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Valorization of biodiversity: phytochemistry, biological activities of two wild olive subspecies

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JURY

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الملخص

في إطار تثمين الموارد الطبيعية "النباتات البرية والمستوطنة"، تم تحديد نوعين فرعيين من أشجار الزيتون البرية في الجزائر، أحدهما يعرف باسم Oleaster "*Olea europaea* L.var. sylvestris"، منتشر بشكل رئيسي في الشمال، والأخر يعرف باسم أليو أو آليوان بين الطوارق "*Olea europaea* subsp.*laperrinei*"، وينتشر في الصحراء الوسطى. يتناول الجزء الأول من الدراسة الآلية التشريحية والفيزيائية التي تم تطويرمن النوعين للتكيف مع ظروفهم البيئية. بينت الدراسة النسيجية والقياسات الفسيولوجية أن شجرة زيتون Laperrine قد أظهرت تغيرات تشريحية وفسيولوجية كبيرة مقارنة بشجرة الزيتون الاخرى. بينما ركز الجزء الثاني للدراسة على تحديد الملامح الفينولية لمستخلصات الأوراق المائية والميثانولية من النوعين الفرعيين عن طريق تحليل لكروماتوغرافيا السائلة عالية الأداء ،الدي سمح بتحديد العديد من المركبات الفينولية حيث ظهر oleuropein كمركب رئيسي في جميع المستخلصات، حيث أظهرت المستخلصات الصحراوية وجود الكمية الأكبر منه. أظهرت جميع المستخلصات نشاطاً معتبرا مضاداً للجذور ، ولكن تم تحديد أعلى نشاط في مستخلصات السلالات الصحراوية، كما وجد الميثانول كمذيب استخلاص أفضل من الماء. أما الجزء الثالث فيتعلق بالدراسة الكيميائية والنشاط المضاد للبكتيريا للزيتين الأساسيين، وكشف تحليل كروماتوغرافيا الغاز مع كاشف تأين اللهب عن تحديد 31 مركباً حيث كان α-pinene المكون الرئيسيي في *Olea. europaea* subsp. *laperrinei* 29 مركبًا و Nonanal حدد كمكون رئيسي في الزيتون الآخر، أظهر كلا الزيتين المتطايرين نشاطًا فعالًا مضادًا للبكتيريا، في حين أن النوع المستوطن لديه نطاق تثبيط أعلى حيث *P. aeruginosa* و *B. subtilis* وجدت كسلالات حساسة و *K.pneumoniae* هي المقاومة. الجزء الأخير يعتمد على تثمين القيمة الغذائية لزيوت الزيتون البكر الممتازة من خلال تحليلها الكيميائي، و تثمين قيمتها العلاجية من خلال نشاطها المضاد للبكتيريا. سمحت النتائج باكتشاف 22 مكونًا في*Olea europaea* L.var. sylvestris. حيث تم تحديد, palmitic acid ,oleic acid, squalene,linoleic acid كعناصر سائدة، في حين تم تحديد 14 مكونا في الزيتون المستوطن الذي كانت فيه oleic، squalene acid, linoleic acid ,palmitic acid هي المكونات الرئيسية. تمثل الأحماض الدهنية غير المشبعة الجزء السائد في الزيتين. بالإضافة إلى ذلك، تشير النتائج إلى أن تأثير الزيتين البكر الممتازين على البكتيريا موجبة الجرام أكبر منه على البكتيريا السالبة الغرام، حيث اظهر زيت *Olea europaea* subsp. *laperrinei* أعلى نطاق تثبيط مع جميع السلالات البكتيرية التي تم اختبارها، وكانت *B.subtilis,P.aeruginosa ,B.subtilis,S. aureus(MRSA)*, هي السلالات الحساسة ، في حين كانت *K. pneumoniae* و*E.faecalis* هي السلالات المقاومة لكلا الزيتين.

الكلمات المفتاحية : *Olea europaea* L.var. sylvestris ,*Olea europaea* subsp. *laperrinei*, الآليات التشريحية والفيزيائية ؛ المكونات الكيميائية، الأنشطة البيولوجية.

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Abstract

As part of the valorization of natural resources “wild and endemic plants”, two subspecies of the wild olive trees that have been inventoried in Algeria, one of which is commonly referred to Oleaster "*Olea europaea* L.var. sylvestris", spreads mainly in the northern region, and the other is known as ālew or āliwan among the Tuareg Berbers "*Olea europaea* subsp. *laperrinei*", spreads in the central Sahara. The first part deals with physio-anatomical mechanisms developed to adapt to their environmental conditions. The histological study and the physiological parameters measured revealed that the laperrine olive tree exhibited significant anatomical and physiological changes compared to the Oleastre tree. The second part focuses on studying of phenolic profiles of aqueous and methanolic leaf extracts from the two subspecies by high-performance liquid chromatography analysis which allowed the identification of several phenolic compounds with oleuropein as the main compound in all extracts, with Saharan subspecies extracts showing a higher level. All the extracts showed significant antiradical activity, but a high value was detected in the Saharan subspecies extracts with an IC50 value near to those exerted by the butylated hydroxytoluene, Methanol was found as a better extraction solvent than water. The third part relates to a phytochemical study and the antibacterial activity of the two essential oils, the gas Chromatography coupled with flame ionization detector analysis revealed the identification of 31 compounds where α-pinene main components in *Olea europaea* subsp. *laperrinei* volatile oil and 29 compounds with nonanal as major constituents in the Oleaster, both volatile oils showed an efficient antibacterial activity, with the endemic one has a higher range of inhibition, in which *P. aeruginosa* is the sensitive strain and *K. pneumoniae* is the resistant one. The last part is based on the valorization of the nutritional value of their extra virgin olive oils, through their chemical analysis, and their therapeutic value through their antibacterial activity. The results allowed the detection of 22 components in *Olea europaea* L.var. sylvestris oil, with squalene, oleic acid, palmitic acid, linoleic acid, as predominant components, while 14 components were identified in the endemic one, in which palmitic acid, linoleic acid, oleic acid, squalene were the main components. Unsaturated fatty acids represented the dominant class in the two oils, where the linoleic acid was apparent by high percentage and significant amount for the first time comparable with the previous study that was conducted on olive oil. In addition, the results indicate that the effect of the two extra virgin oils on gram positive bacteria is greater than on gram negative bacteria, with *Olea europaea* subsp. *laperrinei* oil exhibited the highest range of inhibition with all bacterial strains tested.

Key words: *Olea europaea* L.var. sylvestris**,** *Olea europaea* subsp. *laperrinei***,** physio-anatomical mechanisms, phytochemistry , biological activities.

Résumé

Dans le cadre de la valorisation des ressources naturelles « plantes sauvages et endémiques », deux sous espèces d'olivier sauvage ont été inventoriées en Algérie, dont l'une est communément appelée Oleaster "*Olea europaea* L.var. sylvestris", principalement distribuée dans la région du nord, et l'autre est connue sous le nom d'ālew ou d'āliwan chez les Berbères touaregs "*Olea europaea* subsp. *laperrinei*", répartie dans le Sahara central.La première partie de l'étude est basée sur leurs mécanismes physio-anatomiques développés pour s'adapter à leurs conditions environnementales. L'étude histologique et les paramètres physiologiques mesurés ont révélé que l'olivier de Laperrine présente des changements physio-anatomiques significatifs par rapport à l'olivier Oléastre. La deuxième partie s'est concentrée sur la détermination des profils phénoliques des extraits aqueux et méthanoliques des feuilles des deux sous-espèces par l'analyse de Chromatographie liquide à haute performance qui a permis l'identification de plusieurs composés phénoliques, l'oleuropéine étant le composé principal dans tous les extraits, les extraits de la sous espèces sahariens montrant un niveau plus élevé. Tous les extraits ont montré une activité anti-radicalaire significative et les valeurs élevées ont été détectée dans les extraits de la sous-espèce saharienne avec une valeur IC50 proche de celle de hydroxytoluène butylé, le méthanol s'est révélé être un meilleur solvant d'extraction que l'eau. La troisième partie, concerne une étude phytochimique et l'évaluation de l'activité antibactérienne des deux huiles essentielles, l'analyse de chromatographie en phase gazeuse couplée à un détecteur à ionisation de flamme a révélé l'identification de 31 composés dont le quel α-pinène est le composant majoritaire dans *Olea europaea* subsp. *laperrinei* et 29 composés ont été détectés chez l'Oleaster dont le nonanal est le principal constituant. Les deux huiles volatiles ont montré une activité antibactérienne efficace, tandis que l'huile de la sous espèces endémique a un effet d'inhibition plus élevée, dans laquelle *P. aeruginosa* est la souche sensible tandis que *K. pneumoniae* est la résistante. La dernière partie est basée sur la valorisation de la valeur nutritionnelle de leurs huiles d'olive extra vierges, à travers leur analyse chimique, et de leur valeur thérapeutique à travers leur activité antibactérienne. Les résultats ont permis la détection de 22 composants dans l'huile d'*Olea europaea* L.var. sylvestris, avec le squalène, l'acide oléique, l'acide palmitique, l'acide linoléique, comme composants prédominants, tandis que 14 composants ont été identifiés dans la sous-espèces endémique, dans lesquels l'acide palmitique, l'acide linoléique, l'acide oléique et le squalène étaient les principaux composants. Les acides gras insaturés représentaient la classe dominante dans les deux huiles, où l'acide linoléique était apparent par cette quantité significative pour la première fois comparable à l'étude précédente menée sur l'huile d'olive. De plus, les résultats indiquent que l'effet des deux huiles extra vierges sur les bactéries gram positives est plus important que sur les bactéries gram négatives, l'huile d'*Olea europaea* subsp. *laperrinei* a présenté la gamme d'inhibition la plus élevée avec toutes les souches bactériennes testées.

Mots clés: *Olea europaea*L.var. sylvestris ; *Olea europaea* subsp. *laperrinei*; mécanismes physio-anatomiques, photochimie, activités biologiques.

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The scientific products

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Abreviation list

DNA: Deoxyribonucleic acid.

ABA : Abscisic acid.

RMF : Root mass fraction.

ROS : Reactive oxygen species.

RONS : Reactive oxygen and nitrogen species

OLE : Olive leaf extract.

TEAC : Trolox equivalent antioxidant capacity.

FRAP : Ferric reducing antioxidant power**.**

ISO : International standard organization.

AFNOR : French standardization association.

EO : Essential oil

CT : Canopy temperature.

DSI: Drought susceptible index.

EC : Electrical conductivity.

LA : Leaf area.

RWC: Relative water content.

LWL: Leaf Water Loss.

SLW: Specific Leaf Weight.

CCI: Chlorophyll content index**.**

RFC : Folin-Ciocalteu reagent.

AlCl3 : Aluminum trichloride .

HPLC -DAD : High-performance liquid chromatography with diode-array detection **.**

DPPH : 1,1-diphenyl-2-picrylhydrazil radical.

BHT : Butylated hydroxytoluene.

IC50 : Half-maximal inhibitory concentration.

GC/MS : Gas Chromatography /mass spectrometry.

GC/DIF: Gas Chromatography coupled with flame ionization detector.

Rt : Retention times.

KI: kovats index.

CBS : SFAX Biotechnology center.

NIST: National institute of standards and technology.

PCA : Principal component analysis.

ANOVA: analysis of variance.

TPC: Total phenolic contents.

TFC: Total flavonoids contents.

UFA: Unsaturated fatty acids.

EVOO: Extra virgin olive oils.

MRSA: Staphylococcus aureus.

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Introduction

Introdution

Algeria is characterized by significant ecosystem diversity and is divided into three types of environments that are distinguished by relief morphology, and climate. As a result, we distinguish two Mediterranean regions the Tell in the north and the Steppe (comprises both the Saharan Atlas and the high plateaus), however the south or “desert” is a part of the Arabian Saharan region. From the North to the South of Algeria, from forests, maquis and matorrals to semi-arid and arid steppes and then towards desert ecosystems, following a variability of rainfall ranges, due to the interaction of the Mediterranean climate with the relief of the Atlas, creating a north-south decrease in rainfall and an increase in dry season length. This explains its notable degree of endemism and biological diversity, notably the great diversity of floristic heritage (**Meddour *et al.*, 2023).**

Among them, the olive tree, *Olea europaea* L., is an extremely significant floristic heritage entity with agro-economic and environmental importance in Algeria **(Dsasi, 2018),** in which two forms of the wild olive tree that have been inventoried, one of which is commonly referred to as oleaster “*Olea europaea* L.var. sylvestris [Mill.] Lehr”, mainly in the northern region **(Breton &Bervillé, 2012),** and the other is known as ālew or āliwan among the Tuareg Berbers, it is an endemic subspecies was detected in the central Sahara which is “*Olea europaea* subsp. *laperrinei* [Batt.& Trab.]Cif.” **(Chaker, 2013)**, and represents a fundamental element of the ecosystem of the Hoggar mountains (**Besnard & Baali-Cherif, 2009**).

These two wild olives are characterized by great plasticity to colonized semi-arid and arid regions (**Arenas-Castro *et al.*, 2020**). As a result, the role of Oleastre in olive cultivation may be crucial going forward, especially given the predicted significant reduction in productivity and growing area suitable for olives due to climate change **(Fanelli et *al.*, 2022).** In order to select new olive cultivars with a proven potential for adaptation, and higher olive oil quality **(Baccouri *et al.*, 2010)**, it is imperative that the wild olive resources be thoroughly valorized and effectively conserved **(Fanelli *et al.*, 2022).** Additionally, the laperrine’s olive tree, due to its adaption to an exceptionally dry environment, offers a potentially interesting genetic resource for enhancing the drought tolerance of the Mediterranean olive tree **(Besnard *et al.*, 2012).**

Medicinal and aromatic plants are popular in all areas of the country and are passed down through rural populations among generations and passed down orally among older and illiterate women **(Ilbert *et al.*, 2016),** these plants are used by the Tuaregs to treat themselves in the absence of doctors in certain isolated regions of the hoggar, and in Kabylia by mountain

people to heal themselves when the roads are closed due to snowfall **(Mokkadem, 2004).** Among them olive tree which has an important medicinal value **(Addab *et al*., 2020)**, the low incidence of diseases and maintenance of health has been linked to frequent use of the two main olive tree products: olive oil and olive fruits. These products include considerable amounts of compounds with health advantages **(Hashmi *et al*., 2015).** Despite Oleaster yields high-quality oil, especially for pharmacological applications **(Bouarroudj et *al*., 2016),** little attention has been attracted to the production of this oil in Algeria, because it is more expensive **(Aumeeruddy-Thomas &Caubet, 2017)**, and has a lower oil yield than cultivated one **(Bouarroudj et *al*., 2016).** Regarding the laperrine'soil is not valued; neither its agroeconomic nor medicinal worth has been predicted or studied.

Olive leaves have also been used in traditional medicine to treat many human diseases **(Özcan &Matthäus, 2017).** Owing to their high phenolic contents, olive leaf extract has become one of the most efficient sources of plant polyphenols with significant antioxidant potential **(Brahmi *et al*., 2012)**. Moreover, the accumulation of phenolic compounds is linked to a plant's defensive mechanism against solar radiation and its capacity to block UV-B rays (**Di Ferdinando *et al*., 2014).**

The volatile oils of olive leaves contain of several important chemical compounds that exert their beneficial health effects **(Brahmi *et al.* 2012).** Only a few studies have examined the chemical profile and beneficial health effects of olive essential oil, most of which have been conducted on cultivated olive in Tunisia **(Brahmi *et al*., 2012),** Italy **(Popovi *et al*., 2021),** Turkey **(Vural &Akay, 2021)** and Algerian cultivars **(Boukhebti *et al*., 2015),** while no research has been conducted on the volatile fractions of wild olive in Algeria and only one study has been performed worldwide in Portugal on *Olea europaea* L. ssp. sylvestris **(Makowska *et al*., 2017).**

From the perspective of the valorization of wild plants, Our thesis is part of the research work adopted by Valorization of Natural Biological Resources Laboratory (Ferhat Abbas Sétif 1 University), which aims to contribute to better understanding of our floristic heritage and to valorize it. In this context two wild olive samples were fixed in this research: *Olea europaea* L. var. sylvestris and *Olea europaea* subsp. *laperrinei*. Despite their value cited above, the physio-anatomical mechanisms developed, as well as the chemical profile, and biological activities of their leaf extracts, essential oil, and extra virgin oil, remain less known or unknown like in our case of *Olea europaea* subsp. *laperrinei*, because the majority of them were related to cultivated olives as previously mentioned.

The objective of this study is to characterize and valorize the two wild olive resources in Algeria using the following frameworks:

- The study of their physiological and anatomical mechanisms developed to survive in their environmental conditions.
- The determination of the phenolic extract leave's profiles and the valorization of these extracts as a natural antioxidant through evaluation of their antiradical potentials.
- Chemical characterization of their essential oils and extra virgin oils (EVOO), as well as the valorization of their medicinal value through the evaluation of their antibacterial activity.
- Describe how the different habitat factors of the two olives affect the chemical profiles of their leaf extracts, essential oils, and extra virgin oils, and the role of those chemicals in the ecophysiological adaptation to these conditions.

Our research was divided into three distinct chapters as follows:

- The first chapter is devoted to bibliographical concepts and divided into three parts (the first: a brief review of the two specimens, and their genus, and family; the second: the drought stress and its effect on *Olea europaea* L.; the third: on the antioxidant systems and secondary metabolites in olive extracts; the fourth: an overview of essential oils and extra virgin oils.
- The second chapter describes the plant material used, and all the techniques on which it is based the physiological and anatomical study, the quantitative and qualitative characterization of the extracts; the chemical analysis of essential oils and extra virgin oils; as well as the methods used in the assessment of biological activities (antibacterial and antioxidant activity).
- Third chapter the results are presented with a discussion, and finally a general conclusion is presented with some proposed perspectives.

CHAPTER I

BIBLIOGRAPHICAL CONCEPTS

Part I: Brief review of the Oleaceae family, genus *Olea* and *Olea europaea* L. complex

I-1- Brief description of the Oleaceae family

I-1-1Taxonomy of Oleaceae family

Oleaceae, or the family of dicotyledons, is a medium-sized group of woody plants that, has recently been inserted into the Lamiales order through numerous molecular studies (**Wagstaff & Olmstead, 1997; APG, 1998**) in (**Wallander & Albert, 2000**). The family comprises approximately 600 species distributed across 30 genera (**Cronquist, 1981**; **Grohmann, 1981; Bamford & de Wit 1993; Muzzalupo *et al*., 2014**), which are traditionally separated into two sub-family, Jasminoideae (with one to several erect ovules perlocule and 4-12 petals) and, Oleoideae (two pendulous ovules perlocule and 4 petals) **(Rohwer, 1996; Kim& Jansen, 1998; Besnard, 2002).**

According to the recent studies performed by **Wallander & Albert (2000) and Green (2004)**, through non-molecular (morphology, anatomical and chemical criteria) and molecular (phylogeny based on DNA sequences) data, have revealed that the Oleaceae family is currently divided into five tribes including, Myxopyreae, Fontanesieae, Forsythieae, Jasmineae and Oleeae. This later monophyletic tribe being classified into four subtribes: Oleinae (12 genera), Fraxininae (*Fraxinus*), Ligustrinae (*Syringia* and *Ligustrum*), and Schreberinae (*Schrebera* and *Comoranthus*), the Oleeae tribe is equivalent to subfamily Oleoideae **(Kim &Jansen, 1998).**

The paraphyletic Jasminoidea is divided into four tribes: Myxopyreae (*Myxopyrum, Nyctanthes*, *Dimetra)*, Fontanesieae (*Fontanesia)*, Forsythieae (*Forsythia)*, and Jasmineae (*Myxopyrum, Nyctanthes*, *Dimetra)* **(Kim & Jansen, 1998)**. Within the Jasminoidea subfamily, the genus Jasminum is the largest ,with over 200 species. Based on the findings of abovementioned studies, the different tribes and genera within the Oleaceae family was arranged in **(Figure 1).**

The composition of the Oleaceae family in genera and species differ depending on the authors:

- **Rohwer (1996)** reported that this family contains 25 genera and approximately 500 to 600 species.

- The oleaceae comprises approximately 600 species and 24 genera **(Wallander &Albert, 2000; Green, 2002; Green, 2004; Besnard *et al*., 2009)**.

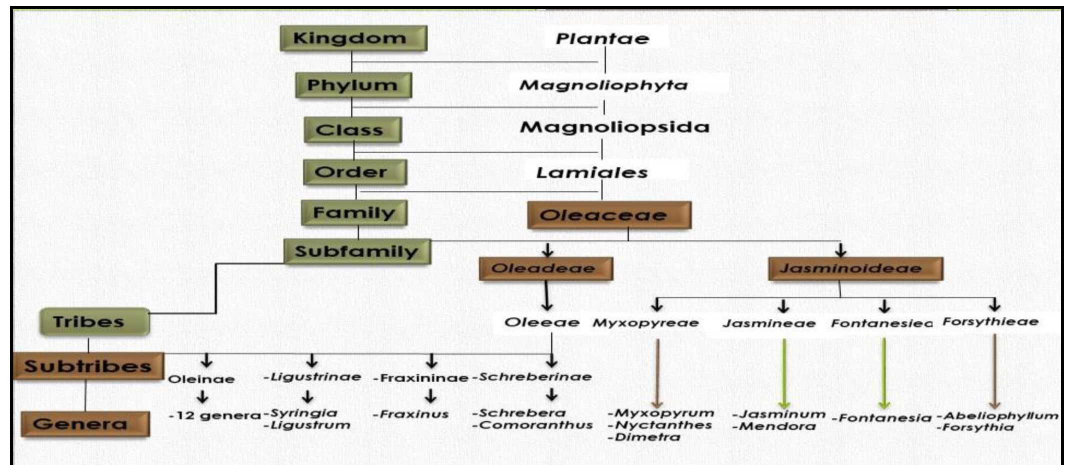


Figure 1: Taxonomy of the Oleaceae family and the phylogenetic tree of its tribes **(Wallander & Albert, 2000)**.

I-1- 2-Botanical Description of Oleaceae family:

I-1- 2-1-Vegetative characters:

The Oleaceae is a monophyletic group well characterized by morphological synapomorphics that are frequently Trees (eg; *Olea europapea* L.), shrubs (eg; *Menodora sp*), rarely woody climbers (eg; *Jasminum sp*), however there are herbaceous species (eg; *Dimetra craibiana*). This family may be deciduous or evergreen **(Wallander & Albert, 2000).** The leaves are frequently opposite leaves or rarely alternative simple or compound (trifoliate or imparipinnate), petiolate with leaf margins that are entire crenulate or rarely serrated, without stipules (**Simpson, 2019; Green, 2004**).

I-1-2-2-Floral characters

- Flowers usually actinomorphic, bisexual or unisexual (plants sometimes dioecious) subdivided into two parts:

- Non-reproductive parts (floral perianth parts): the perianth was generally tetrameric and dichlamydeous. It consisted of two parts, one of which was the calyx, which was small with 4–15-valvate lobes, calyx rarely absent. (eg; *Fraxinus sp*.) . The other part was the corolla, which was frequently sympetalous and usually had 4-12-lobed (eg; *Jasminum*), with a variety of lobe arrangements, such as imbricate, valvate or convolute, and rarely corolla wanting.

- Reproductive parts: the male reproductive portion androecium with 2 stamens, rarely 4, epipetalous or sympetalous when the stamens are adhere to the Corolla tube; Anthers are dithecal. The Pollen grains were tricolpate. The female reproductive portion such as gynoecium included two united carpels that form a syncarpous, superior bilocular ovary with 2-lobed stigma and terminal style.The ovary often has 2 ovules per loculus, but occasionally has 1 to 4, with either anatropous or hemitropous form. Fruit**:** this family shows a substantial diversity of fruits berry, capsule, drupe or samara **(Simpson, 2019; Green, 2004)**.

I-1-3- Phylogenetic analysis of the Oleaceae family

The basic chromosome number varies among family and tribes in which, Oleoideae is Monophyletic group. They all have x = 23(2n=46) **(Taylor, 1945; Green, 2004)**. In contrast, jasminoideae subfamily schowed varying low basic chromosome numbers (x =5, 11, 12, 13, or 14) **(Taylor, 1945)**.

I-1-4-Distribution

Wide-spread family throughout all continents except the Antarctic, in both northern temperate and southern subtropical ecosystems, from low to high altitude **(Wallander & Albert, 2000; Green, 2004)**.

- Some genera are broadly distributed and occur on more than one continent **(Mitchell *et al.*, 2014),** such as**:** *Jasminum* (200 species) in the tropical and subtropical parts of the old world; *Fraxinu*s (45-50species), mainly the temperate and subtropical regions of the Northern Hemisphere, and *Olea* (more than 40species) tropical and subtropical parts of the old world.
- Genera with restricted distribution **(Wallander & Albert, 2000),** including**:** *Dimetra* (1 endemic species) Thailand; *Abeliophyllum* (1 endemic species) Korea, and *Hesperelaea* (1 endemic species) Northwest Mexico.

I-1-5-Importance of the Oleaceae family

Oleaceae species are culturally important, including ornamental species such as; Lilacs (*Syringa*), Forsythias, Roènes (*ligustrum*), Filaires, and Alaverts (*Phillyrea*), as well as fragrances plants (eg; *Jasminum*). Furthermore, it has an economic importance is due to the production of oil and fruits (eg; *Olea europaea* L.), and timber, as well as some species

cultivated for used as drupes and leaves, providing food sources to wild animal (eg*;Fraxinus)*. Additionally, single species of Oleaceae might coexist with several other species as an example Fraxinus excelsior which associated almost 1000 other species (eg; fungi, insects, birds) (**Argenson *et al*., 1999; Wallander & Albert, 2000; Dupin *et al*., 2020).**

I-2-General data about the Genus *Olea*

The genus "*Olea*" appears from the Greek "elaia" and the Latin "*Oleum*"(**Kaniewski *et al*., 2012; Hashmi *et al*., 2015) ;** however, it is additionally known as "Olivo" in Spanish, "Olive" in English, French, and German, "Oliva" in Russian, Latin, and Italian, "Zaitun" in Arabic **(Khan & Khan, 2016; Msomi & Simelane, 2017).** The genera *Olea* belong to the monophyletic family of Oleaceae, and are classified into the sub-family Oleideae within the tribe Oleeae (**Wallander & Albert, 2000; Bernard *et al*., 2002**), which includes 33 species and 9 sub-species classified into three sub-genera, based on morphological characteristic *(***Green, 2002***).*

Tetrapilus contains 23 species is the most distinctive group whith greater diversity, (**Green, 2002; Besnard, 2009),** main features defining sub genus Tetrapilus are the absence of peltate scales and the corolla tube being longer than the corolla lobes (**Besnard, 2009**). The subgenus Olea has two sections (*Olea* and *Ligustroides*): section *Olea* composed exclusively by the olive complex (*Olea europaea* L.), in which six subspecies have been identified and section *Ligustroides* (8 species), the indumentum and the panicle position are the main characteristics employed for differentiating these two sections (**Vargas *et al*., 2001**; **Green, 2002**). Additionally, Paniculatae includes only one taxon (*Olea paniculata)* which is differentiated from the subgenus *Olea* by the presence of domatia on the undersides of the leaves and of both axillary and terminal panicles **(Green, 2002; Besnard & Baali-Cherif, 2009**).

I-2-1- Morphological features of the Genus *Olea*

Table 1: Distinctive morphological characters of *Olea* subgenus and sections. (**Green, 2002; Bernard *et al*., 2002; Besnard,2009)**

Subgenus	Flower characters				Inflorescence position	Leaf characters		
	Corolla tube	Stigma	Dioecy	Calyx tube Stricture		Leaf blade margins	Peltate scales	Domatia in axils of veins
Subgenus *Tetrupilus*	Longer than the corolla lobe	Shortly bilobed or capitate	Present Or Absent	/	Axillary	Entire or serrate	Absence	Absence
	Equal or							

Subgenus *Paniculatae*		shorter than the corolla lobe	Capitate	Absent	/	Axillary and terminal	Entire	Presenc e	Presence
Subg enus *Olea*	**Section *Olea***	Equal or shorter than the corolla lobe	Capitate	Absent	+ or - membranous	Axillary (or subterminal)	Entire	Presenc e	Absence
	Section *Ligustroides*	Longer than the corolla lobe	Capitate	Absent	+or – coriaceous	Terminal (sometime axillary)	Entire	Presenc e	Absence

I-2-2- Distribution

Table 2: Geographical distribution of *Olea* subgenus and its sections.

	Subgenus		Taxon	Geographical distribution
Genus *Olea*	**Subgenus *Tetrupilus***		23 species (Green ,2002 ; Bernard *et al* ., 2002 ;Besnard,2009)	South Eastern Asia (Green ,2002 ; Bernard *et al* ., 2002 ;Besnard,2009)
	Subgenus *Paniculatae*		*Olea paniculata* (Green ,2002 ; Bernard *et al* ., 2002 ;Besnard,2009)	from Pakistan to New Caledonia (Green 2002 ;Besnard ,2009)
	Subgenus *Olea*	**Section *Olea***	*Olea europaea* L. complex (6 subspecies) (Vargas et al. 2001; Green, 2002, Bernard *et al* ., 2002 ;Besnard,2009).	From China at East Southern Africa. Saharan mountains, Macaronesia Mediterranean basin (Green ,2002 ; Bernard *et al* ., 2002 ;Besnard,2009)
		Section *Ligustroides*	8 species (Vargas et al. 2001; Green, 2002, Bernard *et al* ., 2002 ;Besnard,2009).	Central and Southern Africa (Green, 2002 ; Bernard *et al* ., 2002 ;Besnard,2009)

I-3-General features of the *Olea europaea* L.complex

I-3-1-Systematic and distribution of olive tree

The *Olea europaea* L. complex is included in the *Olea* section and is the most popular member of the genus *Olea* (**Kaniewski *et al* ., 2012),** with six subspecies recognized **(Vargas *et al*., 2001; Médail *et al*., 2001; Green , 2002; Besnard *et al*., 2009)**. Studies of the level of specific ploidy for these 6 subspecies revealed that they are all diploid with the exception of *cerasiformis* (4x), *maroccana* (6x); the other subspecies are reported to be diploid **(Besnard *et al*., 2008)** ,with cases of triploidy in laperrinei (2x-3x) **(Baali-Cherif *et al*., 2007; Besnard *et al*., 2008; Besnard, 2009).**

- Subsp. *cerasiformis* (Webb & Berth.) Kunk. & Sund: endemic to Madeira **(Figure 2).**

- ❖ Subsp. *cuspidata* (Wall.) Ciferri (1942): the most widespread subspecies in the world extend from the Arabian Peninsula through eastern and southern Africa, as well as from Southeast Asia to southwest China **(Figure 2).**
- ❖ Subsp.*europaea*: includes two varieties: Var europaea (cultivated olive) and Var sylvestris = oléastres (wild olive), (Miller lehr): present in the Mediterranean basin **(Figure 2).**
- ❖ Subsp. *guanchica* P. Vargas *et al*.(2001): endemic to the Canary Islands (Figure 2).
- ❖ Subsp. *laperrinei* (Batt. &Trab.). Ciferri (1942): distributed only in the Sahara region in the Saharan massifs (Hoggar, Jebel Marra in Algeria) **(Figure 2).**
- ❖ Subsp. *maroccana* (Creut. & Burd) P. Vargas *et al*.(2001): located in the southwest of Morocco (Agadir's mountains) **(Figure 2).**

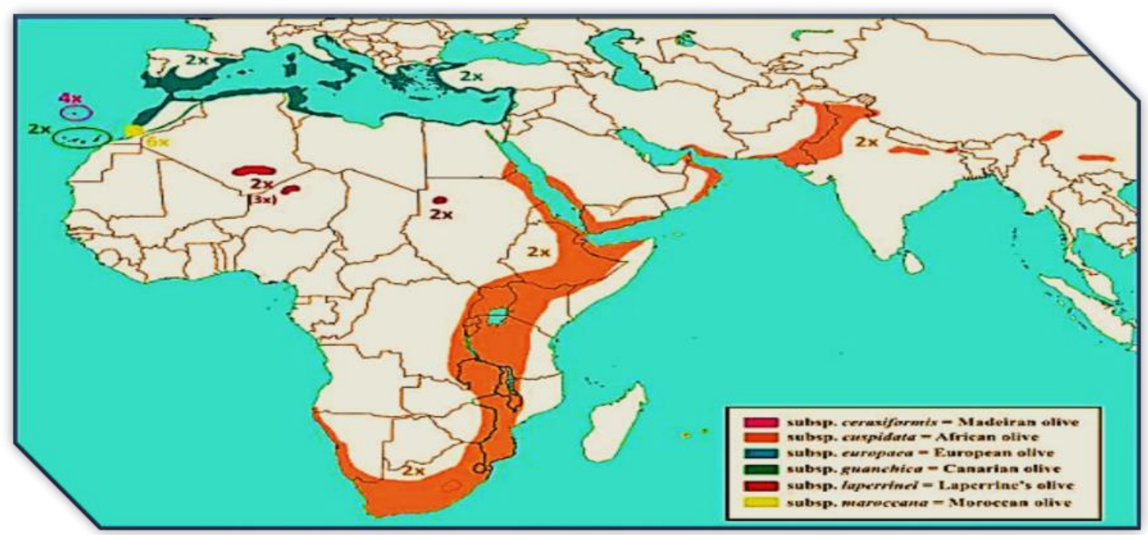


Figure 2: Distribution of the complex *Olea europaea* L. (six subspecies) worldwide **(Rubio de Casas *et al*., 2006**).

I -3-2-Morphological characteristics of the olive trees

I -3-2-1- General appearance

The olive tree is a long-lived evergreen that reaches 1000 years of age, or more. Olive trees possess a titanic resistance that makes it practically immortal. Despite of cold winters and very hot and dry summers they continue to grow, bearing fruit that nourishes and heals **(Ivan, 2005)**. Additionally it aholds historic importance in the context of religion; it is cited in the Koran **(Msomi& Simelane, 2017).**

I -3-2-1-Aerial part

- **Trunk**: straight and circular with a large diameter, as it ages; it deforms and emerges cords that give a tormented appearance characteristic of the olive tree. The cords develop into mother branches, and the secondary branches appear on them. In addition, branches are installed to give the developed and varied features of the tree, and all depends on the form of growth either horizontally or vertically **(Loussert &Brousse, 1978).** The color of the trunk varies depending on the age of the tree; up to its tenth year, the trunk is smooth and gray-green, before changing to a dark gray to gray-brown hue **(Rugini, 1998)**.
- **Leaves**: are narrow, opposite, lanceolate, or linear sometimes ovate, the leaf margin is entire and has acute tips, with short petiole. The blade is glabrous on the upper surface and the underside has a silvery appearance due to the presence of covert hairs (**Argenson *et al*., 1999; Ivan, 2005**). Leaves dimensions range from 4-10 cm in length and 1-3 cm wide with 5–11 primary veins on either side of the midrib. **(Ali, 1982; Guillen *et al*., 1993**). According to **Loussert & Brousse (1978)**, olive leaves have longevity of around three years and exhibit distinctly xerophytic characteristics associated with the hypostomatic characteristic. In which the stomatal density fluctuates depending on the variety and cultivars for adapting to variations of the environmental conditions **(Abdulrahman & Al-Bamarny, 2020).**
- **Flowers**: are arranged i in small clusters, creamy white in color, with feathery bubbles, varying in length from 1 to 2 cm **(Guillen *et al*., 1993)**. Blooms in the spring they have a standard stricture, consisting of 4 fused sepals, 4 fused petals, 2 stamens and 2 carpels, according to **Loussert & Brousse (1978)** and **Argenson *et al*. (1999)** the floral formula is as follow:

$$4\ (S) + 4\ (P) + 2E + 2C$$

Quezel & Santa (1963) reported that the number of flowers per inflorescence is a varietal character, however generally the inflorescences realized by 15 flowers in axillary clusters of 1-year wood, are functionally unisexual or bisexual a result of the formation of two distinct sorts of flowers a perfect flower with both male and female sexes and a staminate flower **(Bernie *et al*., 2006)**, it flowers from May to June **(Boucher *et al*., 2011)**. However, the floral parts can undergo anomalies: atrophy of

the stamens, the carpels, or deformation of the style all of which can cause infertility **(Loussert & Brousse, 1978).**

- **Fruit :** is an ovoid drupe with green color to black at maturity **(Shu, 1996)**, with variable dimensions between 1 and 3cm **(Loussert & Brousse, 1978; Argenson *et al*., 1999)**, and weighs about 12 to 20 grams at maturity **(Garcia-Gonzalez *et al*., 2010),** it is smaller in the wild olive than in cultivate one **(Shu, 1996)**. It consists of three distinct parts, the first of which is a leathery Epicarpe with stomata **(Mathew, 2011)** covered with a waxy material impermeable to water **(Loussert & Brousse, 1978)**, and fleshy mesocarp and rich in stored fat **(Loussert & Brousse, 1978; Chiez, 1982)**, and a woody endocarp consisting of a solid core that forms an envelope around a single seed, which in favorable conditions, will give a new olive tree. The form of the core differs between varieties **(Loussert &Brousse, 1978).** Drop when ripe, if fruit isn't harvested in the "on" year, trees tend toward alternating bearing. Olives need to mature in 6-8 months, but table olives are picked sooner when they are solid, while oil olives remain on the trees until the oil content reaches 20–30% **(Ivan, 2005)**.

I-3-2-2-Root system: the olive tree has a very extensive root system **(Pagnol, 1996)**, that extends under the trunk at a depth of 50 to 70 cm **(Loussert &Brousse, 1978)**. Even though they may extend 2-3 m below the surface in deep alluvial soils **(Carr, 2013)**. Their development (number and extent of roots) depends on pedologic and climatic conditions, as well as on the mode of multiplication of the tree **(Villa, 2003)**, therefore root system is either: pivoting if it comes from seed and in light soils or fibrous if it is obtained by cuttings and in heavy soils **(Ben Rouina, 2001)**.

I-3-3-Physiological characteristics

I-3-3-1-The development of olive trees

According to **Loussert & Brousse (1978)**, four distinctive phases in the life of an olive tree over years, as summarized in **Figure 3**:

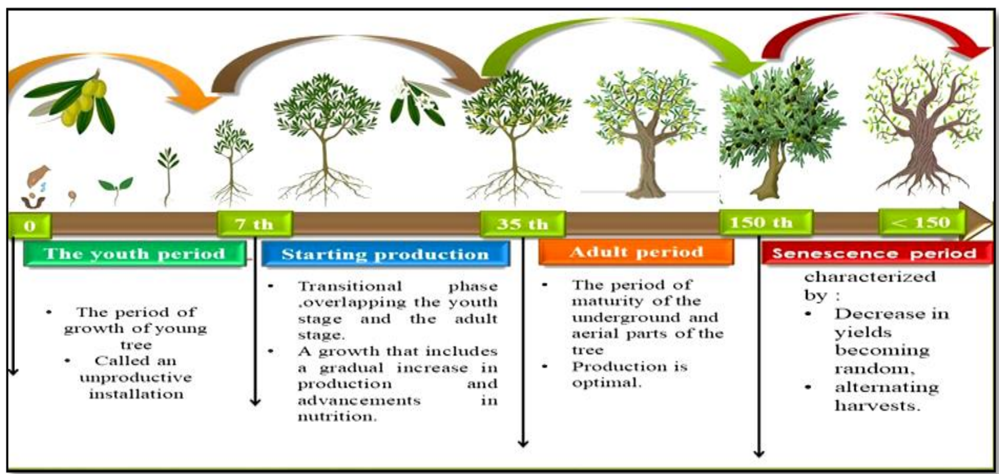


Figure 3: Life cycle of olive tree (**Loussert & Brousse, 1978**).

I-3-3-2-Annual life cycle

The phenological events of plants occur annually, but because of environmental conditions, their period may change each year. According to **Lousert &Brousse, 1978** the climatic conditions and the olive tree's adaption area, have a significant impact on its vegetative cycle.

According to **Loussert &Brousse (1978)** and **Daoudi (1994)**: the chronologies of vegetative cycle as follows **(Figure 4):**

Figure 4: Annual life cycle of olive tree (**a** :Winter rest**; b:** Vegetative awakening**; c:** Formation of floral clusters**; d :**Buds swellinge**; e:** Corollas differentiation **f:** Start blooming ; **f1** : Full bloom; **g** : Fructification; **h**: Fruit set**; h1:** Fruit enlargement**; h2:** Fruit ripening) (**Loussert & Brousse, 1978**) .

I-4-*Olea europaea* subsp.*laperrinei* (Batt &Trab)

I-4-1- Classification of *Olea europaea* subsp.*laperrinei* (Batt &Trab)

The classification proposed by **Cronquist (1981**), of the laperrine olive tree, called Aleou by the Tuaregs

Kingdom: Plantae

Sub- Kingdom: Tracheobionta

Phylum: Magnoliophyta

Sub- Phylum: Magnoliophytina

Class : Magnoliopsida

Sub-class : Asteridae

Order : Scrophulariales → **According to APG II (2003)** → **Order** : Lamiales

Family : Oleaceae

Genus : *Olea*

Species : *Olea europaea* (Linné, 1753)

Sub-species : *Olea europaea* subsp.*laperrinei* (Batt. et Trab.) Cifferi.

I-4-2-General features of the tree

It is rare to find a free foot and this tree has a generally typical bushy appearance with multiple shoots, (3 to 6 on average) with an average height varying from 1.5 to 4 m in height. Whose trunk has a diameter of approximately 0.4 to 0.5 m **(Benichou, 1962),** due to the difficult conditions of the Saharan climate the flowering events of Laperrine olive are rare **(Besnard, 2008),** the majority of trees do not flower every year **(Besnard *et al*., 2007b)** as well as, concerning fruiting only a few trees produce fruit (**Baali-Cherif & Besnard, 2005)** . This tree can resist high altitudes of 1400 and 2700 m (**Besnard *et al*., 2007b**), and extremely poor rainfall conditions with average annual rainfall varying from 50 to 100 mm/year (**Besnard *et al*., 2009**), the populations of this tree are fragmented and difficult to reach due to its locations in wadi beds and cliffs on volcanic rocks **(Table 3**), **(Baali-Cherif &Besnard, 2005).**

Table 3: Morphological characters *Olea europaea* subsp.*laperrinei* according to **Baali-Cherif (2007)**

Morphological characters of the vegetative parts		Morphological Characters of the reproductive parts		
Branches	**Leaves**	**Inflorescences**	**Flowers**	**Fruits**
	-Shape :	Length:2-4 cm	Corolla diameter :	Shape :

-Slender and flexible	Entire leaf margin and opposite Lanceolate or linear	Number of clusters per branch : 6-10	4-5mm	Ovoid drup dry Weight (g) 0,006-0,008
-The tree's center:vigorous branches with exuberant foliages	Length : 4 - 6 cm 8 -15 times longer than the wide (Quézel & Santa,1963).	Number of flower per clusters :6-8		Dimension : 5-7 mm
-Tree's periphery : very short branches with very small leaves	Width : 0,5 - 0,7 cm Petiole length :0,2 - 0,5 cm	Flexible cluster, with main axis that has 2- 4 secondary branches	Peduncle length : 1-1,7mm	The epicarp changes from a tender green color to a blackish color When ripe The endocarp consists of a very hard core
	The color : adaxial face : green abaxial face : silver the midrib : flat	The color : white		

I-4-3-Distribution

Olea europaea subsp.*laperrinei* (Batt &Trab), or laperrine's olive tree, also known as the southern olive tree, this endemic taxon, naturally adapted to the arid areas of the Saharan mountains ranges **(Besnard *et al* ., 2012).** It represents an important genetic resource for the quality of its resistance to drought and has been described as heliophilic and xerophytic subspecies. Has been observed in Algeria only in the central Sahara (**Quézel & Santa,1963**), in Ahaggar, Taessa, Abeleheg, Hoggar, Tefedest, Tassili and Mouyedir and their distribution area extends from southern Algeria to north-eastern Sudan via Niger (**Green, 2002; Baali-Cherif *et al*., 2007**). **Besnard *et al*. (2007b)**, suggests that the Hoggar (Southern Algeria) is an important reservoir of genes for the taxon and may be its center of origin because it contains a great genetic diversity compared to other massifs and this notion was previously justified by **Wickens (1976)**, who stated that this taxon was introduced to Jebel Marra in Sudan by pigeons or by human from Hoggar settlements.

I-5-*Olea europaea* L.var.sylvestris (Miller lehr)

I-5-1-Taxonomy of *Olea europaea* L.var.sylvestris

The taxonomic classification of the species according to **Cronquist (1981**):

Kingdom: Plantae

Phylum: Magnoliophyta

Sub- Phylum: Magnoliophytina

Class: Magnoliopsida

Sub-class: Dialypetals

According to APG II (2003); Ghedira (2008)

Order: Scrophulariales → **Order**: Lamiales

Family: Oleaceae

Genus: Olea

Species: *Olea europaea* (Linné, 1753)

Sub-species: *O.europea* subsp.*europaea* var. sylvestris

I-5-2-General aspects of oleaster trees

Wild olive trees (*Olea europaea* L.var. sylvestris), called oleaster, are considered to be one of the oldest trees in the world **(Kassa *et al.*, 2019)**. It is a characteristic component of Mediterranean flora, in particular of the thermo-Mediterranean bioclimatic stage **(Baali-Cherif & Besnard, 2005),** which can attain up to 10 m in height, has a short and stocky tortuous appearance **(Arab *et al.*, 2013)**, frequently appearing in the form of a shrub. The oleaster differs from the cultivated olive tree by the presence of short and thorny, almost quadrangular shoots **(Terral & Arnold-Simard, 1996)**. It has several reed branches with opposite branchlets **(Ali, 1982)** and greyish bark **(Sarwar, 2013).** According to **(Lousert &Brousse, 1978),** there are several types of oleasters which differ in habit, leaf shape, flowering and fruiting times, including true oleasters (wild forms) and feral olive (domestic returned to wild) which results from hybridization between true oleasters and cultivars **(Table 4) (Zohary & Hopf, 1994).**

Table 4: Morphological features of *Olea europaea* L.var. sylvestris as described by **Baali-Cherif (2007).**

Morphological characters of the vegetative parts		Morphological Characters of the reproductive parts		
branches	**Leaves**	**Inflorescences**	**Flowers**	**Fruits**
Robust branch with exuberant foliages	Forme : entire, ovoid, leathery or oblong	Length : 3- 6cm	Color : white	Shape : Ovoid drup
	Adaxial face : gray green Abaxial face : silver	Number of clusters per branch : (8-15)	Corolla diameter : 5-6 mm	Dry Weight 0,35-0,50g
	Length : 5- 8 cm 2 to5 times longer than the wide (Quézel & Santa,1963)	Number of flower per clusters (8-10)	Peduncle length : 1-2 mm	Dimension:4-6 mm
	-Width :1- 1,4cm -Petiole length : 0,3-0,6cm -The midrib : Protruding			-The epicarp changes from green toblack when ripe -The endocarp consists of fusiform hard core

I-5-3-Distribution

This taxon is most frequently found in dry "xirophile" lands and forests in the Mediterranean region. Its current range is the Maghreb, southern Iberian Peninsula, Mediterranean islands, southern Italy, the Peloponnese, the coasts of Greece, Turkey, the Levant and northern Libya, much more sporadically on the northern Mediterranean coast **(Zohary *et al.*, 2012).**

Part II: Impact of drought stress on *Olea europaea* L.

II-1-Drought stress

Soil degradation and drought constitute major obstacles to plant growth, plants are frequently subject to extreme conditions of water potential, temperature, and salinity, which result in various types of stress (**Hopkins, 2003**).The onset of drought manifests it self through the combination on the one hand, the restriction of soil water availability and, on the other hand, the increase in evaporative demand **(Poormohammad Kiani, 2007)** environmental stress,notably water stress is one of the main environmental limitations of Mediterranean vegetation (**Di Castri, 1981**). Seriously restrict plant growth and plant productivity (**Wang *et al*., 2011**). As they constitute a permanent threat to the survival of the latter (**Hopkins, 2003**), the plant's response to stress essentially depends on the intensity of the stress, its duration, the phase of the plant's development, and its state when stress is detected **(Yokota *et al*., 2006; Demirevska *et al*., 2009)**. The development of adaptive mechanisms by the plant to handle stress, maintain their water status, and tolerate dehydration (**Oukara *et al*., 2017**). Tolerance to water stress is manifested by modification of plant processes such as physiological, biochemical and morphological characteristics **(Doré *et al*., 2006; Zid & Grignon, 1991; shakeel *et al*., 2011**; **Oukara *et al*., 2017).**

Additionally, it is defined as a decrease in the relative water content in plant tissues and osmotic potential, as well as a loss of turgor and stomata closure **(Braham, 1997).** As a result, photosynthesis is reduced and plant enzymatic processes generally slow down, all of which contribute to the reduction in growth **(Kasraoui *et al*., 2004)**.It is the result of a temporary decrease in water availability to plants **(Chaves & Oliveira, 2004)**.

II-2- Different mechanisms employed by plants to manage water deficits

Plants respond to reduced water availability by employing different strategies, which can be divided into three separate mechanisms:

II-2-1-Drought escape

Plants can shift and/or shorten their vegetative cycle over time (**Amigues *et al*., 2006**).With this strategy, plants complete their life or growth cycle before severe water stress occurs **(Tardieu, 2005**; **Basu *et al*., 2016).**

II-2-2-Drought avoidance

It is a conservative strategy that allows the plant to avoid water deficit by avoiding tissues dehydration by reducing transpiration or increasing water uptake, **(Tardieu, 2003; Tardieu,**

2005; **Antunes *et al*., 2018**). Water absorption is optimized by a larger allocation towards the development of the root system (**Jackson *et al*., 2000**), reducing transpiration by stomatal regulation «stomatal closure induced by abscisic acid (ABA) », in which the closure of stomata reduces carbon assimilation and leads to slowed photosynthesis of transpiring leaf surface area by slowing tissue growth and falling of aged leaves (**Chaves *et al*., 2010; Muller *et al.*, 2011; Osmolovskaya *et al* ., 2018 ; Li *et al.*, 2018**).

II-2-3-Drought tolerance

It consists of maintaining the important physiological functions of plant despite water deficits (**Amigues *et al*., 2006**). As a result of the the osmotic adjustment phenomenon, the plant reduces its water potential by increasing the concentration of solutes and protective proteins to maintain cellular turgor ,which prevent stomata from closing, preserving physiological functions such as photosynthesis (**Osmolovskaya *et al* ., 2018**)

II-3-Adaptative strategies to drought stress

Acclimation of plants to water deficit is the result of different events, which lead to adaptive changes such as changes in plant morphology, anatomy, physiology and antioxidant response:

II-3-1-Morphological adaptation

Under stress conditions, the processes of plant adaptation differ from one species to another, resulting in morphological and physiological as well as biochemical changes in both aboveground and underground plant parts. Generally, when water availability is limited, trees develop their root systems (**Markesteijn &Poorter, 2009**), by increasing the root/shoot ratio and the rooting depth, in which the growth of the root part is considerably more important than the aerial part (**Wu & Cosgrove, 2000; Poorter *et al*., 2012; Brunner *et al*., 2015**), because the roots are less sensitive than the shoots to growth inhibition caused by low water potential, (**Wu &Cosgrove, 2000**). These growth mechanisms are strongly influenced by the degree of the stress. Under moderate stress, Plants respond with a small increase in root mass fraction (RMF) while maintaining their aboveground growth, However, under extremely stressful conditions, plants show a significant increase in RMF, mainly as a result of a a decrease in stem growth (**Poorter *et al*., 2012 ; Brunner *et al*., 2015**).

In this context, the olive root system emerges very parallel to the soil, making it better suited to absorbing water intermittent rainfall than the water of deep layers (**Fernández &Moreno, 1999**). Concerning drought adaptive mechanism of the root part olive tree adjustments were made to the root organogenesis, including the number, diameter, and depth of roots. Olive

root growth and dispersion are significantly influenced by soil properties **(Connor &Fereres, 2005)**, the total root system is more important in dry than humid conditions **(Brito *et al.*, 2019)**.In this manner deeper roots are developed to access more soil volume which enables them to use water reserves up to the deeper layers **(Assunta, 2010),** to maintain transpiration under stressful conditions and reduce the intensive drying of the upper soil layers through hydraulic redistribution from deeper layers to the surface **(Nadezhdina *et al.*, 2015).**

Water deficits slow the installation of new aerial organs and reduce the growth of preexisting organs **(Gaufichon *et al.*, 2010).** The leaf is the most reactive and adaptable organ to environmental stress **(Nevo *et al.*, 2000; Marchi *et al.*, 2008).** When a drought occurs decreasing plant water content, the cells shrink, and relaxes resulting in loss of turgor **(Farooq *et al.*, 2012)**, which consequently reduce the leaf water potential **(Farooq *et al.*, 2009)** in **(Brito *et al.*, 2019)** and the speed of division of cells constituting plant tissues **(Granier *et al.*, 2000)**. The modification of the appearance of the leaf depends on the stage of development at which the stress is applied, if imposed in an early stage of the cell expansion inhibits cell expansion and reduces leaf area while leaves will senesce and can fall off, if imposed after a substantial leaf area has developed **(Farooq *et al.*, 2012)**. In the other hand, a decrease in the number of branches and growth rate could influence the number of leaves **(Farooq *et al.*, 2012; Shakeel *et al.*, 2011).**

II-3-2-Anatomical adaptation

Due to plant anatomical plasticity under drought stress an important adaptation mechanism was implemented to mitigate drought stress and various features appeared, such as the decrease in the size of vascular tissue and some major vascular bundles in the midrib, as stimulation of bulliform cell expansion, the increased in stomatal and chloroplast density, and reduction of stomatal size and leaf thickness **(Taratima *et al.*, 2020; Taratima *et al.*, 2021).**

Olive leaf posses particular anatomical structure that gives them more resilient to harm caused by drying ,including high mesophyll compactness, organized along sclereids in spongy parenchyma and two or three palisade layers connected to the upper epidermis **(Briton *et al.*, 2019)**, in addition to the reduction in the leaf area plated with a waxy layer and stellar trichomes concealing. There is an increase in non-glandular leaf hairs and appearance of the small and abundant stomata on the abaxial leaf surface **(Bosabalidis & Kofidis, 2002; Briton *et al.*, 2019).**

II-3-3-Physiological adaptation

- **Stomata closure:**

The first and foremost effect of drought is established on cell growth due to the reduction in turgor pressure which is controlled by a signals cascade «ex; Abscisic acid (ABA), cytokinins » which is generated from roots to shoots inducing the loss of turgor pressure leading to stomata closure, which is an important physiological adaptation to water deficits. This strategy is known as drought avoidance **(Shakeel *et al*., 2011).**

- **Osmotic adjustment:**

Other mechanisms may be implicated when drought persists for prolonged periods and the avoidance strategies are insufficient for sustaining plant growth In this case, plants might decrease their osmotic potential and maintain cell turgor by accumulating organic and inorganic solutes and protective proteins (known as an osmotic adjustment), cell wall hardening, ROS detoxification, and metabolic changes were also observed. This strategy is referred to as drought tolerance **(Shakeel *et al*., 2011; Osmolovskaya *et al*., 2018).**

Olive trees have demonstrated evidence of osmoregulation as a stress avoidance mechanism and exhibit a strong capacity for osmotic adjustment under drought conditions. To resiste to these stress the tree should decrease its osmoticum potentiel (**Rieger, 1995 in Massenti *et al*., 2022**). This enables the establishment a high water gradient between roots and plant which allows the extraction of water from the soil (**Xiloyannis *et al*., 1999, Dichio *et al*., 2006**). Different mecanisms of osmotic regulation have been developed by the accumulation of organic compatible solutes in olive leaf and root cells including sugars, sugar alcohols, amino acids. These solutes not only acumulate to maintain osmotic adjustment, but also enable the metabolic machinery to continue functioning by protecting cellular proteins and membranes. Olive tree have proven that cell wall elasticity is another factor that helps in osmotic adjustment (**Bacelar *et al*., 2009 in Breton *et al*., 2019**). This factor provides both maintaining turgor pressure and protecting against cell wall breakage, because of their capacity to shrink more readily under stress (**Joly& Zaerr, 1987**).

II-3-4-Biochemical adaptation

II-3-4-1-Reactive oxygen species (ROS) in plants

As an early line of defense against biotic and abiotic stresses, also referred to as oxidative stress, plants produce reactive oxygen species (ROS) such as oxygen ions, free radicals, and

peroxides. ROS are a natural by-product of normal oxygen metabolism and are crucial for cell signaling **(Shakeel *et al*., 2011)**.On the other hand, ROS levels rise dramatically under water stress, resulting in oxidative stress and subsequent oxidative damage, including DNA fragmentation, protein degradation, and ultimately cell death **(Apel &Hirt, 2004).**

II-3-4-2-Antioxidant enzymes in plants

The regulation of the antioxidant system is one of the most relevant mechanisms against oxidative stress caused by ROS, in which plants have an internal defensive system catalyzed by enzymes developed to avoid damage caused by (ROS) to guarantee normal cell function **(Horváth *et al*., 2007)**.The olive tree developed specific mechanisms to protect its photosynthetic systems, such as an increase in non-enzymatic antioxidant mechanisms including the accumulation of phenolic compounds and carotenoids as well as certain enzymatic antioxidant mechanisms **(Brito *et al* .,2019)** . According to a study result performed by **Abdallah *et al*.(2017)**, olive trees exposed for the first time to drought established memory of ROS which is translated by high levels of ROS signaling to keep the antioxidant system on alert.

Part III: General data on oxidative stress, antioxidant systems and secondary metabolites

III-1-General informations about free radicals and oxidative stress

III-1-1-Free radicals

Early in the 20th century, chemists first introduced the concept of free radicals, defining them as intermediate organic and inorganic molecules with various of suggested definitions **(Engwa, 2018)**. Free radicals are chemical species (molecule, part of a molecule or an atom) that can exist independently, having one or more unpaired electrons in their atomic orbital (single electron on the peripheral layer of the molecular skeleton) **(Halliwell & Gutteridge, 2015; Engwa, 2018)** .This species is therefore characterized by instability **(Afonso *et al*., 2007),** and high reactivity. In order to reach a more stable state, these radicals can act as a reducer that gives a free electron to other molecules, or behave as an oxidant that can extract an electron from neighboring molecules.The majority of these radicals have a very short half-life (6−10 s) in biological systems despite their high reactivity **(Young, 2001).**

III-1-2-Free radical generation

Free radicals can originate from either extracellular or intracellular sources:

- Extracellular sources: some organic substances in the atmosphere have the ability to produce free radicals through non-enzymatic reactions with oxygen, including environmental pollution (aromatic hydrocarbons, pesticides...), extreme temperatures, radiation (X-rays, -rays), heavy or transition metals (eg ; Cd, Hg, Pb, As, metal ions such as Fe2+ and Cu+), microbial infections, medications, and their metabolites.
- Intracellular sources include operations carried out during normal metabolism in living organisms, including several cell organelles; such as mitochondria, peroxisomes, and the endoplasmic reticulum, as well as numerous enzymes, metabolism of fatty acids, and cell phagocytic activity **(Engwa, 2018 ; Martemucci *et al* ., 2022).**

III-1-3- Oxidative stress

In moderation, the body can benefit from free radicals.The antioxidant/prooxidant balance is thought to be in equilibrium under typical conditions. If this is not the case, then either linked to the deficit of antioxidants or an overproduction of radicals, then the excess of these radicals is called oxidative stress. In addition , oxidative stress can translate into the inability of the

body to defend it self against the attack of reactive oxygen and nitrogen species (RONS) **(Sies, 2015)**, the primary cell molecules can be affected through excessive production of ROS and/or RNS which can lead to the denaturation of proteins with a decrease in their activity, enzymatic inactivation and alterations of amino acids, DNA filament fragmentation, base mutation, and altered gene expression, as well as glucose autooxidation,lipid peroxidation and alteration of membrane functions **(Engwa, 2018; Martemucci *et al.*, 2022).**

III-2-Antioxidants

An antioxidant is a molecule that reduces or prevents the oxidation of other substances, also defined as "any substance which, in low concentration relative to the substrate probably oxidized, prevents or slows down oxidation of this substrate". Additionally, it is a molecule that can neutralize active forms of oxygen and maintain non-cytotoxic levels of free radicals levels at cell and organism **(Halliwell, 1999; Santos-Sánchez *et al.*, 2019).**

This functional definition covers a wide range of substances, including enzymes with specific catalytic properties and small water- or lipid-soluble molecules. This physicochemical variety allows the presence of antioxidants in all compartments of the body, whether intracellular, membrane, or extracellular **(Carocho &Ferreira, 2013; Godic *et al.*, 2014).**

III-3-Antioxidant activity of olive leaf extract

Several studies have demonstrated the powerful free radical scavenging ability and antioxidant activity of olive leaf extract (**Cebe *et al.*, 2012; Makowska-Wąs *et al.*, 2017**). Olive leaves are considered an inexpensive, sustainable, and abundant source of antioxidants **(Lafka *et al.*, 2013).** Generally, leaf extract has higher antioxidant activity than vitamin C and vitamin E (**Suliman *et al.*, 2013**), as in the case of olive extracts (**Benavente-Garcıa *et al.*, 2000**). The antioxidant properties of olive leaves are mainly linked to the bioactive compounds that they contain, notably oleuropein and hydroxytyrosol which have powerful antioxidant properties (**Goldsmith *et al.*, 2018**), in which oleuropein showed antioxidant effects on intestinal mucosal damage **(Alirezaei *et al.*, 2014)** and on human breast cancer cell lines (**Bulotta *et al.*, 2011**). According to **Jemai *et al.* (2008a**), during the peroxidation of liver lipids, hydroxytyrosol exhibits antioxidant capacities by increasing the activities of (CAT) and (SOD). The other coumpounds identified in OLE have a hright antioxidant activity such as, rutin, apigenin **(Sharififar *et al.*, 2009),** tyrosol (**Visioli &Galli, 2002**), caffeic acid (**Adjimani &Asare, 2015**), and luteolin (**Tsimogiannis *et al.*, 2007**).

The high antioxidant capacity is not due only to single components, and combined effects of flavonoids, oleuropeosides, and substituted phenols **(Suliman *et al.*, 2013)**. Similar to olive extract which is composed of an array of phenolic alcohols, phenolic acid flavonoids, and oleuropein, it exerts significant antioxidant capacities by reducing lipid peroxidation in the liver exposed to stress **(Dekanski *et al.*, 2009)**, and increases renal glutathione concentration, which reduces nephrotoxicity **(Tavafi *et al.*, 2012)**

III-4- Analytical methods for determining antioxidant activity *in vitro*

Many methodologies are available to evaluate the antioxidant activity of substances or natural extracts. The methods are based on the use of free radicals such as TEAC, DPPH, TRAP, or metal ions for the FRAP test **(Popovici *et al.*, 2009)**. The most widely used tests have been written.

III-4-1-DPPH radical Scavenging Assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a characteristic purple radical **(Alsaleem *et al.*, 2020).** It has an unpaired electron on a nitrogen atom (DPPH radical form). This electron becomes paired in the presence of an antioxidant accompanied by the change of color to yellow, therefore reduced form (DPPH-H), which can be quantified by UV/Visible spectroscopy from 515 nm to 518 nm as a function of time **(Popovici *et al.*, 2009),** in order to follow the Scavenging kinetics **(Di Meo *et al.*, 2013).** DPPH is one of the first free radicals used to study the structure-antioxidant activity relationship of phenolic compounds **(Popovici *et al.*, 2009)**.The DPPH scavenging mechanism can follow two types of mechanisms on the one hand by the transfer of atoms and on the other hand, by the transfer of electron **(Figure 5) (Chen *et al.*, 2004; Delattre, 2007)**. This test is also simple and very easy to implement because it only requires with the radical reagent DPPH and the antioxidant **(Sirivibulkovit *et al.*, 2018).**

Figure 5: Chemical structure of stable DPPH and its reduced form (DPPH-H) **(Gülçin, 2012).**

III-4-2-Test TEAC (Trolox Equivalent Antioxidant Capacity)

This method provides an evaluation of the capacity of a compound trapping the radical cation ABTS $^{\bullet +}$ (2, 2'-azino-bis-3-ethylbenzthiazoline -6-sulfonic acid), which is colored blue-green transformed into a blue to green color during the Formation of ABTSH can be monitored by UV/Visible absorption spectrophotometry in the range of 600–750 nm. The particularity of this method is that the capacity of an antioxidant is expressed in relation to Trolox **(Karadag *et al*., 2009)**, which is a chemical analog of vitamin E **(Christen, 2000).**

III-4-3-Ferric Reducing Antioxidant Power (FRAP)

Method used to evaluate of antioxidant activity based on colorimetric electron transfer for the reduction of ferric iron (Fe+3) present in the K3Fe(CN)6 complex into ferrous iron (Fe+2), this reduction is done in the presence of an antioxidant (**Pellegrini *et al*., 2003**),and the reaction is reflected by a change of color from yellow color of (Fe+2) to blue-green of (Fe+2) **(Figure 6) (Chou *et al*., 2003).** The intensity of this color was measured by spectrophotometry at 593 nm **(Thomas, 2016).**

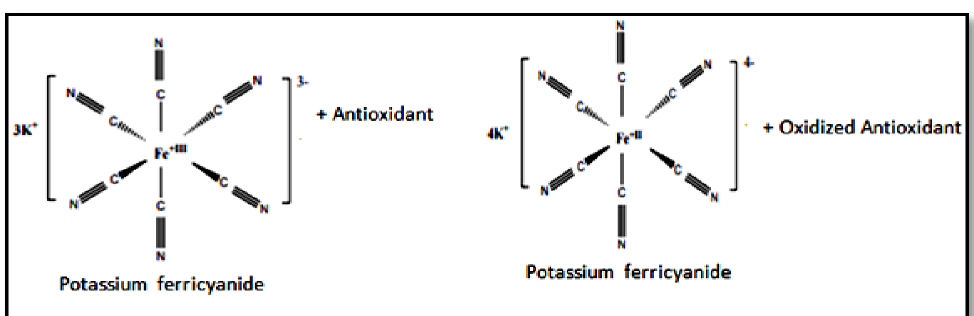


Figure 6: Reaction mechanism of the FRAP test **(Gülçin, 2012)**

III-5-Secondary metabolites

Secondary metabolites are frequently described as molecules that are not only essential for the maintenance of plant life processes, but also are fundamental substances for the defense and adaptability of plants against external attacks. Primary metabolites (such amino acids, lipids, and carbohydrates) are converted into a variety of secondary metabolites in higher plants **(Akula& Ravishankar, 2011),** and they are divided into various chemical classes principally alkaloids, terpenes and phenolic compounds **(Raven, 2009)**. The physiological and developmental stage of the plant have a significant impact on the synthesis of these

substances **(Rao &Ravishankar, 2002)**. Also they are essential frequently for providing defense against environmental stresses. In addition, they are important for adaptability in plant stress physiology **(Seigler, 1998)**. Additionally, secondary metabolites contribute to giving plants their distinct odors, colors, and tastes **(Akula& Ravishankar, 2011)**.

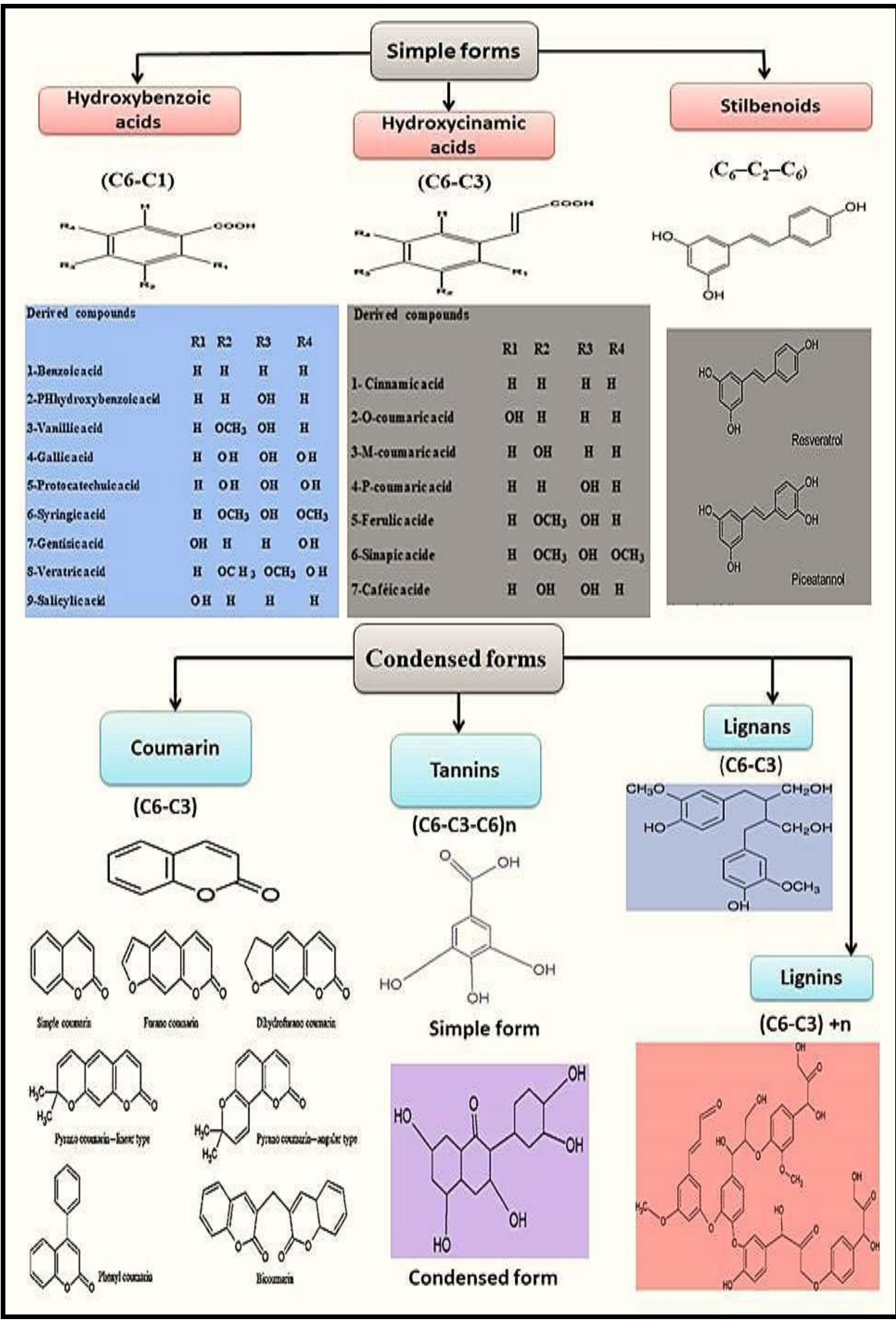
III-5-1- Phenolic compounds

Phenolic compounds often known as polyphenols are phytomicronutrients **(Dacosta,2003)** and the most commonly distributed secondary metabolites, ubiquitously present in the plant kingdom and accumulate in all plant parts including the roots, stems, leaves, flowers, and fruits **(Lattanzio,2013)**. Approximately 8,000 compounds have been identified as belonging to the immense family of polyphenols, which have been identified due to their structural features diversity **(Figure 7) (Dacosta, 2003)**.

According to **Raven et al. (2007),** phenolic compounds are distinguished by having an aromatic core to which at least one hydroxyl group (-OH) is attached and they are divided into two major classes based on the number of carbon atoms in the primary skeleton: non-flavonoids and flavonoids **(Hanasaki *et al*., 1994; Dacosta, 2003).**

III-5-1-1-Non-flavonoids compounds

Hopkins (2003) reported that non-flavonoids compounds mostly consist of phenol acids, lignins and tannins as shown in the **(Figure 7).**



Derived compounds

	R1	R2	R3	R4
1-Benzoicacid	H	H	H	H
2-PHhydroxybenzoicacid	H	H	OH	H
3-Vanillicacid	H	OCH_3	OH	H
4-Gallicacid	H	OH	OH	OH
5-Protocatechuicacid	H	OH	OH	OH
6-Syringicacid	H	OCH_3	OH	OCH_3
7-Gentisicacid	OH	H	H	OH
8-Veratricacid	H	OCH_3	OCH_3	OH
9-Salicylicacid	OH	H	H	H

Derived compounds

	R1	R2	R3	R4
1- Cinnamicacid	H	H	H	H
2-O-coumaricacid	OH	H	H	H
3-M-coumaricacid	H	OH	H	H
4-P-coumaricacid	H	H	OH	H
5-Ferulicacide	H	OCH_3	OH	H
6-Sinapicacide	H	OCH_3	OH	OCH_3
7-Caféicacide	H	OH	OH	H

Figure 7: Basic structure of simple forms **(Dykes &Ronney, 2006)** and Condensed forms of phenolic acids and their main compounds derived **(O'Connell &Fox, 2001)**.

III-5-1-2-Flavonoides compounds

The term flavonoid brings together numerous natural compounds divided into several families, the most important of which are flavones and isoflavones. These pigments give plants their colors **(Bruneton, 1999).** Constitute the largest class of phenolic compounds. More than 4000 compounds have been identified, of which approximately 50% of polyphenols. These compounds have a basic structure formed of 2 benzene nuclei A and B connected by a nucleus C which is a pyranic heterocycle ($C_6C_3C_6$) **(Lobestein, 2010).** The different classes of flavonoids depending on the structure and oxidation state of the central nucleus C, flavonoids include the following classes: flavanones, flavones, flavanols (catechins), flavonols, flavonols, isoflavones, and anthocyanins **(Arrabi *et al.*, 2004)**.

III-5-2-Phenolic compounds identified in olive leaves

According to the literature, the amount of phenolic compounds in olive leaves varies considerably from 2.8 mg/g **(Altiok *et al.*, 2008)**, to 198 mg/g, **(Djenane *et al.* 2019)**, although in some instances it can even exceed 250.2 mg/of dry matter. **(Mylonaki *et al.*, 2008).** According to the study carried out by **Brahmi *et al.* (2013) and Djenane *et al.* (2019)** the variability of the concentration of phenolic compounds in olive leaves is linked to several factors, including factors specific to the species such as the variety, age of the plantations, maturity, and post-harvest, as well as environmental factors that include climatic conditions, edaphic conditions. in addition the concentration of phenolic compounds can vary due to storage conditions before and after extraction and operating conditions such as the method of preparing olive leaf extracts.Quantification techniques and also depending on the extraction solvents **(Lafka *et al* ., 2013).**

The phenolic composition of olive leaves has been the subject of literature. A range of phenolic compounds have been identified through chemical analysis of olive leaf extract. The phenolics compound already identified in olive leaf extracts were shown in **(Table 5).**

Table 5: Phenolic compounds frequently identified in olive leaf extract

Chemical classes	Phenolic compounds	Structures	References
Phenolic acids	Caffeic acid	HO HO COOH	Kawsar *et al.*(2008) ;Dekanski *et al.*(2009) ; Omar(2010) ; Tavafi *et al.* (2012) ; Abaza *et al.* (2015) ; Makowska-Wąs *et al.*(2017) Djenane *et al.* (2019), Mechchate *et al.*(2020)

	Vanilic acid	HO–C6H3(OCH3)–COOH	Kawsar *et al*.(2008);Omar(2010);Abaza *et al*.(2015) ;Makowska-Wąs *et al*.(2017) ; Djenane *et al*.(2019) ; Mechchate *et al*.(2020)
Phenolic alcohols	Tyrosol	HO–C6H4–CH2CH2–OH	Omar(2010);Tavafi *et al*.(2012) ;Abaza *et al* .(2015) ;Makowska-Wąs *et al*.(2017) ; Djenane *et al*.(2019) ;Mechchate *et al*.(2020)
	Verbascoside		Omar(2010);Vogel *et al*.(2015) Abaza *et al* .(2015) ; Makowska-Wąs *et al*.(2017) ; Djenane *et al*.(2019) ; Mechchate *et al*.(2020)
Flavonoids	Luteolin		Dekanski *et al*.(2009) ; Omar(2010);Vogel *et al*.(2015) ; Makowska-Wąs *et al*.(2017) ; Djenane *et al*.(2019) ; Mechchate *et al*.(2020)
	Quercetin		Dekanski etal (2009) ; Omar (2010);Olayinka *et al*.(2015) ; Makowska-Wąs *et al*. (2017) ; Djenane *et al*.(2019) ; Mechchate *et al*.(2020)
	Rutin		Omar(2010);Makowska-Wąs *et al*. (2017) ; Djenane *et al*.(2019); Mechchate *et al*. (2020).
	Apigenin		Dekanski *et al*.(2009) ; Omar(2010);Vogel *et al*.(2015) ; Makowska-Wąs *et al*.(2017) ;Djenane *et al*.(2019) ; Mechchate *et al*.(2020).
Secoiridoides	Oleuropein		Benavente-Garcia *et al*.(2000) ;Al-Azzawie *et al*.(2006) ;Altiok *et al*.(2008) Dekanski *et al*.(2009) ;Omar(2010) ;Tavafi *et al*.(2012) Vogel *et al*.(2015) ; Afaneh *et al*.(2015), Alhamdz *et al*.(2015) ;Qadir *et al*.(2016) Makowska-Wąs *et al*.(2017) ; Djenane *et al*.(2019) ;Mechchate *et al*.(2020).
	hydroxytyro-sol		Erbay *et al*.(2010) ; Omar(2010) ;Tavafi *et al*.(2012) ; Vogel *et al*.(2015) ;Makowska-Wąs *et al*.(2017) ; Djenane *et al*.(2019) ; Mechchate *et al*.(2020).

Numerous plant species belonging to different families include phenolic acids, phenolic alcohols, and flavonoids, however, only the Oleaceae family contains secoiridoids **(Díez&Adamos, 1997)**. Oleuropein, demetiloleuropein, ligstroside, and nüzhenide are the compounds categorized as secoiridoids in olives.Oleuropein is the primary component of olive within the secoiridoid structure **(Mazza, 1993)**. Additionally according to **Altiok *et al.* (2008) and Goldsmith *et al.*(2018)**, oleuropein, is the main phenolic of olive leaves, accounts for 29% of the components in olive leaf extract.

III-5-3- Biological effects of phenolic compounds

III-5-3-1-Functions of plant phenolics compounds

Plant phenolics are thought to play crucial physiological role because they may attach to certain proteins and enzymes, and also change the enzymatic balances in the process; they participate in redox chains and alter a number of operations related to morphogenesis, respiration, and growth. Additionally, polyphenols are often involved in the defense mechanisms of plant; the biosynthesis of these substances is a response to biotic and abiotic stress including pathogen infection, insect and herbivore predation, and nutritional poverty, as well as an increase in the formation of free radicals and other oxidative species in plants **(Lattanzio, 2013)**. Plant phenolics are regulated by genetic factors involved in the stress response, including morphological, biochemical, and physiological change **(Figure 8)** **(Calatayud *et al*., 2013)**.

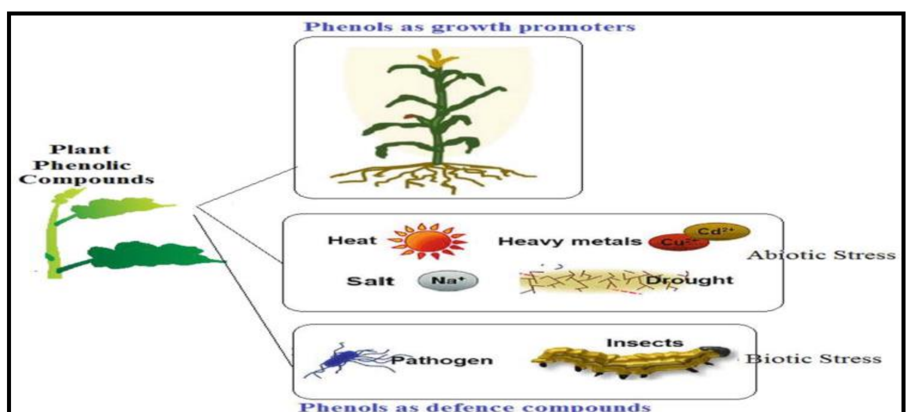


Figure 8: Overview of the function of plant phenolic compouds (**Prastyusha, 2003**).

III-5-3-2-Advantages of polyphenols in human health

Many plants are used in phytotherapy, and their effects are often attributed to the phenolics chemicals that contain. These chemicals have biological effects that are benefitial to human health. Polyphenols are potent antioxidant agents, the position of hydroxyl groups on aromatic nuclei **(Halliwell, 1994; Rolland, 2004)** and the capacity of aromatic compounds to support electronic delocalization are two structural factors that influence the potency of polyphenols as antioxidants. Additionally, polyphenols can exert their effects through a variety of strategies, including inhibiting the enzymes responsible for oxidative stress, chelating the metal ions that generate reactive oxygen species, and safeguarding antioxidative defense systems **(Halliwell ,1994)**.

Due to the high levels of antioxidants contained in these compounds, polyphenols have anti-aging effects **(Pandey &Rizvi, 2009)**, which explains why anti-aging cosmetic pruducts contain them **(Menaa *et al*., 2014),** in addition, several polyphenols have anti-inflammatory effects **(Sakurai *et al*., 2010)**, and a significant antibacterial activity is exhibited by these compounds **(Ghnimi, 2015).** Polyphenols have an anti-diabetic effect that may influence glycemia levels through a variety of mechanisms, such as the inhibition of gut absorption of glucose or the uptake of glucose by peripheral tissues **(Pandey &Rizvi, 2009).**

Flavonoids are typical phenolic compounds, that have been shown to exibit several pharmacological and biochemical activities, including their function as potent metal chelators and free radical scavengers and antioxidants with the ability chain-breaking. The biological advantage of flavonoids includes reducing the activity of harmful metals that cause oxidative stress, such as iron and copper it is interesting to note that flavonoids are known to possess antioxidant-dependent vitamin C-sparing and vitamin C-stabilizing properties **(Kandaswami & Middleton, 1994)**. Concerning their pharmacological effects, flavonoids have Neuroprotective effects, such as Alzheimer disease **(Pandey &Rizvi, 2009)**, their effect can be explained by inhibiting the acetylcholinesterase (AChE) which is the target enzyme in the treatment of Alzheimer's disease **(Brühlmann *et al*., 2004).** Other flavonoids classes has also been reported to possess anticancer property like Quercetin **(Karakaya, 2004; Pandey &Rizvi, 2009).**

The therapeutic value of olive leaves is associated with the presence of oleuropein, which has a variety of pharmacologic and health-promoting properties**(Al-Azzawie *et al*., 2006)**, including, anti-inflammatory activity **(Puel *et al*., 2006,Vogel *et al*., 2015),** hypoglycemic and cardioprotective effect **(Al-Azzawie *et al*., 2006)**, anti-cancer effects **(Sedef *et al*., 2009)**, neuroprotective effect **(Sun *et al*., 2017)**, and antimicrobial activity **(Bisignano *et al*., 1999)**. Additionally, it possesses powerful antioxidant activity–**(Benavente-Garcia *et al*., 2000)**. These findings suggest that oleuropein can be extracted from olive leaves as a renewable natural resource **(Savournin *et al*., 2001; Briante *et al*., 2002; Bouaziz & Sayadi, 2005).**

III-5-4- Analysis of phenolic compounds

III-5-4-1-High-Performance Liquid Chromatography (HPLC)

- **Principle of the proposed technique**

According to **Panaiva (2006)** and **Ali (2022),** high-performance liquid chromatography analysis consists of exploiting the interactions of solutes with two phases, one mobile phase and the other stationary phase under high pressure. The latter solutes are separated according to several parameters such as the affinity of the solute in the eluent, the polarity or the electric charge. After separation, the solutes are identified and measured using a detector coupled to the chromatographic column. The mode of operation of HPLC is described in detail below. Thus, at a given moment, the mixture to be separated is injected into the inlet of the column, and is entrained by the mobile phase **(Figure 9).** The constituents of the mixture are then collected and identified according to their adsorption and desorption rates. Chromatography results show a representative plot of constituent (peak) according to their retention time at the outlet of the column.

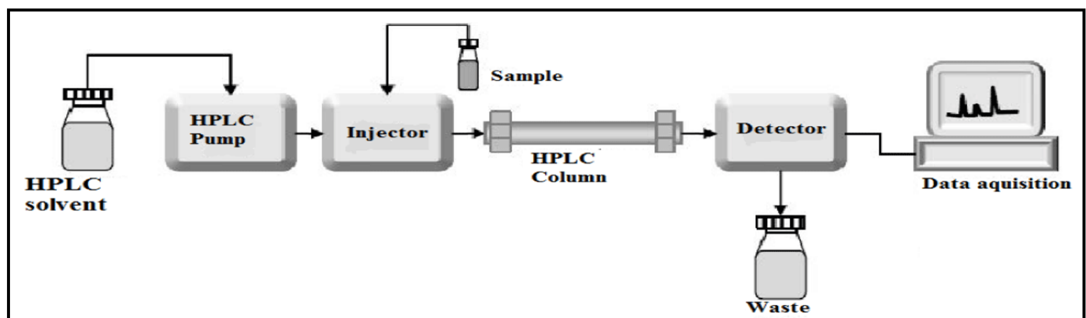


Figure 9: Component of high-performance liquid chromatography HPLC **(Ali, 2022).**

- **Qualitative and Quantitative analysis**

It makes it possible to distinguish among the various components of a mixture. The retention times (Rt in min) were compared to commercial standards chosen under identical conditions. The chromatogram's peak areas are calculated as the basis for the quantitative analysis. These areas correspond to the product concentrations that were examined. It is possible to quantify a particular compound by comparing its area to that of an appropriate standard. A calibration curve showing the area as a function of concentration was created for this purpose. The subsequent injection of the same volume (v) of the sample to be assayed makes it possible, using the measurement of the peak area reported on the calibration curve, to determine the mass or concentration of the sample **(Panaiva ,2006).**

Part IV: Overview of essential and fixed oils

IV-1-Definition of essential oils:

The term “essential oil” was coined in the 16th century by Swiss physician Parascelsus von Hohenheim to designate the active compound of a natural remedy. Today there are approximately 3000 essential oils (**El Kalamouni *et al*., 2010**). Many authors have attempted to define essential oils as follows:

- Following ISO (the International Standards Organization) , and AFNOR (the French Standardization Association) it is defined as follows: “Product obtained from a vegetable raw material, either by steam distillation or by mechanical processes from the epicarp of Citrus, or by dry distillation” **(ISO, 1997; AFNOR (1986,2000).**
- Tenscher *et al*. (2005) defined as: a mixture of lipophilic, volatile and often liquid compounds synthesized and stored in specialized cellular structures.
- As defined by **Naeem *et al*. (2018)** these compounds are concentrated hydrophobic liquids containing volatile compounds obtained from plants.
- As stated by **Kar *et al*. (2018)** complex compounds characterized by a strong odor and are formed from various plant metabolites. They are clear and soluble in lipid/organic solvents and have a density lower than that of water.

IV-2-Physical and chemical properties of essential oils

Aromatic plants are plant species that produce essential oils that are synthesized by plants for defense, signalling, or as part of their secondary metabolism **(Bauer *et al*., 2008; Steward, 2005)**. They play important roles in plant defense against both biotic and abiotic stresses and serve as chemical signals allowing the plant to control or regulate its environment (ecological role) such as, the attraction of pollinating insects repellent to predators (**Pavela, 2015; Zuzarte& Salgueiro, 2015; Dhifi *et al*., 2016**), inhibition of seed germination, or communication between plants (emission signals chemically signaling the presence of herbivores. EOs contained in herbs are responsible for different scents that plants emit **(Dhifi *et al*., 2016)**. Aromatic plants are distributed worldwide and belong to a limited number of families including, Asteraceae, Cupressaceae, Lamiaceae, Lauraceae, Rutaceae, Myrtaceae, Piperaceae, and Poaceae (**Svobod& Greenaway, 2003;Bruneton *et al*., 2004**). These EO are complex mixtures that may contain over 300 different compounds **(Dhifi *et al*., 2016)**. Althought they have common physical properties, they can vary depending on their chemical composition **(Caissard *et al*., 2004).**

- **Lipophilic**: poorly soluble or insoluble in water but soluble in alcohol, ether, and fixed oils **(Dhifi *et al.*, 2016) ,**whose solubility is total in fatty oils which represent their best solvents, it is high in alcohols and in organic solvents (**Franchomme& Pénoël,2001).**
- **Liquid**: are generally liquid at room temperature **(Dhifi *et al.*, 2016),** however, there are exceptions; viscous, solid, or even a solid-liquid mixture is producted when extracted from star anise **(Kaloustian &Hadji-Minaglou, 2012).**
- **Volatile**: are volatile, this is what makes them steamable and particularly fragrant. This characteristic also differentiates them from fatty or “fixed” vegetable oils. If the latter leaves indelible stains, the trace left by the essential oil tends to disappear almost quickly. As volatility is closely linked to chemical composition, monoterpenes are, for example, much more volatile than sesquiterpenes **(Fernandez& Chemat, 2012)** .
- **Colors:** most essential oils have an almost imperceptible yellow. They darken aging (oxidation), but there are some exceptions , where the flow of blue oils from annual tansy, the green of bergamot zest, and the red (*Satureja sp*.). In some extreme cases, aged and oxidized essential oils present a significant toxic risk **(Fernandez &Chemat, 2012 ; Faucon, 2012).**

IV-3-Essantial oil generation: Plants may secrete essential oils through secretory glands placed either internally (inside the plants) or externally (on the plant surface) (**Svoboda& Greenaway, 2003**). They are generated by a variety of plant organs such as the root part (rhizomes, and roots) or aerial part (flowers, herbs, buds, leaves, fruit, bark, seeds, and wood). Because they are toxic to cells, they can accumulate in particular histological structures including glandular trichomes, secretory cavities, and resin ducts (**Asbahani *et al* .,2015 ; Fahn,2001**). The synthesis of essential oils can be affected by many factors, such as biotic and abiotic (exogenous source), as well as by the stage of a plant's overall growth and certain organs (endogenous source) (**Sangwan *et al*., 2001 ;Gobbo-Neto &Lopes** ,**2007**). Many parameters including ontogeny, rate of photosynthetic activity, photoperiod, nutrition, humidity, salinity, soil nature, storage structures and growth regulators, affected the production of essential oils both quantitatively and qualitatively (**Sangwan *et al*., 2001**),as well as there are numerous reports on differences in oil content and composition in aromatic plants due to seasonal variation. Microclimatic factors such as temperature, rainfall distribution and geographical features especially altitude also contribute to differences in

chemotype of certain essential oil bearing plants. The type and nature of oil constituents and their individual concentration levels are important attributes particularly in terms of biological activities of the essential oils (**Bakkali *et al*., 2001).**

IV-4-Advantages of essential oils and their applications

IV-4-1- Phytotherapeutic and Pharmacological activity:

As a result of their potential as medicinal agents, essential oils are used as decongestants for the respiratory system as peppermint oil **(Naeem *et al*., 2018).** During recent years many reports have confirmed various activities of essential oils, such as antimicrobial activity **(Essien *et al*., 2018)**, anti-inflammatory activity **(Kar *et al*., 2018),** and antioxidant activity due to the bioactive components of essential oils **(Lesgards *et al*., 2014).** These properties are the result of their chemical composition **(Kar *et al*., 2018).**

The pharmaceutical industry also uses essential oils in a growing number of forms (spray complexes, lozenges, capsules, toothpastes etc.). These preparations containing essential oils meet the requirements for plant-based medicines, and are used to enhance the sensory qualities of pharmaceutical drugs. The primary application of essential oils in pharmaceutical is aromatherapy Moreover, they can be used as balms, compresses and creams **(Naeem *et al.,* 2018).**

IV-4-2-Application of essential oils

Essential oils are widely used as flavor enhancers in many areas of the food industry **(Garneau,2005)** ,and are also included in the composition of foods in the form of herbs or spices.Their great antimicrobial activity **(Fernandez &Chemat ,2012)**, and antifungal activity **(Kumar *et al*.,2008)** allow them to be the preservatives of foods with excellence.

The global natural perfume market has experienced significant growth because of the increasing use of natural fragrances such as essential oils due to their numerous health benefits such as aromatherapy **(Campolo *et al* .,2017)**, in contrast to artificial fragrances which include chemicals that can cause endocrine disruptors and carcinogens **(Ramezani *et al* .,2008).** Essential oils are also used in cosmetic products as preservatives because of their biological properties **(Fernandez &Chemat ,2012).**

Some reviews on the potential of essential oils in the agricultural industry as repellents and insecticides have been published owing to their phytotoxicity, and they are intended to replace

synthetic products. The stored product sector is the perfect candidate for developing of new EO-based alternative pest control strategies. Products based on essential oils have already confirmed their effect on stored product protection **(Ramezani *et al*., 2008;Campolo *et al*., 2017).**

IV-5- Chemical composition of essential oils

IV-5-1-Terpenoids

Terpenoids are a set of molecules with very varied structures, ranging from simple carbon chains to complex cyclic arrangements. All of these molecules have one thing in common: they all come from the condensation of isoprene subunits C5H8 **(Thormar, 2011).**

The classification of terpenoids is based on the number of isoprene units **(Figure 10),** is pentacarbon molecules that comprise them **(Thormar, 2011; Dewick, 2002).**

There are more than 30,000 different terpenes, making it the largest group of natural products known. Monoterpenes are by far the most represented, followed by sesquiterpenes **(Thormar, 2011).** In nature, terpenes exhibit various chemical functions: alcohols, oxides, aldehydes, ketones, carboxylic acids, and esters **(Breitmaier, 2006).**

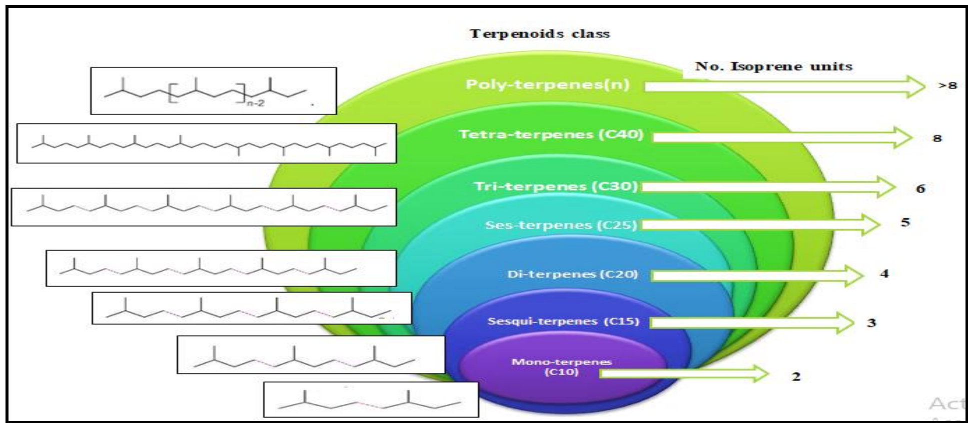


Figure 10: Classification of terpenoids based on number of isoprene units **(Mosquera *et al*., 2021).**

IV-5-2-Aromatic compounds

Aromatic compounds are phenylpropane derivatives (C6-C3), which are much less present in the composition of essential oil, but they are considered an important set because they are generally responsible for the organoleptic characteristics of essential oils. Very often, these are phenols (Eugenol, chavicol) present in the essential oil of clove (Eugenol), alcohols (cinnamic alcohol) present in the Balsam of Peru essential oil, aldehydes (cinnamic aldehyde) found in cinnamon essential oil, methoxy derivatives (Anethol, estragol, elemicin) such as

fennel essential oil (Anethole) or methylene dioxy (apiole, myristicin, safrole) which make up the essential oil of parsley (apiole) **(Mayer, 2012).**

IV-5-3-Compounds of various origins

Depending on the recovery method used, essential oils can contain various aliphatic compounds, generally of low molecular mass which can be produced during hydrodistillation: hydrocarbons (linear or branched, saturated or unsaturated), acids (C3 to C10), alcohols, aldehydes, acyclic esters and lactones **(Bruneton, 1995; Ghestem *et al*., 2001**).

IV-6-Extraction techniques

Once the plant raw material has been identified, it is appropriate to use an extraction methode adapted to obtain it. Currently, distillation remains the reference method.

IV-6-1-Hydrodistillation

This method, which is one of the oldest and simplest methode , has recently been abandoned in favor of new distillation processes for reasons of quality and production cost **(Duval, 2012**). Today, it is a standardized method, whether for the extraction of essential oils or for quality control **(AFNOR, 1996).**

According to **Bousbia (2011),** the process consists of immersing the vegetable raw material in a still filled with water placed on a heat source until it boils. Boiling water penetrates the plant cells so that the water vapor carries away the plant's volatile substances. This steam is collected and condensed. The essential oil made up of these different volatile substances separates by gravity from water with which it is immiscible **(Figure 11).**

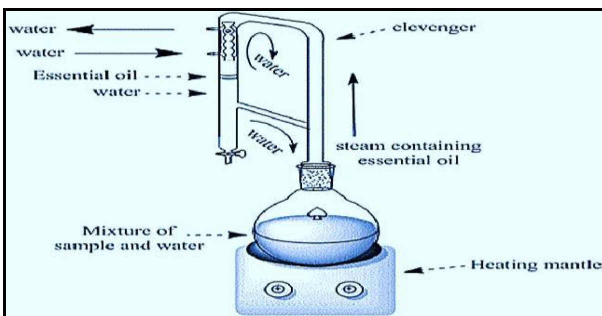


Figure 11: Hydro-distillation Clevenger apparatus system **(Samadi *et al* ., 2017).**

The heat applied to the plant raw material allows the cells to burst and the molecules contained therein to be released. An azeotropic mixture is formed comprising water and

volatile molecules whose boiling temperature is close to 100°C while the boiling temperature of aromatic molecules alone is often much higher **(Lucchesi, 2005)**.

IV-6-2-Steam training technique

Steam distillation is a more recent variant of distillation in which no direct contact is made between plant matter and water. Here, water vapor is produced in a separate boiler, then injected at the base of the still in which the plant is located. The steam rises into the still and passes through the plant. In the same way as in hydrodistillation, we witnessed a bursting of the cells and the formation of a mixture of azeotrope, recovered at the top of the tank and condensed **(Lucchesi, 2005).**

IV-7-Analysis and identification of essential oils:

The characterization of essential oils is an essential step in their valorization. It is therefore necessary to develop rapid, reliable and appropriate analytical tools to identify and quantify the different constituents of these complex mixtures.

IV-7-1-Analysis of essential oils by gas chromatography (GC)

Gas chromatography (GC) is an analytical method that allows the separation of volatile compounds or compounds probably vaporized by heating without decomposition. It is essential for its acceptable cost, automation possibilities, robustness, reproducibility and modularity **(Fernandez & Chemat, 2012).** GC is based on the sharing of the analyte between a mobile gas phase and a phase (liquid or solid) immobilized on the surface of an inert support **(Skoog *et al*., 2003).** GC comprises of three main parts according to **INSA (2015)**:

- **Injector:** the sample is introduced with a microsyringe, through an elastomer septum, into the vaporization chamber. The injector has a dual function. The sample is brought to the vapor state and then brings it into the gas flow at the top of the column.

- **Column:** placed in a temperature-regulated enclosure. It comes in the form of a silica tube, rolled up on it self and with a length ranging from 1 to more than 60 m. Entrained by an inert carrier gas; the analytes studied are separated according to their ability to interact with the stationary phase.

- **Detector:** this module make it possible to detect or even identify compounds at the column outlet.

The identification of a substance can be facilitated by knowing its retention time which is a characteristic value for a given stationary phase. the retention time, specific to each compound which depends on the operating conditions (nature of the stationary phase, temperature programing, aging of the column), does not represent a sufficient basis for identification **(Bicchi *et al* ., 2008).**

IV-7-2-Chromatographic Retention indices

The identification of a compound cannot be based solely on the knowledge of its retention time which is a value linked to the nature of the stationary phase and the experimental conditions used. Retention indices, which are more reliable than retention times, are preferred for essential oils analysis. They are calculated from the retention times of a range of linear alkane standards at constant temperature (Kovats Index, KI) **(Kovats, 1965)** or by temperature programing (Retention Indices, RI) **(Souici *et al*., 2007; Lourici &Messadi, 2009).** For each constituents of a mixture, the retention indices are calculated on polar and nonpolar columns. Then, they are compared with those of reference compounds. However, for the same compound, it is common to note discrepancies when comparing the retention indices obtained in the laboratory with those in the literature, particularly for compounds eluted at the end of the column and in the case of the polar column **(Joulain, 1994).**

IV-7-3-Gas Chromatography coupled to Mass Spectrometry (GC/MS)

The coupling of GC with mass spectrometry (CG-MS) in electronic impact mode (SM-IE) is the most widely used technique in the field of essential oils. The principle of mass spectrometry consists of bombarding a molecule with electrons that will be fragmented; the different positive fragments obtained constitute the mass spectrum of the molecule. This technique provides structural information from the observed fragmentations but allows us to know, in most cases, the molar mass of a compound. It makes it possible to identify compounds by comparing their mass spectrum with those of reference compounds contained in commercial computerized spectral libraries **(Adams, 2007).** In most cases, the use of mass spectrometry and retention indices calculated on two columns with different polarities in CPG, allows the identification of a large number of constituents in essential oils **(Bicchi *et al*., 2008).**

IV-7-4-Gas Chromatography coupled with Flame Ionization Detector (GC/DIF)

The flame ionization detector (FID) is the most popular detector. The operating principle of the proposed method is as follows. When the solute is burned in a hydrogen flame, it is

formed by the combustion of CO, furthermore CO2 which are then ionized into CO^+ CO_2^+. These ions are collected by an electrode, often in the shape of a cylindrical grid centered around the axis of the flame and brought to a potential varying between 100 and 300 V. The ionic current is then amplified and recorded. It is a universal detector that responds to any organic compound: (it does not respond to permanent gases as well as to a certain number of mineral and organic gases: CO, CO2, COS, CS2, SO2, H2O, NH3, NO, N2O, HCOOH, HCHO, SiCl4). This detector is very sensitive but requires very stable gas flow rates. The very weak current resulting from the detection is strongly amplified and transformed into a voltage measurable by an electrometer. The peak area varies according to the quantity of compound eluted in a linear relationship over a very wide range of concentrations. Moreover the response (signal/mass) of the FID detector is quite comparable for many organic compounds, and the GC/FID is very useful for quantitative and in particular the semi-quantitative analysis of essential oils (by direct normalization of the areas of the chromatogram without any prior calibration) (**Bouderdara, 2013).**

IV-2- Olive oil

IV-2-1-Definition Olive oil and its beneficial effects

Olive oil is a fruit juice from the olive tree considered as versatile product, one of the components of the Mediterranean diet recommended by many dieticians, it is very well known for its virtues and beneficial effects on human health **(Figure 12).** it has acquired a place in the research of its medicinal and cosmetic properties, also has a heritage dimension, a market value and a use value **(Serra, 2009; Terral *et al*., 2009),** thanks to its physicochemical characteristics and organoleptic products which are defined by the commercial standard of the International Olive Council **(COI, 2009)** and at European level, by Annex I of Regulation (EEC) No. 2568/91 (EEC, 1991. Owing to these characteristics the oil sector becoming a strategic economic sector that plays an important role internationally, but olive production and the quality of the oil extracted depend very strongly on the cultivar (**Ouaouich &Chimi, 2007),** which gives an olive oil with its sensory profile **(Demnati, 2008).**

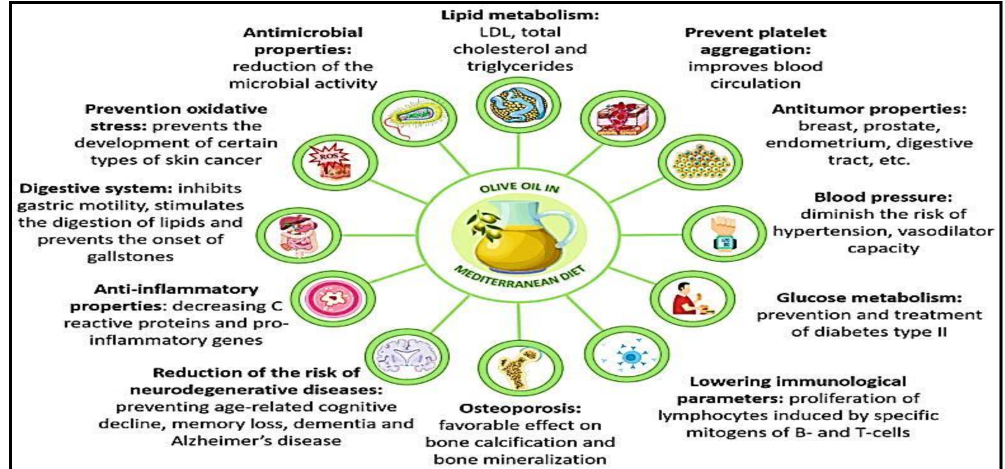


Figure 12: Beneficial and health effects of olive oil **(Jimenez-Lopez *et al*., 2020).**

IV-2-2-Production of olive oil

IV-2-2-1- Olive oil extraction

IV-2-2-1-1- Preliminary operations

This step corresponds to the olive harvest and is the first step that must be performed carefully respecting the period and the method of picking **(Amouretti& Comet, 1985)**, it is performed when the fruits reach their physiological maturity stage. Several harvesting techniques have been adopted, and these are performed either by picking, digging, or by mechanical means using pneumatic combs or shakers, and manual harvesting remains the best technique, because it preserves the fruit and consequently produces quality oils **(Iddir, 2020).** The transport of olives is the second stage in which olives are transported in appropriate containers

such as wooden or plastic containres that allow good ventilation of the olives without the risk of crushing. On the other hand, transporting olives in jute bags is not very suitable, because this method inevitably causes damage to the drupes, especially if they are very ripe. They are the source of the biological processes that alter oil quality (**Argenson *et al*., 1999).** Storage is necessary when the reception rate is greater than the crushing capacity, the duration must be as short as possible, 2 to 5 days maximum, because prolonged storage represents a main cause of deterioration in the quality of the oil, unused, damp musty and rancid). a long shelf life leads to fermentation, an increase in the acidity level, the peroxide index, as well as the deterioration of the organoleptic properties of the oil **(Chimi *et al*., 2007),** leaf stripping is necessary to avoid too greenish coloring of the oil this operation can be carried out manually or using a vibrator sieve very often accompanied by vacuum cleaners.

IV-2-2-1-2-Grinding

Constitutes the first mechanical phase of the extraction it self, in this operation, the olive tree is subjected to actions that cause rupture of the cell walls and membranes resulting in the release of cell juices and oil. Two types of crushers are more widespread: stone grinder (traditional installations) and hammer crusher (modern installations) **(Iddir, 2020)**.

IV-2-2-1-3- Malaxing

This operation aims to homogenize the dough and detach the lipid cells; increase the percentage of free oil and assembly of oil droplets **(Boskou, 2006; Iddir, 2020).**

IV-2-2-2- Extraction processes

IV-2-2-2-1-Batch processes (Pressure extraction)

Once the olive paste which is composed of pomace and a must containing the oil and vegetable oils. is homogenized and coalescence is carried out, the following step consists of the separation of the two solid-liquid phases by simple pressure, in which the solid phase (the remains of the stones, the skin and the pulp of the olives devoid of its oil) and the liquid phase (contains oil). Oil is separated from vegetable waters by natural decantation **(Hamlat ,2022)**.

IV-2-2-2-2-Continuous processes (Extraction by centrifugation)

Olive oil extraction is performed in successive phases. These are horizontal centrifuges subject to constituent differences according to two or three phases and separation and carried out according to differences in specific weight:

- **Continuous three-phase system:** using a horizontal centrifuge called a “decanter”, which works continuously, the liquid and solid phases are separated. These centrifuges are called three-phase because they separate: the pomace the oil the vegetable oils For

good oil-water and vegetable oil-oil separation, the oily phase and the aqueous phase each undergo vertical centrifugation **(El Hajjouji *et al.*, 2007).**

- **Continuous two-phase system:** in this process the decanter separates the oil and mixes the pomace and vegetation water in a single phase of pasty consistency called wet pomace or 2-phase pomace. This two-phase decanter allows slightly higher oil yields to be obtained than those obtained by the conventional three-phase decanter and the press system **(Chimi, 2006).**

IV-2-3-Chemical composition of olive oil

Like all vegetable oils, olive oil is composed of a saponifiable fraction (triglycerides, phospholipids, etc.) made up of fatty acids and their derivatives, and an unsaponifiable fraction or minor components **(Figure 13),** which includes sterols, alcohols aliphatics, pigments, hydrocarbons, aromatic compounds, tocopherols and phenolic compounds **(Berra, 1998).** The chemical composition of olive oil largely depends on the fruit variety, agronomic conditions, degree of maturity, extraction processes and storage conditions **(Dugo *et al.*, 2004).**

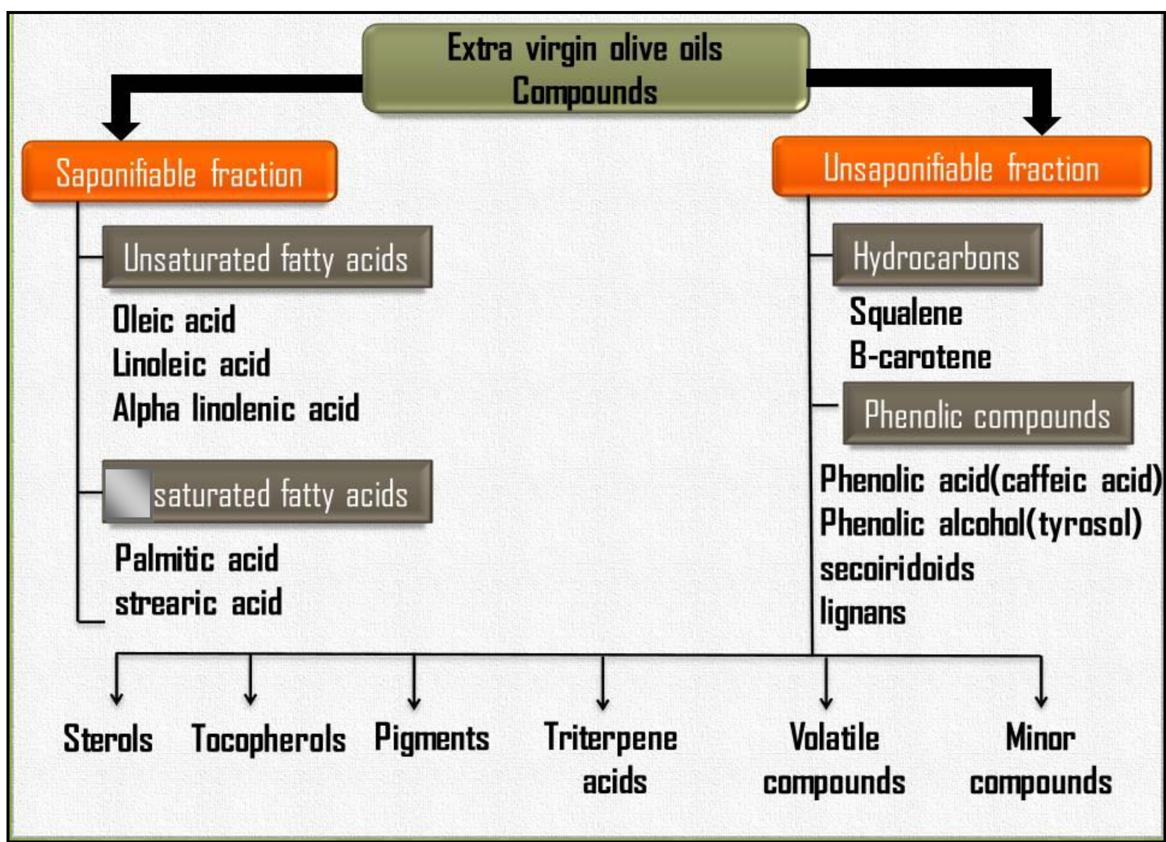


Figure 13: Main chemical compounds of olive oil **(Joshi &Zanwar, 2021).**

CHAPTER II

MATERIALS AND METHODS

I-Biological Material

The aerial parts (stems, leaves, fruits) of two wild subspecies of *Olea europaea* L. harvested, during the month of February 2021 for the laperrine olive and in the spring for the other (leaves, stems), while the fruits that were collected during the beginning of November concerning the subspecies that collected in the central Sahara Hoggar "Tamanrasset" in the case of *Olea europaea* subsp.*laperrinei*, and in the beginning of December for the other subspecies that were sampled from the Sétif region in the case of *Olea europaea* L .var sylvestris. For each one, two stations were prospected, the lists of the stations under investigation and the geographic coordinates are shown in (**Table 6, Figue 14**).

Identification of specimens: subspecies were identified using:

-Identification Keys: were provided by **Ozenda (2004)** and **Quézel &Santa (1962–1963)** keys.

- Online Herbariums: by comparing them to specimens from online herbariums, such as ENSA (National Superior School of Agronomy; Algeria) and MPU (Herbarium of North African Vascular Plants of Montpellier; France)

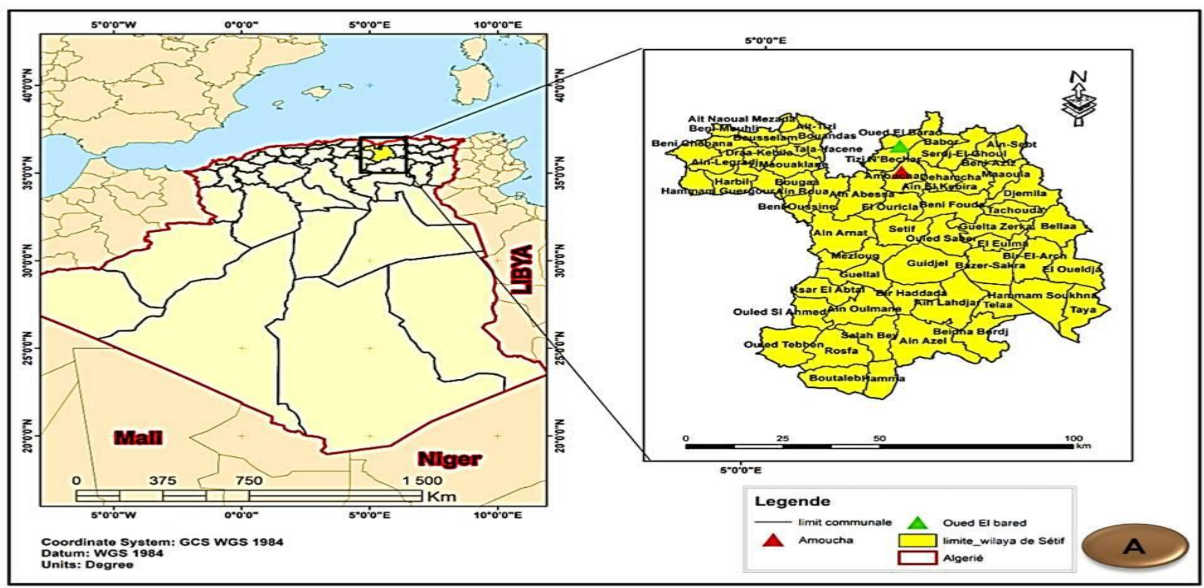
- Online Databases: Tela Botanica and APD (African Plant Database)

The identification of subspecies was also confirmed by Pr: Tarek Hamel, at the University of Annaba. Reference specimens for *Olea.europaea* subsp. *laperrinei* and *Olea europaea* L.var. sylvestris have been deposited in the herbarium of the Valorization of Natural Biological Resources Laboratory (L.V.R.B.N) of the Faculty of Nature and Life Sciences, Ferhat Abbas University. The specimens were registered under the numbers 23 /OEL 27 and 23 /OES 28.

Table 6: Altitude and geographic locations of the sampling sites of *Olea europaea* L.var. sylvestris and *Olea europaea* subsp.*laperrinei.*

Plant	***Olea europaea* subsp. *Laperinie***		***Olea europaea* L.var. sylvestris**	
Harvest region	Mountainous massifs of high plateau ‘ Setif ’ (Figure 14)		Massifs of Hoggar ‘Tamanrasset’ (Figure 14)	
Harvest sites	**Amoucha**	**Oued Elbared**	**Ilman**	**AkarAkar**
Longitude (East)	05° 41 E	05° 40 E	05°47 E	05 °70 E
Attitude (North)	36 °38 N	36° 37 N	23°21N	23 °05N
Altitude(m)	693m	814m	1952m	1861m
Harvest	20Č	21 Č	24 Č	22Č

temperature (air temperature)				
Harvest time	14:00 h	13:00 h	13:30 h	12:00 h
Bioclimatic stage	Belongs to the semi-arid bioclimatic stage with a cold winter		Belongs to the Saharan bioclimatic stage with a hot winter	
sampling substrate characteristics	Olive populations are frequently difficult to reach due to high mountain ranges, notably in the Oued El Bared station (Figure 15).		Olive populations are often difficult to reach because of their rocky, granitic sampling substrate (they grew on volcanic rocks), accompanied by coarse sand, for example the Ilaman station, the olive populations grew on a wadi bed (Wadi Ilaman) (Figure 15).	



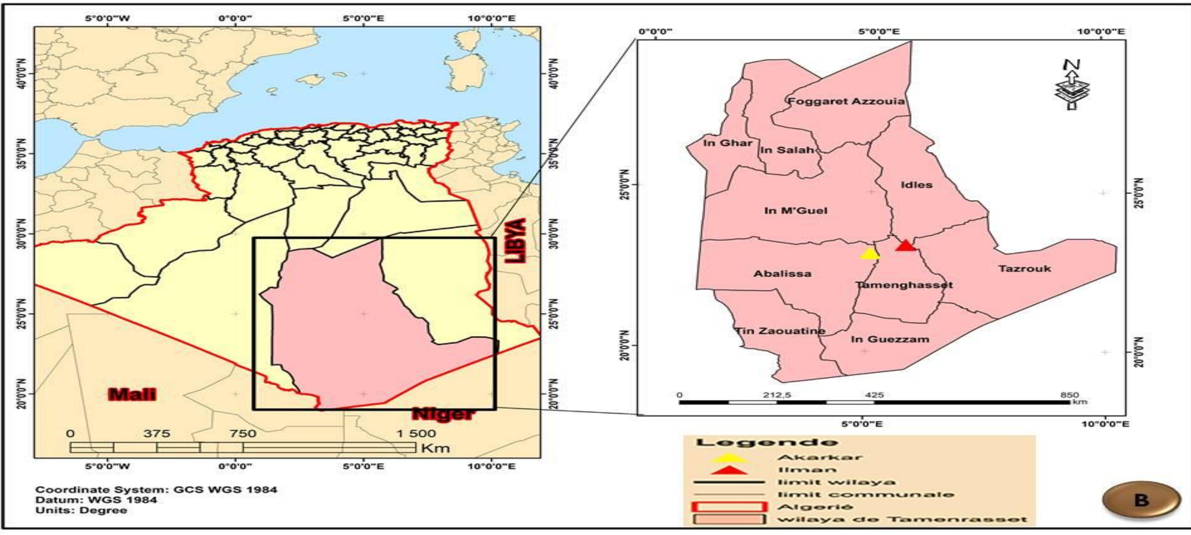


Figure 14: Location of the harvesting sites (A) of *Olea europaea* L.var. sylvestris, and (B) of *Olea europaea* subsp.*laperrinei.*

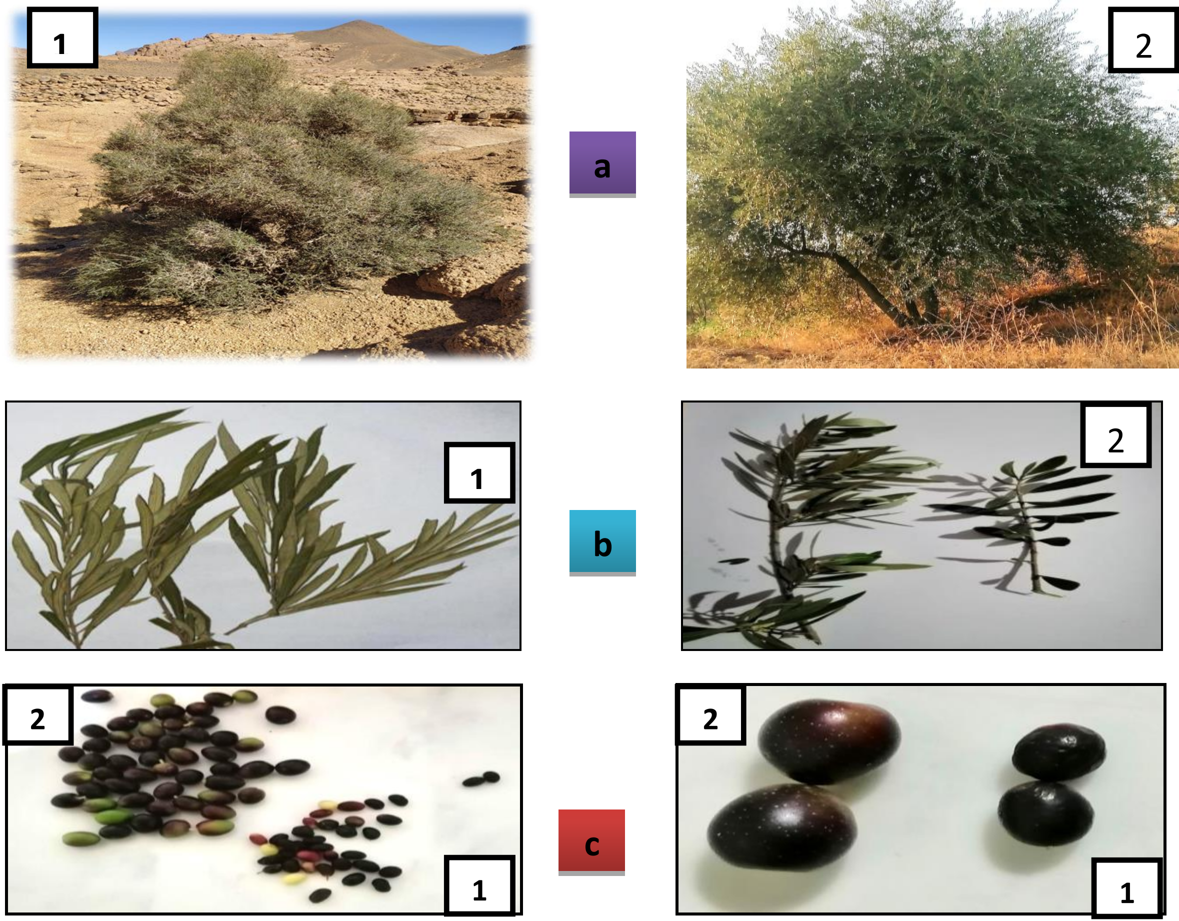


Figure 15: Different parts of the two subspecies, a:Tree, b :Leaves,c : Fruits, (1) : *Olea europaea* subsp.*laperrinei*, (2) : *Olea europaea* L.var. sylvestris.

- **Selection of plants**

Our work is part of the promotion of wild biodiversity and natural products from these food and medicinal plants. The subspecies under investigation were selected based on a review of the literature, which indicated that these subspecies have either not been investigated at all or have been rarely investigated. Through this study, we aimed to contribute to a better understanding the capacity of resistance to water stress, chemical and phenolic composition and biological activities of these spontaneous subspecies.

II-Physiological and anatomical study for assessing the effects of drought stress

II-1-Physiological parameters

The purpose of the current study was to compare two subspecies of olive tree, of *Olea europaea* subsp. *laperrinei* and *Olea europaea* L.var. sylvestris are located in two different bioclimatic stages; one of them belongs to the Saharan bioclimatic stage (Tamanrasset sites) and the other from the semi-arid bioclimatic stage (Setif sites) in terms of water stress rate,

physiological responses, and adaptive potential, by applying the following physiological parameters :

II-1-1-Canopy temperature [CT]:

Canopy temperature is an integrative trait that reflects the plant water status or the resultant equilibrium between the root water uptake and shoot transpiration **(Berger *et al.*, 2010),**in which the water is released in the form of vapor through the process of transpiration, which allows the temperature of the aerial parts of the plant to be regulated and helps it get rid of the absorbed heat in the form of Light radiation **(Oulmi ,2015).**The canopy temperature was determined using the «Teletemp Corp., Fullerton, CA, Modèle AG-42» infrared thermometer (**Figure 16**), as used in the study of **Patel *et al.* (2001)**. The average of three readings was obtained for each leaf. Three duplicates were used for each measurement. The measurements were made at midday; plants are exposed to high temperature, which activate their resistance mechanisms. The difference between the air temperature (Ta) and canopy temperature (Tc) is known as canopy temperature depression (CTD), and it can be calculated using the formula used by **Akkuzu *et al.* (2010):**

$$\Delta T = Ta - Tc$$

Figure16: Measurement of canopy temperature using infrared thermometer.

II-1-2-Drought susceptibility index [DSI]:

The cell destruction test is one of the few experiments applied at the plant cell level to estimate the physiological effects of stresses on the cell. This test is based on estimating the injury caused by heat shock by measuring electrical conductivity, which shows the measurement of chemicals leaking from the affected tissues into distilled water **(Oulmi ,2015).** The drought susceptibily index (membrane stability index MSI) was determined according to the method described by **Merbah *et al.* (2020)**. This index was determined after cutting 1-cm2 leaf pieces which were washed three times with distilled water

to remove adhering dust which may affect the test results, before being placed in a tube containing 10 ml of distilled water; each tube contained a sample of 10 pieces of the leaves and manually shaking the tubes periodically and leaving them at laboratory temperature for 24 hours. The electrical conductivity (EC1) was then recorded using a Conductivity Meter (Figure 17). Subsequently, the same samples were placed in a water bath at a temperature of 100C̊, for 60 minutes a second reading (EC2) was then taken (**Bastam, 2013; karimi *et al*., 2018; Merbah *et al*., 2020) (Figure 23).** The membrane stability index (membrane permeability) was calculated by the following formula:

$$\mathbf{DSI}\ (\%) = \frac{EC1}{EC2} \times \mathbf{100}$$

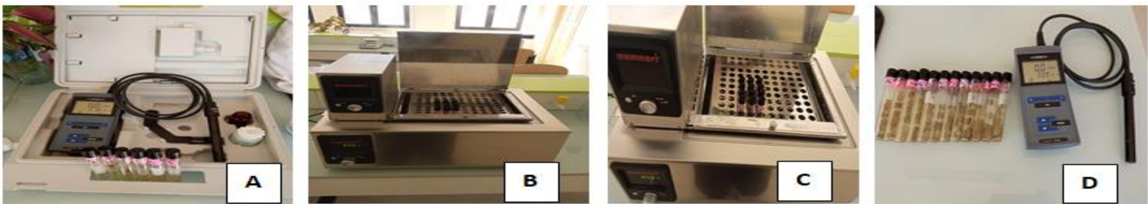


Figure 17: Determination of membrane stability index, **A**: record of electrical conductivity (EC1)**; B** and **C**: place of the same samples in a water bath; **D**: record of the electrical conductivity (EC2).

II-1-3-Leaf area [LA]:

The leaf area index is an essential variable in method- estimates of the plant canopy reaction to global changes in the environment, which makes it indispensable for many investigations of the interaction between the atmosphere and vegetation **(Awal *et al*., 2004).** Therefore, it is valuable in the assessment of the relationship between plants, soil, and water, plant protection strategies, and heat transmission in plants **(Pandey &Singh, 2011**).The mean leaf area was estimated using the formula proposed by **Awal *et al*. (2004)** and **Pandey *et al*. (2011)**:

$$\mathbf{LA\ (cm^2)=0.771\times(l\ xI)}$$

Where;

LA: Mean area of a leaf (in cm^2).

L: Mean length of five leaves expressed in cm.

I: Mean width of five leaves expressed in cm and **0.771** is the regression coefficient of the estimated area through a millimeter sheet.

II-1-4-Relative water content [RWC]:

The RWC is directly related to the water status of plants **(Bacarrillo-López *et al* .,2021)**, and referred to the hydration level (actual water content) relative to its maximal water holding capacity at full turgidity **(Mahlare *et al*., 2003)**.After collection the leaves was instantly weighed to determine their fresh weight. The leaves were then placed in a test tube containing distilled water, in the absence of light and at a laboratory temperature, for 4 h in order to obtain the saturation weight, after wiping off excess water with absorbent paper drying. The sample was then dried in an oven at 65°C for 16 h to obtain the dry weight. Subsequently the relative water content is calculated according to the relationship mentioned by **Cansev (2012); Gouiâa *et al.*(2013)** and **Karimi *et al*. (2018):**

$$\text{RWC (\%)} = \frac{FW - DW}{TW - DW} \times 100$$

RWC: Leaf relative water content;
FW: Fresh weight;
DW: Dry weight;
TW: Turgid weight.

II-1-5-Leaf water loss [LWL]:

Specific leaf area (SLA) and specific leaf weight (SLW) are reciprocal to each other. So leaves with lower SLA and/or higher SLW are thicker, while leaves with higher SLA and lower SLW are thinner, SLW is reported to be related to drought tolerance in several crops and has been suggested as a selection criterion for breeding programs targeting low rainfall areas. Genotypes with a high SLW (thick leaves) have leaves with a small surface area to volume ratio, which is thought to be an advantage in using water efficiently **(Amanullah, 2015)**. Leaf water loss, also known as rate water loss [RWL], was calculated (g/g) using the following relationship according to **(Berka & Aïd, 2009)** with some modifications**:**

$$\text{LWL (mg cm}^{-2}\text{ min}^{-1}) = \frac{(PF1 - PF2)}{SF \times 30}$$

LWL: Leaf water loss;
FW1: Fresh weight at time 0;
DW2: Dry weight after 30 m;
SF: Leaf area.

II-1-6-Specific Leaf Weight [SLW]:

According to **karimi *et al*. (2018),** this value, calculated by dividing the dry weight of leaves by their leaf area**:**

$$SLW(mg/cm^2) = \frac{DW\ (mg)}{LA\ (cm^2)}$$

SLW: Specific leaf weight

DW: Leaf dry weight

LA: Leaf area.

II-1-7-Chlorophyll content index [CCI]:

Chlorophyll decreasing it is an indicator of drought stress, thus when plants are exposed to limited water availability, several changes occur in their photosynthetic pigments, such as Chlorophyll (**Mickky *et al*., 2018**). That is why we calculated this index to evaluate the effect of stress on the chlorophyll contents in the two olives. A portable, non-destructive, and lightweight instrument (CCM-200 plus; Opti-Sciences Tyngsboro, MA, USA) was used to estimate the chlorophyll content index for olive leaves (**Figure 18**). Measurements were performed on the fifth fully developed leaf by counting down from the top of the olive branch. Three readings were recorded for each leaf and the average. Three duplicates were used of each measurement (**Khaleghi *et al*., 2012; Esmaeil pour *et al*., 2015).**

Figure 18: Measurement of leaf chlorophyll content using a portable CCM-200.

II-2-Anatomical Study:

II-2-1-Preparation of histological sections: the cross sections of young fresh stems and leaves were chosen from the two subspecies of the olive tree "*Olea europaea* subsp.*laperrinei* and *Olea europaea* L.var.sylvestris", the different steps ordered manner are summarized in **Figure 19**.

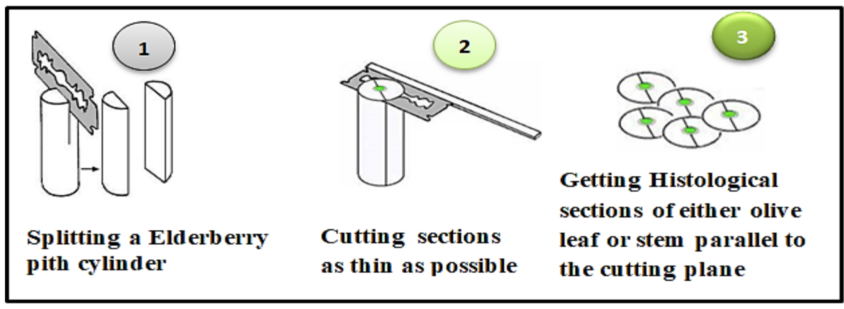


Figure 19: Different steps for preparing histological sections.

II-2-2-Histological double-staining procedure

A histological study of anatomical sections of the leaves and stems of the two subspecies was performed using the double staining technique **(Prat, 2007).** The sections were placed in successive solutions; first, in diluted bleach then in diluted acetic acid to destroy the cellular contents where the cell walls were respected by successively acting with green iodine and carmine, while the cellulosic walls turned pink, and the walls of lignified or sclerotized cells turn green, the experimental protocol detailed in **figure 20**.

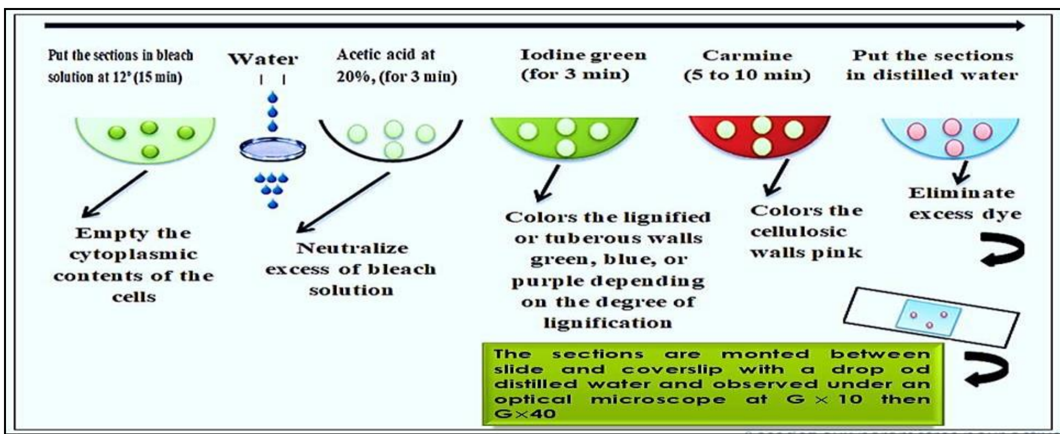


Figure 20: Different steps for double-staining histological sections.

III-Identification and quantification of phenolic compounds and evaluation of antioxidant activity

III-1-Obtaining plant extracts

The extraction of active components from plants, using standard procedures and selective solvents is a crucial step in medicinal plant research **(Azwanida, 2015; Handa *et al.*, 2008).** One technique that is frequently used in this regard is maceration, which has been labeled the "Green method" **(Vongsaka *et al.*, 2013)**, despite this, it has been proposed that as being more applicable than other contemporary extraction techniques **(Dhanani *et al.*, 2013)**.

III-1-1-Aqueous extraction

The water extracts was prepared for our investigation using maceration; it was adjusted in accordance with the method of **Bougandoura *et al.* (2012).** 5 g of dried leaves from each plant were dissolved in100 ml of distilled water. After maceration for 24 h in the dark at ambient temperature (approximately 20 °C) **(Figure 21),** the mixtures were filtered through Wattman filter paper (N°1) to remove the grounds from the filtrate. The operation is repeated a second time with 50ml of the same solution and the filtrates obtained were added and

lyophilized, the extracts were then powdered and kept refrigerated (4°C) until used, the extraction procedure is illustrated in **figure 22**.

III-1-2-Methanolic extraction

Identical to the aqueous extraction, the methanolic extract of the two plants were prepared from 5g of the dry leaves, were macerated in 100 ml of hydroalcoholic solution (20% distilled water - 80% methanol) at room temperature and in the dark for 24 and 48 h (Figure 21). The mixture was then filtered on Wattman paper (No.1). Using 50ml of the same solution, the procedure was repeated. The extracts were collected by Filtration, which were then evaporated to a dry states **(Djeridane *et al.*, 2006).** The dry extracts were stored at -18 °C until use, and the extraction procedures are illustrated in **figure 22**.

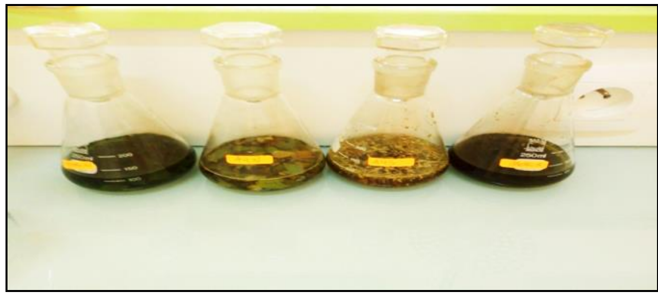


Figure 21: Maceration of two olive leaves in water and methanol.

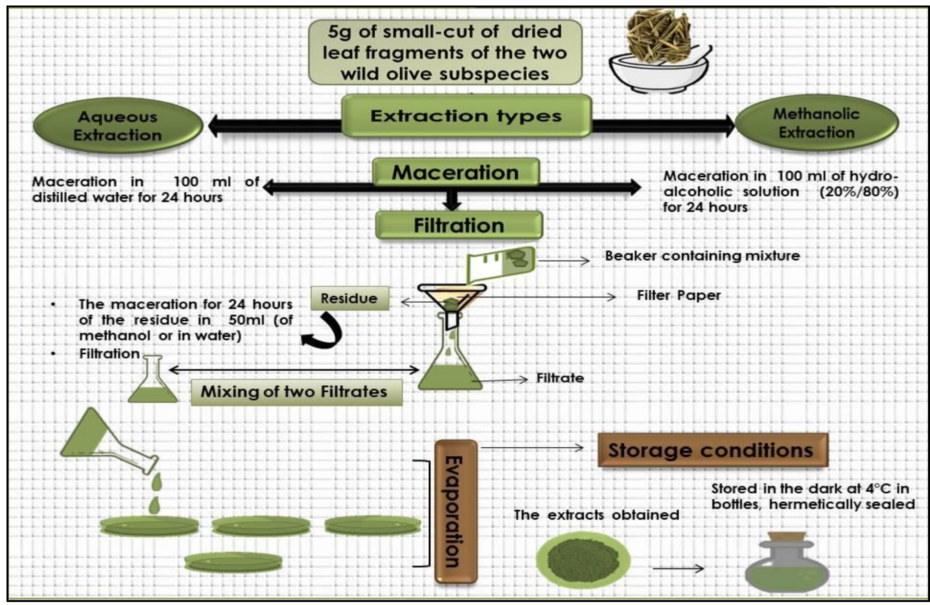


Figure 22: Schematic of different extraction steps.

III-1-3-Plant extraction yield determination

Based on the dry weight of 5g of the leaves, the yield of dried extracts was determined using the following formula:

$$Y\% = (M1 / M2) X 100$$

Where:

Y%: Yield of dried extracts

M1: Extract mass in g after solvent evaporation

M2: Dry plant material mass in grams (**Bouaziz *et al*., 2008**).

III-2- Quantitative analysis of extracts

III-2-1- Total phenolic content

The total phenolic content in the different extracts was determined by the method using the method of Folin- Ciocalteu reagent. The phenols present in the different extracts undergo an oxidation reaction in the presence of Folin- Ciocalteu reagent (RFC), resulting in the appearance of a blue discoloration that is proportional to the concentration of polyphenols contained in olive leaf extracts **(Singleton *et al*.,1999).**

The total polyphenol content is determined by extrapolation on the standard curve, which was established from a series of dilutions (10 -100 µg/ml) of a stock solution (100 µg/ml) of gallic acid serving as a reference. Put 0.1ml of each extract in test tubes, then added a volume of 0.5 ml of 50% Folin-Ciocalteu reagent; shake vigorously then left to act for 1 min before adding 1.5 ml of 20% sodium carbonate. After 2 h of incubation at ambient temperature and in the dark, the absorbances were read from the spectrophotometer at 760 nm. The same operation was carried out for gallic acid at different concentrations by introducing 100ml of the latter into a series of tubes and adding the other reagents. The standard is represented by methanol with added Folin-Ciocalteu and sodium carbonate. The results are expressed as mg equivalent of gallic acid/g of extract.

III-2-2-Determination of total flavonoids

Using the aluminum trichloride $AlCl3$ method, which is based on the formation of a flavonoid complex with aluminum chloride, the total flavonoid content was determined. A volume of 1 ml of $AlCl32\%$ solution (prepared in methanol) was added to 1 ml of sample solution (prepared in methanol), then they are mixed and shaken vigorously. After 30 min of incubation at room temperature, the absorbance was measured at 430 nm. The yellow coloring indicated that the extracts contained flavonoids. The same operation is carried out for quercetin at different concentrations by introducing 1 ml of the latter into a series of tubes and

adding 1 ml of 2% AlCl3, the quercetin was used as a standard for calibration. The results are expressed as mg equivalent of quercetin / g of extract **(Kosalec, 2004).**

III-3-HPLC-DAD analysis for identifying the active biomolecules

Chromatographic analyses were performed to determine the phenolic chemicals in each extract, using an AGILENT series 1260 HPLC-DAD machine, under the following conditions of operation:

Diode array detector, autosampler, column thermostat, and quaternary pump, with different types (A 1260 DAD VL) (1260 TCC) and (1260 Quat pump VL), all of which are included in the instrument. Separation by chromatography was performed on a column with particle size (100mm× 4.6mm x 3.5 µm) with Agilent type ZORBAX Eclipse XDB-C18.

The elution settings were: a flow rate of 0.5 mL/min, sample injection volume of 10 µL, column temperature of 40°C, mobile phases A and B of 0.1% acetic acid in water and 100% acetonitrile, respectively. The elution program was as follows: A; 0–22 min, 10%–50% B; 22–32 min, 50%–100% B; 32–40 min, 100% B; 40–44 min, 100–10% B. The re-equilibration process took six minutes.

The samples were identified at 254, 280, and 330 nm in the DAD detector scan range of 190 to 400 nm. A volume of 5 µl was injected into each sample and reference standard. Furthermore, the phenolic compounds of each sample were identified by comparing their peak areas with those of the internal standards. The results are expressed as mg/g and (mg/ml). **Figure 23** shows the various procedures use in the identification and quantification of the detected chemicals.

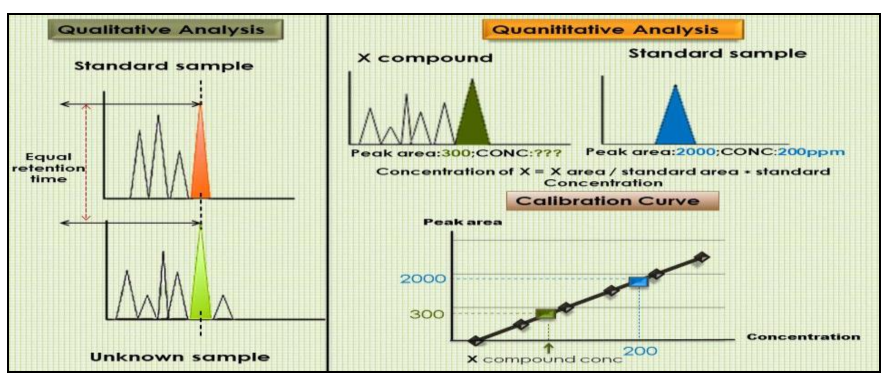


Figure 23: Methods used for the identification and quantification of compounds detected via HPLC analysis.

III-4-Evaluation of antioxidant activity using DPPH test

A slight modification was made to the method published by **Kulsic *et al*.(2004)** in order to assess the anti-radical scavenging activity in *vitro,* using the 1,1-diphenyl-2-picrylhydrazil (DPPH) radical (0.004% in methanol). After dissolving 4 mg of DPPH in 100 ml of methanol, 1 ml of a 0.004% (w/v) DPPH solution was added to 1ml of extracts, prepared at different concentrations for each extract (20ug/ml; 40ug/ml; 60ug/ml; 80ug/ ml; 100ug/ml). The mixtures were shaken vigorously and then, incubated in the dark at laboratory temperature during 30 min.

Positive control: BHT (Butylated hydroxytoluene), whose absorbance was measured under the same conditions as the samples, whereas the negative control (the standard) consisted of 1mL of methanol and 1mL of DPPH solution (0.004%), all determinations were performed in triplicate. The optical density (DO) was measured at 515 nm and recorded .The percentage of radicals scavenging activity of DPPH of each extract was calculated as follow:

$$PI\% = [A_{control} - A_{sample} / A_{control}] \times 100$$

Where:

$A_{control}$: optical density of the control;

A_{sample}: absorbance of the positive control or samples after 30 min.

Antioxidant activity results are expressed as IC50 value (µg extract/ml) (concentration providing 50% inhibition of DPPH radicals), which were calculated graphically by interpolation from linear regression analysis (from the regression equation which is represented by the percentage of trapping as a function of the concentrations of the plant extract), a greater ability to scavenge free radicals is indicated by a lower IC50 value. **Figure 24** provides an illustration of the DPPH test process.

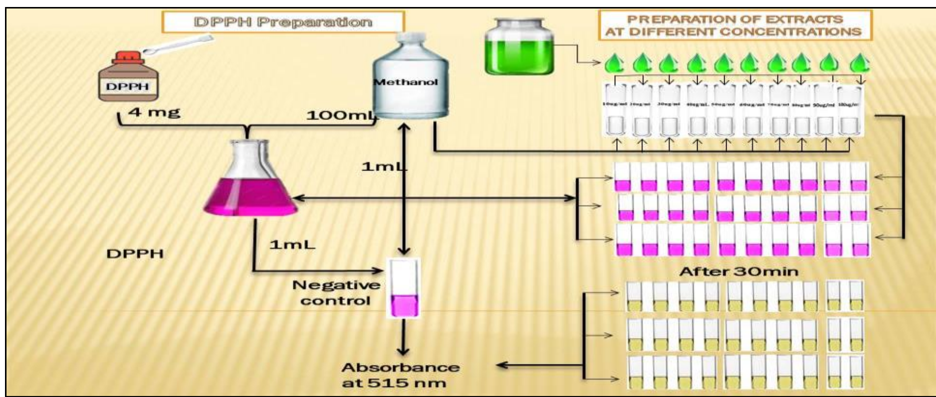


Figure 24: Protocol used to evaluate the antioxidant activity using DPPH test.

IV- Essential oil and extra virgin oil extraction and the assessment of their antibacterial activity

IV-1-Essential oil extraction

The isolation of essential oils from the two wild olive subspecies was ensured by hydrodistillation, using a Clevenger-type apparatus. This method was based on the power of water vapor in transporting essential oils. 400 g of each sample of dried plant material and 500 ml of distilled water were placed in a flask. When the mixture reaches a boil, the essential oil-laden vapors rise to the condenser in the assembly through the vertical tube. Oil droplets accumulated in the tube previously filled with distilled water fell back into the little buret. The water slowly returns to the heated tank through the diagonal conduit while the oil floats on top. After boiling, the extraction process took three hours to complete, the obtained essential oils were stored in colored glass tubes that were enclosed, light-protected, and kept at 4°C until use.

IV-1-2-Extraction yield determination

The yield of essential oils is expressed by the ratio of the quantity of essential oils obtained after distillation of biomass expressed as a percentage calculated by the following formula:

$$\mathbf{Y\% = \frac{EO\ M}{PM\ M} \times 100}$$

Y% : Essentail oils yields ;
EO M: Essentail oil Mass (g);
PM M: Plant matter Mass (g).

IV-2-Extra virgin olive oils isolation

The two wild olives oils were extracted using traditional method based on cold pressing. The different extraction steps are illustrated in **Figure 25**.

Figure 25: Protocol used for the extraction of extra virgin olive oils.

IV-3-Chemical analysis of essential oils by gas chromatography (GC):

The chemical analysis of essential oils was carried out at SFAX BIOTECHNOLOGY CENTER "CBS" in Tunisia at the level of a specialized unit (US19CBS03): Analytical Services Provider Unit (UPSA).

IV-3-1-Gas Chromatography coupled to Mass Spectrometry (GC/MS) Analysis: The isolated essential oils from the two subspecies were analyzed using Gas Chromatography on a Hewlett Packard G 6890 apparatus, equipped with an agilent mass spectrophotometer with a direct capillary interface and fused silica capillary column Agilent 19091S- 433,in order to separate and identify the constituents of essential oils .

IV-3-2-Gas Chromatography coupled with Flame Ionization Detector (GC/DIF) Analysis: The quantitative determination of essential oils was performed by gas chromatography Hewlett-Packard Model 7890 equipped with a flame ionization detector (CPG/FID). To obtain the same elution order with GC/MS, simultaneous auto-injection was done on a duplicate of the same column, applying the same operational conditions for the standardization of the retention time. The operating conditions were as follows:

- **Operating conditions**

- Capillary column: HP-5MS Phenyl Methyl Siloxane (30m x 250μm x 0.25μm film thickness).
- Carrier gas: Helium was used at about 20ml/min, pulsed spinless mode and the injector size were 10.0μL/min.
- Ionizing energy: The mass spectral detector was operated in electron impact mode with ionizing energy of 70 eV scanning from *m/z* 35.0 to 550.0.

- The ion source temperature was 230°C and the quadruple temperature was 150°C.
- Oven programing:
- The GC temperature program was started at 190°C for 15min, and then increased to 280°C a rate 4°C/min.
- The detector and injector temperature were set to 280°C and 250°C respectively.
- MS Acquisition parameters [Scan Parameters]: Low Mass: 35.0; High Mass: 550.0.
- GC Injector: Injection Volume 1.00 microliters.

In our study, the programing conditions of the two gas chromatographers were constant, therefore, the retention time of compounds remained constant. The compounds were identified by comparing their mass spectra with those of authentic standards that were stored on the National Institute of Standards and Technology **(NIST, 2002)** and those described by **Adams (2007)**. Additionally, their Kovats retention indices were compared to a homologous series of n-alkanes **(Gallori *et al*., 2001).**

IV-4-Chemical Analysis of extra virgin olive oils by gas chromatography coupled to Mass Spectrometry (GC-MS): chemical analysis of extra virgin olive oils was carried out at the SFAX BIOTECHNOLOGY CENTER “CBS” in Tunisia at the level of a specialized unit (US19CBS03): Analytical Services Provider Unit (UPSA).

The two extra virgin olive oils isolated were analyzed using Gas Chromatography on a Hewlett Packard G 6890 apparatus, equipped with an agilent mass spectrophotometer with a direct capillary interface and a fused silica capillary column Agilent 19091S- 433, in order to separate and identify the extra virgin oils constituents. The operating conditions employed were as follow:

- **Operating conditions**

- Capillary column: HP-5MS phenyl methyl siloxane (30m x 250µm x 0.25µm film thickness).
- Carrier gas: Helium was used at approximately 1.0 mL/min, pulsed spinless mode and the injector size were 10.0µL/min.
- Ionizing energy: The mass spectral detector was operated in electron impact mode with ionizing energy of 70 eV scanning wavelengthts of *m/z* 35.0 to 550.0.
- The ion source temperature and quadruple temperature were 230°C and 150°C.

- Oven programing :
- The GC temperature program started at 190°C for 15min, was then elevated to 280°C at rate 4°C/min.
- The detector and injector temperature were set to set at 280°C and 250°C respectively.
- MS Acquisition parameters [Scan Parameters]: Low Mass: 35.0; High Mass: 550.0.
- GC Injector: Injection volume 1.00 µL.

IV-5-*In vitro* assessment of the antibacterial activity of the two essential oils and the two extra virgin olive oils

IV-5-1- Disk diffusion method

According to **Rahal** (**2005**) the disk diffusion method "aromatogram method" was used with a little modification to evaluate the antibacterial activity of *Olea europaea* var.sylvestris and *Olea europaea* subsp.*laperrinei* essential oils and extra virgin olive oils.

IV-5-1-1-Principle

The principle of the aromatogram method consists to place sterile disks of 6 mm in diameter on the surface of an Muller-Hinton agar that has been poured into a petri dish then and has been already inoculated with a pure bacterial culture, then impregnated with a precisely defined amount of essential oils to be tested, and antibiotics in order to compare the results. Following incubation, a halo of inhibition of microbial growth appeared around the disk containing the essential oil and extra virgin olive oil , signaling biological activity, the results were read by measuring the diameter (in mm) of inhibition observed **(Figure 26).**

IV-5-1-2-Bacterial strains employed

The seven bacterial strains referenced were used to assess the antibacterial activity of the essential oils from the two subspecies. Three Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Klebsiella pneumonia* ATCC 700603), and four Gram–positive bacteria (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 43300 and *Enterococcus faecalis* ATCC 51299) and concerning the extra virgin olive oils were tested with the same bacterial strains. The strains used were acquired from the bacteriology laboratory of the University Hospital Center (CHU) Sâadana Abdenour of Setif, Algeria as well as from the Pasteur Institute of Algeria. Gentamicin (GENT), an antibiotic, was used as a positive control.

IV-5-1-3-Culture Media Preparation

After preparation and sterilization, the Muller Hinton agar was evenly dispersed in 90 mm diameter sterile petrie plates with a 4 mm thickness. The solution was allowed to cool and solidify at room temperature on the work surface.

IV-5-1-4-Preparation of essential oils and extra virgin oils dilutions

The essential oils used are in their pure state so that they can used them in the aromatogram technique, the following dilutions: 1/2, 1/4, 1/10 (v/v) in Hexan were prepared. The extra virgin oils were diluted were prepared 1/1, 1/2, 1/4, 1/10(v/v).

IV-5-1-5-Inoculum preparation

The bacteria to be tested were cultured on a nutrient agar (isolation) that was favorable for their growth and then incubated in the dark at 37°C for 18 hours. in order to obtain young and well-isolated colonies, turbid suspensions were prepared by taking 3 to 4 well-isolated and identical colonies using a platinum loop placed in 5 ml of sterile physiological water with 0, 8% NaCl and then stirred with a vortex. A first reading of the concentration of the suspension is taken using a spectrophotometer at an absorbance of 625 nm and must be between 0.08 and 0.1.If one of the values found at the first reading was not included in the interval, it was adjusted either by adding physiological water if it is greater than the maximum value, or by adding colonies, if it is less than the minimum value. The prepared inoculum for more than 15 minutes.

IV-5-1-6-Streaking bacteria and disc deposition

Within 15 minutes after adjusting the density of the bacterial suspension. The swab was introduced into the bacterial suspension and spread over the entire dry agar surface, from top to bottom, in tight streaks in a motion, zigzag by rotating the box approximately 60°, after each application, to obtain an equal distribution of the inoculum. The goal of each application is to have a uniform distribution of the inoculum.

Using bunsen burner forceps, sterile discs 6 mm are placed on the previously inoculated agar. The disks are loaded with 10 µl of the different dilutions of the essential oils and extra virgin oils using a micropipette. Other disks impregnated with 10 µl of Hexan are used as negative controls. Gentamicin used as positive control. The test was carried out in triplicate, let diffuse for 30 min, and finally incubated at 37°C for 24 hours in the oven.

IV-5-1-7-Reading the results

The absence of clear zones around the disc indicates no inhibitory effect of the essential oil or extra virgin oil, but the presence clear zones around the disc, indicates the presence of inhibitory activity. In this case, the inhibition diameter around the discs was measured and expressed in mm, followed by the interpretation of the sensitivity of bacteria to essential oils or extra virgin oils according to the scale of **Ponce *et al.* (2003)(Table 7)**.

Table 7: Diameter scale of Bacterial sensitivity

Inhibition diameter	**Species sensitivity**
< 8 mm	– Not sensitive
9-14mm	+ Sensitive
15-19 mm	+ + Very sensitive
> 20 mm	+ + + Extremely sensitive

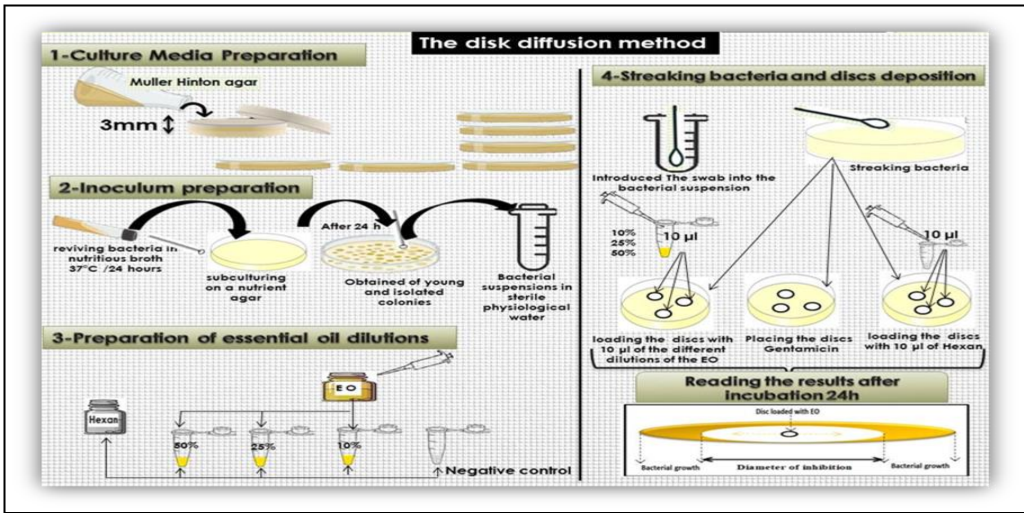


Figure 26: Assessment of antibacterial activity using the disk diffusion method.

IV-7- Statistical techniques for data analysis

The experimental data obtained in the yield, total polyphenols, and total flavonoids and in the evaluation of the antiradical activity were expressed by means ± S.D.

a- Principal component analysis (PCA)

This multi-variable analysis was carried out to visualize the relationships of the variables (the physiological parameters measured) with the axes that represent in present study the two olive populations, to extract information on the level of the water stress exerted on the two subspecies, as well as the PCA analysis biplot was developed to identify the species under stress between the two species tested using the **“R Core Team, 2020” s**oftware**.**

b- Analysis of variance (ANOVA)

The analysis of variance was carried out using **"Costat 6.400, 1998"** Software and the comparison performed at 95% (P = 0.05) was used to determine if there were any significant differences in:

a- Responses of the two subspecies in the two regions to stress, according to physiological parameters measured.

b- Phenolic and flavonoids contents and antioxidant activities of aqueous and methanolic extracts of the subspecies studied in this study.

c- The data of the antibacterial activity between of two essential oils were investigated and between the results of the antibacterial activity of the two extra virgin wild olive oils.

CHAPTER III
RESULTS AND DISCUSSION

Part I: Physiological morphological ,and anatomical responses of *Olea europaea* L. subspecies to drought stress

I-1-Physiological responses of the two subspecies of *Olea europaea* L.

I-1-1-Variance analysis (ANOVA) of the physiological tests measured of the two subspecies between their stations in each region

I-1-1-1- Variations in the physiological tests results of *Olea europaea* L.var.sylvestris between the two northern collection sites

The results show that the difference in the physiological test values of the two northern stations (Amoucha and Oued El Bared),was not considerable for the following tests: relative water content (RWC), leaf area (LA), leaf water Loss (LWL), specific leaf weight (SLW) and drought susceptible index (DSI).

The only exceptions were the canopy temperature (CTD) and chlorophyll content index (CCI), for which Oued El Bared station had the highest values (4.15 c°) and (7.23%) respectively **(Table 8 and Figure 27).**

Table 8 : Variability of several physiological tests obtained from *Olea europaea* L.var.sylvestris between the two stations Amoucha and Oued El Bared

CollectionStations	RWC (ns)	LA (ns)	LWL(ns)	SLW(ns)	DSI(ns)	CTD(***)	CCI(**)
-Amoucha -Oued El Bared	83.89(a) 82.12(a)	4.19(a) 3.74(a)	11.16(a) 9.83(a)	11.21(a) 13.08(a)	73 .51(a) 73.46(a)	1.24(b) 4.15(a)	6 .47(b) 7.23(a)
Mean	83.01	3.96	10.49	12.15	73.49	2.69	6.85
Min	82.12	3.74	9.83	11.21	73.46	1.24	6 .47
Max	83.89	4.19	11.16	13.08	73 .51	4.15	7.23
LSD $_{5\%}$	6.71	1.22	2.96	4.72	2.82	0 .40	0.33

ns: non significant, *: significant at (p= 0.05), **: significant at (p= 0.01), ***: significant at (p= 0.001)

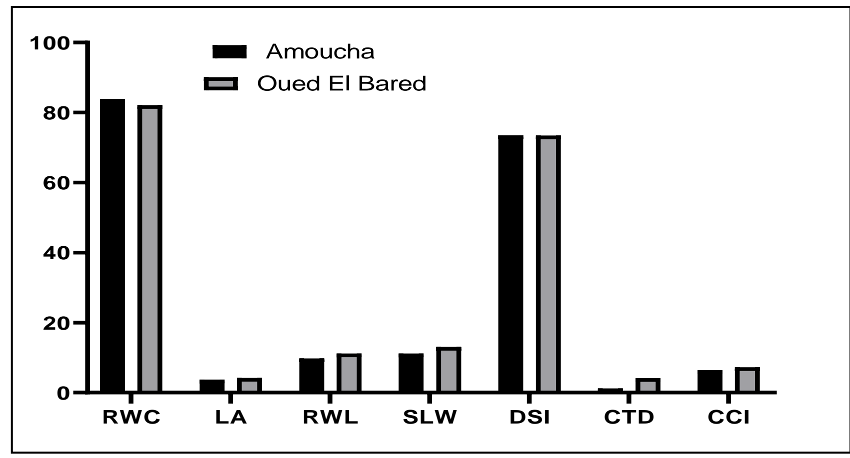


Figure 27: Variations of several physiological tests calculated from *Olea europaea* L.var. sylvestris in the two Northern stations "Amoucha and Oued El bared".

I-1-1-2-Variations in physiological tests results of *Olea europaea* subsp. *lapperinie* between the two southern stations

The results show that there was a significant difference between the two southern stations Akar-Akar and Ilaman concerning the RWC, LWL, and DSI. The Ilaman station had the highest RWC and DSI tests values compared to Akar-Akar station. in contrast, the results of the tests RWL represented a greater value in the Akar-Akar station. There were variations in the other tests between the two study locations, but they were not statistically significant **(Table 9 and Figure 28).**

Table 9 : Variability of several physiological tests obtained from *Olea europaea* subsp. *laperinie* between at the two stationsAkar-Akar and Ilaman

Collection Stations	RWC (*)	LA (ns)	LWL (*)	SLW (ns)	DSI (***)	CTD (ns)	CCI (ns)
-Akar-Akar -Ilaman	68.19(b) 74.19(a)	1.36(a) 1.33(a)	4.85(a) 3.73(b)	11.60(a) 10.81(a)	43 .68(b) 64.83(a)	4.33(a) 4.86(a)	3 .94(a) 2.64(a)
Mean	71.19	1.35	4.29	11.21	54.25	4.60	3.29
Min	68.19	1.33	3.73	10.81	43 .68	4.33	2.64
Max	74.19	1.36	4.85	11.60	64.83	4.86	3 .94
LSD $_{5\%}$	3.77	0.29	1.03	1.65	4.57	1 .72	1.97

ns: non significant, *: significant at (p= 0.05), **: significant at (p= 0.01), ***: significant at (p= 0.001)

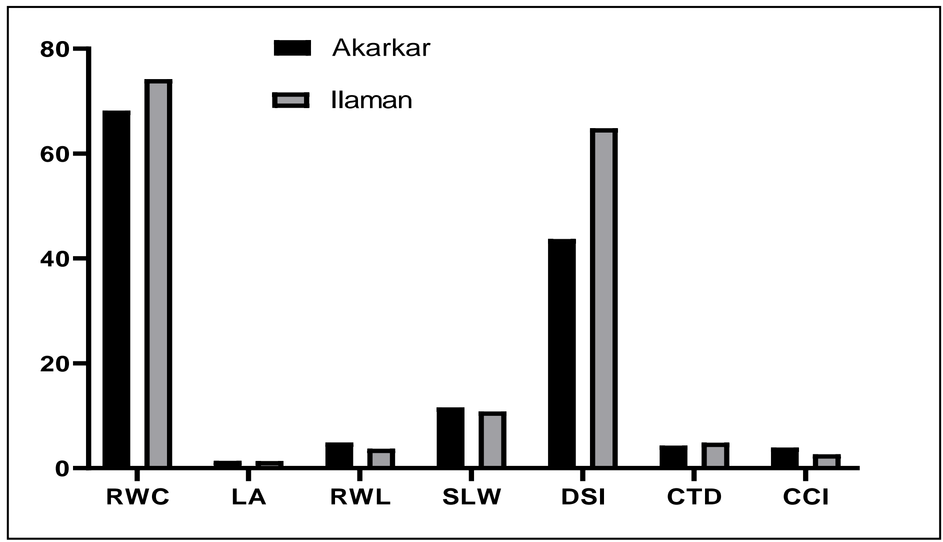


Figure 28: Variations of several physiological tests calculated from *Olea europaea* subsp.*laperrinei* at the two southern stations"Akar-Akar and Ilaman**".**

I-1-2-Variance analysis (ANOVA) of physiological tests results from the two subspecies therefore between the two northern and the southern regions

The findings indicate that there are notable differences between the two subspecies (*Olea europaea* subsp.*laperrinei* and *Olea europaea* L.var. sylvestris), there was a difference between the two regions, southern (Akar-Akar and Ilaman) and northern (Amoucha and Oued El Bared) respectively **(Figure 29). Table 10** shows that the northern region had the highest values for the RWC, LA, RWL, DSI, and CCI tests, while the southern regions had the highest values for the CTD test. Additionally, a difference, although not statistically significant, between the two regions for the SLW test.

Table 10 : Comparative study of stress rates between the two subspecies, originating from the northern and southern regions using physiological tests

Subspecies studies	**RWC** (***)	**LA** (***)	**RWL** (***)	**SLW** (ns)	**DSI** (***)	**CTD** (***)	**CCI** (***)
- *O. e.* sylvestris from North region	83 .01(a)	3 .97(a)	10 .50(a)	12.15(a)	73 .49(a)	2.69(b)	6.85 (a)
-*O. e. laperrinei* from South region	71.19(b)	1.35(b)	4.30(b)	11.21(a)	54.25(b)	4.60 (a)	3.29(b)
Station effect	13,46	0.14	0.03	0.85	333.95	8.90	0.22
Region effect	419,12	20.57	115.45	2.65	1109.63	10.83	38.04
Interaction station region*	45 ,32	0.17	4.49	5.29	336.99	4.24	3.17
Cv %	13,12	14.78	13.24	13.38	2.63	15.14	12.30
LSD 5%	3,20	0,52	1.30	2.08	2.23	0.74	0.83

ns: non significant, *: significant at (p= 0.05), **: significant at (p= 0.01), ***: significant at (p= 0.001)

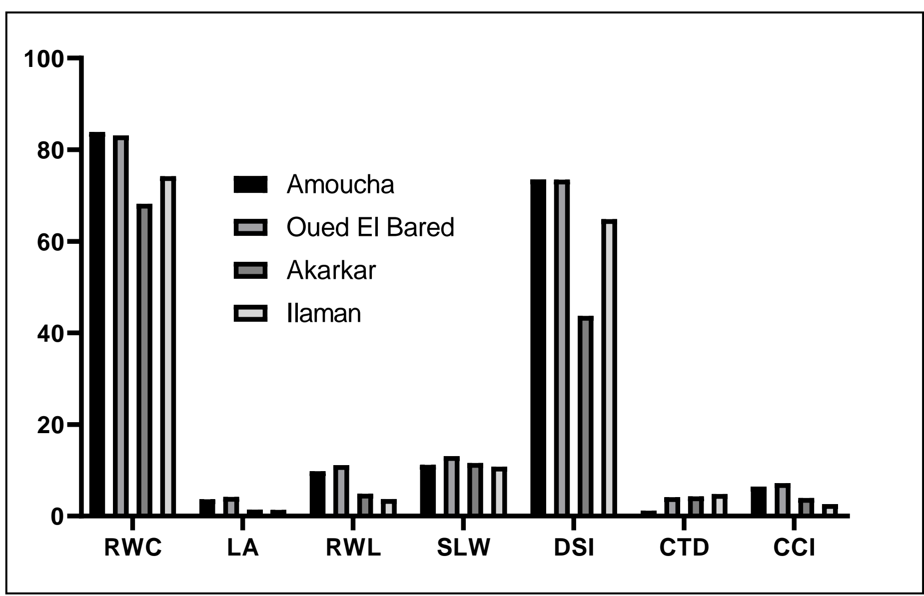


Figure 29: Variation in physiological tests results between the two harvest regions

I-1-3-Principal component analysis (PCA)

A principal components approach was developed in our study to combine between the different physiological tests calculated from the two subspecies harvested from different sites in the two northern and southern regions to extract information on the most stressful subspecies, in this multivariable analysis, each index is considered as a quantitative variable to be explained.

Principal component analysis showed that the first two axes explain respectively 73.39 and 18.32% **(Table 11; Figure 30).** They explain all the information with a percentage of 91.71%,also this analysis shows that the folowing tests namely RWC, LA, RWL, DSI and CCI (r = 0.940; 0.997; 0.983; 0.808 and 0.956 respectively) were significantly and positively correlated with PC1(axe1), while SLW and CTD (r = 0.812 and 0.645 respectively) were significantly and positively correlated with PC2 (axe2).

Table 11: Correlations of seven measured physiological indices with the two first axis

Axis	% of var	Physiological indices						
		RWC	LA	RWL	SLW	DSI	CTD	CCI
PC1	73.39	0,940	0,997	0,983	0,582	0,808	- 0,621	0,956
PC2	18.32	- 0,262	0,074	0,146	0,812	- 0,275	0,645	0,188

The biplot predicts that the two sites Amoucha and Oued El Bared (northern region) are positively linked to PC1 (coordinates: 2.070 and 2.452) and characterized by high values of RWC, LA, LWL, DSI and CCI, thus PC1 is the axis of non-stress conditions (**Table 12 and Figure 30**). The sites of Akar-Akar and Ilamane experiences are negatively linked to the same axis (coordinates: - 2.368 and - 2.154) and characterized by lower values of these physiological indexes, therefore PC1 the stress axis in the case of the southern region, there is a difference in the specific weight leaf and the temperature between the two regions but this difference could not differentiate the regions.

Table 12: Coordination of different harvest sites with the two first components

Axis	Northern region		Southern region	
	Amoucha	Oued El bared	Akar-Akar	Ilaman
PC1	2,070	2,452	- 2,368	- 2,154
PC2	- 1,519	1,424	0,676	- 0,582

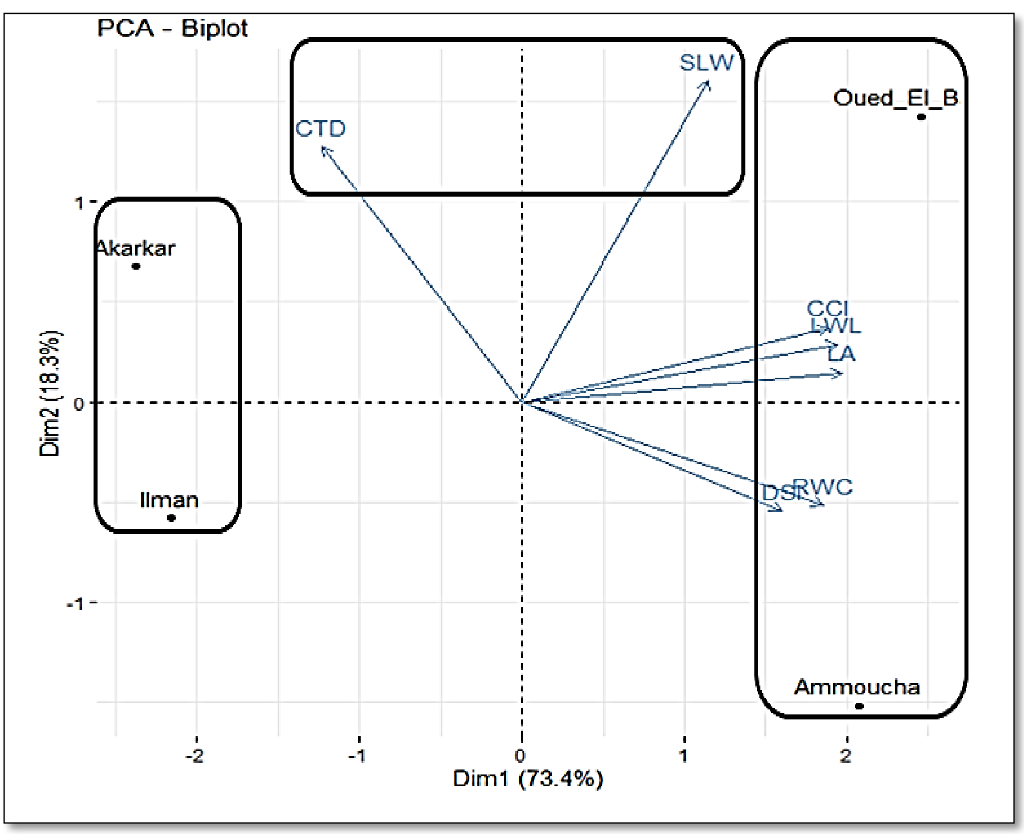


Figure 30: Biplot of principal component analysis of physiological index of two olive subspecies; *Olea europaea* subsp.*laperrinei* and *Olea europaea* L.var. sylvestris according to seven drought tolerance indexes.

I-4-Discussion

Despite the two collection stations of *Olea europaea* L.var. sylvestris belong to the same bioclimatic stage, but there is a difference which has been noted concerning the temperature and Chlorophyll content indices,the difference in temperature value can be explained by the difference of exposure of the two stations to the sun light **(Djeridane *et al*., 2006),** which the station of Oued El Bared was well lit, wind speeds were significant in the Oued El Bared station during the measurement period, suggesting that wind may have an influence on canopy temperatures.Since aerodynamic resistance slowly decreases with increasing wind speed, the canopy temperature was less affected but the wind speed at lower speeds had more effect (**Akkuzu *et al*., 2010**), while the variability of chlorophyll content can also be explained by sun light and soil, as previously mentioned, sun light was more important in the Oued El Bared station which accounts for their height chlorophyll content because ,sun light is an essential factor for the synthesis of chlorophyll, which in turn requires a number of elements from the soil, such as phosphorous (P) and nitrogen (N). As a result, the soil condition should also affect the levels of chlorophyll (**Li *et al*., 2018).**

Olea europaea subsp.*laperrinei* showed a differed in three physiological tests between the two southern stations, with the Ilaman site exhibiting higher values for the membrane stability index and relative water contents. The closing of the stomata produces a considerable reduction in the progressive water loss by evapotranspiration, consequently maintaining the amount of water in leaves, which explains this increase in RWC and the decrease in RWL (**Berka&Aïd, 2009**), the high DSI value can be explained by leakage, which occurs when cell membranes are damaged and become more permeable under drought stress conditions (**Dwivedi *et al*., 2018**). In contrast, the Akar Akar station demonstrates a lower RWC value and the highest rate of water loss, which may be explained by the fact that the leaves in this case did not limit their stomata closure, which increases transpiration and, consequently reduces the water content **(Berka&Aïd, 2009**).

The species of arid regions are resilient to severe rainfall fluctuations and dry conditions (**Berka&Aïd, 2009**), because they are armed with constitutive morphological and anatomical modifications that help conserve water (**Kar *et al*., 2011**). Olive trees are one of the species that adequately enhance the semi-arid and arid zones of the Mediterranean basin through its hardiness and flexibility of adaptation **(Gouiâa *et al*., 2013).** One of the morphological adaptations of plants to different environmental conditions is their shape and size of leaves

(**Poorter** ***et al*****., 2012; Catoni** ***et al*****., 2017).** The leaf is the most reactive and adaptable organ to environmental stress (**Nevo** ***et al*****., 2000; Marchi** ***et al*****., 2008**).

Additionaly, the leaf area index is an essential variable in method estimates of plant canopy reaction to global changes in the environment, making it indispensable for many investigations of the interaction between the atmosphere and vegetation **(Awal** ***et al*****., 2004).** Therefore, it is valuable for assessing of plant nutrition, the relationship between plants, soil, and water, plant protection strategies, and heat transmission in plants **(Pandey &Singh, 2011).** The leaf structure more accurately depicts the consequences of water stress than do the stem and roots **(Guerfel** ***et al*****., 2009).**

According to the study findings, RWC and LA were higher in values in *Olea europaea* L.var. sylvestris than that of *Olea europaea* subsp.*laperrinei.* The reduction in water contents and leaf area of the Saharan subspecies can be explained by drought stress, in which the occurrence of drought decreases plant water content, the cells shrink and relax resulting in loss of turgor **(Farooq** ***et al*****., 2012),** which consequently reduces the leaf water potential **(Farooq** ***et al*****., 2009),** and decreases the speed of division of cells constituting plant tissues **(Granier** ***et al*****., 2000),** as a result, it inhibits cell expansion and decreases leaf area **(Farooq** ***et al*****., 2012**). The decrease of leaf area under water stress is an adaptative strategy developed by plants to prevent a higher rate of transpiration and to decrease radiation-absorbing surfaces as a result of a water deficit (**Karimi** ***et al*****., 2018**). Generally the reduction of leaf water potential in the olive tree is accompanied by an accumulation of soluble sugars and proline to osmotically adjust to its environment (**Gouiâa** ***et al*****., 2013**). In addition, not only water stress but also altitudinal gradients are often accompanied by changes of water gradient, which has a clear effect on the plasticity of leaf structures **(Liua** ***et al*****., 2021).**

Concerning the RWL value**,** *Olea europaea* subsp.*laperrinei* has a lower rate due to the modification of the micromorphological characteristics of the leaf under stress **(Kröber** ***et al*****., 2015**), as previously indicated the restriction in the expansion of the leaf area in response to drought stress is an adaptation mechanism to minimize water loss **(Karimi** ***et al*** **., 2018).** Furthermore, **Guerfel** ***et al*****. (2009)** and **Gouiâa** ***et al*****. (2013)**, noticed that under conditions of high temperature, olive plants can reduce excessive water loss by closing their stomata **(Guerfel** ***et al*****., 2009),** as well as the size and number of stomata will be reduced therefore the rate of transpiration to achieve a dynamic equilibrium through these conditions **(Ait-Said** ***et al*****., 2005; Guerfel** ***et al*****., 2009, Ennajeh** ***et al*****., 2010**). Therefore, the elevated rate of water

loss noticed in *Olea europaea* L.var. sylvestris can be explained by the size of their high leaf area and their stomata architecture.

A lack of water leades to plant tissue degradation, and changes the shape and function of membranes **(Dwivedi *et al*., 2018).** The ability of a cell membrane to remain stable and function optimally under limited water conditions is a crucial factor in plant drought resistance and temperature adaptation. Since the membrane serves as the first line of defense and contains several heat-sensitive sensors that assist the plant in activating its defense system before it is exposed to heat shock **(Borhan & Ghassemi-Golezani, 2015)**. In the current study, the height value of DSI was observed in *Olea europaea* L.var. sylvestris this high value can be explained by leakage, which occurs when cell membranes are damaged and become more permeable **(Dwivedi *et al*., 2018)**. In contrast the lowest value of the membrane stability index was observed in *Olea europaea* subsp.*laperrinei* subspecies which is indicates of the plant resistance to drought.

Olea europaea L.var. sylvestris had higher chlorophyll contents than the saharan subspecies, certain factors might explain this variability ,such as environmental challenges and notably drought stress which causes the decrease of chlorophyll **(Agastia *et al*., 2000).** In addition, many investigations have noticed that high altitude affects plant leaves by thickening cuticles, and barrier tissues while decreasing the rate of photosynthesis **(Mathur *et al*., 2014; Ahmad *et al*., 2016; Kuster *et al*., 2016).** Furthermore, climate can affect the synthesis of chlorophyll as high temperatures which can damage a series of enzymatic reactions that are essential in chlorophyll synthesis **(Li *et al*., 2018)**, as well as precipitation, water, and the production of chlorophyll are tightly connected, in particular, the lack of water in leaves affects the production of chlorophyll and accelerates its degradation, since water plays the role of a solvent for mineral salts in order to absorb them by plants and considered as the transmit through which nutrients are transported in plants. Additionally, soils should play an important role in regulating chl because chlorophyll synthesis requires many elements from soils such as nitrogen (N) and phosphorous (P) **(Li *et al*.,2018)**, thus the decrease in chlorophyll content can be due to the deficiency of mineral salts in soil**(Tränkner *et al*.,2018)**.

The two subspecies showed a significant difference in the canopy temperature index which is more important for *Olea europaea* subsp.*laperrinei*, this increase is mainly related to drought stress as mentioned above this taxon under drought conditions was reducing its relative water content and tends to close its stomata in order to reduce transpiration rates. As transpiration

decreases, the leaf temperature increases **(Borhan & Ghassemi-Golezani,2015)**. Significance of less evaporative cooling due to a decrease in the vaporization of water molecules. As a result, the temperature of the leaf rises, which elevates the saturation vapor pressure of water molecules inside the leaf **(Konrad *et al*.,2021)**.

I-5-Anatomical study

Morphological similarities and variations were observed between olives grown in their different natural habitat, where the leaf of *Olea europaea* var. sylvestris is small, simple, thick, subsessile, lanceolate, and pointed at the apex relatively **(Figure 15b).** Nervation of the leaf is reticular. The upper surface is dark-green and shiny, while the lower surface shimmers silver and tomentous (densely pilous), this is because leaf hairs were more abundant on the abaxial surface of the leaf in young and mature leaves **(Figure 15b; 36a,b).**

The morphological characteristics of leaves of *Olea europaea* subsp. *laperrinei* are somewhat similar, but they differ from it in other characteristics **(Figure 15b)** as their leaves have a smaller leaf area and appear pale green with increased leaf thickness, the main vein (Midrib) does not appear clear "it is flat in shape for Laperrine leaf and is salient in shape for the Oleaster leaf" **(Figure 32a,b; 35a,b,c),** in addition, the density of leaves hairs "peltate scales" in both surfaces was much higher in the Saharan sample **(Figure 33a,b; 36a,b),** and the same goes to the number of stomata which increased in the desert sample **(Figure 33c,d; 36c,d)**. The same observation was recorded for the braches and stems, which have less thikness and width than the Saharan tree **(Figure 15b).**

No significant differences were observed between the anatomical study performed on young fresh stems, where it noted that both cross sections in stem of two subspecies consisting of pith, vascular stacks, cortex, and epidermis layer which is more thicker and contains of trichomes "peltate scales" where it was more dense in the Laperrine stem **(Figure 31a,b,c; 34a,b,c).**

Concerning to the cross sections of leaves, the results showed that both cross section samples consisting of epidermis that appeared thicker, more cutinized and an increase in trichomes were observed in the desert sample **(Fig. 32c,d; 35b,c)**, also we noted that there is a difference in the shape and width of the main vein which is smaller and very narrow for Laperrine leaf, while it's appearance is salient in shape and larger for the Oleaster leaf **(Figure 32a; 33a,b).**), also we noted that there is a difference in the shape and width of the main vein which is smaller and very narrow for laperrinei leaf, while it's appearance is salient in shape and larger for the Oleaster leaf **(Figure 32a; 33a,b).** The palisade parenchyma was thiker with laperrine leaves (it consists of three layers), whereas in oleaster leaves, it consists of two layers **(Fig.32 a,b,c; Fig. 34a,b,c).**

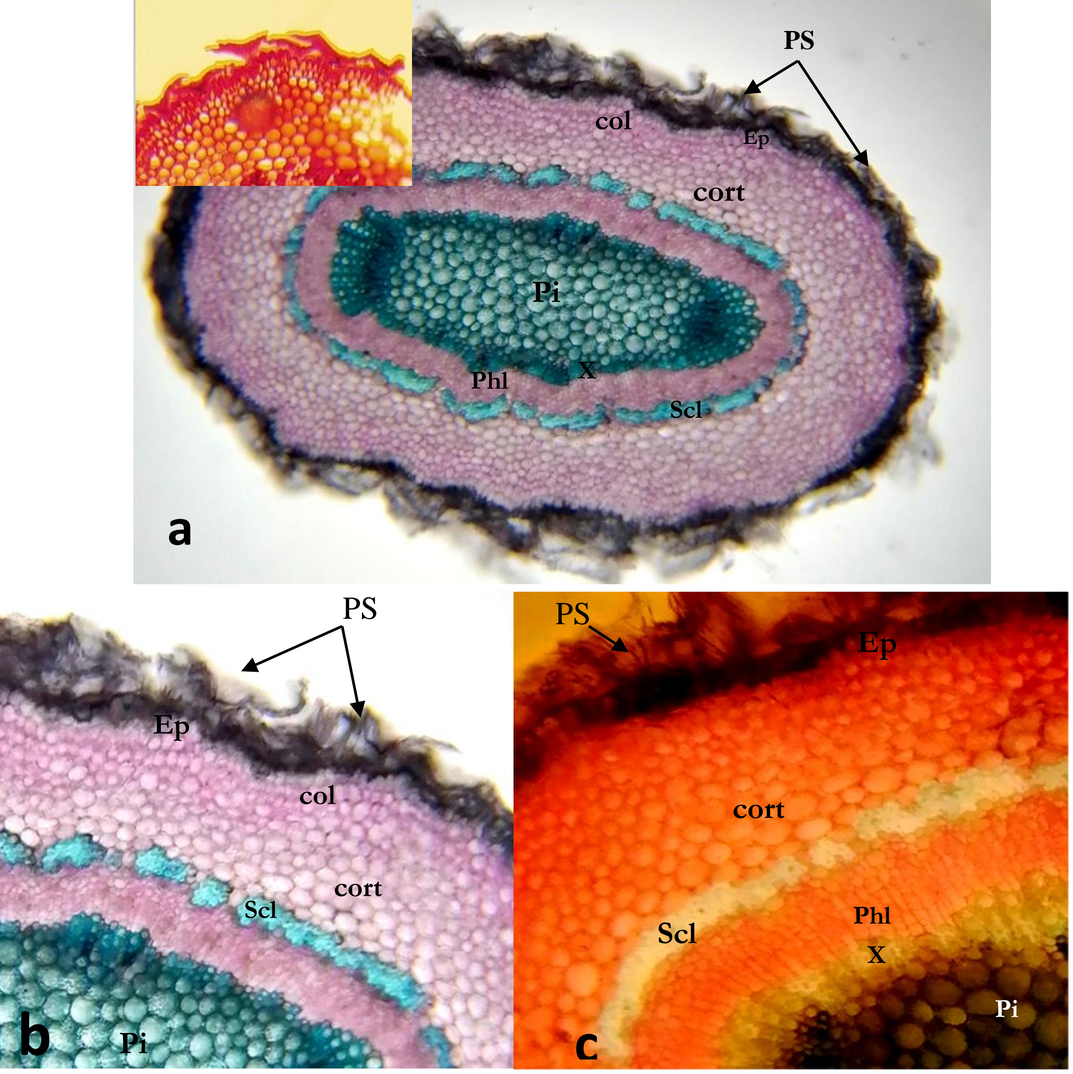


Figure 31: (a, b, c): Cross sections in the stem of *Olea europaea* subsp. *laperrinei* (×100)
a, b, c: general views showing different tissues, structures and positions of the trichomes, PS: peltate scales, Ep: Epidermis with thick cuticle, Col: Collenchyma tissue Cor: cortex, scl: Sclerenchyma tissue, Phl: phloem, X: Xylem, Pi: Pith.

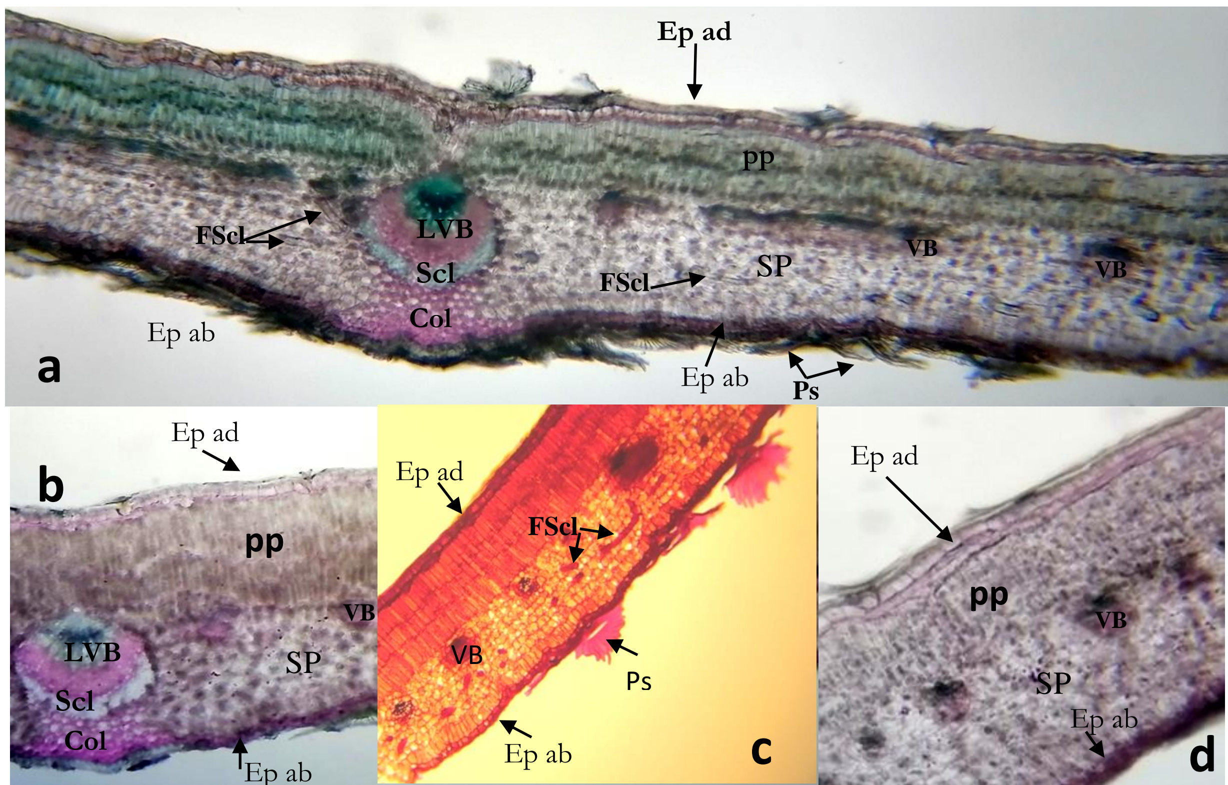


Figure 32: Cross sections in fresh leaves of *Olea europaea* subsp. *laperrinei* (×100)
A,b,c: general views showing different tissues (×100); Ep: Epidermis on both Upper surface and underside of leaf (EPad: epidermis adaxial, EPab: epidermis abaxial), LVB: Larger vascular bundle, VB: Vascular bundle, Col: Collenchyma tissue, Scl: Sclerenchyma tissue, SP: Spongy parenchyma, PP: Palisade parenchyma, FScl: Sclerenchyma fibers (filamentous scleroids), PS: Peltate scales.

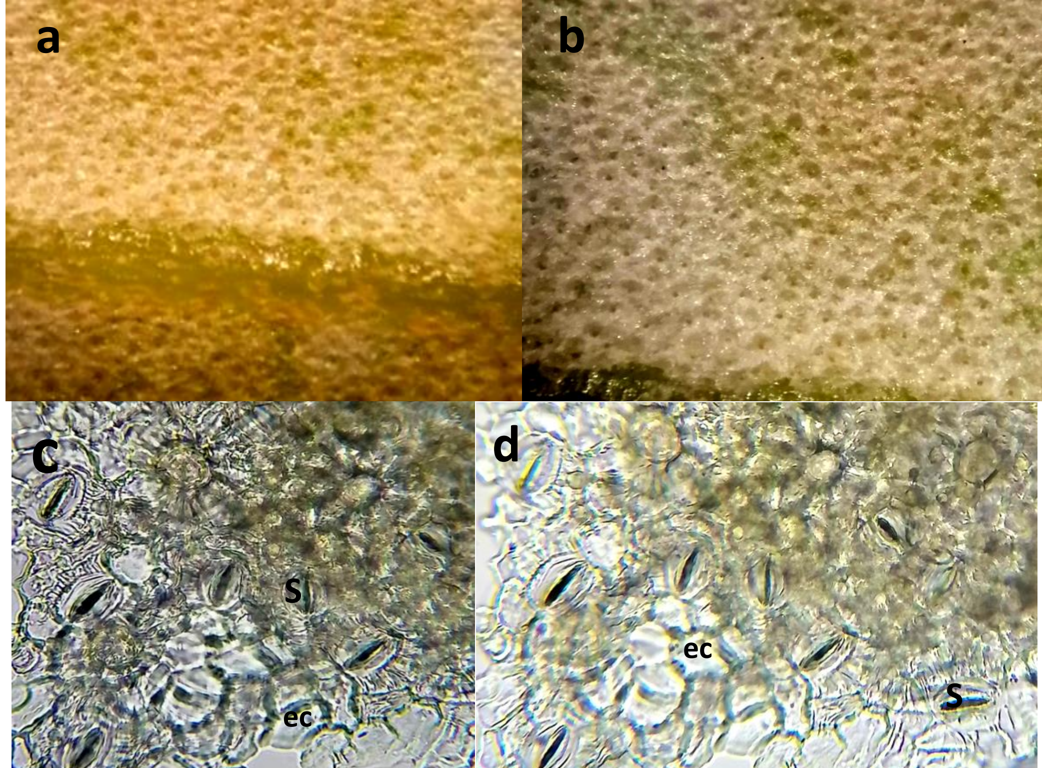


Figure 33: Trichomes and stomas on young leaves of *Olea europaea* subsp. *laperrinei* (×10)
a: Upper surface of leaf; b: underside of leaf; c, d: Paradermal section of the lower epidermis: "ec: epidermal cell, s:stomata"

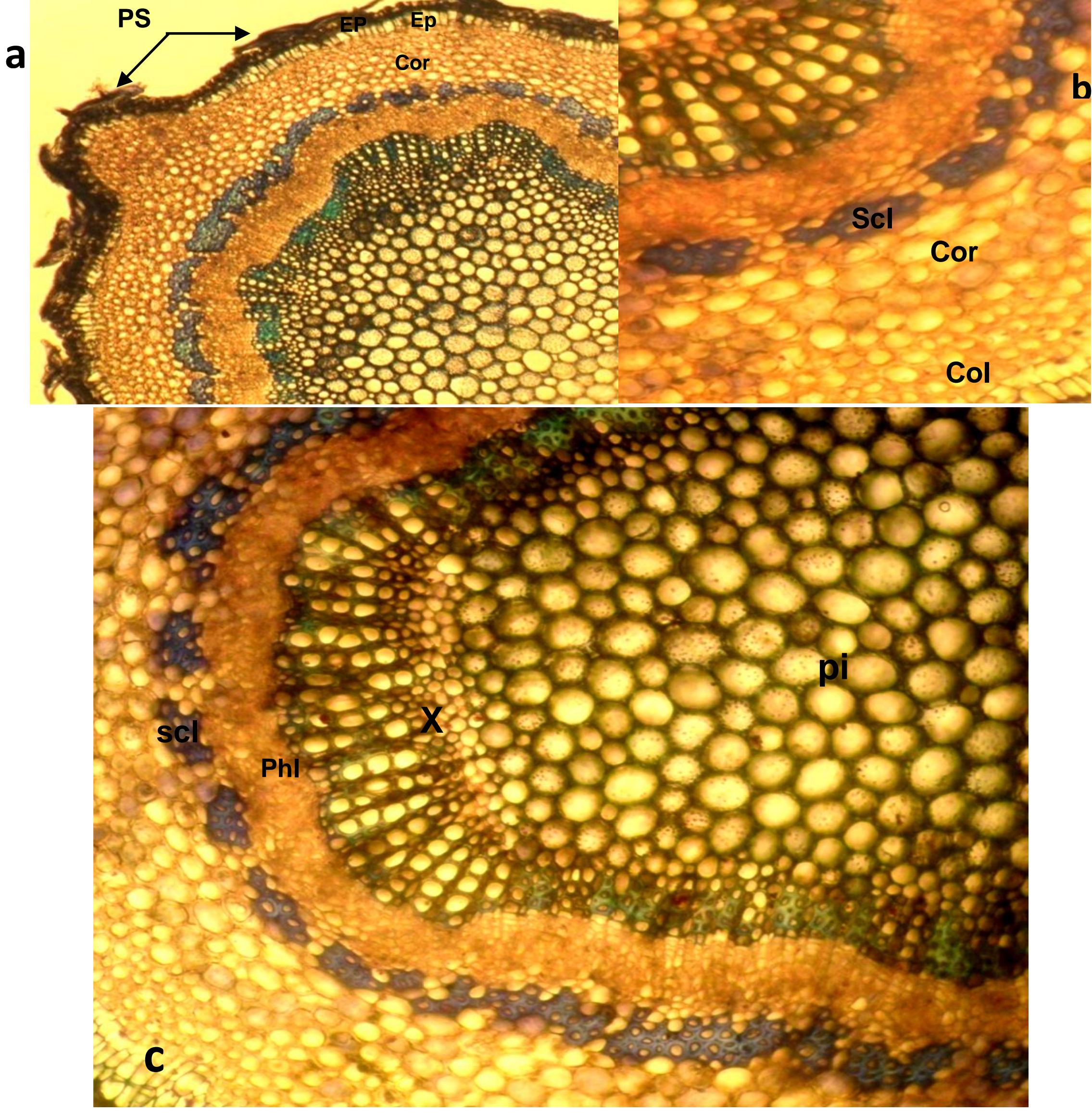


Figure 34: Cross sections in stem of *Olea europaea* var. sylvestris (×100)
a, b, c: general views showing different tissues, structures and positions of the trichomes, PS: peltate scales, Ep: Epidermis with thick cuticle, Col: Collenchyma tissue Cor: cortex, scl: Sclerenchyma tissue, Phl: phloem, X: Xylem, Pi: Pith.

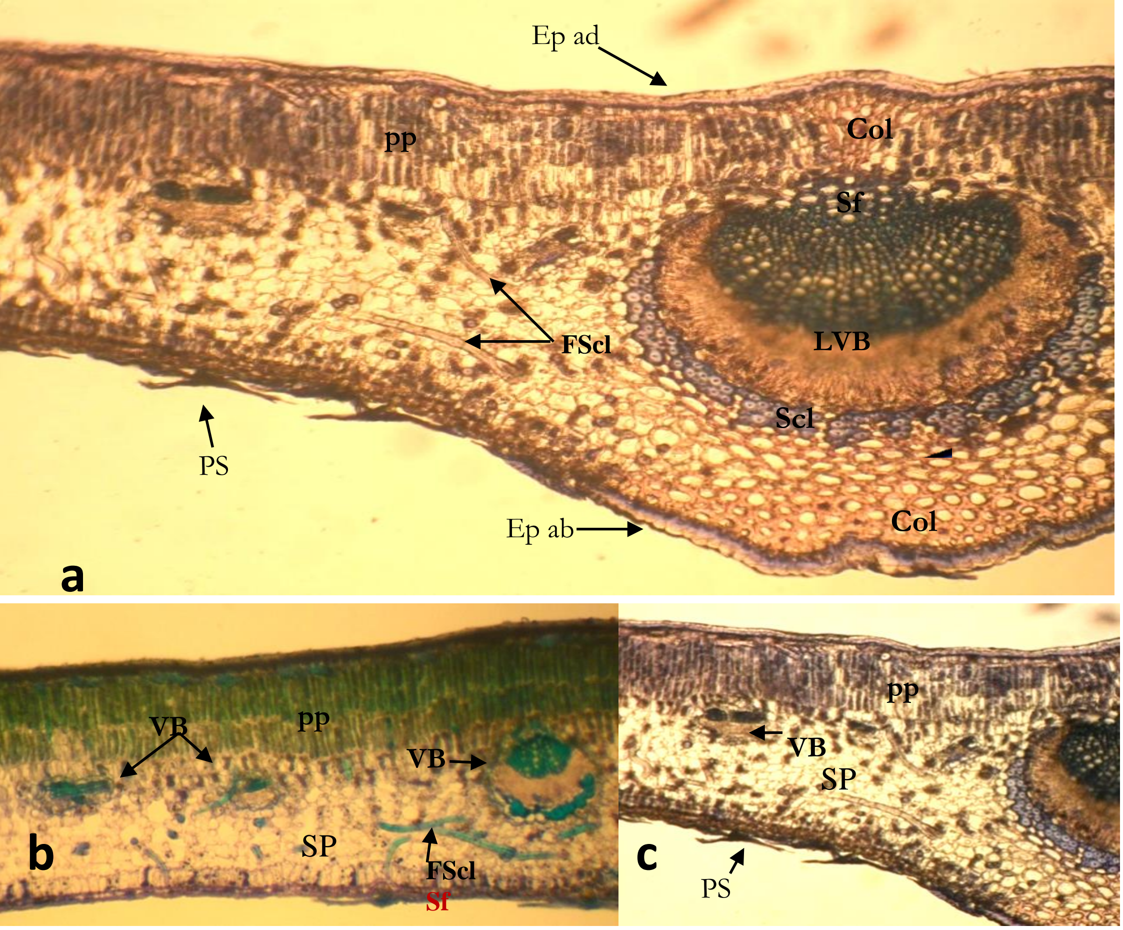


Figure 35: Cross sections in leaf of *Olea europae* avar. sylvestris.
A,b,c: general views showing different tissues (×100); Ep: Epidermis on both Upper surface and underside of leaf (EPad: epidermis adaxial, EPab: epidermis abaxial), LVB: Larger vascular bundle, VB: Vascular bundle, Col: Collenchyma tissue, Scl: Sclerenchyma tissue, SP: Spongy parenchyma, PP: Palisade parenchyma, FScl: Sclerenchyma fibers (filamentous scleroids), PS: Peltate scales.

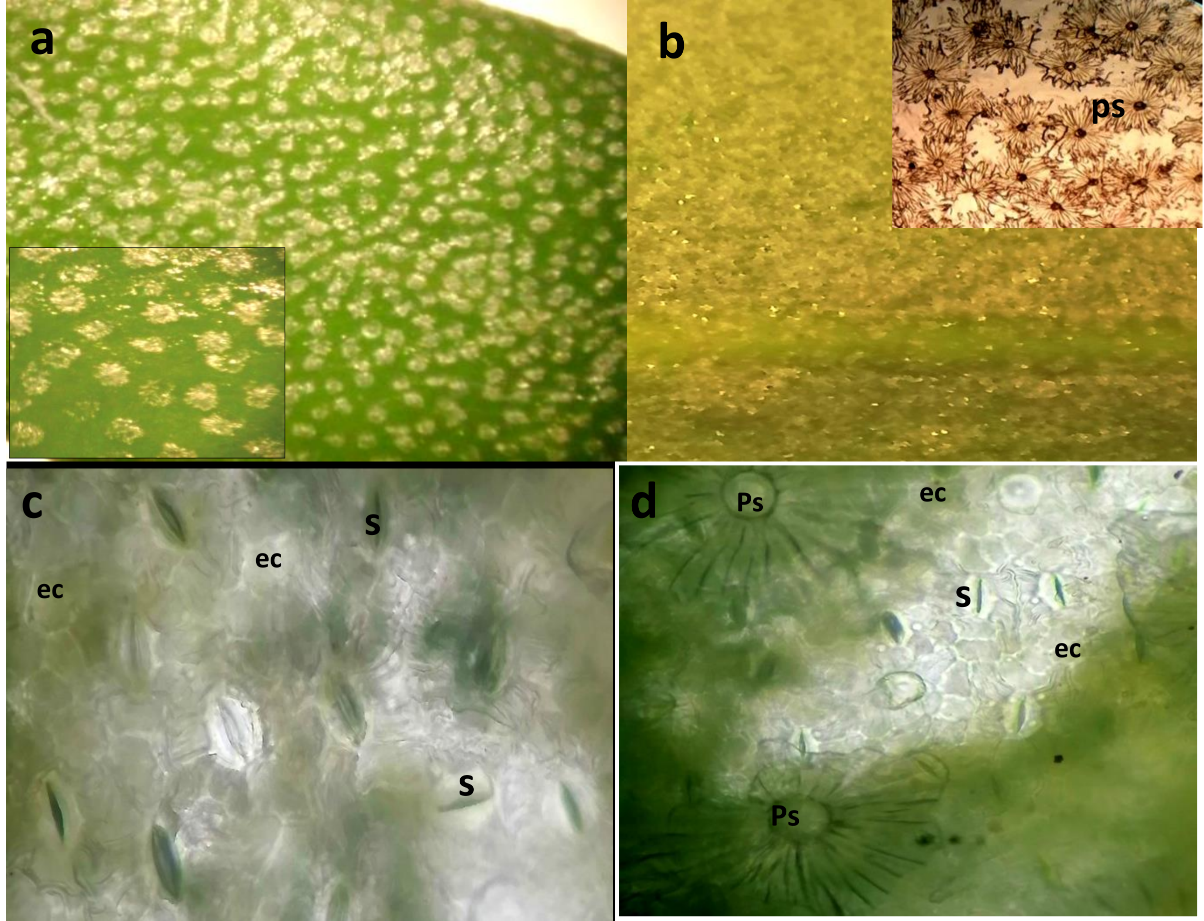


Figure 36: Trichomes and stomas on young leaves of *Olea europaea* var. sylvestris (×100); (a) Upper surface of leaf; (b) underside of leaf; c and d: Paradermal section of the lower epidermis: "ec: epidermal cell, s: stomata; Ps: peltate scales"

I-5-1-Discussion

Anatomical analysis of the leaves and stems of *Olea europaea* var. sylvestris is very rare subject, and there is just one study obtainable on *Olea europaea* subsp. *laperrinei* **(Baali-Cherif *et al*., 2007).**

The obtained data from the morpho-anatomical study indicated that there is a difference in stem and leaf cross sections of the two olives investigated, these variation can be attributed to the intensity of stress, its duration, the phase of the plant's development, and its state when stress detected **(Yokota *et al*., 2006; Demirevska *et al*., 2009)**, therefore, it can be deduced that under the drought stress exerted in the Saharan region the lapperine olive tree was armed by particular anatomical structure that gives them more resilience to harm caused by drying, this information was confirmed by **(Briton *et al*., 2019),** compared to *Olea europeae* var. sylvestris that sampled in less stressful condition.

In this context, stem cross sections indicated that there are some differences between the two samples, where the lapperine olive shows up more of trichomes "peltate scales" and consisting of epidermis that appeared thicker and more cutinized. These results agree with those of **Baali-Cherif *et al.* (2007).**

Additionally, *Olea europaea* subsp. *laperrinei* was appeared with reduction in the leaf area compared to the other, which was explained by environmental stress wereby the leaf was considered the most reactive and adaptable organ to stressful conditionss **(Nevo *et al.*, 2000; Marchi *et al.*, 2008),** whereby leaf structures more clearly indicate the impacts of water stress than do stems or roots **(Edziri *et al.*, 2021).** According to **Fatima *et al.* (2022)** under drought stress the modifications in anatomical, morphological, and physiological features enable leaves to endure for extended periods of time. Among the anatomical aspects that appear in our obtained was the increase in the leaf thickness of laperine leaves. Plants use this essential trait as their first line of defense against environmental stress and as a way for them to protect themselves **(Wang *et al.*, 2020)**, thicker leaves may be able to retain greater leaf water contents, enhance leaf hydraulic conductivity, and store more water **(Wang *et al.*, 2011). Ennajeh *et al.* (2010)** and **Ahmad *et al.* (2023)** explained the increase of leaf thickness during the drought by the increases in stomata and in trichome density, as well as by the reduction in epidermal cell diameter. These later characteristics were seen in our sample of *Olea europeae* subsp. *laperrinei,* where an increase in trichome density "peltate scales" and a reduction in the size of the stomata were noted compare to other sample, Many authors have confirmed the development of these traits during drought **(Bosabalidis & Kofidis, 2002; Briton *et al.*, 2019; Taratima *et al.*, 2020; Taratima *et al.*, 2021).**These anatomical structures enable plants to sustain optimal water conditions and tolerate certain levels of dryness **(Rugini *et al.*, 2016)** , including the height density of peltate scales, which mainly serve to safeguard the stomata and reduce transpiration-related water loss (**Bosabalidis & Kofidis 2002; Besnard *et al.*, 2012.; Briton *et al.*, 2019**).

II-Identification and quantification of phenolic compounds and evaluation of antioxidant activity

II-1-Extraction yield of crude extracts

Based on the extracted yield values, methanol was found to be a more effective solvent than water, notably, the methanolic extract of *Olea europaea* subsp.*laperrinei* produced a higher yield of 37.20 ±6.47%, while the methanolic extract of *Olea europaea* L.var. sylvestris resulted in a yield of 35.60 ± 3.18%. Similarly, the Saharan subspecies shows a greater extraction yield (25.60 ±0.23%) when employing water as a solvent in compared to the Mediterranean variety (22.20 ± 2.24%). **Table 13** and **Figure 37**show the percentage yields of various extracts from dehydrated plant materials.

Table 13: Yields, total polyphenols, and total flavonoids in aqueous and methanolic extractsof *Olea europaea* L.var. sylvestris and *Olea europea* subsp.*laperrinei* aerial parts

Plants samples	Extract type	Yield (%)	Total polyphenols (mg GAE/g Ext)[1] ***4	Total Flavonoids (mg QE/g Ext)[2] ***5
***Olea europaea* L.var. sylvestris**	Methanolic extract	35,60 ± 3,18	18,13 ± 1.04	14.30 ± 0.47
	Aqueous extract	22,20 ± 2,24	17,57 ± 0.34	12.98 ± 0.99
Olea europaea* subsp. *Laperrinei	Methanolic extract	37,20 ± 6,47	26,75 ± 2 .16	22.83 ± 0.13
	Aqueous extract	25.60 ± 0,23	18,93 ± 1.17	15.24 ± 0.27

1)*mg GAE/g Ext:mg of Galic Acid Equivalent/ g of dried Extract;*
2) *mg QE/g Ext:mg of Quercetin Equivalent/g of dried Extract*
3)*Each value in the table presented as mean ± standard deviation (n=3)*
4)*Results were compared using ANOVA from CoStat Software p<0.001.****; LSD 0.05 = 2.530
5)*Flavonoids Results were compared using ANOVA from CoStat Software p<0.001.**** ; LSD 0.05 = 1.074

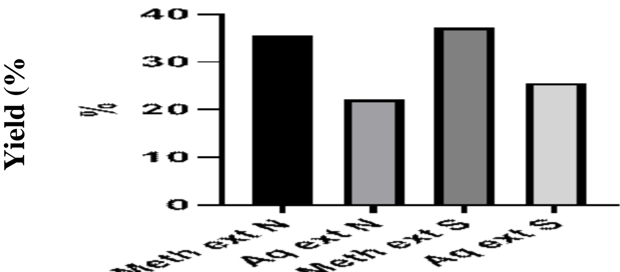


Figure 37: Yields of different extracts obtained from thetwo northern and southern subspecies.*Abbreviations: AqN :* subsp.var.sylvestris *aqueous extract; AqS :*subsp.*laperrinei aqueous extract; MethN :* subsp.var.sylvestris *methanolic extract; MethS :* subsp.*laperrinei methanolic extract.*

II-2-Quantitative analysis of the two subspecies extracts

II-2-1-Total phenolic content of the two subspecies extracts

The determination of the total contents (TPCs) was carried out according to the Folin-Ciocalteu method. Gallic acid was used as a standard and the TPC is expressed in milligrams of gallic acid equivalents per gram of extract (mg EAG/g extract). The calibration curve was obtained for gallic acid at different concentrations; it shows a proportionality of the absorbance as a function of the concentrations **(Figure 38).**

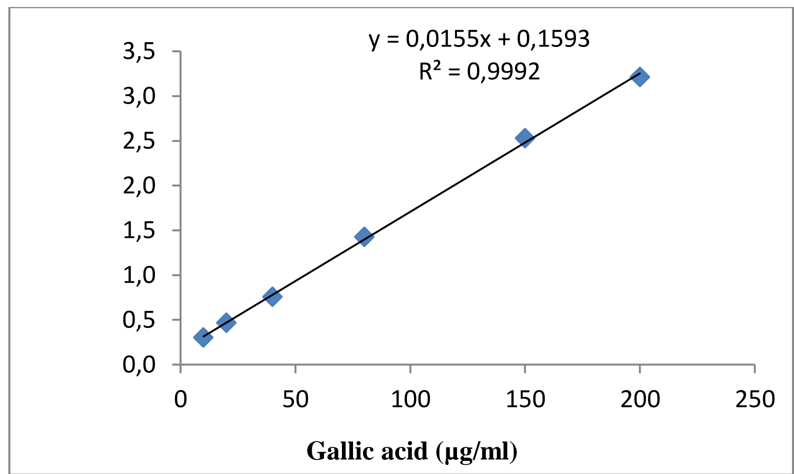


Figure 38: Standard calibration curve of Gallic acid used for polyphenols determination.

The TPCs of *Olea europaea* L.var.sylvestris and *Olea europaea* subsp.*laperrinei* in both aqueous and methanolic extracts were measured. The obtained data are illustrated (**Table 13, Figure 39**).

These findings demonstrate that the leaves of both subspecies can be are rich sources of polyphenols. Moreover, the TPCs of the four leaf extracts under investigation differed significantly depending on the solvent used; methanolic extracts were found to have higher phenolic component quantities than water extracts. As previously mentioned, the sample from the Saharan region had the highest concentration of phenols in its methanolic and aqueous extract (26,75 ± 2.16 mg GAE/g Ext) and (18,93 ± 1.17 mg GAE/g) respectively, compared to the sample from the Mediterranean region, which had significantly lower amounts (18,13 ± 1.04 mg GAE/g) and (17,57 ± 0.34 mg GAE/g) respectively.

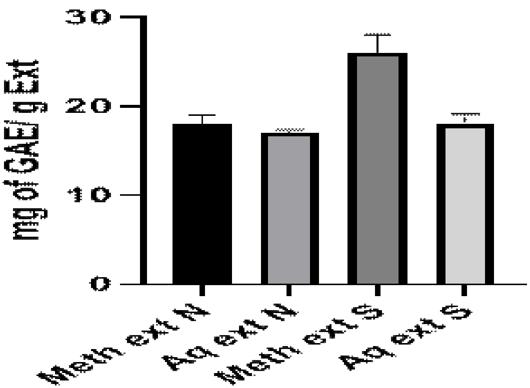


Figure 39: Total polyphenol content, expressed as milligrams of Gallic acid equivalent per gram of extract, for various extract samples; (Aq**:** aqueous extract; Meth: methanolic extract; S: Saharan subspecies; N: Northern subspecies).

II-2-2-Total flavonoid contents of the two subspecies extracts

The total flavonoids contents (TFCs) of various extracts was carried out using the aluminum trichloride method (AlCl3) and the standard was quercetin **(Figure 40).** The flavonoid content is expressed as milligram of quercetin equivalent per gram of extract (mg QE/g extract).

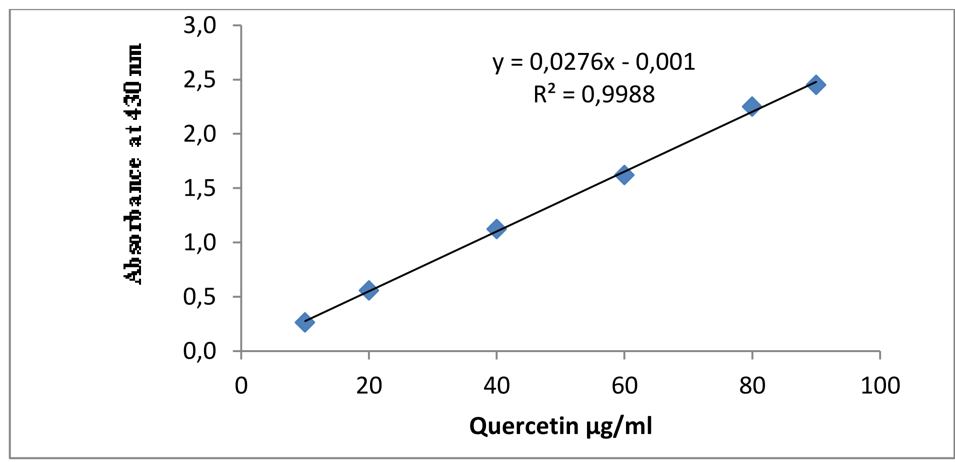


Figure 40: Standard calibration curve of Quercetin used for flavonoids determination.

The amounts of flavonoids in the extracts under investigation are summarized in (**Table 13, Figure 41**).

Accordingly, the TFC in the tested extracts ranged from 12.98 ± 0.99 to 22.83 ± 0.13 mg QE/g of extract, In relation to solvents ,the methanolic extract of the two subspecies had greater flavonoids contents than the aqueous extract, in addition, *Olea europaea* subsp.*laperrinei* extracts demonstrated the highest flavonoids levels in both methanolic and

aqueous extracts (22.83 ± 0.13 mg QE/g Ext),(15.24 ± 0.27 mg QE/g Ext) respectively, whereas ,the methanolic or aqueous extract of *Olea europaea* var.sylvestris showed the lowest concentrations (14.30 ± 0.47 mg QE/g Ext) (12.98 ± 0.99 mg QE/g Ext).

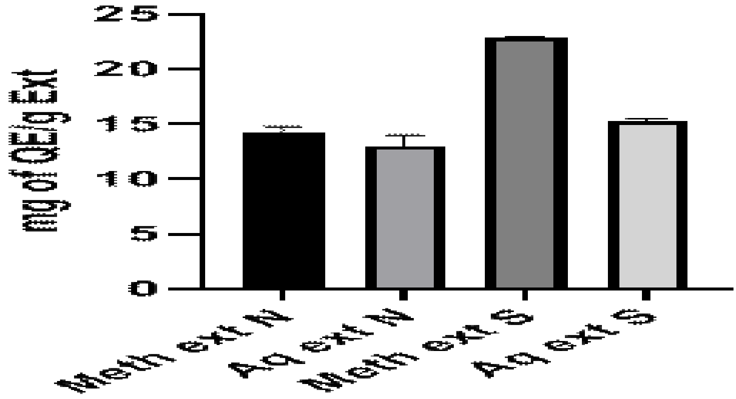


Figure 41: Total flavonoids of every extracted sample; (Aq: aqueous extract; Meth: methanolic extract; S : Saharan subspecies ;N :Northern subspecies).

II-3-HPLC-DAD analysis of the two subspecies extracts

The identification of individual polyphenols present in the extracts was performed using data from HPLC/DAD analyses, identified polyphenols represented different contents that corresponded most closely to the injected standards. The polyphenolic profile of the extracts was examined using 17 controls.The information on the phenolic compounds included in each extract is shown in **Tables 14 and 15**.

The extracts under study have various contents that were categorized on important molecular features such as substituted phenols (hydroxytyrosol and tyrosol), phenolic acids (vanillic acid, caffeic acid, gallic acid, p-Coumaric acid, Chlorogenic acid), flavones (luteolin, Quercitin, Apigenin), and secoiridoids (oleuropein and verbascoside). Among these, sixteen and seventeen phenolic compounds were detected in the aqueous and methanolic extract issues from *Olea europaea* L.var. sylvestris respectively. On the other hand, 14 and 11 compounds were identified in the aqueous and methanolic extract issues from *Olea europea* subsp. *laperrinei* respectively **(Tables 14,15).**

Regarding the methanolic extract of *Olea europaea*L. var. sylvestris, oleuropein (142.773 mg/g) was the predominant constituent, following by Luteoline-7-glucoside and Apigenin-7-glucoside. The minor phenolic compounds included Gallic acid (0.121 mg/g) and Luteolin (0.078 mg/g). In the aqueous extract, Oleuropein (11.586 mg/g) was the main constituent,

followed by Rutin, hydroxytyrosol, and Luteoline-7-glucoside **(Tables 14).**

Table 14: Identification of phenolic compounds in *Olea europaea* L.var. sylvestris leaf extract by HPLC-DAD chromatographic separation

Aqueous extract					Methanolic extract			
N°	TR	Compounds	Concentration (mg/ml)	Concentration (mg/g)	RT	Compounds	Concentration (mg/ml)	Concentration (mg/g)
1	7.072	Gallic acid	0.0024	0.165	7.076	Gallic acid	0.0014	0.121
2	10.149	**Hydroxytyrosol**	**0.0752**	**5.186**	10.151	Hydroxytyrosol	0.0222	1.93
3	11.489	**Chlorogenic acid**	**0.0508**	**3.503**	11.492	Chlorogenicacid	0.0334	2.904
4	12.857	Tyrosol	0.0467	3.220	12.855	Tyrosol	0.0157	1.365
5	13.579	Caffeicacid	0.0184	1.268	13.576	Caffeicacid	0.0113	0.982
6	14.090	Vanillicacid	0.0049	0.337	14.290	Vanillicacid	0.0062	0.539
7	15.363	**Rutin**	**0.1029**	**7.096**	15.357	**Rutin**	**0.0741**	**6.443**
8	15.742	Verbascoside	0.0272	1.875	15.983	**Verbascoside**	**0.0643**	**5.591**
9	16.387	**Luteoline-7-glucoside**	**0.0716**	**4.937**	16.382	**Luteoline-7 glucoside**	**0.0963**	**8.373**
10	17.061	p-Coumaric acid	0.0015	0.103	17.225	p-Coumaric acid	0.0027	0.235
11	18.057	**Apigenin-7-glucoside**	**0.0517**	**3.565**	18.050	**Apigenin-7-glucoside**	**0.0773**	**6.721**
12	18.109	Ferrulicacid	0.0229	1.579	18.238	Ferrulicacid	0.0178	1.547
13	18.841	**Oleuropein**	**0.1680**	**11.586**	18.827	**Oleuropein**	**1.6419**	**142.773**
14	21.154	Naringinin	0.0110	0.785	21.164	Naringinin	0.0298	2.591
15		Luteolin	n.d.	-	22.964	Luteolin	0.0009	0.078
16	23.335	Quercitin	0.0274	1.889	23.435	Quercitin	0.0033	0.287
17	25.804	Apigenin	0.0027	0.186	26.386	Apigenin	0.0029	0.252
∑			**0.6853**	**47.262**			**2.1015**	**182.40**
n.d. : not detected								

According to *Olea europaea* subsp. *laperrinei*, the principal components of the methanolic extract were oleuropein (276.157 mg/g), luteoline 7glucoside (10.557 mg/g), and rutin (8.471 mg/g). On the other hand, the primary constituents of the aqueous extract were oleuropein (28.07 mg/g), rutin (13.40 mg/g), and quercitin (8.41 mg/g) **(Tables 15).**

The two subspecies extracts share a common characteristic: the most prevalent components of the aqueous extracts were rutin and oleuropein, however, the predominent components of the methanolic extracts were oleuropein and luteoline7glucoside.

As mentioned in **Table 14 and 15**, there are differences in both the amount and number of identified compound in different extract types and subspecies studies; whereas *Olea europea* subsp. *laperrinei* is prevailing in relation to the quantity of these constituents, but *Olea europaea* L. var. sylvestris extracts are dominating in terms of the number of identified compounds.

II-4-Determination of antioxidant activity by DPPH free radical scavenging activity the two subspecies extracts

Table 15: Identification of phenolic compounds in *Olea europaea* subsp. *laperrinei* leaf extract by HPLC-DAD chromatographic separation

	Aqueous extract				Methanolic extract			
N°	RT	Compounds	Concentration (mg/ml)	Concentration (mg/g)	RT	Compounds	Concentration (mg/ml)	Concentration (mg/g)
1	10.185	Hydroxytyrosol	**0.0525**	5.25	-	Hydroxytyrosol	**n.d.**	-
2	11.496	Chlorogenicacid	0.0428	4.28	11.494	Chlorogenicacid	0.0299	4.271
3	12.830	Tyrosol	0.0235	2.35	-	Tyrosol	**n.d.**	-
4	13.791	Caffeicacid	0.0102	1.02	-	Caffeicacid	**n.d.**	**-**
5	14.051	Vanillicacid	0.0046	0.46	-	Vanillicacid	**n.d.**	-
6	15.321	**Rutin**	**0.1340**	**13.40**	15.335	**Rutin**	**0.0593**	**8.471**
7	16.008	Verbascoside	0.0426	4.26	15.946	Verbascoside	0.0482	6.885
8	16.311	**Luteoline-7-glucoside**	**0.0611**	**6.11**	16.341	**Luteoline-7-glucoside**	**0.0739**	**10.557**
9	17.025	p-Coumaric acid	0.0183	1.83	17.324	p-Coumaric acid	0.0187	2.671
10	17.988	Apigenin-7-glucoside	0.0479	4.79	17.995	**Apigenin-7-glucoside**	**0.0354**	**5.057**
11	18.064	Ferrulicacid	0.0256	2.56	18.194	Ferrulicacid	0.0156	2.228
12	18.799	**Oleuropein**	**0.2807**	**28.07**	18.782	**Oleuropein**	**1.9331**	**276.157**
13	21.034	Naringinin	0.0090	0.90	20.683	Naringinin	0.0340	4.857
14	23. 303	**Quercitin**	**0.0841**	**8.41**	-	Quercitin	**n.d.**	-
15		Apigenin	n.d.	-	-	Apigenin	**n.d.**	-
Σ			**0.8368**	**83.680**			**2.2481**	**321.153**

n.d. : not detected

The spectrophotometric approach was used to assess the antioxidant activity of the methanolic and aqueous extracts of two subspecies, and the positive control (BHT), against the very stable free radical DPPH. This was done by monitoring the radical's reduction, which was accompanied by a color change from violet to yellow (DPPH-H), which can be measured at

515 nm. The antiradical activity of plant extracts increased in parallel with the increase of the concentration of the different extracts and BHT used control (0 to 100 µg/ml).

The amount of antioxidants required to reduce the initial concentration of DPPH radicals by 50% in 30 min (IC50) was used to determine the free radical scavenging effects.The curves of the inhibition percentages as a function of concentrations were used to graphically determine the IC50 (IC50= (50-b) /a) **(Figure 42),** in which, the IC50 value is inversely proportional to the antioxidant activity.

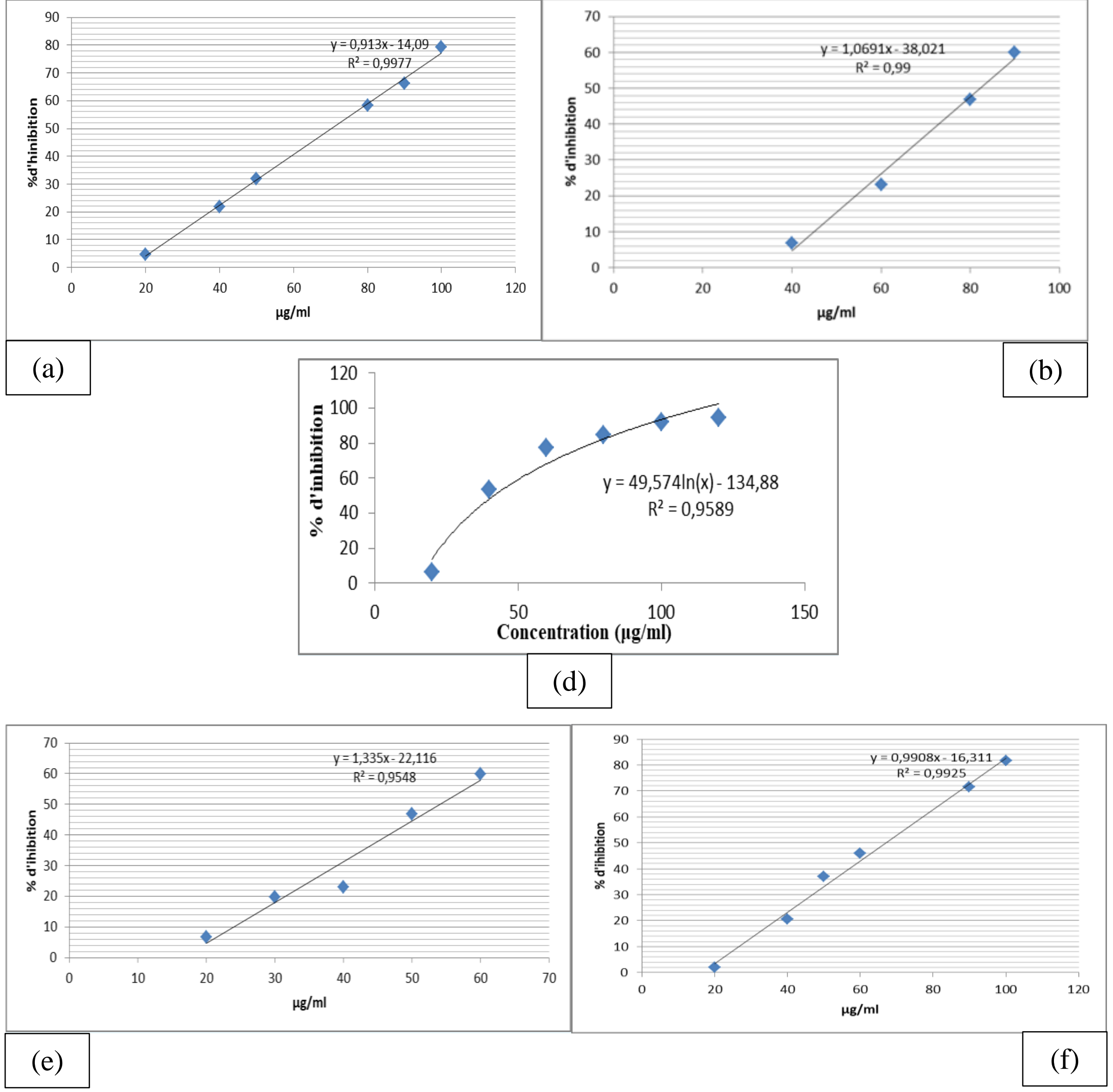


Figure 42: Variation of DPPH inhibition as a function of extracts concentration (a):aqueous extract and (b): Methanolic extract of ; *Olea europaea* L.var. sylvestris; (d): the BHT; (e): aqueous extract (f): Methanolic extract; of *Olea europaea* subsp.*laperrinei*

Based on our data, the antioxidant activity measured by the DPPH radical scavenging activity fluctuated between 54.01 ± 0.46 to 82.33 ± 0.50 µg/mL **(Table 16; Figure 43) .** The endemic saharan subspecies *Olea europaea* subsp. *laperrinei,* showed the highest capacity to neutralize DPPH radicals in both methanolic and aqueous extracts, with IC50 values of 54.01 ± 0.46µg /ml and 66.97 ± 0.41 µg /ml, respectively. On the other hand, extracts from *Olea europaea* var. sylvestris were found to have the least amount of antioxidant activity, with values of 70.19 ± 2.09 µg/ml for the methanolic extract and 82.33 ± 0.50 µg/ml for the aqueous extract. Increased antioxidant activity is indicated by a decreased IC50 value, therefore, *Olea europaea* subsp. *laperrinei* antioxidant activity was roughly similar to that of the positive control (BHT) (41.65 ±0.00 µg/mL), whereas BHT control antioxidant activity was almost twice as high as *Olea europaea* L.var. sylvestris. The higher antiradical activity was observed in both subspecies under investigation in the methanolic extracts than in the aqueous extracts.

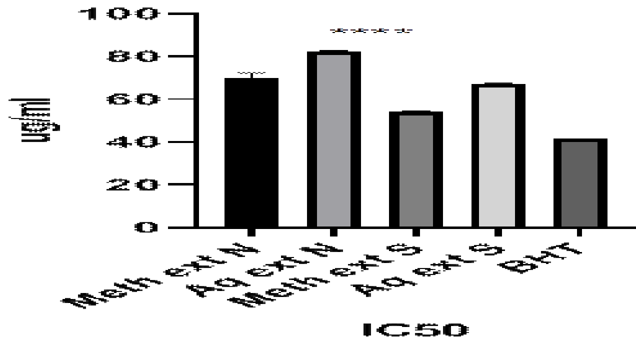


Figure 43: Comparison of the antiradical activity of the extracts of the two subspecies with that of positive controls BHT. Values are means of three repetitions ± SD.

Table 16: In vitro antiradical activity of the methanolic and aqueous extracts of the two subspecies investigated and the positive control (BHT).

Samples	Extract type	DPPH ***IC_{50}(ug/ml) (1)
***Olea europaea* L. var. sylvestris**	Methanolic extract	70.19 ± 2.09
	Aqueous extract	82.33 ± 0.50
Olea europaea* subsp. *Laperrinei	Methanolic extract	**54.01 ± 0.46**
	Aqueous extract	66.97 ± 0.41
BHT		**41.65±0.00**

(1)Each value is represented as mean ± SD (n=3).
*(2)ANOVAnotes:*Significant at P <0.05;**Significant at P <0.01;*** Significant at P < 0.001.*
*(3) Results were compared using ANOVA from CoStat Software p<0.001.***;LSD 0.05 = 1.87*

II-5-Discussion

To the best of our knowledge, only a few investigations have been conducted on the bioactivities of leaf extract issues from wild subspecies of *Olea europaea* L., as in our case of subsp. *laperrinei* only one study has been conducted, because the majority of them were related to cultivated olives. In addition, the present study is the first, to investigate the antioxidant activity and quantify the total phenolic and flavonoid content of two wild subspecies of *olea europaea* L.in Algeria.

Several relationships can be observed in our research when comparing the values acquired from the two subspecies investigations,the yield data, total phenolic content, and flavonoid contents,as well as the antioxidant activity of all the extracts evaluated indicate that the results exhibit a comparable dynamic variation,in which their greatest values were seen when methanol was used as a solvent rather than water.In their research, **Abaza *et al.* (2015)** validated that the phenolic content and extract yields were affected by the extracting solvents used,and it has been noted that methanol produces the highest yields**.**According to **Addab *et al.* (2020)** investigation, water also plays an an important function in the extraction process of polyphenols by enhancing their diffusion in the tissues of plants,on the other hand, they stated that differences in yield findings is not just attributable to the type of solvents used, however can be related to a number of factors, including; specimen composition, pH, temperature, and extraction duration. Furthermore, yield extraction is influenced by the location and timing of harvest**.**

As previously stated, the yield of extract leaves from *Olea europaea* subsp.*laperrinei* was higher than that of *Olea europea* L.var. sylvestris. The values obtained were comparable to those reported by **Arab *et al.* (2020)**, who noticed that the extraction yield of wild olive is 38,74% **.** However, our value was more significant than those of other authors who found a lower yield **(Luís *et al*., 2012; Addab *et al*., 2020).**

Overall, the findings of this study make abundantly evident that the two wild olive subspecies leaf extracts contains substantial levels of phenols and flavonoids. According to the extraction solvents, the two methanolic extracts had a higher amount of phenolic components than the water extracts. The methanolic extract of the *Olea europaea* subsp.*laperrinei* that spreads from the Sahara contains a very high concentration of these phenolic compounds compared to other extracts. Polar solvents are the most effective extraction media for phenolic compounds **(Badarinath *et al*., 2010)**, as evidenced by the

heighest concentrations of phenolic detected in all of our extracts.These findings agree with several of studies that examined the methanolic extracts of olive leaves from Tunisia, Malta, France, and Serbia **(Stankovic *et al.*, 2017),** as well as the Chemlali variety from western Algeria **(Debib& Boukhatemm, 2017)** and the Meski cultivar from Tunisia **(Orak *et al.*, 2019).** Other researches (**Luís*et al.*, 2012; Saiah *et al.*, 2016; Himour *et al.*, 2017**) have reported the highest concentrations of these phytochemicals, which differs from our results. Moreover, our data show that flavonoids comprise the majority of polyphenol compounds, which is consistent with the finding of **Chu *et al.* (2000)** suggestion**.**

An HPLC-DAD approach was used to determine the bioactive contents of the different extracts investigated and assess their potential impact on the antioxidant potential; a variation in the quantity and number of identified chemicals was found between extract types and subspecies studies.Where *Olea europaea* subsp.*laperrinei* dominates in relation to the quantity of these components, whereas *Olea europaea* L.var. sylvestris extracts are predominant in terms of the number of noticed compounds; as a result, we can infer that not just the number of identified compounds but the quantity and structure of these phenolics, can affect the bioactivity that the plant provides **(Debib& Boukhatemm ,2017)**, like in our case of the saharan subspecies. The dominance of the oleuropein compound was a characteristic shared by all extracts. This result is in accordance with other studies that found oleuropein the be the most common phenolic compound when examining various varieties of *Olea europaea* L. across various extract types; water and methanolic extract **(Edziri *et al.*, 2019),** methanolic and ethanolic extracts **(Monteleone *et al.*, 2021).** Additionally according to **Altiok *et al.* (2008) and Goldsmith *et al.* (2018),** oleuropein, is the main phenolic of olive leaves, accounts for 29% of the components in olive leaf extract.The therapeutic value of olive leaves has been associated, with the presence of oleuropein, which has a variety of pharmacological and health-promoting properties**(Al-Azzawie *et al.*, 2006).**

Free radicals are the primary cause of many human diseases, including cancer and Alzheimer's disease. Antioxidant chemicals can donate hydrogen atoms or chelate metals to destroy free radicals. Therefore, the use of commercial antioxidants is necessary; many of these are made synthetically, including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). It has been reported that these synthetic antioxidants are harmful. Consequently, interest in naturally occurring antioxidants found in a variety of plants is increasing **(Salah*et al.*, 2012).** In this regard, there has been an increase in interest in the chemical constituents of olive leaves. Olive leaves are an inexpensive raw material that can be

used to create products with additional value (**Briante *et al*.,2002).** This research was conducted to confirm the ability of leaf extracts issued from wild olives to synthesize secondary metabolites, assess their effectiveness they were as natural antioxidants, and investigate how geographic variations might affect the chemical composition and bioactivity of different wild olive subspecies.

Our findings indicate that the antioxidant capacity of the two wild olive studies is high when compared to numerous other published results of *Olea europea* L. in Algeria 95.70±2.46 µg/ml **(Saiah *et al*., 2016),** France (113.30±1.12 µg/ml), and Serbia (94.39±1.03 µg/ml) **(Stankovic *et a*l., 2017)**.Other studies, however, demonstrated a high activity to our obtaining, such as in Algerian Chemlali olive leaves with 40µg/ml **(Debib& Boukhatemm ,2017),** and in *olea europea* L from Portugal with 48.77 ± 2.35 mg/L **(Luís *et al*., 2012)**.Additionally,the methanolic extracts from the Saharan subspecies contribute to an increase in the scavenging activities of free radicals more than the other extracts, which is directly related to their higher levels of polyphenols **(Luís *et al*.,2009; Olmo-García *et al*., 2018; Nicolì *et al*., 2019)**. These polyphenols can inhibit the oxidation of macromolecules and prevent the synthesis of free radicals **(Acker *et al*., 1995).** The structure of phenolic compounds, particularly the number and location of hydroxyl groups in relation to functional carboxyl groups **(Addab *et al*.,2020),** can explain the variability in antioxidant activity observed between the leaf extracts of the two wild olives. These findings suggest that oleuropein is the main contributor to the height antioxidant properties of olive leaf extracts **(Benavente-Garcia *et al*., 2000; Goldsmith *et al*. 2018; Addab *et al*., 2020)** which were present high amounts in all of our extracts; notably laperrine methanolic extract, as well as this compounds that it is a well-known antioxidant derivative **(Orak *et al*., 2019)**. In addition, other findings suggest that oleuropein can be extracted from olive leaves which are considered a renewable natural resource **(Savournin *et al*., 2001; Briante *et al*., 2002; Bouaziz & Sayadi, 2005).**

The high antioxidant capacity is not due only to single components, but also due to the combined effect of flavonoids, oleuropeosides, and substituted phenols **(Talal *et al*., 2011)**. Some studies showed that other phenolic compounds that can be involved in the antioxidant activity of olive leaf extract such as rutin, had good effects as antioxidants due to their ability to reduce oxidative stress, in high-cholesterol-diet-fed **(Augustyniak *et al*., 2010; Ghasemzadeh *et al*.,2010).** Besides, luteolin may have preventive benefits against the appearance of diabetes-related cardiac dysfunction by minimizing oxidative stress **(Wang *et***

***al*., 2009),** rutin and luteolin were among the predominant compounds found in all our extracts. Moreover, our results confirm the prior reports that indicate olive leaves are a good source of many antioxidants **(Bouaziz &Sayadi, 2005, Ranalli *et al*., 2006)**.

in which the stressful one originated from the Saharan region was dominant in both types of extracts than the less stressful one from the northern region, it may be used to illustrate how secondary metabolites assist plants in adjusting to the ecological conditions in their environment **(Khan *et al*.,2011),** which, during stress, the results showed a rise of those metabolite's synthesis and accumulation notably the phenolic compounds,this finding may corroborate the notion that phenolics are essential for *Olea europea* L. ecophysiological adaptation to particular ecological circumstances,in which synthesis and accumulation of those metabolites, mainly the phenolic compounds increased during stress It could support the suggestion that phenolics could play a key role in the ecophysiological adaptation of *Olea europea* L.to the specific ecological conditions **(Debib& Boukhatemm, 2017)**, furthermore, **Edziri *et al*. (2019)** postulated that a variety of factors, such as genetic heritage, geographic location, soil composition, climate, altitude, rainfall, and types of olive variety, could account for the notable variations seen in the methanolic extracts of the leaves of four cultivars of olives from Tunisia.In addition, the kind of solvent employed during the extraction procedure **(Zaïri *et al*., 2020).** As demonstrated in our work, these variables may have a direct impact on the chemical profile and, consequently, their therapeutic benefits.

III-Essential oil extraction and antibacterial activity assessment

III-1-Essential oils yield

Extraction of essential oils from the aerial parts of the two olive subspecies by hydrodistillation yielded a greenish liquid. The yields of dried leaves of *Olea europaea* subsp. *laperrinei* and *Olea europaea* L.var. sylvestris were 0.19% and 0.17% (w/w), respectively.

III-2-Chemical profile of volatile oils

The composition of the volatile oil was determined by Gas Chromatography Mass Spectrometry (GC-MS) and Gas Chromatography with Flame Ionization (GC-FID) analysis. The results of the analysis of essential oils allowed the identification of 31 components for *Olea europaea* Subsp. *laperrinei*, representing an average of 93.33% of the total oil, whereas 29 components were detected by representing 93.54% of *Olea europaea* L.var.sylvestris oil. The identified chemicals and their relative abundances are listed in their order of appearance in **Table17** and **Table18**.

III-2-1-Chemical profile of *Olea europaea* L.var. sylvestris volatile oil

The predominant component of *Olea europaea* L.var. sylvestris volatile oil was nonanal, which with an average rate of 11.82%. Moreover it was followed by theaspirane A (9.81%), 3-hexen-1-ol, benzoate (9.31%), β-damascenone (6.87%), α-pinene (6.32%), farnesol (6.10%), and geranylacetone (5.25%). Other low concentration chemicals were detected, including β-ocimene (4.20%) and dl-limonene (3.56%).

Olea europaea L. var. sylvestris volatile oil was rich with monoterpene hydrocarbons (22.75%) which represent the predominant groups of compounds identified in this essential oil, followed by Norisoprenoids (19.94%) and Aldehyde (14.88%) which were the next most common groups of compounds, while the least amount of identified components in the essential oil were found in the following chemical classes such as oxygenated monoterpenes (10.08%), oxygenated sesquiterpenes (7.51%), and sesquiterpene hydrocarbons (0.98%) **(Table17).**

Table 17: Chemical profile of volatile oils derived from *Olea europaea* L.var. sylvestris aerial parts

N°	Compounds	RT	KIb	KLc	(%)
1	α.-Thujene	7.687	927	921	0.15
2	**α-Pinene**	7.875	932	928	6.32
3	β-Pinene	9.057	978	971	1.65
4	α.-Phellandrene	9.838	1002	999	2.55
5	δ-3-Carene	10.004	1008	1004	0.39
6	p-Cymene	10.416	1033	1036	1.08
7	dl-Limonene	10.551	1029	1024	3.56
8	Eucalyptol	10.616	1030	1028	1.80
9	β-Ocimene	10.916	1038	1033	4.20
10	γ-Terpinene	11.416	1060	1056	0.47
11	**Nonanal**	12.762	1080	1086	11.82
12	Neo-Allo-Ocimene	13.49	1130	1124	2.38
13	4-Terpineol	14.95	1180	1176	0.37
14	2-Decenal	17.33	1252	1254	2.66
15	**Theaspirane A**	18.339	1315	1309	9.81
16	**β –Damascenone**	20.615	1385	1374	6.87
17	trans-Caryophyllene	21.545	1420	1416	0.60
18	Geranylacetone	22.302	1453	1447	5.25
19	β –Ionone	23.193	1485	1477	3.26
20	α-Farnesene	23.650	1504	1509	0.38
21	Croweacin	24.109	1457	1456	2.68
22	Farnesol	25.021	1690	1684	6.10
23	**3-Hexen-1-ol, benzoate**	25.23	-	-	9.31
24	Spathulenol	25.486	1640	1648	0.45
25	Unknown	25.6	-	-	2.15
26	Apiol	26.515	1621	1617	2.9
27	Viridiflorol	27.25	1612	1619	0.96
28	2-Pentadecanone	31.126	1698	1701	3.04
29	Octadecanal	34.573	2021	2028	0.38
	Total				**93.54**
Chemical classes of the constituents					
Monoterpene Hydrocarbons					22.75
OxygenatedMonoterpenes					10.08
Sesquiterpene Hydrocarbons					0.98
Oxygenatedsesquiterpenes					07.51
Aldehyde					14.88
Norisoprenoids					19.94
Othercomponents					17.40

RT: Retention Time; KIc: Kovats Retention Index; KIb: LiteratureKovatsRetention Index.

III-2-2-Chemical profile of *Olea europaea* subsp. *laperrinei* volatile oil

Regarding the chemical composition of the volatile oil from *Olea europaea* subsp. *laperrinei*, α-pinene found to be the predominant component with an average rate of 16%of the total oil, followed by β-ocimene (12.82%), dl-limonene (8.20%), nonanal (8.18%), β-pinene (4.73%), croweacin (4.64%) ,and α-phellandrene (4.62%).The remaining constituents, such as farnesol (3.44%), Neo-allo-ocimene (2.66%), and p-cymene (2.3%) were found in trace levels.

Monoterpene hydrocarbons are the predominant groups of identified compounds in this essential oil at a rate of 55.74%, followed by aldehydes (12.82%) which was the second most common group, in contrast oxygenated monoterpenes (10.47%) and oxygenated sesquiterpenes (06.32%) were the chemical classes that had the least number of components detected in this essential oil **(Table18).**

Table 18: Chemical profile of volatile oils derived from from *Olea europaea* subsp. *laperrinei* aerial parts

N°	Compounds	RT	KIb	KLc	Concentration (%)
1	α –Thujene	7.593	927	921	0.88
2	**α –Pinene**	7.804	932	929	16
3	Camphene	8.193	953	948	0.008
4	**β-Pinene**	9.004	978	971	4.73
5	β-Myrcene	9.404	988	985	0.69
6	α-Phellandrene	9.804	1002	999	4.62
7	δ-3-Carene	9.963	1008	1003	1.53
8	α –Terpinene	10.15	1018	1010	0.31
9	p-Cymene	10.416	1033	1016	2.3
10	**dl-Limonene**	10.551	1029	1024	8.20
11	Eucalyptol	10.616	1030	1028	4.32
12	**β –Ocimene**	10.916	1038	1033	12.82
13	γ –Terpinene	11.416	1060	1056	1
14	α –Terpinolene	12.286	1087	1083	0.54
15	Linalool	12.680	1100	1098	1.35
16	**Nonanal**	12.780	1080	1091	8.18
17	Neo-Allo-Ocimene	13.49	1130	1126	2.66
18	4-Terpineol	14.95	1180	1176	1.73
19	α-Terpineol	15.36	1192	1187	0.71
20	2-Decenal	15.69	1252	1249	1.82
21	β –Damascenone	20.615	1385	1379	1.64
22	β –Caryophyllene	21.552	1420	1416	0.42
23	Germacrene	23.103	1499	1494	0.41
24	Aromadendrene	23.25	1442	1462	0.82
25	α-Farnesene	23.650	1504	1511	0.45
26	Croweacin	24.109	1457	1472	4.64
27	Farnesol	25.033	1690	1679	3.44
28	Spathulenol	25.486	1640	1652	0.45
39	Caryophyllene oxide	25.6	1580	1569	0.44
30	Apiol	26.515	1621	1619	4.23
31	β-Eudesmol	27.17	1650	1647	1.99
	Total				**93.33**
Chemical classes of the constituents					
monoterpene Hydrocarbons					55.74
Oxygenated monoterpenes					10.47
sesquiterpene Hydrocarbons					02.10
Oxygenated sesquiterpenes					06.32
Aldehydes					12.82
Other components					5.87

RT:Retention Time; KIc: Kovats Retention Index; KIb: Literature Kovats Retention Index

III-3- Antibacterial activity assessment of the two olives essential oils

Disc diffusion essays were performed to evaluate the in vitro antibacterial activity of the two essential oils, by measuring the mean growth inhibition zone diameters of seven bacteria strains at different essential oils concentrations **(Table 19)**, Gentamicin was used as a positive control in this analysis.

The findings indicate that among all the bacterial strains tested, the essential oil of *Olea europaea* subsp. *laperrinei* exhibited the highest range of inhibition zone diameters. *P. aeruginosa* and *B. subtilis* demonstrated extreme sensitivity, with the latter showing a higher range of inhibition diameters than *Olea europaea* L.var. sylvestris essential oil, with inhibition diameters varying between 20.33 ± 0.58 mm and 17.67 ± 1.15 mm, in addition *E. coli, S. aureus* ATCC 25923, *S. aureus* ATCC 43300, and *E. faecalis* exhibit moderate sensitivity to the two essential oils, however, *K. pneumoniae* was extremely resistant to the two essential oils. Compared to the other doses, the greatest activity for all bacterial strains tested was observed at a 1/2 dilution of the two oils **(Table19).**

Table 19: Diameter of inhibition (mm) of essential oils from *Olea europaea* L.var. sylvestris and *Olea europaea* subsp. *Laperrinei*

	***Olea europaea* L.var. sylvestris**			***Olea europea* subsp. *Laperrinei***			**Control (mm)**
Concentration (W/W)	**1/10**	**1/4**	**1/2**	**1/10**	**1/4**	**1/2**	**Gentamicin**
***S. aureus* ATCC25923**	-	08,33 ±0,58	11,67± 1,15	12,00±1,00	13,67±1,15	15,33±1,53	24±0
***E.coli* ATCC 25922**	09,33+0,58	10,67 ±1,15	11,00±1,00	12,33±0,58	13,67±1,15	14,67±0,58	22±0
***K. pneumonia* ATCC700603**	-	-	-	-	-	-	15±0
***E. faecalis* ATCC 51299**	07,67+0,58	09,33±0,58	11,33±1,15	10,67± 0,58	12,67±0,58	14,67±0,58	21±0
***S. aureus* AT CC 43300**	-	08,67±0,58	10,33±0,58	11,00±1,00	12,33±0,58	15,67±1,53	23±0
***P.aeruginosa* ATCC 27853**	-	08,67±0,58	11,00±1,00	13,33±0,57	12,33±0,58	20,33±0,58	27±0
***B.subtilis* ATCC6633**	10,33+0,58	14,33 ±0,57	11,67± 1,15	13,33±0,57	14,33±0,57	17,67±1,15	30±0

III-3-1-Analysis of variance (ANOVA) of antibacterial activity of the two essential oils

The results of antibacterial activity were subjected to statistical analysis (ANOVA).According to the results of this statistical analysis, the interaction between the doses, the essential oils, and the bacterial strains ,was extremely significant (P < 0.001) **(Table 20).**

Table 20: Main effects and interactions of the two essential oils**.**

Sources	Df	MS	F	P
Main Effects				
Bacteria	6 2473.020563	412.17009	808.30244	.0000 ***
Dosses	34486.49513	1495.4984	2932.8062	.0000 ***
Oils	2490.3088235	245.15441	480.76974	.0000 ***
Interaction				
Bacteria* dosses	18 241.0010823	13.388949	26.256927	.0000 ***
Bacteria* oils	12 215.5980392	17.966503	35.233921	.0000 ***
Dosses* oils	6 224.2009804	37.36683	73.279698	.0000 ***
Bacteria* dosses* oils	36 163.2156863	4.5337691	8.8911269	.0000 ***

Notes:*Significant at P <0.05;**Significant at P <0.01**;*** Significant at P < 0.001.**

Bacterial sensitivity significantly varied, according to the statistical analysis. According to their extreme sensitivity to the two essential oils investigated and their higher inhibition diameter (17, 54 mm) *B. subtilis* bacteria , was classified in the category "a"followed by, the strain *P. aeruginosa* categorized as belonging to group "b" and has a higher sensitivity. *E. coli, S. aureus* ATCC 43300, *E. faecalis,* and *S. aureus* were categorized in ranges b, c, de, and e, respectively, and have moderate sensitivity to both *Olea europaea* L .var. sylvestris and *Olea europaea* subsp. *laperrinei* essential oils. In contrast, the resistant strain of *K. pneumonia* was designated as category (f) in the list **(Table 21).**

Table 21: Sensitivity of the two essential oils against all bacterial strains tested

Rank	Bacteria	Mean(mm)	n	Significant ranges
1	*B.subtilis* **ATCC6633**	17.54	24	a
2	*P.aeruginosa* **ATCC 27853**	15.37	24	b
3	*E.coli* **ATCC 25922**	14.54	24	c
4	*S. aureus* **ATCC 43300**	13.71	24	d

5	*E.faecalis* **ATCC 51299**	13.46	24	de
6	*S. aureus* **ATCC 25923**	13.17	24	e
7	*K. pneumoniae* **ATCC700603**	3.83	24	f

Note: LSD 0.05 = 0.40

Statistical analysis of oils dilutions effect and antibiotic on the inhibitory zone of bacterial tested, revealed that category "a" contains the antibiotic Gentamicin which had the most powerful antibacterial activity, followed by class "b" of 1/2 dilution essential oils with moderate activity, while the 1/4 and 1/10 dilutions had minimal antimicrobial activity **(Table 22).**

Table 22: Effects of oil dilutions and antibiotic on the inhibitory zone of bacteria tested

Rank	**DOSES**	**Mean (mm)**	**N**	**significant ranges**
1	Gentamicin	23.19	42	a
2	½	12	42	b
3	¼	10.02	42	c
4	1/10	7.14	42	d

Note: LSD 0.05 = 0.30

Concerning the antibacterial activity potential, for all evaluated bacterial strains, the essential oil extracted from the Saharan population had a greater potential for growth bacterial inhibition than the essential oil extracted from the Northern population **(Table 23).**

Table 23: Effectiveness of two essential oils from several sampling locations

Rank	**Station**	**Mean**	**n**	**significant ranges**
1	**Tamanrasset**	14.86	84	a
2	**Setif**	11.31	84	b

Note: LSD 0.05 = 0.21

III-4-Discussion

There are only a few researchs on the chemical profile and antibacterial properties of the essential oil from some varieties of *Olea europaea* L. have been found **(Halouie *et al.*, 2010),** but no research has been conducted on either *Olea europaea* subsp. *laperrinei* or *Olea europaea* L. var. sylvestris in Algeria. Due to the lack of studies on the chemical profile and antimicrobial activity of our investigated wild subspecies, the obtained results were compared with those results of the species *Olea europaea* L.

The results showed that both subspecies essential oils generated important yield. This yield is comparable to that produced by the dried leaves of the Tunisian Chemlali cultivar, which ranges from 0.1 to 0.22% **(Brahmi et *al*., 2012).** In contrast ,other hydrodestilated leaves gave less yield of essential oil compared to our result,such as leaves of *Olea europea* L. from the north of Algeria produce 0.01% of essential oil yield **(Boukhebti *et al*.,2015)**,as well as fresh leaves of *Olea europaea* L. from Tunisia **(Halouie *et al*.,2010)** and fresh leaves of the three *Olea europaea* L. cultivars Chemlali which gave a yield ranging between 0.07 and 0.16% (**Campeol *et al*.,2003**), thus according to this result we can deduce that the essential oil yield extracted from the fresh leaves, was inferior to those obtained from the dried leaves**.**

The variation in essential oil yield either between our subspecies studied or with the other studies can refer to many factors including geographical and environmental conditions **(Sangwan *et al*., 2001),** harvesting period, because following around a third of the essential oils evaporate into the air **(Aboukhalid *et al*., 2017).**

According to the chemical composition of the most prevalent component of var. Sylvestris essential oil, is a nonanal, which accounted for 11.82% of the oil. This was followed by theaspiranea A and 3-hexen-1-ol,benzoate .These results were comparable to those of *Olea europea* L. essential oil from different cultivars in the Mediterranean basin; Such as **Brahmi *et al*. (2012)** observed that among Tunisian cultivars, the Chemchali cultivar had a notable rise in 3-hexanol benzoate (16%), whereas the Neb jewel cultivars predominantly revealed nonanal (10.0%), furthermore, **Brahmi *et al*. (2015)** found that the most prevalent class of chemicals in the leaf volatile oil with the Chetoui Tunisian variety was Aldehydes dominated by nonanal. On the other hand, when compared the Italian cultivars, the essential oils from various cultivars in Italy revealed, that nonanal was the main component in the Cipressino cultivar (11.8%),moreover, it was found that theaspirane (9.6%) was one of the main components of the Leccino cultivar **(Campeol *et al*., 2003).**

Similar results were obtained from another investigation of the essential oils of four Italian cultivars, indicating that aldehydes were the most prevalent chemical class detected among all cultivars. They reported that the well-known green leaf volatile (Z)-3-hexen-1-ol, which contributes to the "odor" of leaves, was present in 7.30% of Oblica cultivars , Furthermore, it was shown that nonanl (11.15%) was the most prevalent nonanal compound in Frantoio cultivars and they determined that all four cultivars contained theaspirane A, with cv. Lastovka has the highest percentage (9.17–12.21%) **(Popovi *et al*., 2021).** The volatile oil profile of two varieties, Chemlal and Sigoise, in two different regions of

Algeria, demonstrated that samples from the Mediterranean area had a significant percentage of nonanal; notably the Sigoise variety exhibited 11.7% comparable to our findings **(Tlili *et al.* 2022).**Numerous investigations demonstrated the therapeutic value of nonanal compound as an antibacterial and antifungal agents **(Kubo *et al.*, 1995)**and that they were mainly responsible for the antidiarrheal activity**(Zavala-Sánchez *et al.*, 2002).**

The predominant class of constituents in the volatile fraction of *Olea europaea* subsp. *laperrinei* leaves was monoterpene hydrocarbons (55.74%), with α-pinene as the major component, this data is similar to that of **Haloui *et al.* (2010)**, who found that monoterpene hydrocarbons was the dominant group of constituents (55.16%) and α-pinene (52.70%) as the main component in Tunisian olive essential oil, additional *Olea europaea* L. cv. fresh leaves from Turkey consisted mainly of α-pinene (9.82%) **(Vural& Akay, 2021)**. Nevertheless, our findings are in contradiction with the results of **Boukhebti *et al.* (2015)**, who determined that palmitic acid (14.71%) dominates the chemical composition of *olea europaea* L.essential oils.Many researchers have exhibited the insecticidal, spasmolytic, antibacterial, and anticholinesterase characteristics of α-pinene **(Lee *et al.*, 2001; Savelev *et al.*, 2003).** Additionally, this substance was found to have antistress properties, allowing to lessen rats' hyperthermia caused by stress **(Akutsu *et al.*, 2000).**

These differences in the chemical composition of *Olea europeae* L. essential between the two wild olives can be explained by many factors including variety, processing details, storage conditions, and different environmental factors such as geographic distribution **(Prenzler *et al.*, 2002; Temime *et al* ., 2006 a,b),** and altitude sites **(D'Imperio *et al.*, 2003).**

In various bioassays, *Olea europaea* L.essential oil has been shown to exibit greater anti-bacterial and anti-fungal activities in vitro. This is related to the higher concentration of volatile components **(Medina *et al* .,2006)** and at room temperature, a high susceptibility to both Gram-positive and Gram-negative bacteria was shown by the olive essential oil, additionally, it increase plasma membrane permeability, which causes greater fluid loss from bacterial cells **(Chang *et al.*, 2001)**, it may also reduce microbial respiration **(Lee *et al.*, 2001).**

The results of this study revealed that the two volatile oils have efficient antibacterial activity toward Gram-positive and Gram-negative bacteria strains,notably to *P. aeruginosa* and *B. subtilis,* and also the two essential oils have a moderate inhibitory effect on *E. coli,*

S. aureus ATCC43300*, E. faecalis,* and *S. aureus* ATCC 25923.On the flip side*, K. pneumonia* has no significant effect on the two essential oils, and the antimicrobial activity olive leaves were also analyzed by **(Vural & Akay, 2021),** they found that olive essential oil has remarkable antibacterial activity towards*B.cereus* ,*E.faecalis*,*E.coli,P.aeruginosa, S.aureus as* well as founding that *K. pneumoniae* has a low sensitivity. **Brahmi *et al.* (2012)** also revealed that the olive essential oil has an efficient antibacterial activity to *E.faecalis*, *S. aureus, E. coli, P. aeruginosa* , while our results are more important than those found by **Boukhebti *et al.* (2015)** for *P.aeruginosa, S. aureus* ATCC 25923*, B. subtilis* and *Escherichia coli* ATCC 25922 which reported that *E. coli* and *B. subtilis* are the most resistant to the essential oil.

Our findings indicate that *Olea europaea* subsp. *laperrinei* essential oils are superior to those of *Olea europaea* L.var. sylvestris,primarily owing to their abundance in monoterpenes, which account approximately 70% of total oil notably α-pinene which appears in greater amounts in the Saharan subspecies than in the other subspecies,due to α-pinene broad potential in antimicrobial therapy,which can be used either alone or in combination with other antibiotics to prevent the growth of bacteria , they are also bacteriostatic and bactericidal **(Borges *et al.*, 2022)**. Furthermore , this substance causes the lipid layer of bacterial plasma membranes to be perturbed, which changes the permeability of the membrane and allows intracellular materials to leak out **(Trombetta *et al.*,2005).**

IV- Extra virgin olive oil extraction and the evaluation of their antibacterial activity

IV-1- Extra virgin olive oil yield

Extraction of extra virgin olive oils from the two wild olives using the traditional method gave green dark colored oil from *Olea europaea* L.var.sylvestris, in contrast the *Olea europaea* subsp. *laperrinei* generated yellowish oil, with twice the yield of *Olea europaea* L.var.sylvestris despite the same quantity of fruits from the two wild olives were extracted **(Figure 44).**

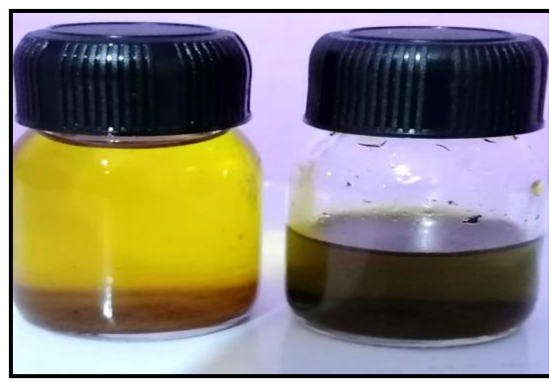


Figure 44: The extra virgin olive oils generated from the two wild olives.

IV-2-Chemicals composition of two extra virgin olive oils (EVOOs)

Based on the chromatographic analysis results of the two extra virgin olive oils (EVOOs). 22 components from *Olea europaea* L.var. sylvestris oil were detected; these components represent an average of 98.61% of the total oil,whereas14 components were identified in *Olea europaea* Subsp. *laperrinei* oil making up an overall percentage of 98.56%. Table 24 summarizes the identified compounds and their relative according to their abundances in their order of appearance.

IV-2-1- Chemical profile of *Olea europaea* L.var. sylvestris EVOO

The predominant component of *Olea europaea* L.var. sylvestris EVOO, was squalene, which had a rate of 27.87%, followed by oleic acid (17.27%), palmitic acid (14.31%), linoleic acid (6.87%). Other low concentration compounds were detected including apiole (2.81%), tyrosole (2.45%) and stearic acid (1.19%) **(Table 24).**

IV-2-2- Chemical profile of *Olea europaea* Subsp. *laperrinei* EVOO

The chemical composition of EVOO obtained from *Olea europaea* subsp. *laperrinei*, the most prevalent component was identified was palmitic acid, which accounted for 27.98% of the total oil. Linoleic acid (25.99 %), oleic acid (17.43 %), squalene (13.43%), β–sitosterol (5.66%), were the next most dominant components. The remaining constituents, such as palmitoleic acid (2.61%), stearic acid (1.77%), lauric acid (0.24 %) and myristic acid (0.23%) were detected in trace amounts **(Table 24).**

Table 24 : Chemical composition of extra virgin olive oils extracted from *Olea europaea* var. sylvestris and *Olea europaea* subsp. *laperrinei*

***Olea europaea* L .var. sylvestris**					***Olea europaea* subsp. *laperrinei***			
N°	**TR**	**Compounds**	**Chemical Formula**	**%**	**TR**	**Compounds**	**Chemical Formula**	**%**
1	5.981	Tyrosole	C8H10O2	2.45	6.616	Dillapiole	C12H14O4	0.18
2	6.228	Allyltetramethoxy benzene	C13H18O4	0.49	7.022	8-Heptadecene	C17H34	0.08
3	6.275	Spathulenol	C15H24O	0.55	9.716	Myristic acid	*C14:0*	0.23
4	6.610	Apiole	C12H14O4	2.81	12.198	n-Pentadecanoic acid	C15:0	0.11
5	8.416	Hydroxytyrosol	C8H10O3	1.19	14.551	Glucose	C6H12O6	0.19
6	9.116	D-Ribofuranose	C5H10O5	0.76	14.998	Palmitoleic acid	C16:1	2.61
7	9.328	D-Fructose	C6H12O6	2.15	15.8	**Palmitic Acid**	C16:0	**27.98**
8	9.669	Sorbopyranose	C6H12O6	1.42	23.76	**Linoleic acid**	C18:2	**25.99**
9	10.19	Glucofuranose	C6H12O6	1.54	24.4	**Oleic acid**	C18:1	**17.43**
10	10.61	Nd	-	1.1	25.15	Stearic acid	C18:0	1.77
11	11.45	α-DMannopyranose	C6H12O6	2.88	35.49	Lauric acid	C12:0	0.24
12	11.55	d-Xylose	C5H10O5	1.71	35.491	nd		2.66
13	11.76	Glucopyranose	C6H12O6	1.01	38.52	**β –Sitosterol**	C29H50O	5.66
14	14.52	Glucose	C6H12O6	3.35	39.596	**Squalene**	*C30H50*	**13.43**
15	15.5	**Palmitic Acid**	C16:0	**14.31**		-		-
16	18.104	Nd	-	5.84		-		-
17	23.76	**Linoleic acid**	C18:2	6.87		-		-
18	24.09	**Oleic acid**	C18:1	**17.27**		-		-
19	25.009	Stearic *acid*	C18:0	1.19		-		-
20	26.78	Tetracosane	C24H50	1.06		-		-
21	32.444	Pentacosane	C25H52	0.79		-		-
22	39.520	**Squalene**	*C30H50*	**27.87**		-		-
∑				**98.61%**				**98.56%**

IV-2-3- Different fractions of the two EVOOs

The different fractions of the two EVOOs extracted from *Olea europaea* L .var. sylvestris and *Olea europaea* subsp. *laperrinei* are listed in **(Table 25).**

The saponifiable fraction is the predominant fraction of the identified compounds of *Olea europaea* subsp.*laperrinei* EVOO contains a total of 76.36%, and within this fraction unsaturated fatty acids (UFA) represent a larger proportion (46.03%) than to saturated fatty acids (SFA) (30.33%). In contrast, the unsaponifiable fraction had the lowest amount of compounds (19.54%), with hydrocarbons and sterols being the most prevalent classes at of 13.51% and 5.66% respectively. On the other hand, EVOO from *Olea europaea* var. sylvestris showed that the unsaponifiable fraction represented the main fraction (52.03%) with hydrocarbons as a dominant class (30.21%), while the saponifiable fraction had lower amounts with a rate of 39.64%, in which the unsaturated fatty acids had a higher level (31.58%) than the saturated fatty acids (8.06%).

According to these findings *Olea europaea* subsp. *laperrinei* contained higher amounts of UFA and SFA than *Olea europaea* L .var. sylvestris. However, the latter poses a high level of other minor compounds, such as hydrocarbons, phenolic compounds, and volatile compounds with the exception of sterols which are absent.

Table 25: Fractions of the two EVOOs extracted from *Olea europaea* L .var. sylvestris and *Olea europaea* subsp. *Laperrinei*

Fractions	Saponifiable fraction		Unsaponifiable fraction				
	Saturated fatty acids	Unsaturated fatty acids	Hydrocarbons	Phenolic compounds	Sterols	Volatile compound s	Other minor compounds
***Olea europaea* var. sylvestris**	8 .06%	31.58%	30.21%	3 .64%	0%	3.36%	14.82%
∑	39 .64%		52.03%				
Olea europaea* subsp. *Laperrinei	30 .33%	46.03%	13.51%	0%	5.66 %	0.18%	0.19%
∑	76.36%		19.54%				

IV-2- Evaluation of the antibacterial activity of the two EVOO

Using disc diffusion essays, the antibacterial activity of the two EVOOs was assessed in vitro by measuring the mean growth inhibition zone diameters (mm) of the seven bacterial strains at various EVOO doses. The antibiotic gentamicin was used as a positive control in this study **(Table 26).**

The results indicate that the effect of the two EVOOs on positive bacteria is greater than that on negative bacteria. In addition, the EVOO from *Olea europaea* subsp.*laperrinei* exhibited the highest range of inhibition among all bacterial strains tested compared with to other EVOO. S. aureus (MRSA), *P.aeruginosa, S. aureus* and *B.subtilis* are the sensitive straines to

the two EVOO.In which *Olea europaea* L.var.sylvestris shows a smaller inhibition diameters range compared to the other one, with inhibition diameters varying between (15.33±0.57; 14.66±1.55; 14±1.00; 12.66±0.57) respectively. Furthermore *E.coli* ATCC exhibited a moderate sensitivity to the *Olea europaea* subsp. *laperrinei* EVOO and no effect was observed with the other EVOO. However, *K. pneumonia* and *E.faecalis* was extremely resistant to the twoEVOO. The greatest activity for all bacterial strains tested was observed at pure doses of both EVOOs compared the other dilution.

Table 26: Inhibition diameter zones (mm) of the two EVOOs from *Olea europaea* L.sylvestris and *Olea europaea* subsp. *laperrinie* oils.

	***Olea europaea* L.var. sylvestris**				***Olea europea* subsp. *laperrinei***				
Concentration (W/W)	**Pure**	**1/2 1/10**	**1/4**		**Pure**	**1/2**	**1/4**	**1/10**	**Gentamicin**
S. aureus ATC C 43300 (MRS A)	13.33 ±1.15	10.66±0.57	9.33±0.57	0±0.0	15.33±0.57	11.66±0.57	12.33±0.58	0±0.0	24±1.00
***S. aureus* ATCC25923**	13.33±0.57	10±0.00	0±0.0	0±0.0	14±1.00	11.66±1.53	11.33±1.16	10.66±0.57	22±1.00
B.subtilis ATCC6633	11±1.00	9±1.00	0±0.0	0±0.0	12.66±0.57	11.33±1.52	0±0.00	0±0.0	30±1.00
E.coli ATCC 25922	7.66±0.57	0±0.0	0±0.0	0±0.0	9.77±0.57	0±0.0	0±0.0	0±0.0	22±1.00
***K. pneumonia* ATCC700603**	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	25±1.00
***P.aeruginosa* ATCC 27853**	13±1.00	11.33±0.57	0±0.0	0±0.0	14.66±1.55	11.66±0.57	0±0.0	0±0.0	27±1.00
***E.faecalis* ATCC 51299**	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	21±1.00

Ⅳ-2-1- Analysis of variance (ANOVA) of antibacterial activity of the two EVOOs

ANOVA was used to analysis performed on the antibacterial activity data. The statistical analysis revealed a highly significant ($P < 0.001$) interaction between the two EVOO, bacterial strains and the doses **(Table 27).**

Table 27: Main effects and interactions between the two EVOOs.

Sources	Df	MS	F	P
Main Effects				
Oils	61.88571429	61.885714	141.26087	.0000 ***
Doses	14982.02857	3745.5071	8549.5272	.0000 ***
Bacteria	1960	326.66667	745.65217	.0000 ***
Interaction				
Doses* oils	4 25.59047619	6.397619	14.603261	.0000 ***
Bacteria* oils	6 128.9142857	21.485714	49.043478	.0000 ***
Bacteria* doses	24 1904.571429	79.357143	181.1413	.0000 ***
Bacteria* doses* oils	24 193.9428571	8.0809524	18.445652	.0000 ***

Notes:*Significant at P <0.05;**Significant at P <0.01;***** Significant at P < 0.001.**

The statistical analysis revealed significant variation in bacterial sensitivity. According to their extreme sensitivity to the two essential oils investigated and their higher mean inhibition diameter (12.06 mm), followed by, the other strains with a higher sensivity such as *S. aureus* (MRSA) which classified in the category "a", and *S. aureus* strain categorized in group "b", then *P.aeruginosa* and *B.subtilis* were classified in the same category "c".

E. coli, K. pneumonia, and *E. faecalis,* were categorized in ranges "d","e" and "f" respectively, which are the resistant strains to both *Olea europaea* L .var. sylvestris and *Olea europaea* subsp. *laperrinei* EVOOs **(Table 28).**

Table 28: Sensitivity of the two EVOOs on all bacterial strains tested

Rank	Bacteria	Mean(mm)	n	Significant ranges
1	*S. aureus* ATCC 43300 (MRSA)	12.06	30	a
2	*S. aureus* ATCC 25923	11.47	30	b
3	*P.aeruginosa*	10.47	30	c
4	*B.subtilis*	10.4	30	c
5	*E. coli* ATCC	6.13	30	d
6	*K. pneumonia*	5	30	e
7	*E.faecalis*	4.2	30	f

Note: LSD 0.05 = 0.33

The statistical analysis of the effects of EVOO dilutions and antibiotic on the inhibitory zone of bacterial tested revealed that category "a" contains the antibiotic Gentamicin had the most powerful antibacterial activity, followed by class "b" of pure EVOO with moderate activity ,the sensitivity of the bacteria decreases with increasing dilution, therefore 1/2, 1/4and 1/10 dilutions had minimal antimicrobial activity **(Table 29).**

Table 29: EVOOs dilutions and antibiotic effects on the inhibitory zone of tested bacteria

Rank	DOSES	Mean (mm)	n	Significant ranges
1	Gentamicin	24.43	42	a
2	pure	8.90	42	b
3	1/2	6.21	42	c
4	1/4	2.36	42	d
5	1/10	0.76	42	e

Note: LSD 0.05 = 0.28

Concerning antibacterial potential, for all evaluated bacterial strains, EVOO extracted from the Saharan population had a greater potential for growth bacterial inhibition than the EVOO extracted from the Northern population (**Table 30).**

Table 30 : Effectiveness of two EVOOs from several sampling locations

Rank	Station	Mean	N	Significant ranges
1	EVOO of *Olea europea* L.sylvestris	9.08	105	a
2	EVOO *Olea europea* subsp.*laperrinie*	7.99	105	b

Note: LSD 0.05 = 0.18

IV-3-Discussion

Limited knowledge of the chemical composition and antibacterial potential of wild olive oils either in Algeria or worldwide has been found, only some studies have been conducted on *Olea europaea* L. var. sylvestris, but no research has been done on the endemic subspecies *Olea europaea* subsp. *laperrinei*.

Our findings indicated that the two wild olives that were extracted had different colors, green dark colored oil from *Olea europaea* L.var. sylvestris and a yellowish oil from the other. **Jimenez-Lopez *et al.* (2020)** , explained these color differences of the EVOOs by the number of pigments, as increasing the number of pigments elevates the greenish-yellow color and vice versa.

As previously mentioned, unsaturated fatty acids, of which oleic acid is the predominant FA of *Olea europaea* L.var. Sylvestries EVOO, accounted for 17.27%. Oleic acid was followed in order of dominance by palmitic and linoleic acids. Since our research include the percentage of all oil fractions, the results mentioned below have higher fatty acid concentrations because their analysis only evaluated the composition of fatty acids. Our findings were similar to those btained from *Olea europea* L.var. sylvestris oil from different locations in the Mediterranean basin, in that oleic acid was the predominant constituent, but not in terms of their respective amounts, such as in **Baccouri *et al.* (2008)**, who detected that oleic acid was the main FA in VOO from some selected wild olives in Tunisia, with an average of 48.4% to 71.1%. Moreover, **Hannachi *et al.*(2013)**, were found that the main FA of nine Tunisian wild olives was oleic acid and its contents varied from 47.03% to 71.55%. As well as **Bouarroudj *et al.*(2016)**, obtained that the oleic acid was the major FA in four

Algerian oleaster populations originating from Bejaia, with values ranging from 64.7% to 76.1% . Addionally, the oleic acid is the main FA source (67.79%) in both ssp. maroccana and var. sylvestris oils, followed by palmitic acid (18.66%) and linoleic acid (8.20%) (**Elgadi *et al*., 2021**).Similar results were obtained from another investigation, in Northeastern Portugal, with the oleic acid (68.9–70.6%) being the most abundant FA, followed by palmitic acid, (ranging from 14.2% to 15.2%), and linoleic acid (varying from 7.9% to 9.9%) **(Rodrigues *et al*., 2020)**, as well as in the Spanish wild olive, the oleic acid is the major FA (76.41 % to 78.07%) followed by palmitic acid (13.47 % to13.69 %) **(Espínola *et al* ., 2021).**

Numerous researchs have indicated an association between the beneficial effects of olive oil and oleic acid **(Perez-Martinez *et al*., 2011; Kabaran, 2018).** In addition, phenolic compounds, primarily hydroxytyrosol and tyrosol, have a multitude of beneficial effects, such as anti-oxidant activity, antimicrobial, and anti-inflammatory properties ,as well as regulating a variety of signaling molecules involved in inflammation, cell adhesion, growth, apoptosis, and aging, and they are considered cardioprotective and neuroprotective agents **(Flori *et al*., 2018).**

Additionally, unsaturated fatty acids (UFAs) made up the majority of the chemical composition of *Olea europaea* subsp. *laperrinei* EVOO. However, the quantity and variety of FAs in this subspecies' EVOO differ from this of *Olea europaea* L.var. sylvestris, which produces only three FAs (oleic acid, palmitic acid, and linoleic acid). In contrast, many FAs, such as palmitic acid, linoleic acid, oleic acid**,** palmitoleic acid, stearic acid and lauric acid, were detected in *Olea europaea* subsp. *laperrinei* EVOO, with the latter exhibiting higher fatty acids contents in common with the other. This subspecies differs from the other, where oleic acid is one of the dominant FAs but not the dominant one, in this case palmitic acid is the major FA flowed by linoleic acid, then oleic acid. Our results were similar to the results of numerous investigations that were done on the chemical composition of the other subspecies of *Olea europaea* L., which revealed that oleic acid, palmitic acid, and linoleic acid were the major Fas, but did not agree with those later on in terms of the amounts of each one, such as, the oil of *O. europaea* subsp. *cuspidata* from Kenya, oleic acid was found to be the highest at 44.3%, followed by linoleic acid (33.3%) and palmitic acid (12.1%) **(IOC, 2019) . Elgadi *et al*. (2021)**, also found that oleic acid (63.22 to 67.79%) was the main fatty acid, followed by linoleic acid (8.20 to 19.81%) and palmitic acid (10.65 to 19.02%) in *Olea europaea* subsp. *maroccana* oil. Our oil contains high concentrations of linoleic acid, which has been shown in numerous studies to have many health advantages, such as **Ramsden *et al*. (2012),** reported

that vegetable and seed oils containing linoleic acid are the most frequent sources of polyunsaturated fats in the human diet, also noted that linoleic acid is an omega-6 fatty acid which represents a principal component of human tissues. Linoleic acid applied topically is thought to be beneficial for treating melasma patients (**Lee *et al*., 2002).** It has also been linked to a lower risk of hypercholesterolemia **(Ramsden *et al*., 2021)** and atherosclerosis **(Das, 2021).** Additionally it reduces headache pain when used in combination with omega-3 fatty acid supplements **(Mercola &Adamo, 2023).** It has also been demonstrated that omega-6 linoleic acid has a hypolipidemic effect when combined with palmitic acid **(Ahmad & Beg, 2016).**

The two EVOOs investigated generated a greater amount of UFA than SFA, whereas *Olea europaea* subsp. *laperrinei* EVOO, has a more important amount of UFA which represents an average of 46.03% of UFA, than *Olea europaea* L.var. sylvestris (31.58%). Owing to its high concentrations it can be regarded as a source of UFA, and as such, it offers a variety of therapeutic benefits (**Amiot, 2014**), including protection against cardiovascular disorders (**Kabaran, 2018**). Numerous studies have shown that a diet high in unsaturated fat (UFA) raises HDL cholesterol and lowers TG levels, making it useful for treating hypercholesterolemia **(Schwingshackl & Hoffmann, 2012).**

The variability in the chemical profiles of EVOOs between the two wild olives were attributed to a variety of factors, including pedoclimatic conditions and olive variety (**Lombardo *et al*., 2018**), other factors that may have affected the composition but were considered into account in our study include storage conditions, harvest timing and extraction **(Jimenez-Lopez *et al*., 2020).**

Overall, the results of this study clearly show that the two EVOOs have effective antibacterial activity, with the greatest activity observed against grame positive bacteria as compared to grame negative bacteria. Several authors, including **Medina *et al*. (2006)** and **Boukhebti *et al*. (2020)**, have confirmed these findings. According to **Janakat *et al*. (2015),** the resistance of the gram negative to olive oil related to their outer cell (wall) membrane which offered their protection, which gram positive bacteria lack.

As previously stated, *S. aureus* (*MRSA*), *P.aeruginosa, S. aureus* and *B.subtilis* were the sensitive straines, however *K. pneumoniae* and *E. faecalis* were the resistant strains to the two EVOO investigated, the antimicrobial activity olive oil was also analyzed by **Hussain *et al*. (2014),** they found a similar result that *S.aureus, P. aeruginosa* showed a heigh antibacterial

activity and *E.faecalis* as resistant bacteria, while it showed an important antibacterial activity of *K. pneumonia* which was resistant in our results. Furthermore, **Boukhebti *et al.* (2020),** noticed that olive oil has a remarkable antibacterial activity toward *B.subtilis, S. aureus* and *E. coli* as well as *K. pneumonia* and *P.aeruginosa* were the resistant bacteria.

According to our findings, the EVOO extracted from *Olea europaea* subsp. *laperrinei* has the highest potential for inhibition of all bacterial tested, which is mainly due to their abundance of fatty acids which was greater in this oil for 76.36%, than in the other oil, where the majority of them are absent. Many previous studies have reported that FAs are antibacterial agents that cause partial solubilization and membrane fission in order to disrupt bacterial cell membranes **(Yoon *et al.*, 2018).** According to **Desbois &Smith (2010)**, FAs affect oxidative phosphorylation and the electron transport chain. In addition to disrupting the creation of cellular energy, and it caused the limitation of enzyme activity, hinders nutrition uptake, produces harmful peroxidation and auto-oxidation of the breakdown products, or directly lyses bacterium cells.Many other authors confirmed the antibacterial properties of FAs **(Yang *et al.*, 2017; E. Ivanova *et al.*, 2017 ;Kumar *et al.*, 2020; Casillas-Vargas *et al.*, 2021).** Notably palmitic acid, linoleic acid, oleic acids, lauric acid **(Huang *et al.*, 2010 ; Yoon *et al.*, 2018)**. β –Sitosterol is another compound that appeares in *Olea europaea* subsp. *laperrinei* EVOO and is absent in the other, and it has been shown in the literature to have a strong antibacterial potential **(Alawode *et al.*, 2021).**

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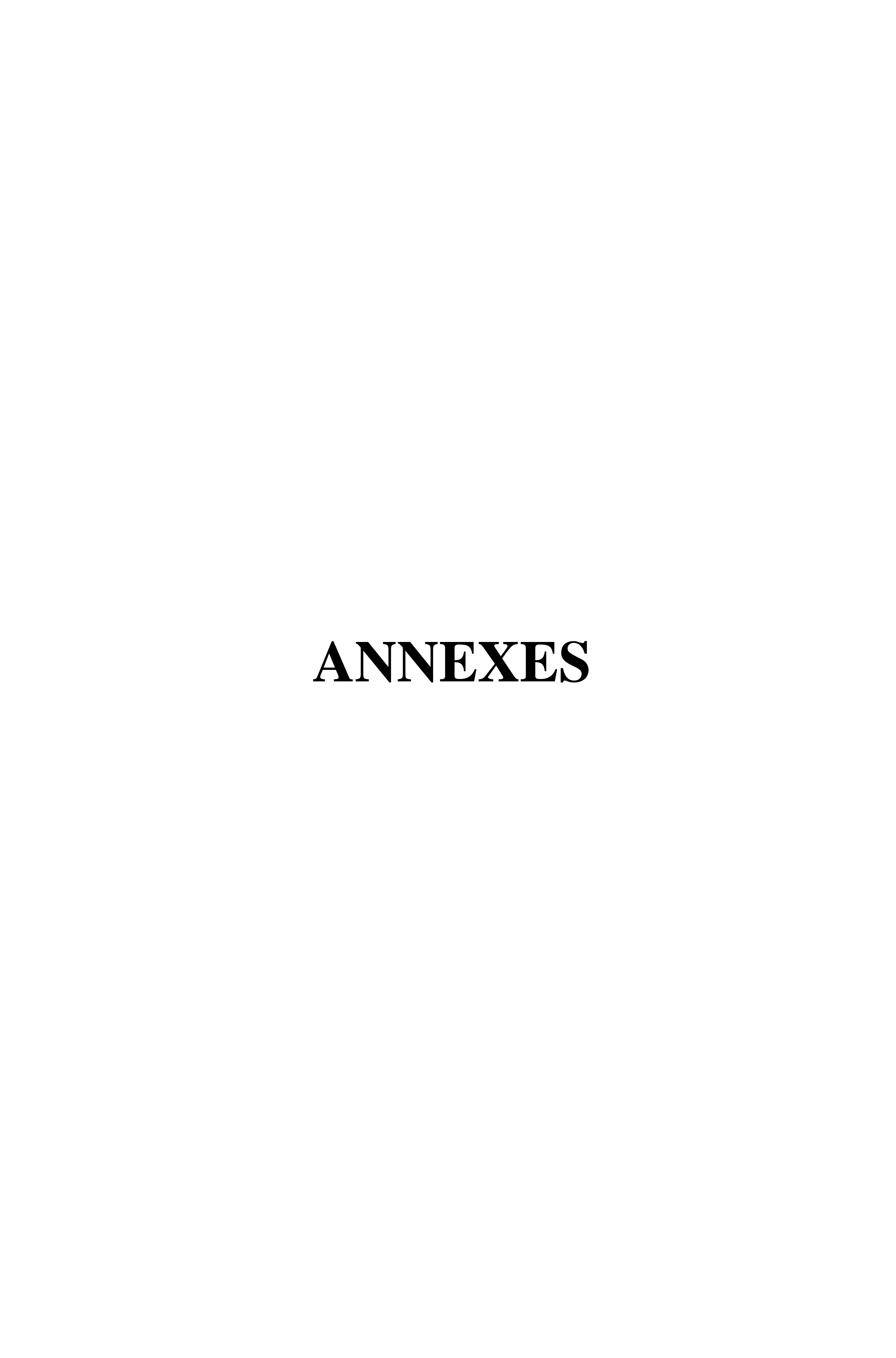
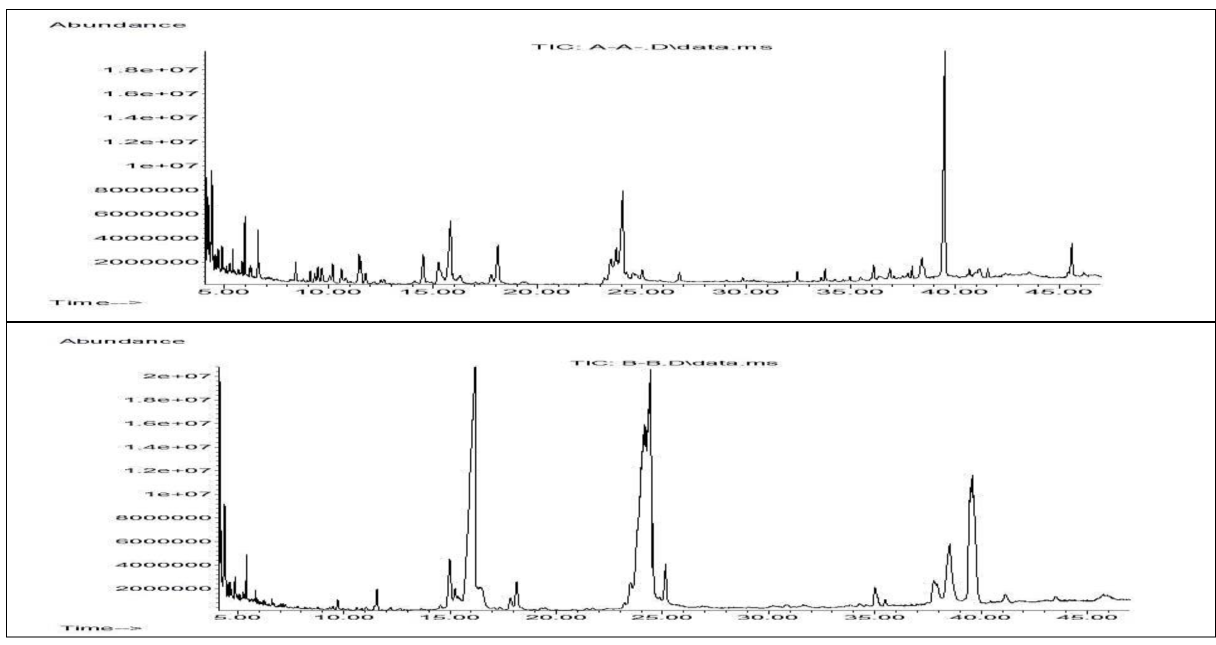
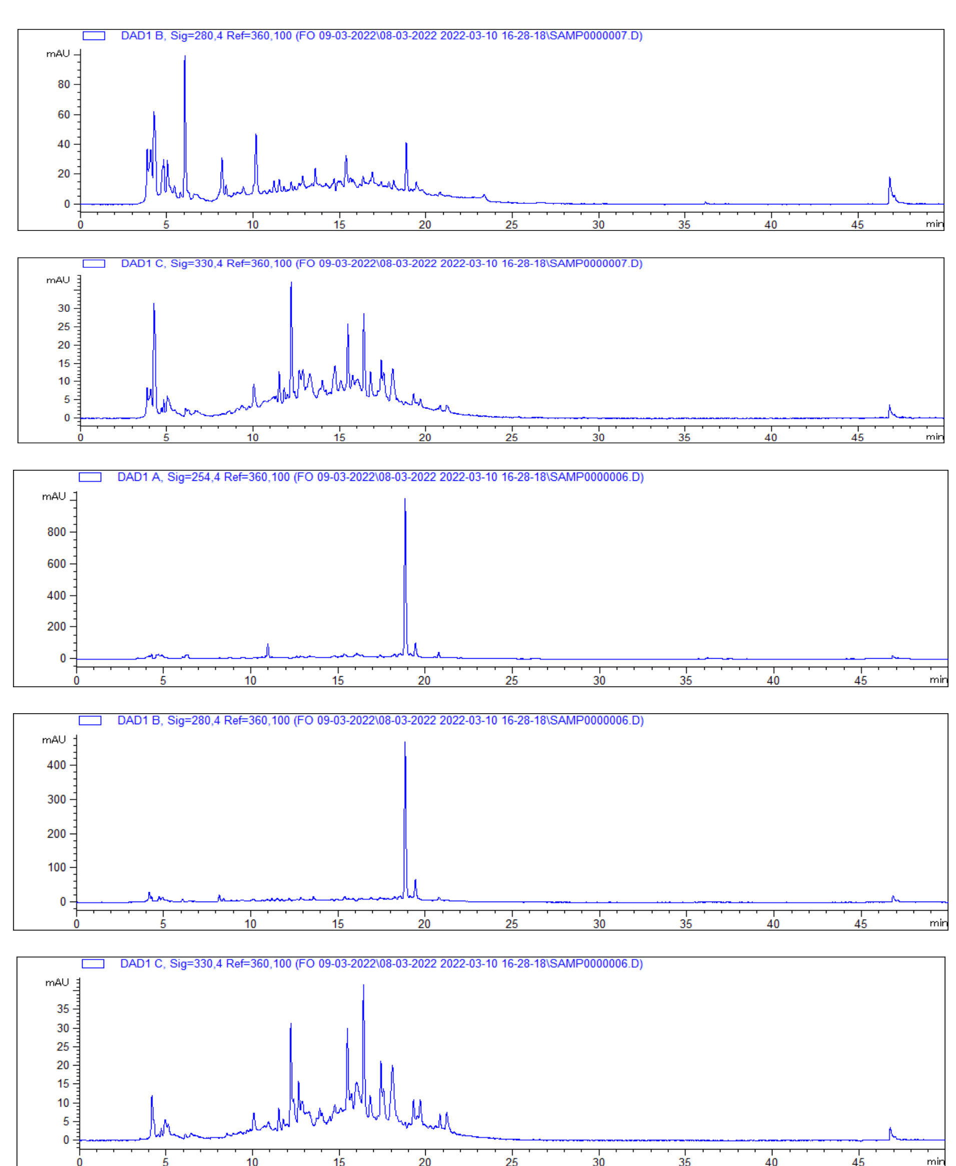
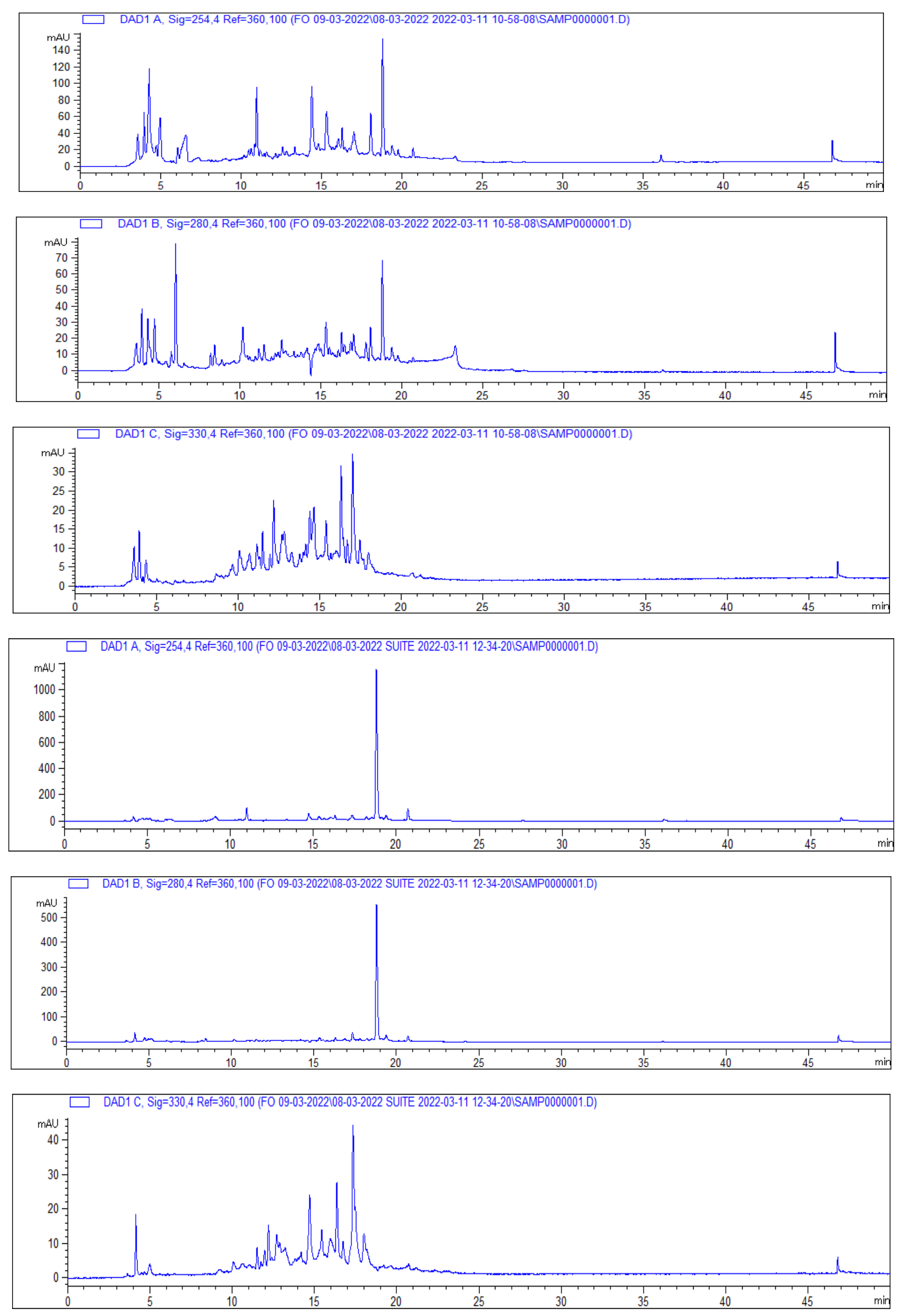


ANNEXES



Annexe 1 : Gas chromatograms of extra virgin olive oils





Annexe 2: HPLC analysis chromatograms of the different extracts .

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Chemical composition and antimicrobial activity of essential oils of two wild olive subspecies *Olea europaea* L.var. sylvestris and the endemic olive *Olea europaea* subsp. *lapperinie* from Algeria

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Chemical composition and antimicrobial activity of essential oils of two wild olive subspecies *Olea europaea* L.var. sylvestris and the endemic olive *Olea europaea* subsp. *lapperinie* from Algeria

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ABSTRACT

Two wild olive subspecies are fixed in this research: *Olea europaea* L.var. sylvestris and *Olea europaea* subsp. *laperrinei* despite its ecological value, the chemical composition of subsp. *laperrinei* oil remains unknown. The samples were harvested from the different geographical area. Gas Chromatography-Mass Spectrometry (GC-MS) and Gas Chromatography-flame-ionization detection (GC-FID) analysis of *Olea europaea* L. var. sylvestris allowed the identification of 29 compounds oil with Nonanal (11.82%), theaspiranea A (9.81%), 3-hexen-1-ol,benzoate(9.31%) as a major constituents, while in the subspecies of the Saharan region 31 compounds were separated, where α-pinene (16%), β-Ocimene (12.82%), dl-Limonene (8.20%) were the main components. The results of the disc diffusion method showed that the two volatile oils have efficient antibacterial activity but, subsp. *laperrinei* essential oil has a higher range of inhibition, in which *P. aeruginosa* and *B. subtilis* showed an extreme sensitivity, while the *K. pneumoniae* bacterium shows a great resistance to the two essential oils.

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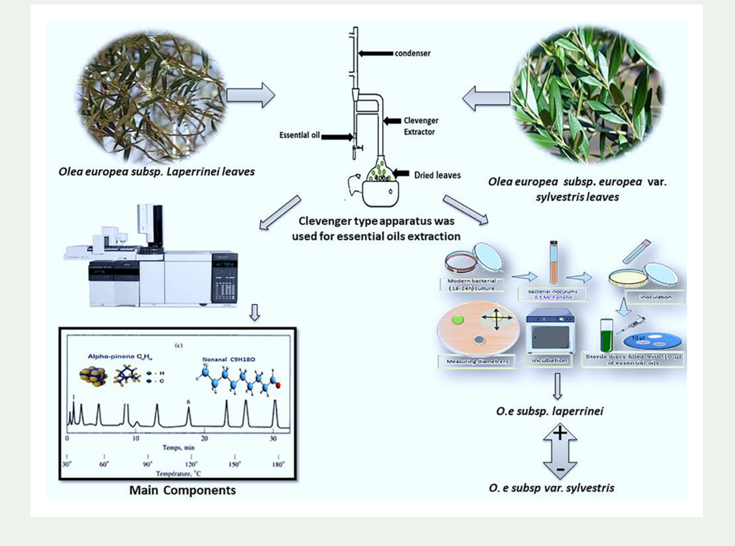

1. Introduction

Olea europaea L. tree, popularly known as the olive tree (Nunes et al. 2016), belongs to the *Oleaceae* family which is native from Mediterranean countries, and is one of the ancient crops in the world (Romero-García et al. 2014), an evergreen tree or shrub that can be found in both cultivated and wild populations all around the Mediterranean region (Branislava et al. 2007). The two principal olive tree products, both olive fruits, and olive oil contain significant amounts of chemicals with health benefits, a low incidence of diseases and health maintenance has been related to the regular consumption of these products (Hashmi et al. 2015), also it has already been utilised as a significant component in shampoos, face and body creams, lotions and soaps (Cicerale et al. 2009).

Predominantly olive fruits and olive oil, but also olive leaves, have been used in traditional medicine to treat cancer, hypertension, arrhythmia and intestinal muscle spasms (Özcan and Matthäus 2017) and supposed to be the source of several phytochemicals which have antioxidant, anti-inflammatory, antibacterial (Borges et al. 2020), antiviral (Micol et al. 2005) and anti-tumor properties (Abaza et al. 2007).

The volatile fractions of olive leaves consist of multiple important chemical constituents which justify their beneficial health effects (Brahmi et al. 2012), the chemical profile of *Olea europeae* L. essential oil has been previously studied and it was found that the chemical composition varied depending on a variety of parameters, including environmental conditions, geographic distribution, cultivar, processing details, storage conditions (Temime et al. 2006a, 2006b), altitude sites (D'Imperio et al. 2007). *Olea europaea* L. essential oil is mostly composed of aldehydes, which Nonanal was present in significant proportions (Campeol et al. 2001, 2003; Ben Abdeljelil et al. 2017). It also contains terpenoids, which α-pinene is the predominant component in this class, while these substances exhibit antimicrobial activity as well as antioxidant and antifungal activity (Brahmi et al. 2012).

Typically, plant essential oils are frequently utilised as synthetic material substitutes (Golestan et al. 2016; Khorshidian et al. 2018), also with broad-spectrum antibacterial properties that are effective against a variety of pathogenic organisms (Upadhyay et al. 2010b). Literature revealed that essential oils of *Olea europaea* L. are greatly effective in inhibiting the activity of both gram-negative and gram-positive bacteria (Lee et al. 2007; Brahmi et al. 2013), this great activity is due to the chemical composition of essential oil (Jallali et al. 2022) such as terpenoids (Djoukeng et al. 2005; Brahmi et al. 2013), whereas, these phytochemicals give it medicinal value, especially in terms of its pharmacological potential (Upadhyay et al. 2010a).

The *Olea europaea* L. complex is the most popular member of the genus Olea in which six subspecies are recognised (Green 2002). In the present research two wild subspecies of *Olea europaea* L. were investigated, including *Olea europaea* subsp. *laperrinei* and *Olea europaea* subsp. *europaea*; this latter was grouped into two forms, one wild (var.sylvestris [Mill.] Lehr) and the other cultivated (var. sativa) (Breton and Bervillé 2012). Due to our objectives which are based on the study of wild forms, the wild variety of this subspecies which is *Olea europaea* subsp. *europaea* var. sylvestris has been fixed, while actually known as *Olea europaea* L. var. sylvestris, harvested from the northern region and *Olea europaea* subsp. *laperrinei*, an endemic subspecies taken from the central Sahara of Algeria.To the best of our knowledge, the present paper is the first to evaluate the chemical composition and the antimicrobial potential of essential oils from these two wild species in Algeria. Only reports on cultivated olive have been found, they focus on Italian cultivars (Campeol et al. 2001), Tunisian (Haloui et al. 2010), Turkish (Vural and Akay 2021), Indian (Upadhyay 2014) and Algerian (Boukhebti et al. 2015; Tlili et al. 2021).

In this respect, the present study aims to investigate and compare the chemical composition and antimicrobial potential between the two wild subspecies, in different environmental conditions.

2. Results and discussion

2.1. Chemical composition of volatile oils

The Hydro-distillation of the aerial parts of the two specimens has generated a liquid with a greenish colour.The dried leaves of *Olea europaea* L.var. sylvestris and *Olea europaea* subsp. *laperrinei* gave 0.17% and 0.19% (w/w) of yields respectively. The GC-MS and GC-FID analysis was used to determine the volatile oil composition, the chemical composition of the studied samples is reported in supplementary material (Tables S1 and S2), 29 components were identified by representing 93.54% of var. sylvestris oil, while subsp. *laperrinei* essential oil allowed the identification of 31 components, corresponding to 93.33% of the total essential oil.

The most abundant constituents of var. sylvestris essential oil was determined to be nonanal the predominant component with a rate of 11.82%, followed by theaspiranea A (9.81%) and 3-hexen-1-ol,benzoate (9.31%). Other compounds were found in minimal percentages, such as β-ocimene (4.20%) and dl-limonene (3.56%). Monoterpene Hydrocarbons (22.75%), Norisoprenoids (19.94%) and Aldehyde (14.88%) were the dominating groups of compounds in this essential oil; however, Oxygenated

Monoterpenes (10.08%), Oxygenated sesquiterpenes (7.51%) and Sesquiterpene Hydrocarbons (0.98%) were the last components of the essential oil.

Concerning the chemical profile of the volatile fraction from subsp. *laperrinei*, α-pinene represent the main component with a percentage of 16%, then ranks secondly β-Ocimene (12.82%), dl-Limonene(8.20%) and Nonanal (8.18%), the other constituents were detected in trace amounts including p-Cymene(2.3%). This essential oil is rich in Monoterpene Hydrocarbons (55.74%), Aldehydes (12.82%), Oxygenated Monoterpenes (10.47%) and Oxygenated sesquiterpenes which represent a rate of (06.32%).

The data obtained from this study revealed that the essential oils from the two subspecies produced a significant yield. This yield is similar to those obtained from Tunisien Chemlali cultivar's dried leaves (Brahmi et al. 2012); another study showed that the fresh leaves essential oil yield, was inferior to those obtained from the dried leaves (Brahmi et al. 2012; Boukhebti et al. 2015).

The Results of the phytochemical profile of *Olea europaea* L.var. sylvestris essential oil showed that a nonanal is the most abundant constituent of this oil; this result is in accordance with the results obtained in *Olea europaea* L. essential oil from Chemchali Tunisian cultivars (Brahmi et al. 2012, 2013); also, in Chetoui Tunisian variety (Brahmi et al. 2015), in addition, from Italian cultivars (Campeol et al. 2001, 2003; Popović et al. 2021). Nonanal was identified as the compound responsible for the antidiarrhoeal activity (Miguel et al. 2002; Medina et al. 2006).

The main constituent of the volatile fraction from *Olea europaea* subsp. *laperrinei* leaves were α-pinene, this data obtained is similar to Haloui et al. (2010) in the Tunisian olive essential oil. Many authors have demonstrated that α-pinene has insecticidal, spasmolytic, antibacterial, and anticholinesterase properties (Lee et al. 2001; Savelev et al. 2003). This substance was also discovered to have anti stress potential (Akutsu et al. 2002). However the results obtained are not according to Boukhebti et al. (2015). These variations of the chemical profile of *Olea europeae* L. essential oil are related to the different environmental conditions such as geographic distribution, cultivar, processing details, storage conditions (Temime et al. 2006a, 2006b), altitude sites (D'Imperio et al. 2007).

2.2. Antibacterial activity

Disc diffusion essays were performed with the two essential oils to measure growth inhibition zone diameters at different concentrations with all bacteria strains (Supplementary material, Table S3).The results show that the essential oil of subsp. *laperrinei* has represented the higher range of inhibition zone diameter from all strains tested, *P. aeruginosa* and *B. subtilis* showed extreme sensitivity and higher for the latter than var. sylvestris oil with inhibition diameters varying between 20.33 ± 0.58 mm and 17.67 ± 1.15 mm, *E. coli, S. aureus* ATCC 43300*, E faecalis,* and *S. aureus* ATCC 25923 are moderately sensitive to the two essential oils; while the *K. pneumoniae* bacterium shows great resistance to the two essential oils. For all bacterial strains examined the highest activity was shown at a 1/2 dilution of the two oils than the other doses.

According to Statistical analysis, the interaction between the doses, the essential oils, and the bacterial strains were very highly significant ($p < 0.001$) (Supplementary material, Table S4). It also showed a significant variation in bacterial sensitivity (Supplementary material, Table S5). *B. subtilis* bacteria classified in the category 'a' is very sensitive to the two essential oils with an average inhibition diameter of 17, 54 mm followed by *P. aeruginosa* which is classified in group 'b' with a higher sensitivity, *E. coli, S. aureus* ATCC 43300*, E. faecalis*, and *S. aureus* classified in range b, c, de, e respectively with an average sensitivity to both essential oils, while the *K. pneumonia* was listed as category (f), representing the resistant strain. The antibiotics gentamicin classified in category 'a' with significant antibacterial activity followed by ½ of diluted essential oils, while, with the diluted essential oils (1/4 and 1/10), there was a limited antimicrobial activity (Supplementary material, Table S6), the inhibition of bacterial growth is more important for the essential oil obtained from the Saharan population than extracted from the Northern population on all bacterial strains tested (Supplementary material, Table S7).

In various bioessays, *Olea europaea* L. essential oil has demonstrated greater anti-bacterial activity *in vitro*. Related to the higher concentration of volatile components (Medina et al. 2006) and at room temperature, a high susceptibility to both Gram-positive and Gram-negative bacteria was shown by the olive essential oil, additionally, increase plasma membrane permeability, which causes greater fluid loss from bacterial cells (Chang et al. 2001), also it may reduce microbial respiration (Lee et al. 2001). The results of this study revealed that the two volatile oils have efficient antibacterial activity towards Gram-positive and Gram-negative bacteria strains, the antimicrobial activity olive leaves essential oil were also analysed by many authors and showed a remarkable antibacterial activity (Brahmi et al. 2012; Boukhebti et al. 2015; Vural and Akay 2021).

3. Experimental section

See supplemental material.

4. Conclusion

The chemical composition and antimicrobial activities of the essential oil obtained from the two wild subspecies were tested for the first time in this research, the clear distinction was recorded on chemical composition and antimicrobial activity between the two subspecies studied, In fact, a significant difference was found in both chemical compositions and antimicrobial activity of the two oils. Furthermore, var. sylvestris represents a lower number of chemical constituents with nonanal the most abundant, on the other hand, subsp. *laperrinei* represents the most number of chemical constituents with α-pinene as the major constituent; the richness of Saharan essential oil, that gave a high potential of antibacterial activity against all tested bacterial strains comparing with var. sylvestris essential oil, it can also be deduced that there is a variations in the chemical compositions of the produced volatile oils, in particular the number of constituents as well as the abundance of the major components

between the two subspecies studied. Therefore, environmental and climatic conditions have been shown a significant influence on the synthesis of main chemicals.

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Author contributions

Collecting plant samples SB;HB;AO; the extraction of the essential oils and the Antibacterial activity was carried out by SB and YM; the GC-MS and GC-FID analyses and treatment of results were performed by SB, HB, AO and NCA; writing of the original draft by SB, writing, review and editing by SB, HB, AO, NCA and YM . All authors revised and agreed the manuscript before submission.

Disclosure statement

The authors report that they have no conflicts of interest.

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Original article

Identification of bioactive compounds and determination of totalphenolic and flavonoid contents in leaf extracts originated from the Algerian desert *Olea europaea* subsp. *laperrinei* and *Olea europaea* subsp. *europaea* var. *Sylvestris* and evaluation of their potential as antioxidants

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Abstract. *Background and aim:* since ancient times, *Olea europaea* L. Notably, the olive leaf has been used in ethnopharmacology to treat fevers and malaria. Currently, this aerial part aroused the interest of researchers around the world in the fields of medicine and pharmacology due to their beneficial effects on human health, including anti-hypertensive hypoglycemic, hypocholesterol, antimicrobial properties, as well as utilized to prevent Alzheimer's disease and to provide protection from colon, breast, and ovarian cancers. Additionally,has a great antioxidant potential due to their high phenolic content.Despite the knowledge of bioactivities of olives that have been frequently reported, the majority of them were related to cultivated olives, whereas wild olives are even less recognized or unknown like our case subsp. *laperrinei*. The purpose of this work was to realize a comparative evaluation of the phytochemical profile, total phenolic, and flavonoids contents, as well as the antioxidant potential of both aqueous and methanolic extract from two subspecies such as *Olea europea* subsp.*laperrinei* from Sahara and *Olea europaea* var. *sylvestris* from north of Algeria has been done. In order to understand how environmental stress exerted on these wild species affects the capacity to synthesizesecondarymetabolites as well as antioxidant potential. *Methods:* Chromatography liquid with High-performance (HPLC) was used to identify and quantify the constituents of subspecies, the total phenolic and flavonoids content in the extracts was determined using Folin-Ciocalteu and spectrophotometric method respectively, and the antioxidant activity was analyzed in vitro using DPPH scavenging method. *Results*: HPLC analysis showed that Oleuropein is the main compound in all extracts in which the Saharan extracts showed a height level (276.157 mg/g), furthermore, all the extracts obtained showed reasonably high total phenolic and flavonoid contents and good radical scavenging activity notably those from subsp.*laperrinei* were more important. Furthermore, the highest values were obtained using methanol as solvents than water. *Conclusions:* The results also showed that wild olives have very high antioxidant potentials and it could be deduced that their leaves can under stress conditions increase the synthesis of bioactive as in the case of subsp. *laperrinei* compared to var. *sylvestris* from no stressful condition. Also, demonstrate the value of wild olive leaves as a natural antioxidant.

Key words: *Olea europaea* L, subsp. *laperrinei*, var. *sylvestris*, phytochemical profile, total phenolic, flavonoids contents, antioxidant potential

Introduction

Medicinal plants are the main source of medicines, due to the abundance of what is known as secondary metabolites (1). For this reason, people all around the world, turn to traditional medicine as an alternative to modern therapies. In which the pharmaceutical industry demonstrates that natural products continue to be a very valuable source for the production of new complex organic molecules that frequently exhibit pharmacological properties, such sources are becoming more and more important (2,3). In Algeria, many plants are traditionally used to treat many diseases, among these plants is *Olea europaea* L. (1,4,5) which is included in the *Oleaceae* family (6), it is one of the most essential fruit trees in Mediterranean regions, it is frequently employed in traditional medicine (7,8).

Olive leaves are a copious by-product generated by the olive oil industry and olive tree pruning (6). These aerial parts are currently attracting growing attention in the vast field of medicine and pharmacology (9), due to their high phenolic contents, olive leaf extract becomes one of the most effective sources of plant polyphenols with high antioxidant potential (10). Typically, the Phenolic compounds in olive leaves are numerous and of diverse nature, which the major groups of phytochemicals in *Olea europaea* L. leave extracts such as the phenolic compounds (11) and polyphenolic compounds (secoiridoids and flavonoids) (10,12).

In effect, the active constituent and frequently reported of olive leaf extract is Oleuropein a secoiridoid with great antioxidant activity in vitro (13). The health-promoting properties of this compound have been extensively studied (14), principally for their antioxidant properties and therapeutic benefits such as antimicrobial and antiproliferative activities (15,16), furthermore Flavonoids are a widely distributed group of polyphenol compounds that are recognized as antioxidants in diverse biological systems (17), it has possessed anti-inflammatory, anti-allergic, antiviral and antiproliferative activities (18,19), as well as hypoglycemic properties (20), however, the variability in qualitative and quantitative of total phenolic compounds and evidently the magnitude of the antioxidant capacity of olive leaves extracts might depend on several factors mainly rests upon the environmental conditions (21), the olive cultivars/varieties analyzed (22,23), as well as the extraction method/solvent (22-24).

Free radicals are a major contributor to the emergence of different human diseases, including cancer, Alzheimer's disease, neurological disorders, cardiac reperfusion… ect (25), in this context using synthetic antioxidants is a necessity for reducing oxidative stress but the extensive use of these additives in the food industry, exhibit genotoxic, carcinogenic effects (26,27) and hemorrhaging (28). In contrast, using natural antioxidants derived from plants does not induce side effects (28, 29) and has greater advantages over using synthetic antioxidants (30). As a result, it has become a necessity for the pharmaceutical and cosmeceuticals industry the shift towards natural products (31).

Olive leaves are classed as a source of several antioxidants (32), for the purpose of valorization of wild olive leaves as cheap and natural antioxidants we conducted this study, the current study was carried out to comparatively analyzed, the total phenolic and flavonoid content, to identify and quantify some of them by HPLC method, as well as to evaluate the antioxidant activity of both aqueous and methanolic leaves extracts of two wild subspecies of *Olea europaea* L. which belongs to two different bioclimatic levels such as *Olea europaea* subsp. *europaea* var. *sylvestris* from the northern region is characterized by a Mediterranean climate with dry summers and wet winters and *Olea europaea* subsp. *laperrinei* an endemic subspecies from the Saharan region in Algeria has a hyperarid climate with summers that are exceptionally long, and with a very low precipitation rate, In order to explain how environmental conditions affect the plant's capacity to synthesize secondary metabolites.

In light of our knowledge, only a few research have been made on the chemical profile and the antioxidant of leaves extracts issues from wild subspecies of *Olea europaea* L., furthermore no data were also provided on the antioxidant activity and the amount of total phenolic and flavonoid compounds of the Saharan endemic subspecies "*Olea europea* subsp. *laperrinei*", as well as is the first to investigate the comparative evaluation between two wild olive subspecies in Algeria.

Materials and methods

Sources and processing of plant material

The aerial parts of two wild species were collected in February 2021, were collected at two locations: *Olea europaea* subsp. *europaea* var. *sylvestris* was harvested from the northern region in Algeria "Setif, Oued El bared" (36° 37 N, 05° 40; 814 m), while *Olea europaea* subsp. *laperrinei* was collected from massifs of Hogar which are located in "central Algerian Sahara Tamanrasset (23°21N, 05°47 E; 1952m), (Figure 1) and (Figure 2).

The samples were identified at Laboratory of (L.V.R.B.N), University of Setif 1. Voucher specimens were deposited in the herbarium of the Department of Ecology and Biology, Setif University, Algeria. Aerial parts of the plant material were dried at ambient temperature under obscurity.

Determination of plant extract yield

The yield of dried extracts based on a dry weight basis as obtained from 5g of the leaves was calculated from the equation (A):

$$yield\% = \frac{W1(g)}{W2(g)} * 100 \ldots\ldots\ldots\ldots \text{(A) (33)}$$

W1: the weight of the extract after the solvent evaporation in grams

W2: the weight of the dry plant material in grams.

Preparation of aqueous extracts

The extraction from plants is an important step in the separation of medicinally active portions notably, bioactive constituents using selective solvents through standard procedures (31, 34), in which maceration is a technique widely used in medicinal plants research (34), However has been suggested by Vongsaka et *al*. (35) as more applicable, compared to other modern extraction methods, is known as the "Green method" (36). In our study water extract was obtained by maceration (modify (too according to Bougandoura et *al*. (37) method. 5g of dried leaves of each plant were extracted with 100ml of distilled water for 24 hours at room temperature (around 20 °C), The whole is filtered through filter paper N° 1 in order to separate the grounds from the filtrate. The aqueous extract was

Figure 1. Olive tree of *Olea europaea* subsp. *laperrinei* and *Olea europaea* subsp. *europaea* var. *sylvestris*. (a. Tree of *Olea europaea* subsp. *laperrinei*; b. Tree of *Olea europaea* subsp. *europaea* var. *sylvestris*).

Figure 2. Olive tree branch with leaves of *Olea europaea* subsp. *laperrinei* and *Olea europaea* subsp. *europaea* var. *sylvestris*. (a. leaves of *Olea europaea* subsp. *laperrinei*; b. leaves of *Olea europaea* subsp. *europaea* var. *sylvestris*)

powdered by lyophilization and stored at refrigeration (4°C) until analysis.

Preparation of methanolic extract

The areal parts of the samples were cut into very small pieces and macerated in 80 %methanol for 24 and 48 hours at laboratory temperature. The ratio of dried plant to solvent is 1:10 w/v. The extract was collected by filtration and evaporated to dryness under a vacuum (38). The dry extract was conserved at -18 °C until used.

Determination of total phenolic content

The total phenolic content of plants extracts was determined spectrophotometrically according to Foline-Ciocalteu method (39). A volume of 0.5 Ml of 50% Folin-Ciocalteu reagent was mixed in a test tube containing 0.1mL of the extract and homogenized. The resultant mixture was allowed to react for 1 min and 1.5 mL of 20% Na2CO3 was added. It was mixed thoroughly and incubated in the dark for 2 h and then the absorbance was recorded at 760 nm using a visible light spectrophotometer (Spectronic 20 genesys TM). A Gallic acid was used as a standard for the calibration curve. The total phenolic compounds content was expressed as mg equivalent of Gallic acid per gram of extract (mg GAE/gE).

Determination of total flavonoids content

Total flavonoids content was determined using a spectrophotometric method based on the formation of flavonoid complex with aluminum chloride. A volume of 1 ml of 2% $AlCl_3$ solution prepared in methanol was added to 1 ml of sample solution at room temperature. After 30 min of incubation the absorbance was measured at 430 nm using a visible light spectrophotometer.

The yellow color indicated that the extracts contained flavonoids. Quercetin was used as a standard for calibration. Total flavonoid content was calculated as mg equivalent Quercetin per gram of extract (mg EQ/GE) (40).

Evaluation of antioxidant activity in vitro

Anti-radical scavenging activity was evaluated using 1,1-diphenyl-2-picrylhydrazil (DPPH) radical (0.004% in methanol) according to the method described by Kulsic *et al*. (41) with a minor modification. In order to prepare 0.004% (w/v) DPPH solution, 4mg of DPPH was dissolved in 100mL of methanol. Concerning the sample solutions 4mg of aqueous and methanolic extracts were dissolved in 1mL of distilled water and methanol. In parallel, serial dilutions were performed in order to prepare different concentrated solutions for each extract (20ug/mL; 40ug/mL; 60ug/mL; 80ug/mL; 100ug/mL), then one milliliter of extracts prepared at different concentrations was added to 1mL of DPPH-methanol solution. The mixtures were shaken vigorously and left standing in the laboratory conditions for 30 minutes in the dark. The optical density (DO) was measured using a Spectrophotometer at 515 nm against the blank. The blank consisted of 1mL of methanol and 1mL of DPPH solution (0.004%), and BHT (Butylated hydroxytoluene) was used as a positive control.

All determinations were performed in triplicate. The optical density was recorded and the percent of inhibition (PI) was calculated according to the mathematical formula (B):

$$PI\% = \frac{(A0 - At)}{A0} \times 100, \text{ where} \qquad (B),$$

A0: optical density of the blank (control) at starting time.

At: optical density of the sample after 30 min.

Antioxidant activity results are expressed as IC50 value (μg extract/mL) (concentration providing 50% inhibition of DPPH radicals), which was calculated graphically by interpolation from linear regression analysis.

Identification of active biomolecules by HPLC-DAD analysis

In order to detect and quantify the phenolic compounds in the different extracts, we evaluated them by Chromatographic analyses, which were achieved on an Agilent series 1260 HPLC-DAD instrument (Agilent, Waldbronn, Germany). The instrument includes a 1260 Quat pump VL quaternary pump, an online degasser, 1260 ALS auto sampler, 1260 TCC column thermostat and 1260 DAD VL diode array detector. Chromatographic separation was done in a ZORBAX Eclipse XDB-C18 column (4.6 mm × 250 mm I.D., 3.5 μm particle size). The elution conditions were as follows: mobile phase A (0.1% acetic acid in water) and mobile phase B (100% acetonitrile), flow rate of 0.5 mL/min, sample injection volume of 10 μL, and operating temperature 40°C. The running gradient was as follows: A; 0–22 min, 10%–50% B; 22–32 min, 50%–100% B; 32–40 min, 100% B; 40–44 min, 100–10% B. Re-equilibration duration lasted 6 min. DAD detector scanned from 190 to 400 nm and the samples were detected at 254, 280, and 330 nm. The injection volume was 5 μl for every sample and reference standards. Congruent retention times compared to standards were used to identify the peaks. Phenolic chemicals were quantified using HPLC by comparing peak areas with those used as internal standards. data were represented as mg/g and (mg/ml).

Statistical analysis

The average and standard deviation were used to express the results. Data were statistically analyzed, to determine if there are any significant differences between the aqueous and methanolic extracts as well as between subspecies studied in this research, which were statistically analyzed by ANOVA using the statistical package CoStat, with the criterion of P values <0.05.

Results

Plant extract yield

The percent yields of different extracts from dry plant materials were reported in (Table 1, Figure 3).

Table 1. Yield, total polyphenols and total flavonoids in aqueous and methanolic extracts of the aerial parts of *Olea europaea* subsp. *europaea* var. *sylvestris* and *Olea europea* subsp. *laperrinei*.

Plants amples	Extract type	Yield (%)	Total polyphenoles (mg GAE/g Ext)[1]	Total Flavonoids (mg QE/g Ext)[2]
Olea europaea* subsp. *europaea* var. *sylvestris	Methanolic extract	35,60 ± 3,18	18,13 ± 1.04	14.30 ± 0.47
	Aqueous extract	22,20 ± 2,24	17,57 ± 0.34	12.98 ± 0.99
Olea europea* subsp. *laperrinei	Methanolic extract	37,20 ± 6,47	26,75 ± 2 .16	22.83 ± 0.13
	Aqueous extract	25.60 ± 0,23	18,93 ± 1.17	15.24 ± 0.27

mg GAE/g Ext:mg of Galic Acid Equivalent/ g of dried Extract; mg QE/g Ext:mg of Quercetin Equivalent/g of dried Extract; each value in the table presented as mean ± standard deviation (n=3)

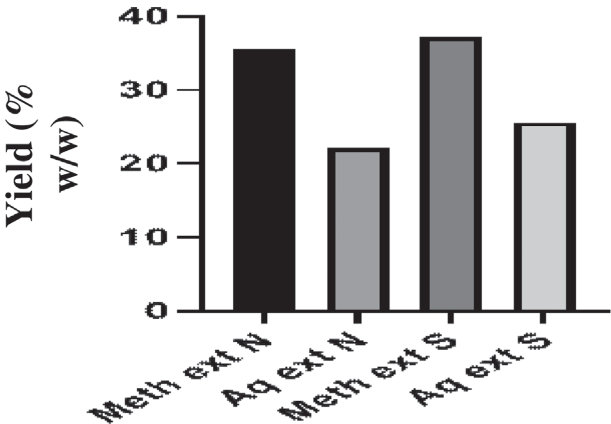

Figure 3. Yields of different extracts obtained from var. *sylvestris* and subsp. *laperrinei*. (abbreviations: AqN: aqueous extract from Northern subspecies; AqS:aqueous extract from Saharan subspecies; MethN: methanolic extract from Northern subspecies; MethS:methanolic extract from Saharan subspecies).

According to the yield values of the extracts obtained, it was observed that methanol as a solvent gives best yields than water and methanolic extract from *Olea europea* subsp. *laperrinei* gives a greater yield of (37.20 ±6.47%), followed by methanolic extract of *Olea europaea* subsp. var. *sylvestris* (35.60 ± 3.18 %). In the same way, extraction yield using water as a solvent, also is higher in the Saharan subspecies subsp. *laperrinei* (25.60 ±0.23%) than the variety from the Mediterranean region (22.20± 2.24%).

Total phenolic contents

Total phenolic contents were determined for aqueous and methanolic extracts of *Olea europaea* var. *sylvestris* as well as *Olea europea* subsp.*lapperinie*. The total quantity of phenols in the analyzed extracts are measured using the Folin–Ciocalteu, according to the gallicacid calibration curves figure 4. The values obtained for the total phenolic compounds are presented in (Table 1, Figure 5).

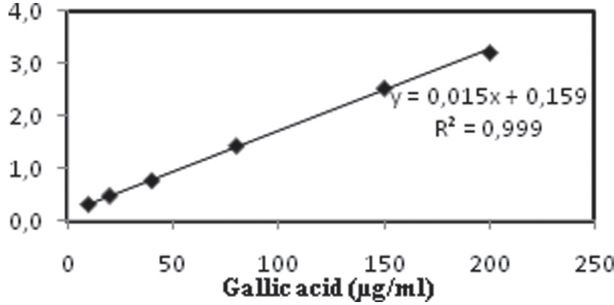

Figure 4. Gallicacid calibration curve used for polyphenols determination.

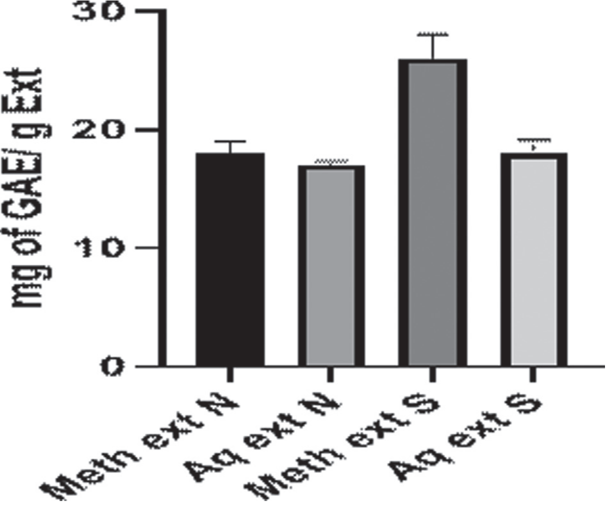

Figure 5. Total polyphenol content of different extracts samples expressed as mg of Galic Acid *Equivalent/g of extract.* (abbreviations: AqN: aqueous extract from Northern subspecies;AqS:aqueous extract from Saharan subspecies; MethN: methanolic extract from Northern subspecies; MethS: methanolic extract from Saharan subspecies).

Results suggest that the leaves extracts of both subspecies can be arich source of polyphenols, in addition, the phenolic contents varied considerably between the four leaves extracts studied, in relation to the solvent used the methanolic extracts shown to contain higher amounts of phenolic components than water extracts. As mentioned above, concerning the samples studies the greatest quantity of phenols were observed in the methanolic and aqueous extracts of the sample from the Saharan region (26,75 ± 2 .16mg GAE/g Ext), (18,93 ± 1.17 mg GAE/g) respectively, whereas the methanolic and aqueous in the sample from the Mediterranean region showed a muchlower concentration of phenols (18,13 ± 1.04 mg GAE/g), (17,57 ± 0.34 mg GAE/g) respectively.

Flavonoid concentration

The concentration of flavonoids in various extracts was determined using a spectrophotometric method based on the formation of flavonoid complex with aluminum chloride, the content of flavonoids was expressed in terms of quercetin equivalents (mg quercetin per gram of extract) (Figure 6), the summary of quantities of flavonoids in the examined extracts is reported in Table 1 and Figure 7. Overall, The concentration of flavonoids in the tested extracts ranged from 12.98 ± 0.99 to 22.83 ± 0.13 mg QE/g of extract, regarding to extracts, the methanolic extract of the two subspecies having greater contents of flavonoids compared to the aqueous extract, in fact, the extract of *Olea europaea* subsp. *laperrinei* showed the highest concentration of flavonoids in either a methanolic or aqueous (22.83 ± 0.13 mg QE/g Ext) ,(15.24 ± 0.27 mg QE/g Ext), while methanolic or aqueous extract of *Olea europaea* var. *sylvestris* showed a lowest concentrations (14.30 ± 0.47 mg QE/g Ext)(12.98 ± 0.99 mg QE/g Ext).

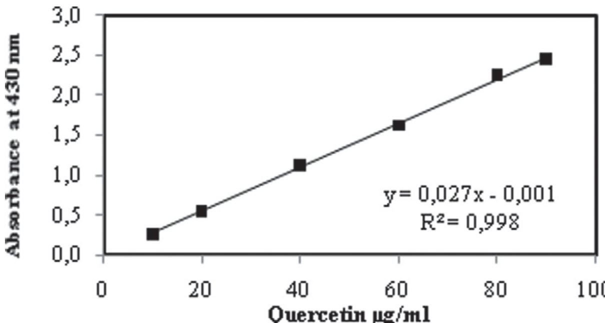

Figure 6. Quercetin calibration curve for flavonoids determination.

Antioxidant activity of plantextracts

The antioxidant activity of the methanolic and aqueous extracts of two subspecies and the positive control (BHT)against very stable free radical DPPH was evaluated using a spectrophotometric method by following the reduction of this radical which was accompanied by a color change from violet to yellow (DPPH-H), which can be measured at 515 nm. The ability of plant extracts to reduce DPPH radicals was determined by the decrease in their absorbance at 515 nm. Free radical scavenging effects results were defined as the amount of antioxidants necessary to decrease the initial DPPH radical concentration by 50% in 30 minutes (IC50), a lower IC50 value indicates higher antioxidant activity (Figure 8).

The obtained results for antioxidant activity evaluated by DPPH radical scavenging activity ranged from

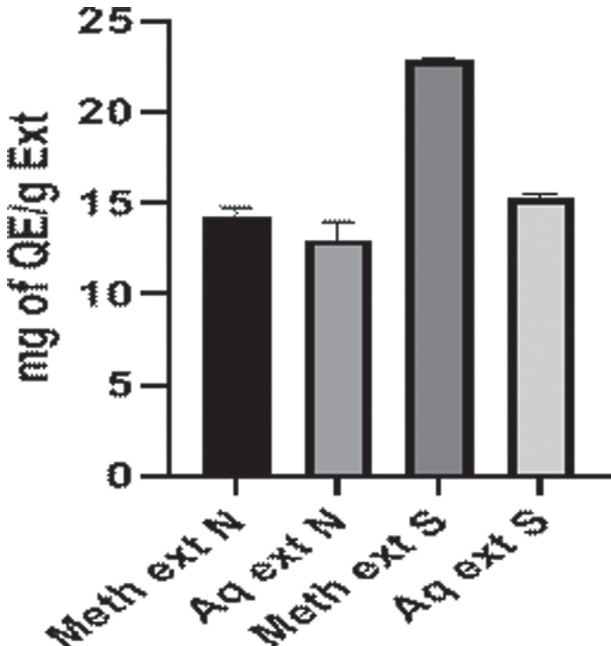

Figure 7. Total Flavonoids content of different extract samples expressed as mg of Quercetin *Equivalent/g of extract*. (abbreviations: AqN: aqueous extract from Northern subspecies; AqS:aqueous extract from Saharan subspecies; MethN: methanolic extract from Northern subspecies;MethS: methanolic extract from Saharan subspecies)

54.01 ± 0.46 to 82.33 ± 0.50 µg/mL Table 2. The highest capacity to neutralize DPPH radicals was found in the methanolic and aqueous extracts of endemic Saharan sub species *Olea europea* subsp.*laperrinei* with an IC50 value of 54.01 ± 0.46µg /ml and 66.97 ± 0.41 µg /ml respectively, these concentrations are near those exerted by positive control (BHT). In contrast, the minutest antioxidant activity was determined for extracts from *Olea europaea var. sylvestris* with values of 70.19 ± 2.09µg /ml (methanolic extract) and 82.33 ± 0.50 µg /ml (water extract) approximately two folds of that of BHT (Figure 9).

HPLC-DAD analysis of plant extracts

Identification and quantification of individual polyphenols Present in the extracts were carried out by using data from HPLC/DAD analyses, the data of phenolic compounds of each extract are listed in Tables 3 and 4.

The contents identified in all the extracts studied are numerous and diverse nature, grouped according to major molecular characteristics such as substituted phenols (hydroxytyrosol and tyrosol), phenolic acids (vanillic acid, caffeic acid, gallicacid, ferrulic acid, p-Coumaric acid, Chlorogenic acid), flavones (luteolin, Quercitin, Apigenin) and secoiridoids (Oleuropein and verbascoside) in which sixteen and seventeen phenolic compounds were identified in aqueous and methanolic extract issue from *Olea europaea* var. *sylvestris* respectively, in contrast, the aqueous and methanolic extract issue from *Olea europea* subsp. *laperrinei* showed fourteen and eleven compounds respectively.

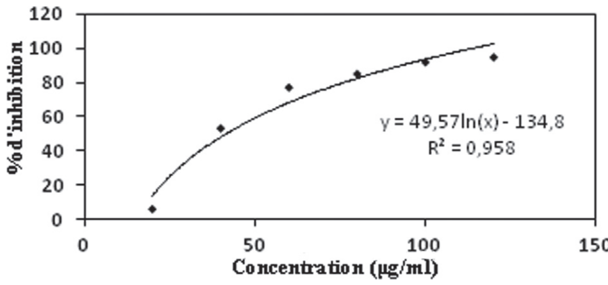

Figure 8. DPPH scavenging effect of standard antioxidant BHT.

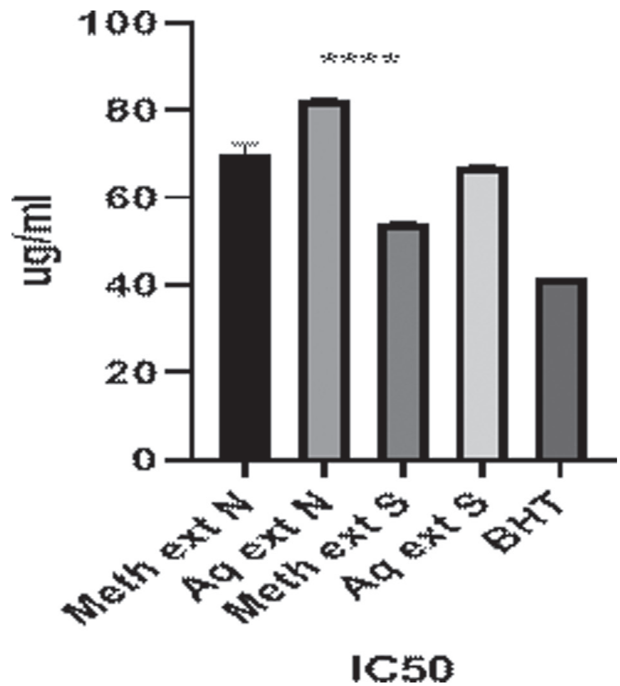

Figure 9. Comparison of antioxidant activity (IC_{50}) of different extracts with BHT.

Table 2. In vitro antioxidant activities of investigated *Olea europaea* subsp. *europaea* var. *sylvestris* and *Olea europaea* subsp. *laperrinei* leaves extracts, and positive control (BHT).

Samples	Extract type	DPPH $^{***}IC_{50}$(ug/ml) (1)
Olea europaea* subsp. *europaea* var. *sylvestris	Methanolic extract	70.19 ± 2.09
	Aqueous extract	82.33 ± 0.50
Olea europea* subsp. *laperrinei	Methanolic extract	**54.01 ± 0.46**
	Aqueous extract	66.97 ± 0.41
BHT		**41.65 ± 0.00**

(1) Eachvalueisrepresented as mean ± SD (n=3).
(2) Results were compared using ANOVA from CoStat Software p<0.001.***

Table 3. Phenolic compounds evaluated by HPLC-DAD expressed in (mg/ml)/(mg/g) of *Olea europea* subsp.*europea* var. *sylvestris* leaves extract.

Olea europaea* subsp. *europaea* var. *sylvestris								
Aqueous extract					**Methanolic extract**			
N°	**TR**	**Compounds**	**Concentration (mg/ml)**	**Concentration (mg/g)**	**RT**	**Compounds**	**Concentration (mg/ml)**	**Concentration (mg/g)**
1	7.072	Gallic acid	0.0024	0.165	7.076	Gallic acid	0.0014	0.121
2	10.149	**Hydroxytyrosol**	**0.0752**	**5.186**	10.151	Hydroxytyrosol	0.0222	1.93
3	11.489	**Chlorogenicacid**	**0.0508**	**3.503**	11.492	Chlorogenicacid	0.0334	2.904
4	12.857	Tyrosol	0.0467	3.220	12.855	Tyrosol	0.0157	1.365
5	13.579	Caffeicacid	0.0184	1.268	13.576	Caffeicacid	0.0113	0.982
6	14.090	Vanillicacid	0.0049	0.337	14.290	Vanillicacid	0.0062	0.539
7	15.363	**Rutin**	**0.1029**	**7.096**	15.357	**Rutin**	**0.0741**	**6.443**
8	15.742	Verbascoside	0.0272	1.875	15.983	**Verbascoside**	**0.0643**	**5.591**
9	16.387	**Luteoline7 glucoside**	**0.0716**	**4.937**	16.382	**Luteoline7 glucoside**	**0.0963**	**8.373**
10	17.061	p-Coumaric acid	0.0015	0.103	17.225	p-Coumaric acid	0.0027	0.235
11	18.057	**Apigenin-7-glucoside**	**0.0517**	**3.565**	18.050	**Apigenin-7-glucoside**	**0.0773**	**6.721**
12	18.109	Ferrulicacid	0.0229	1.579	18.238	Ferrulicacid	0.0178	1.547
13	18.841	**Oleuropein**	**0.1680**	**11.586**	18.827	**Oleuropein**	**1.6419**	**142.773**
14	21.154	Naringinin	0.0110	0.785	21.164	Naringinin	0.0298	2.591
15		Luteolin	n.d.	-	22.964	Luteolin	0.0009	0.078
16	23.335	Quercitin	0.0274	1.889	23.435	Quercitin	0.0033	0.287
17	25.804	Apigenin	0.0027	0.186	26.386	Apigenin	0.0029	0.252
∑			**0.6853**	**47.262**			**2.1015**	**182.40**

n.d.: notdetected.

Concerning var. *sylvestris* methanolic extract, Oleuropein (142.773mg/g) was the major compound followed by Luteoline7glucoside and Apigenin-7-glucoside, while Luteolin (0.078 mg/g), Gallic acid (0.121 mg/g) were the minor phenolic compounds, also Oleuropein (11.586 mg/g) represented the predominant component of the aqueous extract followed by Rutin, hydroxytyrosol and Luteoline-7-glucoside. Regarding the methanolic extract of subsp. *laperrinei*, Oleuropein (276.157 mg/g), Luteoline7glucoside (10.557 mg/g) and Rutin (8.471mg/g) were the major compounds, in the other hand the main compounds in aqueous extract as Oleuropein (28.07 mg/g), Rutin (13.40 mg/g) and Quercitin (8.41 mg/g).

The characteristic shared between the two subspecies' extracts is that oleuropein and rutin are the most abundant component of the aqueous extracts, whereas Oleuropein and Luteoline7glucoside was the predominant in the methanolic extracts.

As described in (Table 3 and 4), the number and quantity of compounds are different between extracts types and subspecies studies, which var. *sylvestris* extracts are predominant in terms of the number of identified compounds, but subsp. *laperrinei*is dominating relative to the amount of these constituents.

Discussion

To the best of our knowledge, only a few investigations have been made about the bioactivities of leaf extracts issues from wild subspecies of *Olea europaea*

Table 4. Phenolic compounds evaluated by HPLC-DAD expressed in (mg/ml)/(mg/g) of *Olea europea* subsp. *laperrinei* leaves extract.

Olea europea **subsp**. *laperrinei*								
Aqueous extract					**Methanolic extract**			
N°	**RT**	**Compounds**	**Concentration (mg/ml)**	**Concentration (mg/1g)**	**RT**	**Compounds**	**Concentration (mg/ml)**	**Concentration (mg/1g)**
1	10.185	Hydroxytyrosol	**0.0525**	5.25		Hydroxytyrosol	n.d.	
2	11.496	Chlorogenicacid	0.0428	4.28	11.494	Chlorogenicacid	0.0299	4.271
3	12.830	Tyrosol	0.0235	2.35		Tyrosol	n.d.	
4	13.791	Caffeicacid	0.0102	1.02		Caffeicacid	n.d.	
5	14.051	Vanillicacid	0.0046	0.46		Vanillicacid	n.d.	
6	15.321	Rutin	**0.1340**	**13.40**	15.335	Rutin	**0.0593**	**8.471**
7	16.008	Verbascoside	0.0426	4.26	15.946	Verbascoside	0.0482	6.885
8	16.311	Luteoline7 glucoside	**0.0611**	**6.11**	16.341	Luteoline7 glucoside	**0.0739**	**10.557**
9	17.025	p-Coumaric acid	0.0183	1.83	17.324	p-Coumaric acid	0.0187	2.671
10	17.988	Apigenin-7-glucoside	0.0479	4.79	17.995	Apigenin-7-glucoside	0.0354	**5.057**
11	18.064	Ferrulicacid	0.0256	2.56	18.194	Ferrulicacid	0.0156	2.228
12	18.799	Oleuropein	**0.2807**	**28.07**	18.782	Oleuropein	**1.9331**	**276.157**
13	21.034	Naringinin	0.0090	0.90	20.683	Naringinin	0.0340	4.857
14	23. 303	Quercitin	**0.0841**	**8.41**		Quercitin	n.d.	
15		Apigenin	n.d.	-		Apigenin	n.d.	
∑			**0.8368**	**83.680**			**2.2481**	**321.153**

n.d.: not detected.

L. in addition, the present paper is the first, to investigate the antioxidant activity and quantify the total phenolic and flavonoid content of subsp. *laperrinei*, as well as, the comparative evaluation between two wild olive species in Algeria.

In the comparison between the values obtained from the two subspecies studies, different relations can be found In our study. the analysis of the yield results, total phenols, and flavonoid content in all the extracts assessed suggests that the results follow a variation with a similar dynamic,it was also noticed that the highest values were obtained using methanol as solvents than water. Numerous studies show also that extracting solvents influenced the extract yields and phenolic content, Moreover, has been reported that high yields are obtained with methanol (24). Water also plays an essential role in the extraction process of polyphenols by increasing their diffusion in the tissues of plants. The disparity in yield results is not related only to the type of solvents but could be caused by several of parameters, such as pH, temperature, extraction time, and sample composition. Additionally, the location and time of the harvest influence the yield extraction (1)**.** In our results the extract leaves issue from subsp. *laperrinei* has a greater yield than var. *sylvestris*, the values obtained were similar to those obtained byArab and Yahhiaoui (42), while other authors found less yield (1,43).

Many human diseases, such as cancer, and Alzheimer's disease, are mainly caused by free radicals. The Antioxidant compound can deactivate the free radicals by chelating metals or donating hydrogen atoms. The use of commercial antioxidants is therefore required and many of them, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are produced synthetically. However, these synthetic antioxidants are reported to be toxic. As a result, there is an

increasing interest in natural antioxidants that are present in many plants (44). In this context, the interest in the olive leaf and its chemical composition has recently been increasing. In fact, Olive leaves are considered a cheap raw material that can be used as a source of high-added-value products (45). This research was done to confirm the ability of leaf extracts issued from wild olives to synthesize secondary metabolites, to evaluate their capacity as natural antioxidants, as well as to examine how geographic differences could influence the chemical composition and bioactivity of wild olives.

In general, the results obtained in this work indicate clearly that leaf extracts obtained from the two wild olive subspecies contain high concentrations of phenols and flavonoids. According to the solvent used the Methanolic extracts of the two subspecies were shown to be containing higher phenolic component levels compared to water extracts. in relation to the two wild subspecies examined the methanolic extract of the subsp.laperrinei spread from Sahara contains a very high proportion of these phenolic compounds. These height concentrations of phenolic found in all our extracts can be explained by the type of solvent. Therefore, polar solvents were the best extraction media for phenolic compounds (46). These amounts found are in agreement with numerous research which have been done on methanolic extract of olive leaf from Tunisia, Malta, France, Serbia (47) and in Chemlali variety from west of Algeria (48) as well as Meski cultivar from Tunisia (59). In contrast to our results, other studies determined the height concentration of these phytochemicals (43, 50, 51), additionally according to the results, flavonoids represent the major fraction of polyphenol compounds; this result is in accordance with those suggested by Chu et *al*. (17).

On another side, the extracts from the Saharan region also induce an increase in the scavenging activities of free radicals more than those from the northern region related directly to their higher levels of polyphenols (22,52,53), these later can prevent the synthesis of free radicals and opposing the oxidation of macromolecules (54,55), the variability of the antioxidant activity observed between leaf extracts of the two wild olives can be explained by the structure of the phenolic compounds, notably by the number and the position of the hydroxyl groups compared to the functional carboxyl groups (1). Therefore, the antioxidant potential of the subspecies studies is height compared to many other published results in Algeria (51), France and Serbia (47), while other studies showed a high activity to our finding (43, 48), furthermore, our results are added to the other reported (32, 56, 57) to confirm that olive leaves are a source of several antioxidants.

As previously noted, the variations in the antiradical activity observed for the various extracts studied related to the bioactive content, in order to identify and quantify those chemicals and estimated their possible influence on the antioxidant potential an HPLC-DAD technique is employed, a difference in the number and quantity of compounds was detected between extracts types and subspecies studies, which var. *sylvestris* extracts are predominant in terms of the number of identified compounds while subsp. *laperrinei* is dominating relative to the amount of these constituents, consequently we can deduce that, like in the case of subsp. *laperrinei*, not only the number of identified compounds but also the quantity and structure of these phenolics can influence the bioactivity ensured by the plant (48). The characteristic shared between all extracts that is Oleuropein predominant compound, the results of other researchers studying different species of *Olea europaea* L. in various extract types were in line with that Oleuropein is the most abundant phenolic compound in water extract, methanol extract (49) and also in both methanolic and ethanolic extracts (58), therefore we can deduce that Oleuropein would be responsible for great antioxidant activities (13) observed in our subspecies, moreover our results confirm that it is a well-known antioxidant derivative (59).

The HPLC analysis showed the richness of all extracts by oleuropein, a well-known antioxidant derivative that could explain the highest antioxidant activity found (59). The health-promoting properties of this compound have been extensively studied principally for their antioxidant properties and therapeutic benefits, such as antimicrobial and antiproliferative activities (15, 16). Several studies suggest that the phenol extract with high hydroxytyrosol content obtained from olive leaves (*Olea europaea* L.) increased the oxidative stability of different food lipids (butter and lard) (60). Interestingly, some studies showed that other phenolic

compounds can be involved in the antioxidant activity of olive leaf extract for example, rutin has good effects such as antioxidant, and anti-aging, their beneficial effects are attributed to their ability to reduce oxidative stress, in high-cholesterol-diet-fed (61, 62). Besides, luteolin may have preventive benefits against the appearance of diabetes-related cardiac dysfunction by minimizing oxidative stress (63); moreover, gallic acid also possesses beneficial effects on human health and decreases oxidative stress (64).

The variability seen in the phytochemical profile and antioxidant potential between sub-species studies, as well as the predominant of the stressful one, subsp. *laperrinei*" in both types of extracts than the Mediterranean subspecies, may be explained the role play by secondary metabolites in the process of plants adapting to the ecological condition in their environments (65), in which synthesis and accumulation of those metabolites, mainly the phenolic compounds increased during stress It could support the suggestion that phenolics could play a key role in the ecophysiological adaptation of *Olea europea* L. to the specific ecological conditions (48), additionally, Edziri et *al*. (50) hypothesized that the significant differences observed in leaves methanolic extracts of four olive cultivars from Tunisia can be explained by many factors including genetic origins, geographical region, soil composition, environmental climate, altitude, rainfall and the amounts and type of phenol contents may vary according to the olive variety. Furthermore, the type of solvent used through the extraction protocol (66). These factors may directly affect the chemical profile and, as a result, their therapeutic effects, as in our study.

Conclusion

Olive leaves are widely applied in different fields due to their wide range of bioactivities. Moreover, the valorization of Algerian genetic heritage, especially wild olives and Saharan endemic subspecies which are unknown until this day like our case of subsp. *laperrinei* is becoming a necessity to demonstrate their importance, by studying their bioactive content.

From the results of this preliminary study, the optimal extraction procedure for the dried leaves of the two samples is maceration with methanol than water. It gives a high extract yield, the maximum total phenolic and flavonoid contents, the greatest antioxidant activity as well as the main active components. Moreover, it could be confirmed that not only cultivated but also wild olive leaves are considered a source of natural antioxidants due to their height amounts of total phenolic and flavonoid content and good radical scavenging activity which are detected preceding. Despite wild olive "subsp. *laperrinei*" spread in arid regions (Sahara) but containing a high amount of these phytochemicals and Greater antioxidant activity which is similar to that presented by BHT than var. *sylvestris* which collected from a habitat with more favorable conditions.

In addition, the variability demonstrated between the two subspecies explained clearly the role of habitat factors in the production of these secondary metabolites and on the other hand, the role of the secondary metabolites in the ecophysiological process of plants to adapting in stressful conditions (lower precipitation, higher temperatures). As a result, it has become a necessity for the pharmaceutical and cosmeceuticals industry the shift towards natural products.

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الملخص

في إطار تثمين الموارد الطبيعية "النباتات البرية والمستوطنة"، تم تحديد نوعين فرعيين من أشجار الزيتون البرية في الجزائر، أحدهما يعرف باسم *Olea europaea* L.var. sylvestris "Oleaster"، منتشر بشكل رئيسي في الشمال، والأخر يعرف باسم أليو أو آليوان بين الطوارق "*Olea europaea* subsp.*laperrinei*"، وينتشر في الصحراء الوسطى. يتناول الجزء الأول من الدراسة الآلية التشريحية والفيزيائية التي تم تطويرمن النوعين للتكيف مع ظروفهم البيئية. بينت الدراسة النسيجية والقياسات الفسيولوجية أن شجرة زيتون Laperrine قد أظهرت تغيرات تشريحية وفسيولوجية كبيرة مقارنة بشجرة الزيتون الاخرى. بينما ركز الجزء الثاني للدراسة على تحديد الملامح الفينولية لمستخلصات الأوراق المائية والميثانولية من النوعين الفرعيين عن طريق تحليل *لكروماتوغرافيا السائلة عالية الأداء* ،الدي سمح بتحديد العديد من المركبات الفينولية حيث ظهر oleuropein كمركب رئيسي في جميع المستخلصات، حيث أظهرت المستخلصات الصحراوية وجود الكمية الأكبر منه. أظهرت جميع المستخلصات نشاطاً معتبرا مضاداً للجذور ، ولكن تم تحديد أعلى نشاط في مستخلصات السلالات الصحراوية، كما وجد الميثانول كمذيب استخلاص أفضل من الماء. أما الجزء الثالث فيتعلق بالدراسة الكيميائية والنشاط المضاد للبكتيريا للزيتين الأساسيين، وكشف تحليل *كروماتوغرافيا الغاز مع كاشف تأين اللهب* عن تحديد 31 مركباً حيث كان α-pinene المكون الرئيسيي في *laperrinei* subsp. *Olea. europaea* 29 مركبًا و Nonanal حدد كمكون رئيسي في الزيتون الآخر، أظهر كلا الزيتين المتطايرين نشاطًا فعالًا مضادًا للبكتيريا، في حين أن النوع المستوطن لديه نطاق تثبيط أعلى حيث *P. aeruginosa* و *B. subtilis* وجدت كسلالات حساسة و *K.pneumoniae* هي المقاومة. الجزء الأخير يعتمد على تثمين القيمة الغذائية لزيوت الزيتون البكر الممتازة من خلال تحليلها الكيميائي، و تثمين قيمتها العلاجية من خلال نشاطها المضاد للبكتيريا. سمحت النتائج باكتشاف 22 مكونًا في*Olea europaea* L.var. sylvestris. حيث تم تحديد, palmitic acid ,oleic acid, squalene,linoleic acid كعناصر سائدة، في حين تم تحديد 14 مكونا في الزيتون المستوطن الذي كانت فيه oleic،palmitic acid, linoleic acid, acid squalene هي المكونات الرئيسية. تمثل الأحماض الدهنية غير المشبعة الجزء السائد في الزيتين. بالإضافة إلى ذلك، تشير النتائج إلى أن تأثير الزيتين البكر الممتازين على البكتيريا موجبة الجرام أكبر منه على البكتيريا السالبة الغرام، حيث اظهر زيت *Olea europaea* subsp. *laperrinei* أعلى نطاق تثبيط مع جميع السلالات البكتيرية التي تم اختبارها، وكانت *B.subtilis,P.aeruginosa ,B.subtilis,S. aureus(MRSA)*, هي السلالات الحساسة ، في حين كانت *K. pneumoniae* و*E.faecalis* هي السلالات المقاومة لكلا الزيتين.

الكلمات المفتاحية : *Olea europaea* subsp. *laperrinei* ,*Olea europaea* L.var. sylvestris, الآليات التشريحية والفيزيائية ؛ المكونات الكيميائية، الأنشطة البيولوجية.

Abstract

As part of the valorization of natural resources “wild and endemic plants”, two subspecies of the wild olive trees that have been inventoried in Algeria, one of which is commonly referred to Oleaster "*Olea europaea* L.var. sylvestris", spreads mainly in the northern region, and the other is known as ālew or āliwan among the Tuareg Berbers "*Olea europaea* subsp. *laperrinei*", spreads in the central Sahara. The first part deals with physio-anatomical mechanisms developed to adapt to their environmental conditions. The histological study and the physiological parameters measured revealed that the laperrine olive tree exhibited significant anatomical and physiological changes compared to the Oleastre tree. The second part focuses on studying of phenolic profiles of aqueous and methanolic leaf extracts from the two subspecies by high-performance liquid chromatography analysis which allowed the identification of several phenolic compounds with oleuropein as the main compound in all extracts, with Saharan subspecies extracts showing a higher level. All the extracts showed significant antiradical activity, but a high value was detected in the Saharan subspecies extracts with an IC50 value near to those exerted by the butylated hydroxytoluene, Methanol was found as a better extraction solvent than water. The third part relates to a phytochemical study and the antibacterial activity of the two essential oils, the gas Chromatography coupled with flame ionization detector analysis revealed the identification of 31 compounds where α-pinene main components in *Olea europaea* subsp. *laperrinei* volatile oil and 29 compounds with nonanal as major constituents in the Oleaster, both volatile oils showed an efficient antibacterial activity, with the endemic one has a higher range of inhibition, in which *P. aeruginosa* is the sensitive strain and *K. pneumoniae* is the resistant one. The last part is based on the valorization of the nutritional value of their extra virgin olive oils, through their chemical analysis, and their therapeutic value through their antibacterial activity. The results allowed the detection of 22 components in *Olea europaea* L.var. sylvestris oil, with squalene, oleic acid, palmitic acid, linoleic acid, as predominant components, while 14 components were identified in the endemic one, in which palmitic acid, linoleic acid, oleic acid, squalene were the main components. Unsaturated fatty acids represented the dominant class in the two oils, where the linoleic acid was apparent by high percentage and significant amount for the first time comparable with the previous study that was conducted on olive oil. In addition, the results indicate that the effect of the two extra virgin oils on gram positive bacteria is greater than on gram negative bacteria, with *Olea europaea* subsp. *laperrinei* oil exhibited the highest range of inhibition with all bacterial strains tested.

Key words: *Olea europaea* L.var. sylvestris, *Olea europaea* subsp. *laperrinei*, physio- anatomical mechanisms, phytochemistry , biological activities.

Résumé

Dans le cadre de la valorisation des ressources naturelles « plantes sauvages et endémiques », deux sous espèces d'olivier sauvage ont été inventoriées en Algérie, dont l'une est communément appelée Oleaster "*Olea europaea* L.var. sylvestris", principalement distribuée dans la région du nord, et l'autre est connue sous le nom d'ālew ou d'āliwan chez les Berbères touaregs "*Olea europaea* subsp. *laperrinei*", répartie dans le Sahara central.La première partie de l'étude est basée sur leurs mécanismes physio-anatomiques développés pour s'adapter à leurs conditions environnementales. L'étude histologique et les paramètres physiologiques mesurés ont révélé que l'olivier de Laperrine présente des changements physio-anatomiques significatifs par rapport à l'olivier Oléastre. La deuxième partie s'est concentrée sur la détermination des profils phénoliques des extraits aqueux et méthanoliques des feuilles des deux sous-espèces par l'analyse de Chromatographie liquide à haute performance qui a permis l'identification de plusieurs composés phénoliques, l'oleuropéine étant le composé principal dans tous les extraits, les extraits de la sous espèces sahariens montrant un niveau plus élevé. Tous les extraits ont montré une activité anti-radicalaire significative et les valeurs élevées ont été détectée dans les extraits de la sous-espèce saharienne avec une valeur IC50 proche de celle de hydroxytoluène butylé, le méthanol s'est révélé être un meilleur solvant d'extraction que l'eau. La troisième partie, concerne une étude phytochimique et l'évaluation de l'activité antibactérienne des deux huiles essentielles, l'analyse de chromatographie en phase gazeuse couplée à un détecteur à ionisation de flamme a révélé l'identification de 31 composés dont le quel α-pinène est le composant majoritaire dans *Olea europaea* subsp. *laperrinei* et 29 composés ont été détectés chez l'Oleaster dont le nonanal est le principal constituant. Les deux huiles volatiles ont montré une activité antibactérienne efficace, tandis que l'huile de la sous espèces endémique a un effet d'inhibition plus élevée, dans laquelle *P. aeruginosa* est la souche sensible tandis que *K. pneumoniae* est la résistante. La dernière partie est basée sur la valorisation de la valeur nutritionnelle de leurs huiles d'olive extra vierges, à travers leur analyse chimique, et de leur valeur thérapeutique à travers leur activité antibactérienne. Les résultats ont permis la détection de 22 composants dans l'huile d'*Olea europaea* L.var. sylvestris, avec le squalène, l'acide oléique, l'acide palmitique, l'acide linoléique, comme composants prédominants, tandis que 14 composants ont été identifiés dans la sous-espèces endémique, dans lesquels l'acide palmitique, l'acide linoléique, l'acide oléique et le squalène étaient les principaux composants. Les acides gras insaturés représentaient la classe dominante dans les deux huiles, où l'acide linoléique était apparent par cette quantité significative pour la première fois comparable à l'étude précédente menée sur l'huile d'olive. De plus, les résultats indiquent que l'effet des deux huiles extra vierges sur les bactéries gram positives est plus important que sur les bactéries gram négatives, l'huile d'*Olea europaea* subsp. *laperrinei* a présenté la gamme d'inhibition la plus élevée avec toutes les souches bactériennes testées.

Mots clés: *Olea europaea*L.var. sylvestris ; *Olea europaea* subsp. *laperrinei*; mécanismes physio-anatomiques, photochimie, activités biologiques.

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