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Evaluation of the Biological Activities of Extracts from Selected Plant Species Used in Herbal Medicine in Algeria

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Dedications

I dedicate this work.

To you, my dearest parents, for your infinite love and the sacrifices that words can never fully express. May Allah surround you with his protection and bless you with long-lasting health and happiness.
To my beloved brothers, Naoufel and Mohammed, and to my sweet little sister, Ritage, may this humble testimony carry all my love and deepest admiration for you. May Allah safeguard your steps and grant you a long and beautiful life.

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This achievement is not mine alone; it is a reflection of your love, your hope, and your endless encouragement.

With all my heart,
Your daughter, sister, wife, mother, and friend,
Khadidja

Abstract

This study presents the first investigation of the chemical composition, antioxidant and antibacterial properties of two *Ononis* species native to the northeastern Sahara of Algeria; *Ononis angustissima* Lam. subsp. *filifolia* Murb. and *Ononis aurasiaca* Förther & Podlech. The latter is an endemic species of the Aures Mountains.

Crude compounds were sequentially extracted from the aerial parts of the plants using petroleum ether (PE), dichloromethane (DCM), and methanol (Me-OH). The total phenolic content (TPC) and total flavonoid content (TFC) were quantified through spectrophotometric methods, while essential oils were extracted via hydrodistillation and analyzed by gas chromatography-mass spectrometry (GC-MS). The results showed that the DCM extract of *O. aurasiaca* contained high levels of TPC and TFC. GC-MS analysis identified 44 compounds (93.4%) in *O. aurasiaca* essential oil, with major constituents including dodecanal, hexahydrofarnesylacetone, 2-tridecanone, phytol, 1-heneicosene, and n-heneicosane. In *O. angustissima*, 34 compounds (91.6%) were identified, with linalool, hexahydrofarnesylacetone, β-eudesmol, α-cadinol, and T-cadinol being the main constituents.

The antioxidant activity of the all extracts was evaluated *in vitro* using three different methods. All crude extracts demonstrated significant DPPH scavenging activity, particularly the DCM extract of *O. aurasiaca*, which had an IC_{50} value of 24.22 ± 0.28 µg/ml. Essential oils showed the most pronounced activity in inhibiting β-carotene discoloration, notably the essential oil of *O. angustissima*, achieving 91.35 ± 0.06%. However, all extracts showed only moderate to weak iron-reducing activity in the phenanthroline test.

The antibacterial activity was assessed both *in silico* and *in vitro*. The results of molecular docking of the ten major compounds of the essential oils indicated that phytol, hexahydrofarnesylacetone, T-cadinol, and β-eudesmol could be effective inhibitors against three targeted bacterial proteins. *In vitro* antibacterial tests conducted on four strains by disc diffusion methods, revealed that the extracts were primarily effective against Gram-positive strains, specifically *Staphylococcus aureus* ATCC 25923 and ATCC 43300, while showing limited impact on Gram-negative strains, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922.

Key words: *Ononis aurasiaca* Förther & Podlech, *Ononis angustissima* Lam. subsp. *filifolia* Murb., endemism, crude extract, essential oil, chemical composition, antioxidant activity, molecular docking, antibacterial activity.

الملخص

يعتبر هذا العمل الدراسة الأولى للتركيب الكيميائي وخصائص مضادات الأكسدة والمضادات البكتيرية لنوعين من نبات *Ononis* الأصليين في شمال شرق الصحراء الجزائرية: *Ononis angustissima* Lam. subsp. *filifolia* Murb. و*Ononis aurasiaca* Förther & Podlech، هذا الأخير هو نوع مستوطن في جبال الأوراس.

تم استخلاص المركبات الخام من الأجزاء الهوائية للنباتات بشكل متسلسل باستخدام الإيثر البترولي (PE) وثنائي كلوروالميثان (DCM) والميثانول (Me-OH)، ثم تم قياس محتوى الفينولات الكلية (TPC) و الفلافونويدات الكلية (TFC) باستخدام الطرق الطيفية، في حين تم استخراج الزيوت الأساسية بواسطة التقطير المائي وتحليلها بواسطة كروماتوغرافيا الغاز مقرونة بمقياس الطيف الكتلي (GC-MS). حيث أظهرت النتائج أن مستخلص ثنائي كلوروالميثان الخاص بـ *O. aurasiaca* يحتوي على قيم مرتفعة من الفينولات والفلافونويدات الكلية. في المقابل حدد تحليل GC-MS 44 مركباً (93.4٪) في الزيت الأساسي لـ *O. aurasiaca*، وتمثلت المركبات الرئيسية في dodecanal، hexahydrofarnesylacetone، 2- tridecanone، phytol، 1-heneicosene، و n-heneicosane. بالنسبة لـ *O. angustissima*، تم تحديد 34 مركباً (91.6٪) حيث المكونات الرئيسية كانت هي: linalool، hexahydrofarnesylacetone، β-eudesmol، α-cadinol، و T-cadinol.

بالنسبة لنشاط مضادات الأكسدة، فقد تمت التجربة على جميع المستخلصات مخبريا، باستخدام ثلاث طرق مختلفة. أظهرت جميع المستخلصات الخام نشاطاً كبيرا في اقتناص الجذور الحرة DPPH، خاصة مستخلص ثنائي كلورو ميثان الخاص بـ *O. aurasiaca* إذ بلغت قيمة IC_{50} 24.22 ± 0.28 ميكروغرام/مل. في المقابل أظهرت الزيوت الأساسية فعالية أكبر في تثبيط فقدان لون β-carotene، خصوصاً زيت *O. angustissima* الأساسي الذي حقق نسبة تثبيط 91.35±0.06٪. أما في اختبار phenanthroline، تبين أن جميع المستخلصات تملك نشاطاً متوسطاً إلى ضعيف في عملية ارجاع الحديد.

تم أيضا تقييم النشاط المضاد للبكتيريا بطريقتين: افتراضيا عن طريق المحاكاة الحاسوبية ومن ثم مخبريا. نتائج عمليات الإرساء الجزيئي للعشرة مركبات الرئيسية للزيوت الأساسية أشارت إلى أن: phytol، hexahydrofarnesylacetone, T-cadinol, β-eudesmol يمكن أن تكون مثبطات فعالة لثلاثة بروتينات بكتيرية مستهدفة. كما كشفت اختبارات النشاط المضاد للبكتيريا مخبريا بتقنية الانتشار على القرص والتي أجريت على أربعة سلالات، أن المستخلصات كانت فعالة بشكل أساسي ضد السلالات موجبة الجرام، وخاصة *Staphylococcus aureus* ATCC25923 و*Staphylococcus aureus* ATCC43300، بينما أظهرت تأثيراً محدوداً على السلالات سالبة الجرام *Pseudomonas aeruginosa* ATCC27853 و*Escherichia coli* ATCC25922.

الكلمات المفتاحية: *Ononis angustissima* Lam. subsp. *filifolia* Murb.، *Ononis aurasiaca* Förther & Podlech، الاستيطان، المستخلصات الخام، الزيت الأساسي، التركيب الكيميائي، نشاط مضادات الأكسدة، الإرساء الجزيئي، النشاط المضاد للبكتيريا.

Résumé

Ce travail représente la première étude de la composition chimique, des propriétés antioxydantes et antibactériennes de deux espèces d'*Ononis* originaires du nord-est du Sahara Algérien ; *Ononis angustissima* Lam. subsp. *filifolia* Murb. et *Ononis aurasiaca* Förther & Podlech, cette dernière étant une espèce endémique des montagnes des Aurès.

Les composés bruts ont été extraits séquentiellement à partir des parties aériennes des plantes en utilisant de l'éther de pétrole (PE), du dichlorométhane (DCM) et du méthanol (Me-OH). La teneur en phénols totaux (TPC) et en flavonoïdes totaux (TFC) a été quantifiée par des méthodes spectrophotométriques, tandis que les huiles essentielles ont été extraites par hydrodistillation et analysées par chromatographie en phase gazeuse-spectrométrie de masse (GC-MS). Les résultats ont montré que l'extrait au DCM de *O. aurasiaca* contenait des niveaux élevés en TPC et de TFC. L'analyse GC-MS a identifié 44 composés (93,4 %) dans l'huile essentielle d'*O. aurasiaca*, dont le dodécanal, l'hexahydrofarnésylacétone, le 2-tridécanone, le phytol, le 1-hénéicosène et le n-hénéicosane sont les principaux constituants. Pour *O. angustissima*, 34 composés (91,6 %) ont été identifiés, avec le linalol, l'hexahydrofarnésylacétone, le β- eudesmol, l'α-cadinol et le T-cadinol étant les plus abondants. L'activité antioxydante de tous les extraits a été évaluée *in vitro* en utilisant trois méthodes différentes. Tous les extraits bruts ont montré une activité de piégeage des radicaux libres DPPH significative, en particulier l'extrait au DCM d'*O. aurasiaca*, qui a une valeur IC_{50} de 24,22±0,28 µg/ml. Les huiles essentielles ont montré l'activité la plus prononcée dans le test de blanchissement du β-carotène, notamment l'huile essentielle de *O. angustissima*, atteignant 91,35 ± 0,06 %. Cependant, tous les extraits ont montré une activité modérée à faible dans la réduction du fer lors du test à la phénanthroline.

L'activité antibactérienne a été évaluée à la fois *in silico* et *in vitro*. Les résultats du docking moléculaire des dix principaux composés des huiles essentielles ont indiqué que le phytol, l'hexahydrofarnésylacétone, le T-cadinol et le β-eudesmol pourraient être des inhibiteurs efficaces contre trois protéines bactériennes ciblées. Les tests antibactériens *in vitro*, réalisés sur quatre souches par la méthode de diffusion sur disque, ont révélé que les extraits étaient principalement efficaces contre les souches Gram-positives, spécifiquement *Staphylococcus aureus* ATCC 25923 et ATCC 43300, tout en montrant un impact limité sur les souches Gram-négatives *Pseudomonas aeruginosa* ATCC 27853 et *Escherichia coli* ATCC 25922.

Mots-clés : *Ononis aurasiaca* Förther & Podlech, *Ononis angustissima* Lam. subsp. *filifolia* Murb., endémisme, extraits brut, huile essentielle, composition chimique, activité antioxydante, docking moléculaire, activité antibactérienne.

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Abbreviations list

Å: Angstrom
Ala: Alanine
$AlCl_3$: Aluminum trichloride
ANOVA: Analysis of variance
Arg: Arginine
Asp: Aspartic acid
ATCC: American type culture collection
BHT: Butylated hydroxytoluene
CAT: Catalase
CPP: ent-copalyl diphosphate
DAHP: 3-deoxy-D-arabino-heptulosonic acid-7-phosphate
DCME: Dichloromethane extract
DMAPP: Dimethylallyl diphosphate
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DPPH: 2,2-diphenyl-1-picryl-hydrazyl
DXP: 1-deoxy-D-xylulose-5-phosphate pathway
ECC: Extractable compound content
EO: Essential oil
ETC: Electron transport chain
Fe^{2+}: Ferrous ions
Fe^{3+}: Ferric ions
$FeCl_3$: Ferric chloride
FPP: Farnesyl diphosphate
GC-LRI: Gas chromatography-linear retention indices
GC-MS: Gas chromatography-mass spectrometry
GGPP: Geranylgeranyl diphosphate
Gln: Glutamine
GPP: Geranyl diphosphate
GPx: Glutathione peroxidase
GTP: Guanosine triphosphate
H_2: Hydrogen
H_2O_2: Hydrogen peroxide
He: Helium
His: Histidine
$HO^{\bullet}$: Hydroxyl radical
HOCl: Hypochlorous acid
HT: Hydrolysable tannins
IC_{50}: Inhibition concentration 50
Ile: Isoleucine
IPP: Isopentenyl diphosphate
IR: Ischemia-reperfusion injury
LC: Liquid chromatography
Leu: Leucine
$LO^{\bullet}$: Alkoxyl radical
$LOO^{\bullet}$: Peroxyl radical
LOOH: Lipid hydroperoxide
LRI: Linear retention indices
Lys: Lysine
Me-OHE: Methanol extract
MEP: Methylerythritol pathway
mM: millimolar
MVA: Mevalonate pathway
N_2: Nitrogen
Na_2CO: Sodium carbonate
$O_2^{-\bullet}$: Superoxide anion radical
O_3: Ozone
OH^-: Hydroxyl anion
OHN: N-3-Oxo-Dodecanoyl-L-Homoserine Lactone

OSB-NCoA: O-succinylbenzoyl-N-coenzyme A.

PA: Proanthocyanidins

Pdb: protein data bank

PEE: Petroleum ether extract

PEP: Phosphoenolpyruvic acid

Phe: Phenylalanine

ROS/RNS: Reactive oxygen and nitrogen species

SASA: Solvent accessible surface area

Sdf: Spatial data file

Ser: Serine

SOD: Superoxide dismutase

TAs: Tropane alkaloids

TCA: Tricarboxylic acid

TCD: Thermal conductivity detector

TF: Total flavonoids

TPP: Total polyphenols

TPS: Terpene synthases

Trp: Tryptophan

Tyr: Tyrosine

Val: Valine

w/w: weight/weight

Y_{EO}: Yield of essential oil

µg QE/mg: Microgram of quercetin equivalents per milligram

µg GAE/mg: Microgram of gallic acid equivalents per milligram

$^1O_2$: Singlet oxygen

Introduction

Introduction

Today, in the context of increasing disease burdens and the alarming rise of antimicrobial resistance, there is growing interest in exploring aromatic and medicinal plants as promising sources of new, safe, and pharmacologically potent biomolecules for pharmaceutical and food applications. Free radicals and oxidative stress are well-established contributors to cellular damage, degenerative disorders, and food spoilage (Sen et al. 2010). Natural antioxidants derived from plant sources offer significant potential as safer alternatives to synthetic preservatives (Lourenço et al. 2019), with phytochemicals such as flavonoids, terpenes, and polyphenols showing promise in mitigating oxidative stress and its health implications. Simultaneously, the decline in antibiotic effectiveness underscores the need for alternative antimicrobial agents, with plant-derived compounds emerging as viable candidates (Abdallah et al. 2023). To accelerate the discovery and development of such bioactive molecules, computational methods including molecular docking and virtual screening, enable cost-effective, rapid assessment of phytochemical–protein interactions, streamlining the identification of compounds with therapeutic potential (Dias et al. 2010). However, achieving reliable and effective plant-based therapeutics demands careful species selection, standardization, and rigorous evaluation of bioactivity, marking a shift toward more holistic and evidence-based approaches in modern medicine.

The genus *Ononis*, a member of the Fabaceae family, comprises numerous species known for their medicinal and ecological significance. Among these, the species of particular interest have demonstrated a wide range of biological activities, making them promising candidates for further scientific researches. Numerous investigations have been conducted in-depth analyses of the chemical composition of *Ononis* species. However, a significant portion of these studies has primarily focused on the composition of aqueous extracts and crude solvent extracts, encompassing acetone, chloroform, ether, ethyl acetate, butanol, hexane, and methanol of some specific species such as *Ononis angustissima* (Bouheroum et al. 2009; Mezrag et al. 2013; Benabderahmane et al. 2014; Guettaf et al. 2016; Mezrag et al. 2017), *Ononis vaginalis* (Abdel-Kader 2001), *O. speciosa* (Barrero et al. 1989), *O. natrix* (San Feliciano et al. 1983; Al-Rehaily et al. 2014; Mhamdi et al. 2014 ; Sayari et al. 2016; Öz et al. 2017; Yerlikaya et al. 2017; Stojković et al. 2020), *O. spinosa* (Öz et al. 2017; Stojković et al. 2020), *O. variegata* and *O. viscosa* (Öz et al. 2017), *O. pubescens* (Jaradat et al. 2017), *O. mitissima* (Besbas et al. 2020), *O. pusilla* (Khouni et al. 2014), and *O. arvensis* (Dénes et al. 2022).

Introduction

By contrast, the essential oils of *Ononis* species have received relatively limited research attention despite the genus's diversity. Existing studies have investigated the essential oils of *O. angustissima* (Mechehoud et al. 2014; Ghribi et al. 2016), *O. natrix* (Khallouki et al. 2002; Elamrani and Benaissa 2010; Al-Mterin et al. 2021), *O. sicula* (Al-Qudah et al. 2014), *O. reclinata* (Casiglia et al. 2017), *O. viscosa* (Erdemgil et al. 2002), and *O. alba* (Zaak et al. 2022).

Various *Ononis* species demonstrate a diverse range of pharmacological properties, including antimicrobial, analgesic, antioxidant, antiproliferative, anticancer, antihypertensive, anti-inflammatory, as well as the ability to accelerate wound-healing effects (Requena et al. 1987; Yılmaz et al. 2006; Altuner et al. 2010; Süntar et al. 2011; Al Qudah et al. 2014; Mhamdi et al. 2014; Ghribi et al. 2015; Öz et al. 2017; Jaradat et al. 2017). These plants have also found use in traditional phytotherapy. For example, *O. spinosa* is employed to treat urinary tract inflammations, kidney stones, wounds, skin issues, and gout (Kırmızıgül et al. 1997; Altanlar et al. 2006; Yilmaz 2006). Various species like *O. spinosa*, *O. arvensis*, *O. hircina*, and *O. antiquorum* have applications for skin irritations, itching, wounds, and dermatitis (Mamedov et al. 2005). *O. sicula* and *O. hirta* are used in wound healing and as antiseptics for skin cancer and cold sores (Taliba and Mahasneh 2010). Additionally, *O. natrix* extracts are recognized for their antirheumatic, diuretic, urolithiatic, and blood pressure-lowering properties (Al-Khalil et al. 1995; Saeed et al. 2003).

Despite this, only two studies have investigated the essential oils of *O. angustissima*, one in southwestern Algeria (Mechehoud et al. 2014) and the other in southwestern Tunisia (Ghribi et al. 2016). The other existing researches have focused on non-volatile extracts (Bouheroum et al. 2009; Mezrag et al. 2013; Benabderahmane et al. 2014; Guettaf et al. 2016; Mezrag et al. 2017). Yet, no dedicated studies have delved into the specific subspecies *Ononis angustissima* Lam. subsp. *filifolia* Murb. Regarding *Ononis aurasiaca*, it has not been previously studied for its chemical composition or bioactivities until now.

Within this framework, the present research aims to explore for the first time the chemical composition and biological activities of two *Ononis* species: *Ononis aurasiaca* Förther & Podlech and *Ononis angustissima* Lam. subsp. *filifolia* Murb. These species are endemic to the northeastern Algerian Sahara and have a history of traditional use among local population.

Introduction

This thesis is grounded in a comprehensive analysis of both secondary metabolites and essential oils from these plants. Employing advanced chromatographic and spectroscopic techniques, the study seeks to elucidate the phytochemical profiles of the selected species, and examines the antioxidant and antibacterial activities of the extracts through a series of *in vitro* and *in silico* assays. To achieve this, following specific objectives have been established:

- Determination of the polyphenol and flavonoid content of the crude extracts of the two plants,

- Analysis of the chemical composition of their essential oils by GC-MS,

- *In vitro* study of the antioxidant activity of crude extracts and essential oils using three different methods: DPPH assay, β-carotene bleaching and Fe^{2+}-phenanthroline test,

- *In silico* study in order to investigate the interaction between the essential oils' principal compounds and selected proteins from bacterial strains using the molecular docking method,

- *In vitro* testing of all extracts as antibacterial agents against three pathogenic strains, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, using the disk diffusion method.

Part I:

Literature review

I. Introduction to the *Ononis* genus, origin and distribution of the specimens

The *Ononis* genus is a large and diverse group of plants within the Fabaceae family, comprises approximately 86 species found mainly in the Mediterranean region, Macaronesia, and neighboring Asia (Ozenda 1958; Förther and Podlech 1991). These plants are typically characterized by yellow flowers, narrow leaflets, and small bushes with branched stems at the base (Ozenda 2004 and Mabberley 2017). The taxonomic treatment of the *Ononis* genus was first presented by Širjaev (1932), who accepted 68 species distributed in two sections according to their distinct peduncles and pod characteristics: Ononis section, characterized by reduced peduncles and pods with few-seeded, and Natrix Grisebach section, characterized by distinct peduncles and multi-seeded pods (Fayed et al. 2019).

The main area of *Ononis* diversity is situated in the south of the Iberian Peninsula and near areas of northern Morocco and Algeria (Turini 2010). Based on the latest revision of *Natrix* section in Macaronesia, North Africa and adjacent West Asia (Förther and Podlech 1991), six subspecies of *Ononis angustissima* Lam. are distributed in North Africa and southern Spain: *O. angustissima* Lam. subsp. *angustissima* and *O. angustissima* Lam. subsp. *longifolia* (Willd.) Förther & Podl. (Canary Islands and Spain), *O. angustissima* Lam. subsp. *polyclada* Murb. (Morocco, Algeria and Tunisia), *O. angustissima* Lam. subsp. *mauritii* (Mayor & Sennen) Förther & Podl. (Morocco and Algeria), *O. angustissima* Lam. subsp. *falcata* (Viv) Murb. (Algeria, Tunisia and Libya) and *O. angustissima* Lam. subsp. *filifolia* Murb. (Algeria and Tunisia).

In Algeria, *O. angustissima* Lam. subsp. *filifolia* Murb. (Figure 2) is primarily found in the eastern part of the northern Sahara (Figure 1), particularly within the Wilaya of Biskra and the surrounding areas (Förther and Podlech 1991). Locally, the aerial parts of this subspecies have traditional uses for hemostatic qualities (Chehma and Djebar 2008) and diabetes treatment (Khacheba et al. 2014).

Ononis aurasiaca Förther & Podlech (Figure 3) is one of the species belonging to the sect. Natrix Grisebach (Förther and Podlech 1991; Turini 2010), which is endemic to eastern Algeria and found only in the Aures region (Figure 1). 3 Km southwestern Banian on the road from Arris to Biskra (Förther and Podlech 1991). It is locally known as "Fizza" and its

aerial parts are traditionally used as a patch on the chest to alleviate symptoms of colds or the flu.

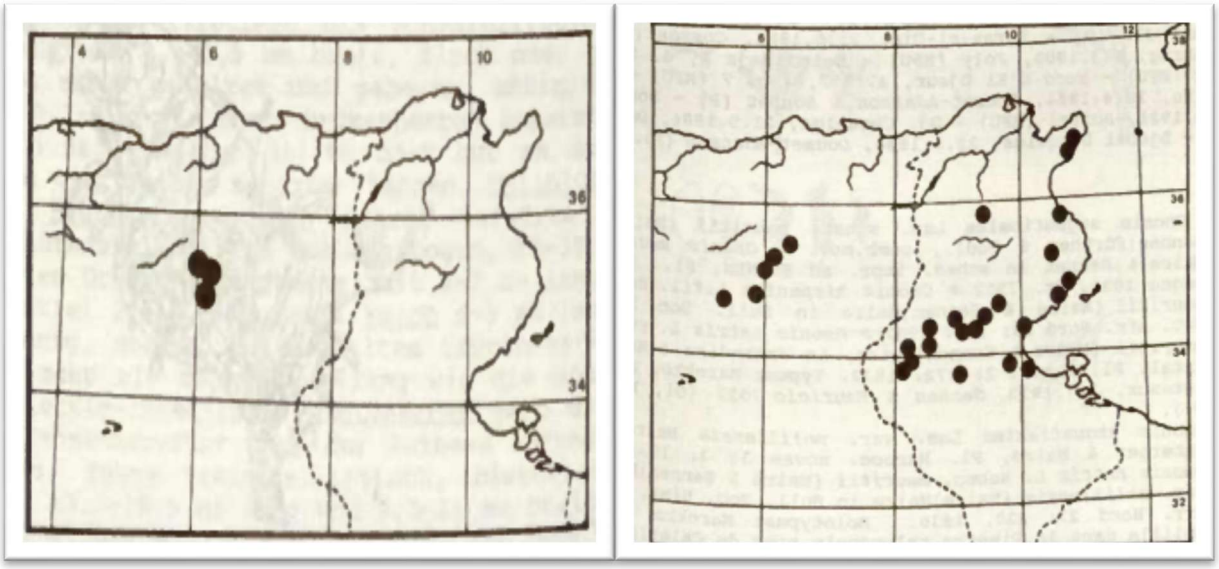

Figure 1: Distribution of *Ononis aurasiaca* (a) and *Ononis angustissima* Lam. subsp. *filifolia* Murb. (b) (Förther and Podlech 1991).

II. Botanical description

i. *Ononis angustissima* Lam. subsp. *filifolia* Murb.

Ononi. angustissima Lam. subsp. *filifolia* Murb. is a non-viscous chamaephyte with erected annual branches forming a dome of 10-40 cm high (Vanden Berghen 1978), characterized with the enduring presence of its dry branches from preceding years. The stems possessed internodes ranging in length from 3 to 19 mm, moderately thick to dense with sessile, sticky, resinous glands, and partially coated in short, long glandular hairs. The leaflets are narrow, linear to thread-like, typically folded and curved, rarely flat, ranging from 4 to 20 mm in length and 0.3-2(2.5) mm in width. The petiole, measures 2-13 mm in length, displaying a loose to moderately dense arrangement with sessile glands and isolated short glandular hairs. Stipules, described as measuring 3 to 16 mm in length and 0.6 to 2.5 mm in width, are moderately to densely covered in sessile, sticky, resinous glands. The calyx is present, spanning 6 to 10.2 mm in length and exhibiting a hairy texture like that found on the petiole.

The inflorescence stalk extends approximately 15- 45 mm culminating in a yellow flower, which appears from February through April. Pods are dense covered with occasional glandular hairs. Seeds are of 1.8 to 2.1 mm long and 1.5 to 1.7 mm wide. The species occurs in semi-desert and dry locations, specifically on sandy and stony soils (Širjaev 1932; Förther and Podlech 1991).

Figure 2: Photo of *O. angustissima* in flowering stage (A). Illustration of the aerial part (B): 1) Leaf-stalk, 2) Flower: a) Pedicel, b) Calyx, c) Flag, d) Wings, e) Keel, f) Stamen, g) Ovary, 3) legume (Pod) (Förther and Podlech 1991).

ii. *Ononis aurasiaca*

Ononis aurasiaca is a perennial semi-shrub that can grow up to 50 cm in high, and characterized by a woody base. It features numerous densely leafy branches with double glandular and thick covering, the glandular hairs on these branches have a length that varies from 0.4 to 1.5 mm, including extremely tiny ones that can reach 0.15 mm. The leaves are trifoliate with 12-33 mm long, usually only one leaflet is present in the inflorescence area, three leaflets in total, including a terminal leaflet that is distinctly stalked. These leaflets are thickly coated in glandular hairs that are 0.3-0.6 mm long, flat or slightly folded, and curled towards the apex of the stem. The species is further characterized by pendulous, linear acuminate and almost cylindrical legumes, measuring 12-18 mm in length and 2.1-2.5 mm in width, these legumes are loosely densely covered with glandular hairs measuring only 0.4-1.5 mm in length. They contain seeds (3-9) that are reniform, measuring 1.6-2.0 mm long and 1.3-1.5 mm wide, with a slightly brown or brown color. Stipules are present with a double glandular coating, measuring 5 to 13 mm long and attached to the petiole. Pedicels of 2.8-4.5 mm in length are also present. The campanulate-funnel-shaped calyx is about 6 to 9 mm in length, features teeth that are more than twice as long as the tube. The corolla is bright yellow, with a thinly purple-nerved flagellum and a distinct smell (Förther and Podlech 1991).

Figure 3: Illustration of *O. aurasiaca* aerial part (A), photo of the species in the flowering stage (B) (Förther and Podlech 1991).

III. Position within the systematic

The placement within the species' systematics was carried out using the following references: Širjaev 1932; Berghen 1978; Förther and Podlech 1991; Turini 2010; https://www.gbif.org/fr/species/3970567.

Table 1: Systematic classification

Kingdom	Plantae	
Phylum	Tracheophyta	
Class	Magnoliopsida	
Order	Fabales	
Family	Fabaceae	
Genus	*Ononis*	
Species	*Ononis angustissima* Lam.	*Ononis aurasiaca* Förther & Podlech
Subspecies	*Ononis angustissima* Lam. subsp. *filifolia* Murb	/

I. Introduction to secondary metabolites

Natural products, also known as secondary metabolites, are compounds not essential for the fundamental life processes of a living cell, unlike primary metabolites. While the term "secondary metabolites" is more precise in describing their biochemical synthesis, it may seem too technical for non-scientific audiences. As a result, both terms, "secondary metabolites" and "natural products," are used interchangeably (Abegaz and Kinfe 2019). These compounds, primarily derived from plants, exhibit variations in quantities within and among plants of the same and different species (Theis and Lerdau 2003; Raghuveer et al. 2015). They serve as valuable sources for the development of medicinal products with diverse applications in treating various infections and diseases. The rich diversity in plant resources contributes a myriad of natural drug materials, demonstrating curative and therapeutic effects. Moreover, plant secondary metabolites play a crucial role as sources for pharmaceuticals, food additives, flavors, and other industrial applications (Tholl 2006). The utilization of traditional knowledge regarding plant materials for disease treatment and prevention has garnered significant attention within the plant-based research community. This increased focus has led to exceptional advancements in drug discovery within the fields of phytochemistry and natural products (Twaij and Hasan 2022).

II. Structure and classification of plants secondary metabolites

Secondary metabolites can be categorized into three main chemically distinctive groups: terpenes, phenolic compounds, and nitrogen-containing compounds (Anulika et al. 2016). A practical approach for their classification involves grouping them based on their specific chemical structures and biosynthesis pathways (Velu et al. 2018). Just as DNA is composed of nucleotides with a unique arrangement of four nitrogen bases and proteins are made from amino acids, secondary metabolites also originate from basic building blocks. These building blocks vary among the different classes of metabolites. For example, mevalonate and methylerythritol phosphate are utilized in the synthesis of terpenoids, which constitute one of the largest families of secondary metabolites. In the case of phenolic compounds, shikimic acid and malonic acid serve as the starting materials in their biosynthesis (Abegaz and Kinfe 2019).

i. Terpenes

Terpenes, also referred to as terpenoids, constitute the largest and most diverse family of natural products. They exhibit a broad range of structures, varying from linear to polycyclic molecules, and encompass a size spectrum extending from the five-carbon hemiterpenes to natural rubber, comprised of numerous isoprene units. All terpenoids are synthesized through the condensation of isoprene units (C5) and are classified by the number of five-carbon units present in the core structure (Raghuveer et al. 2015). Terpene metabolites play a crucial role in plant growth and development, exemplified by their involvement in the production of gibberellin phytohormones. Additionally, these compounds serve as vital tools in various plant interactions with the environment, exhibiting toxicity and acting as feeding deterrents for numerous insects and mammals. Thus they appear to have important defensive role in the plant kingdom (Anulika et al. 2016). Both volatile and non-volatile terpenes are implicated in the attraction of pollinators, in protection against photooxidative stress, in mediating thermotolerance, and indirect defense against microbes (Tholl 2006). Monoterpenes (C10), composed of two isoprene units, and sesquiterpenes (C15), composed of three isoprene units, give rise to many flavor and aromatic molecules such as menthol, linalool, geraniol, and caryophyllene. Furthermore, specific bioactive compounds like diterpenes (C20), triterpenes (C30), and tetraterpenes (C40) exhibit very special properties (Raghuveer et al. 2015). Each terpenoid is composed of 5-carbon (Prenyl diphosphate) building blocks combined to form chains ranging from 10-carbon to 2000–500 000 carbon, as observed in the case of rubber. Saponins, derivatives of terpenes, are steroid and triterpene glycosides, named for their soap-like properties. Another terpene derivative, carotenoids, imparts yellow, red, and orange hues to certain plants, such as carrots (Anulika et al. 2016). Several of these compounds are extensively used in the industry sector, as flavors, fragrances, and spices (Kabera et al. 2014).

ii. Phenolic Compounds

Phenolic compounds derived from plants are one of largest group of secondary plants constituents, synthesized by fruits, vegetables, teas, cocoa and other plants offering specific health benefits. They are characterized by the antioxidant, anti-inflammatory, anti-carcinogenic and other biological properties, and may protect from oxidative stress and some diseases. Phenolics are characterized by the presence of at least one aromatic ring with one or more attached hydroxyl groups. Most of phenolic compounds are polymerized into larger

molecules such as the PA (Proanthocyanidins; condensed tannins) and lignans. Their classification is intricate due to variations in the number and arrangement of carbon atoms, commonly found conjugated to sugars and organic acids. Consequently, the terminology and classification of polyphenols can be complex and confusing. Although all polyphenols have similar chemical structures, there are some distinctive differences leading to their subdivided into two classes: flavonoids and non-flavonoids (Kabera et al. 2014; Raghuveer et al. 2015).

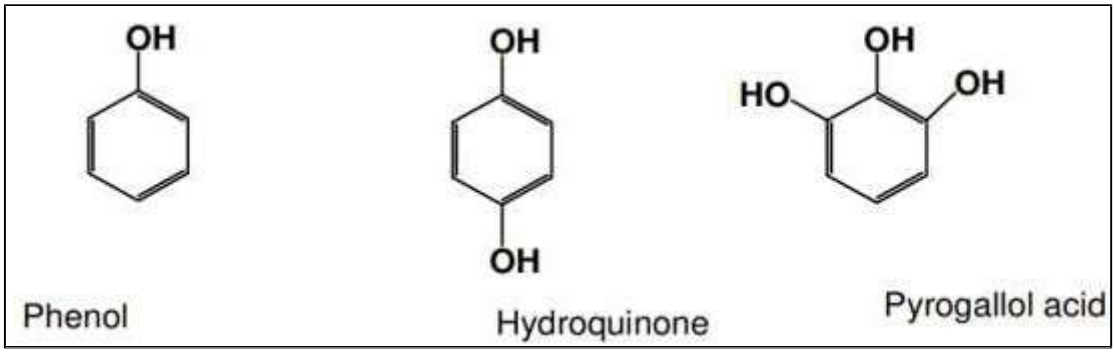

Figure 4: Examples of simple phenolics, C6 (Phenol, hydroquinone and pyrogallol acid) (Kabera et al. 2014).

a. Flavonoids

Flavonoids stand as the first class of phenolic compounds, comprising fifteen carbons with two aromatic rings connected by a three-carbon bridge (Kabera et al. 2014; Raghuveer et al. 2015), they have received great attention during the last two decades due to their potential protective role against various age-related disorders (Abegaz and Kinfe 2019). Flavonoids are water-soluble pigments found in the vacuoles of plant cells, fulfilling many functions such as flower coloration, producing yellow, red or blue pigmentation in petals designed to attract pollinator animals. In higher plants, flavonoids are involved in UV filtration and symbiotic nitrogen fixation. They may also act as chemical messengers, physiological regulators and cell cycle inhibitors (Kabera et al. 2014).

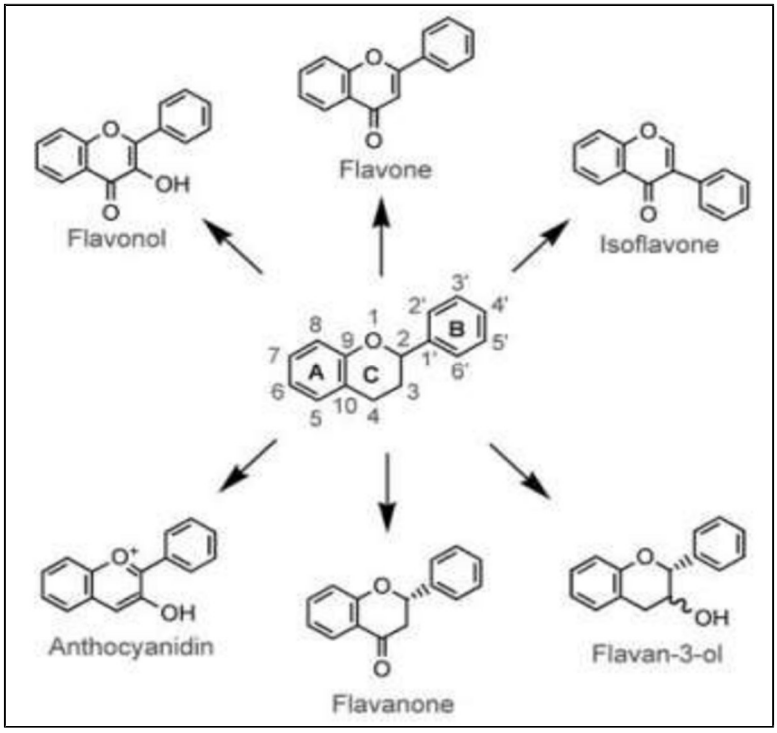

Figure 5: Generic structure of major flavonoids (Raghuveer et al. 2015).

The main subclasses of flavonoids are the flavones, flavonols, flavan-3-ols, isoflavones, flavanones and anthocyanidins (Figure 5), while some flavonoid groups, such as dihydroflavonols, flavan-3,4-diols, coumarins, chalcones, dihydrochalcones and aurones are quantitatively minor components in the diet, they contribute to the overall diversity of flavonoid structures. The basic flavonoid skeleton can have numerous substituents. Hydroxyl groups are usually present at the 4, 5 and 7 positions. Sugars are very common with the majority of flavonoids existing naturally as glycosides. Whereas both sugars and hydroxyl groups increase the water solubility of flavonoids, other substituents, such as methyl groups and isopentyl units, make flavonoids lipophilic (Raghuveer et al. 2015).

b. Non-flavonoids

Figures 6, 7 and 8 present the main non-flavonoids of dietary significance; the C6–C1 phenolic acids, most notably gallic acid, which is the precursor of hydrolysable tannins, the C6–C3 hydroxycinammates and their conjugated derivatives, and the polyphenolic C6–C2-C6 stilbenes (Raghuveer et al. 2015).

b. 1. Phenolic acids

Phenolic acids, also recognized as hydroxybenzoates, are predominantly represented by gallic acid (Figure 6). Gallic acid is the base unit of gallotannins whereas both gallic acid

and hexahydroxydiphenoyl moieties act as subunits of the ellagitannins. Gallotannins and ellagitannins categorized as hydrolysable tannins, can be easily broken down through treatment with dilute acid, releasing gallic acid and/or ellagic acid, whereas condensed tannins are not (Raghuveer et al. 2015).

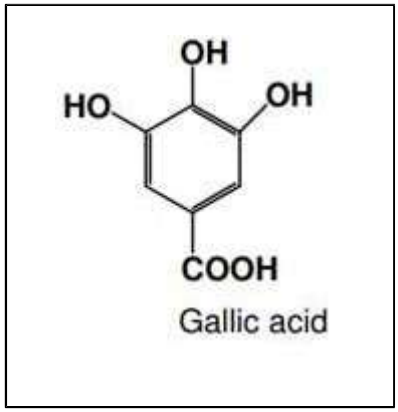

Figure 6: Exemple of C6-C1 phenolics.

b. 2. Hydroxycinnamates

Cinnamic acid, a C6–C3 compound, undergoes conversion into a variety of Hydroxycinnamates (Figure 7). As these hydroxycinnamates are products of the phenylpropanoid pathway, they are collectively referred to as phenylpropanoids. The predominant hydroxycinnamates include p-coumaric, caffeic, and ferulic acids (Raghuveer et al. 2015).

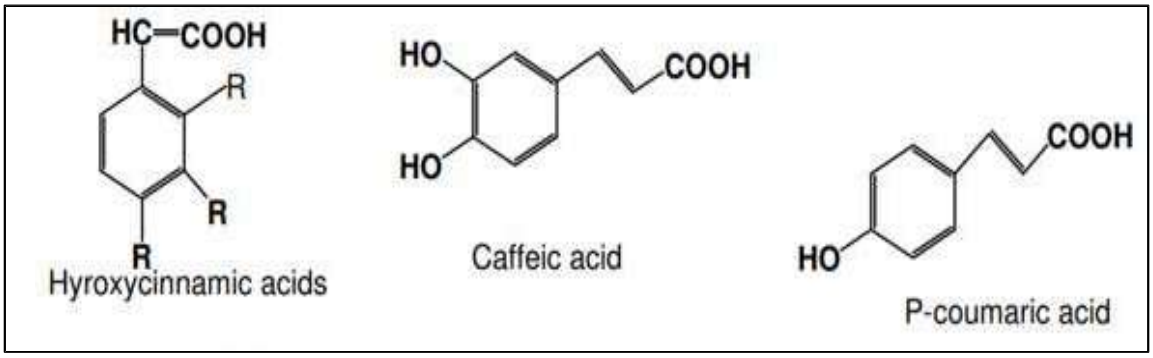

Figure 7: Phenolics with one aromatic ring C6-C3 (Phenylpropanoids): hydroxycinnamic acid, ferulic acid and synaptic acid (Kabera et al. 2014).

b. 3. Stilbenes

Stilbenes, members of the stilbene family characterized by the C6–C2–C6 structure (Figure 8). Stilbenes are phytoalexins, compounds produced by plants in response to attack

by fungal, bacterial and viral pathogens. Resveratrol is the most common stilbene (Raghuveer et al. 2015; Saltveit 2017)

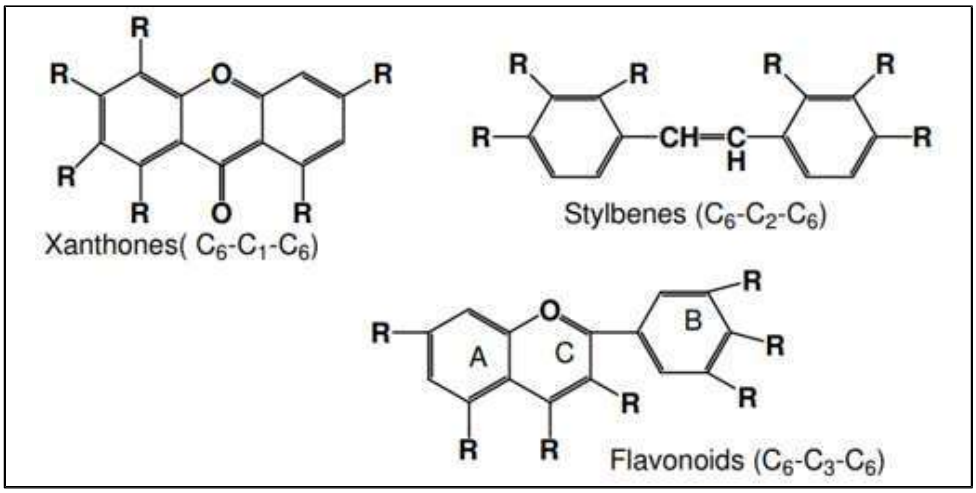

Figure 8: Some phenolics with two aromatic rings (Xanthones: C6-C1-C6, stylbenes: C6- C2-C6 and flavonoids: C6-C3-C6) (Kabera et al. 2014).

c. Tannins

Tannins, derived from the French term "Tanin" meaning tanning substance, represent a class of natural polyphenols. These phenolic compounds have the ability to precipitate proteins and consist of a diverse array of oligomers and polymers. Tannins can form complexes with proteins, starch, cellulose, and minerals. Synthesized through the shikimic acid pathway, also known as the phenylpropanoid pathway, they share this pathway with the production of other phenolics like isoflavones, coumarins, lignins, and aromatic amino acids. Tannins are water soluble compounds with exception of some high molecular weight structures. They are commonly subdivided into two groups: hydrolysable tannins (HT), which include gallotannins, ellagitannins, complex tannins and condensed tannins (PA). Tannins serve as the active principles in plant-based medicines, with literature indicating their use for astringency against diarrhea, diuretic properties against stomach and duodenal tumors, and anti-inflammatory effects (Kabera et al. 2014; Saltveit 2017).

d. Glycosides

The glycosides are characterized by a sugar portion or moiety attached by a special bond to one or non-sugar portions. Many plants store chemicals in the form of inactive glycosides, which can undergo activated through enzyme hydrolysis Consequently, most glycosides can be categorized as prodrugs since they remain inactive until they are hydrolyzed in the large

bowel leading to the release of the aglycone, the biologically active constituent. The classification of glycosides is determined by the nature of the aglycone, which spans a diverse range of molecules such as phenols, terpenes, or steroids. They are heterogeneous, therefore, they are not easy to learn as specific group (Kabera et al. 2014). Various studies have highlighted the therapeutic effects of glycosides, revealing their anticancer, expectorant, sedative, and digestive properties (Martínez et al. 2009; Kuang et al. 2018; Khan et al. 2019)

iii. Nitrogen-containing compounds

A broad array of secondary metabolites features nitrogen within their structures, with notable examples being the alkaloids and cyanogenic glucosides. Alkaloids, a large and diverse group exceeding 15 000 compounds, found in approximately 20% of vascular plant species. Some of them are not entirely distinguishable from amines "ephedrine". The nitrogen atom in these substances is typically part of the heterocyclic ring, a ring that contain both nitrogen and carbon atom. Names of these molecules tend to end in the suffixes –ine or–in (Figure9). Many are derived from amino acids, but others result from modification of various classes of molecules including polyphenols, terpenes, or steroids (Anulika et al. 2016).

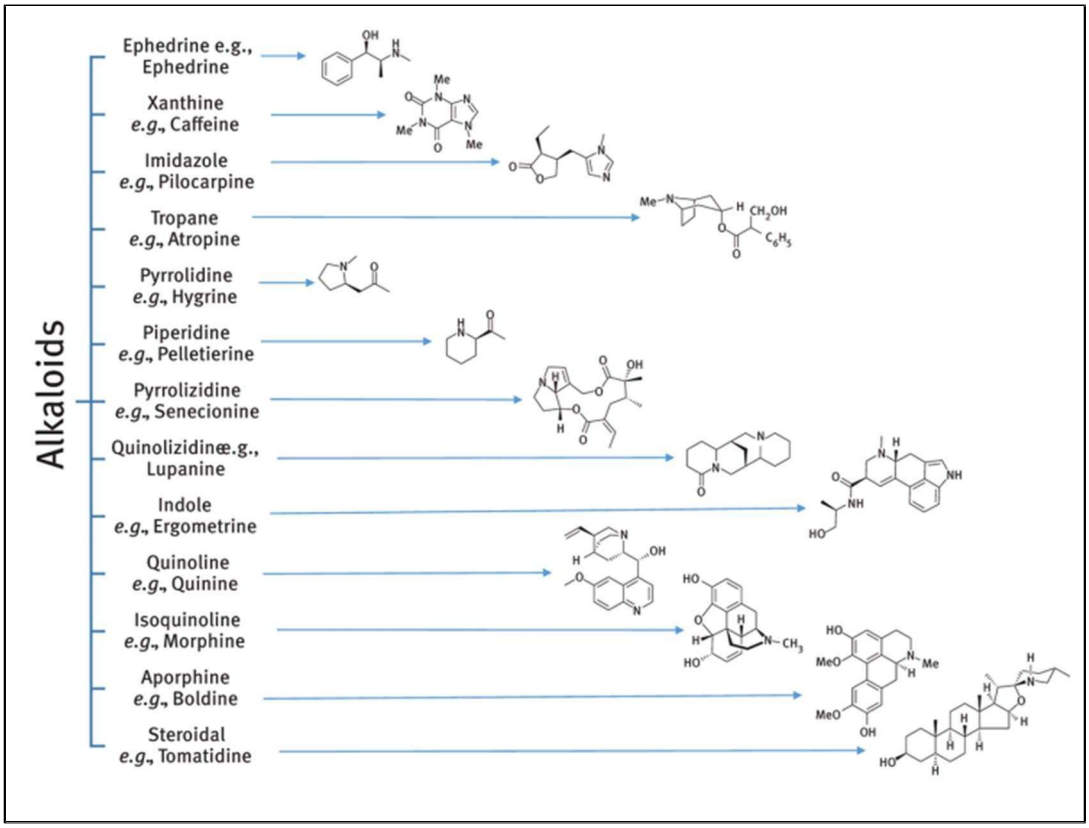

Figure 9: Classification of alkaloids (Abegaz and Kinfe 2019).

As their nomenclature implies, most alkaloids exhibit alkaline properties, at pH value commonly (7.2) (Theis and Lerdau 2003; Raghuveer et al. 2015; Jain et al. 2019). They are often dramatic compounds, having noticeable physiological effects, whether therapeutic or detrimental. The first medically useful example of an alkaloid was morphine, isolated in 1805 from Opium poppy *papaver somniferum*. The alkaloids from the plant origin have important medicinal applications, morphine is used as analgesic, berberine as antibiotic, vinblastine as anticancer and atropine as anti-cholinergic (Raghuveer et al. 2015; Velu et al. 2018). The primary role of alkaloids in plants is often defense against mammals due to their inherent toxicity and deterrence capacity (Anulika et al. 2016). Given their substantial number and structural diversity, alkaloids can be classified into subclasses in various ways, with tropane alkaloids, piperidine alkaloids, xanthine alkaloids, benzyl isoquinoline alkaloids being some examples. The most common classification system is based on the basic heterocyclic chemical entity that appears in the structure of the alkaloid like it is mentioned in figure 9 (Abegaz and Kinfe 2019). Taking tropane alkaloids as an example, characterized by their unique bicyclic tropane ring system, they can be further categorized into three major groups: hyoscyamine/scopolamine, cocaine, and calystegines (Figure 10, 14). Despite sharing a basic structure, tropane alkaloids exhibit significant variations in their biological, chemical, and pharmacological properties (Kohnen-Johannsen and Kayser 2019).

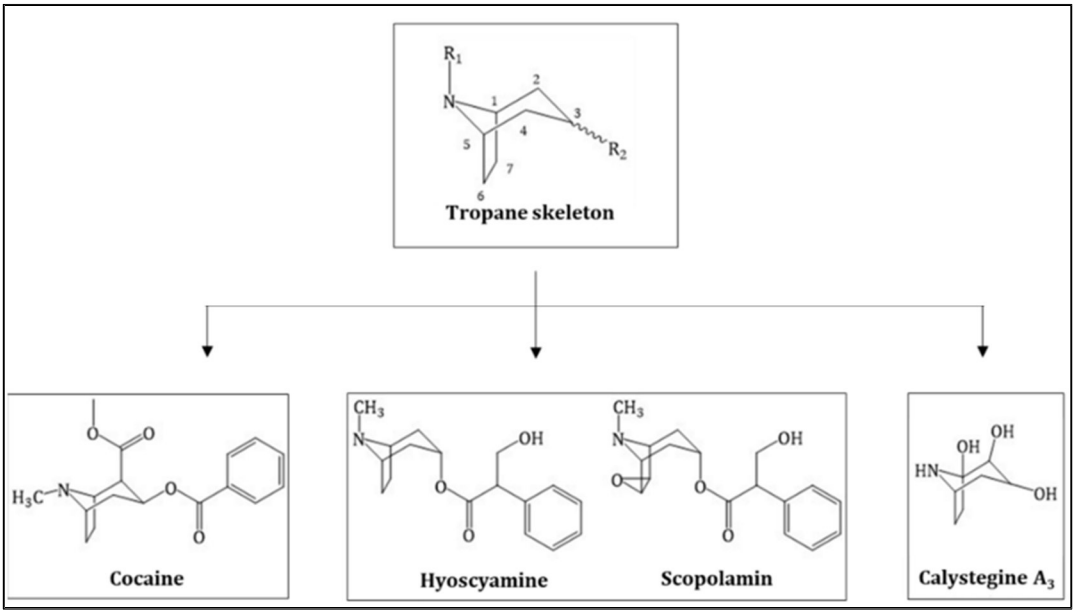

Figure 10: Structure of the tropane skeleton (Green box in figure 14) and the three major groups of TAs derived from this skeleton (Kohnen-Johannsen and Kayser 2019).

III. Biosynthesis of plant secondary metabolites

The biosynthesis of secondary metabolites is characterized by variations among the three main classes involved. Terpenes, for instance, are synthesized from primary metabolites through the mevalonic acid and methylerythritol phosphate (MEP) pathways. In the mevalonic acid pathway, three acetyl-CoA molecules undergo stepwise concatenation to form mevalonic acid. This crucial six-carbon intermediate is subsequently pyrophosphorylated, decarboxylated, and dehydrated, ultimately yielding isopentenyl diphosphate (IPP), the activated five-carbon building block of terpenes. Notably, recent discoveries have identified an alternative route for IPP formation, originating from glycolysis or the photosynthetic carbon reduction cycle through the MEP pathway, operational in chloroplasts and other plastids. Despite ongoing research, some details remain undisclosed. In the biosynthesis of phenolic compounds, two fundamental pathways come into play: the shikimic acid pathway and the malonic acid pathway. The shikimate pathway transforms simple carbohydrate precursors derived from glycolysis and the pentose phosphate pathway into aromatic amino acids, giving rise to diverse phenolic compounds. For nitrogenous compounds, the pathway involves the formation of aliphatic amino acids through the tricarboxylic acid (TCA) cycle (Anulika et al. 2016; Saltveit 2017).

Various pathways are commonly employed for biosynthesis: pentose for glycosides, shikimic acid for phenols, tannins, and aromatic alkaloids, acetate-malonate for phenols and alkaloids, and mevalonic acid for terpenes and alkaloids. As depicted in figure 11, the scheme outlines how metabolites from photosynthesis, glycolysis, and the Krebs cycle are tapped off from energy-generating processes to provide biosynthetic intermediates (Kabera et al. 2014; Velu et al. 2018; Saltveit 2017).

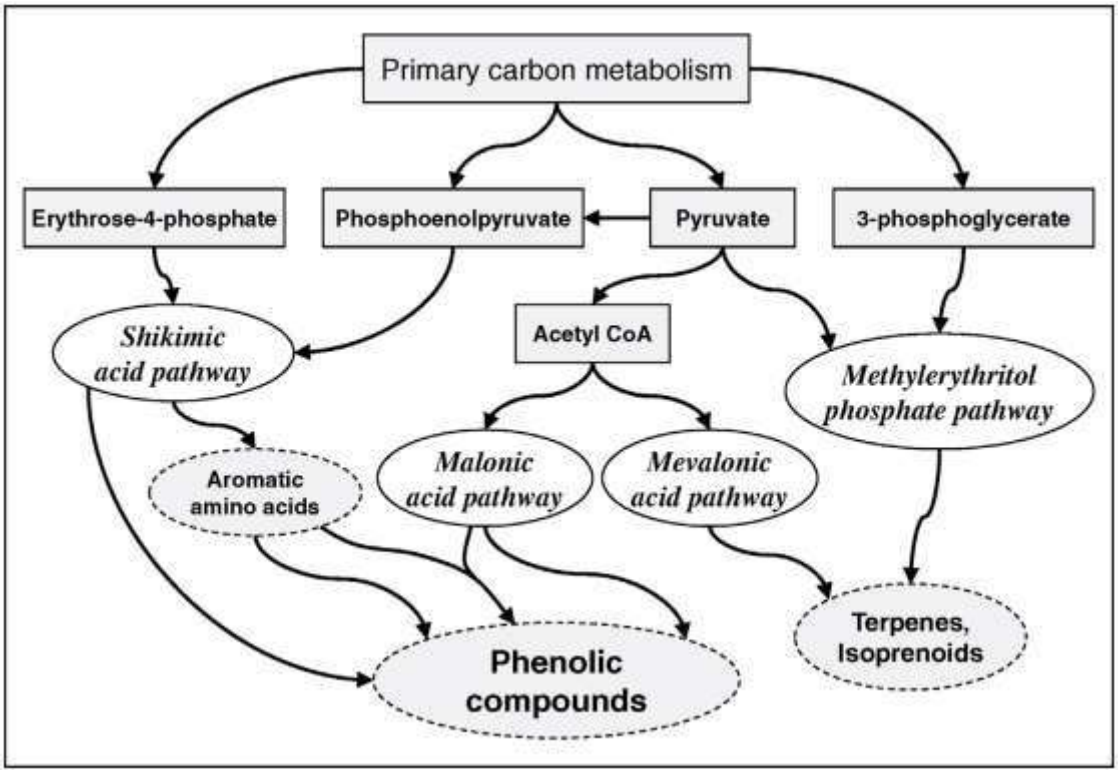

Figure 11: A simplified view of the major pathways of secondary metabolism biosynthesis (Saltveit 2017).

i. Terpenes biosynthesis

The initial substrates for the biosynthesis of the 20 000 terpenes are the simple C5-unit isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). prenyltransferases fuse DMAPP with varying numbers of isopentenyl/l diphosphate (IPP) units to produce the direct precursors of terpenes namely, 1) the linear prenyl diphosphates geranyl diphosphate (GPP, C10), 2) farnesyl diphosphate (FPP, C15), and 3) geranylgeranyl diphosphate (GGPP, C20). As illustrated in figure 12, the principal enzymes facilitating the formation of hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), or diterpenes (C20) from the substrates DMAPP, GPP, FPP, or GGPP are terpene synthases (TPS). Examples of different monoterpene, sesquiterpene, and diterpene synthase products are shown. The enzymatic reactions of all class I terpene synthases initiate with the ionization of the prenyl diphosphate substrate and the formation of carbocation intermediates. Class II diterpene synthases, such as ent-copalyl diphosphate (CPP) synthases, catalyze a protonation-induced cyclization of the substrate GGPP to CPP. Bifunctional (class I/class II) diterpene synthases, such as abietadiene synthase, catalyze an initial cyclization of GGPP to enzyme-bound (+)-CPP, followed by a typical ionization-initiated cyclization of (+)-CPP

and subsequent reaction steps to form abietadiene. The generation of C5-units IPP and DMAPP takes place in separate pathways, the mevalonate pathway in the cytosolic compartment and the methylerythritol phosphate (MEP) pathway in the plastidic. The biosynthesis of FPP and sesquiterpene metabolites occurs primarily in the cytosol, whereas the enzymes responsible for isoprene, monoterpene and diterpene formation are mostly located in plastids. OPP indicates the diphosphate moiety (Tholl 2006; Theis and Lerdau 2003).

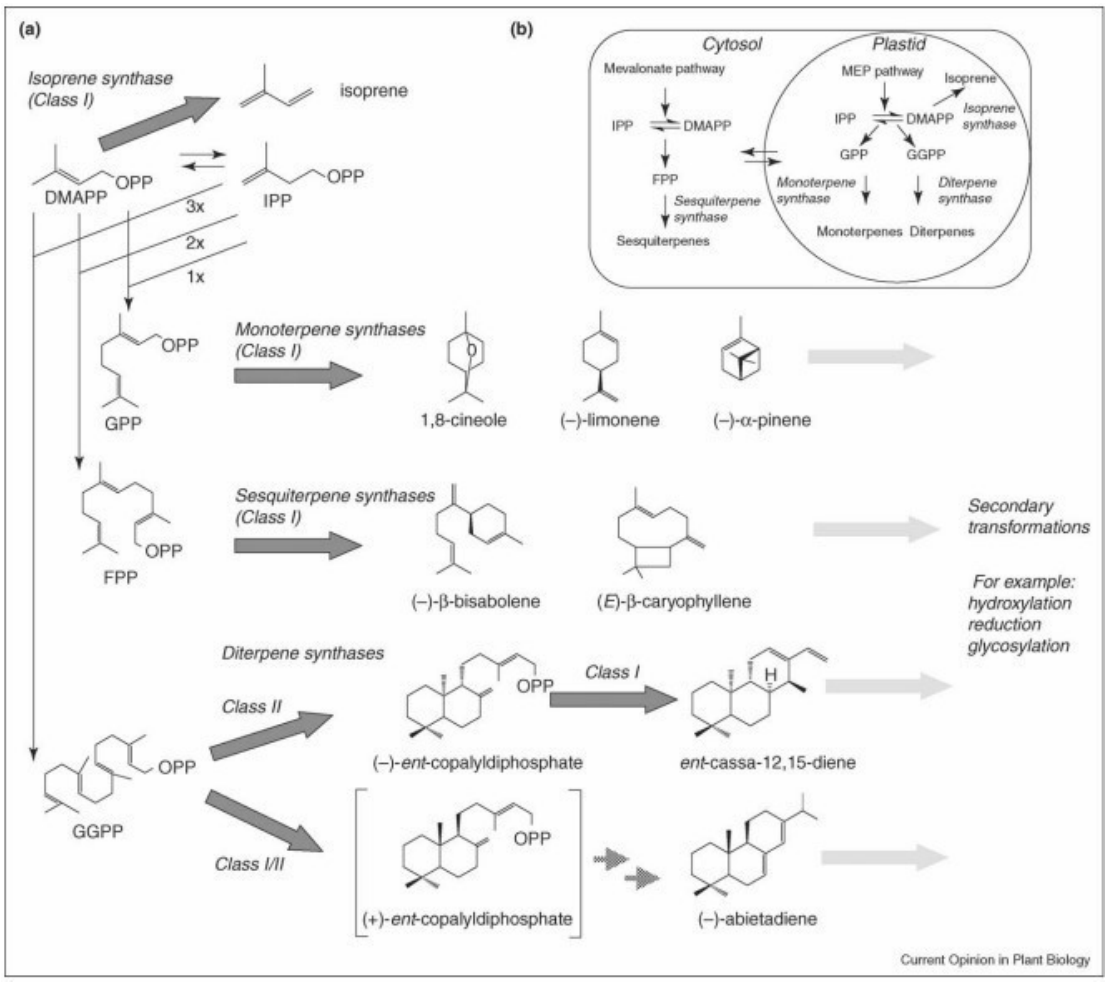

Figure 12: (a) Biosynthesis of different class of terpenes, (b) Compartmentation of terpenes biosynthesis in the plant cell (Tholl 2006).

ii. Phenolic compounds biosynthesis

Of the identified biosynthetic paths, shikimic acid pathway plays a very important role in providing precursors of a large number of aromatic compounds of diverse skeletal patterns and substitutions. This biochemical pathway serves as a pivotal connection between primary and secondary metabolism in higher plants, facilitating the production of aromatic amino

acids as L-tryptophan (Trp), L-phenylalanine (Phe) and L-tyrosine (Tyr) (Figure 13). These amino acids are not only crucial compounents of protein biosynthesis, they also serve as precusors for of a wide variety of plant natural products that play important roles in plant growth, development, reproduction, defense and environmental responses. Trp acts as a precursor of alkaloids. Trp and Phe serve as common precursors of numerous phenolic compounds such as cinnamic acids, phenylpropenes chromones (C6-C3), stilbenes, anthraquinones (C6-C2-C6), flavonoids (C6-C3-C6), lignans (C6–C3–C3–C6) and various important aromatic amino acids (Figure 13). The shikimate pathway comprises of seven enzymatic reactions which begins with an aldol-type condensation of two phosphorylated active compounds; the phosphoenolpyruvic acid (PEP) from glycolytic pathway, and the carbohydrate erythrose-4-phosphate from the pentose phosphate cycle, to give 3-deoxy-D-arabino-heptulosonic acid-7-phosphate (DAHP), and culminates in the formation of chorismic acid, passed through the (–)-shikimic acid. A full set of the shikimate pathway enzymes exists in the plastids, based on experimental evidence and predictions of their subcellular localization (Maeda and Dudareva 2012; Talapatra et al. 2015; Soto-Hernández et al. 2019).

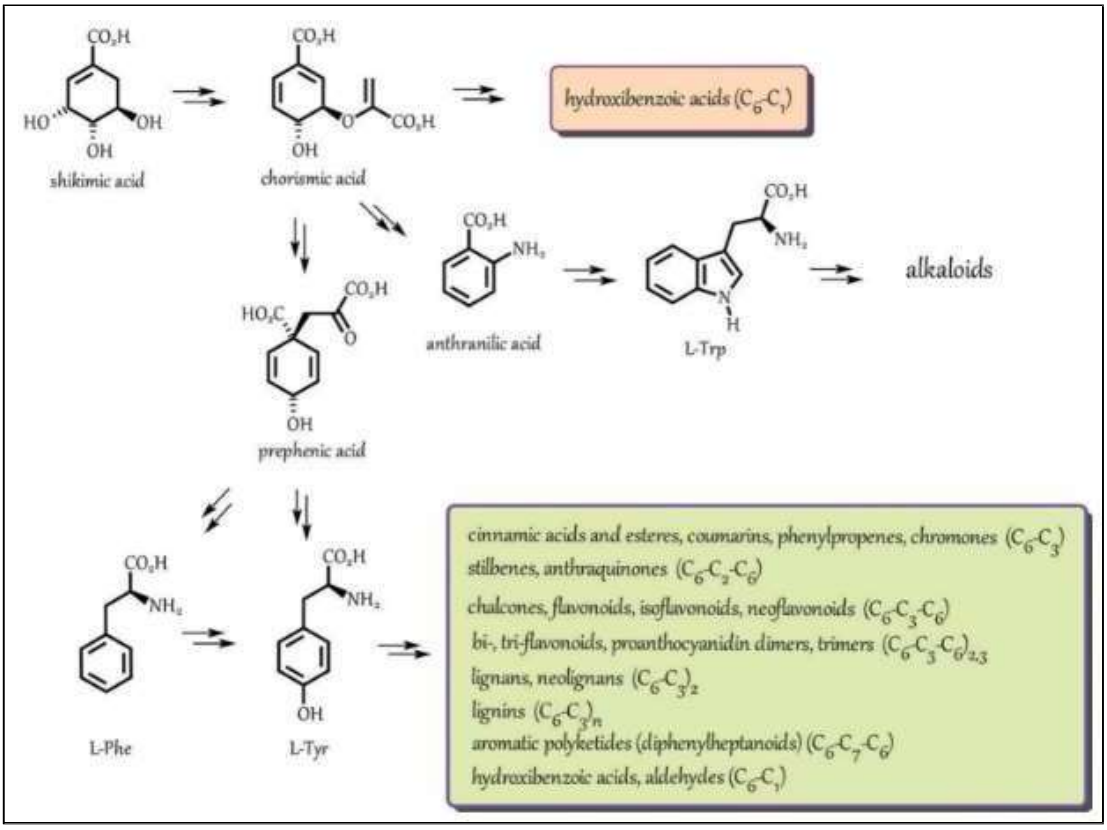

Figure 13: Common precursors shikimic acid and chorismic acid for the synthesis of Phe, Tyr and Trp and diverse phenolic compounds (Soto-Hernández et al. 2019).

iii. Alkaloids biosynthesis

Owing to the considerable diversity observed in alkaloids, both in terms of their structural variations and functional roles, the identification of a unified pathway for their biosynthesis within cellular contexts presents a formidable challenge. Substantial progress has been made in the exploration of alkaloid metabolism over the last half-century, from a combined biochemical, molecular, cellular, and physiological perspective has greatly improved the appreciation for the complex regulation of diverse biosynthetic pathways. Tropane alkaloids (TAs) are an example of a specific class of alkaloid and can be more specifically defined as all molecules that possess a tropane ring system. As illustrated in figure 14, TAs are esters of 3α-tropanole (Tropine), and the tropane skeleton (Green box) serves as the foundation for three major groups of TAs: hyoscyamine/scopolamine, cocaine, and the recently discovered calystegines group. The biosynthesis of the tropane ring system follows a homologous pattern across organisms producing these three TA classes. Initiated with the amino acids ornithine or arginine and their intermediary product putrescine, the process proceeds to the common *N*- methylpyrrolinium cation, precursor of all TAs. This is the branch point of cocaine, hyoscyamine/scopolamine and calystegine as well as nicotine, contributing to the complexity of alkaloid biosynthetic pathways (Facchini 2006; Kohnen-Johannsen and Kayser 2019; Kutchan 2012).

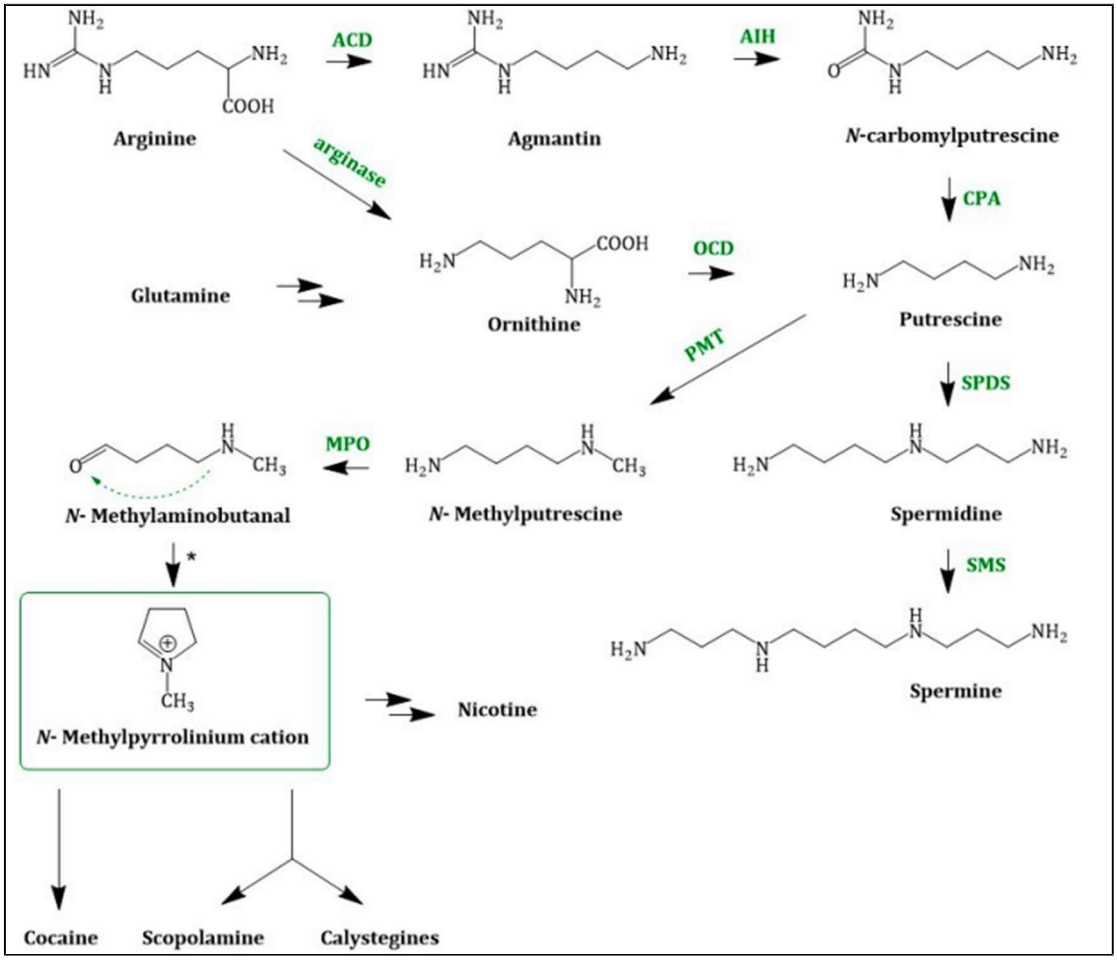

Figure 14: Joint steps of the early TA biosynthesis, ACD (argenine decarboxylase), AIH (argenine deiminase), OCD ornithine decarboxylase, CPA (*N*-carbamoylputrecsine amidase), PMT (putrecsine *N*-methyltransferase), SPDS (spermidine synthase), SMS (spermine synthase), MPO (*N*-methylputrecsine oxidase), ∗ (spontaneous cyclization) (Kohnen- Johannsen and Kayser 2019).

I. Introduction

Plants exhibit the capacity to produce two types of oils, namely fixed oils and essential oils (EOs), the latter being volatile in nature. Fixed oils are comprised of esters formed by glycerol and fatty acids, specifically triglycerides or triacylglycerols. Essential oils, on the other hand, comprise complex combinations of organic compounds, both volatile and semivolatile, and are considered significant secondary metabolites. These oils are generated in numerous plant species through intricate natural pathways involving diverse enzymatic reactions, influencing the distinct aroma, flavor, and fragrance of the plants (Tisserand and Young 2013; Rehman et al. 2016).

Essential oils may consist of approximately 20 to even up to 100 different secondary metabolites, spanning various chemical classes (Carson and Hammer 2011). Although some essential oils may contain 100 to 250 identified components (De Groot 2016), over 3000 essential oils have been extracted from at least 2000 plant species, with 300 holding commercial significance (Moghaddam and Mehdizadeh 2017). Variations in essential oil composition across different plant parts can be attributed, in part, to the existence of distinct secretory structures heterogeneously distributed throughout the plant body, such as oil cells, oil ducts, resin ducts, glands, or trichomes (Glandular hairs). This distribution not only mitigates the risk of autotoxicity but also facilitates the presence of high levels of metabolic components in locations where their defensive and/or attractive functions are crucial (Carson and Hammer 2011; Figueiredo et al. 2008).

Essential oils commonly exhibit organoleptic and physical characteristics. They are typically clear and fluid, with some exceptions being solid or semisolid at room temperature. While most essential oils are colorless or pale yellow, some possess deeper hues, such as blue chamomile and green European valerian. The distinctive aroma of essential oils is contingent upon the plant's organs, species, and origin. These oils are volatile, having a characteristic odor and density less than unity, although exceptions exist. They are displaying a high refractive index and optimal rotation due to the presence of asymmetrical compounds. Despite being hydrophobic, essential oils demonstrate solubility in fats, alcohols, and various organic solvents. Additionally, they are susceptible to oxidation, leading to the formation of resinous products through polymerization (Moghaddam and Mehdizadeh 2017; Hanif et al. 2019).

II. Biosynthesis of essential oils

Essential oils are typically a complex mixtures of volatile terpenes and phenylpropanoids, with some also containing moderate quantities of hydrocarbons or sulfur compounds (Barra 2009). While terpenes and their oxygenated derivatives (terpenoids) are more prevalent and abundant in EOs (Moghaddam and Mehdizadeh 2017), the primary components of EOs are derived through three biosynthetic pathways. These pathways include the mevalonate pathway (MVA) leading to sesquiterpenes, the methylerythritol pathway (MEP), also known as the 1-deoxy-D-xylulose-5-phosphate pathway (DXP), leading to mono- and diterpenes, and the shikimic acid pathway leading to phenylpropanoids. Essential oils represent the final products of terpenoid and phenylpropanoid synthesis, involving a diverse array of enzymes (As detailed in the biosynthesis steps discussed in chapter 2).

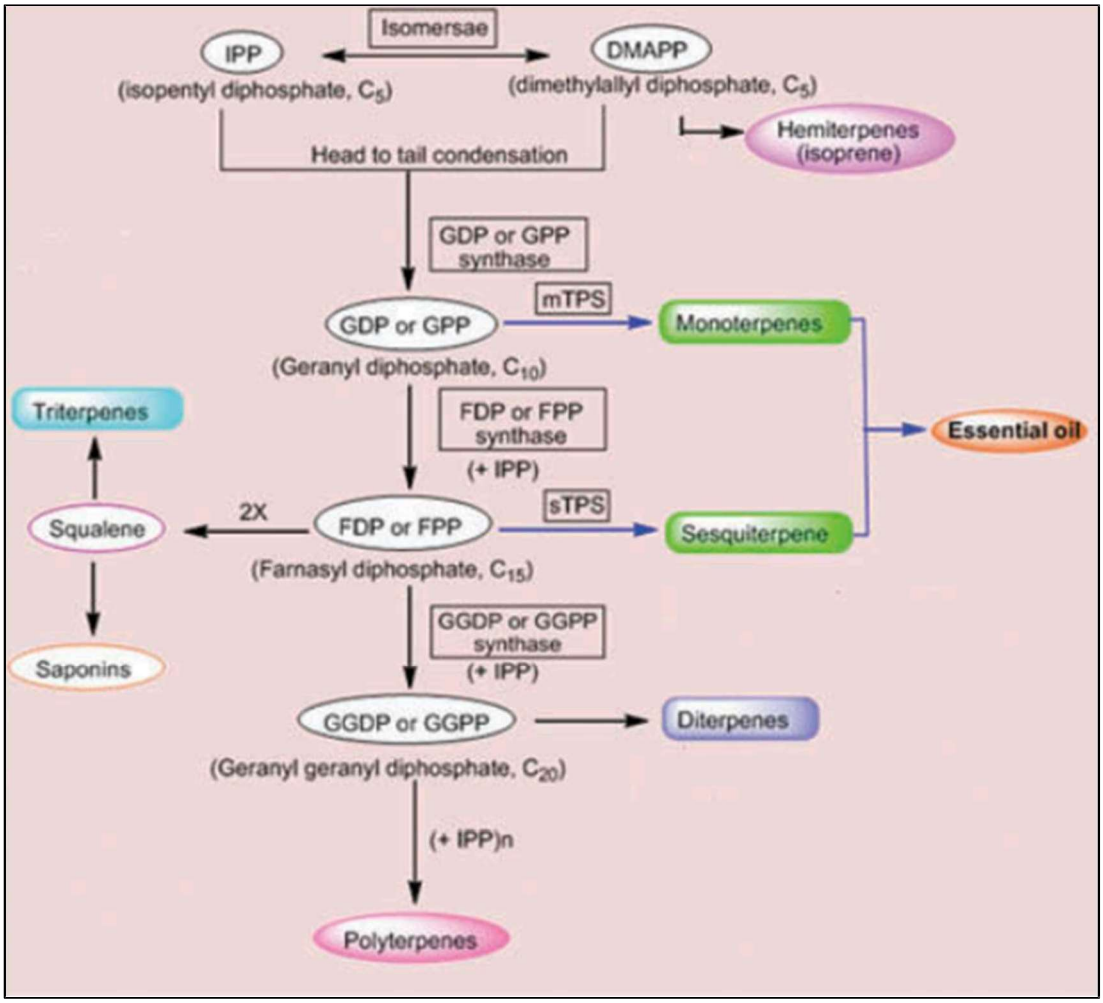

Figure 15: Synthesis of various classes of terpenoids (EOs) in plants (Rehman et al. 2016).

III. Classification of essential oil composition

The constituents of plant EOs fall mainly into two distinct chemical classes: terpenes and phenylpropanoids. Terpene compounds can be divided into two main categories: 1) terpenes with a hydrocarbon structure: mainly the mono-, sesqui-, and diterpenes and 2) their oxygenated derivatives: for instance, alcohols, phenols, aldehydes … Some chemical compounds found in EOs are classified in the table below (Moghaddam and Mehdizadeh 2017).

Table 2: Compounds found in essential oils.

Essential oil compounds		
Hydrocarbon moieties	Terpenoids	Monoterpene Sesquiterpene
	Aliphatic	Open chain Alkanes and alkenes
	Cyclic	Cyclic alkanes and alkenes
Functional groups	Aromatic *Phenylpropanoids*	Benzene ring
	Aromatic alkenes Alcohols Phenols Ketones Carboxylic acids Carboxylic esters Lactones Ethers and oxides Peroxides Furans	Benzene ring
Other compounds	Sulfur compounds Nitrogen compounds	

IV. Factors influencing essential oil composition and production

The presence, yield, and composition of secondary metabolites within plants, encompassing volatile elements and those found in essential oils, can undergo various influences throughout their entire life cycle, from their formation in the plant to the ultimate isolation process (Figueiredo et al. 2008).

Factors impacting the chemical makeup of EOs are categorized as either exogenous or endogenous (Table 3). Exogenous factors, which are environmentally regulated, encompass various abiotic elements known to significantly affect the emission and composition of plant EOs, potentially modify the qualitative and quantitative levels of volatiles in the EOs such as light, precipitation, growing location, and soil characteristics. Conversely, endogenous factors are intricately connected to the anatomical and physiological attributes of plants, contributing to chemical variations among different plant parts and influenced by genetically-related factors (Barra 2009; Prins et al. 2010). Endogenous factors are related to the site of production and accumulation of the EOs within the plant, the age of the plant, and the genetic traits governing the secondary metabolism. Additionally, environmental conditions can influence the DNA of aromatic plants, resulting in variations from chemotypes to distinct genotypes (The chemotype represents a multi-individual chemical variation of a plant's secondary metabolites induced by geographical location) (Barra 2009; Prins et al. 2010; Lima et al. 2021).

Table 3: Factors that influence the production and composition of plant EOs (Figueiredo et al. 2008).

Exogenous factors	Endogenous factors
• Climate/seasonal variations (Light, precipitation). • Geographic variations (Growing site, nature of the soil). • Pollution.	• Physiological variations (Type of plant material and organ development, pollinator activity cycle, type of secretory structure). • Genetic factors and evolution.

V. Analytical techniques for determining essential oil composition

Most analyses of EOs employ chromatographic methods, particularly gas chromatography (GC), to separate individual components for identification using specialized techniques. GC, especially when coupled with mass spectrometry (MS), is highly effective for detecting minor chemical constituents by examining compound fragmentation patterns under ionizing conditions, aiding in structural deduction (Tisserand and Young 2013).

In GC, the separation is depending on the partition or differential distribution of the individual component between the stationary phase (An inner coating of the chromatographic column) and the mobile phase (Gas phase). Achieving this requires the volatilization of the sample at the chromatograph's injection port, typically maintained at or above 200 °C. The EO liquid solution transitions to the gas phase (Using a carrier gas like N2, He, or H2), transporting volatilized substances to the chromatographic column. Here, they interact with a thin polymer film (Of polar, non-polar, or intermediate nature) on the inner walls of the fused-silica capillary column, in a temperature/time-dependent process. Analytes are subsequently identified, and a signal is documented, producing a chromatogram (A graph illustrating signal intensity over time). Ideally, peaks demonstrate a curve shape resembling a Gaussian distribution. The area and height of peaks correspond to the quantity of solute, while the width indicates band spreading within the column. Additionally, the retention time can be associated with the identity of the solute. Consequently, the chromatogram provides qualitative and quantitative analysis information. Various analytical techniques are commonly employed to characterize EOs, including GC with Linear Retention Indices (GC-LRI), GC-MS, Fast GC, GC Enantiomer Characterization, Multidimensional GC, and Liquid Chromatography (LC) techniques (Marriott et al. 2001; Braithwaite and Smith 2012; Stashenko and Martinez 2017).

i. Gas chromatography and Linear Retention Indices in EOs analysis

In the context of analyzing EOs through gas GC, the selection of the capillary column holds significant importance in the comprehensive characterization of the matrix. Factors such as the stationary chemical nature, film thickness, column length, and internal diameter must be carefully considered. Typically, EOs GC analyses are conducted using extended chromatographic columns, ranging from 25 to 60 meters, with internal diameters of 0.20 to

0.32 mm and stationary phase film thicknesses of 0.25 or 0.33 µm (Marriott et al. 2001; Stashenko and Martinez 2017).

It is crucial to note that the degree of separation of compounds on distinct stationary phases can vary significantly. Nonpolar columns generally facilitate boiling-point separations, while polar stationary phases resolve compounds based on their polarity. Given that EO compounds, such as terpenes and their oxygenated derivatives, often have similar boiling points, they may elute within a narrow retention time range on nonpolar columns. To address this limitation, the analytical method can be adjusted by either applying a slower oven temperature rate to widen the elution range of the oil or utilizing a polar stationary phase, as oxygenated components tend to exhibit greater retention than hydrocarbons. In the case of GC analysis using detectors like flame ionization detector (FID) or thermal conductivity detector (TCD), which do not offer structural information of the analyzed molecules, retention data, specifically retention indices, serve as the primary criterion for peak assignment. The retention index system relies on referencing each analyte's position between two n-paraffins bracketing its retention time. Additionally, the index calculation involves linear interpolation based on the carbon chain length of these bracketing paraffins (Marriott et al. 2001; Hüsnü Can Başer and Buchbauer 2015).

ii. Gas Chromatography-Mass Spectrometry

In academic parlance, mass spectrometry (MS) can be defined as the investigation of systems through the generation of gaseous ions, with or without fragmentation, subsequently characterized by their mass-to-charge (*m/z*) ratios and relative abundances (Marriott et al. 2001). The analysis using MS necessitates the ionization of the molecule through the application of accelerated atoms, ions, electrons, or photons, or exposure to a high electrostatic field gradient, or thermal impact, potentially followed by chemical bond cleavage (Stashenko and Martinez 2017).

Mass spectrometry proves to be a widely utilized tool for both routine analytical experiments and fundamental research, owing to various features such as its cost-effectiveness, design simplicity, and exceptionally rapid data acquisition rates. Despite the sample's destruction during MS, the technique exhibits high sensitivity, enabling analysis with minimal amounts of material.

Furthermore, the integration of advanced data acquisition and processing systems, encompassing automated library search techniques, has ensured that the extensive data produced by GC-MS can be effectively managed. This involves comparing unknown spectra with those in a reference MS and employing search algorithms from a library. The use of retention indices, coupled with the structural insights provided by GC-MS, is widely acknowledged and routinely applied to verify the identity of compounds.

II. Bioactivity and uses of essential oils

Essential oils are secondary metabolites typically synthesized by plants to combat infectious or parasitic agents or in response to stress conditions. They have been extensively utilized across various industries, with their primary components, monoterpenes and sesquiterpenes, serving as key agents for flavor, fragrance, and preservatives in foods and beverages, pharmaceuticals (therapeutic action), cosmetics, perfumes, and soaps (Berger 2007; Rehman et al. 2016).

The commercialization of essential oils can be strategically focused on their bioactivity. In this context, ongoing research endeavors aim to discover new applications and uses, demonstrating that essential oils and their constituents possess diverse functional properties. These include anti-mutagenic effects (Mimica-Dukić et al. 2010), neuroprotective, anti- aging, and skin-irritation potential (Ayaz et al. 2017; Rahmi et al. 2021), antitumor effects (Suhail et al. 2011), anti-inflammatory effects (Silva et al. 2003), digestive effects (Jang et al. 2007), as well as antioxidant, antimicrobial, and antiviral activities (Ćavar et al. 2008; Schnitzler et al. 2011). It is noteworthy that the actions of essential oils extend beyond mammals, affecting other organisms such as insects, fungi, bacteria, and viruses (Berger 2007).

I. Introduction

Reactive oxygen and nitrogen species (ROS/RNS) are active substances formed within living systems for specific functions, and they can also be acquired from the external environment. Increased levels of ROS/RNS pose challenges to cells in tissues and organs due to their detrimental oxidative properties. Enzymes, metalloproteins, or small metabolites are the counteractive agents for these reactive species, as antioxidant substances which are produced in vivo or can be obtained externally through dietary sources and medications. Both reactive species and antioxidants play a role in the onset of oxidative stress (Ahmed and Mohammed 2020).

II. Free radicals and reactive oxygen/nitrogen species

In the realm of biology and medicine, the term "free radical" refers to any chemical substance that possesses one or more unpaired electrons in its outer orbitals, enabling it to exist independently. In the most cases, the terms ‘‘free radicals’’ and ‘‘reactive oxygen/nitrogen species’’ are used interchangeably. If in many cases that is correct, in some ones it is wrong (Lushchak 2014; Halliwell and Gutteridge 2015)

Possibly, the most straightforward way to distinguish between these two terms is by analyzing their generation, interconversion, and elimination. These processes are schematically presented in the accompanying figure 16. In living organisms under aerobic conditions, over 90% of consumed oxygen undergoes direct reduction to water by cytochrome oxidase in the electron transport chain (ETC) through a four-electron mechanism, without the release of ROS (Halliwell and Gutteridge 2015). Less than 10% of consumed oxygen follows one-electron successive pathways, resulting in the conversion of molecular oxygen to the superoxide anion radical ($O_2^{-\cdot}$), followed by one-electron reduction and the acceptance of two protons to yield hydrogen peroxide (H_2O_2). This molecule is not a free radical, although it is chemically more active than molecular oxygen and is included in the ROS category. Hydrogen peroxide, upon accepting one more electron, breaks down into the hydroxyl radical ($HO^{\cdot}$) and hydroxyl anion (OH^-). Ultimately, the $HO^{\cdot}$ interacts with one more electron and proton, leading to the formation of a water molecule. In biological systems, this reaction is primarily realized through the abstraction of a hydrogen atom from various compounds, such as proteins and lipids, often initiating chain processes

(Lushchak 2014). To summarize, $O_2^{-\cdot}$, H_2O_2, and $HO^{\cdot}$ collectively are termed reactive oxygen species, but only $O_2^{-\cdot}$ and $HO^{\cdot}$ qualify as free radicals, whereas H_2O_2 does not.

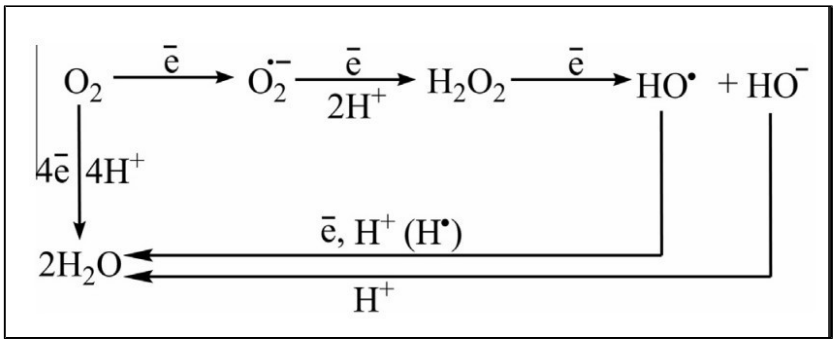

Figure 16: Reduction of molecular oxygen via four- and one-electron pathways.

Reactive oxygen species include also, singlet oxygen ($^1O_2$), peroxyl radical ($LOO^{\bullet}$), alkoxyl radical ($LO^{\bullet}$), lipid hydroperoxide (LOOH), hypochlorous acid (HOCl), and ozone (O_3). In addition to reactive nitrogen, iron, copper, and sulfur species that are also being as part of the spectrum.

Table 4: Reactive oxygen and nitrogen species of biological interest (Ahmed and Mohammed 2020).

Reactive species	Symbol	Half-life (s)	Reactivity / Remarks
Reactive oxygen species:			
Superoxide anion radical	$O^{2-\cdot}$	10^{-6} s	Generated in mitochondria, in cardiovascular system and others.
Hydroxyl radical	$HO^{\cdot}$	10^{-9} s	very highly reactive, generated in Fenton reaction.
Hydrogen peroxide	H_2O_2	Stable	Formed in the body by large number of reactions.
Peroxyl radical	$LOO^{\bullet}$	s	Reactive and formed from lipids, DNA, proteins, etc.
Organic hydroperoxide	ROOH	Stable	Reacts with transit metal ions to produce reactive species.
Ozone	O_3	s	Present in the atmosphere

Table 4: Reactive oxygen and nitrogen species of biological interest (Rest of the table).

Singlet oxygen	$^1O_2$	10^{-4} s	Highly reactive formed during photosensitization and chemical reactions.
Reactive nitrogen species:			
Nitric oxide	·NO	s	Neurotransmitter and blood pressure regulator.
Peroxynitrite	$ONOO^-$	10^{-3} s	Formed from superoxide and nitric oxide.
Nitrogen dioxide	·NO2	s	Formed during atmospheric pollution.

The half-life of some radicals depends on the environmental medium, for example the half-life of NO• in an air saturated solution may be few minutes.

i. Sources of free radicals

Reactive species can arise from both endogenous and exogenous sources. The major mechanism for generating ROS/RNS in aerobic organisms occurs during the reduction of oxygen in the ETC during cellular respiration. Additional contributors to the production of reactive species include xanthine oxidoreductase, peroxisomes (Involved in fatty acid metabolism), and the activation of inflammatory cells (Valko et al. 2006; Bedard and Krause 2007). Exogenous sources of reactive species encompass exposure to environmental factors such as ozone, (Prevalent air pollutant), as well as exposure to ionizing and UV radiations. Metabolism of xenobiotics and alcohol in the liver can further contribute to the production of ROS. Furthermore, various chemical compounds found in cigarette smoke and pesticides can induce oxidative stress in organisms that are exposed to them (Valko et al. 2006; Bedard and Krause. 2007; Ahmed and Mohammed 2020).

III. Antioxidants

i. Definitions and classifications

The term "biological antioxidant" refers to any substance that, when present in lower concentrations than an oxidizable substrate, can prevent and repair damages caused by reactive oxygen species/reactive nitrogen species (ROS/RNS). Both endogenous and

exogenous antioxidants function as "free radical scavengers" enhance immune defense and reducing the risk of diseases, including cancer. These defense systems, found in aqueous and membrane cell compartments, can be enzymatic or non-enzymatic (Gupta et al. 2014; Pisoschi and Pop 2015).

Redox homeostasis of the cell relies on a sophisticated endogenous antioxidant defense system, comprising enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), as well as non-enzymatic antioxidants like glutathione, proteins (Ferritin, transferrin, albumin), and low molecular weight scavengers such as uric acid, coenzyme Q, and lipoic acid (Gupta et al. 2014). Research confirms that exogenous antioxidants from fruits and vegetables, notably including vitamin C and E, carotenoids, and phenolics, complement the activity of endogenous antioxidative defense. Another source of exogenous antioxidants, is dietary supplements that become a valuable source when antioxidants mentioned below (Table 5) are insufficient or lacking in the regular diet (Pisoschi and Pop 2015).

Table 5: Classification of antioxidants.

	Source	**Antioxidant**
Endogenous	Enzymatic antioxidants	SOD, CAT, glutathione-synthesizing enzymes, GPx, GRx, glutathione S-transferase, glutaredoxin, thioredoxin, peroxiredoxin, thioredoxin reductase, sulfiredoxin, methionine sulfoxide reductase, hemeoxygenase, NADPH: quinone oxidoreductase, paraoxonase.
	Non-enzymatic antioxidants	Glutathione, bilirubin, coenzyme Q, estrogens, lipoic acid, melatonin, metal-chelating proteins, ferritin, and uric acid… etc.
Exogenous	dietary antioxidants	Vitamin C, vitamin E, carotenoids, phenolic compounds.
	Laboratory synthesized antioxidants	Antioxidants enzyme mimetics, glutathione precursors, spin traps, and nanoparticles.

ii. Mechanism of action of antioxidants

The antioxidant process operates through two distinct mechanisms: chain-breaking and prevention. In the chain-breaking process, when a radical releases or seizes an electron, it gives rise to a second radical. This subsequent radical perpetuates the process by affecting another molecule, and this cycle continues until either a chain-breaking antioxidant (Such as vitamin C, E, carotenoids, etc.) stabilizes the newly formed free radical, or the radical disintegrates into a harmless product through hydrogen donation, quenching singlet oxygen, peroxide decomposing, oxidative enzyme inhibition, or absorption of UV radiation. In the preventive mechanism, antioxidant enzymes like SOD, CAT, and GPx play a role in averting oxidation by diminishing the rate of chain initiation. This can occur either by scavenging initiating free radicals or by stabilizing transition metal radicals like copper and iron (Gupta et al. 2014; Pisoschi and Pop 2015).

IV. Oxidative stress

Oxidative stress is defined as an imbalance between the presence of ROS/RNS and the organism's ability to counteract their effects through antioxidative defense mechanisms. This condition is characterized by a diminished capacity of endogenous systems to combat oxidative attacks on target biomolecules (Pisoschi and Pop 2015). Several factors contribute to this phenomenon, including elevated levels of both endogenous and exogenous compounds leading to autoxidation and ROS/RNS production, a reduction in the production of antioxidant enzymes and low molecular mass antioxidants, or a combination of these two factors (Lushchak 2014).

Reactive species function as signaling molecules in small quantities, participating in the regulation of cell proliferation, apoptosis, and gene expression through the activation of transcription factors (Pisoschi and Pop 2015). Under normal conditions, the production and elimination of these species are well balanced, maintaining a steady-state level of ROS. However, certain circumstances can disrupt this equilibrium (Figure 17). If the antioxidant systems effectively handle increased ROS/RNS levels under stressful conditions, the situation can be termed "acute oxidative stress," and the ROS level returns to its initial state. Alternatively, when cells are unable to neutralize elevated ROS/RNS amounts, a disturbance in homeostasis occurs, leading to a state known as "chronic oxidative stress." Following oxidative events or changes in the physiological state of organisms, the ROS/RNS level may

not revert to the initial state but stabilizes at a new, termed "quasistationary level," as illustrated in the figure (Lushchak 2014).

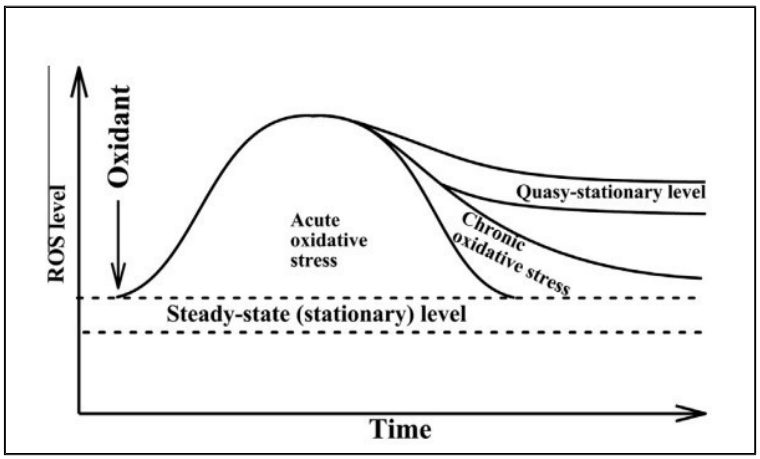

Figure 17: Dynamic of ROS/RNS level under control and stressful conditions in biological systems (Lushchak 2014).

V. Oxidative stress and pathology

At low or moderate levels, ROS/RNS demonstrate positive effects on cellular responses and immune function. However, when present in elevated concentrations, they induce oxidative stress. Free radicals primarily target three key cellular sites. The initial site involves lipid components, leading to the peroxidation of membrane lipids. The second site pertains to cellular proteins, resulting in sidechain oxidation, backbone fragmentation, unfolding, misfolding, and subsequent loss of activity. The third site is the DNA, where the consequences include mutations and the potential development of cancer. Ultimately, these processes may trigger cell apoptosis and necrosis, contributing to the degradation of various cell structures. These detrimental mechanisms play a role in the pathogenesis and pathophysiology of numerous chronic health issues, as outlined in the table below (Valko et al. 2007; Gupta et al. 2014; Ahmed and Mohammed 2020).

Table 6: Disease and related conditions in which ROS/RNS play a contributing role.

Disease	Description	References
Alzheimer's disease	Neurodegenerative disorder characterized by cognitive decline, linked to oxidative damage in the brain	Zhu et al. 2007
Parkinson's Disease	Progressive nervous system disorder, associated with oxidative stress leading to degeneration of dopamine-producing neurons.	Dias et al. 2013
Cardiovascular disease	Hypertension, atherosclerosis, ischemic heart disease, cardiomyopathies and congestive heart failure.	Dhalla et al. 2000
Diabetes and metabolic syndrome	Diabetes, diabetic complications, obesity, and insulin resistance.	Maritim et al. 2003
Neurodegenerative diseases	Apart from Alzheimer's and Parkinson's, conditions like Huntington's disease and amyotrophic lateral sclerosis (ALS) involve oxidative stress	Chen et al. 2012
Pulmonary diseases.	Oxidative stress plays a role in the pathogenesis of chronic obstructive pulmonary disease (COPD) contributing to airway inflammation and tissue damage, asthma, hyperoxia-induced lung injury, and pulmonary toxicity.	MacNee 2001
Hepatic diseases	Alcoholic fatty liver disease, non-alcoholic fatty liver disease, hepatotoxicity, inflammatory bowel disease, and IR injury.	Li et al. 2015
Eye diseases	Cataract, and age-related macular degeneration.	Ung et al. 2017
Skin diseases	UV-induced skin injury, Alopecia areata, scleroderma, Rosacea, contact dermatitis, and Psoriasis.	Baek and Lee 2016

Table 6: Disease and related conditions in which ROS/RNS play a contributing role (Rest of the table).

Cancer	Oxidative stress is implicated in DNA damage and mutations, promoting the development and progression of various cancers.	Matsuda and Shimomura 2013
Aging	Irreversible buildup of ROS-induced damage influences critical aspects of aging, leading to impaired physiological functions, an increased incidence of diseases, and a reduction of life span.	Kregel and Zhang 2007
Chronic Inflammatory Diseases	Conditions like rheumatoid arthritis and inflammatory bowel diseases are associated with persistent oxidative stress and inflammation	Sánchez et al. 2015
Infections	Viral and bacterial infections.	Pohanka 2013

Part II:

Material and methods

I. Plant material and presentation of the harvest region

The two plant species belonging to the *Ononis* genus (Fabaceae) were collected in March 2021 during the flowering stage from two distinct sites within the province of Biskra (Table 7 and figure 18). These regions exhibit characteristics of a dry and hot desert climate classified as "BWh" according to the Köppen classification (Beck et al. 2018). The identification of both species was conducted based on relevant literature sources (Širjaev 1932; Berghen 1978; Förther and Podlech 1991; Turini 2010), and authenticated by Doctor Tarek Benmeddour (Supervisor of this research), department of nature and life sciences, university of Biskra, Algeria, and also verified by Professor Laouar Houcine from the department of natural and life sciences at the university of Sétif, Algeria (Personal communication). Over 40 plants were sampled to ensure representativeness. A reference specimen is deposited in the herbarium of the laboratory of genetics, biotechnology, and valorization of bioresources at the University of Biskra, under the code PHA–F015–S3–2021 for *O. aurasiaca* and FAB-F015-S6-2021 for *O. angustissima* Lam. subsp. *filifolia* Murb.

The aerial parts were cleaned with tap water and subsequently dried at room temperature in the shade for a month. The plant material employed primarily comprises leaves, flowers, and pods.

Table 7: Geographic coordinates of harvest sites

Harvest Region	Longitude	Latitude	Altitude	Distance from Biskra
El Hadjeb	5° 35′ 49″ E	34° 47′ 25″ N	150 m	14.1 km
M'Chouneche (Baniane)	6° 03' 00" E	34° 59' 00" N	559 m	41.0 km

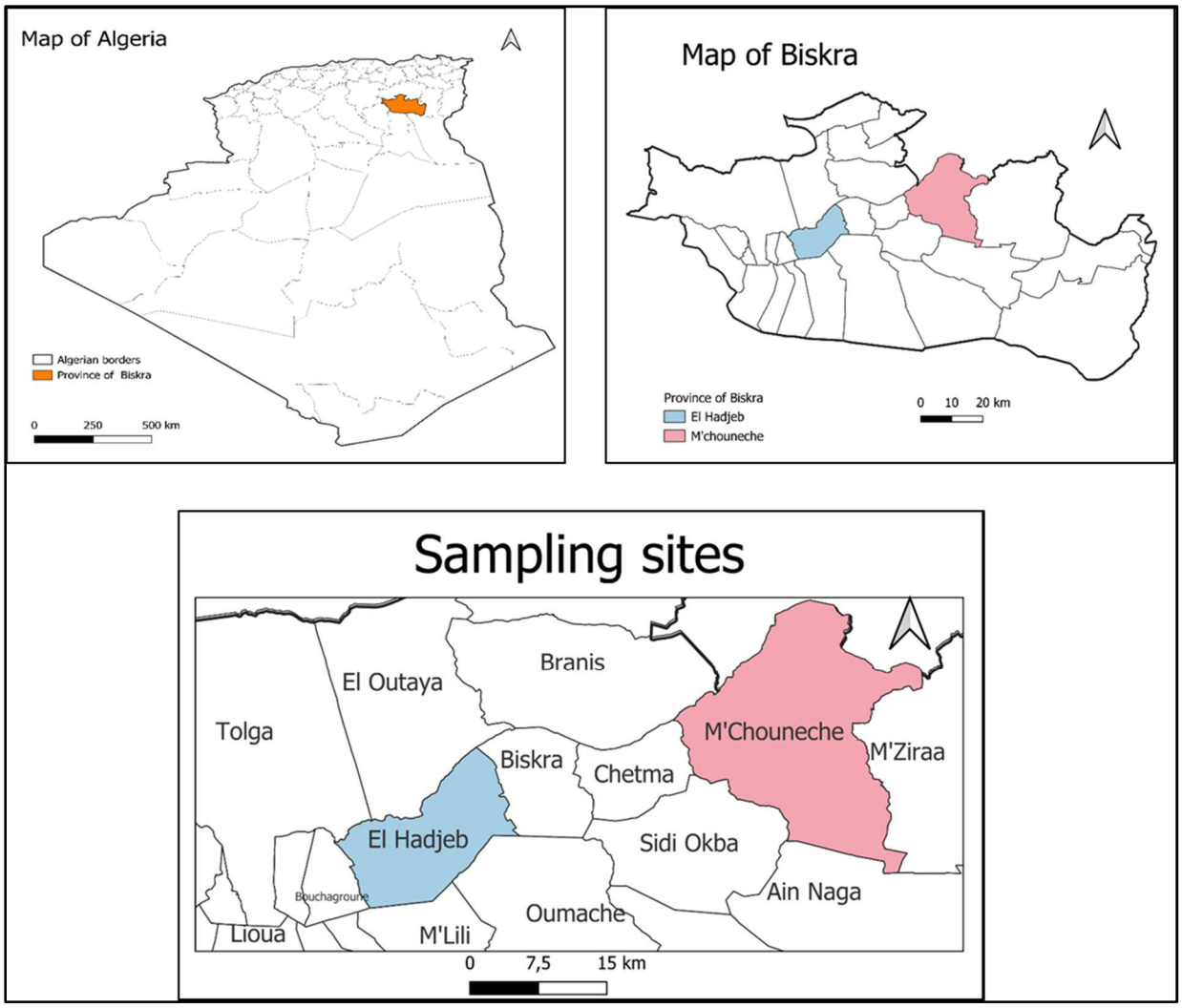

Figure 18: Localization of the harvest region (Original map).

II. Preparation of extracts

i. Liquid-solid extraction using organic solvents

The dried aerial part was coarsely ground and sieved through a 1 mm mesh. 100 g of the obtained powder were successively macerated in solvents of increasing polarity (1500 ml each): petroleum ether (PE), dichloromethane (DCM), and methanol (Me-OH), respectively (Biochem Chemopharma, Cosne sur Loire, France), following the method described by Yakhlef et al. (2011) with modifications. The operation was repeated three times for each solvent and each plant to thoroughly deplete the plant material. Maceration was carried out in darkness at room temperature under continuous agitation for 24 hours. The mixture was filtered each time, and the obtained filtrates were combined and concentrated using a rotary evaporator (Heidolph). The final residue was then dried in an oven at 40°C until the weight

of the crude extract was stabilized. The extract was subsequently stored in opaque, hermetically sealed vials at 4°C until further use (Figure 19).

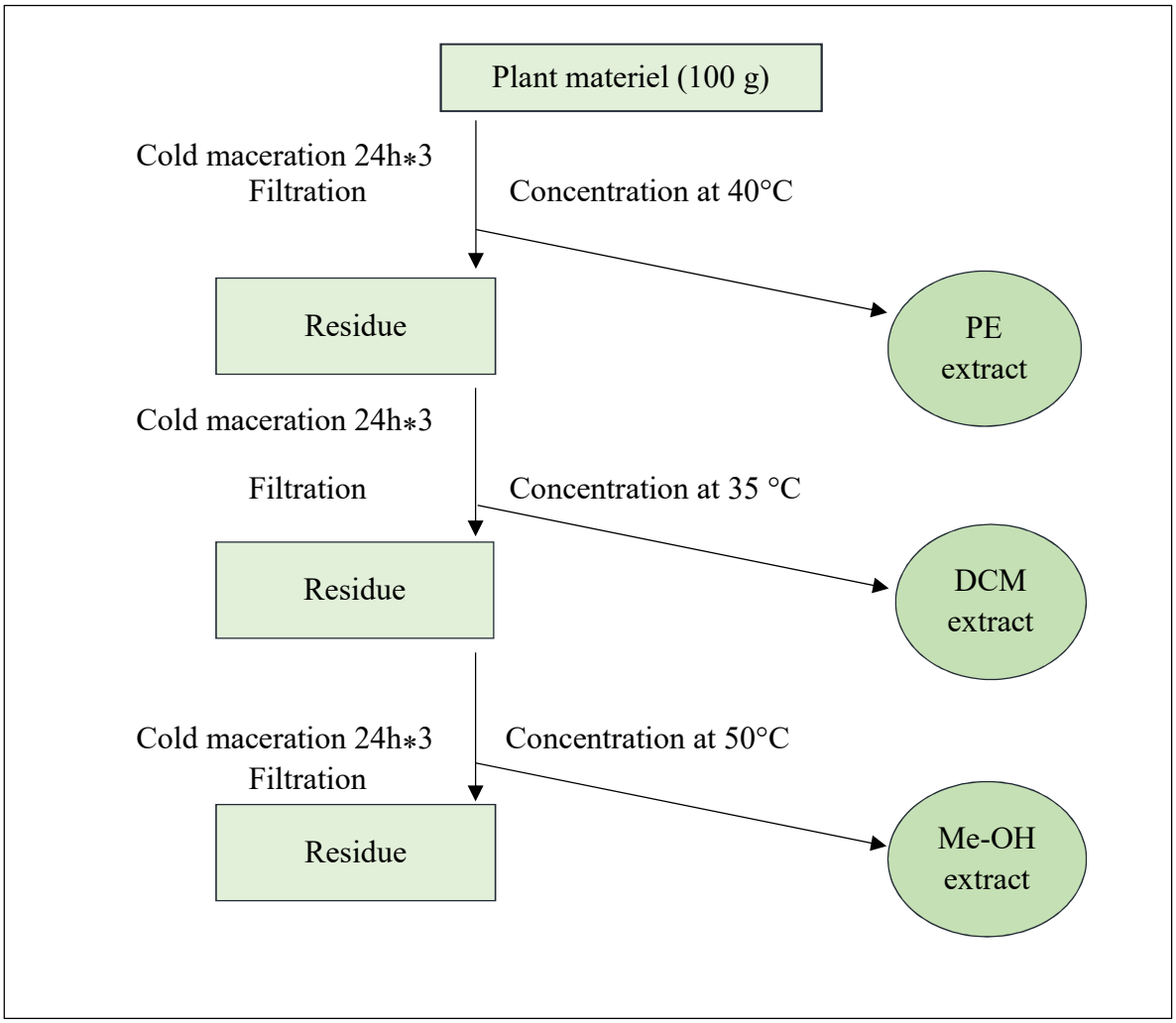

Figure 19: Schematic representation of the liquid-solid extraction protocol using organic solvents with increasing polarity; PE, DCM and Me-OH.

ii. Extraction of essential oils

Essential oils are obtained through the distillation of the dried aerial parts of plants. The extraction process, pioneered by Clevenger, was conducted over a four-hour duration, and this procedure was reiterated multiple times, utilizing 1 kg of dried material for each cycle. The plant material is placed within a flask positioned atop a flask filled with distilled water (2000 ml). The water vapor passing through the flask ruptures the plant cells and, through a chemical reaction, carries certain components of the essential oils along with it. Following the condensation of vapors and the addition of petroleum ether (Biochem Chemopharma,

Cosne sur Loire, France), the resulting oils were recovered through decantation. Subsequent to the solvent evaporation, the essential oils were stored at 4 °C in opaque glass tubes.

iii. Determination of extraction yield

The yields of organic extracts (ECC) and/or essential oils (Y_{EO}) were determined as a percentage (%) relative to the weights of the dry plants used, according to the following formulas:

$$ECC\ (\%) = \frac{OE\ (g)}{PP\ (g)} * 100 \qquad Y_{EO}\ (\%) = \frac{EO\ (g)}{PM\ (g)} * 100$$

Where:

- **ECC**: **E**xtractable **C**ompound **C**ontent

- **OE**: Weight of the **O**rganic **E**xtract obtained after the removal of all solvent traces

- **PP**: Weight of the dry **P**lant **P**owder used for liquid-solid extraction

- **Y_{EO}**: **Y**ield of **E**ssential **O**il

- **EO**: Weight of the **E**ssential **O**il obtained by hydrodistillation

- **PM**: Weight of the **P**lant **M**aterial intended for hydrodistillation.

III. Determination of total polyphenols and flavonoids in crude extracts

i. Determination of total polyphenols

Principle

The Folin-Ciocalteu method is widely employed to ascertain the total polyphenol content. This method involves a colorimetric reaction based on the reduction of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_4$) during the oxidation of phenols, resulting in the formation of a blue chromophore complex composed of tungsten (W_8O_{23}) and molybdenum (Mo_8O_3) oxides (Blainski 2013).

The intensity of the coloration produced is contingent upon the solution's pH, is proportionate to the quantity of polyphenols in the analyzed extract, and is measurable in the visible spectrum at a wavelength around 760 nm (Bueno 2012).

Procedure

A calibration curve was prepared with precise concentrations (0-200 µg/ml) of gallic acid (Sigma–Aldrich, Germany), under the same experimental conditions as the six crude extracts. Subsequently, 1000 µl of the 10% Folin-Ciocalteu solution (Sigma–Aldrich, Germany) was added to 200 µl of the extract or standard antioxidant. After agitation and a 3-minute rest, 800 µl of Na_2CO_3 solution (7.5%) was added (Sigma–Aldrich, Germany). The entire mixture was incubated for 5 minutes at 50 °C in darkness, and the absorbance was read at 760 nm against a blank without extract (Dinçer et al. 2013).

The total polyphenol content was determined from the regression line of the calibration curve ($y = a\ x + b$), and the results are expressed in micrograms equivalent of gallic acid per milligram of powdered extract (µg GAE/mg).

ii. Determination of flavonoides

Principle

The quantity of total flavonoids in the crude extracts was determined using the aluminum trichloride method (Ghedadba et al. 2014). This method is commonly employed for the quantification of flavonoids in plant extracts. Aluminum trichloride ($AlCl_3$) is utilized to form a covalent bond with the hydroxyl (OH) groups of flavonoids, resulting in a yellow coloration. The intensity of the formed color is directly proportional to the concentration of flavonoids present in the extract and can be measured through spectrophotometry at 430 nm.

Procedure

A quantity of 1 ml of each extract and standard was added to 1 ml of 2% $AlCl_3$ solution (Sigma–Aldrich, Germany). Subsequently, absorbance was measured after an incubation period of 20 minutes at room temperature and in the absence of light, in comparison to the blank. The concentration of flavonoids was determined from the calibration curve range ($y = ax + b$) established with quercetin (0-40 µg/ml) (Sigma–Aldrich, Germany). The results were expressed in micrograms equivalent of quercetin per milligram of powdered extract (µg QE/mg).

IV. Chemical composition analysis of essential oils using Gas Chromatography-Mass Spectrometry (GC-MS)

Essential oils were diluted to 0.5% in HPLC-grade n-hexane and subsequently injected into a GC-MS apparatus. GC-MS analyses were conducted using an Agilent 7890B gas chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an Agilent HP-5MS capillary column (30 m × 0.25 mm; coating thickness 0.25 µm) and an Agilent 5977B single quadrupole mass detector.

The analytical conditions were as follows:

- Programmed oven temperature from 60 to 240 °C at a rate of 3 °C/min; injector temperature, 220 °C; transfer line temperature, 240 °C.
- Helium carrier gas at a flow rate of 1 ml/min. The injection volume was 1 µl, with a split ratio of 1:25.
- Acquisition parameters were as follows: full scan mode; scan range: 30–300 m/z; scan time: 1.0 s.

The identification of constituents relied on comparing retention times with those of authentic samples, juxtaposing their linear retention indices to the n-hydrocarbon series. Computer-assisted comparisons were also employed using commercial mass spectra libraries (NIST 14 and ADAMS 2007) and in-house libraries developed at the Department of Pharmacy, University of Pisa, via Bonanno Pisano 6, 56126 Pisa (PI), Italy. These libraries were constructed from pure substances and known components of commercial essential oils, along with literature MS data (Stenhagen et al. 1974; Masada 1976; Swigar and Silverstein 1981; Jennings and Shibamoto 1982; Davies 1990; Adams 2007).

V. Antioxidant activity assessment

i. DPPH free radical scavenging test

Principle

The antioxidant capacity of the extracts was determined using the method described by Al- Qudah et al. (2014) with modifications. This involved assessing the extracts' ability to scavenge the free radical DPPH (2,2-diphenyl-1-picryl-hydrazyl). DPPH is a stable radical with a lone electron, exhibiting a violet coloration (DPPH•). In the presence of an antioxidant, the color tends to decrease due to the reduction of free electrons and the

formation of a stable yellow compound, DPPH-H. The decolorization is proportional to the antiradical activity of standard or natural antioxidants (Hepsibha et al. 2010).

Procedure

The anti-radical activity of various extracts and essential oils from both plants was assessed based on the DPPH free radical scavenging reaction in comparison to BHA (Beta Hydroxy Acid), ascorbic acid, and quercetin, Ascorbic Acid, and Quercetin (Sigma–Aldrich, Germany). Different concentrations were prepared for standards, crude extracts and essential oils. To 1.5 ml of these solutions, 0.5 ml of the DPPH• solution (0.1 mM) was added (Sigma–Aldrich, Germany). The mixture was then incubated in darkness for 30 minutes, and the absorbance was subsequently measured at 517 nm against a blank. The DPPH radical scavenging capacity was calculated using the following equation:

$$\text{Anti-radical activity (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] * 100$$

A: Absorbance

ii. β-carotene bleaching assay

Principle

The β-carotene/linoleic acid method is a commonly employed simplified model for assessing the inhibitory activities against lipid peroxidation. β-carotene, a natural pigment and a precursor of vitamin A, exhibits strong biological activity but is susceptible to degradation when exposed to free radicals generated during the oxidation of linoleic acid in an emulsion, resulting in its discoloration or bleaching. The efficacy of the tested compound as an antioxidant is determined by its ability to trap free radicals, thereby slowing the bleaching of β-carotene. Antioxidant activity is often quantified through spectrophotometry by measuring the rate of color loss in the reaction mixture at specific time intervals. A slower rate of color change indicates strong antioxidant activity (Hsu et al. 2003; Athamena et al. 2010; Ghedadba et al. 2014).

Procedure

The assessment of the β-carotene bleaching activity of various extracts involved monitoring the decrease in absorbance over time using the method outlined by Tepe et al. (2006) with slight modifications. Specifically, 2 mg of β-carotene (Sigma-Aldrich) was dissolved in 1 ml of chloroform, followed by the addition of 25 µl of linoleic acid (Sigma-Aldrich) and 200 mg of Tween 40. The solvent was completely evaporated using a rotary evaporator under pressure (Heidolph), and then 100 ml of hydrogen peroxide was added with vigorous stirring. Subsequently, 350 µl of different extracts or essential oils (2 mg/ml) were added to 2.5 ml of the previously prepared emulsion. The same procedure was applied to the positive control (BHA). The negative control or blank was prepared by adding 350 µl of methanol. The reaction mixture was incubated in darkness at room temperature, and discoloration was monitored for 48 hours at 490 nm. The antioxidant activity (AA) was calculated using the following formula:

$$AA\ (\%) = [A_{48h}\ (\text{sample}) / A_{48h}\ (\text{BHA})] * 100$$

iii. Phenanthroline test for reducing activity

Principle

The reducing power of an extract is assessed in this assay by evaluating its ability to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). The Fe^{2+} ions then form a stable red-orange complex with phenanthroline, known as the Fe^{2+}-phenanthroline complex. The intensity of the coloration is directly proportional to the concentration of Fe^{2+} ions, which in turn is linked to the antioxidant activity of the extract. Higher antioxidant activity results in a stronger reduction of Fe^{3+} ions to Fe^{2+} ions, leading to a more intense color formation. This method provides a quantitative measure of antioxidant capacity and can be determined through spectrophotometry at 510 nm (Roy et al. 2011).

Procedure

The reducing activity was determined following the method described by Szydlowska-Czerniak et al. (2008). In brief, 10 µl of various extracts and/or BHA (Standard antioxidant)

at different concentrations were mixed with 50 µl of 0.2% ferric chloride ($FeCl_3$) (Sigma-Aldrich). Then, 30 µl of 0.5% phenanthroline (Biochem-Chemopharma) were added, followed by 110 µl of methanol. The mixture was incubated in darkness at 30 °C for 20 min. Absorbance was measured at 510 nm against a blank without extract. The result is expressed in terms of A0.5 (µg/ml), corresponding to the concentration with an absorbance of 0.5.

VI. *In silico* molecular docking: insights into antibacterial activity

Molecular docking involves positioning a compound (Ligand) in a way that allows it to interact with a protein (Receptor). This method was utilized to examine the binding affinity and types of interactions of selected phytochemicals with receptor proteins (Hatai and Banerjee 2019).

i. Ligand preparation

The 3D chemical structures of the main compounds in the essential oils of *O. aurasiaca* and *O. angustissima* were obtained from the PubChem library (https://pubchem.ncbi.nlm.nih.gov/). These files were downloaded in sdf (Spatial Data File) format and then converted to pdb (Protein Data Bank) format using the Open Babel UGI molecule format converter. The energy minimization of the ligands was performed using the PyRx tool.

ii. Selection of receptors as protein targets

MenB (PDB ID 3T88) from *E. coli* is a 1,4-dihydroxy2-naphthoyl-CoA synthase, an enzyme involved in menaquinone (Vitamin k2) biosynthesis in some Gram negative and most Gram-positive bacteria, which plays a crucial role in their electron transport chain. Mammalian cells cannot synthesize menaquinone, and thus the enzymes in the biosynthetic pathway of bacterial menaquinone are potential targets for developing novel antibacterial drug (Li et al. 2011).

FtsZ (PDB ID 3WGN) from *S. aureus*, a protein that hydrolyzes GTP, is a prokaryotic homolog of tubulin and plays a crucial role in cell division (Matsui et al. 2014). In many bacteria, FtsZ polymerizes at the division site to form the Z-ring, which acts as a scaffold for recruiting other division proteins and possibly generating the force needed for cell constriction. Due to its essential role in cell division, FtsZ has become a prime target in the search for new antibiotics (Xiao and Goley 2016).

LasR (PDB ID 2UV0) is involved in quorum sensing, a process where many Gram-negative bacteria use auto-inducer molecules to coordinate population-wide activities. This communication regulates the production of pathogenic virulence factors and antimicrobial resistance. The quorum sensing system of *P. aeruginosa*, an opportunistic pathogen responsible for many deaths among cystic fibrosis patients and other immunocompromised individuals, is the most extensively studied. Understanding the activation and inhibition mechanism of LasR offers a foundation for discovering or designing new quorum sensing inhibitors (Bottomley et al. 2007).

iii. Receptors preparation

Crystal structures of target proteins were obtained from RCSB PDB database (https://www.rcsb.org/). The proteins were prepared for docking, by removing water molecules, other associated ligands, and any unusual chains proteins in BIOVIA Discovery Studio. Then, polar hydrogens were added, and charges were assigned using the USCF Chimera tool, after which the structures were saved in pdb format.

iv. Molecular docking analysis

Molecular docking simulation was performed using PyRx software (Virtual screening tool, Version 0.8) developed by Trott and Olson (2010). Ligands and receptors were initially in pdb format and converted to pdbqt format within PyRx. Docking sites on target proteins were identified by creating grid boxes with specific dimensions (Angstrom) and centers for receptors 3T88, 3WGN, and 2UV0 (Table 8). The present tool predicts docking results by calculating the energy values for each ligand, and the resulting ligand/receptor complexes were visualized using Discovery Studio Visualizer to identify specific atomic interactions between the tested compounds and protein amino acids.

Table 8: Spatial information from grids formed around selected proteins.

Receptor	Grid box	X	Y	Z
3T88	Center	- 20.9818	23.0783	- 20.2801
	Dimension (Å)	83.5731	67.9903	86.9595
3WGN	Center	-8.7011	1.9534	-17.8831
	Dimension (Å)	67.5428	70.8221	100.4447
2UV0	Center	53.6957	25.8034	37.0438
	Dimension (Å)	36.2346	47.1842	48.2063

VII. *In vitro* antibacterial activity

Four bacterial strains, comprising two Gram-negative strains (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) and two Gram-positive strains (*Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus* ATCC 43300), were used for investigation. These strains were sourced from the isolate collection at Benbadis Hospital University Center in Constantine, Algeria.

The in vitro antibacterial activity was evaluated using the agar disk diffusion method in Mueller-Hinton (MH) medium (Biochem Chemopharma, Cosne sur Loire, France), following the protocol described by Benmeddour et al. (2015). The agar plates were inoculated using bacterial suspensions prepared freshly from cells in their exponential growth phase, at a final concentration of approximately 10^6 colony-forming units (CFU)/ml. Each extract was tested at three concentrations, prepared with dimethyl sulfoxide (DMSO), with DMSO (Biochem Chemopharma, Cosne sur Loire, France) and Gentamycin serving as negative and positive controls, respectively. The experiment was replicated three times for validation. The inhibitory effect of the extracts was determined by measuring the diameters of inhibition zones.

Statistical analysis

Statistical analyses were performed using STATISTICA software (Version 8.0, StatSoft Inc. 2007). Mean values, accompanied by their respective standard

deviations (n=3), were utilized to represent the results. Subsequently, the data underwent a factorial analysis of variance (ANOVA), and means were compared utilizing the Fischer LSD test at a significance level of P = 0.05.

Part III:

Results and discussion

I. Extraction yields analysis of the two *Ononis* species extracts

The liquid-solid extraction of the aerial parts of both plants through maceration in organic solvents of increasing polarity, as well as hydrodistillation through steam entrainment, resulted in the acquisition of six crude extracts and two essential oils. The extraction efficiency, as denoted by color and extraction yield (ECC and Y_{EO}) are presented in table 9 as a percentage relative to the initial weight of the plant material (w/w).

The acquired outcomes unequivocally illustrate that both species encompass varying concentrations of extractable compounds contingent upon the solvent, spanning from 0.889% to 21.913%. Polar extracts manifest elevated yields by mass in comparison to non-polar extracts. These observations are congruent with the findings of Jaradat et al. (2017), wherein the methanolic crude extract of *O. angustissima* exhibited a greater weight yield than hexane (23% and 8.48%, respectively). Corroborating this notion, another investigation by Valyova et al. (2008) ascertained that polar fractions (Ethyl acetate 1.16%) of *O. spinosa* demonstrated a significant extractable compound content (ECC) in contrast to non-polar fractions (Petroleum ether 0.41%).

Regarding the essential oils obtained, they exhibit a strong odor with low extraction yields (0.015% for *O. angustissima* and 0.012% for *O. aurasiaca*). This result is relatively low compared to subsequent studies. To the best of our knowledge, *O. aurasiaca* has never been studied before. A comparison is made with other species within the same genus. The essential oil yield of *Ononis angustissima* collected in Tunisia was 0.04 w/w, nearly three times higher (Ghribi et al. 2016). Mechehoud and colleagues studied the essential oils of *O. angustissima* collected in the region of Bechar in 2014, obtaining a rate 48 times higher than ours. The results of this current study are also far from those of *O. natrix* from Morocco, where a range of 0.7 to 1.3% was found (Elamrani and Benaissa 2010). Extraction from freshly collected *O. natrix* and *O. sicula* in Jordan produced pale yellow oils with yields of 0.21% and 0.18%, respectively, which are again distant from our results (Al-Qudah et al. 2014).

However, it is challenging to compare yield results as this parameter is relative and depends on various factors; such as the use of species from distinct geographic and climatic origins, the harvesting zone and conditions, the method of plant material drying, and the extraction protocol, among others (Sefidkon et al. 2006; Sellami et al. 2011; Margeretha et al. 2012; Rahimmalek and Goli 2013).

Table 9: Extraction yield and characteristics of crude extracts and essential oils.

Plant	Crude extract			Essential oil	
	Extract	Color	ECC %	Y_{EO} %	Color
O. aurasiaca	PEE	Dark green	3.939	0.012	Dark brownish yellow
	DCME	Dark green	3.200		
	Me-OHE	Green	11.356		
O. angustissima	PEE	Dark yellowish to green	0.889	0.015	Brown
	DCME	Dark green	14.567		
	Me-OHE	Light brown	21.913		

II. Quantification of total polyphenols and total flavonoids in crude extracts

The quantitative analysis of crude extracts, prepared successively using organic solvents of increasing polarity, through spectrophotometric assays aimed at determining the content of total polyphenols and total flavonoids. The assays were conducted following the Folin Ciocalteu reagent method (Dinçer et al. 2013) and the Aluminum trichloride method (Ghedadba et al. 2014) respectively. Gallic acid and quercetin were employed as standards (Figures 20 and 21).

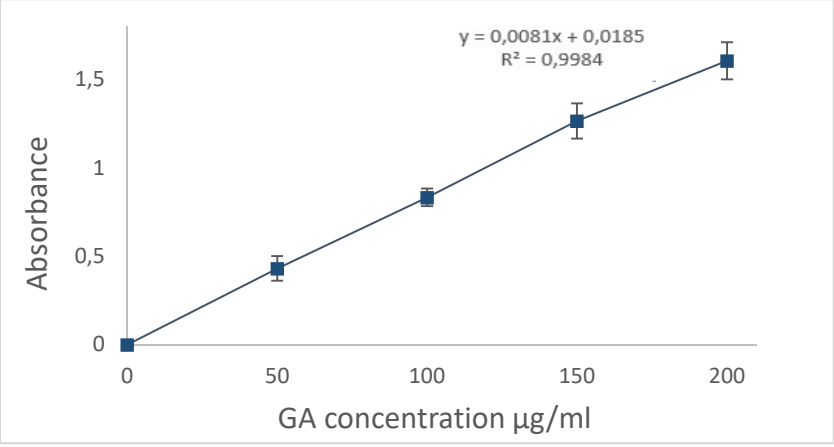

Figure 20: Calibration line for total polyphenols. Each value represents the mean ± SD (n=3)

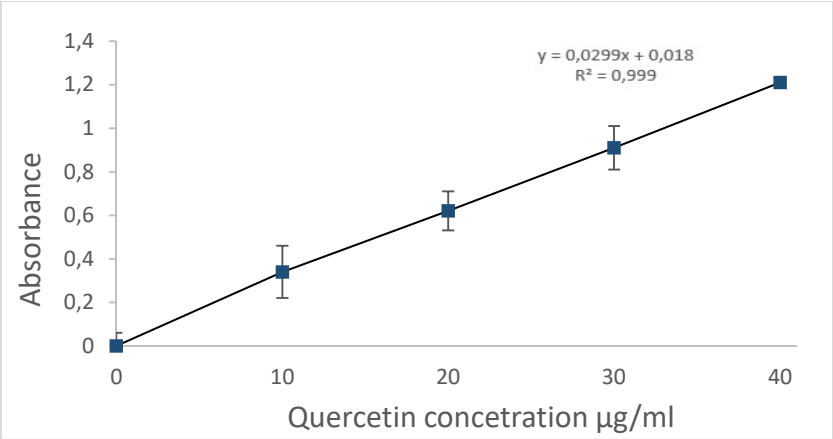

Figure 21: Calibration line for total flavonoids. Each value represents the mean ± SD (n=3)

The assay results reveal the presence of these two secondary metabolites in all extracts, albeit with differential responses to the solvent employed (Table 10). The DCME of *O. aurasiaca* exhibits notably high levels of TPP and TF, measuring 365.62 ± 0.85 (µg GAE/mg) and 74.98 ± 0.03 (µg QE/mg) respectively. Conversely, the lowest quantities are recorded for Me- OHE and PEE with 56.98 ± 0.48 (µg GAE/mg) and 16.12 ± 0.06 (µg QE/mg) respectively. Regarding *O. angustissima*, TPP are also found in higher concentrations, 121.17 ± 0.24 (µg GAE/mg) and 116.23 ± 0.84 (µg GAE/mg) in DCME and Me-OHE compared to PEE. This suggests that these phenolic compounds are more soluble in DCM and Me-OH solvents compared to PE, primarily due to the solubility degree of these compounds in the extraction solvent, which itself depends on their chemical nature in the plant, ranging from simple to highly polymerized compounds (Mahmoudi et al. 2013).

Table 10: Content of total polyphenols and total flavonoids in crude extracts from the aerial parts of both plants.

Plant	Extract	TPP content (µg GAE/mg)	TF content (µg QE/mg)
O. aurasiaca	PEE	$73.03^{a} \pm 0.34$	$16.12^{a} \pm 0.06$
	DCME	$365.62^{c} \pm 0.85$	$74.98^{d} \pm 0.03$
	Me-OHE	$56.98^{a} \pm 0.48$	$36.86^{b} \pm 0.39$
O. angustissima	PEE	$64.38^{a} \pm 0.41$	$43.21^{bc} \pm 0.47$
	DCME	$121.17^{b} \pm 0.24$	$58.93^{cd} \pm 0.01$
	Me-OHE	$116.23^{b} \pm 0.84$	$30.84^{ab} \pm 0.42$

Each value represents the mean ± SD (n=3). Values with same superscript letters within a column are not significantly different (Fisher LSD, P=0.05).

This study represents the first report on the phytochemical profile of *O. aurasiaca* species. The quantity of TPP in Me-OHE is akin to that obtained with *O. natrix* (51 mg GAE/g) (Mhamdi et al. 2014). Comparative analysis of TPP content within the same extract and that found in Bulgarian *O. spinosa* led to the deduction that it is fourfold higher at 25.03 ± 1.63 mg GAE/g (Valyova et al. 2008). Regarding TF, this extract appears notably richer compared to Me-OHE of *O. natrix* studied by Sayari et al. (2016) and Amri et al. (2015), with proportions of 1.5 ± 0.22 (mg QE/g) and 8.09 ± 0.30 (µg QE/mg), respectively.

The results concerning TPP content in the DCME of *O. angustissima* are in concordance with those of *O. alba poir* (129.0 ± 10.19 mg GAE/g). Additionally, their TF quantity (98.26 ± 8.91 mg EQ/g) surpasses our findings. Regarding *O. aurasiaca*, the TF levels are also lower, in contrast to the abundant presence of TPP in this extract (Zaak et al. 2020).

The quantity of TPP and TF in the PEE determined in both species examined within this study proves to be higher than that observed in *O. mitissima* (52.11 ± 0.65 mg GAE/g, 27.27 ± 0.41 mg QE/g), except for the TF concentration of *O. aurasiaca*, which was the lowest of all (Besbes et al. 2020).

III. Chemical composition analysis of essential oils

The chemical composition of the essential oils from *O. angustissima* and *O. aurasiaca* was determined using gas chromatography-mass spectrometry (GC- MS). The identified compounds are listed in tables 11 and 12 along with their respective percentages and linear retention indices (LRI). Compound identification relied on comparing retention times and linear retention indices to a series of n- hydrocarbons. Additionally, commercial mass spectral libraries (NIST 14 and ADAMS 2007) as well as in-house developed libraries derived from pure substances and commercially available essential oils of known composition were utilized, supplementing the MS literature data.

The analysis allowed for the identification of 34 compounds, accounting for 91.6% of the total composition of the essential oil of *O. angustissima* (Table 11). The oxygenated sesquiterpenes are the predominant compounds characterizing this oil (32.6%), among which β-eudesmol (6.6%), α-cadinol (6.4%), and t-cadinol (6.1%) stand out as major components. Another significant class is the apocarotenoids (21.4%), primarily represented by hexahydrofarnesylacetone (14.8%). Oxygenated monoterpenes and sesquiterpene hydrocarbons are identified in considerable quantities (17.3% and 14.7%, respectively). Limonene (2.3%) and methyl eugenol (0.8%) are the only compounds identified representing monoterpene hydrocarbons and phenylpropanoids, respectively.

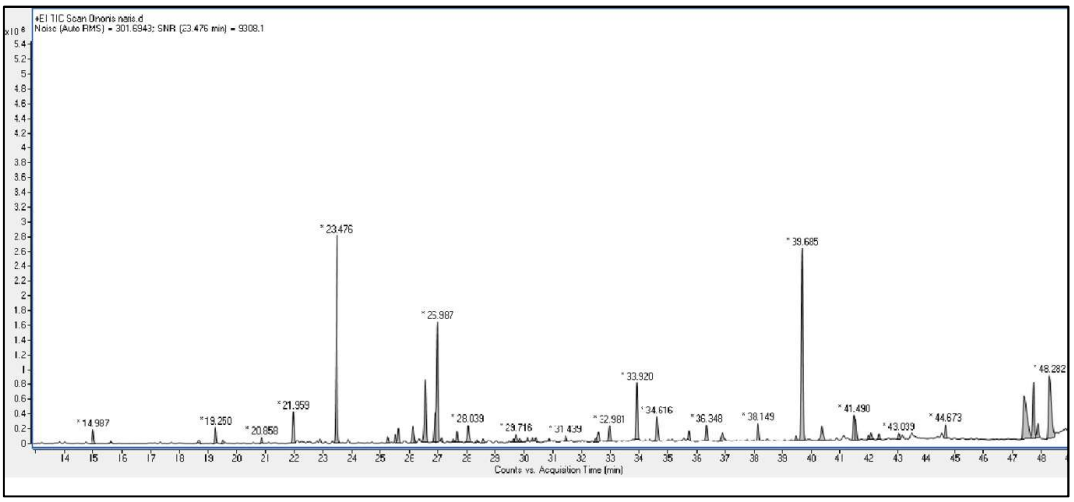

Figure 22: GC-MS chromatogram of *O. aurasiaca* and *O. angustissima* essential oils.

Table 11: Chemical composition of the essential oil of *Ononis angustissima* Lam. subsp. *filifolia* Murb.

	Constituents	LRI*	Percentage %
1	Limonene	1032	2.3
2	Fenchone	1089	1.8
3	Linalool	1101	6.2
4	4-terpineol	1179	1.2
5	α-terpineol	1191	2.5
6	endo-fenchyl acetate	1221	1.0
7	Nerol	1227	0.9
8	Carvone	1244	0.9
9	Geraniol	1256	2.8
10	4-vinylguaiacol	1314	1.1
11	α-copaene	1377	0.8
12	(E)-β-damascenone	1382	1.6
13	Methyl eugenol	1403	0.8
14	(E)-geranylacetone	1455	1.3
15	γ-muuroline	1478	3.3
16	3,4-dehydro-β-ionone	1486	1.4
17	(E)-β-ionone	1487	1.5
18	Valencene	1493	0.8
19	2-tridecanone	1496	1.4
20	α-muurolene	1499	1.3
21	trans-γ-cadinene	1514	2.3

22	δ-cadinene	1524	3.9
23	Dihydroactinidiolide	1536	0.8
24	α-cadinene	1538	0.8
25	α-calacorene	1543	1.5
26	(E)-nerolidol	1564	4.5
27	1,10-di-epi-cubenol	1615	1.7
28	1-epi-cubenol	1629	3.8
29	T-cadinol	1642	6.1
30	α-muurolol	1646	2.1
31	β-eudesmol	1650	6.6
32	α-cadinol	1655	6.4
33	cis-α-santalol	1682	1.4
34	Hexahydrofarnesylacetone	1845	14.8

	Monoterpene hydrocarbons	2.3
	Oxygenated monoterpenes	17.3
	Sesquiterpene hydrocarbons	14.7
	Oxygenated sesquiterpenes	32.6
	Phenylpropanoids	0.8
	Apocarotenoids	21.4
	Others	2.5
	Total identified	**91.6**

* Linear retention indices on a HP-5-MS capillary column.

The essential oil of *O. aurasiaca* appears to contain 44 compounds, constituting 93.4% of the total oil composition (Table 12). Among these, Apocarotenoids (15.6%) emerge as the most dominant class, with hexahydrofarnesylacetone (14.4%) being a major constituent. Sesquiterpene hydrocarbons (11.8%) are also present, with β-selinene (5.1%) being the primary compound, followed by diterpenes (9.0%), mainly comprised of phytol (8.3%). Additionally, this composition includes monoterpene and oxygenated sesquiterpene fractions (0.3% and 2.4% respectively), with the latter class solely represented by trans-sabinyl acetate. The remaining 54.3% of the total composition primarily consists of non-terpene derivatives, wherein dodecanal (14.6%), 2-tridecanone (8.7%), and 1-heneicosene (7.9%) are the major compounds.

Moreover, this composition differs from that of *O. angustissima* Lam. subsp. *filifolia* Murb. in terms of profitability, despite sharing three classes of compounds in common: oxygenated monoterpenes (17.3% vs 0.3%), sesquiterpenes (47.3% vs 14.2%), and apocarotenoids (21.4% vs 15.6%). It is clear that *O. angustissima* is richer, as it also contains monoterpene hydrocarbons and phenylpropanoids. The major components confirm this observation, with hexahydrofarnesylacetone (14.8%), β-eudesmol (6.6%), α-cadinol (6.4%), linalool (6.2%), and t-cadinol (6.1%) found in *O. angustissima* compared to dodecanal (14.6%), hexahydrofarnesylacetone (14.4%), 2-tridecanone (8.7%), phytol (8.3%), and 1-heneicosene (7.9%) in *O. aurasiaca*.

Given that this is the first study conducted on the plant *O. aurasiaca*, the discussion is based on results from studies on other species belonging to the same genus. This species is found to have a low proportion of sesquiterpenes, either sesquiterpene hydrocarbons or oxygenated sesquiterpenes (14.2%), compared to *O. angustissima* collected in Tunisia, where sesquiterpenes constitute the major class of compounds at 39.5% (Ghribi et al. 2016). The same pattern is observed with *Ononis angustissima* Lam. subsp. *filifolia* Murb. evaluated in this study, where results clearly show their richness in sesquiterpenes, constituting almost half of the total composition of the essential oil (47.3%). These two oils shared other compounds but with different quantities. However, some major compounds are minor or absent in the Tunisian plant, for example: hexahydrofarnesylacetone (14.8 vs 3.5%, respectively), β-eudesmol (6.6 vs 0.0%), α-cadinol (6.4 vs 0.7%), and linalool (6.2 vs 0.0%). Conversely, 2-tridecanone (1.4 vs 9.3%), α-eudesmol (0.0 vs 22.4%), acetophenone (0.0 vs 7.4%), and dodecanal (0.0 vs 4.7%) are found in the reverse situation. Nevertheless, it can be concluded that these two plants are very close in terms of the chemical composition of their essential oils.

Table 12: Chemical composition of the essential oil of *Ononis aurasiaca*.

	Constituents	LRI*	Percentage %
1	Decanal	1205	0.9
2	β-cyclocitral	1222	0.1
3	trans-sabinyl acetate	1298	0.3
4	Undecanal	1307	1.1
5	p-vinylguaiacol	1313	0.3
6	1-undecanol	1373	2.1
7	Dodecanal	1408	14.6
8	(E)-geranylacetone	1456	0.5
9	2-methyltetradecane	1462	1.0
10	β-chamigrene	1476	1.3
11	γ-muurolene	1477	0.4
12	β-selinene	1487	5.1
13	α-selinene	1495	1.7
14	2-tridecanone	1496	8.7
15	α-muurolene	1499	0.3
16	Tridecanal	1513	0.3
17	trans-γ-cadinene	1514	0.8
18	δ-cadinene	1524	1.6
19	Cubenene (syn. cadina-1,4-diene)	1533	0.3
20	α-cadinene	1538	0.3
21	(E)-nerolidol	1566	0.2

22	Dodecanoic acid	1567	0.7
23	(Z)-3-hexenyl benzoate	1568	0.3
24	Hexyl benzoate	1579	0.4
25	2-tetradecanone	1598	0.3
26	Humulane-1,6-dièn-3-ol	1618	0.5
27	T-cadinol	1641	0.4
28	α-cadinol	1652	1.3
29	2-pentadecanone	1698	2.3
30	Tetradecanoic acid	1762	0.9
31	n-octaecane	1800	1.2
32	Neophytadiene	1839	0.3
33	Hexahydrofarnesylacetone	1844	14.4
34	benzyl salicylate	1866	1.3
35	n-nonadecane	1900	1.8
36	2-heptadecanone	1901	1.2
37	(E,E)-farnesylacetone	1919	0.6
38	Methyl hexadecanoate	1927	0.4
39	Isophytol	1949	0.4
40	n-eicosane	2000	1.1
41	1-heneicosene	2097	7.9
42	n-heneicosane	2100	4.4
43	2-nonadecanone	2101	1.1
44	Phytol	2114	8.3

Oxygenated monoterpenes	0.3
Sesquiterpene hydrocarbons	11.8
Oxygenated sesquiterpenes	2.4
Diterpenes	9.0
Apocarotenoids	15.6
Non-terpene derivatives	54.3
Total identified	**93.4**

* Linear retention indices on a HP-5-MS capillary column.

A significant difference was observed in the composition of *O. angustissima* essential oil analyzed in this study compared to another Algerian one growing in the Bechar region. Only ten compounds out of the 24 identified by Mechehoud et al. (2014) were found in the oils of both plants. According to their findings, the major compounds present were phytol (17.4%), (Z,Z)-farnesol (8.8%), β-eudesmol (7.5%), δ-cadinene (5.1%), and valencene (5.0%). This variance can be attributed, as suggested by Förther and Podlech (1991), to the utilization of two different subspecies. Specifically, in the southwest of Algeria, *O. angustissima* Lam. subsp. *polyclada* Murb. and *O. angustissima* Lam. subsp. *mauritii* Förther & Podl are the two subspecies present in this region. The subspecies *Ononis angustissima* Lam. subsp. *filifolia* Murb. is endemic to the Algerian-Tunisian region, with its presence in Algeria limited to the eastern zone of the northern Sahara, while *O. aurasiaca* is endemic to Algeria, found solely in the Aures region.

Khallouki and colleagues (2002) studied *Ononis natrix* species collected in Morocco, revealing a total of 25 compounds, with only five of them identified in the oils analyzed in this study, albeit in varying concentrations. These compounds include α-muurolene, γ- muurolene, γ- cadinene, δ-cadinene, and β-selinene in *O. aurasiaca*, and linalool, α- terpineol, γ- muurolene, α-muurolene, and β-eudesmol in *O. angustissima*. Comparing two Jordanian *Ononis* species, *O. natrix* and *O. sicula*, with our *Ononis* species, they were found to be rich in sesquiterpenes, whether oxygenated or hydrocarbon sesquiterpenes, with percentages of 57.81% and 86.42%, respectively. However, they were found to be poor in monoterpenes

(1.84% and 0.79% vs 19.6% and 0.3%) for *O. angustissima* and *O. aurasiaca*, respectively, noting that the latter is devoid of monoterpene hydrocarbons, considering these oils were extracted from fresh plant material. Among the 85 compounds identified in total in the essential oils of *Ononis natrix*, (2E,6E)-farnesol (18.83%), dodecanal (12.58%), and 2-phenyl ethyl tiglate (5.20%) were the major constituents. In *Ononis sicula*, selin-11-en-4-α-ol (12.76%) and intermedeol (10.74%) were the principal compounds among the 77 compounds identified in total (Al-Qudah et al. 2014).

The chemical composition of essential oils from another endemic *Ononis* species in Turkey, *Ononis viscosa* L., may exhibit similarities to that of *O. aurasiaca*. Out of a total of 40 identified compounds, nine are shared between them. Conversely, only three compounds are found in the oil of *O. angustissima*, with hexahydrofarnesylacetone and dodecanal being the major constituents across all three plants (Erdemgil et al. 2002).

These findings highlight significant chemical diversity within different species of the *Ononis* genus worldwide, attributed to the presence of various chemical classes with associated major compounds, albeit in different proportions. These results align with the classifications proposed by Förther and Podlech (1991) and Turini et al. (2010), which are based on botanical and genetic criteria.

IV. Assessment of antioxidant activity

i. Assessment of DPPH free radical scavenging activity

The scavenging activity of various extracts obtained from the aerial parts of the two plants *O. aurasiaca* and *O. angustissima* towards DPPH• radicals, was compared with that of other standard antioxidants such as ascorbic acid, quercetin, and BHA. The results obtained demonstrated a concentration-dependent activity profile for both the extracts and the controls. As depicted in figures 23, 24, and 25, all extracts exhibited good antioxidant activity, albeit lower compared to the positive controls. The DCM and Me-OH extracts of *O. aurasiaca* appear to be potent antioxidants with activities of approximately 95.14 ± 4.36% and 91.71 ± 3.07% at 960 µg/ml, respectively. Quercetin and BHA were able to reduce DPPH radicals with activities of 95.57 ± 0.25% and 93.27 ± 0.27%, respectively, at only 60 µg/ml. The other crude extracts and essential oils showed a range of activity between 60.92 ± 4.04% and 80.54 ± 0.52%, respectively.

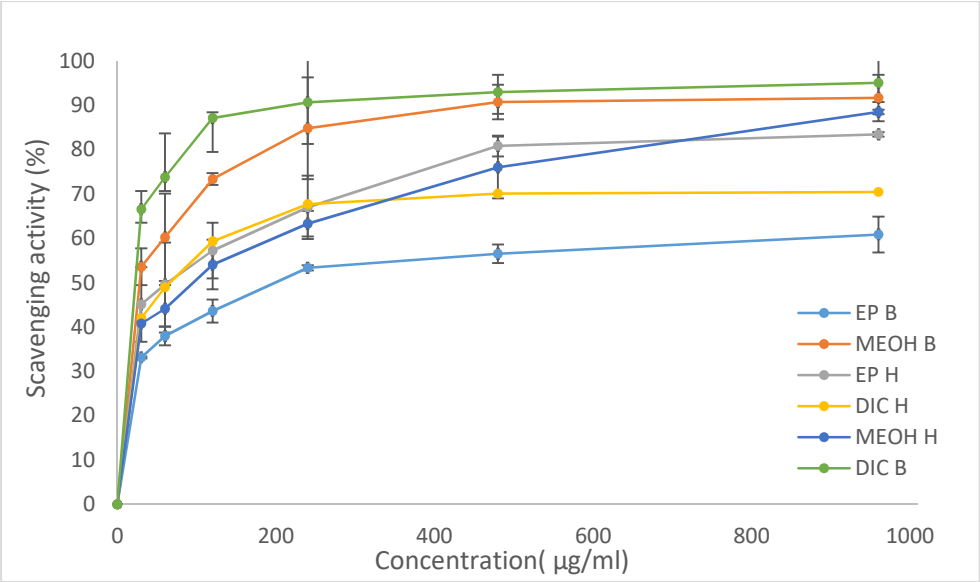

Figure 23: Scavenging activity of crude extracts of *O. aurasiaca* (B) and *O. angustissima* (H). Values represent the average ± SD (n = 3).

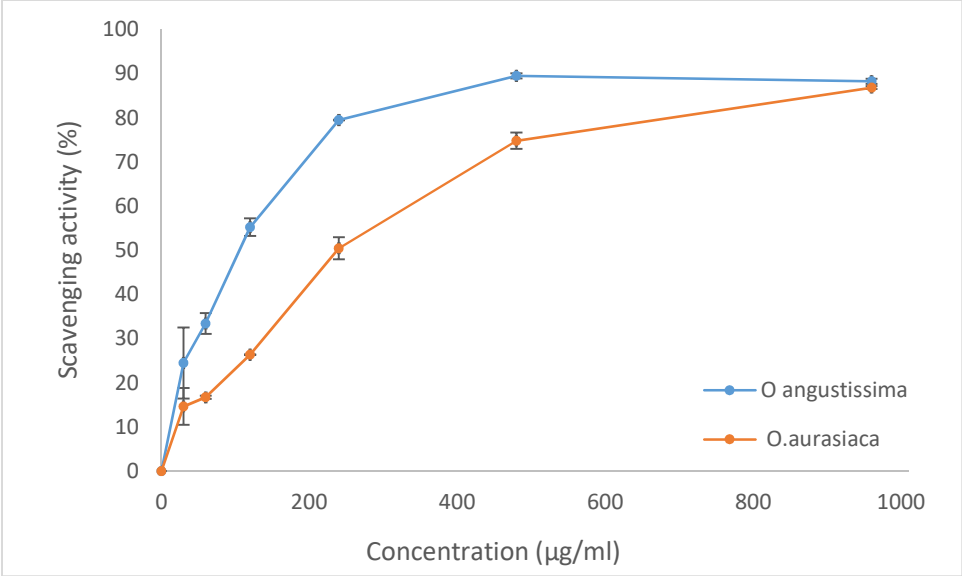

Figure 24: Scavenging activity of essential oils. Values represent the average ± SD (n =3).

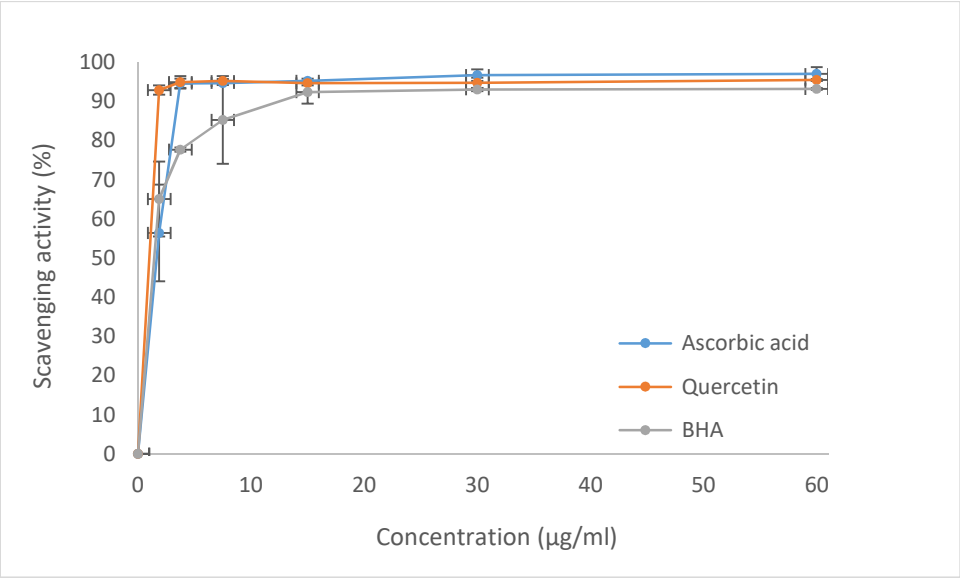

Figure 25: Scavenging activity of standards (Positive control). Values represent the average ± SD (n = 3).

The antioxidant activity results are also expressed in terms of IC_{50} (Table 13), which is defined as the concentration of the extract and/or standard antioxidant capable of scavenging 50% of DPPH radicals in the reaction mixture, where stronger activity corresponds to a lower IC_{50} value.

As shown in the table, the DCME of *O. aurasiaca* exhibited the highest antioxidant activity, being able to scavenge DPPH radicals at a low concentration (IC_{50} = 24.22 ± 0.28 µg/ml). This activity could be attributed to its high content of polyphenols and flavonoids; however, this concentration still remains higher than those of quercetin, BHA, and ascorbic acid, which demonstrated a strong antioxidant activity at very low doses (IC_{50} = 1.15 ± 0.23 µg/ml, 1.55 ± 0.51 µg/ml, and 2.00 ± 0.35 µg/ml, respectively). The Me-OHE of the same species also showed good activity, followed by the DCME of *O. angustissima* (IC_{50} = 30.16 ± 0.91 µg/ml and 44.22 ± 1.53 µg/ml, respectively). Overall, all organic extracts exhibited considerable radical-scavenging activity compared to essential oils, especially that of *O. aurasiaca*, which demonstrated the lowest potential (IC_{50} = 231.87 ± 2.25 µg/ml). The activity of the crude extracts is further explained by their contents of PPT and FT. The depicted data in the figure 26, showcases the ranking of effectiveness, with the following sequence: $DCME_B$ holds the highest rank, followed by Me-OHE_B, $DCME_H$, PEE_H, Me- OHE_H, PEE_B, EO_H, and finally, EO_B.

Table 13: IC_{50} values for standard solutions and/or extracts in the DPPH test.

Extract / Standard		IC_{50} (µg/ml)
Essential oil	*O. aurasiaca* B	$231.87^{g} \pm 2.25$
	O. angustissima H	$102.30^{f} \pm 2.96$
Crude extract (*O. aurasiaca*)	PEE_B	$81.84^{e} \pm 7.18$
	$DCME_B$	$24.22^{b} \pm 0.28$
	$Me\text{-}OHE_B$	$30.16^{b} \pm 0.91$
Crude extract (*O. angustissima*)	PEE_H	$54.93^{d} \pm 0.68$
	$DCME_H$	$44.22^{c} \pm 1.53$
	$Me\text{-}OHE_H$	$59.18^{d} \pm 3.95$
Positive Control	Quercetin	$1.15^{a} \pm 0.23$
	Ascorbic acid	$2.00^{a} \pm 0.35$
	BHA	$1.55^{a} \pm 0.51$

Each value represents the mean ± SD (n = 3). Values with same superscript letters within a column are not significantly different (Fisher LSD, P=0.05).

Ghribi and colleagues (2015) examined various fractions derived from a hydro-alcoholic crude extract of *O. angustissima* roots collected in Tunisia. The efficacy of their DCM extract was found to be somewhat lower compared to our DCM extract of *O. angustissima* (IC_{50} = 66.87 ± 1.97 µg/ml vs 44.22 ± 1.53 µg/ml, respectively). It is worth noting that according to Förther and Podlech (1991), these two plants belong to the same endemic Algero-Tunisian subspecies (*Ononis angustissima* Lam. subsp. *filifolia* Murb.). This difference in efficacy is mainly attributed to the specific plant parts and extraction methods utilized.

Another species within the same genus, *O. alba Poir* collected from Bejaia, was evaluated for its antioxidant potential. The DCM fraction exhibited significantly lower efficacy compared to our findings, with an IC_{50} of 661.66 ± 6.89 µg/ml (Zaak et al. 2022).

Additionally, the methanolic extract of *O. natrix* collected in Tunisia (A species closely related to *O. angustissima*) was assessed against DPPH radicals, demonstrating strong scavenging activity with an IC_{50} of 29 µg/ml (Mhamdi et al. 2014). This result is nearly comparable to the methanol extract of *O. aurasiaca* investigated in this study and slightly higher than that of the *Ononis angustissima* Lam. subsp. *filifolia* Murb. Conversely, *O. natrix* collected in Morocco appears to be less effective against free radicals, with an IC_{50} of 82.83 ± 0.13 µg/ml for the same crude extract (Amri et al. 2015). The methanolic extract of Jordanian *O. pubescens* represents the most potent activity at 19.41 ± 1.1 µg/ml (Jaradat et al. 2017).

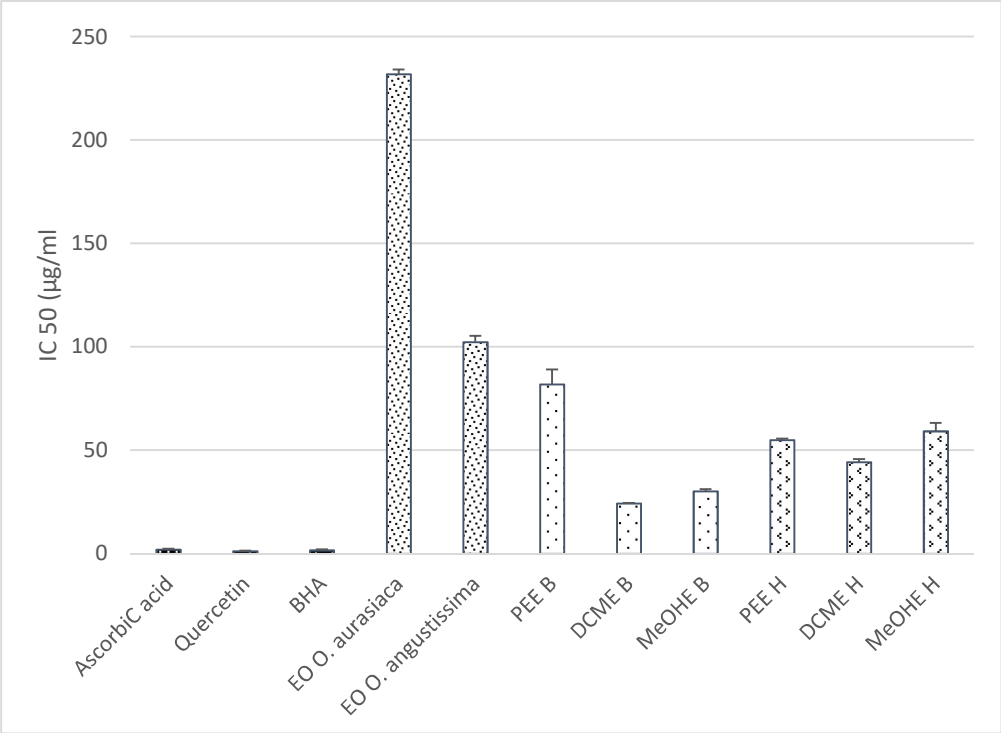

Figure 26: Diagram demonstrating the differences in inhibitory concentration 50 (IC_{50}), between crude extracts, essential oils and standards (Positive controls) in the DPPH assay. Values represent the mean ± SD (n = 3).

ii. Lipid peroxidation inhibition by the β-carotene/linoleic acid method

The inhibition of lipid peroxidation represents a widely employed method for assessing antioxidant potential, hinging upon the oxidative degradation of β-carotene in conjunction with linoleic acid (Madoui et al. 2018). Linoleic acid functions as a radical initiator in this process, precipitating the attack on the β-carotene chromophore and subsequent bleaching (Sayari et al. 2016).

To gauge the capacity of various extracts in attenuating lipid peroxidation, a systematic monitoring of absorbance reduction was conducted at specific time intervals ranging from 1 to 48 hours.

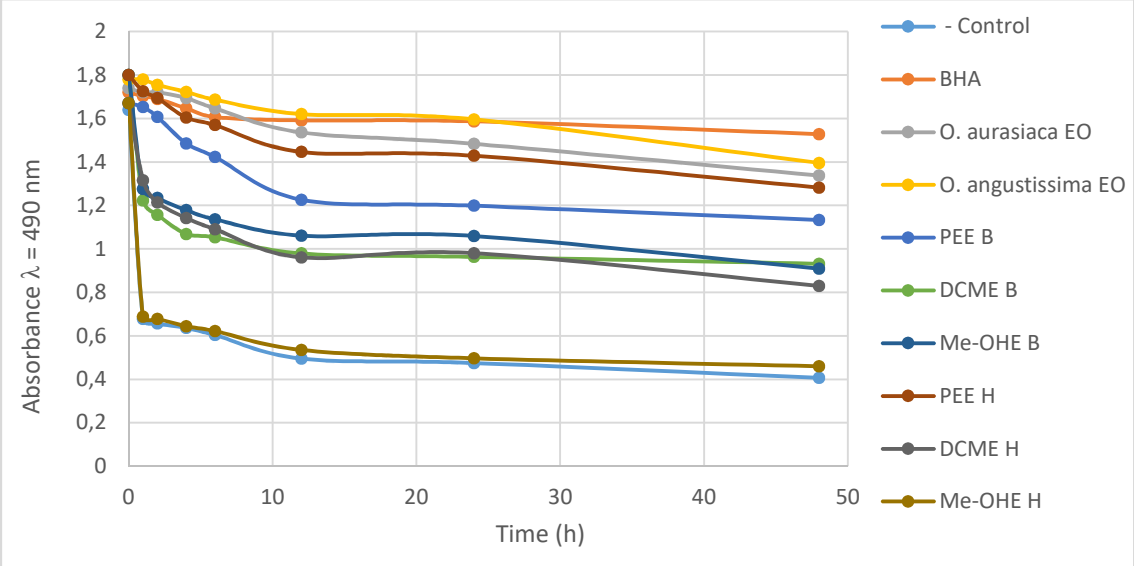

Figure 27: Kinetics of β-carotene/linoleic acid oxidation by crude extracts and essential oils (EO) of *O. angustissima* (H) and *O. aurasiaca* (B) and standard antioxidant (BHA). Values represent the average ± SD (n = 3).

The findings unequivocally underscore the efficacy of essential oils in impeding β-carotene oxidation. Notably, at a concentration of 2 mg/ml, *O. angustissima* essential oil exhibited the most pronounced activity, inhibiting β-carotene discoloration by 91.35 ± 0.06%, akin to the standard antioxidant, BHA, at 89.72 ± 0.03%. Additionally, *O. aurasiaca* oil demonstrated considerable antioxidant potential, estimated at 87.5 ± 0.02%. This was reflected in the observed decline in absorbance values for crude extracts of both species due to β-carotene oxidation, leading to perceptible color degradation of the reaction mixture toward a light orange hue. However, this degradation was comparatively slower for the two PE extracts, indicative of their robust activity (Table 14). Conversely, the Me-OH extract of *O. angustissima* exhibited the lowest antioxidant potency, registering at 30.11 ± 0.01% compared to the negative control. Consequently, it is deduced that apolar extracts exert greater efficacy in the β-carotene-linoleic acid bleaching system than polar extracts.

Table 14: Antioxidant activity (Percentage) exhibited by various extracts of *O. aurasiaca* and *O. angustissima* utilizing the β-carotene bleaching method.

Extract / Standard		AA (%)
Essential oil	*O. aurasiaca* $_{B}$	$87.50^{f} \pm 0.02$
	O. angustissima $_{H}$	$91.35^{g} \pm 0.06$
Crude extract (*O. aurasiaca*)	PEE_B	$74.15^{d} \pm 0.04$
	$DCME_B$	$60.99^{c} \pm 0.01$
	$Me\text{-}OHE_B$	$59.49^{c} \pm 0.01$
Crude extract (*O. angustissima*)	PEE_H	$83.90^{e} \pm 0.01$
	$DCME_H$	$54.32^{b} \pm 0.05$
	$Me\text{-}OHE_H$	$30.11^{a} \pm 0.01$
Positive control	BHA	$89.72^{fg} \pm 0.03$
Negative control	Blank	$26.63^{a} \pm 0.01$

Each value represents the mean ± SD (n = 3). Values with same superscript letters within a column are not significantly different (Fisher LSD, P=0.05).

The findings of this study align with those of Sayari et al. (2016), who examined the antioxidant properties of *O. natrix* extracts, revealing that the chloroform extract exhibited the highest efficacy in preventing β-carotene bleaching compared to polar extracts, achieving a 71.82% efficacy. Similarly, Guettaf et al. (2016) explored the antioxidant capabilities of *O. angustissima*'s aqueous extract, yielding results inferior to the nonpolar extracts discussed herein, registering a 54% efficacy. These investigations collectively corroborate Frankel et al.'s (1994) assertion regarding the superior performance of lipophilic antioxidants in oil- water emulsions compared to hydrophilic counterparts. This enhanced efficacy stems from their concentration at the lipid-water interface, where they impede lipid radical formation, thereby arresting β-carotene oxidation. Conversely, hydrophilic antioxidants remain dispersed in the aqueous phase, limiting their efficacy in scavenging linoleate radicals.

Limited research exists regarding the capacity of *Ononis* genus species to mitigate lipid peroxidation, and to date, no studies have investigated the effectiveness of essential oils in

preventing linoleic acid peroxidation. This study represents a pioneering effort in evaluating the bleaching inhibition activity of β-carotene by essential oils extracted from *O. angustissima* and *O. aurasiaca*. Both species exhibit notable effectiveness in this regard. Despite variations in compound yields, these oils share commonalities in three compound classes. GC-MS analyses reveal *O. angustissima*'s richness compared to *O. aurasiaca*, particularly in monoterpenic hydrocarbons and phenylpropanoids, contributing significantly to its antioxidant potential. Conversely, *O. aurasiaca*'s essential oil contains distinctive non-terpenic derivatives such as dodecanal, 2-tridecanone, and 1-heneicosene, believed to originate from fatty acid metabolism, thus likely responsible for inhibiting linoleic acid peroxidation. This elucidates the observed antioxidant capacity of *O. aurasiaca* essential oil in the β-carotene bleaching assay.

iii. Assessment of reducing power using the Fe^{+2}-phenanthroline test

In this assessment, the capacity of an extract to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) is gauged, employing 1,10-phenanthroline as a ligand that forms a stable red-orange complex with the resulting ferrous ions, exhibiting peak absorbance at 510 nm (Roy et al. 2011).

The reducing potential of all extracts, notably essential oils, was markedly inferior to that of BHA, as evidenced by color variations during experimentation. While BHA produced a vivid red-orange hue, essential oils yielded a paler shade, darkening slightly in the case of *O. aurasiaca*'s DCME, which displayed the highest activity. This heightened activity is likely due to their elevated levels of TPP and TF. Crude extracts of *O. aurasiaca*'s Me-OH, and *O. angustissima*'s PE and DCM, exhibited the ability to reduce Fe^{3+} ions with an $A_{0.5}$ around 200 µg/ml, whereas other extracts demonstrated diminished efficacy (>200 µg/ml) (Table 15).

Table 15: Reducing power of different *O. aurasiaca* and *O. angustissima* extracts in the Fe^{+2}-phenanthroline test.

Extract / Standard		$A_{0,5}$ (µg/ml)
Essential oil	*O. aurasiaca* B	$795.48^{g} \pm 0.02$
	O. angustissima H	$848.23^{h} \pm 0.07$
Crude extract (*O. aurasiaca*)	PEE_B	$275.83^{e} \pm 0.03$
	$DCME_B$	$95.59^{b} \pm 0.01$
	$Me\text{-}OHE_B$	$200.03^{c} \pm 0.02$
Crude extract (*O. angustissima*)	PEE_H	$206.65^{c} \pm 0.09$
	$DCME_H$	$230.35^{d} \pm 0.10$
	$Me\text{-}OHE_H$	$470.55^{f} \pm 0.02$
Positive control	BHA	$9.37^{a} \pm 0.04$

Each value represents the mean ± SD (n = 3). Values with same superscript letters within a column are not significantly different (Fisher LSD, P=0.05).

According to our current knowledge, a scarcity of studies exploring the antioxidant properties of essential oils derived from the *Ononis* genus utilizing the Fe^{+2}-phenanthroline method. Guchu et al. (2020) conducted research on *Acacia hockii*, a plant belonging to the Fabaceae family (The same family as our two species), which demonstrated a considerable antioxidant capacity in its methanolic extract unlike what was observed in our study. Srief et al. (2023) examined non-polar extracts and essential oil from *Mentha piperita*, revealing no antioxidant activity in either the extracts or the oil. This discrepancy could be attributed to the fact that polar solvents have a higher affinity for phenolic content compared to non-polar solvents, and essential oils are non-polar substances.

Several *Ononis* species, including *O. alba Poir*. (Zaak et al. 2022), *O. natrix* (Mhamdi et al. 2014; Sayari et al. 2016; Al-Mterin et al. 2021), *O. pubescens* L. (Jaradat et al. 2017), *O. mitissima* L. (Besbas et al. 2020), and *O. arvensis* L. (Dénes et al. 2022), have been investigated for their potential as sources of antioxidants, particularly through the extraction using organic solvents. Evaluation of the antioxidant properties of these species involved

various methodologies such as DPPH, β-carotene bleaching, and phenanthroline, all indicating their capability to combat free radicals effectively.

Furthermore, the current body of research on the antioxidant activity of essential oils predominantly focuses on species taxonomically distant from the *Ononis* genus, exhibiting distinct chemical compositions.

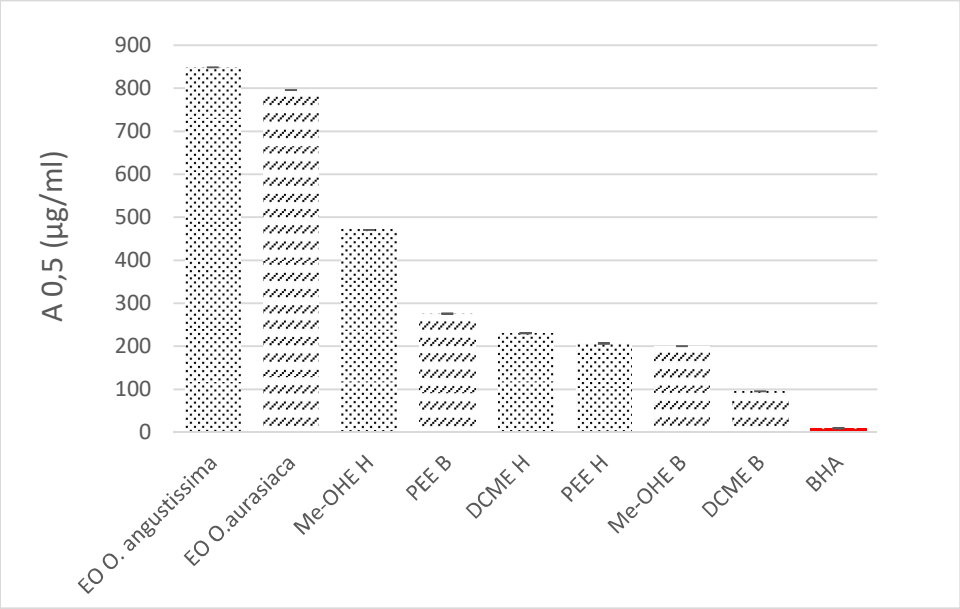

Figure 28: Diagram of reducing power of crude extracts, essential oils and positive control (BHA) in the Fe^{+2}-phenanthroline assay. Values represent the mean ± SD (n = 3).

V. Structure-activity relationships

To enhance comprehension of the mechanisms by which plant extracts mitigate free radicals, an examination of their fundamental compounds' structures is imperative. Mezrag et al. (2017) observed that Flavonoids extracted from *O. angustissima* exhibit substantial scavenging capabilities due to the abundance of free OH groups on their aromatic rings. Consequently, discussions have revolved around elucidating structure-activity relationships, which entail discerning the correlation between the capacity of phenolic compounds to scavenge free radicals and the quantity and positioning of hydroxyl groups attached to the phenyl ring (Cai et al. 2006; Schulz et al. 2011; Załuski et al. 2015).

Concerning terpenes (Such as phytol, β-selinene, and linalool in the cases of *O. aurasiaca* and *O. angustissima* essential oils, respectively), the explanation for their antioxidant prowess

necessitates consideration of additional structural factors. Spectrophotometric investigations have explicitly demonstrated the significant antioxidant activity of terpenes possessing conjugated double bonds on the isoprene units, as opposed to substances lacking this structural feature (Wojtunik et al. 2014). Monoterpenes, being lipophilic compounds found in the volatile fractions of essential oils, have been observed to exhibit direct antioxidant activity, particularly those rich in non-phenolic compounds. For instance, dodecanal, the principal compound of *O. aurasiaca* essential oil, was identified as capable of donating hydrogen atoms to peroxyl radicals (Baschieri et al. 2017). Compounds exhibiting these structural attributes generate relatively stable resulting antioxidant radicals and swiftly terminate radical chain reactions, thus qualifying as effective direct antioxidants (Wojtunik et al. 2018).

It is noteworthy that the antioxidant efficacy of plant extracts may fluctuate depending on the specific assay methods employed. This variability is often ascribed to disparities in mechanisms of action, sensitivities of reagents, and the intricate nature of phytochemicals or volatile compounds present (Koleva et al. 2002; Schlesier 2002; Munteanu and Apetrei 2021; Srief et al. 2023).

VI. *In silico* analysis of antibacterial activity through molecular docking study

The approach of molecular docking predicts the affinity and the optimal orientation of a ligand within an active site to form a stable complex. This study aimed to identify potential inhibitors by docking the major annotated compounds identified via GC-MS with three target proteins. Docking was conducted using AutoDock tools in PyRx. Interactions analysis were carried out by BIOVIA Discovery Studio Visualizer tools. The resulting binding affinities and interaction patterns are detailed in tables 19, 20, and 21.

Among the ten phytochemicals studied, phytol vs 2UV0 (-8.4 kcal/mol), hexahydrofarnesylacetone vs 2UV0, and t-cadinol vs 3T88 (-8.1 kcal/mol) exhibited the lowest energy values, indicating the highest binding affinities. Conversely, n-heneicosane vs 3T88 (- 4.5 kcal/mol) and Dodecanal vs 3WGN (-4.7 kcal/mol) had the highest energy values, reflecting lower binding affinities

The interaction analysis revealed several types of bonding interactions between the ligands and target proteins, including hydrogen bonds (Conventional Hydrogen bonds and Carbon-Hydrogen bonds) and hydrophobic interactions (Alkyl, Pi-Alkyl and Pi-Sigma interactions). Detailed interaction patterns are depicted in figures from 29 to 43.

Table 16: Docking score and interacting profile of ten principal compounds of *O. aurasiaca* and *O. angustissima* essential oils and the positive controls with MenB (ID 3T88) protein.

Compounds	Binding affinity (kcal/mol)	Hydrogen bond interactions	Hydrophobic interactions
2-tridecanone	-5.4	-	-
β-selinene	-6.0	-	Val A: 108
Dodecanal	-5.2	-	-
n-heneicosane	-4.5	-	-
Phytol	-5.5	-	-
Hexahydro-farnesyl-acetone	-6.3	Gln E: 88	Arg E: 45, Ile E: 131, Val E: 159, **Tyr E: 129****, **Phe A: 270****
β-eudesmol	-7.0	**Gly A: 86**	Ile A: 131, Tyr A: 129, **Phe E: 270****
Linalool	-5.7	-	-
T-cadinol	-8.1	Tyr E: 258	Val A: 136, Leu A: 109, **Leu A: 106**
α-cadinol	-6.9	-	Arg A: 45, Tyr A: 129, Ile A: 131, Val A: 159, **Val A: 44**, **Phe E: 270**, Ala A: 47
OSB-NCoA*****	-9.5	Ser A: 161, **Gly A: 86**, Gly A: 133, Gln A: 88, Ser A: 84, Arg A:45, Lys A: 89, Lys A: 273, **Val A: 44***, Gly A: 85*, Asp A: 87*	**Phe E: 270*****, Tyr A: 139**, **Leu A: 106**, Leu A: 108, Asp A: 139****
Gentamycin	-7.5	Arg A: 230, His E: 104 Gly A: 70*	-

*Carbon Hydrogen interactions, **Pi-Sigma interactions, ***Pi- Pi T-shaped ****Pi-Anion (Electrostatic interactions), ***** o-succinylbenzoyl-N-coenzyme A.

Table 17: Docking score and interacting profile of ten principal compounds of *O. aurasiaca* and *O. angustissima* essential oils and the positive controls with LasR (ID 2UV0) protein.

Compounds	Binding affinity (kcal/mol)	Hydrogen bond interactions	Hydrophobic interactions
2-tridecanone	-6.6	**Tyr F: 56**	Tyr F:47, Ala F:70, **Val F: 76**, **Tyr F: 64**, **Ala F: 50**, Cys F: 79, **Leu F: 40**, **Leu F: 125**, **Ala F: 127**
β-selinene	-7.7	-	Tyr F: 47, **Leu F: 40**, Ala F: 50, Val F:127, Val F: 76, **Leu F: 36**, Ala F: 76, **Tyr F: 64**
Dodecanal	-6.4	-	**Leu F: 36**, **Tyr F: 64**, Phe F: 101, **Tyr F: 56**, Leu F: 110, Ala F: 105, **Trp F: 60**, **Trp F: 88****
n-heneicosane	-7.9	-	**Ala F: 127**, Ala F:70, **Val F: 76**, **Ala F: 50**, **Leu F: 125**, **Leu F: 40**, Tyr F: 147, Cys F: 79, **Leu F: 36**, **Tyr F: 64**, Phe F: 101, **Tyr F: 56**, Leu F: 110, Ala F: 105, **Trp F: 60**, **Trp F: 88****
Phytol	-8.4	**Tyr F: 93**	**Tyr F: 56**, Trp F: 88, Val F: 76, Tyr F: 47, Cys F: 79, **Tyr F: 64**, **Leu F: 36**, Ala 127, Leu F:125
Hexahydro-farnesyl-acetone	-8.1	Gly F: 126	Phe F: 101, Ala F: 105, Leu F: 110, **Tyr F: 56**, **Leu F: 36**, Ala F: 70, Val F: 70, **Ala F: 127**, **Ala F: 50**, **Trp F: 88**, **Tyr F: 64****
β-eudesmol	-7.9	Thr F: 75	Tyr F: 47, **Ala F: 50**, Leu F: 40, **Val F: 76**, **Leu F: 36**, **Ala F: 127**, **Tyr F: 64**
Linalool	-6.2	Tyr F: 47	**Leu F: 125**, Val F: 76, **Ala F: 127**, **Cys F : 79**
T-cadinol	-7.4	-	**Tyr F: 64**, **Ala F: 50**, **Val F: 76**, **Leu F: 36**, **Ala F: 127**, **Tyr F: 56**
α-cadinol	-7.4	-	**Tyr F: 56**, **Val F: 76**, **Ala F: 127**, **Leu F: 36**, **Ala F: 50**, **Tyr F: 64**
OHN***	-8.9	**Trp F: 60, Tyr F: 56**, Asp F: 63, **Tyr F: 93***	**Leu F: 40, Leu F: 125, Ala F: 127, Cys F : 79, Ala F: 50, Val F: 76, Leu F: 36, Tyr F: 64**
Gentamycin	-6.6	Gly F: 54, Ser F: 20, Lys F: 16, Asn F: 49, Glu F: 48, Asp F: 65, Lys F: 16*	-

*Carbon Hydrogen interactions, ** Pi-Sigma interactions, ***N-3-Oxo-Dodecanoyl-L-Homoserine Lactone.

Table 18: Docking score and interacting profile of ten principal compounds of *O. aurasiaca* and *O. angustissima* essential oils and the positive controls with FtsZ (ID 3WGN) protein.

Compounds	Binding affinity (kcal/mol)	Hydrogen bond interactions	Hydrophobic interactions
2-tridecanone	-5.0	-	-
β-selinene	-7.1	-	Val B: 203, Val B: 297, Val B: 307, Leu B: 302
Dodecanal	-4.7	-	-
n-heneicosane	-4.8	-	-
Phytol	-5.8	-	-
Hexahydro-farnesyl-acetone	-5.9	-	-
β-eudesmol	-6.8	-	Val B: 297, Val B: 203
Linalool	-5.7	-	-
T-cadinol	-7.4	Thr A: 309	Val A: 203, Val A: 297, Val A: 307
α-cadinol	-6.9	-	Leu B: 200, Val B: 203, Val B: 297
GTP-gamma-S***	-9.3	Gly B: 72, Gly B: 21, Gly B: 22, Gly B: 110, Ala B: 73, Asn B: 166, Asn B: 25, Arg B: 29, Gly B: 108*, Gly B: 104*	Phe B: 183**
Gentamycin	-8.2	Gly A: 21, Gly A: 22, Gly A: 104, Asp A: 46, Thr A: 109 Gly A: 21*	-

*Carbon Hydrogen interactions, **Pi-Pi T-shaped interactions, ***5'-guanosine-dihosphate-monothiophosphate.

All compounds exhibited suitable binding energy score toward the 2UV0 receptor. Notably, phytol, 2-tridecanone, linalool, and β-eudesmol each formed at least one hydrogen-bond, interacting with residues Tyr F: 93, Tyr F: 56, Thr F: 75 and Tyr F: 47. Multiple hydrophobic interactions were also noted, primarily involving Tyr F:47, Val F:76, Tyr F:64, Ala F:50, and Ala F:127. Molecular docking results indicated that phytol, hexahydrofarnesylacetone, β- eudesmol and n-heneicosane were the top ligands based on their binding energy. Furthermore, Discovery Studio visualization showed that all of them bound competitively to the autoinducer-binding domain of LasR, contrary to Gentamycin (Figure 29). Trp F: 60, Tyr F: 56, Tyr F: 93, Leu F: 40, Leu F: 125, Ala F: 127, Cys F: 79, Ala F: 50, Val F: 76, Leu F: 36, Tyr F: 64 were the common interacted residues (Highlighted in bold in table 17).

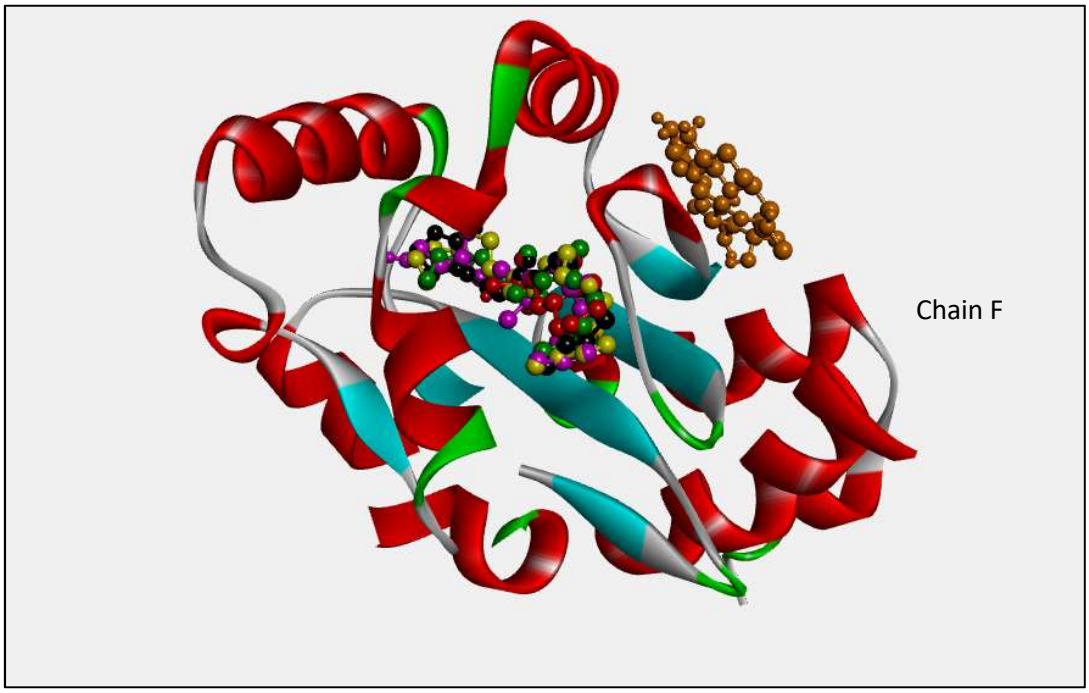

Figure 29: Best pose of top ligands docked to LasR (ID 2UV0) protein: phytol (Purple), β- eudesmol (Red), n-heneicosane (Yellow), Hexahydrofarnesylacetone (Green) compared to co-crystallized OHN (Black) and Gentamycin (Orange).

Molecular docking findings suggest that the ten principal compounds from *O. aurasiaca* and *O. angustissima* essential oils, especially hexahydrofarnesylacetone (Figure 30), n- heneicosane (Figure 31), phytol (Figure 32) and β-eudesmol (Figure 33) act as quorum sensing system inhibitors by inhibiting LasR competitively (Figure 29), potentially offering a new and effective solution against *P. aeruginosa* virulence and antimicrobial resistance.

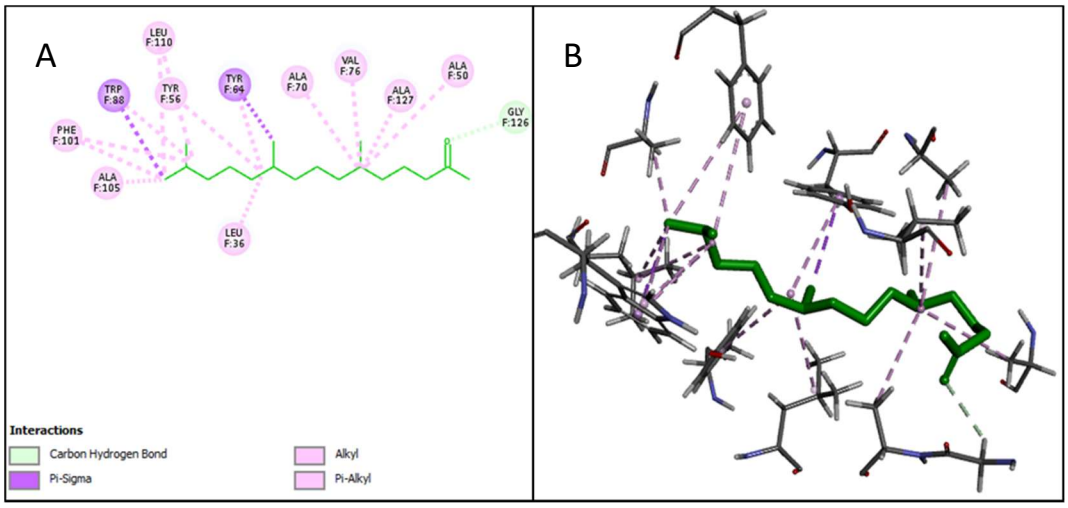

Figure 30: LasR-Hexahydrofarnesylacetone interactions on 2D diagram (A) and on 3D presentation (B).

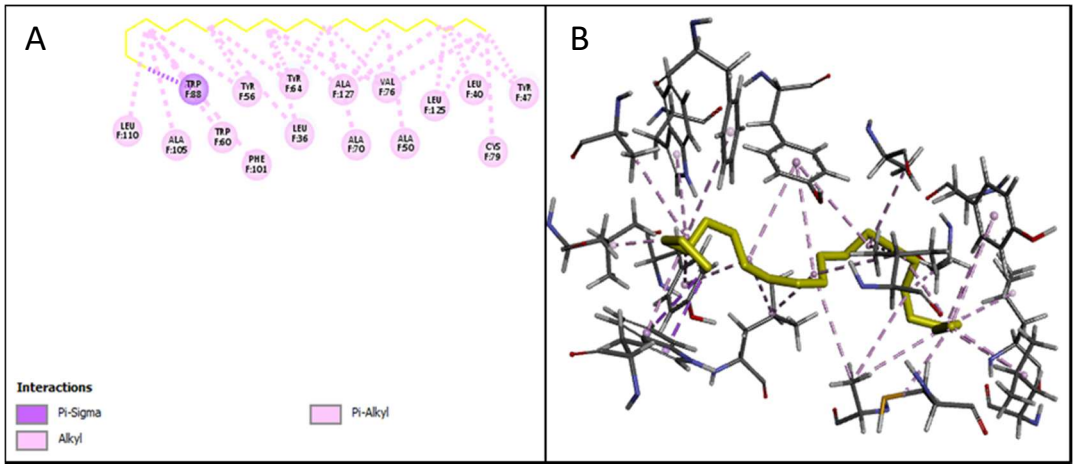

Figure 31: LasR-n-heneicosane interactions on 2D diagram (A) and on 3D presentation (B).

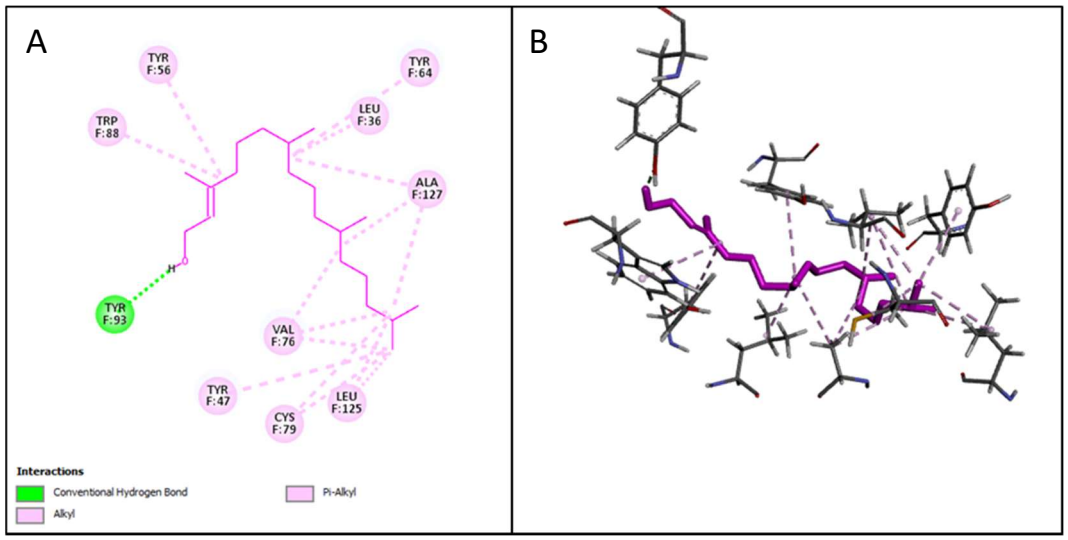

Figure 32: LasR-Phytol interactions on 2D diagram (A) and on 3D presentation (B).

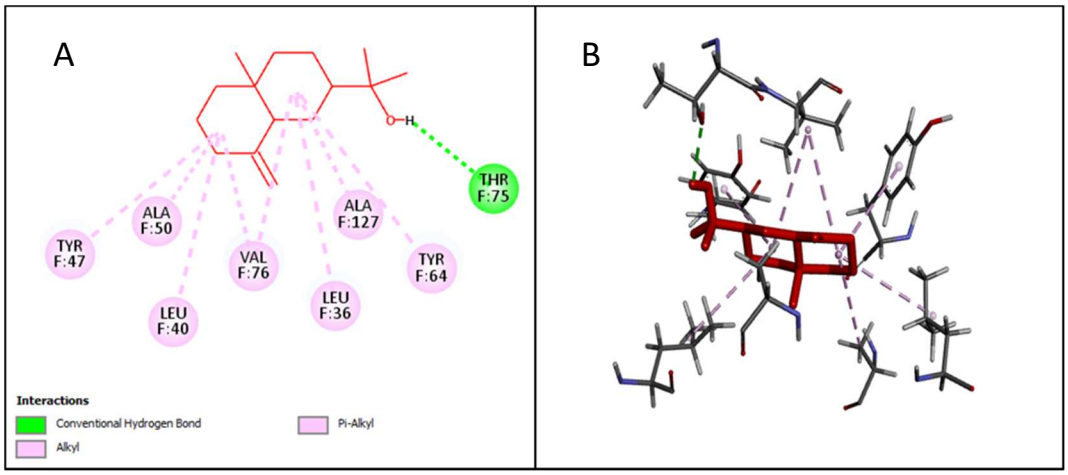

Figure 33: LasR-β-eudesmol interactions on 2D diagram (A) and on 3D presentation (B).

The natural substrate OSB-NCoA exhibited the lowest bonding energy at all (-9.5 kcal/mol), when bound to the MenB (3T88) from *E. coli* (Figure 34), it provides important insight into the catalytic mechanism by revealing the position of all active site residues (Li et al. 2011), through miscellaneous interactions like carbon hydrogen, hydrophobic and electrostatic interactions as detailed in table 16. All main compounds derived from *O. aurasiaca* generally exhibited high binding energy values towards the 3T88 protein, except for hexahydrofarnesylacetone, which displayed lower energy values. Conversely, all main compounds from *O. angustissima*, except Linalool, demonstrated low binding energy values against this receptor through hydrogen bonds and/or hydrophobic interactions.

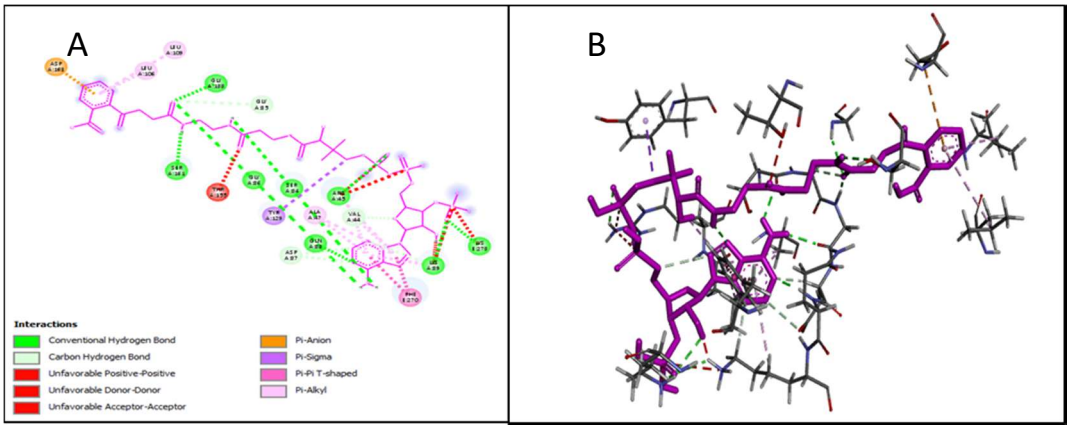

Figure 34: MenB (3T88)-Natural substrat (OSB-NCoA) interactions on 2D diagram (A) and on 3D presentation (B).

t-cadinol and β-eudesmol bound to the active site of the MenB enzyme with an energy of - 8.1 kcal/mol and -7.0 kcal/mol respectively. The common interacting residues as the substrate analogue (OSB- NCoA) were Gly: 86, Phe: 270, Val: 44 and Leu: 106 (Presented in table 16 in bold). In contrast, hexahydrofarnesylacetone interacted with chain E (Figure 35). These outcomes suggest that t-cadinol and β-eudesmol from *O. angustissima* essential oil could effectively inhibit the menaquinone biosynthetic pathway in *E. coli* by inhibiting 3T88 enzyme activity.

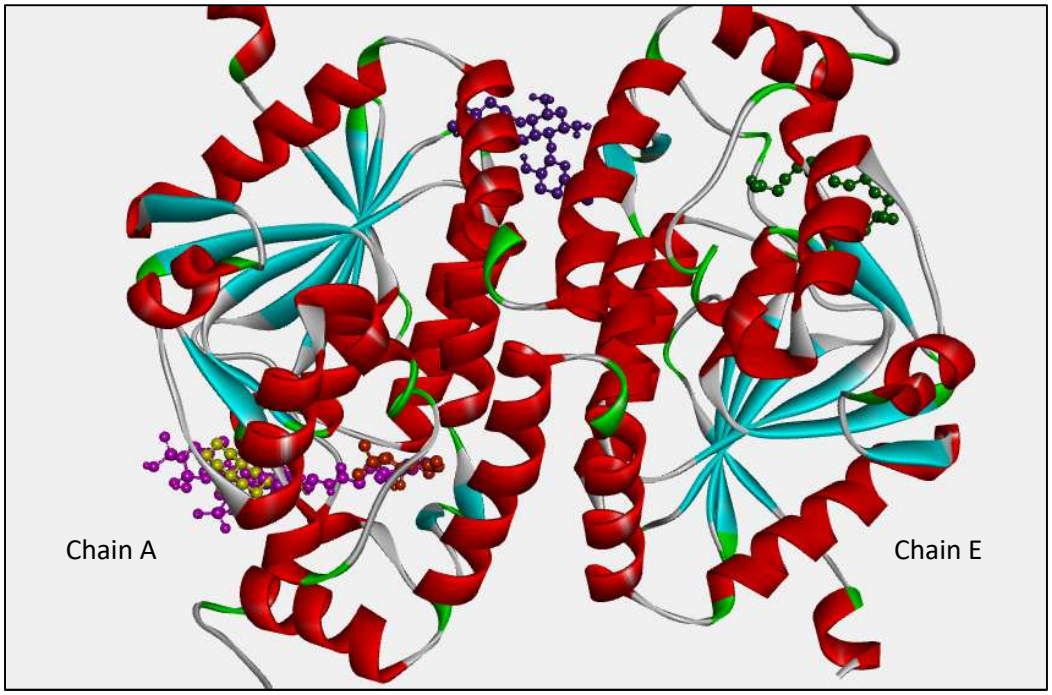

Figure 35: Best pose of top ligands docked to MenB (ID 3T88) protein: t-cadinol (Red), β- eudesmol (Yellow), Hexahydrofarnesylacetone (Green) compared to co-crystallized SON (Purple) and Gentamycin (Blue).

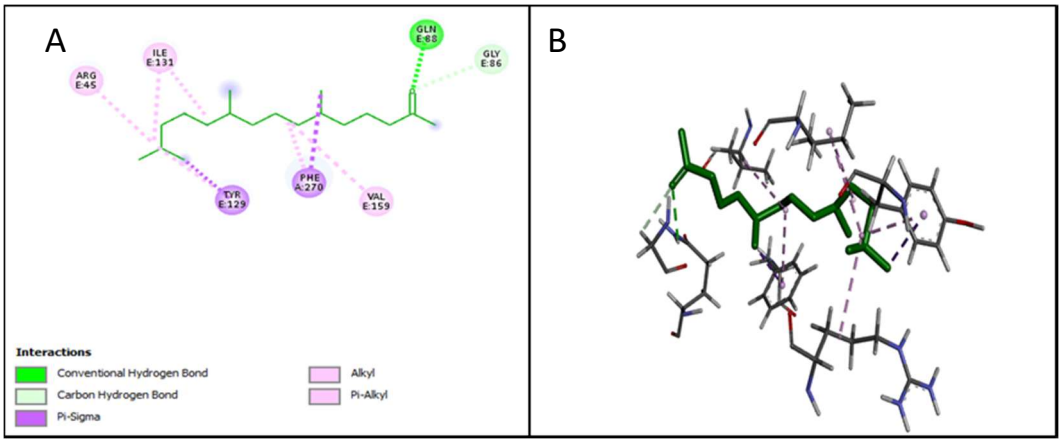

Figure 36: MenB-Hexahydrofarnesylacetone interactions on 2D diagram (A) and on 3D presentation (B).

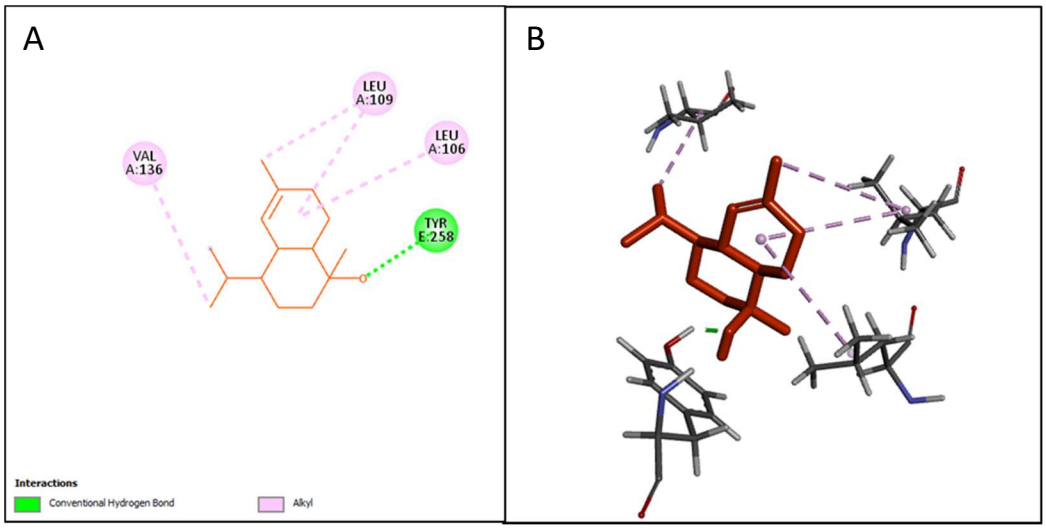

Figure 37: MenB-T-cadinol interactions on 2D diagram (A) and on 3D presentation (B).

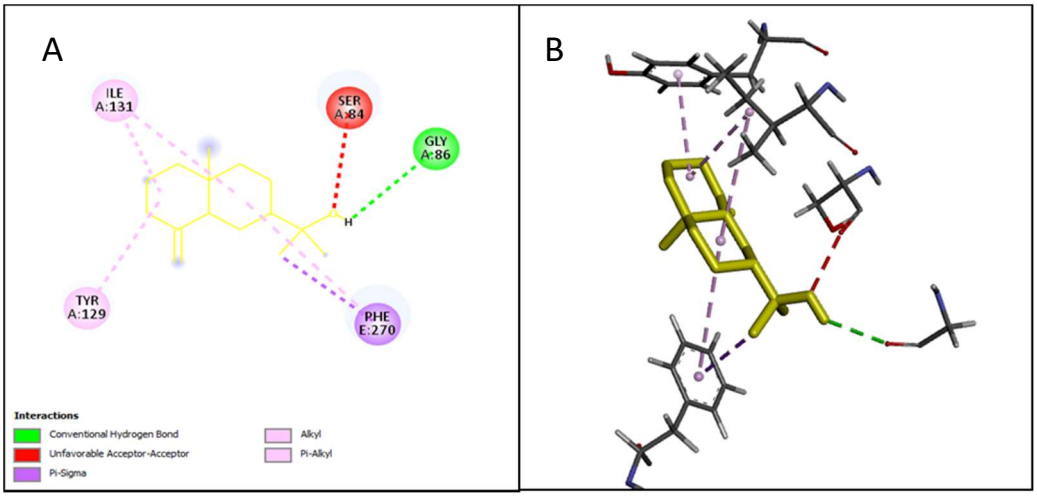

Figure 38: MenB-β-eudesmol interactions on 2D diagram (A) and on 3D presentation (B).

Similar trends were almost observed with the 3WGN protein and *O. aurasiaca* main compounds, none of which showed affinity toward this protein (Table 18), except for β-selinene which displayed low binding energy value (7.1 kcal/mol) and four hydrophobic interactions (Figures 39, 41). The main and common interacting residues were Val: 203, Val: 297 and Val: 307 on either chain A or B. T-cadinol was also found the strongest ligand for this receptor (7.4 kcal/mol), forming a hydrogen bond with Thr A: 309 (Figure 40). However, it is not comparable with the bound GTP-gamma-S and Gentamycin, which displayed several hydrogen bonding interactions with other different amino acids. thus, t-cadinol may interfere with the cell constriction of *S. aureus* by inhibiting the polymerization of FtsZ at the division site.

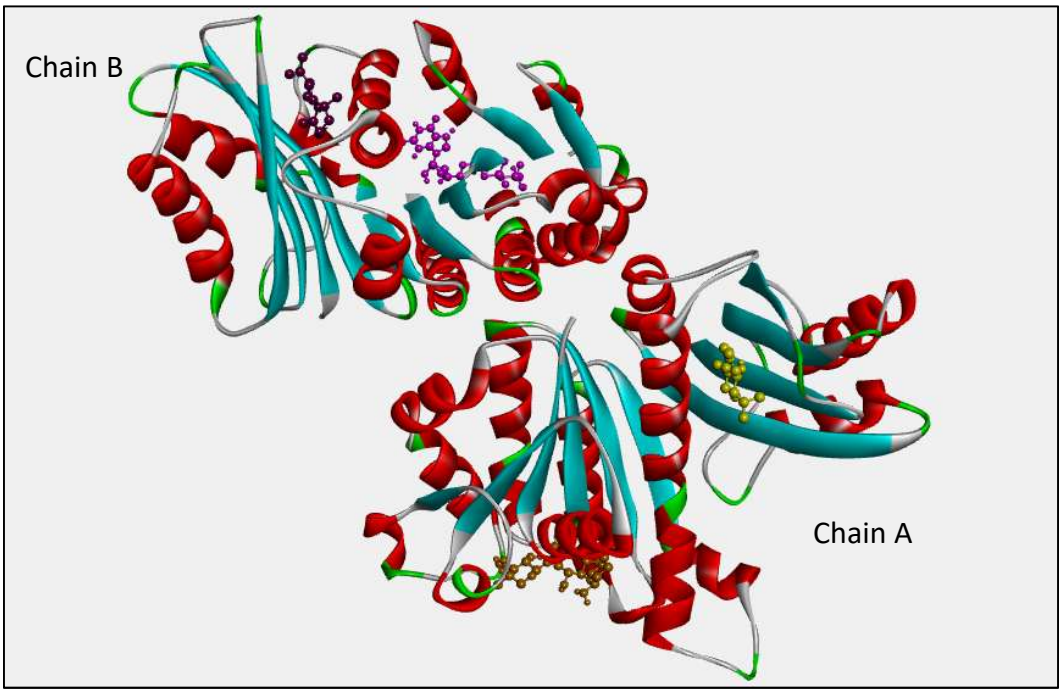

Figure 39: Best pose of top ligands docked to FtsZ (ID 3WGN) protein: β-selinene (Dark purple), t-cadinol (Yellow) compared to co-crystallized GTP-gamma-S (Light purple) and Gentamycin (Orange).

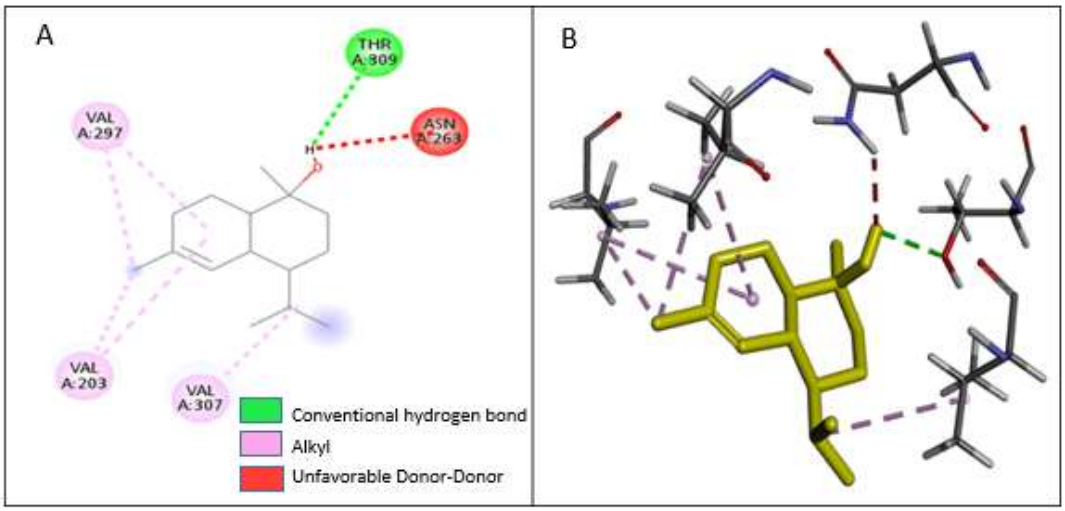

Figure 40: FtsZ-T-cadinol interactions on 2D diagram (A) and on 3D presentation (B).

Gentamycin, used as a positive control, demonstrated binding affinities across all receptors without forming hydrophobic contacts, unlike the tested compounds. It established six classical hydrogen bonds with the 2UV0 receptor (Figure 41) and a mix of classical and non-classical hydrogen bonds with the 3T88 (Figure 42) and 3WGN (Figure 43) receptors, making it the most stable docked compound after the co-crystallized ligands, particularly with the latter two receptors. Additionally, Gentamycin's binding position was found on the opposite side of the

active site of the 2UV0**,** 3T88 and 3WGN receptors, suggesting it may function as an allosteric ligand (Figures 29**,** 35 and 39).

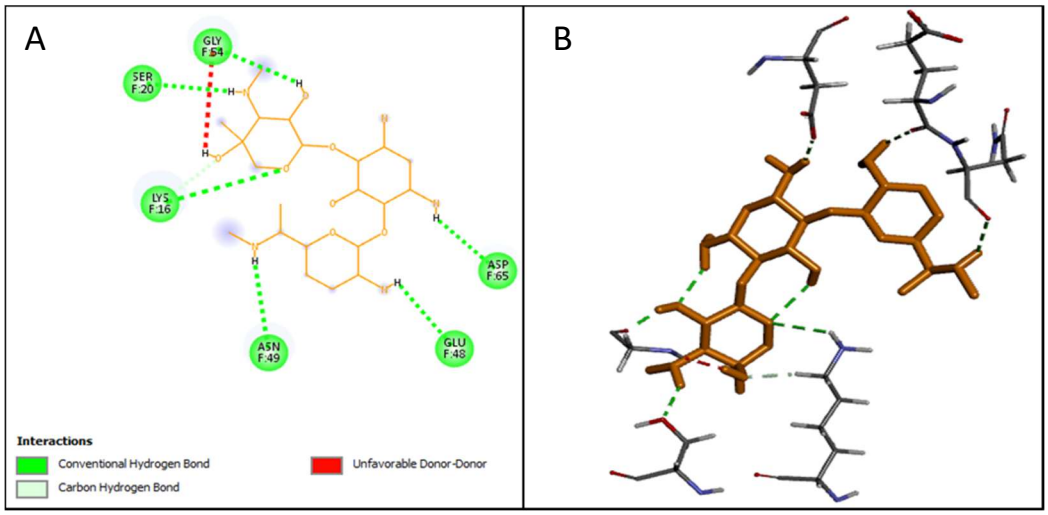

Figure 41: LasR-Gentamycin interactions on 2D diagram (A) and on 3D presentation (B).

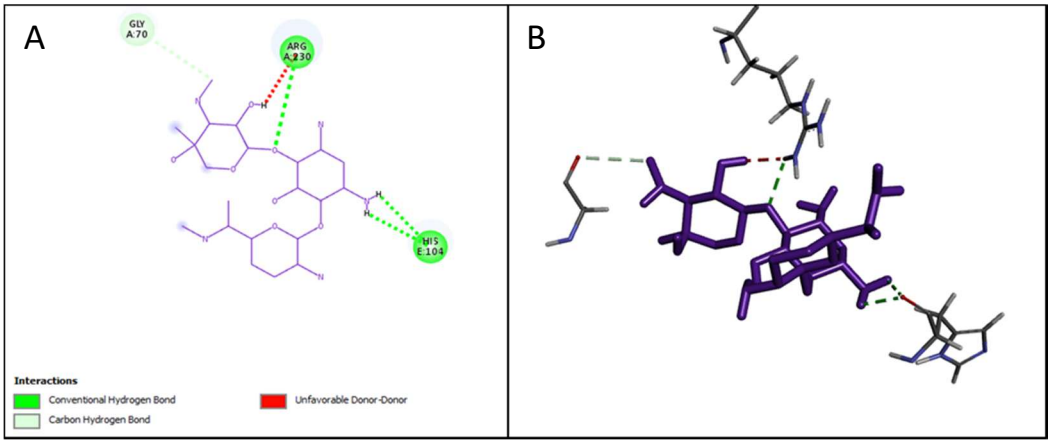

Figure 42: MenB-Gentamycine interactions on 2D diagram (A) and on 3D presentation (B).

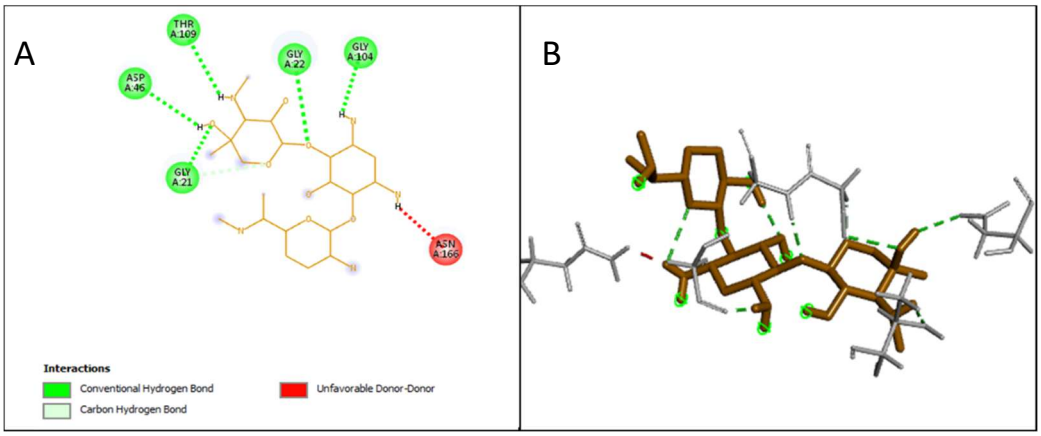

Figure 43: FtsZ-Gentamycin interactions on 2D diagram (A) and on 3D presentation (B).

The *in silico* study conducted for the first time on *O. aurasiaca* and *O. angustissima* essential oils main compounds, concludes that certain ones, particularly phytol, hexahydrofarnesylacetone, t-cadinol, and β-eudesmol could qualify strong inhibitors of the target proteins, given their low binding energy values and favorable interaction patterns. The variety of interactions, including hydrogen bonds and hydrophobic contacts with key active site residues, demonstrates strong binding stability, indicating potential biological activity of these ligands. These findings contribute to the ongoing efforts in drug discovery and warrant further investigation.

One limitation of this study is the reliance on static docking simulations, which do not account for protein flexibility and dynamic behavior. Furthermore, the scoring functions used may not fully capture the complexity of binding interactions. Future studies should incorporate molecular dynamics simulations to better account for protein flexibility.

VII. *In vitro* antibacterial activity investigation

According to the data presented for the first time in table 19 and table 20, the diameter of inhibition zones varied between 8.6 mm and 14.9 mm for Gram-positive strains and between 5.2 mm and 8.8 mm for Gram-negative strains across different dilutions (Figure 44). Utilizing the classification system proposed by Ponce et al. (2003), which categorizes microbial sensitivity into four distinct groups (Resistant, sensitive, highly sensitive, and extremely sensitive) based on inhibition zone diameters, only the Gram-positive *Staphylococcus aureus* strains fell into the sensitive category. However, upon evaluating the inhibition zone diameters at a lower concentration equivalent to 1/100 of oil to DMSO, there were no inhibition zones present for any of the tested strains (Not specifically detailed in tables). Among the tested extracts, the DCME of *O. angustissima* exhibited the most potent effect with an inhibition zone of 14.9 mm followed closely by two essential oils, which displayed nearly equal efficacy. Nonetheless, these results consistently indicated lower inhibition zones compared to those induced by the positive control (Gentamycin), which exhibited a robust effect against all strains.

Table 19: Antibacterial activity of *O. aurasiaca* extracts following the agar disk diffusion method (First study).

Extract	Dilution	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 25923	*S. aureus* A43300
		Gram$^-$		Gram$^+$	
Essential oil	1/1	7.4^{bcdef}±0.6 -	7.3^{bcdef}±0.2 -	13.0^{l}±0.6 +	11.1^{k}±0.3 +
	1/2	7.1^{bcdef}±1.4 -	6.0^{abcd}±0.0 -	11.0^{k}±1.9 +	10.0^{ijk}±0.4 +
	1/20	6.6^{abcde}±0.9 -	5.8^{abc}±0.1 -	9.9^{ijk}±0.5 +	9.2^{ghij}±0.5 +
PEE B	1/1	6.7^{abcde}±0.9 -	5.0^{a}±0.0 -	10.3^{jk}±1.2 +	NT
	1/2	6.8^{abcde}±0.8 -	5.0^{a}±0.0 -	9.4^{ghijk}±1.4 +	
	1/20	5.0^{a}±0.0 -	5.0^{a}±0.0 -	8.3^{efghi}±0.1 -	
DCME B	1/1	7.8^{cdefgh}±0.3 -	5.0^{a}±0.0 -	9.7^{hijk}±1.7 +	NT
	1/2	8.1^{efghi}±0.7 -	5.0^{a}±0.0 -	10.3^{jk}±1.6 +	
	1/20	5.7^{ab}±0.8 -	5.0^{a}±0.0 -	8.8^{fghij}±0.9 +	
Me-OHE B	1/1	7.5^{bcdefg}±0.4 -	5.6^{ab}±0.5 -	9.8^{ijk}±0.6 +	NT
	1/2	7.3^{bcdef}±0.1 -	5.0^{a}±0.0 -	9.6^{hijk}±1.2 +	
	1/20	5.0^{a}±0.0 -	5.0^{a}±0.0 -	5.0^{a}±0.0 -	
Gentamycin		33.4^{m}±0.9 +++	29.4^{m}±1.7 +++	37.2^{m}±0.7 +++	34.1^{m}±1.3 +++

NT: not tested.

Data are presented as mean inhibition zone diameters (mm) ± SD (n=3). Values with the same letter superscripts are not significantly different (Fisher LSD, P=0.05). (-) Resistant, (+) sensitive and (+++) extremely sensitive according to the scale of Ponce et al. (2003).

These results are consistent with other studies on different *Ononis* genus extracts, like the methanolic extract of *O. natrix*, and the dichloromethane and *n*-butanol ones of *O. alba poir*, which were found having no effect on Gram-negative bacteria such as *E. coli* and *P. aeruginosa*. However they have exhibited an effect on Gram-positive strains as *S. aureus* (Mhamdi et al. 2014; Zaak et al. 2020). Furthermore, in a study of Sayari et al. (2016), *O. natrix* leaves extracts were tested against nine Gram-negative and Gram-positive bacterial strains and

consistently with our study; *S. aureus* were the most sensitive tested strain. However, the oil of *O. angustissima*, as examined by Ghribi et al. (2016), demonstrated differing effects in activity against *S. aureus* and *P. aeruginosa* compared to the result of this study, while showing a similar effect against *E. coli*.

Table 20: Antibacterial activity of *O. angustissima* extracts following the agar disk diffusion method.

Extract	Dilution	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 25923	*S. aureus* A43300
		Gram$^-$		Gram$^+$	
Essential oil	1/1	8.8^{fg}±0.4 -	7.1^{bcd}±0.4 -	12.5^{j}±0.7 +	10.4^{i}±0.2 +
	1/2	8.3^{defg} ±0.5 -	6.0^{abc}±0.2 -	10.7^{i}±0.4 +	8.6^{efg}±0.6 -
	1/20	7.5^{cdef}±0.5 -	5.2^{a}±0.0 -	9.4^{hi}±0.8 +	7^{bcd}±0.4 -
PEE H	1/1	7.4^{cdef}±0.2 -	5.0^{a}±0.0 -	8.2^{defg}±0.8 -	NT
	1/2	7.2^{bcde}±0.1 -	5.0^{a}±0.0 -	8.3^{defg}±2.0 -	
	1/20	5.0^{a}±0.0 -	5.0^{a}±0.0 -	5.0^{a}±0.0 -	
DCME H	1/1	8.2^{defg} ±1.0 -	5.0^{a}±0.0 -	14.9^{k}±0.4 +	NT
	1/2	8.7^{efg}±0.2 -	5.0^{a}±0.0 -	12.4^{j}±0.3 +	
	1/20	7.2^{bcde}±1.3 -	5.0^{a}±0.0 -	8.9^{gh}±0.9 -	
Me-OHE H	1/1	7.7^{def}±0.1 -	5.6^{ab}±0.6 -	8.4^{defg} ±0.3 -	NT
	1/2	8.2^{defg} ±1.4 -	5.0^{a}±0.0 -	5.0^{a}±0.0 -	
	1/20	5.0^{a}±0.0 -	5.0^{a}±0.0 -	5.0^{a}±0.0 -	
Gentamycin		33.4^{l}±0.9 +++	29.4^{l}±1.7 +++	37.2^{l}±0.7 +++	34.1^{l}±1.3 +++

NT: not tested.

Data are presented as mean inhibition zone diameters (mm) ± SD (n=3). Values with the same letter superscripts are not significantly different (Fisher LSD, P=0.05). (-) Resistant, (+) sensitive and (+++) extremely sensitive according to the scale of Ponce et al. (2003).

VIII. Validation of computational study through *in vitro* analysis

Upon discussing the *in vitro* results with those obtained from the *in silico* study, several key observations can be highlighted:

- Only *S. aureus* exhibited sensitivity toward *O. angustissima* and *O. aurasiaca* essential oils, whereas *E. coli* and *P. aeruginosa* were found to be resistant *in vitro*.
- The *in silico* study identified β-selinene, (A sesquiterpene hydrocarbon from *O. aurasiaca*) and β-eudesmol, t-cadinol and α-cadinol (Oxygenated sesquiterpenes from *O. angustissima*) as significant inhibitors of FtsZ (3WGN). This finding may directly account for the achieved *in vitro* results.
- *In silico* molecular docking study, demonstrate that all principal compounds effectively inhibited the transcriptional regulators LasR (2UV0). This discrepancy can be attributed to the fact that the *in vitro* study utilized the essential oil in its extracted form (A mixture of compounds), whereas the *in silico* study evaluated each compound separately. As shown in figure 29, the top ligands competitively bound to the autoinducer-binding domain of LasR, potentially explaining the inactivity of the essential oils against the *P. aeruginosa* strain.
- In the case of *E. coli*, all main compounds from *O. angustissima*, except linalool, demonstrated an *in silico* inhibition effect on *E. coli* MenB (3T88) contrary to the *in vitro* findings. This difference could be explained similarly to the first point, or further metrics such as Solvent Accessible Surface Area (SASA), a key parameter in molecular structure studies, are important to validate the *in silico* efficiency of a molecule. SASA describes the area around a macromolecule accessible to solvent molecules, an important indicator for understanding protein folding, ligand binding, and conformational changes. Molecular dynamics can also be a helpful tool to validate or expel a ligand's effectiveness.
- Conversely, all main compounds derived from *O. aurasiaca* were unable to inhibit MenB (3T88) protein, which align with the *in vitro* findings.
- Gentamycin demonstrated high binding affinity and stability across all targets, particularly with 3T88 and 3WGN proteins, aligning with *in vitro* outcomes of its greater effectiveness against *E. coli* and *S. aureus* compared to *P. aeruginosa*.

Conclusion

Conclusion

The study of medicinal plants has garnered increasing interest due to their potential to provide novel therapeutic agents. Among these, the *Ononis* species, known for their traditional medicinal uses, have been less explored in scientific research. This research aimed to provide a comprehensive analysis of the chemical composition and biological activities of two *Ononis* plants, *Ononis aurasiaca* Förther & Podlech and *Ononis angustissima* Lam. subsp. *filifolia* Murb., endemic to Algeria.

The liquid-solid extraction and hydrodistillation processes yielded various extracts and essential oils with differing efficiencies, when polar solvents demonstrating a significant yield in extractable compounds. However, the essential oil yields were low. Quantification of total polyphenols and flavonoids revealed that DCMEs contained the highest levels of these compounds. The GC-MS analysis identified numerous compounds in the essential oils, with *O. angustissima* demonstrating higher yields and a more diverse array in constituents than *O. aurasiaca*.

The antioxidant activity was evaluated *in vitro* using three methods. DCME of *O. aurasiaca* showed great potential in scavenging DPPH radicals. However, the essential oils demonstrated strong activity in lipid peroxidation inhibition. In contrast, all extracts were found having only moderate to weak activity in the phenanthroline test.

The *in silico* antibacterial activity suggests that, specific compounds from essential oils, like phytol, hexahydrofarnesylacetone, t-cadinol, and β-eudesmol could be considered as active compounds, mainly by inhibiting key bacterial proteins.

The *in vitro* antibacterial activity showed that Gram-positive *S. aureus* strains were sensitive to the extracts and essential oils. In contrast, Gram-negative *E. coli* and *P. aeruginosa* were generally resistant. The discrepancy between computational predictions and experimental results for Gram-negative bacterial strains highlight the complexity of whole extract activities tested *in vitro* and the need for further investigation.

This study is the first detailed report on *O. aurasiaca* Förther & Podlech and *O. angustissima* Lam. subsp. *filifolia* Murb., providing an important reference point for future research. Further investigations could focus on isolating and testing individual compounds, exploring synergistic effects, and conducting more advanced *in vivo* studies to fully elucidate the antioxidant and antibacterial potential of these plants. Ultimately, this study laying the groundwork for deeper

research into the biological potential of *Ononis* species, paving the way for their inclusion in plant-based health interventions.

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First report on the chemical composition and the free radical scavenging and antimicrobial activities of the essential oil of *Ononis aurasiaca*, an endemic plant of Algeria

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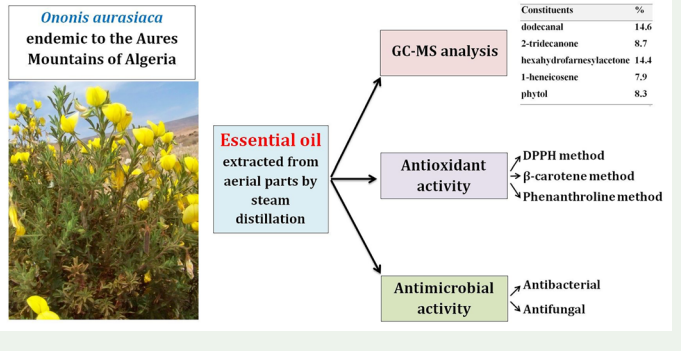
ABSTRACT

This study represents the first investigation of the chemical composition and the antioxidant and antimicrobial activities of *Ononis aurasiaca* Förther & Podlech, a plant species endemic to the Aures Mountains of Algeria. The essential oil of the plant aerial parts was analysed using GC-MS. The *in vitro* antioxidant activity was evaluated using three methods. A total of 44 compounds were identified. The major constituents were dodecanal, hexahydrofarnesylacetone, 2-tridecanone, phytol, 1-heneicosene, and *n*-heneicosane. The oil displayed significant activity in the β-carotene bleaching assay, moderate scavenging activity against DPPH radicals and a low ability to reduce iron ions. Antibacterial tests conducted on four strains revealed effectiveness primarily against Gram-positive strains, specifically *Staphylococcus aureus* ATCC 25923 and ATCC 43300, while showing limited impact on Gram-negative strains, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922. Antifungal activity tests involving two moulds revealed a stronger inhibition against *Scedosporium apiospermum* compared to *Aspergillus niger*.



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1. Introduction

The *Ononis* genus is a large and diverse group of plants within the Fabaceae family, with 86 species found mainly in the Mediterranean region, Macaronesia, and neighbouring Asia (Ozenda 1958; Förther and Podlech 1991). These plants are typically characterised by yellow flowers, narrow leaflets, and small bushes with branched stems at the base (Ozenda 2004 and Mabberley 2017). The taxonomic treatment of the *Ononis* genus was first presented by Širjaev (1932), who accepted 68 species distributed in two sections according to their distinct peduncles and pod characteristics: Ononis section, characterised by reduced peduncles and pods with few-seeded, and Natrix Grisebach section, characterised by distinct peduncles and multi-seeded pods (Fayed et al. 2019). *Ononis aurasiaca* Förther & Podlech is one of the species belonging to the sect. Natrix Grisebach (Förther and Podlech 1991; Turini et al. 2010), which is endemic to eastern Algeria and found only in the Aures region. It is locally known as 'Fizza' and its aerial parts are traditionally used as a patch on the chest to alleviate symptoms of colds or the flu.

Several *Ononis* species have traditionally been used in phytotherapy. For example, *O. spinosa* has been employed for the treatment of inflammatory diseases of the lower urinary tract and kidney stones (Kırmızıgül et al. 1997). It is also used for healing wounds, eczema, other skin disorders, and gout (Altanlar et al. 2006; Yilmaz 2006). The roots of *O. spinosa*, *O. arvensis*, *O. hircina*, and *O. antiquorum* are known to have applications against skin irritations, itching, wounds, and dermatitis (Mamedov et al. 2005). Furthermore, *O. sicula* and *O. hirta* have been utilised for wound healing and as antiseptics in the treatment of skin cancer and cold sores (Talib and Mahasneh 2010a). Extracts of *O. natrix* are used for their antirheumatic, diuretic, urolithiatic, and blood pressure-reducing properties (Al-Khalil et al. 1995; Saeed 2003).

Numerous investigations have been conducted on the chemical composition of *Ononis* species. However, a significant portion of these studies has primarily focused on the composition of aqueous extracts and crude solvent extracts, encompassing acetone, chloroform, ether, ethyl acetate, butanol, hexane, and methanol. Some specific species that have been studied include *Ononis angustissima* (Bouheroum et al. 2009; Mezrag et al. 2013; Benabderahmane et al. 2014; Guettaf et al. 2016; Mezrag et al. 2017), *Ononis vaginalis* (Abdel-Kader 2001), *O. speciosa* (Barrero et al. 1989), *O. natrix* (San Feliciano et al. 1983; Al-Rehaily et al. 2014; Mhamdi et al. 2015; Sayari et al. 2016; Öz et al. 2017; Yerlikaya et al. 2017; Stojković et al. 2020), *O. spinosa* (Öz et al. 2017; Stojković et al. 2020), *O. variegata* and *O. viscosa* (Öz et al. 2017), *O. pubescens* (Jaradat et al. 2017), *O. mitissima* (Besbas et al. 2020), *O. pusilla* (Khouni et al. 2014), and *O. arvensis* (Dénes et al. 2022).

The essential oils of certain *Ononis* species have received relatively little research attention in proportion to the number of species within the genus. For instance, studies on the essential oils of *O. angustissima* (Mechehoud et al. 2014; Ghribi et al. 2016), *O. natrix* (Khallouki et al. 2002; Elamrani and Benaissa 2010; Al-Mterin et al. 2021), *O. sicula* (Al-Qudah et al. 2014), *O. reclinata* (Casiglia et al. 2017), *O. viscosa* (Erdemgil et al. 2002), and *O. alba* (Zaak et al. 2022) have been conducted.

Further research studies have demonstrated a diverse range of pharmacological properties of *Ononis* species. These encompass antimicrobial, analgesic, antioxidant,

antiproliferative, anticancer, antihypertensive, anti-inflammatory activities, as well as the ability to accelerate wound healing (Requena et al. 1987; Yılmaz et al. 2006; Altuner et al. 2010; Talib and Mahasneh 2010b; Süntar et al. 2011; Al-Qudah et al. 2014; Ghribi et al. 2015; Mhamdi et al. 2015; Jaradat et al. 2017; Öz et al. 2017).

Ononis aurasiaca is a perennial semi-shrub that can grow up to 50 cm high with a woody base and trifoliate leaves covered with dense double glands. Its flowers are bright yellow and have a distinct smell (Förther and Podlech 1991). This plant has not previously been studied for its chemical composition or bioactivities until now.

The study aims to conduct a comprehensive analysis of the chemical composition of the essential oil of *Ononis aurasiaca* and evaluate its free radical scavenging and antimicrobial activities. Through the investigation of the unique compounds in this endemic species, the research seeks to advance the understanding of its bioactive properties, pharmaceutical significance, and potential applications.

2. Results and discussion

2.1. Essential oil composition

Since no previous data on the chemical composition and biological activities of this species are available, the discussion is based on the findings of some studies on other species within the same genus.

A low yield of dark yellow oil with a strong odour, about 0.012% (w/w), was obtained. This yield was found to be lower than that of *O. natrix* collected from Jordan (Al-Qudah et al. 2014) and Morocco (Elamrani and Benaissa 2010), which produced pale yellowish oil at a yield of 0.21% (w/w) and light yellowish oil at a yield of 1.0% (m/w), respectively. Similarly, the aerial part of *O. angustissima* from Tunisia (Ghribi et al. 2016) and *O. reclinata* from Italy (Casiglia et al. 2017) yielded light yellow oil at 0.04% (w/w) and oil with a pleasant smell at 0.18% (w/w), respectively.

The identified compounds are listed in Table S1 (supplementary material), along with their relative percentages and linear retention indices (LRI) relative to the *n*-hydrocarbons series. Out of the total volatiles, 44 components were identified, constituting 93.4%. The oil was found to be rich in apocarotenoids (15.6%), with hexahydrofarnesylacetone (14.4%) as the major compound. Sesquiterpene hydrocarbons (11.8%) were also present, with β-selinene (5.1%) as the main constituent, along with diterpenes (9.0%), mainly consisting of phytol (8.3%). Additionally, compounds identified included oxygenated monoterpenes (0.3%), and oxygenated sesquiterpenes (2.4%). Among the latter, *trans*-sabinyl acetate was the only identified compound. The remaining 54.3% of the total composition was consisted mainly of non-terpene derivatives. Among them, dodecanal (14.6%), 2-tridecanone (8.7%), and 1-heneicosene (7.9%) were the major compounds.

The presence of non-terpene compounds alongside terpenoids and apocarotenoides suggests a complex metabolic network in *Ononis aurasiaca*, with potential crosstalk and interconversions among different biosynthetic pathways.

Sesquiterpene hydrocarbons, like β-selinene, are synthesised from mevalonate pathway and methylerythritol phosphate pathway (Sacchettini and Poulter 1997), which are key pathways involved in the production of isoprenoid compounds in

plants. These pathways also contribute to the biosynthesis of diterpenes, such as phytol. Despite their high structural diversity, terpenes are derived from two isomeric precursor molecules, dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP), which are derived from isoprene units (Dewick 2002; Vranova et al. 2012; Böttger et al. 2018). β-selinene is biosynthesized from IPP and DMAPP through the conversion of geranyl diphosphate, which is the precursor of monoterpenes, to farnesyl diphosphate, the precursor of sesquiterpenes (Schwab et al. 2008). Phytol, the major diterpene in *O. aurasiaca* essential oil, is derived from chlorophyll during degradation (Schulz et al. 2011). It acts as a precursor for the biosynthesis of different compounds, including hexahydrofarnesylacetone (Letaief et al. 2021), which is the predominant apocarotenoid. The non-terpene derivatives identified in the essential oil, such as dodecanal, 2-tridecanone, and 1-heneicosene, may have diverse biosynthetic origins. These compounds could arise from fatty acid metabolism and subsequent oxidative reactions or other biochemical pathways within the plant (Radulović et al. 2009).

O. aurasiaca oil was found to have a significantly higher proportion of sesquiterpene hydrocarbons than *O. angustissima* collected in Tunisia (Ghribi et al. 2016). On the other hand, the percentage of oxygenated sesquiterpenes was much lower in *O. aurasiaca* (2.4%) than in *O. angustissima* (33.2%), where this class dominated the oil. Furthermore, the essential oil of another *O. angustissima* population, collected from the Bechar region in Algeria (Mechehoud et al. 2014) had only 24 components, with β-selinene (2.7%) and α-muurolene (1.2%) as the main ones. Interestingly, there are six subspecies of *O. angustissima* Lam. distributed in North Africa and southern Spain, as identified in the latest revision of the Natrix section in Macaronesia, North Africa and adjacent West Asia (Förther and Podlech 1991). Two Jordanian species of the same genus, *O. natrix* and *O. sicula* (Al-Qudah et al. 2014), as well as *O. reclinata* from Italy (Casiglia et al. 2017), exhibited a higher abundance of oxygenated sesquiterpenes compared to *O. aurasiaca*, which contains only one oxygenated sesquiterpene, α-cadinol. It is worth noting that the oil of *O. aurasiaca* does not contain any coumarin isopimpinellin, a class of compounds that has been detected in significant quantities in the oil of *O. reclinata* (Casiglia et al. 2017). On the other hand, the essential oil of *Ononis viscosa* subsp. *breviflora*. from Turkey (Erdemgil et al. 2002) contained 40 constituents, with results comparable to those of *O. aurasiaca* especially for the major identified constituents hexahydrofarnesylacetone and dodecanal. Also, the dominant compounds in *O. natrix* from Morocco (Elamrani and Benaissa 2010) are apocarotenoids, including farnesyl acetone and geranyl acetone. Additionally, another Moroccan population of *O. natrix* (Khallouki et al. 2002) exhibited 26 compounds, five of which are also present in *O. aurasiaca*, albeit in different percentages, including α-muurolene, γ-muurolene, γ-cadinene, δ-cadinene, and β-selinene. Similarly, the essential oil of *O. alba* Poir. from Algeria, as reported by Zaak et al. (2022), demonstrated low percentages of oxygenated monoterpenes and oxygenated sesquiterpenes, along with a high proportion of non-terpene derivatives. This observation aligns with the composition of the oil from *O. aurasiaca*.

The chemical diversity among *Ononis* species worldwide is significant, both due to the presence of various chemical classes and related principal compounds in different amounts. These findings support the classification based on botanical and genetic criteria (Förther and Podlech 1991; Turini et al. 2010).

Various factors contribute to shaping the properties of the essential oil, including intrinsic conditions within the plant such as genetics, development stage, physiological and biochemical pathways, as well as extrinsic factors such as environmental and experimental conditions. These factors collectively influence the yield, composition, and percentages of individual components in the essential oil, and considering them can provide further insights into the study results (Figueiredo et al. 2008; Moghaddam and Mehdizadeh 2017).

2.2. Antioxidant activity

2.2.1. DPPH method

According to the DPPH assay (supplementary material), the essential oil and reference compounds presented a concentration-dependent free radical scavenging activity, as shown in Figure S1 (Supplementary material). However, the essential oil exhibited lower antioxidant activity than the reference compounds. At the highest tested concentration (240 µg/ml), the essential oil inhibited DPPH radicals by 51.002 ± 4.159%, while quercetin, BHA and ascorbic acid showed higher inhibition percentages, 98.462 ± 0.311%, 96.235 ± 1.069% and 98.379 ± 0.830%, respectively. Furthermore, the essential oil required a higher concentration (IC_{50}= 231.87 ± 2.25 µg/ml) to achieve 50% inhibition compared to quercetin (IC_{50}= 1.15 ± 0.23 µg/ml), as reported in Table S2 (supplementary material).

Regarding essential oils, the free radical scavenging capacity of Jordanian *O. natrix* and *O. sicula* essential oils was reported to have IC_{50} values of 54.73 µg/ml and 89.20 µg/ml, respectively (Al-Qudah et al. 2014). However, in the current study, the essential oil of *O. aurasiaca* had a higher IC_{50} value, indicating lower antioxidant activity. The ratio 0.5 ml/1.5 ml of extract/DPPH adopted in the present study, could have influenced the observed antioxidant activity of *O. aurasiaca* essential oil.

2.2.2. β-carotene bleaching method

The assessment of the β-carotene-linoleic acid bleaching activity of *O. aurasiaca* essential oil involved monitoring the decrease in absorbance over time, which indicates the oxidation of β-carotene. The oil exhibited a high antioxidant potential, with an estimated activity of 87.50 ± 0.02% at a concentration of 2 mg/ml, while BHA showed an activity of 89.72 ± 0.03% (Table S2, supplementary material). The observed decrease in absorbance was slower than that of the negative control, which had an activity of 26.63 ± 0.01%.

The presence of non-terpene derivatives, particularly dodecanal known for its strong antioxidant activity (Baschieri et al. 2017), may contribute to the moderate to high antioxidant activity observed in *O. aurasiaca* essential oil, as well as in other essential oils evaluated using the DPPH assay (Al-Qudah et al. 2014; Thomas et al. 2017; Foudah et al. 2021).

Other major constituents of *O. aurasiaca* essential oil, have also been identified in previous studies as potential leading antioxidant agents, such as hexahydrofarnesylacetone (Szewczyk et al. 2016; Venditti et al. 2017; Abd-ElGawad et al. 2019; Elshamy

et al. 2019; Pasdaran et al. 2020; Alilou and Akssira 2021), phytol (Pejin et al. 2014), and 1-heneicosane (Priyadarshi et al. 2018).

2.2.3. Phenanthroline method

The assay used in this study measured the reducing power of an extract by assessing its ability to convert ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). The formation of a stable reddish-orange complex known as the Fe^{2+}-phenanthroline complex, with its absorbance maximum at 510 nm, was used to quantify the reducing activity (Roy et al. 2011).

The reducing activity of *O. aurasiaca* essential oil was found to be markedly lower compared to that of BHA, with values of 751.3 ± 0.015 µg/ml and 0.778 ± 0.2 µg/ml, respectively (Table S2, supplementary material). This finding is consistent with the observation of colour changes during the experiment. The BHA mixture exhibited a bright red-orange colour, while the oil produced a pale hue.

To the best of our knowledge, there is a lack of studies investigating the antioxidant activity of essential oils from the *Ononis* genus using the phenanthroline method. Furthermore, the existing research on the antioxidant activity of essential oils primarily focuses on species that are taxonomically distant from the *Ononis* genus and exhibit distinct chemical compositions (Adefegha et al. 2017; Madouni et al. 2021; Benouchenne et al. 2022; Srief et al. 2023).

Numerous *Ononis* species, such as *O. alba* Poir. (Zaak et al. 2022), *O. natrix* (Mhamdi et al. 2015; Sayari et al. 2016; Al-Mterin et al. 2021), *O. pubescens* L. (Jaradat et al. 2017), *O. mitissima* L. (Besbas et al. 2020), and *O. arvensis* L. (Dénes et al. 2022), have been investigated for their potential as sources of antioxidants, particularly the extracts obtained using organic solvents. The antioxidant properties of these species were evaluated using various methods such as DPPH, β-carotene bleaching, and phenanthroline. All of these species were found to exhibit antioxidant properties against free radicals.

It is worth noting that the antioxidant activity of plant extracts can vary depending on the assay methods used, attributed to differences in mode of action, reagent sensitivities, and the complex nature of phytochemicals or volatile compounds (Koleva et al. 2002; Schlesier et al. 2002; Munteanu and Apetrei 2021).

2.3. Antibacterial activity

As depicted in Table S3 (supplementary material), the assessment of inhibition zone diameters at a low concentration of *Ononis aurasiaca* essential oil, corresponding to a 1:99 ratio of oil to DMSO, revealed a complete absence of inhibition zones for all tested strains.

However, at higher concentrations (1:1) and (1:19), the diameters varied from 9.2 mm to 13 mm for the Gram-positive strains and from 5.8 mm to 7.4 mm for the Gram-negative strains. Applying the classification system of Ponce et al. (2003), which categorises microorganism sensitivity into four groups (resistant, sensitive, highly sensitive, and extremely sensitive) based on inhibition zone diameters, only the Gram-positive *Staphylococcus aureus* strains fell into the sensitive category.

This result is very close to the results of Elamrani and Benaissa (2010), who studied the antibacterial activity of the essential oil of *Ononis natrix*. The oil of this plant gave similar

effect against the two bacterial strains: *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 while its effect was positive on *E. coli* ATCC 25922 compared to *O. aurasiaca*.

However, the oil of *O. angustissima*, as examined by Ghribi et al. (2015), demonstrated differing effects in activity against *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 compared to *O. aurasiaca*, while showing a similar effect against *E. coli* ATCC 25922.

2.4. Antifungal activity

As shown in Figure S2, stronger inhibitory effects against *Aspergillus niger* than *Scedosporium apiospermum* were observed (supplementary material). At a 1/400 concentration, the inhibition rate was 51.64% for *A. niger* while it remained below 16% for *S. apiospermum*. A notable rise in the inhibition rate was observed as the oil concentration in the medium increased.

The antimicrobial activity of an essential oil can often be attributed to its major constituents. In the case of *O. aurasiaca*, five significant compounds (Table S1, supplementary material) have been identified as major components. Notably, these same compounds are common major constituents in essential oils of various other plant species, which have also been evaluated for their antimicrobial activities.

Dodecanal, a predominant component in the essential oils of *Etlingera elatior* (Sukandar et al. 2017), *Machilus kusanoi* and *Machilus zuihoensis* (Ho et al. 2011, 2012), and *Polygonum minus* (Ahmad et al. 2014), has demonstrated a consistent antimicrobial effect against some microorganisms encompassing bacteria belonging to *Staphylococcus* genus and the *Aspergillus* fungi.

Hexahydrofarnesylacetone, found in the oils of *Sagittaria trifolia* (Xiangwei et al. 2006), *Equisetum arvense* (Radulović et al. 2006), *Tilia tomentosa* and *Tilia cordata* (Fitsiou et al. 2007), *Otostegia persica* (Tofighi et al. 2009), *Geranium columbinum* and *Geranium lucidum* (Radulović et al. 2011), *Acantholimon spp.* (Pasdaran et al. 2020), *Stachys laxa* and *Stachys byzantine* (Kiashi et al. 2021), and *Kickxia aegyptiaca* (Abd-ElGawad et al. 2022), has been associated with robust antimicrobial activity. These oils have demonstrated effectiveness against bacterial strains such as *S. aureus* and *P. aeruginosa*, as well as against *Aspergillus* fungi.

2-tridecanone, phytol, and 1-heneicosane have undergone previous biological testing by López-Lara et al. (2018), Ghaneian et al. (2015), and Vanitha et al. (2020), respectively, revealing their potent antimicrobial properties against various microorganisms. Essential oils with high phytol content have additionally displayed significant inhibitory effects against the microorganisms investigated in the current study (Pejin et al. 2014; Lee et al. 2016). Furthermore, heneicosene has been identified as an antibacterial agent in marine *Kocuria* (Shiyamala et al. 2014) and marine *Streptomyces* (Nandhini et al. 2015).

4. Conclusion

In this study, *Ononis aurasiaca*, a spontaneous species endemic to a specific habitat in Algeria, was analysed to determine its chemical composition and free radical scavenging and antimicrobial activities. The results showed that the essential oil of the

plant contained a variety of chemical classes dominated by non-terpene derivatives, apocarotenoids, sesquiterpene hydrocarbons, and diterpenes. The oil exhibited high antioxidant activity in the β-carotene bleaching assay, indicating its potential as a natural antioxidant. However, it showed moderate to low activity in the DPPH and phenanthroline tests. A remarquable antimicrobial effect has been observed only against the Gram-positive bacteria and *Aspergillus niger*.

This study is the first to evaluate the antioxidant and the antimicrobial potential of *O. aurasiaca* essential oil highlighting its promising properties. The low essential oil yield appears to be a characteristic trait and could be attributed to the plant's genetics. The complex chemical profile of the essential oil reflects its intricate secondary metabolism, indicating the presence of various bioactive compounds. Exploring the biosynthetic pathways and regulatory mechanisms can provide valuable insights into the production of these compounds and their potential synergistic effects.

The low yield suggests that other extracts should be investigated for potentially promising secondary metabolites with antioxidant potential or other bioactivities. The significant chemical diversity among *Ononis* species and subspecies worldwide supports their classification based on botanical and genetic criteria.

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First investigation of the chemical composition, antioxidant, antimicrobial and larvicidal activities of the essential oil of the subspecies *Ononis angustissima* Lam. subsp. *filifolia* Murb.

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First investigation of the chemical composition, antioxidant, antimicrobial and larvicidal activities of the essential oil of the subspecies *Ononis angustissima* Lam. subsp. *filifolia* Murb.

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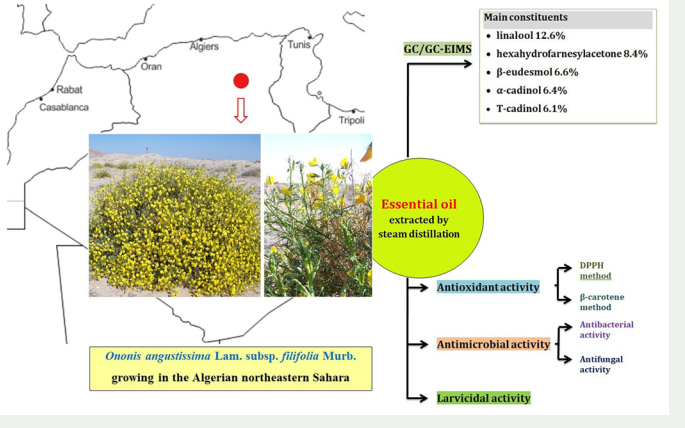
ABSTRACT

This study is the first to explore the essential oil of *Ononis angustissima* Lam. subsp. *filifolia* Murb., a subspecies growing in the Algerian northeastern Sahara. The chemical composition was evaluated by GC/GC-EIMS. Antioxidant activity was evaluated using two methods. Thirty-four (91.6%) individual components were identified. The main constituents were linalool (12.6%), hexahydrofarnesylacetone (8.4%), β-eudesmol (6.6%), α-cadinol (6.4%) and *T-cadinol* (6.1%). The findings provide a chemical basis for understanding relationships between North African subspecies, supporting botanical and genetic classification. The oil exhibited moderate scavenging activity against DPPH radicals (IC_{50} = 102.30 µg/ml) and high activity in the β-carotene bleaching assay (91.346%). Antimicrobial tests revealed effectiveness against Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 and ATCC 43300), limited impact on Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922), and good inhibition against *Aspergillus niger* and *Scedosporium apiospermum*. A notable larvicidal activity was observed against Date Moth, particularly on L2 larvae.



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1. Introduction

The *Ononis* genus, a member of the Fabaceae family, comprises approximately 86 species primarily found in the Mediterranean region, Macaronesia and adjacent areas of Asia (Ozenda 1958; Förther and Podlech 1991). These plants are easily recognisable by their yellow flowers and small, bushy growth with branched stems at the base (Ozenda 2004; Mabberley 2017). Taxonomic treatment of Širjaev (1932) classifies the *Ononis* genus into two sections based on the characteristics of their peduncles and pods: the Ononis section, distinguished by reduced peduncles and pods with few seeds, and the Natrix Grisebach section, characterised by distinct peduncles and multi-seeded pods (Fayed et al. 2019).

The main area of *Ononis* diversity is situated in the south of the Iberian Peninsula and near areas of northern Morocco and Algeria (Turini et al. 2010). Based on the latest revision of *Natrix* section in Macaronesia, North Africa and adjacent West Asia (Förther and Podlech 1991), six subspecies of *Ononis angustissima* Lam. are distributed in North Africa and southern Spain: *O. angustissima* Lam. subsp. *angustissima* and *O. angustissima* Lam. subsp. *longifolia* (Willd.) Förther & Podl. (Canary Islands and Spain), *O. angustissima* Lam. subsp. *polyclada* Murb. (Morocco, Algeria and Tunisia), *O. angustissima* Lam. subsp. *mauritii* (Mayor & Sennen) Förther & Podl. (Morocco and Algeria), *O. angustissima* Lam. subsp. *falcata* (Viv) Murb. (Algeria, Tunisia and Libya) and *O. angustissima* Lam. subsp. *filifolia* Murb. (Algeria and Tunisia).

Numerous studies have conducted in-depth analyses of the chemical composition of various *Ononis* species. Some of these investigations have focused on aqueous and crude solvent extracts, such as *O. arvensis* (Dénes et al. 2022), *O. mitissima* (Besbas et al. 2020), *O. natrix* (San Feliciano et al. 1983; Al-Rehaily et al. 2014; Mhamdi et al. 2015; Sayari et al. 2016; Öz et al. 2017; Yerlikaya et al. 2017) and *O. pubescens* (Jaradat et al. 2017), as well as *O. spinosa* (Öz et al. 2017) and *O. variegata* and *O. viscosa* (Öz et al. 2017). Furthermore, distinct studies have delved into the essential oils of several *Ononis* species, including *O. aurasiaca* (Messaoudi et al. 2023), *O. alba* (Zaak et al. 2022), *O. reclinata* (Casiglia et al. 2017), *O. sicula* (Al-Qudah et al. 2014) and *O. vaginalis* (Abdel-Kader 2001). Additionally, *O. viscosa* (Erdemgil et al. 2002) and *O. natrix* (Khallouki et al. 2002; Elamrani and Benaissa 2010; Al-Mterin et al. 2021) have been examined in separate studies.

Various *Ononis* species demonstrate a diverse range of pharmacological properties, including antimicrobial, analgesic, antioxidant, antiproliferative, anticancer, antihypertensive, anti-inflammatory and wound-healing effects (Al-Qudah et al. 2014; Mhamdi et al. 2015; Öz et al. 2017; Jaradat et al. 2017). These plants have also found use in traditional phytotherapy. For example, *O. spinosa* is employed to treat urinary tract inflammations, kidney stones, wounds, skin issues and gout (Altanlar et al. 2006). Various species like *O. spinosa*, *O. arvensis*, *O. hircina* and *O. antiquorum* have applications for skin irritations, itching, wounds, and dermatitis (Mamedov et al. 2005). *O. sicula* and *O. hirta* are used in wound healing and as antiseptics for skin cancer and cold sores (Taliba and Mahasneh 2010).

However, to date, only two studies have investigated the essential oils of *O. angustissima*, one in southwestern Algeria (Mechehoud et al. 2014) and the other in southwestern Tunisia (Ghribi et al. 2016). The other existing researches have focused on

non-volatile extracts (Bouheroum et al. 2009; Mezrag et al. 2013; Benabderahmane et al. 2014; Guettaf et al. 2016; Mezrag et al. 2017). Yet, no dedicated studies have delved into the specific subspecies *O. angustissima* Lam. subsp. *filifolia* Murb.

In Algeria, *O. angustissima* Lam. subsp. *filifolia* Murb. is primarily found in the eastern part of the northern Sahara, particularly within the Wilaya of Biskra and the surrounding areas (Förther and Podlech 1991). It is a yellow-flowered non-viscous chamaephyte with erected annual branches forming a dome of 10–40 cm high (Berghen 1978), characterised with the enduring presence of its dry branches from preceding years. Locally, the aerial parts of this subspecies have traditional uses for haemostatic qualities (Chehma and Djebar, 2008) and diabetes treatment (Khacheba et al. 2014).

This study endeavours to conduct a comprehensive analysis of the chemical composition inherent in the essential oil of this endemic subspecies and subsequently assess its capabilities in scavenging free radicals as well as its antimicrobial and larvicidal activities. Through an in-depth exploration of the unique chemical constituents present in this plant, this research strives to contribute to the advancement of our understanding regarding its bioactive properties, pharmaceutical relevance and potential applications.

2. Results and discussion

2.1. Essential oil composition

A low yield of light brown oil, redolent of the plant's distinctive scent, was achieved, constituting approximately 0.015% (w/w). In comparison, *O. angustissima* from Algeria (Mechehoud et al. 2014) and Tunisia (Ghribi et al. 2016) produced light yellow oils with yields of 0.73% (w/w) and 0.18% (w/w), respectively. Similarly, *O. natrix* collected from Jordan (Al-Qudah et al. 2014) and Morocco (Elamrani and Benaissa 2010) yielded pale yellowish oil at 0.21% (w/w) and light yellowish oil at 1.0% (m/w), respectively.

The compounds identified, along with their relative percentages and linear retention indices (lri) relative to the n-hydrocarbons series, are detailed in Table S1. The analysis led to the identification of 34 compounds, constituting 91.6% of the total essential oil. Oxygenated sesquiterpenes emerged as the primary chemical class, making up 32.6% of the composition. Notable representatives of this class included β-eudesmol (6.6%), α-cadinol (6.4%) and T-cadinol (6.1%). Oxygenated monoterpenes formed another substantial chemical category, comprising 23.7% of the oil. Linalool (12.6%) was the major component within this group. Apocarotenoids represented 15% of the oil, with Hexahydrofarnesylacetone (8.4%) being the major compound. Limonene (2.3%) and methyl eugenol (0.8%) were the sole identified compounds representing monoterpene hydrocarbons and phenylpropanoids, respectively.

It is noteworthy that except for the presence of a single monoterpene hydrocarbon, limonene, the essential oil of *filifolia* subspecies was found to share similar chemical compound classes with *O. angustissima* collected in Tunisia (Ghribi et al. 2016), but with a notably higher proportion of oxygenated monoterpenes and sesquiterpene hydrocarbons. The percentage of oxygenated monoterpenes was significantly higher at 23.7% compared to 1%. Additionally, the two oils have 17 compounds in common, with 4 of them being major constituents. This substantial chemical similarity suggests the possibility that the Tunisian *O. angustissima* corresponds to the Algerian subspecies *filifolia*.

Conversely, the essential oil of another *O. angustissima* population collected from the Bechar region in Algeria (Mechehoud et al. 2014) shares limited similarities, with just 8 common compounds, including one major constituent, β-eudesmol. This distinction may be attributed to the potential use of a different subspecies of the plant. Indeed, according to Förther and Podlech (1991), in the Southwestern region of Algeria, *O. angustissima* Lam. subsp. *polyclada* Murb. and *O. angustissima* Lam. subsp. *mauritii* (Mayor & Sennen) Förther & Podl. are the two subspecies typically found.

The essential oils extracted from other species within the same genus, however, do not exhibit significant compositional similarities. This includes two Jordanian species, *O. natrix* and *O. sicula* (Al-Qudah et al. 2014), *O. reclinata* from Italy (Casiglia et al. 2017), *O. viscosa* subsp. *breviflora* from Turkey (Erdemgil et al. 2002) and two Moroccan populations of *O. natrix* (Khallouki et al. 2002; Elamrani and Benaissa 2010), as well as *O. alba* Poir. from Algeria (Zaak et al. 2022).

Significant chemical diversity is evident in *Ononis* species globally, attributed to varying chemical classes and principal compounds, supporting botanical and genetic criteria for classification (Förther and Podlech 1991; Turini et al. 2010). Essential oil properties are influenced also by intrinsic factors like genetics and plant conditions, as well as extrinsic factors such as the environment, impacting yield and composition (Moghaddam and Mehdizadeh 2017).

The presence of apocarotenoides alongside mono- and sesquiterpenes suggests a complex metabolic network in *O. angustissima*, with potential crosstalk and interconversions among different biosynthetic pathways.

Monoterpenes and sesquiterpenes, vital isoprenoid compounds in plants, originate from the mevalonate (MVA) and methylerythritol phosphate (MEP) pathways (Sacchettini and Poulter 1997). These terpenes stem from precursor molecules, dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) (Vranova et al. 2012) which condense to form prenyl diphosphates, including geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranyl GPP (GGPP). Specialised enzymes, terpene synthases or cyclases, convert these substrates into terpenes. Further modifications, both enzymatic and non-enzymatic, enhance terpene diversity (Galata et al. 2014). Notably, GPP is a substrate for monoterpenes (Pereira et al. 2018), such as linalool. FPP serves as the precursor for sesquiterpenes like T-cadinol, β-eudesmol and α-cadinol (Jullien et al. 2014).

Apocarotenoid volatiles, essential for diverse functions, result from enzymatic and non-enzymatic carotenoid oxidation (Liang et al. 2021). Plant systems feature multiple carotenoid cleavage dioxygenases (CCDs) responsible for cleaving various carotenoids like β-carotene, yielding apocarotenoids, such as farnesylacetone, ionone isomers, geranylacetone and hexahydrofarnesylacetone (Yahyaa et al. 2013; Lashbrooke et al. 2013; Ilg et al. 2014; Frusciante et al. 2014). These CCDs enable biotechnological synthesis of distinct flavours and fragrances from precursor carotenoids (Serra 2015).

2.2. Antioxidant activity

2.2.1. DPPH method

In the DPPH assay shown in Figure S1, both the essential oil and reference compounds displayed concentration-dependent radical scavenging abilities. However, the essential

oil exhibited lower antioxidant activity compared to reference compounds. At the highest concentration (240 µg/ml), the essential oil inhibited DPPH radicals by about 79.471%, while quercetin, BHA and ascorbic acid showed significantly higher inhibition rates at 98.462%, 96.235% and 98.379%, respectively. The essential oil also required a higher concentration (IC_{50} = 102.30 µg/ml) to achieve 50% inhibition compared to quercetin (IC_{50} = 1.15 ± 0.23 µg/ml), as reported in Table S2. Previous studies reported IC_{50} values of 54.73 µg/ml for Jordanian *O. natrix* and 89.20 µg/ml for *O. sicula* essential oils, indicating that *filifolia* subspecies essential oil exhibited lower antioxidant activity in this study. It is worth noting that the 0.5 ml/1.5 ml extract/DPPH ratio used in this study might have influenced the observed antioxidant activity.

2.2.2. β-carotene bleaching method

The assessment of β-carotene-linoleic acid bleaching activity in the essential oil involved the continuous monitoring of absorbance reduction over time, indicating the oxidation of β-carotene. The oil displayed substantial antioxidant potential, estimated at 91.346 ± 0.06% at a concentration of 2 mg/ml, while BHA exhibited an activity of 89.724 ± 0.03% (Table S2). Notably, the decline in absorbance occurred at a slower rate compared to the negative control, which recorded an activity of 26.63 ± 0.01%.

The presence of mono- and sesquiterpenes, well-known for their strong antioxidant activity, particularly linalool (Gunaseelan et al. 2017; Jabir et al. 2018; Hu et al. 2020; An et al. 2021) and β-eudesmol (Sghaier et al. 2016; Kim 2018; Acharya et al. 2021), likely contribute to the observed antioxidant activity in *filifolia* subspecies essential oil.

Furthermore, the observed antioxidant activity can be attributed to the presence of apocarotenoids, well-known for their potent free radical scavenging abilities (Meléndez-Martínez 2019; Shi et al. 2020). Notably, hexahydrofarnesylacetone, which has been identified in prior research as a promising leading antioxidant agent (Abd-ElGawad et al. 2019; Elshamy et al. 2019; Pasdaran et al. 2020; Alilou and Akssira 2021).

Moreover, the major identified compounds, particularly terpenes, are associated with various therapeutic activities, as reported in studies by Pereira et al. (2018), An et al. (2021), Weston-Green et al. (2021) and Altinoz et al. (2022) for linalool, Han et al. (2017), Mathema et al. (2017) and Kotawong et al. (2018) for β-eudesmol, and Zygmunt et al. (1993), Takei et al. (2006) and Dos Santos et al. (2021) for T-cadinol. These compounds are believed to exert their effects through various mechanisms, including the modulation of detoxifying enzymes involved in oxidative stress responses, telomerase activity reduction, proapoptotic effects enhancement and the inhibition of DNA, RNA, and protein synthesis (Harrison and Quadro 2018; Gao et al. 2021).

Numerous *Ononis* species, including *O. natrix* (Mhamdi et al. 2015; Sayari et al. 2016; Al-Mterin et al. 2021), *O. pubescens* L. (Jaradat et al. 2017), *O. mitissima* L. (Besbas et al. 2020), *O. alba* Poir. (Zaak et al. 2022) and *O. arvensis* L. (Dénes et al. 2022), have been explored for their antioxidant potential, primarily through extractions using organic solvents. Distinct antioxidant assays, such as the DPPH and β-carotene bleaching tests, were employed in these investigations, consistently revealing their abilities to combat free radicals.

It is important to note that the antioxidant activity of plant extracts can vary based on the specific assay methods employed. This variability is often due to differences

in the mechanisms of action, the sensitivities of reagents, and the intricate nature of phytochemicals and volatile compounds present (Schlesier et al. 2002; Munteanu and Apetrei 2021).

2.3. Antibacterial activity

The assessment of antibacterial activity (Table S3) revealed that low concentrations of essential oil (1:99 oil to DMSO ratio) had no inhibition zones for all tested strains. At higher concentrations (1:1 and 1:19 ratios), inhibition zone diameters ranged from 8.6 to 12.5 mm for Gram-positive strains and 5.2–8.8 mm for Gram-negative strains. Applying the classification system proposed by Ponce et al. (2003), which categorises the sensitivity of microorganisms into four groups (resistant, sensitive, highly sensitive and extremely sensitive) based on the size of inhibition zones, only the Gram-positive *S. aureus* strains fell into the sensitive category. These results are consistent with the findings of Elamrani and Benaissa (2010) for *O. natrix* essential oil, which exhibited similar effects against *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 but demonstrated stronger effects on *E. coli* ATCC 25922 compared to *O. angustissima* subsp. *filifolia*. However, the essential oil from Tunisian *O. angustissima*, as studied by Ghribi et al. (2016), displayed varying activity against *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 but showed a similar effect against *E. coli* ATCC 25922.

2.4. Antifungal activity

Aspergillus niger and *Scedosporium apiospermum* were completely inhibited by the reference antifungal, Voriconazole. Figure S2 illustrates significant inhibitory effects of essential oil against both fungi. At a 1/400 concentration, the inhibition rate was 49.78% for *A. niger* and 44.08% for *S. apiospermum*. Notably, as the concentration of the oil in the medium increased, there was a substantial increase in the inhibition rate. These findings align with the inhibitory effects observed in certain essential oils from various plant species, which have demonstrated significant inhibition against the two tested fungi. Examples include essential oils from *Laurus nobilis*, *Oscimum basilicum*, *Cinnamomum zeylanicum* and *Cinnamomum cassia* (Nweze and Okafor 2010; Gogoi et al. 2021; Singh et al. 2021; Brandão et al. 2023; Chau et al. 2023), with linalool being a shared compound, notably a major constituent in the essential oils of *Oscimum basilicum* and *Laurus nobilis*.

The antimicrobial potency of an essential oil is often closely associated with its primary constituents. In the case of *O. angustissima* subsp. *filifolia*, five specific compounds (Table S1) have been identified as major components. Notably, some of these compounds, especially linalool, have been shown to have strong antioxidant and antimicrobial properties.

Linalool is a versatile antimicrobial agent effective against various bacteria, including multi-drug resistant strains such as *E. coli* and *P. aeruginosa* (Zengin and Baysal 2014; Liu et al. 2020). It shows promise in addressing gastrointestinal infections caused by bacteria like *E. coli* and *S. aureus* (Ghosh et al. 2019; Prakash et al. 2019), as well as opportunistic bacteria like *P. aeruginosa* and *Staphylococcus epidermidis* (Soković et al.

2010; Liu et al. 2012). Linalool also exhibits notable antifungal properties, effectively targeting various species, including *A. niger* (Li et al. 2022), *Scedosporium* strains (Shukla 2021), yeast-like fungi and dermatophytes (Máté et al. 2017). Additionally, when vaporised, linalool reduces airborne microbes (Sato et al. 2007) and its antifungal effectiveness is further enhanced when combined with essential oils (Herman et al. 2016). The sesquiterpenes, T-cadinol (Claeson et al. 1992) and β-eudesmol (Acharya et al. 2021) have demonstrated also potential in combatting infectious microorganisms, including *S. aureus*.

The apocarotenoid hexahydrofarnesylacetone, found in various plant oils, including *Otostegia persica* (Tofighi et al. 2009), *Geranium columbinum* and *G. lucidum* (Radulović et al. 2011), *Acantholimon spp.* (Pasdaran et al. 2020), *Stachys laxa* and *S. byzantine* (Kiashi et al. 2021), and *Kickxia aegyptiaca* (Abd-ElGawad et al. 2022), has demonstrated strong antimicrobial activity. These oils have proven effective against bacterial strains such as *S. aureus* and *P. aeruginosa*, as well as against *Aspergillus* fungi.

The mentioned compounds, in particular mono- and sesquiterpenes, exert their antimicrobial effects through several mechanisms. They initially target bacterial cell membranes, causing structural and functional damage by compromising membrane potential and integrity. Additionally, these terpenes inhibit energy-related pathways within bacteria, leading to metabolic dysfunction and the inhibition of vital enzymes (Prakash et al. 2019; Dos Santos et al. 2021; Guo et al. 2021). Terpenes also possess the ability to disrupt fungal morphogenesis, thereby inhibiting fungal growth at the infection site (De Oliveira Lima et al. 2017).

2.5. Larvicidal activity

As depicted in Figure S3, a pronounced larvicidal effect was specifically observed on *Ectomyelois ceratoniae* L2 larvae. At the higher tested concentration (1:1 ratios), the mortality rates after 4 d were 20% for L2 and 12.5% for L3 stages, which further increased to 44% and 33.3% for L2 and L3, respectively, after 5 d.

Previous studies have underscored the potent larvicidal activity of several plants against Date Moth, with *Thymus capitatus* and *Rosmarinus officinalis* essential oils demonstrating efficacy through the direct spray method (Amri et al. 2014). Noteworthy larvicidal activity has also been observed in antifeedant bioassays, specifically with essential oils of *Thymus hyemalis* and *T. algeriensis* (Adouane et al. 2022), as well as *Schinus molle* (Chaaban et al. 2022). Additionally, *Rosmarinus officinalis* essential oils have exhibited considerable larvicidal toxicities in fumigant bioassays (Abada et al. 2020). Adulticidal activity has been noted for *O. basilicum* essential oil, along with *Ruta graveolens* and *Mentha pulegium* (Chaaban et al. 2019).

Some major compounds in the oil of *O. angustissima* subsp. *filifolia* (Table S1) have been recognised for their insecticidal properties. For instance, linalool displayed insecticidal activity against various insect species, including *Tribolium confusum* (Ojimelukwe and Adler 1999), *Aedes aegypti* (Fujiwara et al. 2017) and *Ixodes ricinus* (Tabari et al. 2017). T-cadinol has also shown insecticidal activity against *Aedes albopictus*, *Culex quinquefasciatus* and *Armigeres subalbatus* (Cheng et al. 2009).

Furthermore, essential oils from various plant species rich in T-cadinol, such as *Cinnamomum osmophloeum* (Cheng et al. 2009), *Azorella cryptantha* (López et al. 2012)

and *Chamaecyparis formosensis* (Hsu et al. 2016), have demonstrated insecticidal activity. Essential oils containing β-eudesmol as a major compound identified in *Atractylodes chinensis* (Chu et al. 2011), *Mallotus apelta* (Liu et al. 2014) and *Rhynchanthus beesianus* (Pan et al. 2023), have also shown insecticidal activity. Similarly, essential oils with α-cadinol among the major compounds, such as those from *Schinus areira* (Mattar et al. 2022), *Caryopteris incana* (Gao et al. 2020) and *Zanthoxylum monophyllum* (Pavela and Govindarajan 2017), have been reported to possess insecticidal activity.

Insect olfactory systems, particularly in moths, serve as valuable models for investigating interactions with plant secondary metabolites. The selectivity of olfactory receptors is influenced by processes involving volatiles and odorant receptor proteins (De Bruyne and Baker, 2008).

Active volatile compounds hold the potential to induce paralysis, subsequently leading to the demise of insects. Essential oils also operate through the octopaminergic system, elevating cyclic adenosine monophosphate (cAMP) and calcium levels in nervous cells. Furthermore, specific components compete with octopamine, an invertebrate neurotransmitter, in binding to its receptor (Jankowska et al. 2017).

Furthermore, the volatile constituents exhibit a multifaceted mechanism by potentially targeting pivotal enzymes in cellular metabolism, identified as primary targets for insecticide design. These enzymes encompass acetylcholinesterase (Hung et al. 2022; Mattar et al. 2022; Bi et al. 2023), glutathione S-transferases (Djemâa and Hayette 2023) and cytochrome P450 (Sadeghi et al. 2021; El-Maghraby et al. 2023). This intricate interplay between volatile compounds and enzymatic targets provides a nuanced understanding for potential applications in insect pest control.

3. Conclusion

This study on *O. angustissima*, specifically the subspecies *filifolia* from the Algerian northeastern Sahara, unveiled its distinctive chemical composition, remarkable antioxidant potential, and notable antimicrobial and larvicidal properties.

As the first investigation of this subspecies, the study demonstrates a significant chemical similarity between the Algerian subspecies and the Tunisian species, setting them apart from the population in Southwestern Algeria. This finding aligns with Förther and Podlech's 1991 botanical classification and highlights the potential of combining botanical, genetic and phytochemical studies for precise plant classification. Future research should aim to perform more precise botanical identification to confirm relationships between different studied species and further refine subspecies classification.

The findings imply potential applications in food preservation, cosmetics, and pharmaceutical formulations. Notably, linalool, recognised for its varied biological activities, plays a crucial role in imparting antioxidant, antimicrobial and insecticidal properties to the essential oil.

In addition to exploring biosynthetic pathways and regulatory mechanisms, there is a need to investigate the action mechanisms of major constituents through molecular docking and other *in silico* methods. This will provide insights into compound production, synergistic effects, and their interactions with biological targets, which is essential for the development of potential therapeutic agents and pesticids.

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