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Path

Biotechnology and Molecular Pathology

Theme

Evaluation of the antioxidant activity of leaves extracts of green cypress (*Cupressus sempervirens L.*)

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Dedication

I dedicate this modest work to:

Those who I have loved so much with great affection and I am very proud to have them where all the words in the world cannot express the love and respect I have for them: my dear parents

My dear mother Soumia No dedication can express my respect, my eternal love and my consideration for the sacrifices you have made for me for my education and my well-being. I thank you and Papa Ahmed for all the support and love you have given me since my childhood and I hope your blessing is always with me.

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Rania.

Dedication

Thanks God for his Graces and blessings for everything first.

I dedicate this humble work to my parents, who stood by my side and worked hard in raising me and getting me to where I am now.

I also dedicate it to my little family, my wife who always supports me, and my little daughter.

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Finally, I dedicate it to my coaches & brother (Farouk & Amine), my brother & my training buddy (mustapha) and the entire beni messous Jiu-Jitsu team.

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الحمد لله أولاً على نعمه وبركاته علي في كل شيء.

أهدي هذا العمل المتواضع لوالديّ، اللذين وقفا بجانبي وعملوا بجد لتربيتي والوصول بي إلى ما أنا عليه الآن.

أهديها أيضًا لعائلتي الصغيرة، زوجتي التي تدعمني دائمًا، وابنتي الصغيرة دارين.

أهديها لعمي بوالباني إبراهيم وعائلته المحترمة، وأشكرهم جزيل الشكر على دعمهم.

أهديها لإخوتي : حمزة وعائلته، صلاح الدين، صدام، سرور، أيوب، جمال، علاء، عبد النور، جلال، كريم، عمر، أسامة وياسر.

أخيرًا أهديها لمدربي وأخوي (أمين وفاروق)، أخي ورفيقي في التدريب مصطفى، وكل فريق جيو جيتسو بني مسوس.

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Summary :

The antioxidant are the subject of many works because, in addition to their usage as conservatives in the foodstuffs by replacing synthesis antioxidants, they intervene in the treatment of many diseases.

Within the framework of discovering a new antioxidants from natural sources,we attempted to extract the polyphenols from the *Cupressus sempervirens L*. leaves using different solvents (distilled water, methanol, Aceton and Ethanol), then estimate the total polyphenols from the prepared extracts using Folin-Ciocalteu reagent. The results of total polyphenols estimation revealed the richness of this plant in secondary metabolites, particularly in polyphenols, and that the aqueous extract and the methanolic extract are the richest in total polyphenols, where the best yield was obtained by the methanolic extract (20.4%) followed by the acetone extract (14.96%) and the ethanolic extract (11.48%).

Therefore, *C. sempervirens* L. leaf extract can be used as a natural antioxidant in food, pharmaceutical and cosmetic industries where synthetic antioxidants presently pose a great public health problem.

Keywords: *Cupressus sempervirens L*, leaf extracts, Secondary metabolite, antioxidant activity.

ملخص :

مضادات الأكسدة هي موضوع العديد من الأعمال لأنها بالإضافة إلى استخدامها كمواد محافظة في المواد الغذائية عن طريق استبدال مضادات الأكسدة الصناعية، فهي تتدخل في علاج العديد من الأمراض.

في إطار اكتشاف مضادات أكسدة جديدة من مصادر طبيعية، حاولنا استخلاص البوليفينول من أوراق *Cupressus sempervirens L.* باستخدام مذيبات مختلفة (الماء المقطر والميثانول والأسيتون والإيثانول)، ثم قمنا بتقدير إجمالي البوليفينول من المستخلصات المحضرة باستخدام كاشف فولين- سيوكالتو. أظهرت نتائج التقدير الكلي للبوليفينول ثراء هذا النبات بالمستقلبات الثانوية، خاصة بالبوليفينول، وأن المستخلص المائي والمستخلص الميثانولي هما الأغنى في إجمالي البوليفينول، حيث تحصلنا على أفضل محصول من المستخلص الميثانولي (20.4٪)، يليه مستخلص الأسيتون (14.96٪) ثم المستخلص الإيثانولي (11.48٪).

لذلك يمكن استخدام مستخلص أوراق *C. sempervirens L* كمضاد طبيعي للأكسدة في الصناعات الغذائية والصيدلانية ومستحضرات التجميل حيث تشكل مضادات الأكسدة الاصطناعية حاليًا مشكلة صحية عامة كبيرة.

الكلمات المفتاحية : *Cupressus sempervirens L.*، مستخلصات الأوراق، المستقلبات الثانوية، نشاط مضادات الأكسدة.

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LIST OF ABBREVIATIONS

A: acetone extract

DPPH: 2,2-diphenyl-1-picrylhydrazyl

E: ethanolic extract

ED: (distilled water) aqueous extract

EDTA: ethylenediaminetetraacetic acid

FR: free radicals

GC-MS: Gas chromatography coupled with mass spectrometry

IC50: inhibitory concentrations at 50%

M: methanolic extract.

ROS: reactive oxygen species

INTRODUCTION

Introduction

For thousands of years, humanity has used various plants found in its environment, in order to treat and cure all kind of diseases, these plants represent an immense reservoir of potential compounds attributed to secondary metabolites which have the advantage of being of great diversity in chemical structure and they possess a very wide range of biological activities. However, the evaluation of these activities remains a very interesting task which may be of interest to many studies **(Ogbera *et al., 2010*)**.

The african continent is among the continents that has a great biodiversity in the world, with a very large number of plants used as herbs, natural food and for therapeutic purposes. Many different natural substances have been identified and many have been used in traditional medicine to prevent and treat diseases **(Selim *et al., 2014*)**.

Despite the heterogeneity of the African continent in general and Algeria in particular, there has been little effort devoted to the study of the biological activities attributed to the phenolic compounds of these plants. This is why we were interested in studying *Cupressus sempervirens L.* (The green cypress). A traditional medicinal plant widely used in algeria in terms of the use of its essential oil in traditional treatments, but there are no adequate studies related to the secondary metabolism of its extracts. The dried leaves are used in the treatment of upset stomach, diabetes, inflammation, toothache, laryngitis and as a contraceptive **(Selim *et al., 2014*)**.

Currently, scientific society is highlighting the tragic role of the uncontrollable oxidative process induced by reactive oxygen species (ROS). These oxidants are the direct cause of various disease states such as aging and cancer and indirectly in the peroxidation of lipids in foodstuffs. Whatever the case, the risk is aggravated with the accumulation of these molecules in the body, resulting in a radical reaction chain which degrades the vital biological molecules, as DNA, lipids, proteins and carbohydrates **(Mghezzi *et al., 2016*)**.

Based on this vision, herbal medicine is being revived towards this green wave that produces a host of antioxidants to counter and trap these oxidants.

Indeed, natural antioxidants are the subject of much researchs and a new breath towards the exploitation of secondary metabolites generally and polyphenols, particularly both in health and against pernicious diseases (cancer) and in the food industry. These natural compounds widely distributed in the kingdom are widely sought after for their biological properties: antioxidant, anti-inflammatory, anti-allergic and anti-carcinogenic. Noting that the potent efficiency of these substances in stopping radical reactions by neutralizing free radicals is mainly due to their phenolic structures with the presence of hydroxyl groups **(Koechlin-Ramonatxo *et al., 2006*)**.

This work aims to study the antioxidant activity of leaves extracts of the green cypress *Cupressus sempervirens L*. to answer certain questions and hypotheses, namely :

• The richness of green cypress of algerian biotope in polyphenols ?

• Due to the diversity of bioactive molecules of green cypress do they have an antioxidant property ?

Chapter 1 :
Theoretical part

1. Presentation of cupressus sempervirens L :

1.1. Geographical distribution :

The natural distribution of this cypress is unclear, due to its long horticultural history in the Mediterranean region.

Various authorities attribute its native distribution to the islands of the Aegean Sea (Crete, Samos, Rhodes, Kos and Symi), Cyprus, Turkey, Middle East (Syria, Jordan, Lebanon and Iran), and North-East Africa (Libya , Tunisia), although recent studies on genetic and paleobotanical records suggested that the presence of natural populations from the central Mediterranean.

Today the distribution of C.sempervirens is widespread in all Mediterranean regions and the Middle East, as well as in other regions that have the same Mediterranean climate, notably California, South Africa and southern Australia. It also grows in areas where summers are cooler and wetter.

In natural habitats, C.sempervirens grows in forests, inland valleys and coastal mountains at heights of 500 m to 2000 m **(Belov, 2009; Farjon, 2013**).

Figure 01: Range and distribution of *Cupressus sempervirens L*.

https://doi.org/10.6084/m9.figshare.5101132

1.2. TAXONOMY:

Kingdom: Plantae (Plants)

Subkingdom: Tracheobionta (Vascular plants)

Super division: Spermatophyta (Seed plants)

Division: Coniferophyta (Conifers)

Class: Pinopsida

Order: Pinales

Family: Cupressaceae (Cypress family)

Genus: *Cupressus*

Species: *Cupressus sempervirens L.*

1.3. Botanical description :

Cupressus sempervirens L. is a tree of long life and moderate growth; up to 30 cm in height, a very tall and branching trunk **(Asgary et *al*., 2013)**, with quadrangular branches. Also possessing a gray-brown bark with deeper cracks.

The morphological diversity of the cypress allowed us to distinguish two different appearance forms :

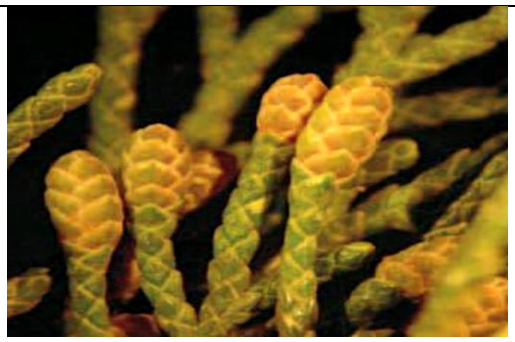
- **Form horizontalis**; characterized by its spreading branches arranged in an irregular crown.
- **Pyramidalis form**; is characterized by its upstanding branches which form a tapered crown **(Boualouana, 2013).**

Figure 2: Cypresses with ascending branches Cypresses with horizontal branches spreading (Original)

The green cypress has dense foliage with dark green leaves; scale-shaped and tightly pressed against the 2 to 5 cm branches formed in opposite-decussate pairs. The trunks of 1 m in diameter, rarely over 2 m, the bark is grey-brown with shallow fissures **(Sebban and Khaldi, 2019).**

It is a monoecious tree; has ovoid yellow male flowers; and globular, female flowers carried by very short twigs.

	
Leaves	Branch



Male folwers

Female flowers

Cones

Axial section of a cone

Seeds

Cypress seeds in an open cone

Figure 03 : Various parts of *Cupressus sempervirens L.* tree

The fruits are ovoid-shaped, about 3 cm in diameter, shiny green when young; then they lignify and turn gray-brown; they are made up of 8 to 12 scales in the shape of a nail head. When the fruits become ripe, they release very small, irregular, ovoid, flattened Pellets, bordered by a narrow wing **(Karine, 1999)**.

The root system well developed, the ability to flourish in both acidic and alkaline soils, and seeds that are easily collected for oil extraction **(Selim et *al* 2014)**.

1.4. Environmental requirements:

Cupressus sempervirens L exist in Mediterranean climates with hot, dry summers and rainy winters, or in semi-arid climates **(Belov, 2009; Farjon, 2013)**.

It is a light demanding species, drought and heat. It grows best in areas with an average annual rainfall of 1000 mm, and the average annual temperature is 15-20° C, but it can withstand an average annual rainfall of less than 600 mm and a drought that lasts for several months (Ducrey et al., 1999), growing with a rainfall rate of only 200 mm per year.

It is often poorly distributed in areas with dry summers. Young plants do not tolerate low temperatures, while adults can survive temperatures down to -20° C. Trees in the forests of Italy and France have shown frost tolerance **(Raddi and Panconesi, 1989).**

These species have the ability to survive covered in snow for several months (Belov, 2009; Farjon, 2009). For optimal growth, clay is needed in the soil, which can vary from acid to alkaline. It prefers well-drained soil and can grow in nutritionally poor soils, but does not grow well in very moist soils rich in organic matter. It cannot grow in shade; it thrives better than other species on rocky, dry and compact soils, although it prefers rich, deep, moist and well-aerated soil with neutral pH, where it is however less competitive.

1.5. Therapeutic interest:

C.sempervirens.L is one of the oldest medicinal plants, this tree is widely used as ornamental plant, due to its resistance to pollution, as well as its wood is widely used in the manufacture of furniture because it is very hard and it does not rot **(karine, 1999)**.

All the parts of this plant are used for industrial interests or as primary material for different products but the most important field is the therapeutic field as the herbal medicine because the Cypress contains several biological constituents which have specific pharmacological properties it is considered like a medicinal plant. The two most used parts are the twigs and the cones **(Riom, 2010)**.

The dry leaves are used in the treatment of stomach pain, diabetes, inflammation, toothache, laryngitis and as a contraceptive **(Selim et *al.*, 2014)**.

C.sempervirens L. has traditionally been used for the treatment of angina and rheumatism **(Zhang et *al.*, 2012)**. In traditional Turkish medicine, the fruits of this plant are used to treat colds and coughs **(Tumen et *al.*, 2011)**.

According to the scientific literature, cypress also has anti-infectious activity, a recent study **(Guinobert et *al.*, 2018)** showed a virucidal effect of the Cupressus sempervirens L. hydroethanolic extract on four viruses causing respiratory tropism in the Human (coronavirus, influenza A-H1N1 virus, parainfluenza virus type 3 and rhinovirus) and three bovine viruses (bovine herpesvirus type 1, bovine respiratory syncytial virus and bovine rotavirus). The authors found that the viruses lost their infectious power. after in vitro contact with the cypress extract for 60 minutes ± 10 seconds at 37 ± 1 ° C. The virucidal effect was recorded on all the viruses tested regardless of their type, naked or enveloped.

Indeed, the cypress is best known for its vasculoprotective and venotonic properties, in particular to treat and prevent varicose veins, hemorrhoids, heavy legs.

2. Plant secondary metabolites:

2.1-general information on secondary metabolites:

One of the major originalities of plants lies in their capacity to produce very diversified natural substances, alongside the classic primary metabolites (carbohydrate, protein, lipid, nucleic acid), frequently accumulate so-called "secondary" metabolites **(Macheix et *al.*, 2005)**.

Primary metabolites are products derived directly from photoassimilates (simple sugars, amino acids, proteins, nucleic acids and organic), which participate in the structure of the plant cell as well as its basic function **(Hopkins, 2003)**.

They are often produced in large quantities but it have relatively low added value. These metabolites are also defined as molecules that are found in all plant cells and necessary for their growth and development **(Raven et *al.* 2000)**.

In contrast, secondary metabolites are not produced directly during photosynthesis, but they are synthesized from primary metabolites as a result of subsequent chemical reactions.

These bioactive molecules are produced in different places of the cell in specific parts of the plant depending on the stage of development (for example during the development of the

seedling, flower, fruit, seed, or root) . In general, the role of the secondary metabolite is linked to its location within the plant **(Pathak et *al*. 1962; Zobel and Brown, 1990)**.

Secondary metabolites are classified into three groups: phenolic compounds, terpenes and alkaloids **(Krief, 2003; Haven et *al*., 2000)**.

3. Phenolic compounds :

3.1. General information on phenolic compounds :

Phenolic compounds or polyphenols (PP) are products of secondary metabolism in plants **(Fleuriet, 1982; Yusuf, 2006)**.

The expression "phenolic compounds" is used for all chemical substances having in its structure an aromatic nucleus, carrying one or more hydroxyl groups.

They participate in the defense of plants against environmental attacks; this is why 80% of phenolic compounds are mainly located in the epidermal tissues of plants.

These are phyto-constituents, generally pigments, responsible for the autumnal colors of the leaves and the colors of the flowers and fruits (yellow, orange, red, …etc).

They are associated with many physiological processes: cell growth, differentiation, organogenesis, bud dormancy, flowering, tuberization. These compounds also play an important role in the food quality of fruits and thus determine their flavor. Polyphenols are distributed in all parts of the plant: roots, stems, flowers and leaves **(Lugasi et *al*., 2003)**.

3.2. The structure of phenolic compounds:

Phenolic compounds are a category of organic molecules specific to the plant kingdom. The term “polyphenols” is frequently used to refer to all the phenolic compounds of plants. while it should be specific only for molecules with several phenol functions.

Therefore, the general designation “phenolic compounds” relates to mono, di and polyphenols, the molecules of which respectively contain one, two or more phenolic functions.

The term "phenol" encompasses approximately 10,000 identified bioactive molecules. The fundamental structural element that characterizes them is the presence of at least one phenolic nucleus (benzene ring) of 6 carbon, which is directly linked at least one free hydroxyl (OH) group or involved in another function: ether, ester or heterosides. Phenolic compounds are responsible for the aroma, color and antioxidant properties of plants.

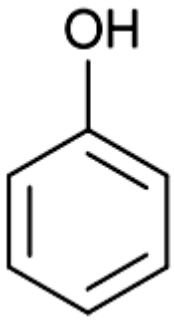


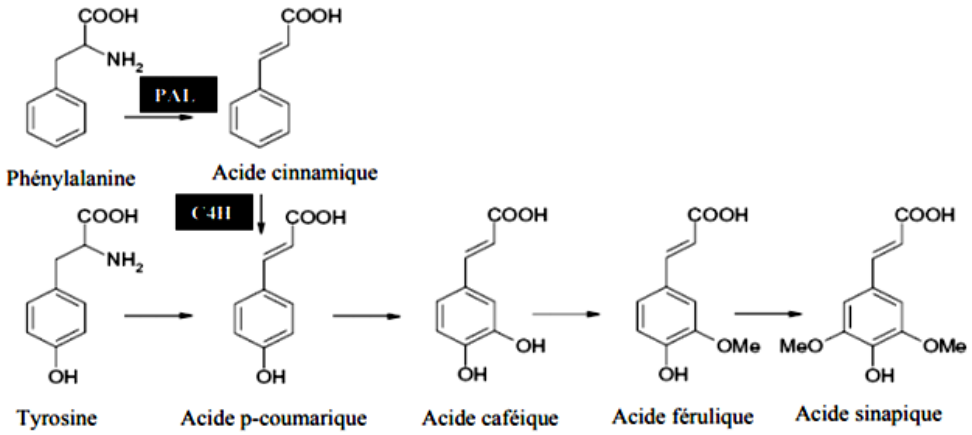
Figure 4: Basic structure of a phenol (Alfa Aesar)

3.3. Biosynthesis of phenolic compounds:

Phenolic compounds are derived by two major metabolic pathways: the shikimic acid pathway and the acetate / malonate pathway **(Macheix et *al*., 2005)**.

➢ Shikimic acid pathway :

The most common pathway is that which, via shikimate (shikimic acid) leads to aromatic amino acids (Phe, Tyr and Trp) then by deamination of the latter to cinnamic acids and to a large number of derivatives, acids benzoates, acetophenones, lignans and lignins, coumarins **(Brunton, 2009)**.



PAL: phenylalanine ammonia-lyase; C4H: cinnmate 4-hydroxylase

Figure 05: Biosynthesis of the most widely distributed phenolic compounds via the shikimate pathway **(Crozier et *al*., 2006)**.

- **Acetate / malonate pathway :**

Glycolysis and β-oxidation lead to the formation of acetyl CoA giving the malonate. It is through this route that the cyclization of the polyketone chains takes place, obtained by repeated condensation of "acetate" units which is carried out by carboxylation of acetyl-CoA. This reaction is catalyzed by the enzyme acetyl-CoA carboxylase **(Akroum, 2010)**.

3.4. Classification of phenolic compounds :

The classification of these substances was proposed by **Harborne in 1980**, on the one hand is based on the number of constituent atoms, and on the other hand on the basic skeletal structure. Three main classes of phenolic compounds are prevalent :

1- The phenolic acids.

2- The flavonoids.

3- The tannins.

4. Main classes of phenolic compounds :

4.1. The simple phenolic acids :

4.1.1. Hydroxybenzoic acids :

• Are derivatives of benzoic acid.

• Have a general basic structure of type (C6-C1)

• Often existing as esters or glycosides.

• The most abundant hydroxbenzoic acids are: benzoic acid, p-hydroxybenzoic acid, protocatechic acid, vanillic acid, gallic acid, syringic acid, salicylic acid, gentisic acid.

4.1.2. Hydroxycinnamic acids :

• Derived from cinnamic acid.

• Have a general basic structure of type (C6-C3).

• Often exist in combination with organic molecules.

• The degrees of hydroxylation and methylation of the benzene ring lead to a significant chemical reactivity of these molecules, the main hydroxycinamic acids: cinnamic acid, P coumarque acid, caffeic acid, ferulic acid, sinapic acid.

4.1.3. Coumarins :

• Coumarins are derived from hydroxycinnamic acids by internal cyclization of the side chain.

• Coumarins frequently have an ecological or biological role.

4.2. The flavonoids :

The flavonoids are compounds consist of basic structure of fifteen carbon atoms, made up of two aromatic rings and a central pyran-type heterocycle, forming a C6-C3-C6 structure **(Figure 06) (De Souza et *al.*, 2004)**.

These are the most abundant compounds among all phenolic compounds. They are involved in the pigmentation of flowers and in the processes of defense against UV radiation, herbivores and microbial attacks **(Korkina et *al.*, 1997)**. Flavonoids are present in a wide variety of foods (fruits and vegetables, cereals, fruit juices, tea and wine, …etc).

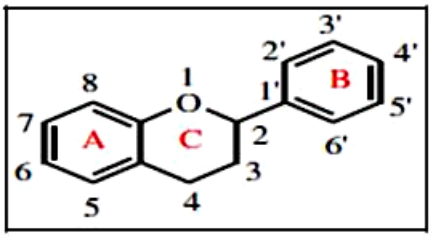


Figure 06: Basic structure of flavonoids **(Korkina et *al.*, 1997)**

4.2.1. Classification of flavonoids :

Depending on the position of the bond of the aromatic ring with the benzopyran radical (chromano), this group of natural products can be divided into three classes: **(Grotewold, 2006)**.

- Flavonoids (2 phenylbenzopyrans),
- Lesisoflavonoids (3-benzopyrans)
- Lesneoflavonoids (4- benzopyranes)

The main categories of flavonoids are defined by :

- The presence or absence of a double bond between carbons 2 and 3 of the C ring, which determines the flatness of the molecule. Flavones, flavonols and derivatives have a double bond and are flat molecules, unlike flavans, flavanones and derivatives.

- The presence of ketones, alcohols and methoxy functions **(Gravot, 2009)**.

4.3. Tannins :

They are high molecular weight phenolic substances, used since antiquity by human for the treatment of skins due to their ability to bind to proteins and to precipitate them, they have a bitter and astringent flavor due to the precipitation of proteins. salivary **(Guignard et *al*., 1985)**. They can sometimes bind to alkaloids **(Paris et *al*., 1981)** chemically, tannins can be classified as:

1. **Hydrolyzable tannins :** which can be degraded by chemical or enzymatic hydrolysis and give a phenolic part which is gallic acid or else elagic acid and a non-phenolic part (glucose, quinic acid).
2. **Condensed tannins :** which are oligomers or polymers of flavone 3-ols, resistant to hydrolysis, and only strong chemical attacks that can degrade them (hot acid treatment) **(Sarni-Manchado et *al*., 2006)**, they are transformed into reddish pigments following the absence of sugar in their molecules **(Paris et *al*., 1981)**.

4.4. Stilbenes :

The occurrence of stilbenes in the human diet is quite low, One of the best natural stilbenes polyphenols that studied is resveratrol (3,4 ', 5-trihydroxystilbene), or trans-resveratrol found largely in grapes, soybeans , peanuts and peanut products **(Pandey et *al*., 2009)**.

5. Properties of phenolic compounds :

Due to the structural diversity of polyphenolic products; these compounds vary considerably in their physicochemical properties, even though they share the common phenolic characteristic **(Tso., 2010)**.

The two fundamental properties that all classes of polyphenols share are :

- The reducing properties which are the basic of the ability of these substances to trap oxygen species (antioxidant activity) and their ability to oxidize **(Monchado et *al.*, 2006)**.
- Complexing properties : the metal complexation of polyphenols is likely to limit the intestinal absorption of metal ions of biological importance such as iron **(Bruneton., 1999)**.

In principle Phenolic compounds can be involved in :

- Certain aspects of the physiology of the plant (lignification, regulation of growth, molecular interactions with certain symbiotic or parasitic microorganisms...).

- In the interactions of plants with their biological and physical environment (relations with bacteria, fungi, UV resistance); either directly in nature or during the conservation after harvest of certain plants **(Fleuriet et *al.*, 2005)**.

Nowadays, polyphenols are widely studied in the medical field where they have been found to have anti-tumor, anti-allergic anti-inflammatory and anti-cancer activities. They are also active in obesity and diabetes **(Dangles., 2006)**.

6. The antioxidant activity of polyphenols :

6.1. The antioxidants :

An antioxidant is a natural or synthetic molecule that is able to inhibit the oxidation of other molecules by intervening at different stages of the oxidation process **(Rahman et *al.*, 2015)**. An antioxidant can therefore: prevent the synthesis of free radicals by inhibiting the initiation of reaction chains or directly deactivate reactive oxygen species (ROS). The antioxidants can be classified according to their modes of action :

- enzymatic systems

- oxidizing enzyme inhibitors

- metal chelators and free radical scavengers.

Antioxidants are a heterogeneous group made up of endogenous antioxidant systems, enzymatic or not, vitamins, trace of elements or even polyphenols **(Thomas., 2016)**.

6.2. Oxidative stress :

Oxidative stress in biological systems is defined as a complex process that occurs due to an imbalance between the production of free radicals (FR) and the body's ability to eliminate these reactive species with the help of endogenous antioxidants and exogenous **(Norma Francenia et *al*., 2019)**, or the shift in the balance between oxidants and antioxidants in favor of oxidants.

6.2. Free radicals :

The free radical (FR) is a molecule or an atom which has one or more unpaired or single electrons, It reacts spontaneously with other atoms or molecules to form a new radical thus causing a chain reaction which is only interrupted when two radicals react to each other.

The radicals are unstable, very reactive species that have an extremely short half-life 10-9 .10-6 sec **(Pierre et *al*., 1997)**.

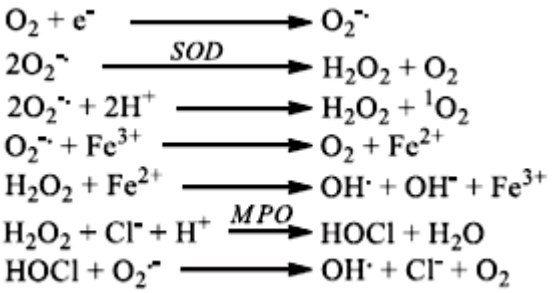
These substances can cause chain reactions involving a number of Steps, each of which forms a free radical that leads to the next step **(Ozcan & Ogun, 2015)**.

➢ The main free radicals :

- The superoxide radical: it arises from the combustion of sugars and fats.

- The peroxynitrite radical: it is produced from nitrogen by white blood cells, these cells contained in the blood and whose main role is to protect the body against infections.

- The hydroxyl radical: it results from the action of solar radiation on our skin, especially when it is not well filtered by the nitrogen layer. It can also be synthesized by reaction of iron or copper with vitamin C. It is to this radical that most of the lesions of aging are attributed. Nitric oxide, oxygen peroxide, singlet oxygen ... are other free radicals that occur at different levels **(Céline, 2004)**.



(*SOD = superoxydedismutase; MPO = myeloperoxidase)*

Figure 07: Main ROS present in the body **(Maurent, 2017)**

6.3. Effects of free radicals

6.3.1. Beneficial effects

Free radicals are known primarily for their deleterious effects, but they are also essential for the functioning of our body. However, the benefits of FR require low concentrations in the cellular medium. Below are some physiological examples involving the necessary presence of free radicals.

- ***Role in muscle contraction :*** They are involved in the mechanism of muscle contraction. Some scientific studies have shown that RLs act on the excitation-contraction coupling at the level of muscle fibers **(Commeauet Matteis, 2017)**.
- ***Immune role:*** They play a role in the course of the immune reaction. They are produced by phagocytic cells to be used in the fight against bacteria and parasites. Phagocytosis is accompanied by a production of reactive oxygen species so sudden and intense that it is known under the name of "oxidative burst", i.e. oxidative explosion **(Commeauet Matteis, 2017)**.

6.3.2. Deleterious effects

The production of excess free radicals can lead to several processes, including mutagenesis, cell transformation, cancer, arteriosclerosis, myocardial infarction, diabetes, inflammatory diseases, central nervous system disorders and cellular aging **(Francenia et *al.*, 2019; Antonio, 2013)**.

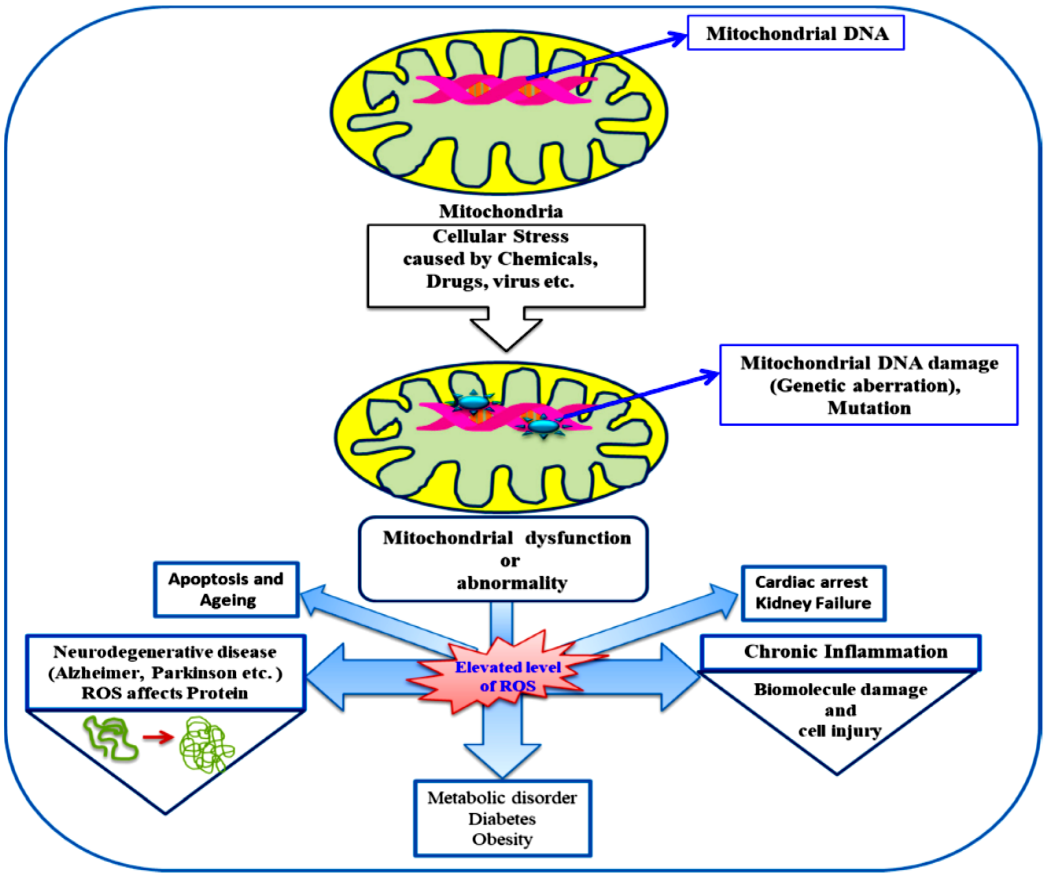


Figure 08: Involvement of oxidative stress and free radicals in diseases **(Singh et *al.*, 2019)**.

The mechanisms of these deleterious effects are listed below :

- **DNA oxidation:** The consequences of free radicals on DNA can participate in mutagenesis, stopping cell divisions by blocking replication mechanisms, stopping protein synthesis by blocking transcription / translation mechanisms, and finally to cell death **(Dizdaroglu et *al.*, 2017)**.
- **Oxidation of proteins:** The modifications of proteins cause the introduction of a carbonyl group in the protein which leads to a structural alteration of the proteins, the consequences of which are major: loss of catalytic function, increased sensitivity to proteases, etc **(Commeauet Matteis, 2017)**.
- **Oxidation of lipids:** Oxidation of lipids, or lipid peroxidation, corresponds to the oxidative deterioration of double bonds of unsaturated fatty acids found in polyunsaturated fatty acids (PUFA). At the cellular level, all the components of the cell are affected and in particular the plasma, mitochondrial and lysosomal membranes. Lipid peroxidation thus induces a disturbance in the structure and

composition of the cell membrane, which is most often manifested by an increase in membrane permeability.

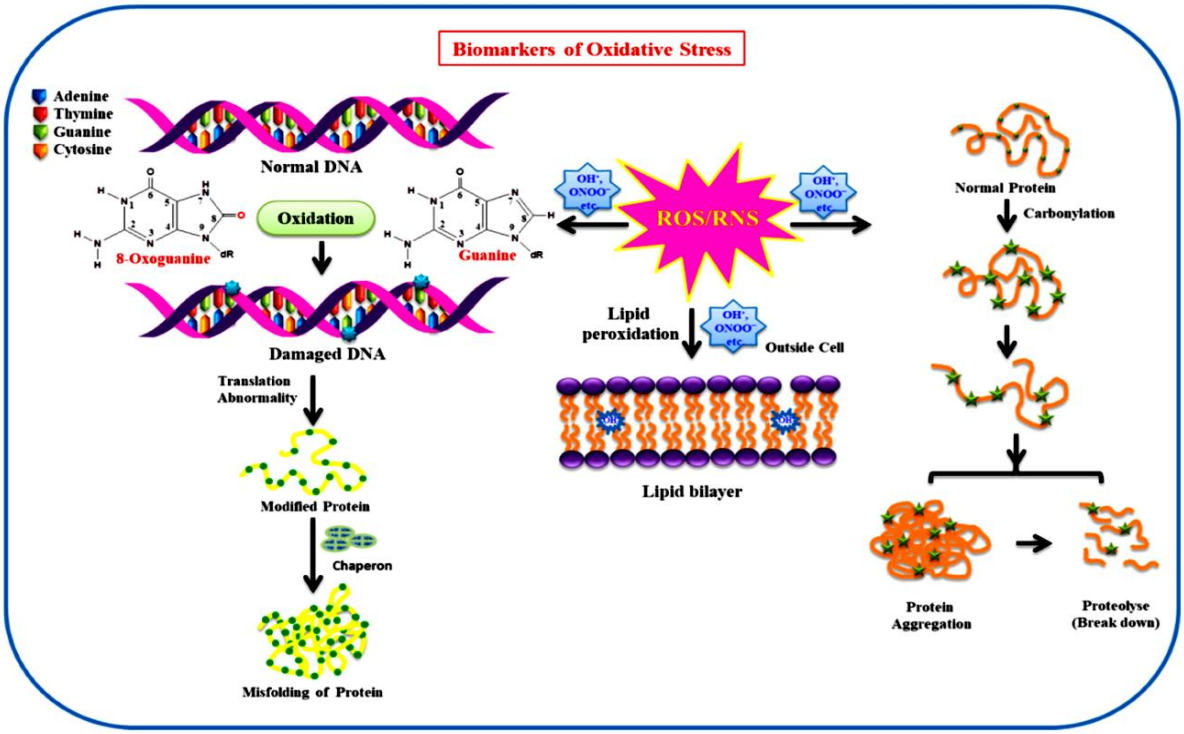


Figure 09: Deleterious effects of free radicals on the organism **(Singh et *al.*, 2019)**

7. Antioxidant defense

Under the term "antioxidant", we regroup together any substance present at low concentration compared to the oxidizable substrate, which is capable of delaying, preventing, neutralizing or reducing the damage of oxidation caused by free radicals in the body and allow to maintain non-cytotoxic concentrations of ROS in the cell **(Halliwell, 1999**).

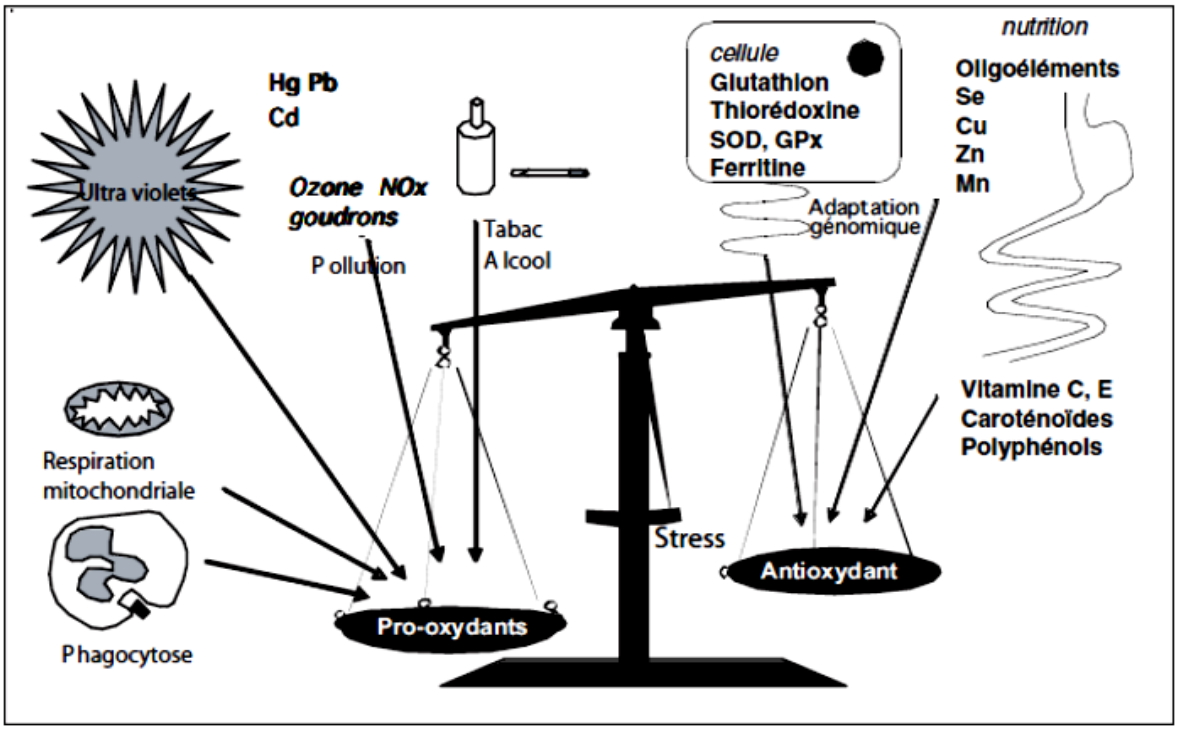


Figure 10: The equilibrium balance between pro and antioxidant systems **(Favier, 2006).**

To protect itself from the deleterious effects of ROS, the body has a complex set of antioxidant defenses. There are two sources of antioxidants: one is provided by the diet in the form of fruits and vegetables rich in vitamins C, E, carotenoids, ubiquinone, flavonoids, glutathione or lipoic acid; The other is endogenous and consists of enzymes (superoxydedismutase, glutathione peroxidase, catalase), proteins (ferritin, transferrin, ceruleoplasmin, albumin) and oxidative damage repair systems such as endonucleases. In addition, there are some trace of elements such as selenium, copper and zinc which are cofactors of antioxidant enzymes **(Haleng et *al.*, 2007)**.

The size of oxidative damage is closely related to the effectiveness of anti-oxidative defenses **(Zeghar and Sahnoun, 2013)**.

8. Antioxidant activity of polyphenols

Typical phenolic compounds possessing antioxidant activity belong to two main classes which are: phenolic acids and flavonoids **(Wojdylo et *al.*, 2007)**.

➢ Phenolic acids :

Hydroxycinnamic acids have a higher antioxidant activity compared to their isotope hydroxybenzoic acids. The greater antioxidant activity of hydroxycinnamic derivatives is linked to the presence of a side propionic chain in place of a carboxylic function, moreover the double bond can stabilize the phenoxy radical by increasing their antioxidant activity.

➢ Flavonoids :

Several modes of action of the antioxidant activity of flavonoids have been described :

• Scavenging activity :

Three proposed mechanisms which phenolic antioxidants can play their scavenging activities :

1- The first mechanism involves the direct transfer of the hydrogen atom from the antioxidant.

2- The second mechanism concerns the transfer of a single electron from the antioxidant to the radical leading indirectly to the abstraction of hydrogen atom.

3- The third mechanism is conditional on the electron transfer sequence to lose the proton.

These three mechanisms can take place in parallel but with different speeds (**Mohajeri and Asemani, 2009)**.

• Chelation of transition metals :

The antioxidant power of phenolic compounds can be preformed by the complexation of transition metals (example: copper and iron). Indeed, the latter accelerate the formation of ROS. **(Laguerre M. et *al.*, 2007)**.

• The inhibitory activity of enzymes :

Flavonoids are known for their ability to inhibit enzymes, in particular, oxidoreductases which involve radical species during their catalytic cycle (lipoxygenase, cyclo-oxygenase, monoxygenase, xanthine oxidase, phospholipase A2, protein kinase) **(Ghedira, 2005)**.

The structural elements necessary for obtaining optimal antioxidant activity have been established by several authors **(Aliaga and Lissi, 2004; Sroka, 2005; Chebil,. 2018)**. It is :

→ the presence of a catechol function on the B ring.

→ the presence of an enone unit in the C ring (double bond between C2 and C3 and the carbonyl function in C4).

→ the presence of hydroxyl group in position 3.

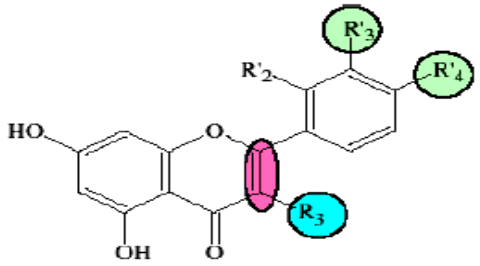


Figure 11: Essential elements for the antioxidant activity of flavonoids **(Chebil, 2018)**

9. Antioxidant activity evaluation methods:

Several methods are available to measure the antioxidant activity of foods and biological systems **(Ali et *al*., 2008; Scherer and Godoy, 2009)**. They can be classified into two groups according to two mechanisms:

- either by the transfer of a hydrogen atom or by the transfer of a single electron **(Sanchez-Moreno, 2002; Huang et *al*., 2005)**. The techniques of the first group are employed to assess lipid peroxidation using a lipid or lipoprotein substrate. The quantification of this property is expressed by measuring the degree of inhibition of oxidation **(Sanchez-Moreno and Larrauri, 1998)**.

So, the methods of the second group are those which intervene in the measurement of the ability of the scavenging of free radicals. They include scanning for hydrogen peroxide (H2O2), hypochlorous acid (HOCl), hydroxyl (OH), superoxide anions (O 2), peroxyl (ROO) and nitric oxide (NO) **(Sanchez-Moreno, 2002)**.

The main tests are summarized in the table below :

Table 01: The main tests of antioxidant activity; Principle, advantages and disadvantages

(Fernandez et *al*., 2012)

Method	Principle	advantage	disadvantages
TEAC (Trolox Equivalent Antioxidant Capacity)	Colorimetric measurement of the electron transfer of an antioxidant onto the ABTS radical cation, expressed in TEAC	Simple method to implement, useful in sreening and in routine	Antioxidant / free radical interference possible, ABTS radical not representative because it's absent from biological systems
Test DPPH (2,2-diphenyl-1-picrylhydryzyl)	Colorimetric measurement (absorbance at 517 nm) of the reducing capacity of an antioxidant in the presence of DPPH free radical, expressed as IC_{50} (concentration required to reduce DPPH by 50%)	Inexpensive full test (commercial DPPH), applicable to simple and complex samples and other techniques (bioautography for example)	Interferences possible, relatively selective (mainly polyphenols), Relatively long (20 min-6h)
ORAC index (oxygen radical absorbance capacity)	Measurement of inhibition of hydroxyl radicals formed by the hydrophilic generator AAPH, thanks to the decrease in fluorescence of fluorescein. Expression in TEAC (comparison with trolox in parallel)	Standardized and commonly accepted method	Relatively expensive method (expensive equipment), long and sensitive to pH
TRAP index (Total radical trapping antioxidant parameter)	Measurement of oxygen consumed during lipid peroxidation. Thermal decomposition of AAPH in the presence of a fluorescence indicator. TRAP value expressed by comparison with trolox	Simple reproducible and sensitive method	Interference between antioxidants and fluorescent indicators, latency period
FRAP (Ferric reducing antioxidant power)	Iron reduction test: measurement of the reduction of a ferric complex to Fe^{+2} by the antioxidant (absorbance measurement at 594 nm)	Fast, inexpensive, repeatable test, applicable to biological solutions and pure antioxidants	Average reliability according to the redox potential of the compounds tested, low pH sometimes incompatible
Index of folin-cio-calteu total phenols determination of	Measurements of total polyphenols (expressed in gallic acid equivalent) and of the reducing capacity of a sample using the folin-ciocalteu reagent. Absorbance at 720 nm proportional to the rate of phenolic compounds	Simple and sensitive method, reproducible	Possible interference because reactive not specified, not applicable to lipophilic compounds and matrices

Chapter 2

Materials & Methods

1. Objective :

In the context of valuing natural extracts of green cypress and looking for natural antioxidants, our study aims to study the chemical constituents, in particular "polyphenols" and to estimate in vitro the antioxidant activity of organic extracts and aqueous extract prepared from the leaves of this plant; *Cupressus sempervirens L*, To achieve our goal we have followed the following steps:

- Extraction and determination of polyphenols from three prepared extracts of green cypress (*Cupressus sempervirens L*) leaves
- Phytochemical study of the prepared extracts
- Evaluation of antioxidant activity by the DPPH test

This study was carried out at the phytopharmacy, plant protection and zootechnics laboratory, Biotechnology Department (SNV Faculty) of Saad Dahlab Blida University 1.

2. The period and the place of the study :

Our study was planned to be carried out in over three months period, ranging from 03/01/2020 to 06/30/2020 at the level of :

• phytopharmacy, plant protection and zootechnics laboratory of the biotechnology department of the University of Blida 1 for the extraction of polyphenols.

• In the Center of Scientific and Technical Research in Physico-chemical Analyzes (CRAPC) of Bou-Ismaïl to carry out high performance liquid chromatography (HPLC) and the antioxidant activity of the prepared extracts , but because of the sanitary situation caused by COVID-19 epidemic we were only able to carry out 20 days of practice so we completed the extraction and dosage of polyphenols part only.

3. Biological material

During this work, the green cypress (*Cupressus sempervirens L*) leaves from the Cupressaceae family were collected to evaluate the antioxidant activity of their phenolic extracts. The medicinal plant in this study was selected on the basis of their use in traditional medicine and their richness in phenolic compounds.

4. Methods :

4.1. Preparation of plant material

4.1.1. Harvest

The leaves of the *Cupressus sempervirens* plant were collected during the month of December 2019 at the level of the biotechnology department (SNV faculty) of Saad Dahlab University of Blida. The geographic location of the harvest site is illustrated in Figure 08. The botanical identification of the collected samples was carried out by experts from the trial garden of El Hamma, Algiers.

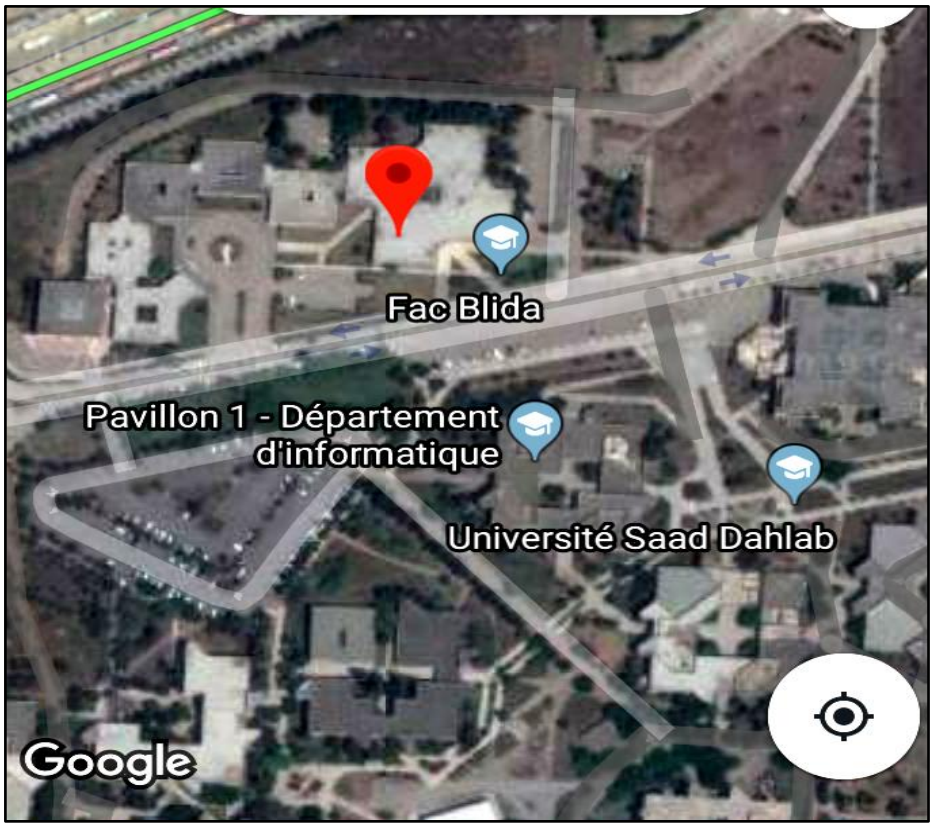


Figure 12: Geographical position of the *Cupressus sempervirens L* leaf harvesting site

4.1.2. the Drying and the grinding

The plant material is freed from debris, washed with water and dried in the open air at room temperature and protected from light for ten days. All these operations make it possible to overcome the degradation of the phenolic compounds.

The advantage of the drying is to avoid any harmful effects due to excess humidity which promotes the development of molds. The dried plant material was then crushed by an electric grinder.

4.1.3. Sieving

When dried, the leaves are reduced to a fairly fine powder using an electric grinder. The latter is sieved to obtain particles of more or less homogeneous sizes. The powder thus obtained is stored in tightly closed jars, protected from light and moisture to use later.

Figure 13: Dried leaf powder (Original 2020)

5. Polyphenols extraction :

The extraction of the polyphenols from the *Cupressus sempervirens* L leaves, was carried out by solid-liquid extraction method in order to release the polyphenols present in vacuolar structures by disruption of the plant tissue and by diffusion. The polyphenols extraction protocol was described by **(Debib and Boukhatem, 2017)** with some modifications to the protocol.

5.1. Preparation of crude extracts :

• Maceration

50g of plant material, crushed so that the contact surface with the solvent is as large as possible and therefore the extraction yield is the best possible, were macerated in 500 ml of the solvent; Aceton (60%) to prepare solution 1 (acetone solution), and to prepare the 2ed solution (ethanol solution), the same experimental steps used previously in preparing the acetone solution are applied, but by using ethanol (60%) instead of acetone. The same for solution 3 and 4 distilled water,or methanol (60%) are added as solvents.

They underwent mechanical agitation for 24 hours using a propeller stirrer at room temperature and in the dark (the maceration flask or beaker was wrapped with aluminum foil), in order to avoid degradation of the polyphenols. The solutions were then filtered through Wattman No. 2 filter paper to separate the filtrate from the grains.

Finally, the filtrates are evaporated to dryness under reduced pressure in a rotary evaporator. The weighed dry residues are taken up in 5 ml of methanol (Figure 14).

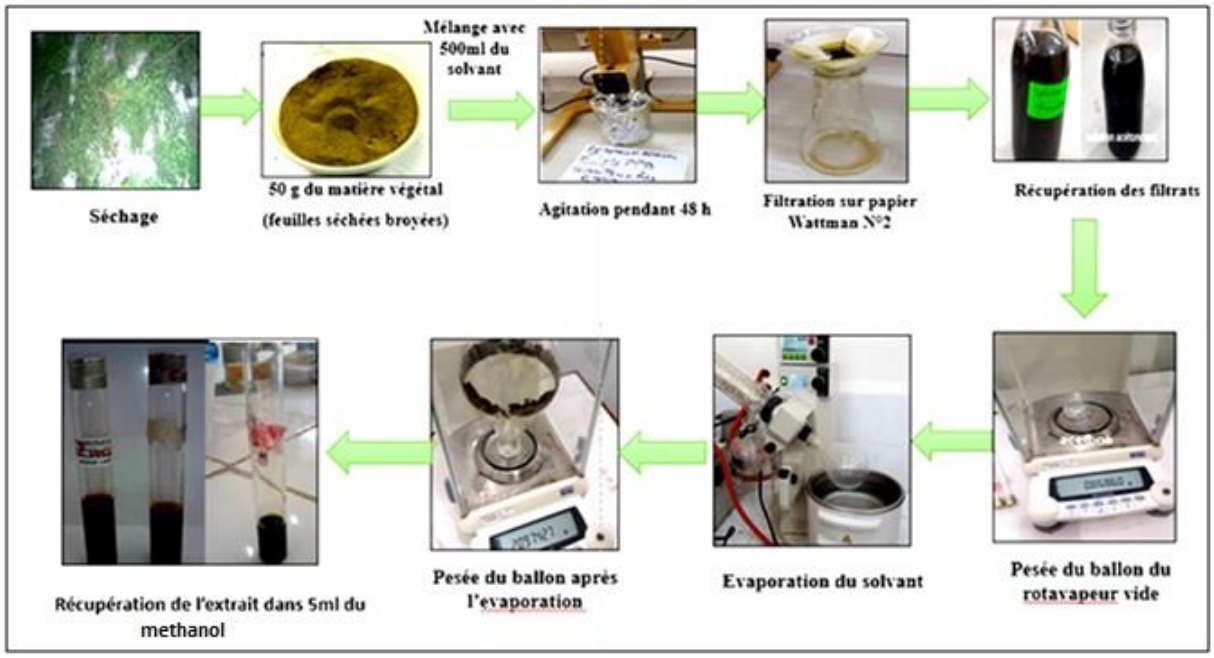


Figure 14 : Protocol for preparing the crude extracts (Originale, 2020).

Chapter 3:
Results and discussion

This chapter is divided into two parts;

Practical part: comprising the extraction of phenolic compounds from *Cupressus sempervirens L.* that we carried out at the phytopharmacy laboratory of the SNV faculty of (Blida University 1).

The second part is theoretical, comprising the expected results of our study, which we have not completed because of the Corona virus (Covid-19) pandemic, so we tried to analyze the results of previous works on the dosage of total polyphenols and the antioxidant activity of phenolic extracts of green cypress *Cupressus sempervirens L.*

1. The yield of phenolic compounds :

The extraction of the phenolic compounds by solvents with different polarity from the plant studied, allowed us to determine the yields of their crude extracts. The results are shown in Figure 15.

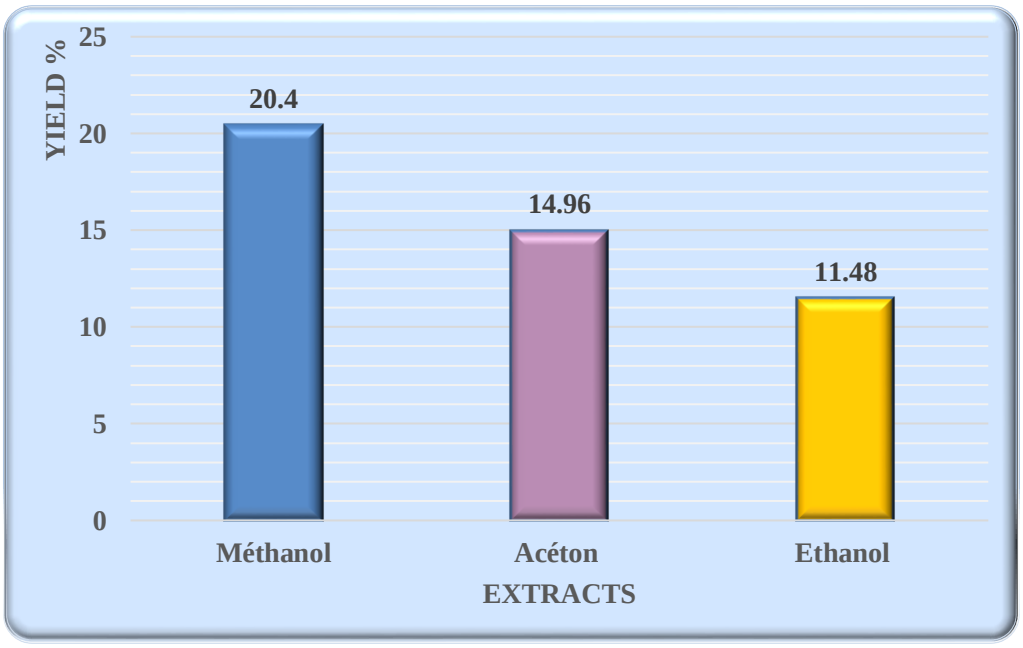


Figure 15: The yield of phenolic compounds of *Cupressus sempervirens L.* crude leaf extracts

Through these obtained results **(Figure 15)**, we note that the best yield was obtained by the methanolic extract (20.4%) followed by the acetone extract (14.96%) and the ethanolic extract (11.48%). These results are close to those found by **(Aliouat and Boudaoud, 2018)** (16٪) in the methanolic extract of the leaves of *Cupressus sempervirens* L, although the extraction method was different (Soxhlet method). On the other hand, maceration gave a low yield of 4.08% according to the same study.

(Mazari, 2009), found that the yield of polyphenols from the extract of the leaves of *Cupressus sempervirens* L is (24.2%). This value is close to that found by **(Hammadache, 2011)** who obtained a yield of extraction of (32.84%). This rate is higher than that reported by **(Ebrahim et al., 2009)** which is (6.09%) by using chloroform as solvent.

These differences obtained in the extraction yields for the same species are linked to several factors. Moreover, studies had shown on the one hand the influence of the extraction technique and environmental factors: the region, the climate, the soil, and on the other hand, the used solvent **(Ebrahimi et al., 2009)**.

According to scientific literature, ethanol and methanol are the best solvents and ultrasonic extraction generally increases extraction efficiency. **(Thongson et al., 2004)** reported that ultrasonic extraction only took 5 minutes to obtain the bioactive components of a medicinal plant **(Thongson et al., 2004)**.

2. Phyto-chemical screening :

The extracts phytochemical composition from *Cupressus sempervirens* L leaves have been studied by several authors. The most recent that of **(Anka et al., 2020)** their results are summarized in Table 02.

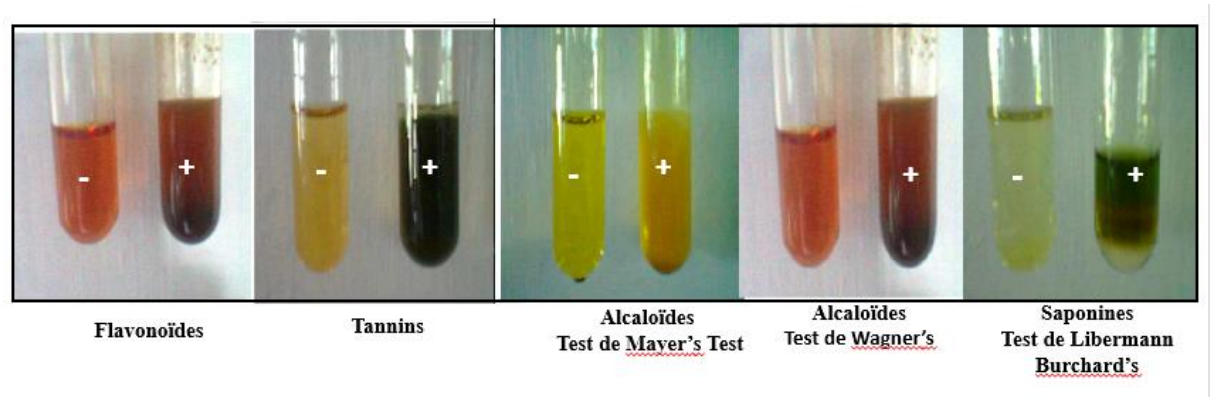


Figure 16: Phytochemical screening results **(Debib, 2014)**.

Table 02: Results of phytochemical tests on the ethanolic extract of *Cupressus sempervirens L.* leaves from two different regions in Lebanon **(Anka et al., 2020).**

Phytochemicals	Beit El-Dein	Jbeil
Phenols	+	+
Alkaloids	+	+
Tannins	+	+
Steroids	+	+
Phlobatannins	-	-
Flavonoids	+	+
Terpenoids	+	+
Glycosides	+	+
Resins	+	+
Quinones	+	+
Saponins	**-**	**-**

These results correspond with those of (**Emami et al 2004)** which revealed the presence of alkaloids, flavonoids, tannins, saponins and phenols. The presence and absence of non-volatile components (fruits and leaves of *Cupressus sempervirens L* .) are summarized in Table 03.

Table 03 : Major non-volatile components of *Cupressus sempervirens L* fruits and leaves

Chemical components	average content Fruit	leaves
Alkaloids	-	-
Flavonoids	++	+++
Saponins	+	+
Tannins	++++	+++
*** Average content was rated from - and +**		

(Alkurdi and Supuka, 2015), showed the existence of coumarins, flavonoids, saponins and tannins in the leaves of *Cupressus sempervirens* L. The abundance of active ingredients gives this plant remarkable pharmacological properties **(Konkon et *al*., 2006)** . This could justify its multiple therapeutic indications. The difference in the chemical composition of the same plant from one region to another can be explained by the influence of several factors on the presence, absence and distribution of different active ingredients such as climate, soil type, water and altitude **(Boughrara, 2016).**

3. Total polyphenol content :

Phenolic compounds are used for the prevention of various diseases which are mainly associated with free radicals. More generally, phenolic compounds have been recognized as antioxidant agents, which slow down degradation due to the effects of oxidation while ensuring better aging, and therefore exhibit medicinal activity and physiological functionsBy referring to the results found in our laboratory; phytopharmacy and plant protection laboratory of the biotechnology department of the University of Blida 1 by our colleagues (**Bouzari and Belkram, 2019)** (figure: 17), the aqueous extract is the richest in total polyphenols (27,68 mg Eq GA / g of extract), followed by the ethanolic extract with a content of (10.25 ± 0.6 mg Eq GA / g of extract).

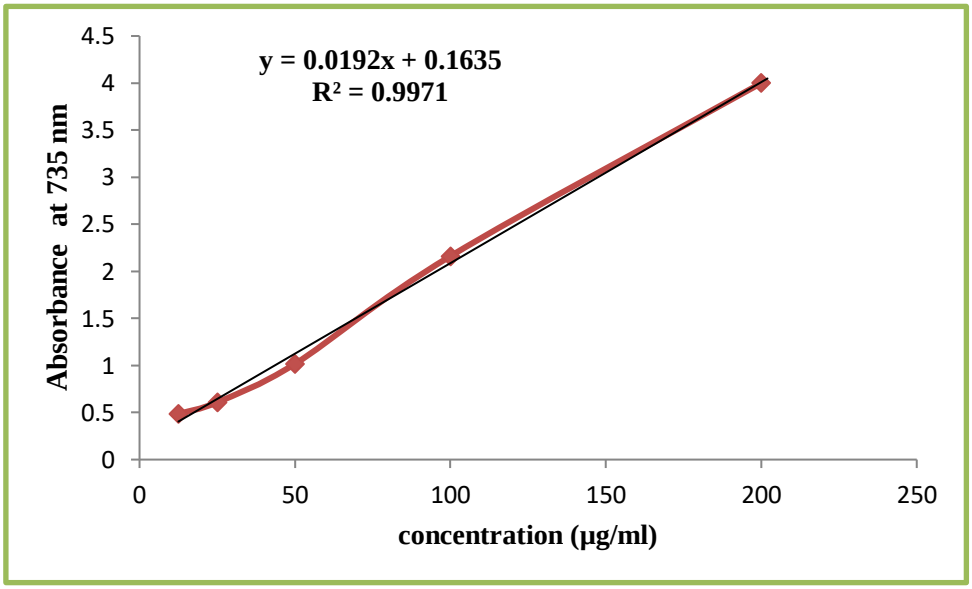


Figure 17: Gallic acid calibration curve **(Debib et *al*., 2014)**

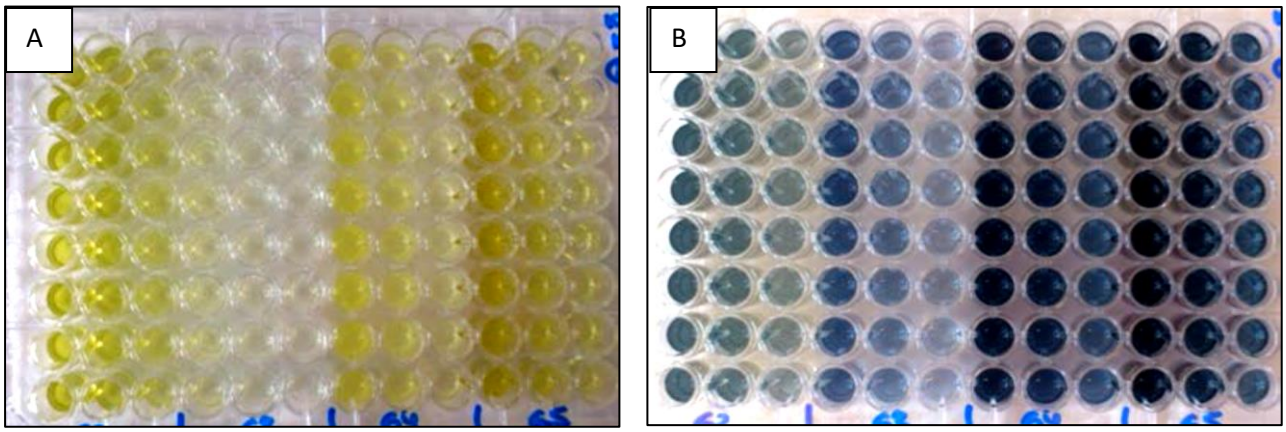


Figure 18: Results of total polyphenols assay ((A) before and (B) after the reaction) **(Merck, 2017)**.

While low levels were recorded in the methanolic extract and the acetone extract (8.3 ± 0.07 mg Eq GA / g extract); (7.54 ± 0.06 mg Eq GA / g extract) respectively. Comparing these results with another study carried out by **(Boudjemaa, 2017)**, on cypress leaves (*Cupressus sempervirens* L) gave values up to (08.70 ± 0.01 ug / mg Eq AG / g of extract) using ethanol and methanol as extraction solvent, the lowest concentration of phenols was measured in ethyl acetate extracts.

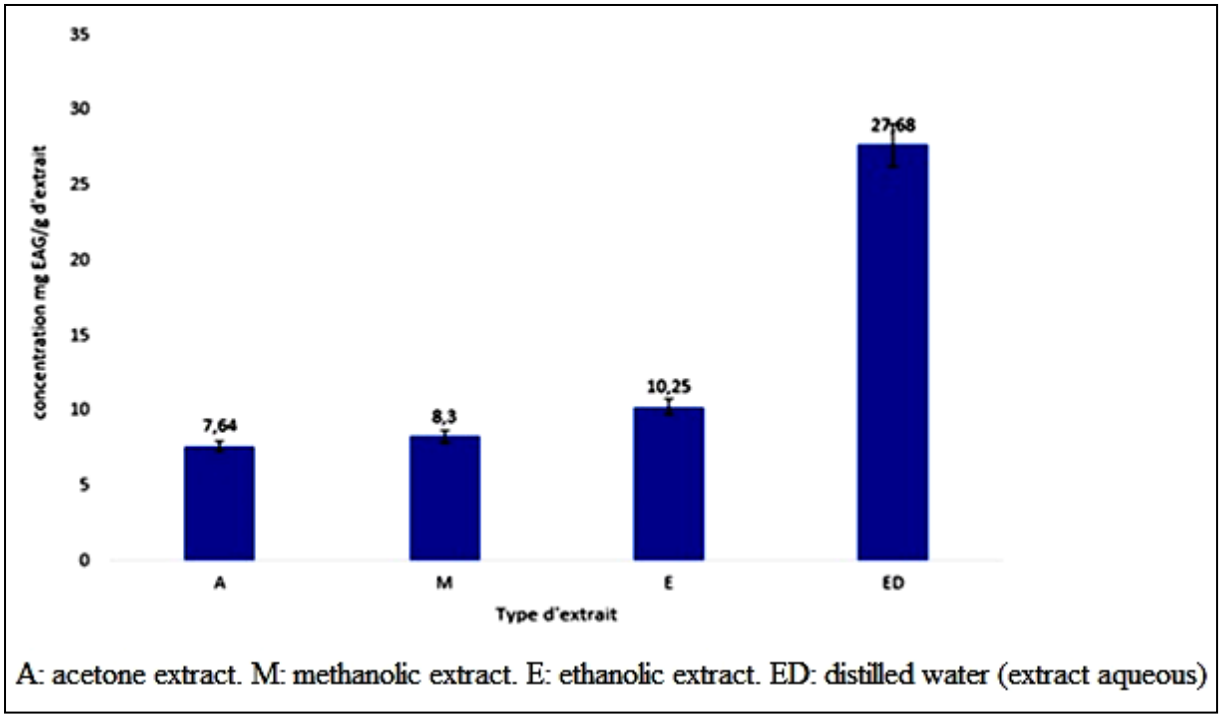


Figure 19: Total polyphenol content of *Cupressus sempervirens L.* leaves extracts **(Bouzari and Belkram, 2019)**

4. The antioxidant activity:

The antioxidant properties of extracts from aromatic plants are mainly attributed to active compounds present in these plants. This may be due to the high proportion of main constituents, but also to the presence of other constituents in small quantities but exhibiting a strong activity or to the synergy between them.

In our study we were unable to complete this part, which is why we analyzed the results of previous studies.

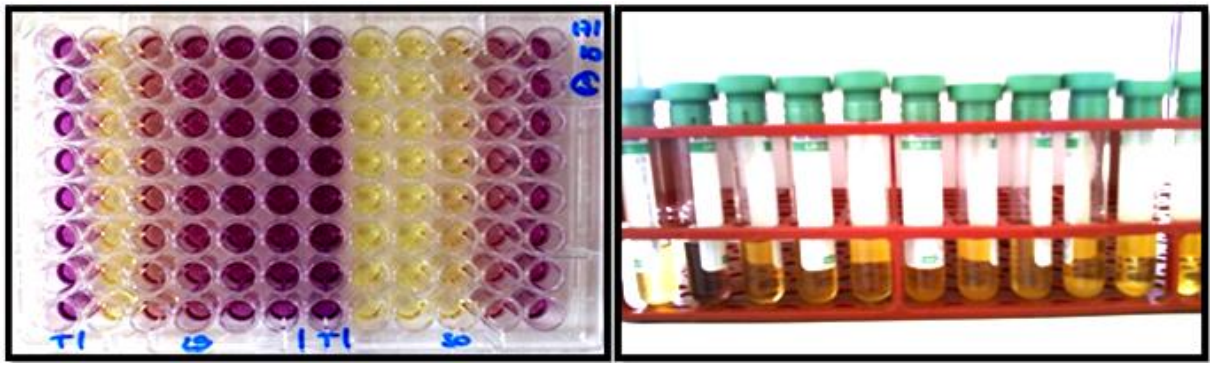
According to **(Sarikurkcu et *al*., 2009)** green cypress leaf extracts remarkably reduced DPPH free radicals and were able to transform its stable purple color to yellow color upon electron abstraction.

The results of **(Anka et *al*., 2020)** showed that the effect of scavenging activity against DPPH increased with increasing the dose of ethanolic extracts in the mountain and seaside samples.

The lowest value of the trapping activity against DPPH was observed at the lowest concentration (100 µg / ml) with a value of 43.55% and 33.78% for the mountain sample and the seaside sample, respectively. This activity increased gradually until reaching 95.05% and 93.56% respectively at the highest concentration (1000 µg / ml) of ethanolic extracts. The two extracts had an IC50 value = 113.17 and 155.75 µg / ml, respectively. All samples tested showed lower DPPH radical reducing activity compared to ascorbic acid (IC50 = 4.1 µg / ml) **(Anka et al., 2020)**.

In another study by **(EL-Seedi et al, 2007)** on an Egyptian variety of C.sempervirens. Antoxidant activity of isolated phenolic compounds compared with α-tocopherol and butylated hydroxy toluene (BHT) as standard antioxidants using the ESR technique. The results showed that the most potent compound is the flavonoid; quercetin (99.75%), followed by rutin (99.68%), caffeic acid (99.43%), p-coumaric acid (95.80%) and then the methanolic extract (75%), these values are higher compared to the α-tocopherol and BHT standards (54.74% and 59.38%) respectively.

The other compounds (Amentoflavone and Cupresseflavone) showed more or less weak antioxidant activities (15.53%, 11.01%) respectively.



A **B**

Figure 20: DPPH scavenging test results (A) in 96-well microplate (B) in tubes **(Debib et al., 2014; Merck, 2017).**

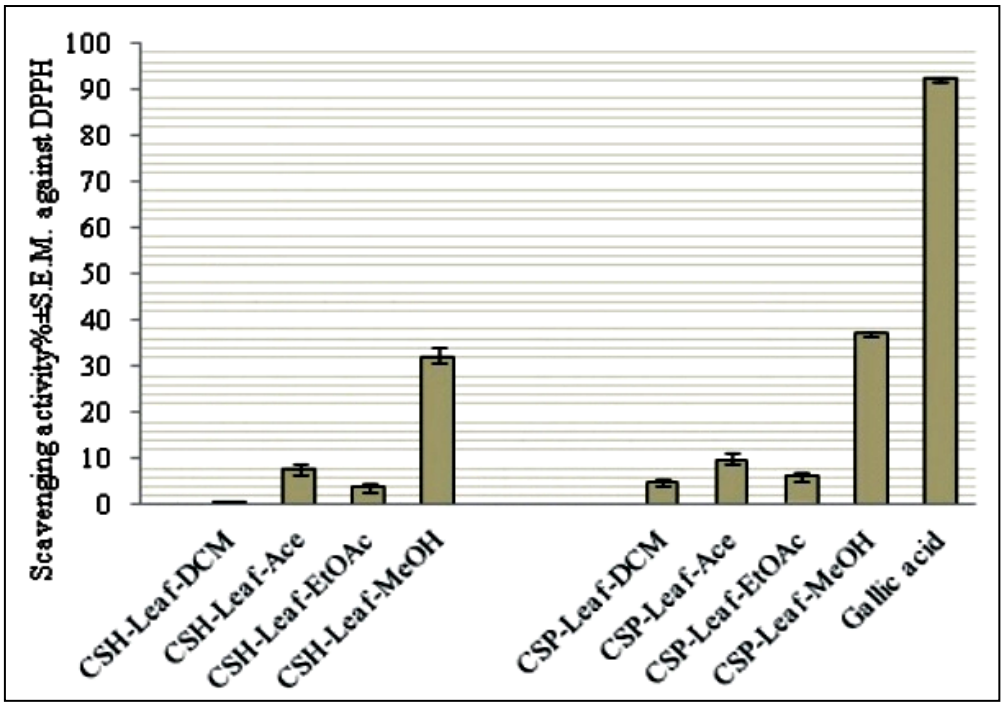
Table 4 : DPPH absorption inhibition (%) **(EL-Seedi et al, 2007)**

No	COMPOSANTS	% D'inhibition
1	DPPH °	0
2	α-Tocopherol	54.74
3	BHT	59.38
4	Methonolic extract	75.68
5	Routine	99.68
6	Quercetin	99.75
7	Caffeic acid	99.43
8	ρ- coumaric acid	95.80
9	Amentoflavone	15.53
10	Cupressuflafone	11.01
11	Essential oil	9.36

DPPH• :2,2-diphenyl-2-picrylhydrazyl ; BHT ; butylated hydroxy toluene

In the study by **(Tumen et *al*., 2012)** on two subspecies (*Cupressus sempervirens var. Horizantalis* and *var. Pyramidalis*) from Turkey, using different methods and solvents.

The methanolic extracts had a more or less moderate trapping activity against DPPH, of approximately (32% and 38%) respectively in horizantalis and pyramidalis, compared to the reference (gallic acid), the activity of which reached (93%). Acetone extracts showed low scavenging activity in horizantalis and pyramidalis approximately (8% and 10%) respectively, while dichloromethane and ethyl acetate extracts showed less potency in both subspecies , the histogram below shows the results of this test.



Dichloromethane (DCM), aceton (Ace), ethyl acetate (EtOAc) and methanol (MeOH)
(CSH: *C.sempervirens var.horizantalis*, CSP: *C.sempervirens var.pyramidalis*)

Figure 21: DPPH radical scavenging activity (inhibition %) of extracts of *C.sempervirens* subspecies and the reference (gallic acid) **(Tumen et *al*., 2012)**

The large variation in IC50 values is due to the efficiency of the extraction method and the power of the solvent used to extract the natural antioxidants. Likewise, we observe the effect of changing the extraction conditions for each study on the antioxidant power of the extract.

Antioxidant activity is often due to the presence of phenolic compounds. However, in several studies the content of phenolic compounds was very low while their antioxidant power is important. This perhaps shows that the extraction of non-phenolic compounds (low specificity of the Folin Ciocalteu test) is responsible for this activity or that the phenolic compounds detected are very active.

A significant amount of polyphenols and flavonoids were detected in Lebanese cypress extracts as revealed in the study by Anka et al., 2020). Therefore, these can be considered as the potential compounds contributing to the strong antioxidant activity in the ethanolic extract of *C.sempervirens* leaves. A positive correlation was recorded between the content of phenol and flavonoids on the one hand; and antioxidant capacity on the other hand.

Therefore, *C.sempervirens* L. leaf extract may act as a potential source of natural antioxidants used in the food, pharmaceutical and cosmetic industries where currently used synthetic antioxidants have been shown to have adverse health effects **(Miguel, 2010)**.

Conclusion

Conclusion :

During this work we were interested in the phytochemical study and the evaluation of the antioxidant activity of the leaves extracts of green cypress (*Cupressus sempervirens L.*), but we could not terminate the practical part due to the COVID 19 corona virus pandemic.

The extraction of phenolic compounds by solvents with different polarity from the plant studied, allowed us to conclude that methanol is the best solvent for extracting polyphenols from green cypress leaves (*cupressus sempervirens L.*) in comparison with acetone and ethanol.

The phytochemical screening carried out by characterization reactions revealed the richness of our plant in Alkaloids, Flavonoids, Tannins, and saponins and other numerous biological constituents. These important results highlight the plant's richness in secondary metabolites and specifically in polyphenols.

The content of phenolic compounds analyzed from several previous studies has allowed us to observe a variability in the recorded rates. These divergences obtained in the extraction yields and the polyphenol content for the same species are linked to several factors. Moreover, studies had shown on the one hand the influence of the extraction technique and environmental factors: the region, the climate, the soil, and on the other hand, the used solvent.

Regarding antioxidant activity, by comparing the results of studies, we found that the extract of the leaves of green cypress (*Cupressus sempervirens L*) is endowed with a powerful antioxidant effect regardless of the extraction method or dosage. Several authors have reported a significant correlation between polyphenols including flavonoids and antioxidant activity.

Therefore, *C.sempervirens* L. leaf extract can be used as a potential source of natural antioxidants used in the food, pharmaceutical and cosmetic industries where the synthetic antioxidants currently used have been shown to have adverse health effects.

It emerges from this study that the results obtained in-vitro remain preliminary and constitute a first step in the search for natural biologically active substances of this miracle plant. For more efficiency, many perspectives can be considered:

• Widen the panel of antioxidant activities in vitro and in vivo and why not other biological tests: anti-tumor, anti-cancer and anti-inflammatory

• Characterize and isolate the active ingredients responsible for these pharmacological properties.

• Use more efficient techniques in order to identify the bioactive secondary metabolites of our extracts and to further study the antioxidant activity in vivo.

• Identify new natural bioactive substances that can respond to different health problems and be an alternative synthetic drug.

• Characterize the quantity of polyphenolic compounds by different separation methods (HPLC, GC-CM).

• Study the toxicity of the extracts to see the possibility of their use.

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Annex

1. Material :

All of the materials, products and chemical reagents used to perform this study are summarized in Table 5.

Table 5: List of equipments, chemicals and reagents used during the manipulation

Glassware	Reagents and chemical products	Consumable material	Equipments
Beaker	Acetone	Whatman filter paper	Scale
Erlenmeyer flask	Ethanol	Aluminum foil	Precision scale
Funnel	Methanol		Stirrer
Test tube	Gallic acid		Oven
Glass bottle	Folin-Ciocalteu reagent		Evaporator

2. Phytochemical study (Evans, 1996; Harbone, 1998) :

The phytochemical screening is a qualifying test which makes it possible to highlight the different chemical groups contained in a plant organ, the results are classified into:

• Frankly positive reaction: + + + +

• Positive feedback: + + +

• Very weak or doubtful reaction: +/-

• Negative reaction: 0

2.1. Flavonoids :

To 5 ml of each extract, a few drops of concentrated hydrochloric acid (HCL) and 0.5g of magnesium (Mg) are added. Leave to act for 3 minutes. An orange or red coloration implies the presence of flavonoids.

2.2. Tannins :

A volume of 2 ml of each extract is mixed with 200 µl of the 1% $FeCl_3$ solution. In the presence of tannins, a greenish or blue - black color develops. The color turns to black brown in the presence of gallic tannins (hydrolyzable tannins) and to greenish blue in the presence of catechetical tannins (condensed tannins).

2.3. Alkaloids :

To 1 ml of each extract, 5 ml of 1% HCl are added, the mixture is heated in a water bath, then each extract is divided into two equal volumes. One volume is treated with Mayer's reagent, the other with Wagner's reagent. The formation of a white or brown precipitate indicates the presence of alkaloids. Mayer's and Wagner's reagents are prepared as follows: **Mayer's reagent:** Dissolve 1.358 g of $HgCl_2$ in 60ml of distilled water and then 5g of KI in 10ml of distilled water. Mix the two solutions and adjust the total volume to 100 ml.

Wagner's reagent: In 75 ml of distilled water, dissolve 2g of KI and 1.27g of I_2. The volume obtained is adjusted to 100 ml with distilled water.

2.4. Saponins :

Libermann-Burchard reaction: To 5 ml of our extracts, we add 5 ml of acetic anhydride ($C_4H_6O_3$) and a few drops of concentrated H_2SO_4. Steroids gives a red coloration with this reaction, while triterpenes gives a green coloration.

3. Antioxidant activity :

3.1. Principle :

DPPH is a stable radical and it exhibits in solution a characteristic absorption at 517 nm which gives it a violet coloration. This color disappears quickly when the DPPH is reduced by a free radical scavenger. We can summarize this reaction by the following equation:

Where (AH) n represents a compound capable of donating a hydrogen to the DPPH radical. (purple) to transform it into a DPPH-H molecule.

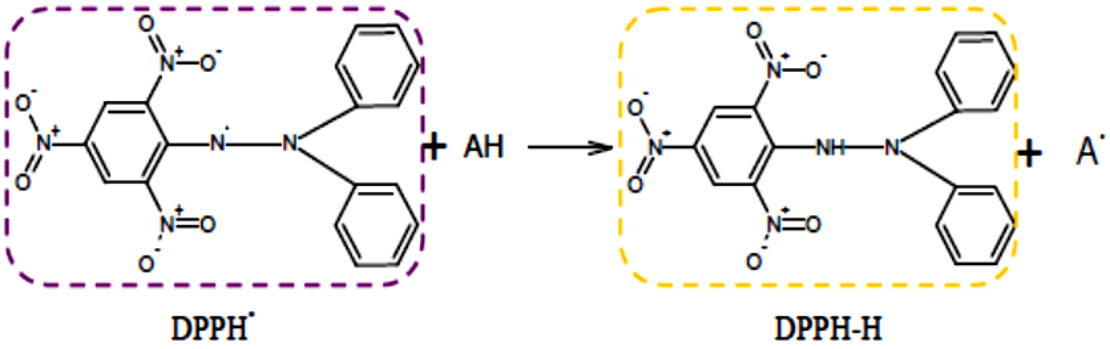

Figure 22: Reaction mechanism occurring during the DPPH • test between the radical species DPPH • and an antioxidant (AH).

3.2. Procedure :

To 1950 µl of the 6.34 10-5M DPPH solution (0.0025g DPPH in 100ml methanol) is added 50µl of each extract at different concentration (1, 2, 3, 4, 5, 6, 7, 8, 9 , 10 mg / ml); For the negative control, mixing 50 µl of methanol with 1950 µl of DPPH.

The blanc of the device is methanol; incubation 30 minutes at room temperature; The reading is taken at 515 nm, compared to the standard which contains ascorbic acid at different concentrations: 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.16, 0.18, 0.2 mg / ml.

● Calculation of inhibition percentages

The percentage reduction of DPPH is given by the following formula:

$$\%\, PR\ of\ DPPH\ = \frac{(Abs\ cont\ - Abs\ samp)}{(Abs\ cont)}$$

-% PR OF DPPH: Percentage reduction or inhibition of DPPH.

-Abs cont: optical density of the DPPH.

-Abs samp: optical density at 30 min after adding the extract.

➢ Calculation of IC50 :

By definition, the IC50 value is the concentration of ascorbic acid or extract which can reduce 50% of DPPH, the latter is determined graphically. The IC50s are calculated graphically by the formula for the regression of the inhibition percentages as a function of different concentrations of the extracts tested using statistical software.