (tastes and odors) (Rodney and all., 2000).

The Phenolics family includes compounds that are non-soluble such as condensed tannins, lignins, cell-wall bound hydroxycinnamic acids, and soluble compounds such as phenolic acids, and flavonoids. All these groups are involved in many processes in plants and animals. Two family, the flavonoids and tannins, are of particular interest because of its multiple roles in plants and its impact on human health.

2.2.1. Flavonoids

Flavonoids represent a very large class of phenolic derivatives (more than 12000 representatives are known), with a variety of functions. Biochemically, however, all flavonoids share a common structure consisting of three rings, (A and B) and heterocyclic ring (C). This carbon structure can be abbreviated C₆-C₃-C₆. According to the IUPAC nomenclature.

The flavonoid family is subdivided into different sub-families such as flavones, isoflavones, flavanones, flavonols, anthocyanins, chalcones. All of these types of compounds comprise three aromatic rings harbouring different substitutions such as methylation and hydroxylation (Nicolas and al., 2005).

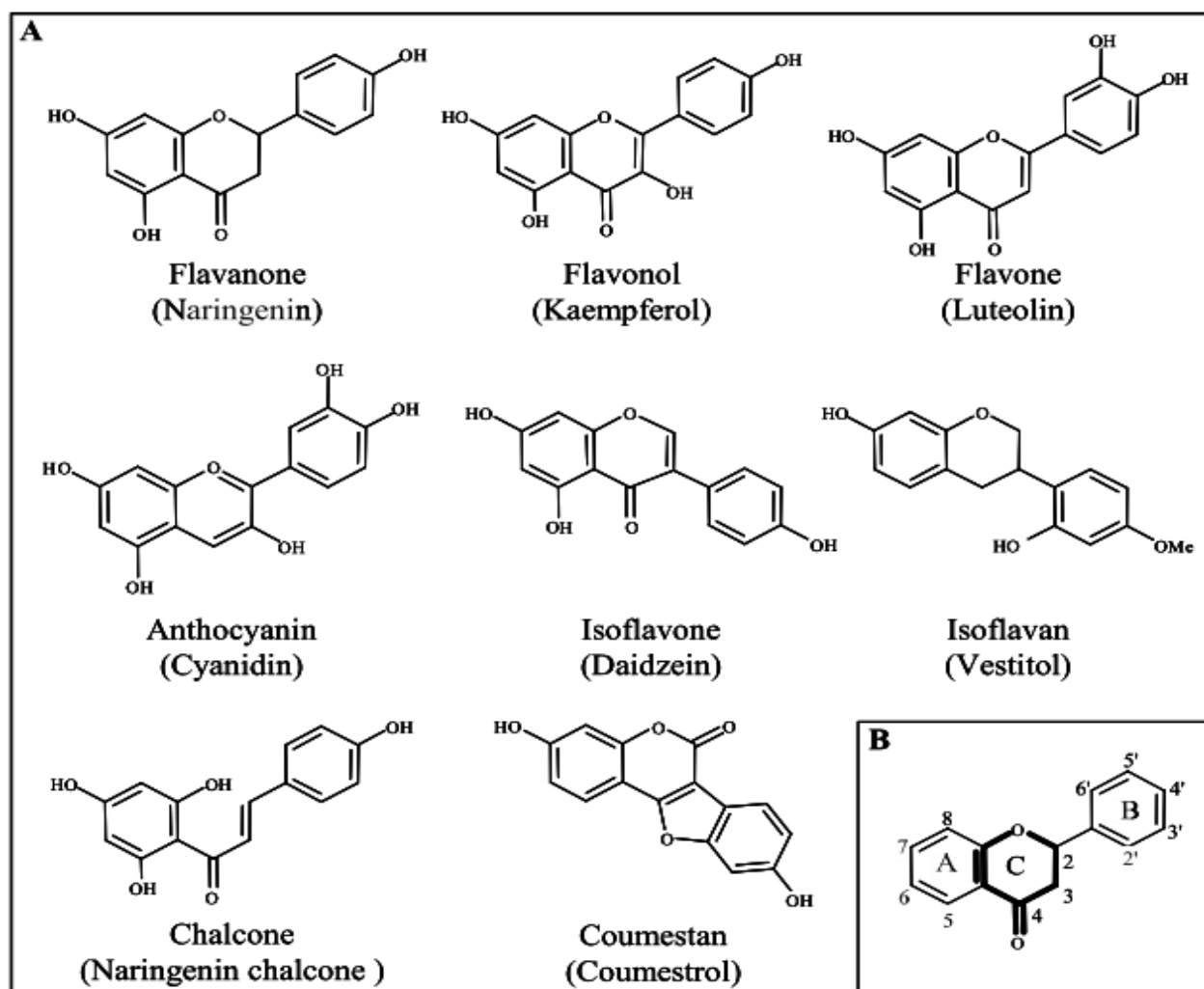


Figure 2: Flavonoids structures (Adapted from Aoki et al., 2000). A: Chemical structure of selected subclasses and biologically active flavonoids. B: General structure of flavonoid compounds. The different flavonoid classes are defined according to the structure of the C-ring and the functions carried by C3 and C4 (in bold).

Flavonoids, which are the product of secondary metabolism of plants, are one the most investigated class of natural products due to their wide range of biological activities, such as anti-inflammatory, oestrogenic, enzyme inhibition, antimicrobial, antiallergic, vascular and cytotoxic antitumour activity, but the antioxidant activity is, without a doubt, the most studied one attributed to flavonoids. This well established antioxidant activity of flavonoids is also

responsible for other biological activities in which the prevention of oxidative stress is beneficial. For example, the anticancer activity of some compounds is due to their ability to scavenge free radicals, thus avoiding the early stages of cancer promotion. Besides this mechanism, flavonoids have also been reported to act as anticancer agents (David et al., 2009).

2.2. 2. Tanins

Tanins are a group of water-soluble polyphenols having molecular weights from 500 to 3,000 which are subdivided into condensed and hydrolysable tannins, and commonly found complexed with alkaloids, polysaccharides and proteins, particularly the latter. On the basis of structural characteristics there are two groups, gallotannins and ellagitannins of hydrolysable tannins (Xiuzhen ,et al 2007).

Tanins are, thus, reported to possess numerous pharmacological properties such as antibacterial, antifungal, antiviral, anti-diarrhoeal, free radical scavenging, immunomodulatory, anti-inflammatory, anti-tumour, and antidote activities. Some, and certainly most, of the beneficial effects which tannins exert as constituents of drugs and herbal remedies may well follow from their interaction with enzymes (proteins) within cell systems

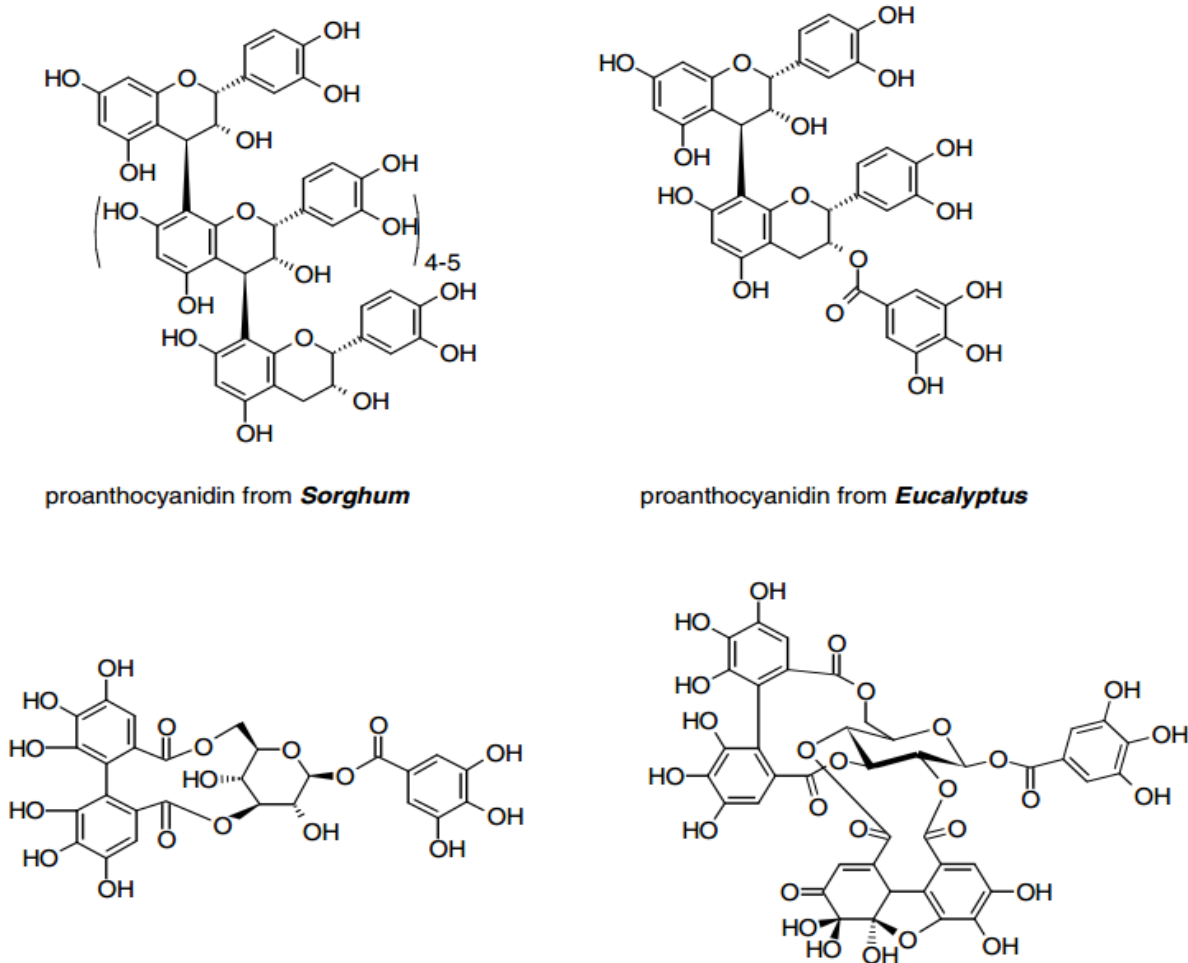


Figure 3: Structures of representative condensed (proanthocyanidins) and hydrolyzable tannins. (Leland et al, 2012).

2.3. Alkaloids

Alkaloids are defined as basic compounds synthesized by living organisms containing one or more heterocyclic nitrogen atoms, derived from amino acids (with some exceptions) and pharmacologically active. The class name is directly related to the fact that nearly all alkaloids are basic (alkaline) compounds. Alkaloids constitute a very large group of secondary metabolites, with more than 12,000 substances isolated. A huge variety of structural formulas, coming from different biosynthetic pathways and presenting very diverse pharmacological activities are characteristic of the group (Sasikumar, 2012).

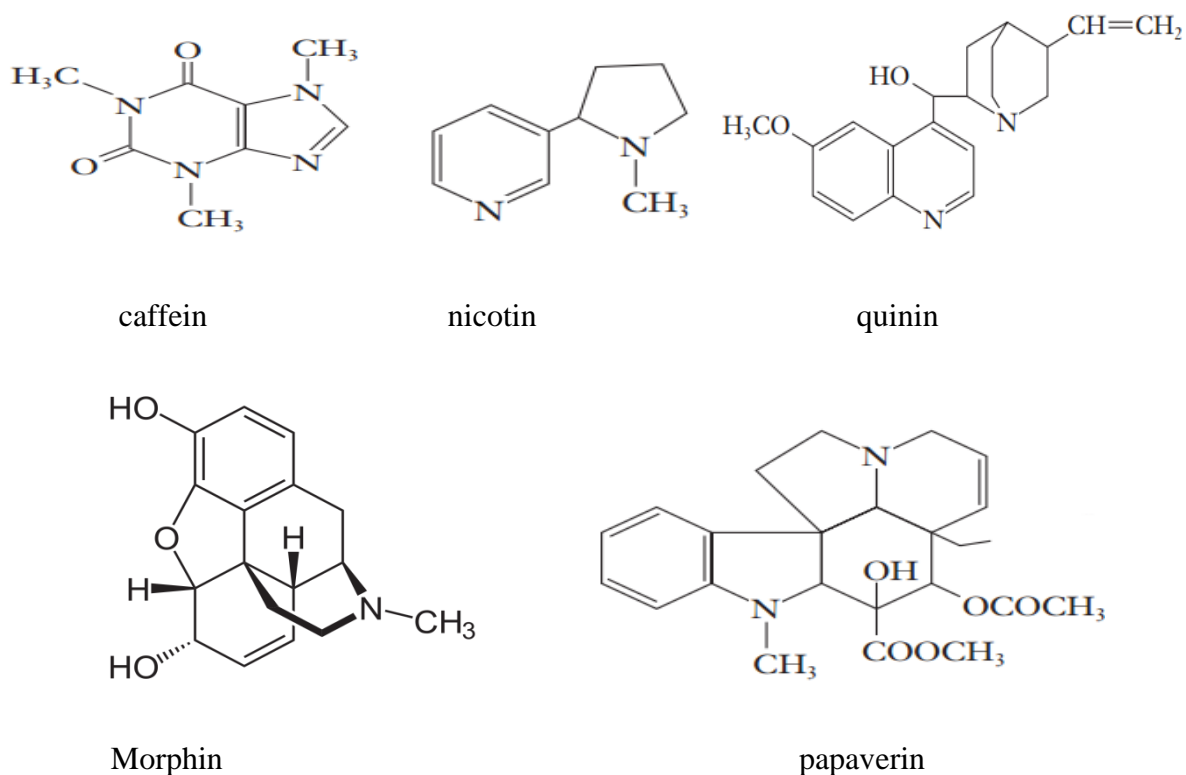


Figure 4: Example of some alkaloids

Alkaloids are associated with a wide range of pharmacological activities. Many are toxic and can cause death, even in small quantities. Some have antibiotic activities and others interfere with behavior patterns, such as antidepressants (reserpine) and hallucinogens (mescaline). It seems alkaloid function in plants and animals is linked to defense mechanisms. Toxicity is a good weapon to inhibit the action of predators, like herbivores (Sasikumar, 2012).

2.4. Essential oil

An essential oil is a concentrated hydrophobic liquid containing volatile aroma compounds from the plant. They are also known as aromatic oils, fragrant oils, steam volatile oils, ethereal oils, or simply as the "oil of" the plant material from which they were extracted (Hesham et al., 2016).

They are complex mixtures of volatile compounds such as terpenes (mostly monoterpenes and sesquiterpenes), phenolics and alcohols. However, the essential oils are highly complex and may include oxygenated compounds. They are oxygenated derivatives of hydrocarbon terpenes such as aldehydes, ketones, alcohols, phenols, acids, ethers and esters. The essential oil is so called because they were believed to represent the quintessence of odor and flavor from the flower kingdom.

Essential oils are projected to protect the plant by acting as antifungal, antibacterial, insecticidal or antiviral components and are also thought to promote the dispersion of seeds and pollens by attracting insects (Laira et al., 2017). Has shown that about 3000 essential oils are known and 300 are commercially important for the pharmaceutical, agronomic, food, sanitary products, cosmetic, and perfume and make-up industries. Essential oils are also used in dentistry and as food preservatives and additives. Some terpenes are potent drugs against diseases such as heart disease, malaria and cancer (Hesham et al., 2016).

2.5 Biosynthesis of secondary metabolites

The biosynthesis of the various three classes of secondary metabolite varies depending on the class involved. The terpenes are biosynthesized from primary metabolite through the pathway of mevalonic acid and methyerythritol phosphate. In mevalonic acid pathway, three molecule of acetyl-COA are joined together stepwise to form mevalonic acid. this key six-carbon intermediate is then pyrophosphorylated, decarboxylated and dehydrated to yield isopentenyl diphosphate(IPP).The IPP is the activated five carbon building block of terpenes.recently,it was discovered that IPP also can be formed from intermediate of glycolysis or the photosynthetic carbon reduction cycle via a separated set of reactions called the methylerythritol phosphate(MEP) pathway that operates in the chloroplast and other plastid. Although all the details have not yet been elucidated, glyceraldehydes-3-phosphate and two carbon atoms derived from pyruvate appear to combine to generate an intermediate that is eventually converted to IPP. In biosynthesis of phenolic compound, two basic pathway are involved, the shikimic acid pathway and the malonic acid pathway. The shikimic acid pathway convert simple carbohydrate precursors derived from glycolysis and the pentose phosphate pathway to the aromatic amino acid. One of the intermediate is shikimic acid, which has given its name to this whole sequence of reaction. The well-known broad spectrum herbicide glyphosphate kill plant by blocking a step in this pathway.

The most abundant classes of secondary phenolic compound in plant are derived from phenylalanine via the elimination of an ammonia molecule to form cinnamic acid. This reaction is catalyzed by phenylalanine ammonia lyase (PAL) perhaps the most studied enzyme in plant secondary metabolite. For the nitrogenous compound, the pathway is the formation of aliphatic amino acid through the TCA cycle (Nwokeji et al 2016).

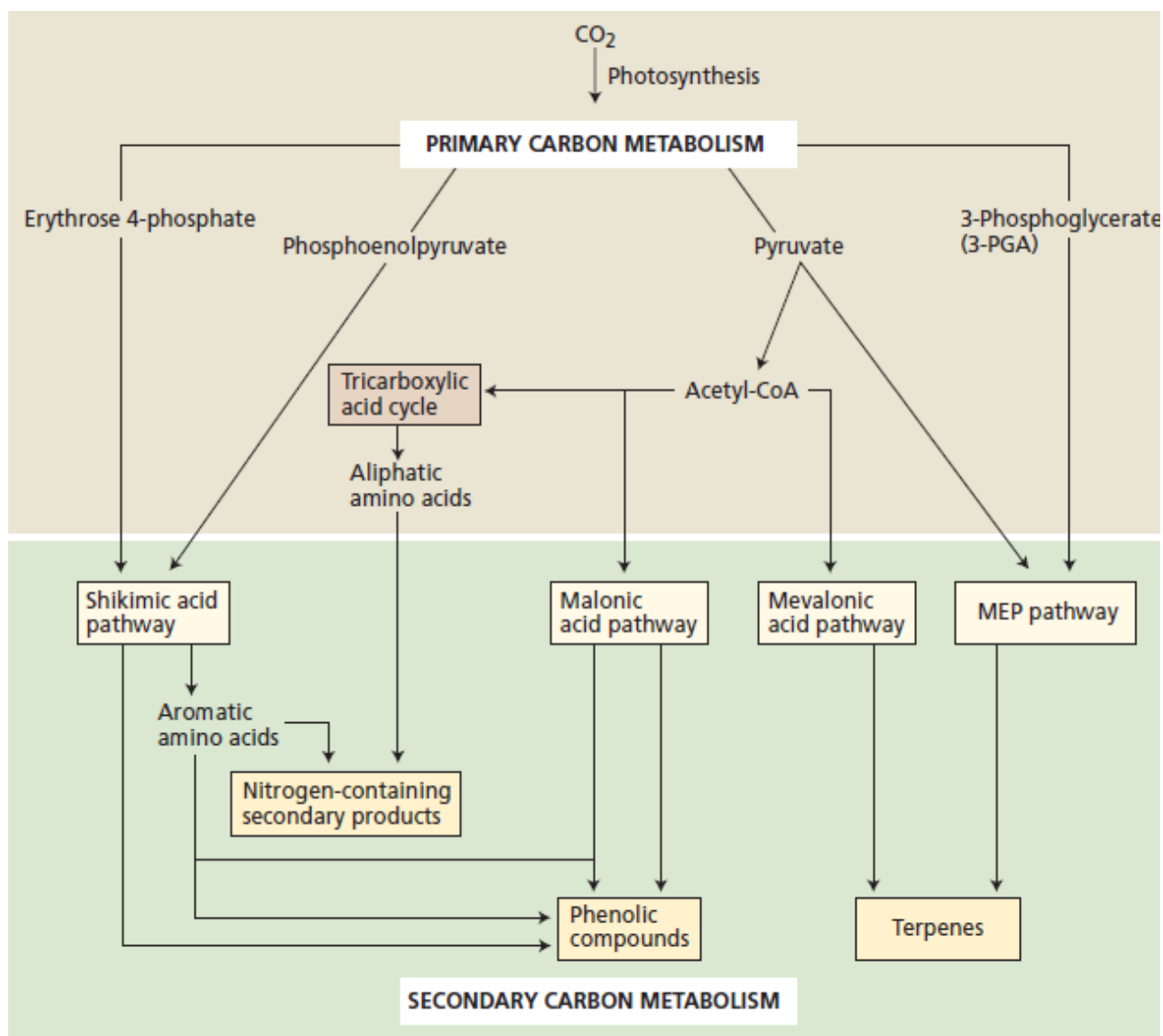


Figure 5: Simplified view of the major pathways of secondary-metabolite biosynthesis and their interrelationships with primary metabolism.

2. Plant profile

2.1. Geographical distribution

Juniperus oxycedrus L. is known as Mediterranean equivalent of the common juniper, native across the Mediterranean region from Morocco and Portugal, southern France and east to Iran, Lebanon and Palestine. It grows on rocky sites from sea level up to 1600 m altitude. Among *J. oxycedrus* interesting subspecies are *J. oxycedrus* var. *badia* H.Gay, distinguished on the basis of larger cones and described from northern Algeria, but also reported for Portugal and Spain and *J. Oxycedrus* subsp. *macrocarpa* Ball., confined to Mediterranean coastal sands, which differs in the broader leaves 2-3 mm wide and larger cones (Floresha and al., 2013).

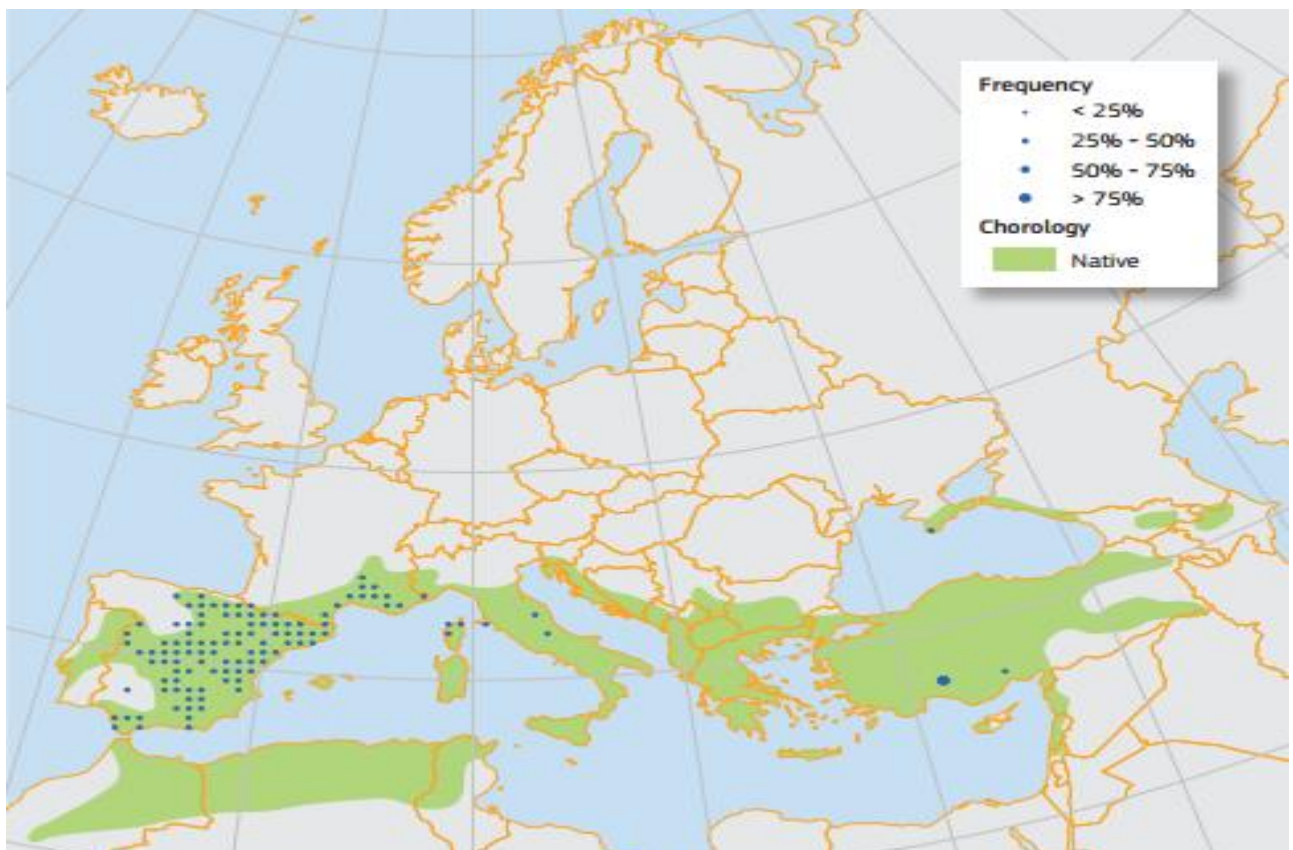


Figure 6: Plant distribution and simplified chorology map for *Juniperus oxycedrus*.

2.2. Botanical description

Juniperus oxycedrus L. (Cupressaceae) is a hardy spreading plant, that vary in size from a low shrub to a tree of about 6 m. The leaves are tiny, narrow and prickly and are reason for people' name of the plant: "Prickly Juniper". The cones are yellow and rounded, and the fruits are berry-like, growing in clusters, and black when ripe. The plant is known as Cade juniper as well, mainly because of the oil that is obtained through destructive distillation of the wood. Cade oil is dark, aromatic oil with a strong smoky smell which is used in some cosmetics and traditional skin treatment drugs, as well as incense (Floresha and al 2013).



Figure 7 : botanical aspect of *Juniperus oxycedrus* L.

2.3. Classification

Kingdom: Plantae

Division: Coniferophyta

Class: Pinopsida

Order: Pinales

Family: Cupressaceae

Genus: *Juniperus L*

Specie: *Juniperus oxycedrus L.*,1753

Vernaculaire Name : Taga

French Name: oxycèdre, genévrier, cade, cadier, petit cèdre

2.5. Importance and usage

The prickly Juniper is suitable for cultivation as an ornamental shrub in southern Europe, where a number of cultivars, especially with more pendulous foliage, are commonly planted in gardens and parks. Its wood is resistant and hard, highly valued for making furniture and other carpentry items. Essential oils are extracted from the branches and leaves by destructive distillation, especially in France and Turkey. This 'oil of cade' is used for medicinal purposes, such as to prepare empyreumatic oil. It has antiseptic and antiparasitic properties. Rectified cade oil is also used as a fragrance component in soaps, detergents, creams, lotions and perfumes (Lara et al., 2016).

3. Biological activities

3.1. Antioxidant activity

3.1.1. Free radicals

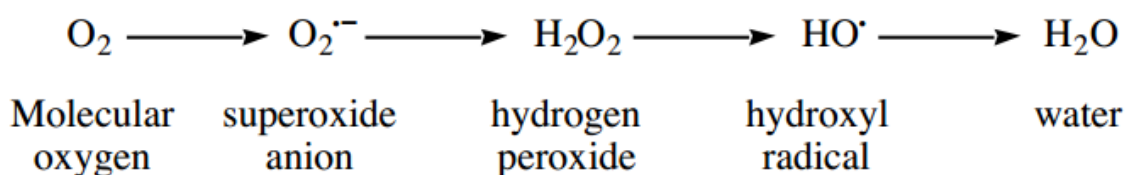
Oxygen is one of the most important molecules on Earth mainly because of the biochemical symmetry of oxygenic photosynthesis and aerobic respiration that can maintain homeostasis within our planet's biosphere. Oxygen can also produce toxic molecules (Reactive Oxygen Species (ROS)) (Mariusz and Sławomir, 2013).

3.1.1.1. Definition of free radicals

A free radical is an atom, molecule, or compound that is highly unstable because of its atomic or molecular structure (i.e., the distribution of electrons within the molecule). This instability makes free radicals very reactive, and they attempt to pair up with other molecules, atoms, or even individual electrons to create a stable compound. To achieve a more stable state, free radicals can “steal” a hydrogen atom from another molecule, bind to another molecule, or interact in various ways with other free radicals (Susinjan, 2015).

3.1.1.2. Production of free radicals in the human body

Free radicals are continuously produced by the body's normal use of oxygen. When cells use oxygen to oxidize molecules rich in carbon and hydrogen (i.e., nutrients) to produce the different forms of energy needed for life. The reduction of molecular oxygen to water is accompanied by a large free energy release that can give rise to free radicals (Reactive Oxygen Species (ROS)). The most important ROS in biological systems is radical derivatives of oxygen. The free radicals have a special affinity for lipids, proteins, carbohydrates and nucleic acids (Battino and al., 1999).



3.1.1.3. Oxidative stress and relevant biological targets for FR and ROS

Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites, so-called oxidants or reactive oxygen species, and their elimination by protective mechanisms, referred to as antioxidants. This imbalance leads to damage of important biomolecules and cells, with potential impact on the whole organism.

Because of their high reactivity, several FR and ROS can rapidly modify either small, free biomolecules (i.e., vitamins, amino acids, carbohydrates, lipids) or macromolecules (i.e., proteins, nucleic acids) or even supramolecular structure (i.e., cell membranes, circulating lipoproteins) (Battino et al., 1999).

The harmful effects of ROS are balanced by the action of antioxidants, some of which are enzymes present in the body. Despite the presence of the cell's antioxidant defense system to counteract oxidative damage from ROS, oxidative damage accumulates during the life cycle and has been implicated in diseases, aging and age dependent diseases such as cardiovascular disease, cancer, neurodegenerative disorders and other chronic conditions (Eboh, 2014)

3.1.2. Antioxidants

The term “antioxidant” refers to any molecule capable of stabilizing or deactivating free radicals before they attack cells.

An ideal antioxidant should be readily absorbed and quench free radicals, and chelate redox metals at physiologically relevant levels. It should also work in both aqueous and/or membrane domains and effect gene expression in a positive way. antioxidants include Vitamin E and C, thiol antioxidants (glutathione, thioredoxin and lipoic acid), melatonin, carotenoids, natural flavonoids, and other compounds. (Eboh Abraham, 2014).

3.2. Antibacterial activity

As you begin to explore the world of microorganisms, one of the first things you'll notice is their extraordinary diversity of structure, function, habitat and applications. Microorganisms (or microbes) inhabit every corner of the globe, are indispensable to life on Earth, are responsible for some of the most deadly human diseases and form the basis of many industrial processes. Yet until a few hundred years ago, nobody knew they existed! (Stuart H, 2005).

Contrary to the synthetic drugs, antibacterial activities of plant origin are associated with lesser side effects and have an enormous therapeutic potential to heal many infectious diseases. The potential for developing antibacterial from higher plants appears rewarding as it will lead to the development of a phytomedicine to act against microbes. Nowadays a number of clinically efficacious antibiotics are becoming less effective due to development of resistance. So, biomolecules of plant origin appear to be one of the alternatives for the control of these antibiotic resistant human pathogens.(Bhakta P,G et al 2009).

Materials and methods

1. Plant material

The leaves of *Juniperus oxycedrus* were collected from Djebel Eltolba of Msila in February 2018. Plant identification was carried out by Dr ZEDAM Abdelghani, botanist at the Agronomy Department, University of Msila (Algeria).

The leaves were dried in the dark, at room temperature then grinded by a mechanical grinder to obtain a powder for increasing the surface of contact with solvent extraction.

In the other side, the bark was collected in April 2018. After 5 days in storage it was manually grinded to small pieces and submitted to hydro-distillation using a Clevenger-type apparatus.



Figure 8: Images of the leaves and the barks.

2. Methods of extraction

2.1. Extraction of the leaves

2.1.1. Extraction by Soxhlet

It was carried out to obtain extracts for the phytochemical screening and evaluation of anti-bacterial and anti-oxidant activities. Leaves were first grinded in a mechanical grinder to obtain the powder of the crude drug which was later. Around 50 g of the powder was packed in a thimble of filter paper prepared manually. The thimble was then inserted into the soxhlet apparatus and extraction was done by using 300 ml for each of methanol and ethanol an 80%. The temperature was maintained at 45°C for both quantities and extraction was continued for 18 hours, then the methanol and ethanol extracts were collected and powder from the thimble was discarded. Then obtained methanolic and ethanolic extracts were dried by rotary flash evaporator, and dried in a vacuum oven so as to obtain thick, viscous mass.

2.1.2. Extraction by Maceration

40 g of dried leaves were macerated using 800 ml methanol and ethanol an 80% at laboratory temperature for 24 hours. Then the extracts obtained were filtered using Whitman filter paper to obtain extracts. The residue left was again subjected to second successive extraction with half amount for both solvents, following previously mentioned procedure to get the second extract. Thus obtained alcoholic extract was concentrated in rotary flash evaporator and dried in a vacuum oven so as to obtain thick, viscous mass. Then the yield value was calculated were used for the phytochemical screening evaluation of anti-bacterial and anti-oxidant activities (Markham , 1982).

2.1.3. Decoction extraction

Ten g of leaves powder was measured and mixed with 100 ml of distilled water and boiled for 1 hour. The liquid extract was then cooled, filtered and dried in the oven at 40 ° C (Diallo et al., 2004).

2.1.4. Infusion extraction

In this process the collection of aqueous extract using 10 g of plant powder placed in 100 ml of boiling water for 3 hours. The liquid extract was then filtered and dried in the oven at 40 ° C (Diallo et al., 2004).

2.1.5. Yield value

$$\frac{\text{Mass of extracts obtained}}{\text{Total mass of crude drug}} \times 100$$

Yield value of each extracts was calculated as:

2.2. Essential oil extraction

The bark was manually grinded to small pieces and submitted to hydro-distillation for 4 h with 800 ml distilled water using a Clevenger-type apparatus.

Yield value

The yield was calculated as follows:

$$\text{Oil\%} = \frac{\text{Weight of the oil}}{\text{Weight of the material taken}} \times 100$$

3. Phytochemical screening

Preliminary screening of all for extracts was done for testing various phytochemicals found in plants. The extracts were tested for the presence or absence of secondary metabolites such as alkaloids, steroidal, flavonoids, tannins and saponins compounds.

These tests were performed according to the method described by Sofowora (1985) and Trease (2002). The tests were based on the extraction with suitable solvents of increasing

polarity and the visual observation of color change or formation of a precipitate after the addition of specific reagents (Sofowora et al., 1985 ; Trease et al., 2002).

3.1. Tests for alkaloids (Dragendraff's Test)

0,2 ml of extract was taken in a test tube with 1,5 ml dilute HCL 2% and 1 ml of Dragendraff's reagent and left for a few minutes. A positive result is indicated by the presence of an orange brown precipitate.

3.2. Test for steroidal compounds (Salkowaski's test)

0,5g of extracts was dissolved in acetic anhydride and then 2 ml chloroform in a test tube. Concentrated sulfuric acid was carefully added on the wall of the test tube to form a lower layer. A reddish brown color at the interface indicated the presence of a steroid ring.

3.3. Test for Tannins

1 ml of extract was added in 2 ml of water in a test tube. 2 to 3 drops of diluted ferric chloride (1%) solution was added and observed for green to blue-green (Cathechic tannins) or a blue-black (Gallic tannins) coloration.

3.4. Test for flavonoids

3.4.1. Test for anthocyanins

To the 5% infused with a more or less dark coloration, 5 ml of 10% H₂SO₄ are added, followed by 1 ml of 10% NH₄OH. If the color increases with acidification, then turns to purplish blue in basic medium.

3.4.2. Free flavonoids

Introduced in a test tube 5 ml of extract, and added 5 ml of hydrochloric alcohol (95% alcohol, distilled water: concentrated HCl) in equal parts, then a few chips of magnesium and 1 ml of isoamyl alcohol. The appearance of a pink-orange (flavones) or purplish pink (flavonones) or red (flavonols, flavononols) indicates the presence of a free flavonoid (genin).

4. Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is a chromatographic technique used to separate the components of a mixture using a thin stationary phase supported by an inert backing. TLC functions on the same principle as all chromatography: a compound will have different affinities for the mobile and stationary phases, and this affects the speed at which it migrates. The goal of TLC is to obtain well defined, well separated spots. Then remove the plates and allow them to dry. The sample spots can now be seen in a suitable UV light chamber.

The extracts were screened on silica gel plates (0.25 mm thickness). Development was carried out with various solvent systems: Tol/AcOEt/MeOH (80/18/2)

After development in the different solvents, the detection was carried out visually in visible light and under UV light (365 nm).

5. Determination of total polyphenols content in plant extracts

Total polyphenols content was determined spectrophotometrically, using the Folin–Ciocalteu reagent according to the method described by Boumerfeg and Baghiani . This method is based on the reduction of a phosphowolframate– phosphomolybdate complex by phenolics to blue reaction products. This reaction occurring under alkaline conditions is carried out with sodium carbonate. Blue coloration is measured at 765 nm and reflects the quantity of polyphenols usually expressed as gallic acid equivalent (GAE).

Briefly, a volume of 200 μ l of the extract was mixed with 1000 μ l of Folin–Ciocalteu reagent (diluted 10% in distilled water). After 4 min, 800 μ l of sodium carbonate solution Na_2CO_3 (75 g/l) was added to the mixture, the reaction mixture was incubated at room temperature for 1h 30 min and the absorbance of the mixture was read at 765 nm, Gallic acid (0-160 μ g/ml) was used as standard for the calibration curve. The total polyphenols content was expressed as microgram of gallic acid equivalents (GAE) per milligram of extract (μ g GAE/mg of extract). All samples were analyzed in three replications.

6. DPPH free radical-scavenging activity

The molecule, 2, 2 – Diphenyl – picrylhydrazyl (DPPH) is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecule do not dimerise, as would be the case with most other free radicals. The delocalization gives rise to the deep violet colour, characterized by an absorption band in methanol solution centered at about 517 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives reduced form with the loss of the violet colour. The pale colour may appear at the end due to still presence of picryl residue, (Maisuthisakul *et al.*, 2007).

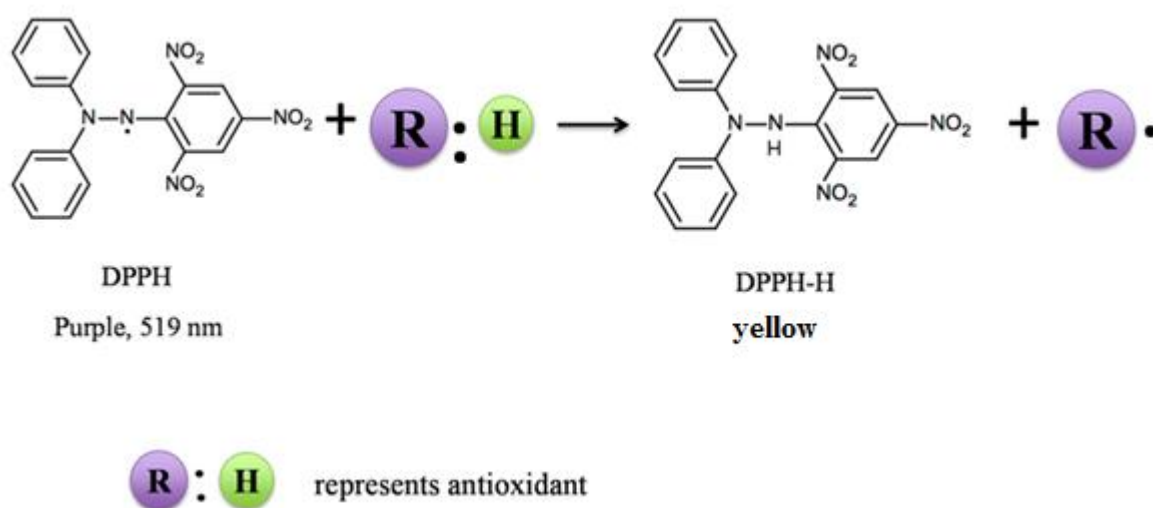


Figure 9: Structure of DPPH and its reduction by the antioxidant.

After dissolving the plant extracts, the solution of DPPH in methanol (0.04 mg/mL) was prepared. 2500 μL of this solution was added to 100 μL of extracts solution at different concentration. The mixture was shaken vigorously and then kept in the dark for 30 minutes at room temperature. Then, the absorbance was measured at 517 nm in a spectrophotometer. All tests were performed in triplicate (Baghiani A, et al 2012). Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula I %.

$$I (\%) = \{(A \text{ blank} - A \text{ sample}) / A \text{ blank}\} \times 100.$$

A blank: Absorbance of the control.

A sample: Absorbance of the reagent with extract.

The scavenging ability of the extracts was expressed as IC₅₀ value, which is the concentration of substrate that causes 50% loss of DPPH activity (color). Low IC₅₀ value indicates strong ability of the extract to act as DPPH scavenger. The three standards antioxidants were using rutin, quercetin and gallic acid.

7. Antibacterial activity

To test the antibacterial property of the sample well diffusion method was used. A positive and negative control was used to compare the results.

7.1. Test organisms

Staphylococcus aureus

Escherichia coli

7.2. Preparation of extract solution for antibacterial activity test

All for of the dried extracts (100 mg) of *Juniperus oxycedrus* that were extracted were dissolved in DMSO to make extract solutions for the antibacterial activity test. For inoculation during the test the stock solution of each extract was freshly prepared.

7.3. Method of antibacterial activity test

In sterile Petri dishes previously poured by media (gelose), a bacterial suspension to be tested was deposited on the surface of the agar plates using a sterile swab. Under aseptic conditions and using a sterile forceps, sterile disks of 6 mm diameter filter paper impregnated with 20µl of essential oil and 15 µl of the various extracts (100 mg) were sterilely deposited on the surface of the media, previously inoculated. The negative control discs are impregnated with distilled water and DMSO. Standard disks containing the reference antibiotic (gentamycin, 10 µg per disc) serve as positive controls. Petri dishes are incubated at 37 ° C for 24 hours (NCCLS, 2001).

7.4. Inhibition zones Measuring

Following 24 hours of incubation of the test plates the clear zones were measured using a ruler. This was done by measuring the entire diameter of the clear zone and the results were recorded.

7.5. Measuring the activity index

The inhibitory effects of the methanolic, ehtanolic, infusion and decoction extracts were calculated and compared by measuring the activity index. This was done by using the following formula:

Activity Index (AI) = Zone of inhibition of extract/ Zone of inhibition of antibiotic.

RESULTS AND DISCUSSION

1. Yield value determination

Yield was calculated and represented in the following table 1.

Table 1: Quantities and yield of *J. oxycedrus* extracts.

| Extracts | InfE | DecE | MeEbS | EtEbS | HMeEbM | HEtEbM | Essential oil |
|--------------------------------|------|------|--------------|-------|--------------|--------|---------------|
| Materiel plant mass | 10 | 10 | 50 | 50 | 40 | 40 | 150 |
| Extracts mass | 1.1 | 1.4 | 11.08 | 8.88 | 9.68 | 8.60 | 0.198 |
| Yield % | 11 | 14 | 22.16 | 17.76 | 19.36 | 17.20 | 0.0198 |

methanolic extract by soxhlet has the highest yield (22.16%), followed by hydromethanolic extract by maceration (19.36%), and then hydroethanolic extract by maceration and ethanolic extract by soxhlet with approximately same results (17.20 and 17.76% respectively), and finally infusion and decoction extracts with lower values.

from point of view yield the percent recovery depends mainly on the type of solvent and the extraction methods being adapted.

The advantage of soxhlet method comparing with the others is that instead of many portions of warm solvent being passed through the sample just one batch of solvent is recycled. In fact the sample phase is always in contact with fresh solvent so enhancing the displacement of target compound from the matrix and the compound are not decomposed due to moderate extraction condition, this is why this method extraction is better than the other methods.

Factors that related with extract solvent that make a solvent is better than another solvent are:

- Solvents with low viscosity have low density and high diffusivity that allows them to easily diffuse into the pores of the plant materials to leach out the bioactive constituents; and
- Solvent polarity (plant constituents and class of aimed secondary metabolite).

2. phytochemical screening resultats

phytochemical screening of *J. oxycedrus* phytochemical is summarized in Table 2.

Table 2: Results of characterization reactions of the main secondary metabolites contained in *J. oxycedrus*.

| compounds | | Results | | | | |
|-------------------|--------------|--------------|------|------|--------|--------|
| | | Powder plant | InfE | DecE | HMeEbM | HEtEbM |
| alkaloids | | + | - | - | + | + |
| tanins | Cathechic | | + | + | + | + |
| | Gallic | | + | + | + | + |
| flavonoids | free | | + | + | + | + |
| | anthocyanins | | + | + | + | + |
| Steroids | | + | + | + | + | + |
| Mucilage | | | - | - | - | - |
| Coumarin | | | - | - | - | - |

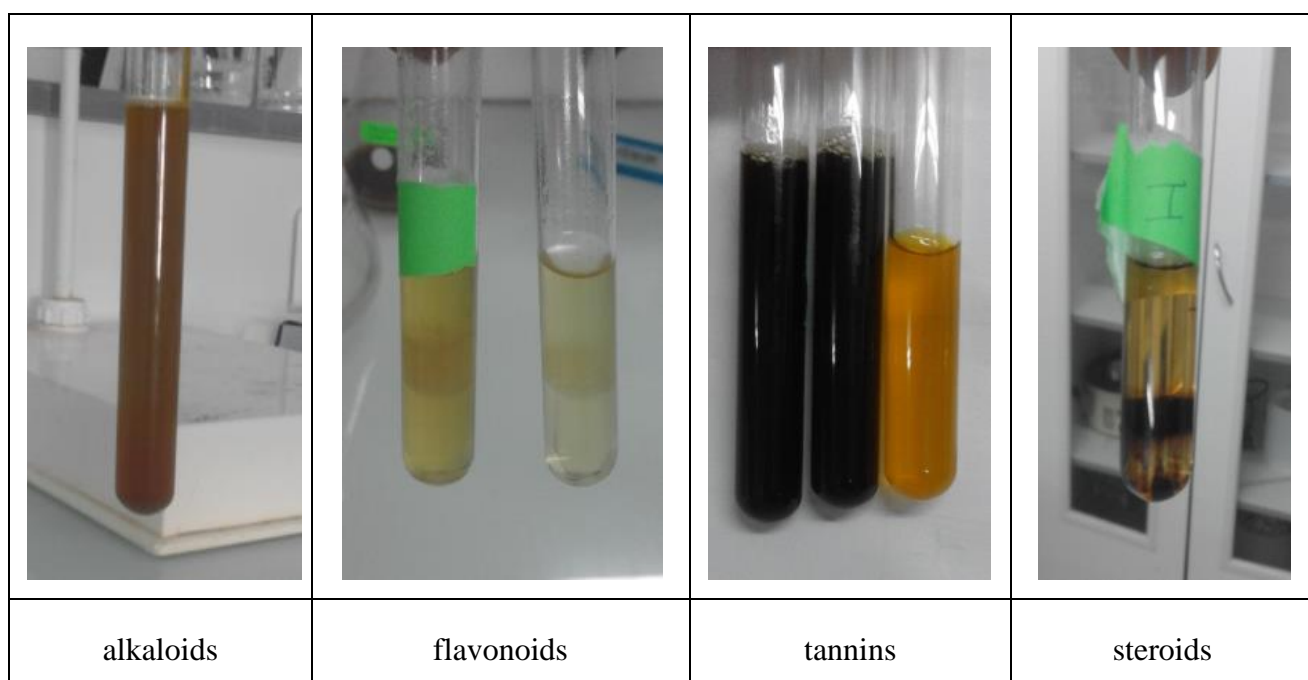


Figure 10: The results images of the characterization reactions of the main secondary metabolites

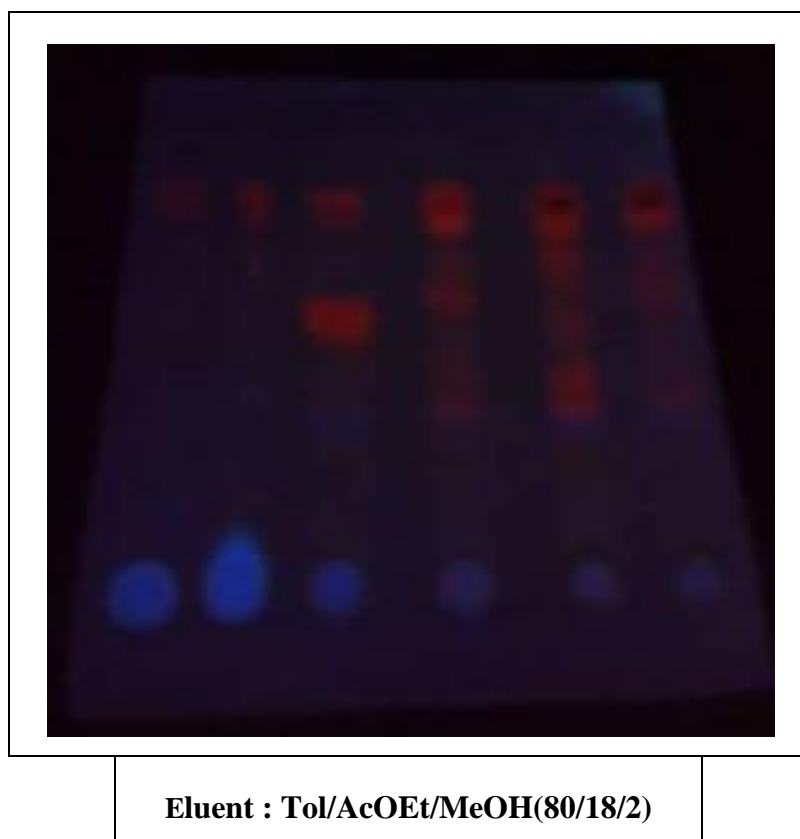
The results reveal the presence of diverse secondary metabolites' like tannins, flavonoids, alkaloids and steroids in the various extract of plant. This diversity explains the large uses in traditional medicine.

3. Thin Layer Chromatography (TLC)

The analysis results of the various extracts obtained from the leaves of *J. oxycedrus* by TLC are presented in fig 11. Solvent systems used to separate several compounds in the extracts (Table 03). Examination under ultraviolet light (365 nm) provides very important information on the structure of molecules. It provides particular indications concerning substitutions. The following table summarizes the relationship between the structure of a flavonic compound and its fluorescence under UV.

Table 3: Results of thin layer chromatography of the extracts.

| Extracts | | | InfE | DecE | HMeME | MHEtE |
|--|--------------|-------|--------|--------|----------------|-----------|
| System Tol/ AcOEt/ MeOH (80/18/2) | Color | Red | Flavon | Flavon | Flavon | Flavon |
| | | Blue | / | / | Flavonol | Flavonol |
| | | Black | / | / | Flavonol 5,6,7 | Anthocyan |

**Figure 11:** TL observed under UV at 365 nm extracts

4. Total phenolic content assay

The evaluation of total phenolic content of different extracts of plant is value in equivalent terms of Gallic acid. Different concentrations of the standards (20 to 160 $\mu\text{g}/\text{mL}$) were plotted versus absorbance. The equation obtained from the linear calibration graph in the studied concentration range for Gallic acid is, $y=0.006x+0.003$, with a correlation coefficient of $R^2=0.986$ (Fig12).

Table 04: Standard curve preparation by using Gallicacid.

| Gallic acid[$\mu\text{g}/\text{ml}$] | Absorbance |
|--|------------|
| 20 | 0.096 |
| 30 | 0.185 |
| 40 | 0.242 |
| 60 | 0.444 |
| 70 | 0.516 |
| 80 | 0.591 |
| 100 | 0.78 |
| 120 | 0.895 |
| 140 | 0.99 |
| 160 | 1.075 |

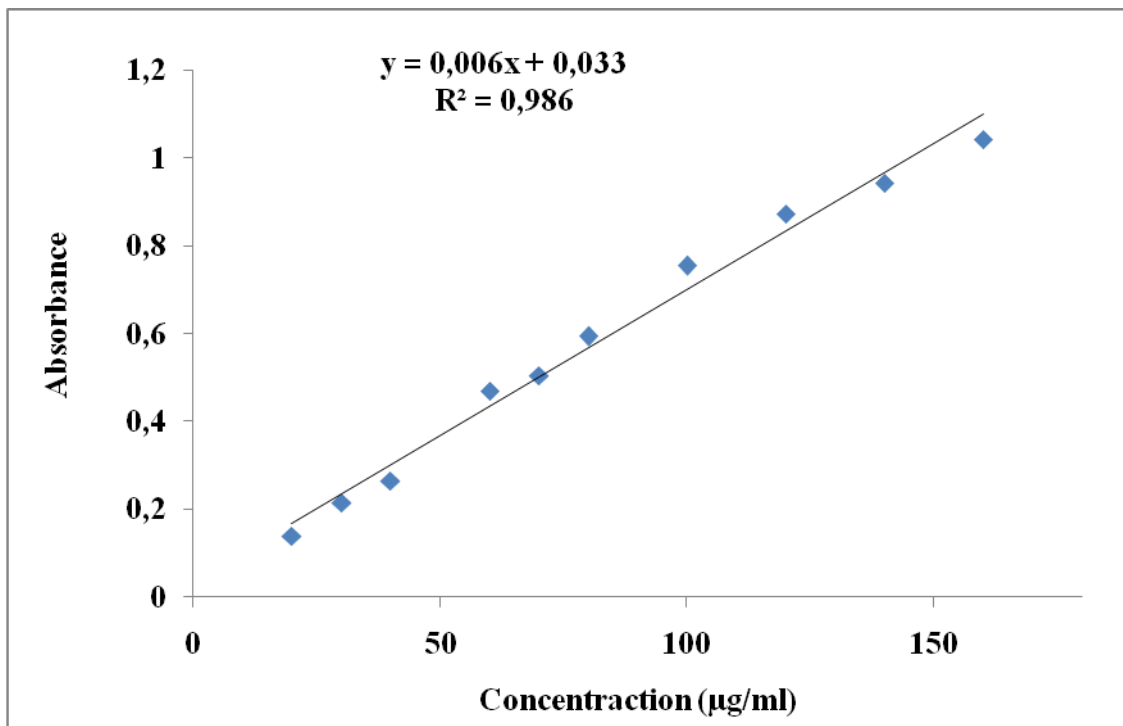


Figure 12: The calibration curve obtained

The results for total polyphenol content of *J. oxycedrus* are presented in fig 13

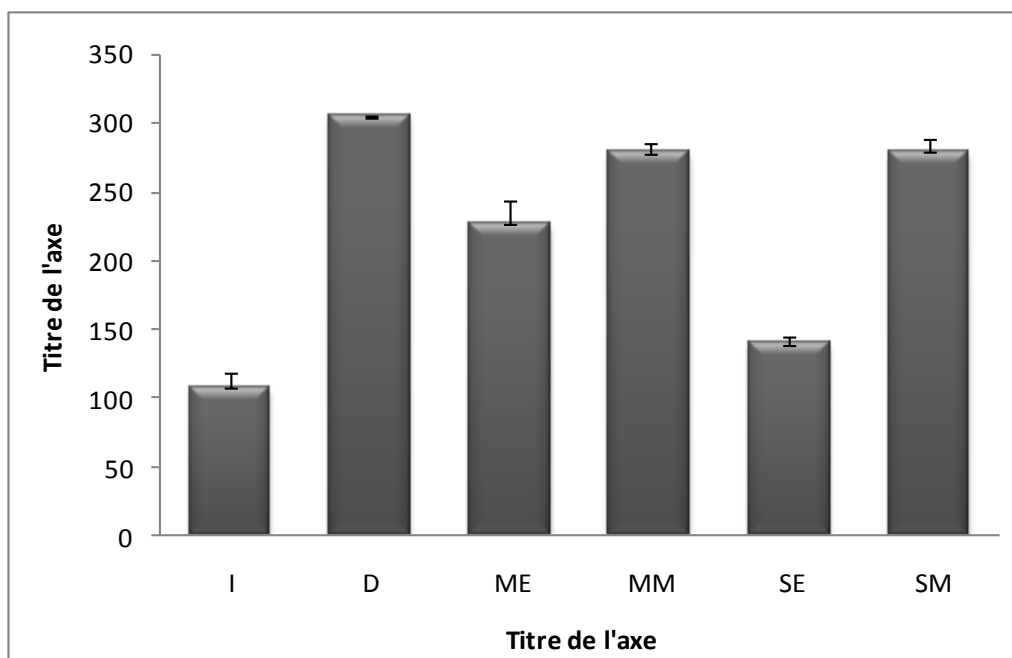
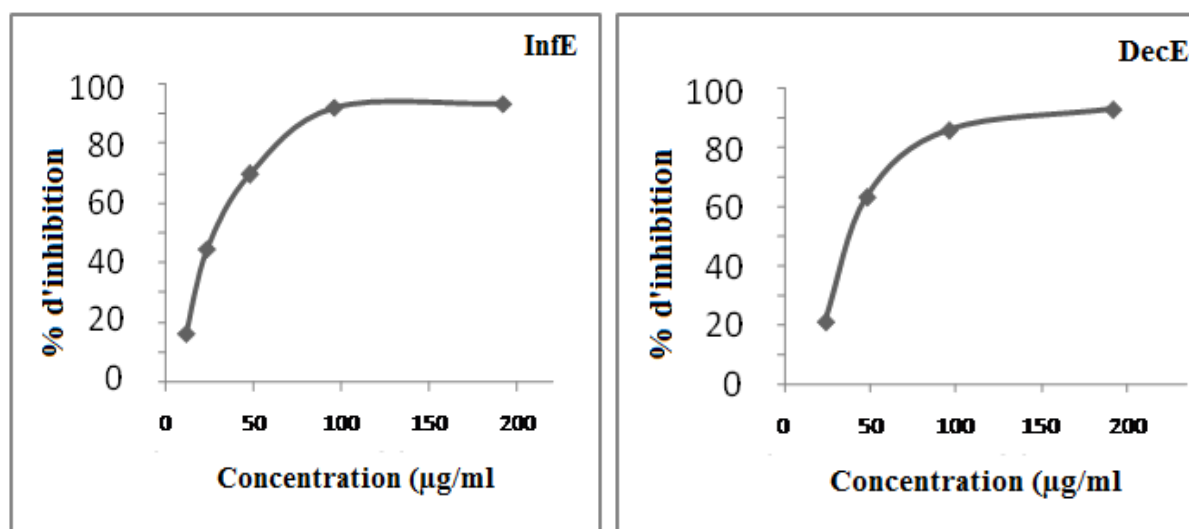


Figure 13: Comparison of total phenolic content of different extracts of *J. oxycedrus*.

Based on the absorbance value of the plant extracts solution reacting with Folin-Ciocalteu phenol reagent and compared with the absorbance values of standard solutions of gallic acid, total phenolics content of the plant extracts was estimated in this order: DecE ($304.54 \pm 0.032 \mu\text{g GAE/g}$) > MeEbS and MeEbM with the same result approximately ($279.29 \pm 8.43 \mu\text{g GAE/g}$) and (279.16 ± 6.35) respectively > EtEbM ($226.8 \pm 16.4 \mu\text{g GAE/g}$) > EtEbS ($139.18 \pm 4.87 \mu\text{g GAE/g}$) > InfE ($107.825 \pm 9.93 \mu\text{g GAE/g}$).

5. DPPH scavenging of extracts of *J. oxycedrus*

Renewed interest in plant antioxidant has emerged during the recent years, probably due to the appearance of undesirable side effects of certain commercial antioxidant. In medicinal plants world, there are a huge number of different types of bioactive compounds with antioxidant activity that play an significant role in terminating the generation of free radical chain reactions. Therefore we interested in to assess the potential of extracts to neutralize free radical by using DPPH (Fig 14).



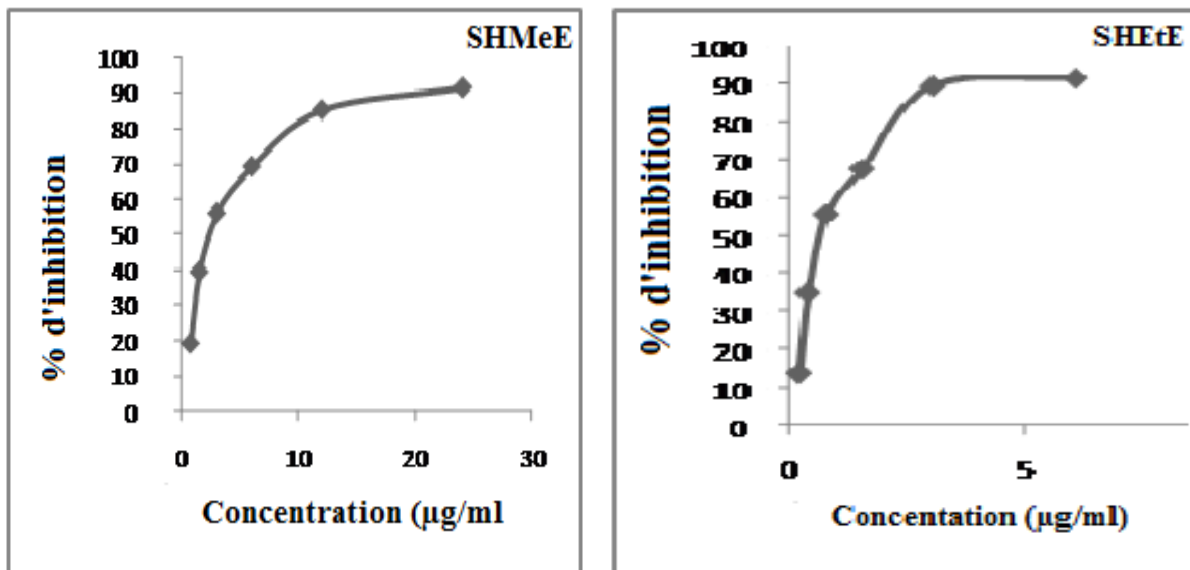


Figure 14: representation of The DPPH scavenging of extracts of *J. oxycedrus*.

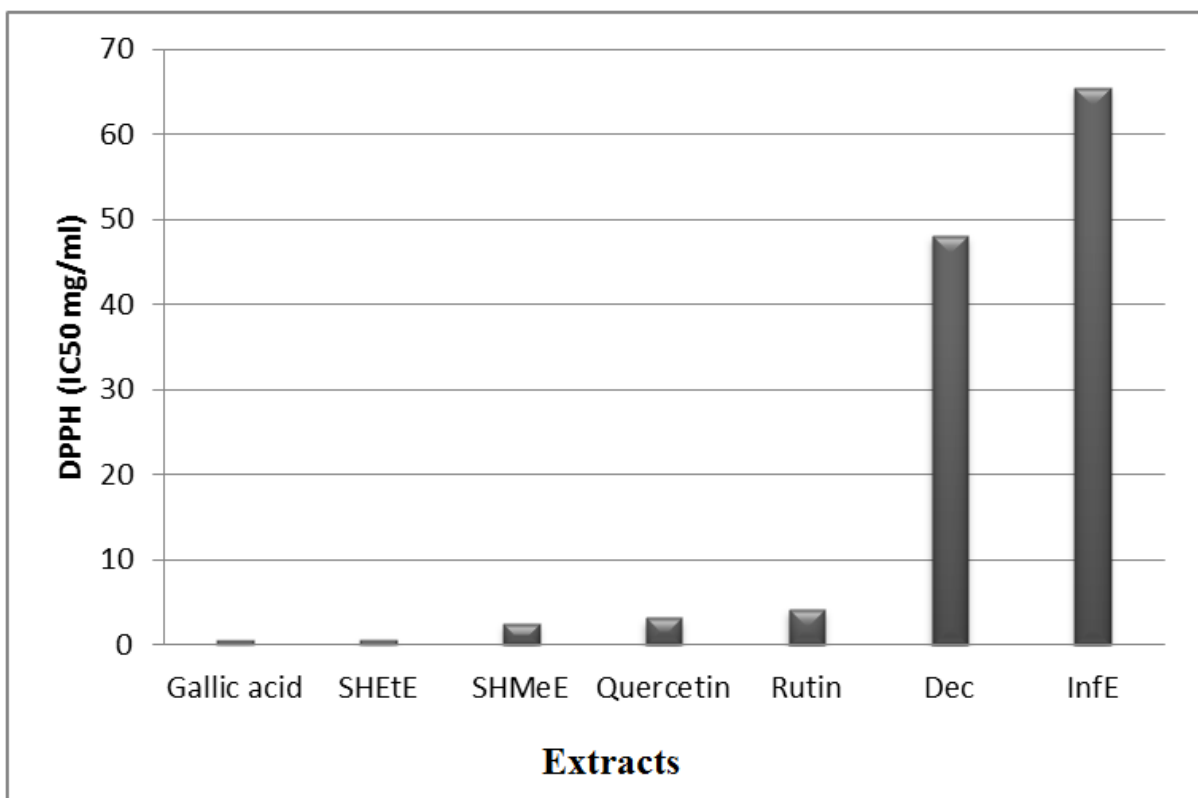


Figure 15: IC₅₀ values of plant extracts for free radical scavenging activity by DPPH method.

One parameter that has been introduced recently for the interpretation of the results from the DPPH method, is the “efficient concentration” or IC_{50} value (otherwise called the IC_{50} value). This is defined as the concentration of substrate that causes 50% loss of the DPPH activity (colour). The IC_{50} of each of the different extracts were determined (fig 15).

The potential of free radical scavenging activities of all extracts of *Juniperus oxycedrus* L. Were in this order: EtEbS>MeEbS>DecE >InfE.

The EtEbS had the highest free radical scavenging activity. It had higher IC_{50} values compared to quercetin and rutin and had approximately the same value with gallic acid. SHMeE showed important antioxidant potential. Finally InfE and DecE with considerable IC_{50} value.

Despite the highest tenor of phenolic compound was found in Dec extract but the Et extract showed better antioxidant activity.

As a result antioxidant activity related not just with tenor of phenolics compound but also by structure of phenolic.

Generally we can conclude that major constituent of phenolics compound in Dec extract up to tannin because this class of phenolic need highly temperature and polar solvent to extract.

The major constituent in EtEbS is flavonoid because this compound who has the potential antioxidant activity.

5. Antibacterial activity test results

Antibacterial activity test for all four types of extract ethanolic, methanolic, infusion and decoction were performed using agar diffusion method. The zone of inhibitions were measured in millimeters (mms) and compared with the zone of inhibitions of antibiotics which were used as positive control. Antibacterial activity of the four different extracts with

positive control has been shown in table 6, and the comparison of zone of inhibition of extracts.

Table 5: inhibition zone of each extract

| Microorganisms | | MeEbS | EtEbS | InfE | DecE | Essential oil |
|------------------------------|----|-------|-------|-------|-------|---------------|
| <i>Staphylococcus aureus</i> | IZ | 11mm | 13mm | 12 mm | 11 mm | 10 |
| | AI | 0.44 | 0.52 | 0.48 | 0.44 | 0.36 |
| <i>Escherichia coli</i> | IZ | - | - | - | - | 9 |
| | AI | - | - | - | - | 0.32 |

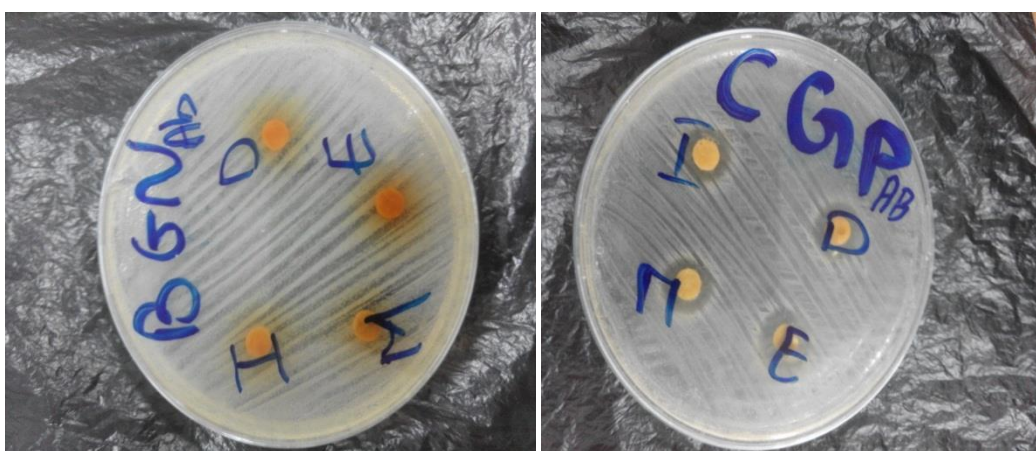


Figure 16: Antibacterial activity of all extract.

- The activity of extract and essential oil on *Staphylococcus aureus* :

For the extracts, EtEbS extract shows a considerable activity with zone of inhibition of 13 mm, followed by InfE with zone of inhibition of 12 mm. While moderate activity was shown by other extract (DecE, SHEtE and) with inhibition zone 11mm. where essential oil has a zone of inhibition with 10 mm.

- The activity of extract and essential oil on *Escherichia coli*:

For the extracts, the effect they have was negative on the growth of *E. coli*, while the effect of essential oil was positive with inhibition zone of 9 mm.

Biological activity of an essential oil is related to its chemical composition. The relation between composition and bioactivity of the essence from the aromatic plants may be attributable both to their major components (alcoholic, phenolic, terpenic or ketonic compounds) and the minor ones present in the oil. It may act together synergistically or antagonistically to contribute to some activity of the tested oil.

The chemical composition of essential oil of *J. oxycedrus* was previously investigated in different regions in the world and the most of these studies indicated that α -pinene was the major component which is known to possess an important antimicrobial activity (Bachir et al., 2017).

Conclusion

Conclusion

The various uses of *J. oxycedrus* in traditional medicine make it an attracting subject for the scientists and researchers that interested in exploring active biological molecules.

The primary phytochemical analysis of ethanolic, methanoic and water extracts revealed the presence of phenol compounds flavonoids, tannins, steroids, and alkaloid, this richest of plant constituent explain the various traditional uses.

Tests by thin-layer chromatography (TLC) showed that the major chemical constituents found in this plant were flavonoids. This result was confirmed by a quantitative analysis based on the dosage of phenolic compounds. The qualitative analysis showed that all extracts are the richest in these molecules.

In result of this study *J. oxycedrus* plant bear a potent antioxidant activity at a concentration level which is related to constituents. Their constituents scavenge free radicals and exert a protective effect against oxidative damage induced to cellular macromolecules because it has shown the presence of number of polyphenols, which are related to their chemical structure and may be responsible of the antioxidant activities

Furthermore this plant also showed an important antibacterial activity, and tests show that the plant extracts may be used effectively as an antibiotic agent against microorganisms such as *Staphylococcus aureus*.

Eventually, the present study reveals the presence of potential antioxidants and antimicrobial properties in the plant extract which could be exploited for pharmaceutical application.

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