الجمهورية الجزائرية الديمقراطية الشعبية

People's Democratic Republic of Algeria

وزارة التعـليـم العالي والـبحـث الـعلـمية

Ministry of Higher Education and Scientific Research

جامـعة الشهيد حمة لخضر الوادي

University of Echahid Hamma Lakhdar – El Oued

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Faculty of Natural and Life Sciences

قسم البيولوجيا الخلوية والجزيئية

Department of Cellular and Molecular Biology

END-OF-STUDIES MEMOIR

Submitted to obtain the academic master's degree

Specialty: Toxicology

THEME

Integrated Evaluation of Medicinal Plants for Antidiabetic Potential: Bridging In Vivo and In Vitro Assessments with Advanced In Silico Analyses, including ADMET Prediction, Molecular Docking, and Molecular Dynamics Simulation

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-University Year 2023/2024 -

Acknowledgments

Primarily, we thank ***GOD***, our creator, for giving us the strength, the will, and the courage to accomplish this modest work.

To our thesis supervisor, Professor ***Lanez Elhafnaoui***, Lecturer Professor (A) at the University of El Oued, for accepting to guide us, for his dynamism, his assistance, and his valuable advice, which have allowed us to progress further in our research.

To our co-supervisor, Miss ***BOUDEBIA Ouafa***, for entrusting us with her confidence by accepting to work on this subject with us. She followed our work with interest while providing us with consistently sound advice, restoring our courage and assisting us through difficult moments and moments of doubt. Also, for her support, attention, and human qualities. For all of this, we wish to express our gratitude to her and particularly thank her deeply.

His attention, Professor ***Lanez Touhami***, director of the VTRS research laboratory at the University of El Oued, for granting us the honor to work in his laboratory, we extend our sincere thanks and appreciation.

We extend our thanks to ***Mr. Ben Omar Mohamed Al Arabi*** for his support and assistance to us in the research and provision of our medicinal plants.

Our thanks also go to the engineer from the VTRS laboratory, ***Mr. TELIBA Ali***, for his valuable support, encouragement, and advice throughout the completion of this work.

We warmly thank ***Dr. ADIAKA*** Aicha for her assistance in the ANTIDIABETIC ACTIVITY tests.

Our gratitude also extends to all members of the VTRS laboratory, especially to the doctoral students ***SAADA Zahra, DJARALLAH Marwa***, and ***BENINE Aida...***

We extend our sincere thanks to all members of the Department of Natural and Life Sciences Laboratory, Hamma Lakhdar University, El-Oued, and to all members of the Emergency Medical Laboratory in El-Oued.

Finally, we would like to express our deep gratitude to everyone who participated in the completion of this thesis.

Dedications

Praise be to Allah, and blessings upon the chosen Prophet, his family, and companions.

*Primarily, I thank Allah who has granted us success in achieving this milestone in our academic journey through this thesis. It is the fruit of effort and success, bestowed upon us by His grace. I dedicate this to **my dear parents**, may Allah protect them and keep them as guiding lights in my path.*

*I extend my gratitude to all the noble family members who supported me, including **my brothers**, **my sister**, and **My brother's wife** , and especially **my aunt's daughter** for standing by my side. I also express my thanks to **my grandparents**, **aunts**, **uncles**, and all extended family members. May Allah continue to be my support throughout my life.*

To my companions on this journey, who shared its moments with me, may Allah protect and guide them.

*I am also thankful to everyone who contributed to the completion of this thesis, especially Mr. **Lanez** and Miss. **BOUDEBIA**, for their support in this important step.*

Thank you all for supporting us in this significant endeavor.

Ouidad

Dedications

Praise be to Allah, who nothing is achieved without His effort and no effort is completed without His grace.

*I dedicate my humble work to the pillar of my family, my role model in life, my grandfather " **Mohammed gaid** "*

*To my father who lighted my path and way in every step, to my support and refuge, my father " **Ibrahim** "*

. Except for those who tirelessly extend their hands during my weakness to those who light my path to success and are a source of my confidence, my dear aunts in my heart

*"**Amale**" "**Naima**" "**Saida**"*

*To my dear mother " **Sabah Ghandir Aoun**"*

*To my brothers "**Khaled**" "**Abdl Hafed**", "**Haitham**", "**Mohammed**"*

*To my sisters "**Basma**", "**Radja**", "**Roudaina**"*

*To the younger ones in the family "**Abdl Rahman,** " "**Musab**", "**Khalifa**"*

*To those souls who have passed away, my grandmother "**Aisha Attia**"*

To all those who taught me a letter during my journey, and Sandy even with a word she uttered to my ears and heart, I rescued it from despair to hope.

Sihame

Dedications

Whoever says, "I am hers," will get her. "And I am hers." And if she refuses, against her will, I will bring her

*I achieved it and today I embrace great glory. I did it after it was impossible. It was harsh and difficult, but I achieved it, praise be to God. That is why I dedicate the fruit of my effort to the one with a fragrant biography and enlightened thought, to the one who encouraged me to persevere throughout my life and my first supporter, **my father**, may God protect him.*

*To the one I prefer over myself, the one who put me on the path of life and melted me with tenderness and love, to the one through which I rise and rely, to the generous heart, **my beloved mother**, may God protect her. to the one with whom I lived the most beautiful moments of my life, to the candles of my path, to those who witnessed with me the troubles of studying and the sleepless nights, **my brothers**, **sisters**, and **my friends**, may God protect them. Finally, I give my regards to my entire family and acquaintances, and to all those who encouraged and supported me, and to my supervising teachers who did not fail to provide me with assistance.*

Khaoula

Abstract

The plant kingdom serves as an exceptional reservoir of bioactive compounds endowed with anti-diabetic properties. This study aims to investigate the anti-diabetic effects of essential oils derived from ***Cotula cinerea*** and ***Origanum majorana L*** both in vivo and in vitro, as well as in silico. Initially, the essential oils were obtained through hydrodistillation, and their respective compounds were characterized using gas chromatography-mass spectrometry (GC/MS) analysis, with *Cotula cinerea* yielding 31 compounds and *Origanum majorana L* yielding 44 compounds.

In vivo experiments using rats that had been given alloxan to induce diabetes were conducted during the study's first phase. Following a 15-day treatment period with 10 µl of the essential oils from *Cotula cinerea* and *Origanum majorana L*, the treatment's effectiveness was evaluated in comparison of control groups, including those using the Acarbose. The results demonstrated that alloxan caused a change in hematological parameters (LYM, GRA), glycemia and hepatic enzyme markers (AST, ALT), triglycerides and kidney function (urea, creatinine, UA).Additionally, alterations in the antioxidant defenses MDA and GSH. Histological examinations also revealed notable liver inflammation, atrophy of the Langerhans islet cells in the pancreas. Nonetheless, the administration of essential oils improved most of the adverse effects of alloxan especially, controlling blood sugar levels.

In the laboratory setting, the inhibition of alpha-amylase activity was demonstrated through the preparation of a starch solution containing essential oils. Findings revealed a notable inhibitory effect of essential oils on alpha-amylase in plant sources, particularly in *Cotula cinerea* and *Origanum majorana L* (IC50=2.432, $-\Delta G$ =27.11) (IC50=3.68, $-\Delta G$ =25.83), as compared to Acarbose.

Within the realm of computer science, an examination of Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) properties was conducted to predict the physicochemical, pharmaceutical, and toxicological characteristics of specific basic compounds present in each oil (more than 10%). This analysis utilized the **SwissADME** and **Protox** servers, with results indicating favorable medicinal attributes of all compounds in contrast to acarbose.

In the **docking**, the basic compounds with a yield of (1%) were selected. For each plant oil, the obtained results confirm their occurrence in the laboratory, indicating negative ΔG values, which translates to a good interaction of the compounds with human amylase.

In the domain of **molecular dynamics simulation**, the compound "trons_thujone" exhibited the most promising interaction with alpha-amylase in comparison to acarbose. Noteworthy results demonstrated minimal conformational changes throughout the simulation, affirming its potential efficacy in diabetes management and suggesting its viability as a prospective therapeutic agent for diabetes treatment.

Keywords: Essential Oil, antidiabetic activity, alloxan, α-amylase, acarbose, swissADME, Docking, IC50, ΔG.

Résumé

Cette étude vise à examiner les effets anti-diabétiques des huiles essentielles dérivées de ***Cotula cinerea*** et d'***Origanum majorana L*** à la fois in vivo et in vitro, ainsi que in silico. Initialement, les huiles essentielles ont été obtenues par hydrodistillation, et leurs composés respectifs ont été caractérisés à l'aide d'une analyse par chromatographie en phase gazeuse couplée à la spectrométrie de masse (GC/MS), *Cotula cinerea* produisant 31 composés et *Origanum majorana L* produisant 44 composés.

Des expériences in vivo utilisant des rats ayant reçu de l'alloxane pour induire le diabète ont été menées lors de la première phase de l'étude. Après une période de traitement de 15 jours avec 10 µl des huiles essentielles de *Cotula cinerea* et d'*Origanum majorana L*, l'efficacité du traitement a été évaluée en comparaison avec des groupes témoins, y compris ceux utilisant l'Acarbose. Les résultats ont démontré que l'alloxane entraînait une modification des paramètres hématologiques (LYM, GRA), de la glycémie et des marqueurs enzymatiques hépatiques (AST, ALT), des triglycérides et de la fonction rénale (urée, créatinine, UA). De plus, des altérations dans les défenses antioxydantes MDA et GSH ont été observées. Les examens histologiques ont également révélé une inflammation notable du foie et une atrophie des cellules des îlots de Langerhans dans le pancréas. Néanmoins, l'administration d'huiles essentielles a amélioré la plupart des effets indésirables de l'alloxane, en particulier en contrôlant les niveaux de sucre dans le sang.

Dans le domaine de l'informatique, une étude des propriétés d'Absorption, Distribution, Métabolisme, Excrétion et Toxicité (ADMET) a été menée pour prédire les caractéristiques physico-chimiques, pharmaceutiques et toxicologiques de composés de base spécifiques présents dans chaque huile(plus que 10%). Cette analyse a utilisé les serveurs **SwissADME** et **Protox**, les résultats indiquant des attributs médicinaux favorables de tous les composés par rapport à l'acarbose.

Dans le domaine de l'informatique, une étude des propriétés d'Absorption, Distribution, Métabolisme, Excrétion et Toxicité (ADMET) a été menée pour prédire les caractéristiques physico-chimiques, pharmaceutiques et toxicologiques de composés de base spécifiques présents dans chaque huile(plus que 10%). Cette analyse a utilisé les serveurs **SwissADME** et **Protox**, les résultats indiquant des attributs médicinaux favorables de tous les composés par rapport à l'acarbose.

Lors du **docking**, les composés basiques avec un rendement de (1 %) ont été sélectionnés. Pour chaque huile végétale, les résultats obtenus confirment leur présence en laboratoire, indiquant des valeurs ΔG négatives, ce qui se traduit par une bonne interaction des composés avec l'amylase salivaire humaine.

Dans la **simulation de dynamique moléculaire**, le composé " trons_thujone" a montré la plus prometteuse interaction avec l'alpha-amylase par rapport à l'acarbose. Des résultats remarquables ont démontré des changements conformationnels minimes tout au long de la simulation, affirmant son efficacité potentielle dans la gestion du diabète et suggérant sa viabilité en tant qu'agent thérapeutique prospectif pour le traitement du diabète.

Mots-clés : Huile essentielle, activité antidiabétique, alloxane, α-amylase, acarbose, swissADME, Docking, IC50, ΔG.

الملخص

يعتبر مملكة النبات مستودعًا استثنائيًا للمركبات الحيوية ذات الخصائص المضادة لمرض السكري. تهدف هذه الدراسة إلى استكشاف المضادة لمرض السكري سواء في ***Origanum majorana L*** و ***Cotula cinerea*** تأثيرات الزيوت الأساسية المستخلصة من الجسم أو في المخبر ا، بالإضافة إلى التحليل الحاسوبي. في المرحلة الأولية، تم الحصول على الزيوت الأساسية من خلال عملية الطيف الكتلي - ، وتم توصيف المركبات الخاصة بها باستخدام تحليل الكروماتوغرافيا الغازيةhydrodistillation التقطير المائي (GC/MS) حيث أظهرت31 *Cotula cinerea* 44مركبًا و *Origanum majorana L* مركبًا.

م إجراء تجارب حية باستخدام الجرذان التي تم إعطاؤها اللوكسان لتحفيز السكري خلال المرحلة الأولى للدراسة. بعد فترة علاج ، تم تقييم فعالية *Origanum majorana L* و *Cotula cinerea* ميكرولتر من الزيوت الأساسية من 10لمدة 15 يومًا باستخدام Acarbose. العلاج مقارنة بمجموعات السيطرة، بما في ذلك تلك التي تستخدم أظهرت النتائج أن اللوكسان تسبب تغييرًا في المعايير ، والدهون الثلاثية ووظيفة الكلى (ALT، AST) ، وسكر الدم وعلامات الإنزيمات الكبدية (GRA، LYM) الهيماتولوجية كما كشفت الفحوصات النسيجية .GSH و MDA بالإضافة إلى تغييرات في الدفاعات المضادة للأكسدة .(UA(يوريا ، كرياتينين ، ومع ذلك ، أدى إعطاء الزيوت الأساسية إلى تحسين .عن التهاب كبدي ملحوظ وضمور خلايا جزيرة لانجرهانز في البنكرياس معظم الآثار السلبية للوكسان خاصة في السيطرة على مستويات السكر في الدم.

في المختبر، تم تحقيق تثبيط ألفا أميليز عن طريق إعداد محلول نشا مع زيوت أساسية. أظهرت النتائج أن الزيوت الأساسية تثبط (IC50=2.432, -ΔG =27.11) *Origanum majorana L- Cotula cinerea* - بشكل كبير انزيم الأميليز في النباتات .

خصائص دوائية جيدة مقارنة بأكاربوز.بالمقارنة مع أكاربوز(IC50=3.68, -ΔG =25.83)

تم اختيار المركبات الاساسية التي مردودها . (1%). لكل زيت من النبتتين .النتائج المتحصل عليها تؤكد تقريبا **docking** في سالبة، مما يترجم إلى تفاعل جيد للمركبات مع ألفا أميليز البشري ΔG تلك التي تم الحصول عليها في المخبر ، حيث تشير إلى قيم

للتنبؤ النظري بالخصائص الفيزيوكيميائية والصيدلانية والسمية للمركبات **ADMET** في الجزء االحاسوبي، قمنا بدراسة خصائص النتائج التي تم الحصول عليها تشير .**Protox**و **SwissADME** ، باستخدام خوادم)اكثر من 10(الأساسية المختارة لكل زيت إلى أن جميع المركبات لها.

، مع ألفا أميليز وأكاربوز. أظهرت النتائج أنه على " trons_thujone "في محاكاة الديناميكا الجزيئية: تم اختيار أفضل مركب، مدى المحاكاة، لم يحدث أي تغيير هيكلي رئيسي، مما يؤكد فعاليته ضد مرض السكري وقد يتم الموافقة عليه كدواء مستقبلي لعلاج السكري.

الكلمات المفتاحية : زيت أساسي، نشاط مضاد لمرض السكري، الوكسان، ألفا أميليز، أكاربوز،

SwissADME, docking, IC50, ΔG.

ABREVIATIONS LIST

ΔG: Binding free energy

A° : Angstrom

Abs: Absorbance

ADA: Association americaine of diabetes

ADMET: Absorption, Distribution, Metabolism, Excretion of drugs and Toxicity

ALP: Alkaline phosphatase

ALT: Aminotransferase

AST: Aminotransferase

C° : Degrees Celsius

Crea: Creatinine

EDTA : Ethylenediaminetetraacetic acid

EOs : Essential Oils

EOCc : Essential oils of *Cotula cinerea*

EOOm : Essential oils of *Origanum Majorana L*

FBS: Fasting blood sugar

g: gramme

GC/MS :. Gas Chromatography-Mass Spectrometry

GRA: Granulocytes

GSH: Glutathione

HGB: Hemoglobin

IC50 : Concentration causing 50% inhibition of an activity

IFD : Induced Fit docking

K : Constante de liaison

LYM: Lymphocytes

MDA : Malondialdehyde

MDS : Molecular Dynamics Simulation

mg : Milligramme

min : Minute

WHO: world Health Organization

pH : Potential hydrogen

PLT: Platelets

RBC: Erythrocytes

RMSD: Root Mean Square Deviation

RMSF : Root Mean Square Fluctuation

SIB: Swiss Institute of Bioinformatics

TG: Triglycerides

UA: Uric Acid

UV :: Ultra-violet

Vis :: Visible

VTRS : Valorisation and Technology of the Saharian Resources Laboratory

WBC: Leucocytes

α : Alpha

β: Beta

µl : Microlitre

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General Introduction

Plant-based substances have recently become of great interest, as they represent significant sources of molecules and bioactive compounds for the design of new products utilized in the pharmaceutical, cosmetic, food industries, and especially therapeutic fields. **(Bekhaoua et al,2019)** Good plants have attracted the interest and curiosity of humans throughout the ages, as they have always been and remain a natural source of treatment, whether in traditional form or in the form of pure active substances. They excel over chemical medicines in their high therapeutic efficacy, as well as their minimal side effects. **(FranswothZ,.1986)**

Algeria is characterized by a rich floral diversity and a likewise rich ethno-medicinal tradition. Several plant-based preparations have been used in Algerian practice for the management of multiple diseases such as diabetes, with claims reinforcing their effects. **(Khacheba et al, 2017**)

Diabetes is a long-term metabolic illness marked by high blood glucose (blood sugar) levels. Serious damage to the heart, blood vessels, eyes, kidneys, and nerves can result from diabetes over time. The most prevalent kind, type 2 diabetes, usually affects adults and is brought on by insufficient or resistant insulin production in the body. Type 2 diabetes has been far more common over the last three decades in all nations, regardless of wealth. Diabetes type 1 is a chronic illness in which the pancreas generates little or no insulin on its own. It was formerly referred to as juvenile diabetes or insulin-dependent diabetes **(WHO., 2024.)**

Patients with type 1 diabetes, like those with type 2, are usually initially treated with dietary adjustments and physical exercise. However, if these measures are insufficient to control blood glucose levels, medications that are antagonists of glucagon-like peptide-1 (GLP-1) receptors, insulin, oral antihyperglycemic agents such as sulfonylureas like Gliclazide which stimulate insulin secretion, or inhibitors of glucose-regulating enzymes such as acarbose which acts as an inhibitor of α-amylase and α-glucosidase, or a combination of these medications may be prescribed **(Erika F. Brutsaert., 2020)**

Polyphenols, and more specifically flavonoids, are known to inhibit the activity of digestive enzymes such as α-amylase and α-glucosidase. This inhibition plays a very important role in the management of diabetes mellitus (c**Khacheba et al., 2014**).

Essential oil plays a significant role in diabetes by inhibiting the activity of digestive enzymes such as α-amylase and α-glucosidase. This inhibition plays a crucial role in the management of diabetes. **(Teng, H. 2017.)**

The present work is dedicated to the phytochemical valorization of two plants that grow in the city of El Oued in Algeria. (*Cotula cinerea _ Origanum majorana L)* Initially, we extracted the essential oil from each plant using the method of water distillation Hydrodistillation By using the gas chromatography-mass spectrometry (GC/MS) analysis method, The components of these essential oils have been identified.. Study in vitro and in vivo and in silico on the inhibitory effect of essential oils on the enzymatic activity of α-amylase, partially responsible for the increase in blood sugar levels and the onset of diabetes .

FIRST PART: BIBLIOGRAPHICAL SYNTHESIS

Chapter one: Medicinal Plants

1. Introduction:

One of the greatest blessings bestowed upon humanity is that it has been endowed with a nature that flourishes with plants and crops in all their colors. (Maathai, W., 2010.) Thus, the Creator has fashioned beauty and benefits within them, as mentioned in numerous places in the Quran. Since the appearance of humans on Earth, the ways of treatment and healing through plants and natural herbs have been known. In ancient times, all diseases and pains were treated with herbs.

With the passage of time and the advancement of civilization, chemically manufactured medicines began to compete with herbal remedies due to progress. (Ghassan M., 1999.) With the advancement of practical application and technology, people gradually became able to abandon herbal medicines in treatment and replace them with chemical drugs. Herbal medicine has now regained people's attention. It has become a topic of discussion among academics, doctors, and patients alike, with both support and opposition.(J.Jeanfils.,1991)

2 . The studied plants:

2 .1 .Cotula cinerea Plant:

2-1 .1 . *Cotula cinerea* Definition:

Cotula cinerea, scientifically known as Sheikh, exhibits a distinctive odor reminiscent of wormwood. Botanically classified as an annular herb, it features dense, lobed leaves and robust, pale bristles. This species blossoms during spring, displaying yellow flowers resembling composite discs. Sheikh thrives abundantly in desert regions, particularly in Arab territories, boasting a rich reservoir of bioactive compounds such as flavonoids, terpenes, and volatile oils (Bohlmann ,F., et al., 1973; Mahran ,G.H .,et al., 1976).

2-1. 2. Vegetative classification of *Cotula cinerea*:

Scientific name: *Cotula cinerea* Del.

Synonymous: Brocchia cinerea (Del) Vis.

Common name: Shihiya, Rubaita, Sheha al-Bal

Table 1: Vegetative Classification of Cotula Cinerea

Species	*Cinerea*
Gender	*Cotula*
Family	Asteraceae (Compositae)
Order	Tubiflorals
Class	Dicotyledons
Branch	Angiosperms
Reign	Plants

2-1. 3. Vegetative description of *Cotula cinerea:*

A diminutive herbaceous plant ranging from 10 to 40 cm in height, possessing a distinctive pungent aroma. (Boning, C., 2010) Adorned with delicate white bristles, its stems are erect or slightly prostrate, exhibiting a cylindrical form tinged with green-yellow hue. The leaves are pale green, ensconced in woolly, dense bristles, notable for their thickness, trifoliate structure, and trifurcation at the apex into 3-5 lobes.

The capitula are diminutive and planar, measuring 6 to 10 cm in diameter. (Naidu, V., 2012) Consisting entirely of tubular florets, they are tetramerous and densely packed, forming a compound inflorescence with an overlaying capitulum. Initially dark, the buds transition to a golden yellow hue upon blossoming. The fruits are naked and meager, as depicted in Figure (1) below. (Ahmed, A.A., et al., 1987; Malinskas, G. A. G., et al., 1987)

Figure 1. Cotula cinerea photograph.

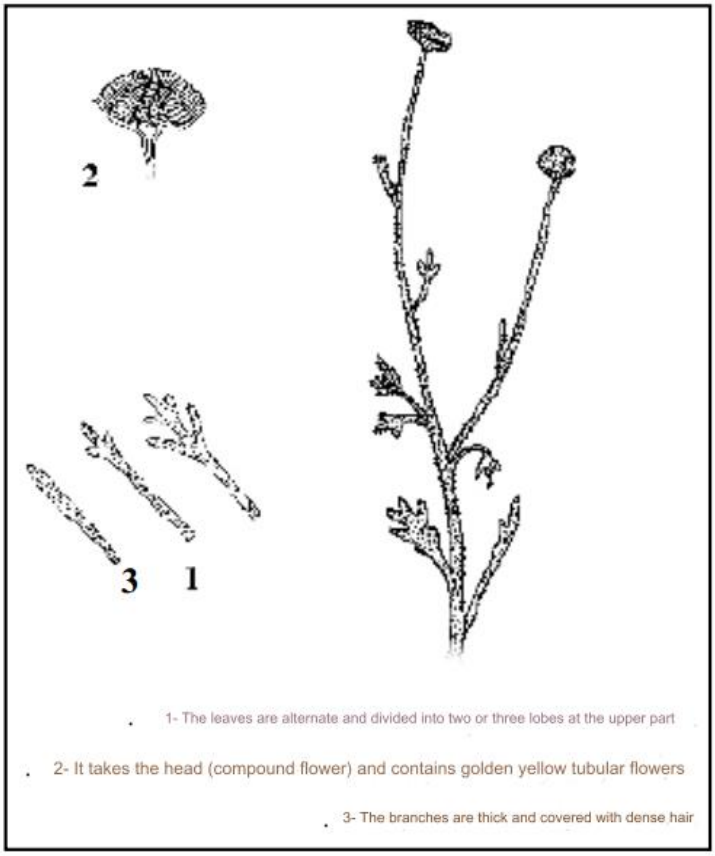

Figure 2. Diagram form of cotula cinerea.

2-1. 4. Geographical Distribution of *Cotula cinerea*:

The distribution of *Cotula cinerea* spans abundantly across the southern hemisphere, encompassing regions such as the Sahara Desert, the deserts of India and Iran in Asia, and the Arabian Peninsula (Rezaei M.B., et al.; G. Fournier'3, H., et al.). In Algeria, it predominantly inhabits desert and semi-arid zones, particularly in the southeastern regions. Figure 3 depicts the geographical distribution of *Cotula cinerea* in Algeria.

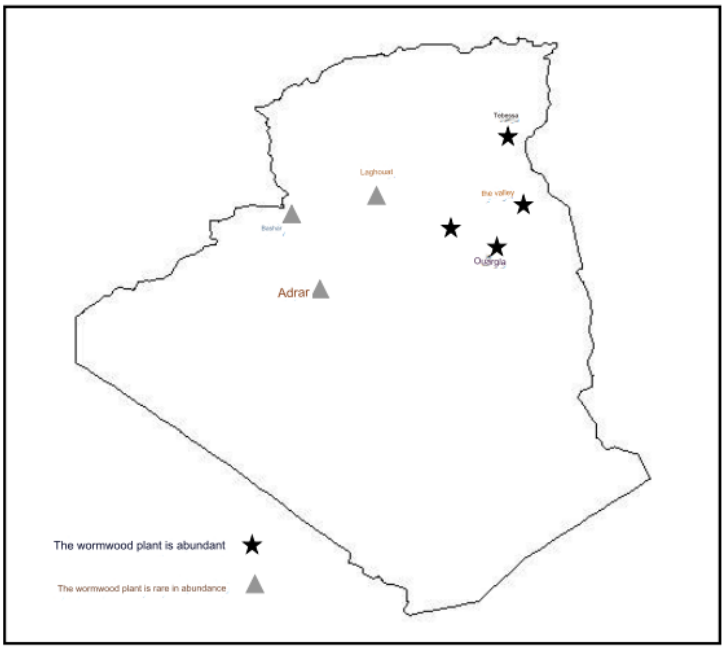

Figure 3. Distribution of *Cotula cinerea* in Algeria.

2-1. 5. Economic and Medicinal Uses of *Cotula cinerea*:

In certain regions, *Cotula cinerea* is employed as a flavoring agent in tea and coffee, as well as a soaking agent for beverages. Additionally, it finds application in traditional medicine for alleviating abdominal pain and aiding digestion (M.Markouk, H.B et al.; Boulahbal F., et al., 2002). Its efficacy against bronchitis is attributed to its cough-suppressing properties, and extracts from *Cotula cinerea* have demonstrated antifungal activity (Bryskier A., et al., 1999). Flavonoids derived from this plant exhibit sedative, anti-inflammatory, and disinfectant effects, while in certain locales, *Cotula cinerea* is utilized for the treatment of stomach and abdominal discomfort (Larpent J.P., et al., 1989).

2.1 . *Origanum Majorana L* Plant:

2. 1-1. *Origanum Majorana L* Definition:

The term "*Origanum*" originates from the fusion of two Greek words, "oros," denoting mountains, and "gonos," signifying joy or pleasure, reflecting its association with the delight of mountainous landscapes renowned for their scenic beauty and profusion in the mountainous regions of the Mediterranean basin (Prena and Neeru Vasudeva., et al., 2015). This botanical specimen is classified as a perennial herb, formerly designated as *Origanum Majorana L* (VagiE, Simandi B., et al., 2002). Exhibiting substantial morphological and chemical diversity, it encompasses forty-nine distinct classifications distributed across the Mediterranean Sea region. Referred to colloquially as sweet marjoram and indigenous to Anatolia (Turkey) and Cyprus, this herbaceous plant thrives extensively in various parts of the Mediterranean territory, particularly in Egypt (Novak J., et al., 2002). Initially utilized by Hippocrates as an antiseptic agent, marjoram serves as an efficacious household remedy for respiratory infections, coughs, and throat irritation (Bremness L., et al., 1994; Yazdanparast R., et al., 2008).

2.1. 2. Vegetative Classification of *Origanum Majorana L*:

Scientific Name: *Origanum majorana L.*

Synonyms: *Marjolaine.*

Common Name: Al-Murdagush.

Table 2: Vegetative classification of *Origanum Majorana L.*

Species	Majorana L
Gender	Oreganum
Family	Lamiaceae
Order	Lamiales
Class	Mangnoliopsida
Branch	Mangnoliophyta

Reign	Plants

2.1.3.Vegetative Description of *Origanum Majorana L*:

This half-perennial shrub exhibits annual growth and is susceptible to cold temperatures. It is an aromatic bush reaching heights of up to 60-30 cm, characterized by multiple square-shaped reddish stems that sprawl to form a dense cluster. (Oakman, H., 1995.).T he stems are straight, weak, hairy, and cylindrical, with green coloration interspersed with red spots (Pimple B P., et al., 2012).

The leaves are soft and simple, featuring a market and oval to oval-rectangular shape, with a green-gray hue and inversely correlated arrangement. Each leaf has a smooth surface adorned with dense hairs, measuring approximately 1.5 to 0.5 cm in length and width. They exhibit dimensions ranging from 0.8-0.2 cm, possess entire margins, an acute apex, a symmetrical and straight base, and are veined. Additionally, small white or pale pink tubular flowers emerge in mid-to-late summer in clusters, with a length of less than 0.3 cm, and are arranged in spikes up to 13 cm long. These flowers are bisexual (A. Blazovics A., et al., 2005).

The plant produces small, dark brown oval seeds that mature from August to September, exhibiting a cylindrical shape. The subterranean roots are longitudinally aligned, occasionally featuring incisions with diameters ranging from 0.6 to 0.2 mm. The external surface of the roots is dark brown, with narrow and deep cracks, serving as indicators of root strength and plant stability. These cracks facilitate water and nutrient absorption and contribute to the plant's structural support in the soil. Long, symmetrical roots with subtle aromatic fragrance contribute to the overall aesthetic appeal and stability of the plant (Singla P., et al., 2014).

Figure 4.*Origanum Majorana L* Photograph

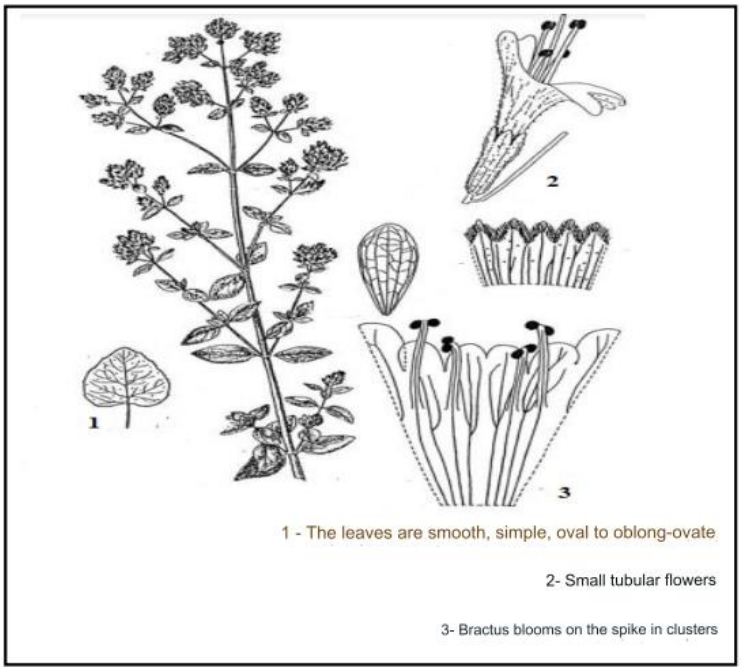

Figure 5. Diagram for *Origanum Majorana L*

2.1. 4. Geographical Distribution of *Origanum Majorana L*:

Origanum Majorana L is primarily found in Turkey and Cyprus, with additional distribution in Mediterranean countries including Lebanon, Iran, and North America. Its growth is notable in the Arabian Peninsula and India, particularly on sunny slopes of meadows, fields, and rocky terrain in dry climates. (Zahran, M., et al., 2010). It is extensively cultivated in the southern regions of Saudi Arabia.

Figure 6 illustrates the distribution of *Origanum Majorana L* in Algeria.

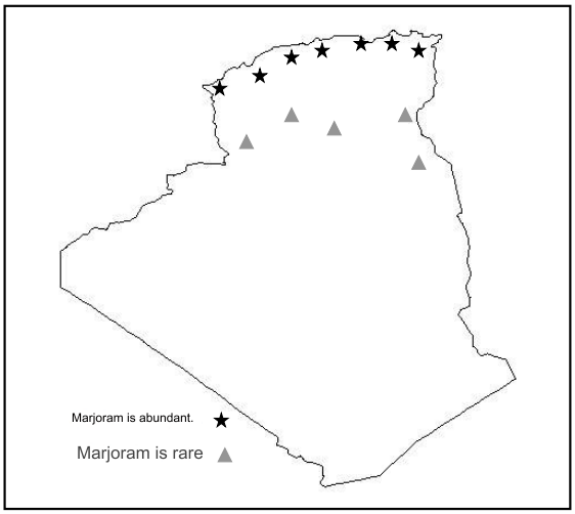

Figure 6. The spread of *Origanum majorana L* in Algeria

2-1. 5. Economic and Medicinal Uses of *Origanum Majorana L*:

Origanum Majorana L exhibits numerous domestic remedies effective in treating chest infections, coughs, sore throats, rheumatic pain, neurological disorders, and heart diseases. (Mancak, M., et al., 2023). These remedies, easily administered at home, provide comfort and alleviate symptoms. Noteworthy among these treatments are:

1. Rest and Relaxation: Adequate rest and sleep are recommended to bolster the immune system and expedite the healing process. (Farmawati, C., et al., 2020).

2. Consumption of Warm Liquids: Ingesting warm beverages such as herbal tea and soup can alleviate congestion and soothe the throat. (McIntyre, A., 2000).

3. Steam Inhalation: Inhaling steam from hot water or utilizing an air humidifier can alleviate congestion and soothe the respiratory tract. (Vathanophas, V., et al., 2019).

4. Honey: Natural honey acts as an antibiotic, soothing sore throats and alleviating coughs. (Kumar, K. S., et al., 2010).

5. Gentle Exercises: Engaging in light physical activities like walking or yoga enhances blood circulation and alleviates rheumatic pain. (Govindaraj, R., et al., 2016).

6. Omega-3 Rich Foods: Consumption of omega-3 rich foods like fatty fish and nuts can reduce inflammation and promote heart health. (Simopoulos, A., 2002).

However, individuals with these conditions should seek appropriate medical advice before implementing any home remedies to ensure efficacy and safety. (Sharifi-R ., et al., 2021).*Origanum Majorana L* finds extensive usage in the food industry as a seasoning for meat and vegetables, although its consumption is advised against during pregnancy due to its potential to stimulate uterine contractions.

Traditionally, the leaves are employed to treat various ailments including diabetes, insomnia, colds, asthma, and panic attacks (Cano J H and Volpato G., et al., 2004). Additionally, the essential oil derived from the leaves imparts a distinctive flavor to dishes. *Origanum Majorana L* is incorporated into a wide array of food products including soups, gravies, meats, fish, canned foods, liqueurs, and beers *(Farrell K T Spices., et al., 1985; Lavrenov V K., et al., 1999).*

3. Previous Studies:

3 -1. Previous Research on the *Cotula cinerea* Plant:

Reviewing the chemical literature concerning *Cotula cinerea* indicates intensive study of several compounds, including lactonic sesquiterpenes (Metwally, M., et al., 1986; Jakupovic, J., et al., 1988; Bohlmann, F., et al., 1989), comarenic ethereal sesquiterpenes (Greger, H., et al., 1985), and monocyclic diterpenes as significant organic compounds. Additionally, triterpenes and characteristic acetylene compounds of the spiroketal enol ether have been identified (Metwally, M., et al., 1986; Bohlmann, F., et al., 1973).

Lotfi et al.'s study conducted vital tests on active compounds within the *Cotula cinerea* plant, revealing a notable absence of alkaloids compared to the presence of flavonoids and glycosides (Lotfi B., et al.). The comprehensive findings are presented in Table 3, detailing active substances in the *Cotula cinerea* plant.

Table 3. Active Ingredients of *Cotula* Plant

Glycosides	turbines and Steroids	tannins	essential oils	Saponics	alkaloids	flavonoids
+	++	++	++	+	-	+++

(+ + +): Highly abundant.

(+ +): Abundant.

(+): Weak.

(-): Absent.

The research team, led by Professor M. Markouk and colleagues from the University of Marrakech in Morocco, investigated the anti-pathogenic activity of *Cotula cinerea*. This plant thrives in sandy and desert soils and is utilized in traditional Moroccan medicine for its anti-inflammatory, sedative, disinfectant, antibacterial properties, and as an aromatic additive in tea (M. Markouk et al.).

A sample of this plant was collected from the Faculty of Science in Mashba, Morocco (Marrakech), and its extract was utilized for experimentation. A trial was conducted using 21 white Swiss mice weighing between 20 and 22 grams. (Mehedi, N ., et al., 2013).

The mice were housed in plastic cages at a temperature of 22 to 24 degrees Celsius and were deprived of food for 14 hours prior to the test. (Van de W., et al., 2002).

The experiment involved administering three different extracts of the plant: ethyl acetate, diethyl ether, and ethanol. The injected doses of these extracts exhibited acute toxicity, resulting in fatalities among some mice and various clinical symptoms in others. (El Kabbaoui, M., et al., 2017). Acetate and n-butanol extracts were also employed.

Table 4. outlines the toxic effects of *Cotula cinerea* plant extracts.

Mortality	Clinical symptoms decrease of weight ,oedema , hair loss,		Oral dose (g/Kg)

			convulsion /No .tested			Number of animals	
EB	**EAc**	**EE**	**EB**	**EAc**	**EE**		**Vehicle control (10mL/Kg)**
0/6	0/6	0/6	0/6	0/6	0/6	6	1
0/6	0/6	0/6	0/6	0/6	0/6	6	2
0/6	0/6	0/6	0/6	0/6	0/6	6	3
0/6	0/6	0/6	0/6	0/6	0/6	6	4
0/6	0/6	0/6	0/6	0/6	0/6	6	5
0/6	0/6	0/6	0/6	0/6	0/6	6	6

EE: Ethyl Ether Extract EAc: Ethyl Acetate Extract EB: n-Butanol Extract

During the toxicity assessment of the extracts, no fatalities occurred among the mice, and their behavior remained normal, even when exposed to doses up to 6 grams per kilogram of body weight. (Mukinda, J., et al., 2007). Therefore, it can be concluded that mice can tolerate concentrations of up to 6 grams per kilogram of their body weight.

The research team, led by Dr. M. Markouk et al., conducted a study on the bacterial inhibitory activity (minimum inhibitory concentration), wherein the bacterial growth following a 24-hour incubation period was assessed. Each test was repeated three times. (Mathur, T., et al., 2006).

The data are presented in Table 5, illustrating the Minimum Inhibitory Concentration (MIC) in micrograms per milliliter (µg/ml) for *Cotula cinerea* extract.

Table 5. Toxicity Efficacy of *Cotula* Plant

Species	**Strain**	**Ethyl acetate extract**	**n- Butanol extract**	**Novobiocine (standard)**

Pseudomonas fluorescens	456-2	200	12	1
Pseudomonas savastanoui	T12-10	200	100	1
Pseudomonas savastanoui	73-29 88	200	50	1
Bacillus sp	VP5	200	25	20
Bacillus breuis	VP7	200	25	20
Bacillus sp	326	200	25	20
Bacillus sphaericus	324	200	200	20
Bacillus sp	459-1	2001	12	20

The results indicated that extraction of *Cotula cinerea* plant in ethyl ether exhibited no activity, while extraction in ethyl acetate demonstrated efficacy against all tested pathogens. (Acheuk, F., et al., 2020). Extraction in n-butanol exhibited very high efficacy against all pathogens. A study was conducted to establish the correlation between extract concentrations and inhibitory activity, revealing a significant increase in the average diameter of the inhibition zone with increasing extract

concentration. A two-way ANOVA test was employed to compare the effect of *Cotula cinerea* extract on antimicrobial properties, revealing significant differences in antimicrobial effectiveness among all plant extracts (Atef, C., et al., 2015).

In a study by Khallouki et al. (2015), the chemical composition of *Cotula cinerea* plant was analyzed for its potential medicinal use in southeastern Morocco. Phenolic compounds in methanol extracts totaled 2.5 ± 79.23 mg/g of dry substance. (Irondi, E., et al., 2018). Neochlorogenic acids, dicafuelkenic-3,5, kryptochlorogenic, chlorogenic, dicafuelkenic-3,4, and dicafuelkenic-5,4 were identified as the main phenolic compounds using HPLC-ESI-MS analysis. The antioxidant capacities assessed through FRAP and DPPH assays were found to be very strong, suggesting potential efficacy in disease prevention according to African traditional medicine, possibly attributed to the high content of flavonoids and phenolic compounds (Khallouki, F., et al., 2015).

In a study by Santiuste et al. (2008), the main constituents of *Cotula cinerea* oil were identified, suggesting its potential use in enhancing the flavor of tea as a substitute for mint. (Fatiha, B., et al). The plant is also utilized in folk medicine for the treatment of pulmonary and tracheal diseases.

The chemical composition and anti-candidiasis properties of *Cotula cinerea* oil were investigated by El-Bouzidi et al. (2011). The oil exhibited potent activity against all tested candida strains, with inhibitory concentrations ranging from 4.7 to 3.2 mg/mL and a minimum concentration required for yeast eradication of 5.9 mg/mL. GC-MS analysis identified 24 compounds in the oil, which may serve as effective antibiotics against resistant candida strains. (Cecchini, C., et al., 2010).

ABDENBI ASMA et al. conducted a study in 2014 on *Cotula cinerea* oil extracted through water distillation from aerial parts in a region near Bashar, Morocco. The oil demonstrated variable antibacterial activity ranging up to 2%, inhibiting approximately seven bacterial strains. The minimum inhibitory concentration of the oil was determined based on the lowest concentration exhibiting significant sensitivity against gut bacteria, with an inhibition zone reaching up to 55 mm (ABDENBI ASMA., et al., 2014).

In a recent review study from 2014, the chemical composition and antibacterial and antifungal activities of *Cotula cinerea* oil, sourced from southern Morocco, were analyzed. Water distillation of pneumatic plant parts yielded the oil, which was analyzed using GC and GC/MS techniques. Key compounds such as ISO-thujanol-3, Santolina triene, and cavor were identified. Antibacterial efficacy

against four bacterial strains was observed, along with antifungal activity against fungal rot (Review, Tutorial, 2014).

3 -2. Previous Studies on *Origanum Majorana L* Plant:

The chemical composition and mineral content of *Origanum Majorana L* leaves were analyzed. The moisture, protein, fat, ash, and carbohydrate content ratios were found to be 66.3%, 18.7%, 8.4%, 6.6%, and 5.7%, respectively. (Pinela, J., et al., 2017). Metal element concentrations, measured in parts per million, were 0.6 for Ba, 5.1 for Fe, 0.039 for K, 0.49 for Co, and 0.01 for Na. Qualitative tests of aqueous and alcoholic extracts revealed the presence of tannins, phenols, flavonoids, saponins, carbohydrates, and alkaloids. Physical and chemical properties, such as combustion and solubility, were investigated, showing partial solubility in polar solvents and complete solubility in non-polar solvents (Maryam A., et al., 2017).

The effect of using *Origanum Majorana L* leaf powder as a preservative for beef at different concentrations (0.5% - 1%) was examined. The beef samples were stored at 5°C for 7-10 days, with changes in meat quality monitored by estimating the peroxide number. The results indicated lower peroxide numbers in samples treated with *Origanum Majorana L* leaf powder compared to fresh samples during the storage period. (Sahunie, A., et al., 2024). The impact on the levels of coliform bacteria and Enterobacteriaceae in the meat was also investigated during the storage period and concentrations mentioned. The findings suggested that the herb contributes to reducing microbial counts in minced meat samples and exhibits antioxidant properties, thereby extending the shelf life of the meat *(Maryam A., et al., 2017).*

4. Essential Oils:

4.1. Generalities About Essential Oils:

4.1.1. Definition of Essential Oils:

Essential oils are defined as volatile oils that evaporate or volatilize without degradation, distinguishing them from fixed oils, which are non-volatile and degrade upon exposure to evaporation or heating (L. Mondello., et al., 1995). They are also referred to as aromatic oils or ethereal oils due to their aromatic aroma and solubility in ether, as well as volatile oils.

Essential oils are complex compounds containing volatile elements found in plants. They are typically colorless or yellowish, non-flammable, susceptible to oxidation in air, and typically liquid at normal conditions. Essential oils are distinguished by their lack of fatty or greasy properties (E. Guentherin., 1972; S. Rahal., 2004) and comprise various plant-derived compounds, which are distilled with steam and separated from water.

The International Standards Organization defines essential oils as compounds obtained from plant materials, either through steam distillation or mechanical extraction from citrus peels, and subsequently separated from the solution through physical means. This definition (D. Lemordant., 1989; K. W. Lee., et al., 2004) is acknowledged by international bodies and is employed in professional contexts as a precise delineation of essential oils.

4.1.2. Storage Sites for Essential Oils:

Essential oils are present in over 2,000 plant species encompassing more than 60 plant families. (Nieto, G., 2017). They can be distributed throughout various plant parts or concentrated in specific organs, with variations in their abundance across different plant species. Typically, essential oils are stored within the cytoplasm of living cells, primarily in liquid form, although some may be bound to glycoside or resin compounds, rendering them non-free and solid. (Ogunsina, O., 2020). Within plant tissues, essential oils are often grouped into specialized structures known as secretory structures.

Flying plant oils are distributed across various plant parts, such as the leaves in mint plants, the petals of flowers like roses and jasmine, or the fruits and peels of citrus fruits. They may be present in multiple plant parts, with their distribution varying in each organ (J. Valnet., 1984; D. Lemordant., 1989; Anonyme., 2000; A. Baaliouamer., 1987; C. R. Karnich., 1994; K. Bauer., et al., 1997; R. L. Shriner., et al.,).

Examples:

Rutaceae:
Essential oils are primarily concentrated in the leaves, flowers, and seeds, exemplified by Citrus aurantium L. (Guzmán, E., et al.,2021).

Myrtaceae:
Essential oils are concentrated in the leaves, as seen in Eucalyptus species. (Elaissi, A., et al., 2012).

Apiaceae:
Essential oils are concentrated within the vascular channels, exemplified by Angelica archangelica. (D'Amelio Sr, F., 1998).

Lamiaceae:
Essential oils are predominantly concentrated in the aerial parts of plants, as observed in species like mint and thyme (F. Abdellatif., et al., 2005).

4.1.3. Physical Properties of Essential Oils:

Although essential oils exhibit variations in their chemical compositions, they share common natural traits when fresh. General characteristics of essential oils include (D. Lemordant., 1989; Anonyme., 2000; D. O. Kennedy., et al., 2002; R. J. Grayer., et al., 2003):

1-Color:

Most essential oils are colorless; however, upon storage, they may oxidize and darken, turning black. (Turek, C., et al., 2013). Rarely, essential oils may exhibit a greenish-blue hue, as seen in chamomile oil, eucalyptus, and some mountain varieties, attributed to the presence of azulene and chamazulene compounds, which impart a green or blue coloration. Additionally, some essential oils may appear yellowish or pale due to bleaching effects (E. Seguin., et al., 2001).

2-Odor:

Essential oils typically possess pleasant fragrances, rarely exhibiting unpleasant or pungent odors. The distinctive aroma of essential oils can often be attributed to specific terpenoids and major compounds, discernible even prior to extraction. (Sell, C., 2020). For instance, the volatile nature of citral in the surrounding air imparts a characteristic scent in plants and trees, such as menthol in mint plants, geraniol in thyme plants, and anethol in aniseed plants (B. A. Arthur Riffer., et al., 1969).

3-Volatility:

Essential oils are characterized by their volatility at normal atmospheric temperatures, distinguishing them from fixed oils that remain non-volatile even when heated. When compared, a drop of essential oil evaporates completely, leaving no residue, while a drop of fixed oil remains on the filter sheet, imparting transparency (B. A. Arthur Riffer., et al., 1969).

4-Solubility:

Essential oils exhibit low water solubility but readily dissolve in most organic solvents, such as petroleum ether and diethyl ether. (Aziz, Z., et al., 2018). Alcohol solubility can reach up to 95%, with the exception of rose oil, which forms an emulsion due to the presence of certain paraffin-type organic compounds. The complete solubility of essential oils in alcohol serves as an indicator of purity, with varying concentrations tested from 95% to 35% diluted with water. The addition of fixed oils may hinder the dissolution of essential oils in alcohol (B. A. Arthur Riffer., et al., 1969; O. Ekundayo., 1988).

5-Specific Gravity:

The specific gravity of essential oils varies depending on their plant sources, typically ranging from 0.1 to 1.7. (Schmidt, E., 2020).Most essential oils have a specific gravity of less than one, causing them to float on the surface of water. However, oils like clove oil and cinnamon bark oil, with specific gravities ranging from 1.02 to 1.07 and 1.03 to 1.04, respectively, sink below the water's surface. The specific gravity value provides insights into the composition of essential oils; values below 0.9 indicate high tertiary and aliphatic compound content, while values exceeding one suggest compounds with chemically diverse aromatic rings (B. A. Arthur Riffer., et al., 1969).

6-Optical Rotation:

Essential oils exhibit optical rotation, which serves as a crucial natural assessment tool to determine purity and detect adulteration. Optical rotation helps differentiate between naturally occurring compounds and industrially synthesized ones. (Sheldon, R., 1993).Additionally, essential oils are predominantly liquid at room temperature, except for rose oil and elemi oil, which solidify. Most essential oils also possess a high refractive index.

4.1.4. Constituents of Essential Oils:

All essential oils comprise a complex mixture of numerous compounds, primarily categorized into two groups. (Dhifi, W., et al., 2016)The first group consists of hydrocarbons, which constitute the major portion of essential oils, while the second group comprises oxygen-containing compounds belonging to organic acid, alcohol, ester, aldehyde, ketone, and ether groups. In minor proportions, these constituents may also include sulfur or nitrogen compounds. The hydrocarbon fraction of essential oils is primarily composed of terpenes (E. Seguin., et al., 2001; Dr. Al-Shahat N., 2000).

1.Terpenes:

Terpenes are natural products found in various plant parts, constituting a significant proportion of secondary metabolites. They contribute to the characteristic pleasant aroma of many plants. The term "terpenes" originates from compounds separable from turpentine oil (B. A. Arthur Riffer., et al., 1969). Most terpenes are volatile oils extracted from plants in a similar manner to essential oils (N. Ramarathnam., et al., 1986).

Terpenes play crucial roles in daily life; for instance, vitamin A, classified as a terpene, is essential for maintaining vision. Studies conducted by Owllach at the University of Göttingen in Germany in 1877 revealed that terpene diversity is determined by the number of isoprene units. The table below illustrates various types of terpenes (E. Seguin., et al., 2001; Ghassan M., 1999).

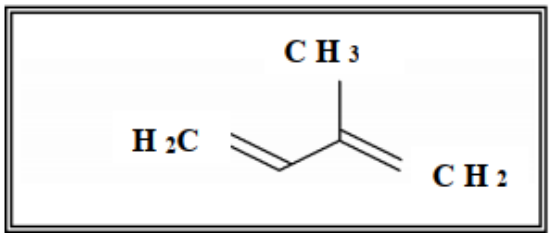

Figure 7: Isobrin unit

Monoterpenes:

Monoterpenes represent one of the most prevalent classes within the plant kingdom (O. Ekundayo., 1988). Comprising two isoprene units, they are characterized by a single ring structure. Among their significant compounds are Limonene and α-Phellandrene.

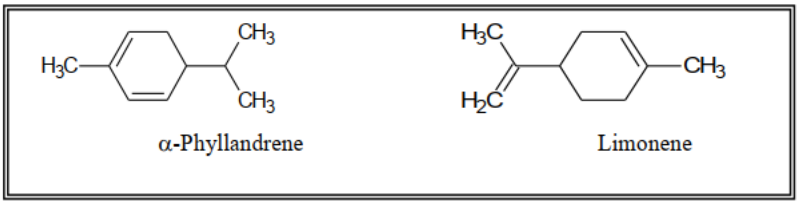

Figure 8. Mono-ring Monoterbines

There are numerous bicyclic compounds such as Sabinene, ß-Pinene, and α-Pinene *(E. Seguin., et al., 2001; R. Paris., 1972)* along with their respective derivatives. Bicyclic monoterpenes are categorized into four groups based on the carbon structure of hydrocarbons. These groups consist of the Thujane Group, the Camphan Group, the Bornane Group, and the Fenchane Group.

Tujane and camphane derivatives are present in cedarwood oil. The primary source is turpentine, which can be extracted from the bark of pine trees.

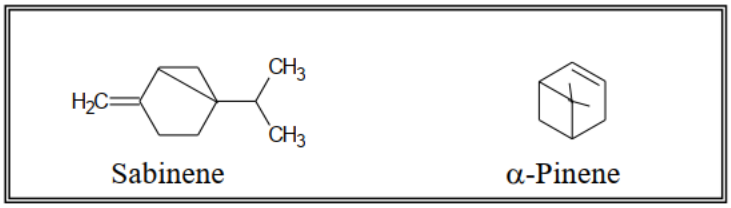

Figure 9. Bicycle Monoterbines

Diterpenes:

These terpenes consist of 20 carbon atoms, equivalent to four isoprene units. (Ruzicka, L., et al., 1953) Examples include phytol (vitol) and vitamin A1.

Sesquiterpenes:

Comprising over 2,500 molecules, each containing three isoprene units with a total of 15 carbon atoms. (Ceausescu, E., 2013.) They are typically categorized by their carbon structure. For instance, cadinene is an example of the first type, while caryophyllene is found in celery oil, representing the second type.

Triterpenes:

An example of triterpenes is esculetin, a precursor in the synthesis of cholesterol.

Tetraterpenes:

These compounds consist of eight isoprene units and are known as carotenoids. Three carotenoids, α-carotene, β-carotene, and δ-carotene, have been isolated. (Riaz, M., et al., 2021)

Polyterpenes:

Natural rubber is a notable example of polyterpenes, found in latex obtained from tropical rubber trees. (Long, Xiangyu, ., et al., 2021) Its molecular structure resembles a series of repeated isoprene units akin to horned structures.

Table 6. Types of Terpenes

Turbine Type	number of carbon atoms	Number of isoprene units
Monocultures	10	2
Half Triple Terpen	15	3

Bilateral terpenes	20	4
Triple Terpen	30	6
Quadruple Turbine	40	8
Multi-turbine	more than 40	more than 8

4.1.5. Benefits and Applications of Essential Oils:

Thousands of years ago, aromatic oils were utilized in ancient Chinese and Egyptian civilizations, as well as in Arab and Islamic cultures. Over time, the use of essential oils has evolved, becoming commonplace in cosmetic institutes and wellness centers, where they are employed in disease treatment as part of a medical regimen aimed at maintaining human health. (Wilson, R., 2002.) Essential oils produced by plants serve various purposes; they attract insects for pollination, augmenting fruit production. Additionally, certain essential oils function as insect repellents or toxins, safeguarding plants from pest infestations. (Regnault-R.,1997) Essential oils facilitate the elimination of metabolic waste and contribute to fortifying the body's physiological systems, including the immune system and circulatory system. For instance, lavender oil aids in promoting hair growth and enhances oxygen uptake while boosting ATP production, vital for cellular energy. The ensuing Table 7 encapsulates the primary benefits and applications of essential oils.

Table 7 . Uses of Essential Oils

medical uses	**other uses**
- Calmant, Sédative.	- Earn some drugs with an acceptable taste and smell.
- Hypotensive.	- Use as a flavor or seasoning on foods.
-Anti-virale.	- Used in perfume and cosmetics.
-Anti-inflammatoire.	- Used in pharmaceutical and soap shapes industry.
-Rebalancing the internal nervous system.	- Worms and parasites repellent.
-Anti-muscle convulsions.	- Insect repellent like mosquitoes.

4.2. Methods of Essential Oil Extraction:

4.2. 1. Distillation:

Distillation techniques represent one of the earliest methods for extracting essential oils, with a history spanning over a century. (Schmidt, E .,2020) The principle behind distillation apparatus operation relies on the vaporization of essential oils from plant materials upon heating. Subsequent condensation of the vapors results in a mixture of essential oils and water, facilitating their separation due to differences in solubility (S.Rahal., 2004; F.Abdellatif., 2005). Each distillation cycle yields a fraction of high-grade, high-quality essential oils. Notably, there are three primary methods of distillation:

1/ Hydrodistillation:

Hydrodistillation, a widely employed technique, involves immersing plant material in a distillation vessel filled with water. (Lawrence, B .,1995) The plant material can either be submerged directly in the water or placed within a mesh container to prevent direct contact with the vessel walls. Typically crafted from stainless steel, copper, or glass, the distillation apparatus is heated either by an open flame or an electric heater. (Das, S ., et al., 2013) This method is suitable for extracting essential oils from heat-resistant dried plants, particularly those with high oil content in various plant parts such as roots, leaves, fruits, and certain flowers.

Table 8 outlines the advantages and disadvantages associated with hydrodistillation.

Table 8. Advantages and Disadvantages of Hydrodistillation

Water distillation advantages	Water distillation defects
- Easy to use. - Low-cost processing. - The machine is simple to make and install. - Do not require a designated place and no conditions Special.	- The impossibility of complete (total) debriefing. - Loss of some decomposable compounds in water, As in the case of oxygen compounds. - It takes a long time to complete the extraction process.

2. Extract with waves spread under pressure:

In this method, the extraction process is conducted under pressure conditions, utilizing microwave radiation. (Jain, T , ., et al., 2009) The application of pressure enhances the extraction efficiency by facilitating the penetration of microwave energy into the plant material, thereby promoting the release of essential oils.

3. Extract with waves placed under aerial pressure:

In this technique, microwave radiation is applied under atmospheric pressure conditions. (Yang, Y ., et al.,2014). The plant material is subjected to aerial pressure, allowing for efficient extraction of essential oils using microwave energy.

4. Water distillation extraction using microwave radiation under vacuum:

This method involves the application of microwave radiation to facilitate water distillation under vacuum conditions. (Li, Hong ., et al.,2019) The use of vacuum enhances the efficiency of water distillation by reducing the boiling point of water, thus promoting the extraction of essential oils from the plant material.

5. Extract with solvent to help microwave radiation:

In this approach, a solvent is added to the plant material to aid in the extraction process using microwave radiation. (Mandal, V ., et al.,2007) The solvent helps to solubilize the essential oils, thereby enhancing their extraction efficiency when exposed to microwave energy.

Species (1), (2), and (3) exhibit superior performance compared to traditional extraction methods. Conversely, type (4) offers greater efficiency than the first three methods, particularly in terms of extraction speed. Type (5) allows for the use of minimal solvent quantities and offers shorter extraction times compared to conventional methods like the Soxhlet extraction technique. However, one notable drawback of species (2), (3), (4), and (5) is their significantly higher cost.

It's worth noting that all these methods reduce extraction time and prevent damage to heat-sensitive compounds (Benkaci-A., et al., 2006; M.A. Ferhat., 2006).

2/Cold Pressing:

This method is specialized for extracting essential oils from citrus fruits due to their susceptibility to oxidation and heat. It involves mechanical or manual extraction under high-pressure water streams. The resulting essential oil is then separated from water using centrifugation (M.A. Ferhat., 2006).

3/Solvent Extraction:

Organic solvent is added to plant material (e.g., jasmine blossom or rose) at room temperature. The plant material is arranged in thin layers to allow the solvent to penetrate cells containing essential oil. The essential oil is dissolved in the solvent, forming a solution of solvent and oil. (El Asbahani, A., et al.,2015) Separation of solvent and oil is achieved through distillation under low pressure. The resulting substance, known as "concrete," is hardened due to the presence of fatty substances attracted by the solvent. Subsequently, the concrete is treated with absolute alcohol at low temperature to remove fatty substances, resulting in "absolute alcohol of essential oils" (M. Vinatoru., et al., 1997; M. Toma., et al., 2001). While this method reduces extraction time and allows for the extraction of heat-sensitive molecules, its use is limited due to its high cost and associated safety and environmental concerns (G.T. Forrest., et al., 2000).

4/Supercritical/Liquid Carbon Dioxide Extraction:

This technique relies on the ability of certain gases, particularly carbon dioxide, to replace compounds such as essential oils, natural colors, and fragrances under critical pressure conditions. (Chakravarty, I., et al., 2021) Carbon dioxide is used in either liquid or gaseous states. When carbon dioxide reaches its critical point (31.4 °C - 73 bar), it becomes supercritical, with a density close to that of a fluid. The efficiency of compound replacement depends on temperature, pressure, and the nature of the solute (E. Guentherin., 2000; F. Abdellatif., 2005). This method is known for its ability to easily separate the solvent and for operating at low temperatures to preserve the integrity of oil components. Carbon dioxide is preferred as a solvent due to its pressure, low temperature, non-flammability, lack of toxicity, and absence of residue, making it popular in the perfume industry (E. Guentherin., 2000; F. Abdellatif., 2005).

5/Maceration Extraction:

This process involves soaking plant parts in solvent for a sufficient duration to dissolve essential oils. (Rao, V ., et al., 2007) Mechanical agitators may be used to prevent clumping and ensure thorough exposure of all plant parts to the solvent. Solvent temperature may be elevated to aid in

melting essential oils, but care must be taken to avoid temperatures that could affect oil quality. The extracted solution is distilled at low temperature and low pressure to separate the solvent for reuse, resulting in the extraction of essential oil (F. Abdellatif., 2005).

CHAPTER TWO: ANTIDIABETIC ACTIVITY

1. Diabetes

1. 1. Definition :

Diabetes is a serious chronic illness that develops when the pancreas is unable to produce enough insulin, the hormone that controls blood sugar, or when the body is unable to use the insulin that is produced Over time, uncontrolled diabetes can lead to hyperglycemia, which can result in serious heart, blood vessel, ocular, retinal, and nerve damage. Over 400 million people worldwide suffer with diabetes. (OMS ,2016).

Diabetes is a class of metabolic diseases with a variety of etiologies that is characterized by Persistent hyperglycemia and disruptions to carbohydrate, lipid, and protein metabolism. processes as a result of inadequate insulin secretion and/or action (DeFronzo et al., 2015).

1. 2. Classification of Diabetes :

Diabetes mellitus is classified into four groups: type I diabetes, type II diabetes, gestational diabetes, and other specific kinds known as secondary diabetes (Luka et al., 2013).

1. 2.1. Type 1 diabetes (formerly known as insulinodependent diabetes).

This accounts for about 10% of diabetes cases and is most common in children or young adults due to auto-immune loss of Langerhans pancreatic cells.

When only 10 to 20 percent of the cells are functioning, hyperglycemia occurs. She is linked to an insulin shortage (KRAZA, 2021).

. 1. 2. 2.Type 2 diabetes (formerly known as diabetes non-insulodependent diabetes, or DNID):

This kind of diabetes is the most common, accounting for around 90% of cases that are diagnosed. It occurs more frequently in adults. Type 2 diabetes is characterized by an increase in insulin sensitivity and abnormalities in the effects of insulin on its target tissues. It is the result of the combination of multiple susceptibility genes, the expression of which is influenced by environmental factors such as excessive consumption of saturated fats, fast-acting sugars, obesity, and sedentary lifestyle. Two major risk factors for developing

type 2 diabetes are family history and membership in a high-risk ethnic group (Haffner, 1998)

1. 2.3.The gestational diabetes:

The gestational diabetes is usually transient and goes away a few weeks after delivery. Women with gestational diabetes have an increased risk of eventually developing type II diabetes (Schaefer-Graf et al., 2002).

1. 2.4.Other types of diabetes: secondary (specific) diabetes:

Secondary diabetes can be caused by a variety of factors, according to the American Diabetes Association (ADA, 1997) and the World Health Organization (OMS, 1999). It can be caused by pancreatic disorders, endocrinological diseases, the use of certain drugs or hazardous substances, genetic anomalies affecting insulin function, and other hereditary diseases that are occasionally associated with diabetes (Chaouki, 2012).

1. 3. Symptoms:

Diabetes is a heterogeneous disease with different presentation and progression between the two types. However, some individuals may not be clearly classified as type 1 or type 2 at the time Of diagnosis (Amara and Benghanem, 2012).

The initial symptoms of diabetes Table 9, especially for type 2 diabetes, can be subtle or unnoticeable. Diabetes can be present for months or even years without symptoms (Amara and Benghanem, 2012).

Table 9: Symptoms of type 1 and type 2 diabetes (Karuranga et al, 2017).

✓ **Symptoms of Diabetes**	
Diabetes Type 1	**Diabetes Type 2**
✓ Excessive thirst and dry mouth	✓ Excessive thirst and dry mouth

✓ Abundant urination	✓ Abundant and frequent urination
✓ Lack of energy, fatigue	✓ Lack of energy, extreme fatigue
✓ Continued faith	✓ Tingling or numbness of the hands and feet
✓ Slow healing of wounds	✓ Recurrent cutaneous fungal infections
✓ Nocturnal enuresis	✓ Slow healing of wounds
✓ Vision impairment	✓ Vision impairment

1. 4. Complications from diabetes:

The two forms of the disease can lead to complications affecting multiple body parts and raise the overall risk of premature death. The potential risks include myocardial infarction, cerebrovascular accident, peripheral neuropathy, limb amputation, optic loss, and nerve lesions (World Health, 2016)

One can think of these complications as**:**

1. 4. 1. Acute complication :

They may arise (as a form of malaise that may extend to a coma) in the event of excessive glycemic modifications, either because the glycemia is excessively high (hyperglycemia)

or because it is excessively low as a result of treatment (hypoglycémie) (Amara and Benghanem, 2012)

1. 4. 2. chronic complications:

Diabetic complications are categorized into two groups: macrovascular complications (coronary artery disease, peripheral artery disease, and cerebrovascular accidents) and microvascular complications (diabetic encephalopathy, neuropathies, and retinal diseases). These complications result from alterations in the microvascular structure, leading to structural and functional changes in the extracellular matrix, which are the primary cause of significant complications in the veins and nerves. These changes involve the synthesis of extracellular matrix proteins and basal membrane erosion, which are pathognomonic characteristic features of diabetic microangiopathies (Kebir, 2018).

1. 5. Pathophysiology:

1.5.1. Type 1 Diabetes Pathophysiology:

The β cells are destroyed by autoimmune mechanisms that create this kind of diabetes. Antibodies directed against antigens expressed on the surface of β cells are produced by T lymphocytes. These cells are destroyed by the combination of the antibody-antigen response and the direct activity of T lymphocytes (Killers) (Perlemuter and Thomas, 2006).

1.5.2.Pathophysiology of type 2 diabetes:

He pathophysiology of type 2 diabetes involves the body's resistance to insulin in affected individuals. Although insulin is still produced, the body no longer reacts to it as well. The pancreas's capacity to generate insulin progressively declines with time. Because of this, whether the cause of the condition is insufficient insulin or the body's improper use of this hormone, managing type 2 diabetes necessitates a steady rise in the types of drugs used in treatment (Favier et al., 2005)

1.6. Factors influencing Type 1 Diabetes:

Environmental factors:

Research has shown that a child's lack of exposure to pathogenic organisms during infancy stunts immune system maturation and raises the risk of having an autoimmune disease (Kekreja and Maclaren, 2002)

Genetic Factors:

Type 1 diabetes is linked to more than 20 distinct sections of the human genome, including the HLA-encoding region on chromosome 6p21 and the insulin-encoding region on chromosome 11p15 (formerly called the DSID2 gene, or in English IDDM2). Depending on the populations under study, different HLA types are linked to diabetes (Arfa et al., 2008).

a. ***Immunological Factors:***

Type 1 diabetes is a slow autoimmune disease mediated by T lymphocytes. Family studies have demonstrated that the destruction of β cells by the immune system (autoantibodies directed against the pancreas) occurs over many years (Langlois, 2008). Hyperglycemia and classical signs of diabetes only appear when 80% of β cells have been destroyed (Dubois, 2010). Type 1 diabetes may be associated with other autoimmune conditions including thyroid diseases, celiac disease, and certain forms of anemia (Carneir and Dumont, 2009).

Viral Infection:

Type 1 diabetes has been linked to a number of viruses, including retroviruses, cytomegalovirus, Epstein-Barr virus, coxsackie viruses, and rubella viruses. By exposing autoantigens, triggering self-reactive lymphocytes, imitating autoantigen molecular sequences that trigger an immune response, or by using other mechanisms, viruses can either directly infect and destroy beta cells or indirectly cause the destruction of beta cells (Erika F. Brutsaert, 2020).

1.7.Factors influencing T2D:

Factors genetic:

Studies involving families have demonstrated the clear role that genetic factors play in the decline of type 2 diabetes: for monozygotes, concordance ranges from 60% to 100%. It is approximately 40% for those who present at the first stage of type 2 diabetes (Simonis-Bik et al., 2010)

a. ***Environmental factor***:

Age, sedentariness, smoking, obesity, especially androgenic obesity, and sedentariness all significantly increase the risk of developing type 2 diabetes (Raverot, 2005).

Issues with insulin sequestration:

the sequestration of insulin is characterized by a biphasic clock Figure 10 .

The β cells of the pancreatic islets of Langerhans ensure the secretion of insulin., She is stimulated by a gradual and acute increase in the extracellular glucose concentration. After a few minutes, the maximum level of sequestration is reached (early peak)..(A second picture appears when the high concentration is maintained (Late submission). This stage lasts while the stimulus is still applied (Magnan et Ktorza, 2005) .

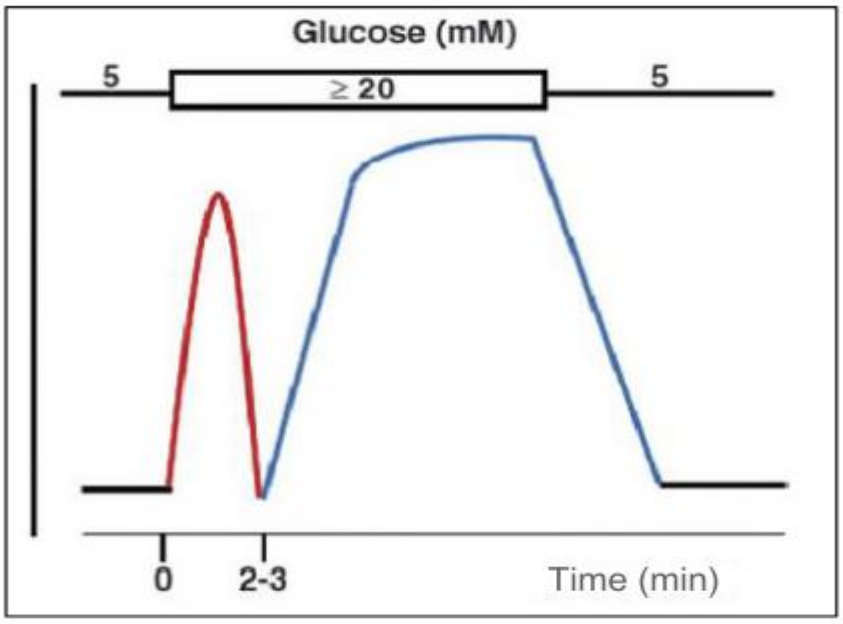

Figure10. Biphasic secretion of insulin in response to a constant glucose stimulus (Magnan and Ktorza, 2005)

Insulin Sensitivity Disorders:

Fasting hyperglycemia and some postprandial hyperglycemias are explained by the liver producing too much glucose due to a reduction in the effects of insulin on insulin-sensitive tissues (muscle, adipose, and liver) (Henri, 2011). Insulin resistance can therefore be identified at the peripheral tissue level, namely in the context of hepatic glucose synthesis and muscle and adipose tissue glucose transport. Hyperglycemia and the excess circulating

free fatty acids or triglycerides accumulated in the muscle worsen this insulin resistance. High amounts of circulating fatty acids also contribute to increased hepatic glucose synthesis (Halimi, 2006).

1.8.treatments:

1.8.1.Medical treatments:

Hypoglycemia medications used orally:

Less than half of the time, oral diabetes medications cause the blood glucose level to return to normal. They have no regressive effect on the installed lesions and are contrary indicated in terms of hepatic and renal insufficiency. Their secondary effects are not to be disregarded. Clinicians managing type 2 diabetes that cannot be well controlled by one regimen have access to five classes of oral anti-diabetics that have hypoglycemic effects through several mechanisms of action.

- **Hypoglycemic sulfonamides (Glibenclamide):** stimulate insulin secretion without influencing its synthesis
  Biguanides (metformin): reduce insulin resistance by promoting insulin action on target tissues, inhibiting hepatic neoglucogenesis, and decreasing intestinal glucose absorption without any effect on insulin secretion.
- **Intestinal α-glucosidase inhibitors represented by acarbose and miglitol:** slow down the digestive absorption of complex carbohydrates.
- **Glitazones**: these are molecules that increase insulin sensitivity and β-cell function.
- **Glinides:** these are insulin secretagogues..

a. *InsulinAdministration.*:

In cases where oral treatment for type 2 diabetes fails in a diabetic patient, it appears that insulin therapy should be initiated as soon as possible to preserve any remaining insulin-sensitive capacity. In such cases, insulin therapy allows for a significant improvement in glycemic control. In instances where oral antidiabetic agents are contraindicated (renal insufficiency, liver...), definitive insulin therapy is clearly necessary.Insulin preparations are classified based on their duration of action, onset time, and peak activity time. Individualization of insulin therapy

should be based on therapeutic goals, lifestyle, diet, age, general health, motivation, ability to recognize hypoglycemia, and self-management skills. Despite all medications, treatments, and hygienic and dietary measures available today, controlling blood glucose levels in a diabetic patient remains challenging. (KRAZA, 2021)

1.8.2.Phytotherapy:

Plants have been used therapeutically in most fields of pathology; this is what we call phytotherapy. The reasons for using plant products generally include the absence of side effects after consuming plants, the absence of chemicals, and the cost-effectiveness of therapy.

Diabetes has been treated by traditional Chinese medicine, by folk medicine, and, in a more formalized manner, by phytotherapy. Numerous experimental studies, conducted in vitro and in vivo, have clearly shown that plants contain active hypoglycemic principles (polyphenols, alkaloids, saponins, coumarins, and terpenoids). There is no evidence-based argument to recommend the use of phytotherapy alone or in combination with conventional treatment to treat hyperglycemia and its complications (Schlienger, 2014). Among the hypoglycemic plants used in Algeria: El bane (Moringa oleifera), Mor et sbor (Aloe vera L), Chih (Artemisia herba-alba Asso), Louben (Boswellia sacra Flueck), Tai lakhdar (Camellia sinensis), Elhalba (Trigonella foenum-graecum L) (Hamza et al., 2009).

1.8.3.Mechanisms of action of medicinal plants:

Pharmacological studies have made it possible to clarify a number of the mechanisms by which medicinal plants with anti-diabetic effects work. Due to the wide range of chemical classes and hypoglycemic constituents included in medicinal plants and their extracts used in diabetes treatment, several mechanisms may be affected (Jarald et al., 2008; Kashikar et Kotkar, 2011; Singh et al., 2012). A few of these mechanisms include: - stimulating insulin synthesis from β cells and/or inducing their regrowth or by reducing the effect of insulin.

- stimulation of β cells through the addition of elements (Cu++, Mg++, Ca++) required for their function, as well as the revitalization and/or hyperplasie of these cells.

-- reduction of glucagon secretion, which results in a decrease in intestinal glucose absorption and/or an increase in peripheral glucose utilization.

-- control of hepatic enzyme activity in terms of stimulating glycogenogenèse and/or inhibiting glycogenolyse.

- They work by inhibiting digestive enzymes including α-amylase and α-glucosidase, which reduced the breakdown of amyloid and oligosaccharides. As a result, they reduce the absorption of glucose at the intestinal level.

- alteration of the renal glucose absorption mechanisms at the proximal contour tube level, as demonstrated for phloridzine (Ehrenkranz et al., 2005).

- by stimulating glucose uptake by adipocytes or muscle cells, or by inhibiting glucose transporters at the intestinal barrier level, hence limiting intestinal glucose absorption.

2. Blood glucose regulation:

2.1.Hormonal regulation of blood glucose:

Coordination of nervous system activities that either stimulate or inhibit hormone and endogenous secretion is how glucose homeostasis is regulated. However, it is generally accepted that the endocrinological system controls blood glucose regulation (Annie Lé Gar.,.2001.) As a result, insulin and counter-regulatory hormones control blood glucose levels. Although growth hormone, cortisol, insulin, and glucagon all have biological activities that can counteract the effects of insulin, glucagon still appears to be the primary hormone in charge of the body's defense against hypoglycemia (Cryer 1997, 1999; Butler et al., 1988; Tuttle et al., 1988). In the absence of glucagon, adrenaline becomes significant. Instead, cortisol and growth hormone have a role in the recovery process after an aiguë hypoglycemia. Thus, the two main hormones regulating blood glucose are insulin and glucagon. Furthermore, according to Lins et al. in 1983, the insulin/glucagon ratio is important because even with a slight increase in glucagonemia, a low drop in insulin concentration makes the liver more susceptible to the effects of glucagon, which increase glucose production and result in hyperglycemia (Annie Lé Gar., 2001)

Insulin role:

It is believed that insulin is the primary pancreatic hormone that allows for the precise regulation of blood sugar levels. She encourages the storage of fat in the adipous tissue and promotes the storage of glucose in the form of glycogen in the muscles and liver. The only hypoglycemic hormone is insulin, which is secreted by the β cells of the Langerhans cells in pancreas.

Glucose is a stimulator of pancreatic insulin secretion :

On the surface of β pancreatic cells, GLUT2, a non-insulinodependant glucose transporter, is expressed. This transporter facilitates the flow of glucose from the blood compartment into the interior of the β-cell. It is the direct detection of glucose by pancreas that will cause a rise in calcium sequestration that is dependent on insulin. The first step is to increase the ATP/ADP ratio by metabolizing the entering glucose. A high intracellular ATP rate will cause the K+ channel to close in response to ATP, causing ATP to be fixed on the protein Kir 6.2 (Cook D.L. et al, 1984). The outcome is a membrane depolarization of the β cell, which is followed by voltage-dependent Ca2+ channel opening (Satin L.S. et Smolen P.D., 1994). The intracellular calcium increase will cause the insulin-containing vesicles to be exocytosed by the use of the SNARE complex (Henquin J-C, 2000) Figure 11.

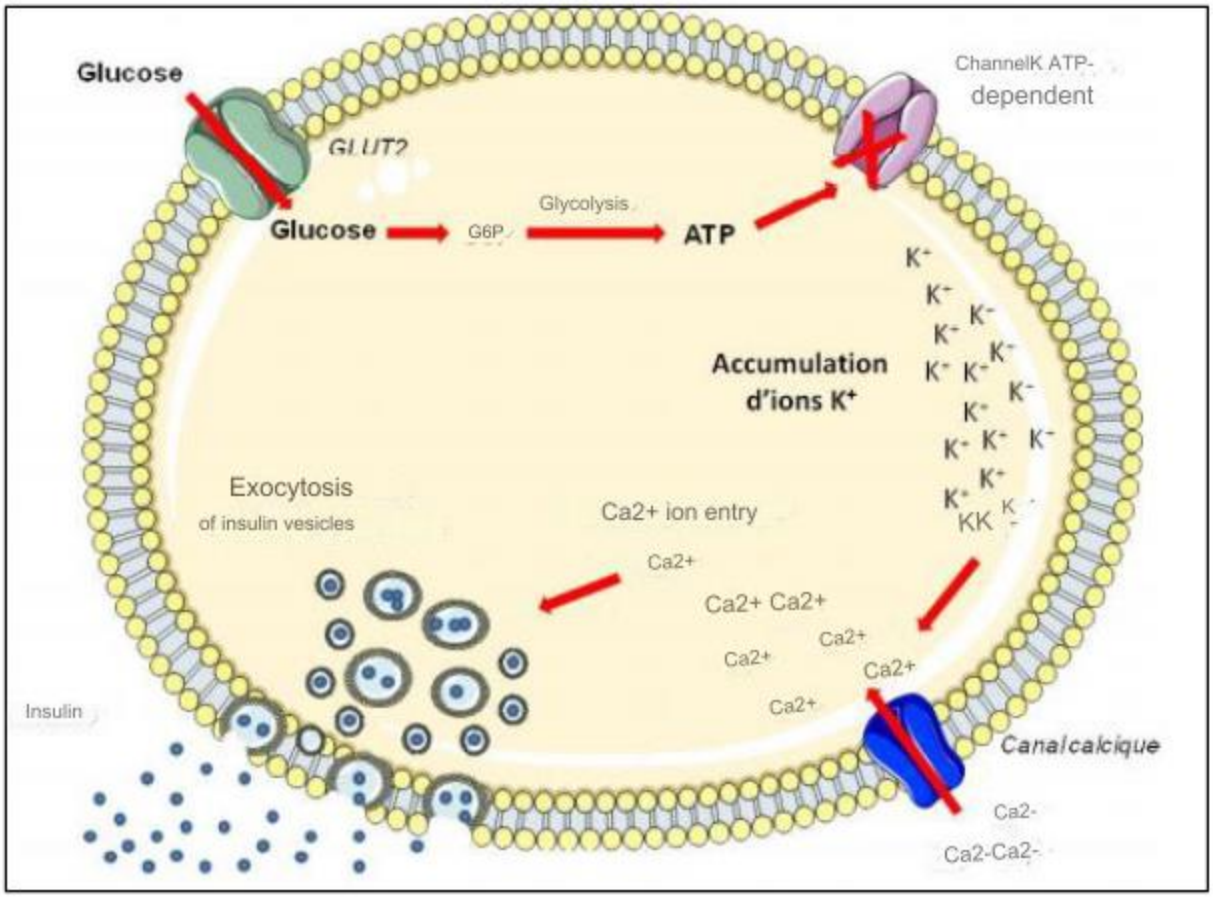

Figure11. Secretion of insulin by the pancreatic β cells (Duparc, 2012).

Signalization of insulin voies:

After being broken down into its constituent parts in the systemic circulation, insulin has anabolic effects due to its interaction with a particular receptor found on the cell membrane surface of three target tissues: muscle, fat, and adipous tissue. This receptor has tyrosine kinase activity that enables it to phosphorylate on tyrosine residues, substratum proteins (IRS: Insulin Receptor Substrates), and adaptor proteins of the SHC family (src homologous and collagen protein) (Van Obberghen E. et al, 2001). The two main pathways of signaling are the phosphatidylinositol-3 kinase (PI3K) pathway, which is involved in metabolic effects, and the MAP kinase pathway, which is involved in cell growth, proliferation, and differentiation processes. The IRS1 and IRS2 are activated by the transmission of the insulinemia signal within the cell (White M.F., 2002). Insuline can cause the transporter GLUT4 to relocate to the membrane surface of muscle and adipocyte cells through the mediation of this signaling (Saltiel A.R. et Kahn C.R., 2001 ; Bryant N.J.

et al., 2002). In the liver, the insulinemia pathway directly activates a group of proteins and transcription factors involved in the glucose metabolism pathways.

Role of Glucagon :

Voies de signalisation de Glucagon :

The 63 kDa membrane glycoprotein (Yengar R et al., 1984) constitutes the glucagon receptor and belongs to the family of G protein-coupled receptors with 7 transmembrane domains. It is present in various tissues, such as the liver, heart, intestines, brain, adipose tissue, and pancreas (Burcelin R et al., 1995).

Le recrutement et l'activation de la petite protéine G associée au récepteur se fera suite à la fixation du glucagon sur son récepteur. G\s and Gq are the two known forms of G proteins that are linked to this receptor (Wakelam MJ., 1986).

It is possible to couple the glucagon receptor to either a Gs or a Gq protein. In the first instance, glucagon fixation will trigger adenylate cyclase activity. The result will be the activation of PKA, which can initiate glycogenolysis in part and activate the transcription factor PGC-1α in another. The fixation of glucagon, in the event of a coupling with a Gq protein, will increase intracellular calcium release by activating the PLC. This rise in calcium levels will contribute, in the same proportion as PGC-1α, to the upregulation of gene expression linked to the regulation of several metabolic pathways (Duparc., 2012).

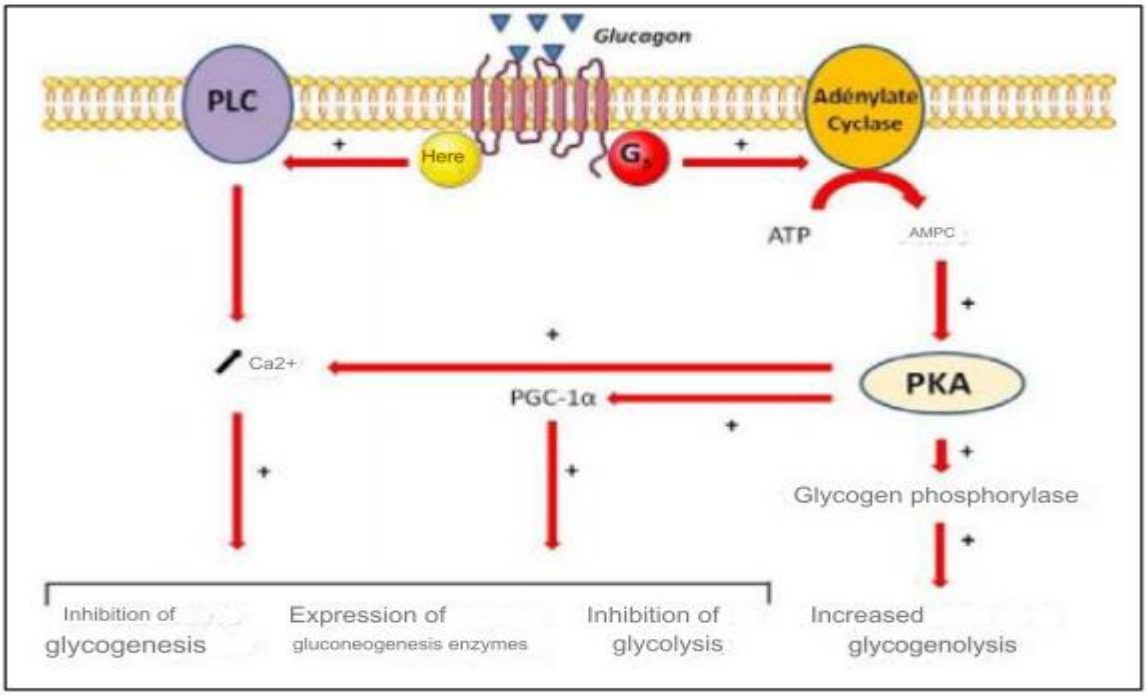

Figure12. Glucagon signaling pathway (Duparc, 2012).

2.2.The enzymatic regulation of glucose metabolism:

2.2.1.L'amylase alpha:

Definition:

Like all enzymes, α-amylase is a macromolecule that belongs to the class of globular proteins (Nouadri, 2011). It is a ubiquitous enzyme that is synthesized in all living things (Merabti, 2006). This endo-enzyme, whose molecular mass ranges from 40 to 70 kDa (Panchal, 1990), hydrolyzes randomly the osidic (1,4) bonds between glycogen, amylose, amylopectine, and amyloid, while excluding the terminal bonds between these chains. She releases glucose, maltose, and most

a. Nomenclature:

- ♣ Coded name: EC 3.2.1.1
- ♣ Common name: alpha-amylase
- ♣ Other name(s): glycogenase, α-amylase; endoamylase; Taka-amylase A, maxilase.

♣ Systematic name: 1,4 – alpha-D-glucane-4-glucano hydrolase (Schamburg and Slzmann, 1991; Dauter et al., 1999; Nouadri., 2011).

b. Types:

Several types of amylase exist, alpha (3.2.1.1), beta (3.2.1.2), and gamma (3.2.1.3) (Yamamoto, 1995). Beta-amylase and gamma-amylase (glucoamylase) are generally of plant or microbial origin, although glucoamylase has been found in certain mammals or aquatic species (Shetty., 2006).importantly, α-dextrines (Kelly et al., 1997).

c. Synthesis Location:

In humans, amylase is mostly found in salivary (amylase S) and pancreatic (amylase P) secrétions. The genes Amy 1 and Amy 2A, which are located on chromosome 1 (Nishide et al., 1986; Horii et al., 1987; Tricoli et Shows., 1984), respectively, code for the human amylases S and P.

The two families that make up the majority of human amylase S are as follows: the proteins of family A are glycosylated and have a molecular weight of 62 kDa, while the proteins of family B are non-glycosylated and have a molecular weight of 55 kDa (Karn et Malacinski, 1978).

d. Structure:

Human pancreatic alpha amylase (HPA) is a monomeric protein that links calcium and chloride (Brayer et coll., 1995). Since the sequence and structure of salivary alpha amylase and pancreatic alpha amylase are similar, they are not discussed separately.Three domains make up the 496 amino acid protein HPA Figure 12.

Domain A (orange and red) is the largest of the three domains and has a central β/a-barrel parallel to eight strands (orange and red; residues 1-99 and 169-404), the active site (Asp197, Glu233, and Asp300), and a chloride-binding site (residues Arg195, Asn298, and Arg337).

Domain B (sky blue; residues 100-168) is the smallest of the three domains and is located on an extended loop between the third β-strand and α-helix of the β-barrel core of domain

A. It consists of a calcium-binding site (residues Asn100, Arg158, and Asp167; His201 from domain A)

Domain C (pink; residues 405-496) occurs at the C-terminus, and its function is not fully understood. It consists of an anti-parallel β structure and is connected to domain A (PDB ID 5td4; Zhang et al., 2016).

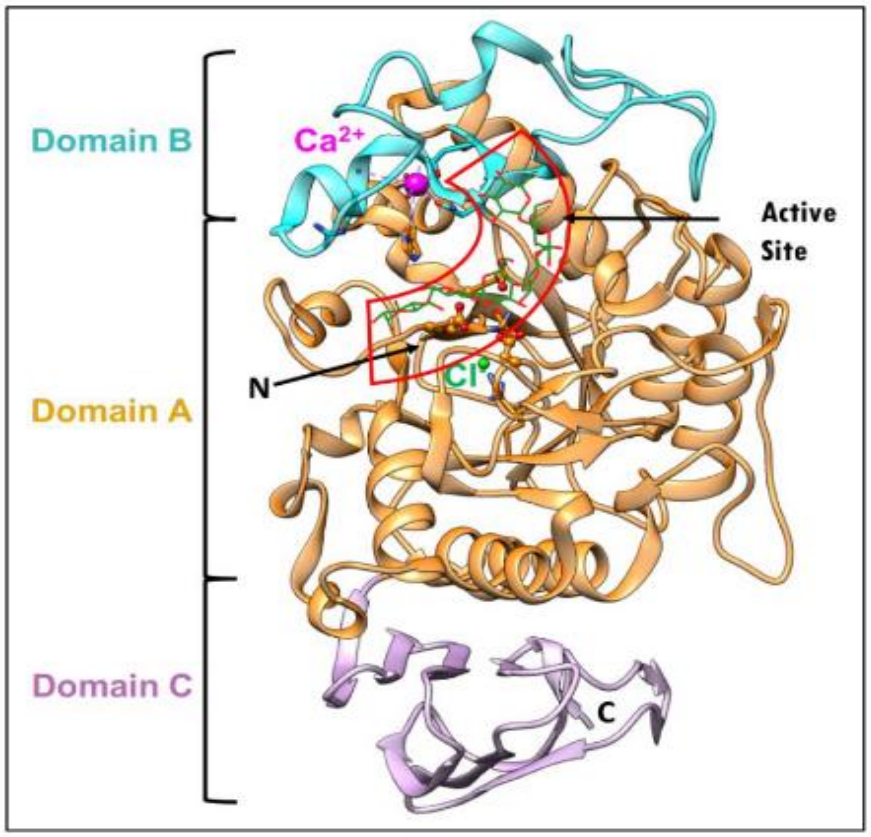

Figure 13. Ribbon diagram of human pancreatic alpha-amylase (HPA) and its subdomains in complex with an octaose substrate (PDB ID 5td4; Zhang et al., 2016).

e. Physical and chemical characteristics of alpha-amylase:

Molecular weights:

The molecular weight of α-amylases varies depending on the species and origin. Between 40.000 and 90.000 daltons are included (Schombury et Salzmann, 1991). The α-amylases found in legumes range in size from 40.000 to 70.000 daltons (Panchal, 1990).

Optimal Temperature:

The ideal temperature range for alpha amylases varies depending on species and origin, and it is between 40°C and 90°C (Schombury et Salzmann, 1991). In fact, bacterial alpha amylase is known for its high thermostability; Bacillus amyloliquefaciens has an optimal temperature range of 70°C to 90°C depending on the application, while fungal alpha amylase has a temperature range of 50°C to 70°C (Sicard, 1982; Panchal, 1990).

Optimal pH:

The alpha-amylase is highly sensitive to pH. Therefore, choosing the ideal pH is crucial for the synthesis of this enzyme (McMahon et al., 1999). As per Kindle (1983), the optimal level of activity can be achieved at pH values ranging from 4 to 8. The optimal pH for bacterial α-amylases is higher than neutrality, whereas the optimal pH range for fungal enzymes is between 4 and 6 (Larpent et al., 1992). According to species, the enzyme needs a pH between 4 and 6 for levures (Panchal, 1990; Avwioroko, 2015).

- ✓ **Effectors :**

  Compounds known as Effectors, also known as activators or Inhibitors, can regulate enzyme activity by acting either directly or indirectly on the enzyme's active site (Garrett et Grisham, 2000).

Generally speaking, inhibitors are nearby structural molecules that either do not react at all or react much more slowly than the substrate. The study of inhibitor effect is utilized to ascertain the catalytic mechanism of an enzyme reaction, to better understand the specificity of an enzyme, and to obtain physical and chemical data on the enzyme's active site (Garrett et Grisham, 2000).

Accordingly, the ions Cu2+, Fe2+, and Hg2+ are competitive inhibitors and structural analogs of activators (Mercier, 1985).

Because they are part of the active site and contribute to the structural stability of the enzyme, the ions Ca2+ and Mg2+ are activators of alpha amylase (Mercier, 1985). However, calcium becomes inhibitory at concentrations over 20 mM (Boel et al., 1990).

Specificity of substrate:

Amidon is the natural substrate of alpha-amylase (Pandey et al., 2000; Alais et al., 2008). The α (1–4) osidic bonds linked to glucose units in the starch molecule form helical chains

of amylose, on which α (1-6) osidic bonds branch short chains of the same composition (Mercier, 1985). The production of α-amylase by several fungal soils (Aspergillus sp., Rhizopus sp., and Penicilium sp.) is controlled by amylase; it involves the induction of the enzyme by its natural substrate (Leveau et Bouix., 1993). Salivary α-amylase continues to digest starch in the small intestine.

f. The function of alpha-amylase:

The primary human source of glucose is the breakdown of amyloid, which starts in the mouth. The salt contains α amylase, which hydrolyzes all α (1–4) glycosidic bonds with the exception of the most extreme and those closest to branching points. When fully cooked food reaches the stomach or the acidity neutralizes α-amylase, the average length of amyloid chains decreases from several million to less than eight hundred units of glucose. Entraînée par β-amylase, qui fonctionne à partir d'extrémité non réductrice, et par α-amylase pancréatique, qui ressemble a l'α-amylase salivaire, l'amidon continue à être digesté dans l'intestin grêle.This enzyme breaks down amidine into a mixture of maltose (a disaccharide), maltotriose (a trisaccharide composed of three glucose residues joined by α (1-4) linkages, and dextrines (an oligosaccharide with α (1-6) branching.Enzymes spécifiques present dans les membranes de la bordure en saïde de la muqueuse intestinale hydrolyse ces oligosaccharides into monosaccharides. Ainsi, une α (1-6) glucosidase détache un par un les résidus glucoses des oligosaccharides Figure 14 (Voet.D et Voet.J., 2005)

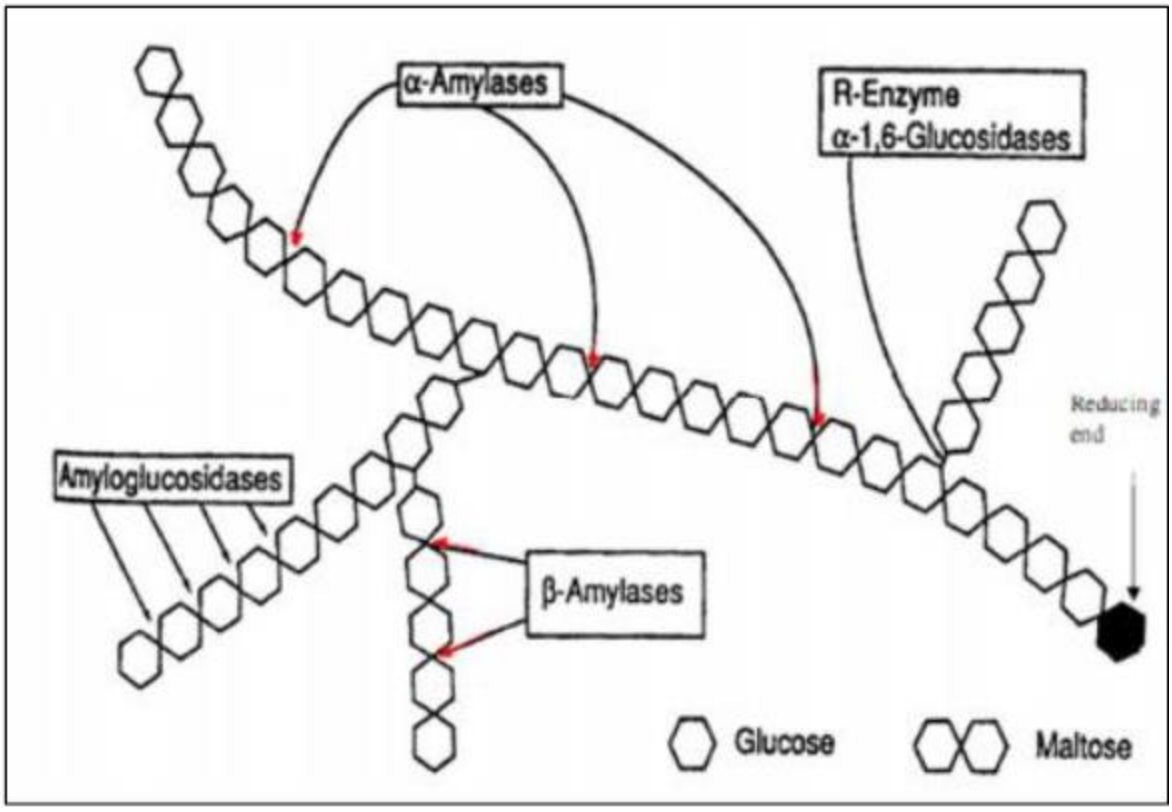

Figure14. Enzymatic Digestion of Starch (Tormo et al., 2004).

Mechanism of action:

Three amino acids from the active site are involved in the catalytic activity of the enzyme: Asp 231 (catalyst), Glu 261 (hydrogen-catalyzing doubler), and Asp 328 (catalyse assistance) (Kolli et al., 2015).

The activity of α-amylase is primarily dependent on temperature and pH. The pH affects the electrical charge of aminated acids present on the catalytic site, which prevents atom-transfer reactions that are required to break ossidic bonds. The synthesis of the enzyme/amidon complex is aided by temperature (Faiveley, 2010) .

The mechanism of action of α-amylases is poorly understood and relies on probability (Sales et al., 2012). It has been described in terms of four steps:

Phase 1: Enzyme/substrat complex formation The enzyme is fixed by affinity either within the chain or on the extrémités réductrices. The amyloid chains are deformed by the complex enzyme/substrat and stabilized by a large number of hydrogen bond connections between

the amino acid residues of the enzyme's fixation site and the polaire (OH) groups of the carbonated chain (Faiveley, 2010).

Phase 2, The attack on the α (1-4) liaison: At the level of the osidique liaison on the catalytic site, the attack is initiated by the glutamic acid, which, in its protonated form (at the optimal pH of the enzyme), provides an atome of hydrogen to the osidique liaison's oxygen atom to break its bond with the carbon-4 carbon of the chain, causing the osidique liaison to rupture.

On the other hand, aspartic acid is ionized at the enzyme's ideal pH, forms an ionic form, and establishes a covalent bond with anomeric carbon, or C1, allowing the first segment of the carbon chain to be released (Khacheba., 2008; Sales et al., 2012).

Phase 3, which is the enzyme's return to its original state, is ensured by an aqueous molecule that hydroxylates the anomeric carbon in order to form a covalent bond with aspartic acid. After the reaction between C1 and the aspartic acid breaks down, the glutamic acid, charged negatively, absorbs a proton from the water molecule. The remaining, unstable group of OH then settles on the anomeric carbon, or C1. This reaction allows the second part of the carbon chain, or etape 4, to be expelled (Khecheba, 2008 ; Devin, 2010; Sales et al., 2012). Since glutamic acid appears to play a significant role in catalysis, this residue could act as a proto donor and function as a general acid catalyser or electrophile. On the other hand, aspartic acid would function as a general bas catalyser or nucléophile on the transfer reactions that allow the chain to break, which is a nucléophile reaction (Muralikrishna, 2005 ; Faiveley, 2010 ; Sales et al., 2012).

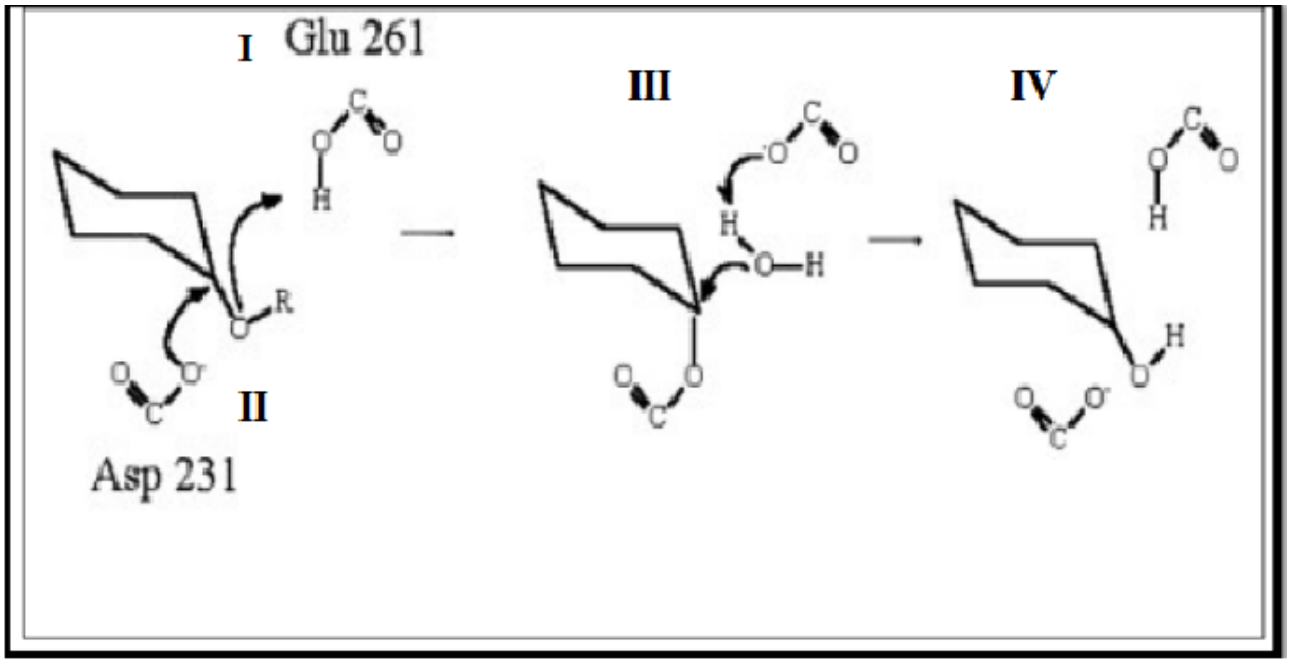

Figure 15. Catalytic mechanism of glycosyl hydrolases (Nielsen et al., 2001)

2.2.2.Alpha-glucosidase:

According to IUPAC-IUBMB nomenclature, α-glucosidases (EC 3.2.1.20, α-D-glucoside glucosehydrolase) are classified as exo-glycosidases that primarily catalyze the hydrolysis of α-1,4-glucosidic bond formation of a substrate (such as glycogen or malto-oligosaccharides, for example) to release α-D-glucoside. Additionally, they have the ability to catalyze trans-glycosylation reactions to create α-D-glucosylated compounds (Kato et al., 2002; Ferrer et al., 2005; Seo et al., 2011).

3.Acarbose:

• ***Mechanism of Action:***

Acarbose is a pseudotetrasaccharide of microbial origin. At the brush border of the intestine, acarbose acts by competitively inhibiting alpha-glucosidases. It thereby reduces the degradation of carbohydrates (di-oligo- and polysaccharides) into absorbable monosaccharides. Acarbose thus reduces postprandial hyperglycemia without causing hyperinsulinemia or weight modification. (BERRIHA, A.,. 2019).

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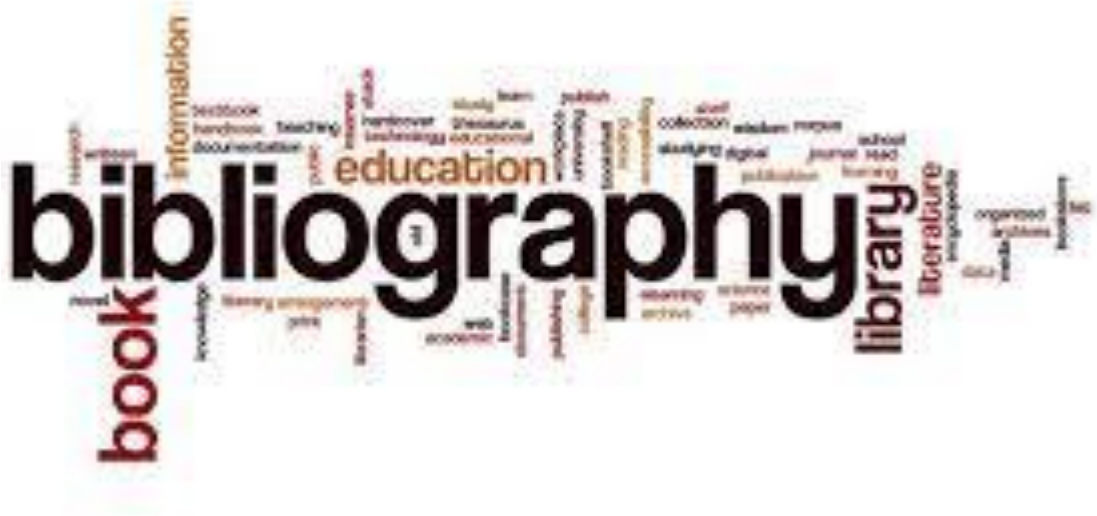
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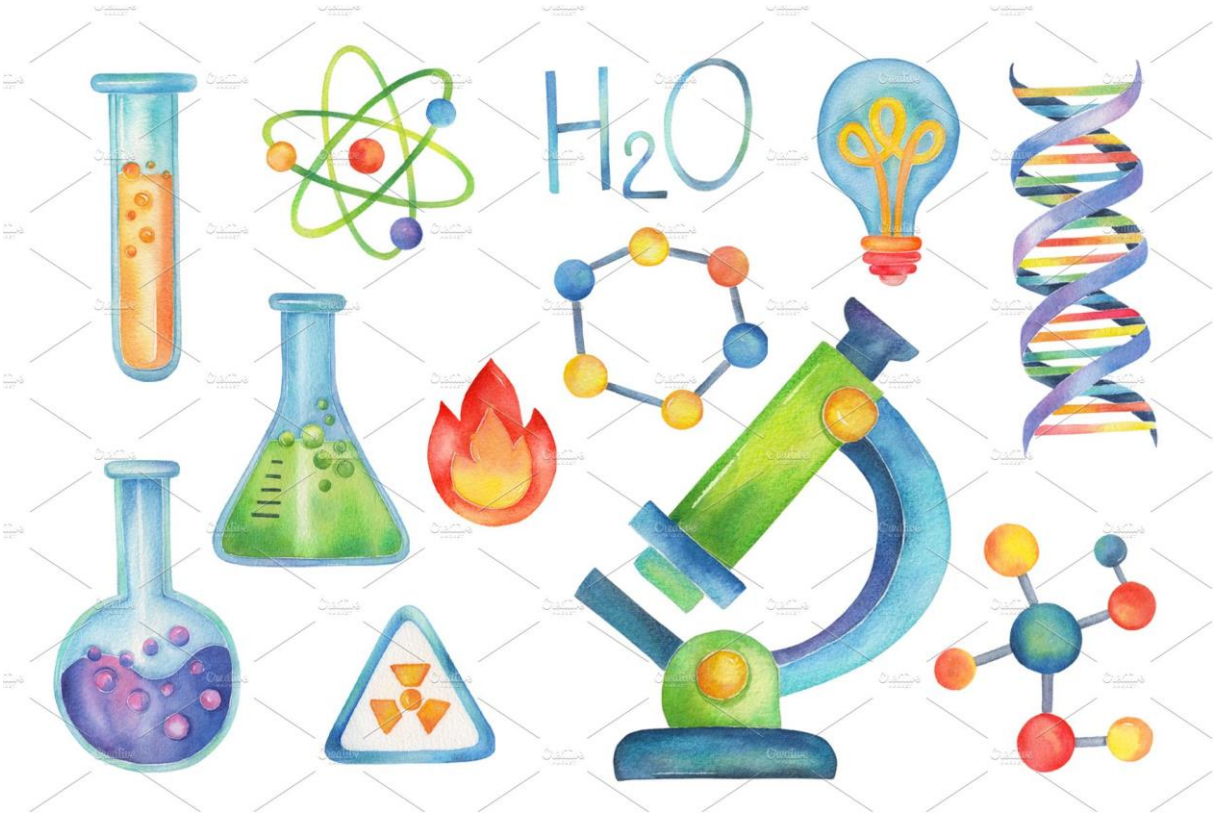
SECOND PART:

EXPERIMENTAL

CHAPTER ONE: MATERIALS AND METHODS



1. Introduction:

This study was conducted collaboratively between two specialized laboratories: the Valorisation and Technology of the Saharian Resources Laboratory (VTRS) at the Faculty of Exact Sciences, Department of Chemistry, University of El Oued, and the Centre de Recherche Scientifique et Technique en Analyses Physico-Chimique (CRAPC) in Ouargla, Algeria. The VTRS lab facilitated essential oil extraction, conducted *in vivo* and *in vitro* assays, and also carried out *in-silico* assays. Meanwhile, CRAPC handled the precise GC/MS analysis of the extracted oils. This collaborative effort ensured thorough and accurate experimentation, combining expertise and resources from both institutions.

2. Plant Material:

2.1. Cotula cinerea

The *Cotula cinerea* plant was harvested at various time intervals ranging from December 2023 to January 2024 in a desert forest area in southeastern Algeria, specifically in Hassi Khalifa region in El Oued province. This region is characterized by the following specifications:

- Geographic Coordinates: Longitude 48°10' East, Latitude 23°09' North.
- Elevation above Sea Level: 44 meters.
- Distance from Sea Level: 290 kilometres.
- Bioclimatic Characterization: Desert.

2.2. Origanum Majorana L:

The *Origanum Majorana L* plant was harvested at different time intervals extending from March 2023 to April 2023 in a desert forest area in southeastern Algeria, specifically in Akfadou region in El Oued province. This region is characterized by the following specifications:

- Geographic Coordinates: Longitude 67°6' East, Latitude 33° North.
- Elevation above Sea Level: 58 meters.
- Distance from Sea Level: 300 kilometres.
- Bioclimatic Characterization: Desert.

3. Chemicals and reagents:

Alpha Amylase:

A solution of alpha-amylase (commercially marketed as Megamylase with a specific activity of 3000 (U. CEIP/cp) at a concentration of 0.5 mg/ml) was prepared in a phosphate saline buffer (pH = 6.4).

Acarbose:

Different concentrations of acarbose solution (commercially marketed as Acorbay 50 mg) were prepared in a phosphate saline buffer (pH = 6.4).

Substrate:

As a substrate, soluble starch was utilized. 250 mg of starch was dissolved in 25 ml of distilled water at a temperature of 50 to 70°C under agitation.

Lugol:

The Lugol solution (KI/I_2; Sigma-Aldrich Chemical) was obtained commercially from Sigma-Aldrich and utilized as a staining reagent in the study. Stored at room temperature in a dark container.

Phosphate Buffer Solution:

The phosphate buffer saline solution was meticulously prepared using sodium dihydrogen phosphate and disodium hydrogen phosphate (Sigma Aldrich) in conjunction with double-distilled water and KCl. The pH was meticulously maintained at 6.4 through the utilization of this phosphate buffer.

4. Materials and Methods:

4.1. Essential Oils Extraction:

4.1.1. Apparatus:

The essential oil extraction process required the use of specialized equipment to ensure accuracy and efficiency. In this study, the following apparatus was utilized:

- ✓ Adventurer – Pro AV53 sensitive balance
- ✓ Heating flask
- ✓ Refrigerant
- ✓ Clevenger apparatus

- ✓ Separating funnel
- ✓ Rotary evaporator

The Clevenger apparatus, a key component in the extraction process, is depicted in Figure 16

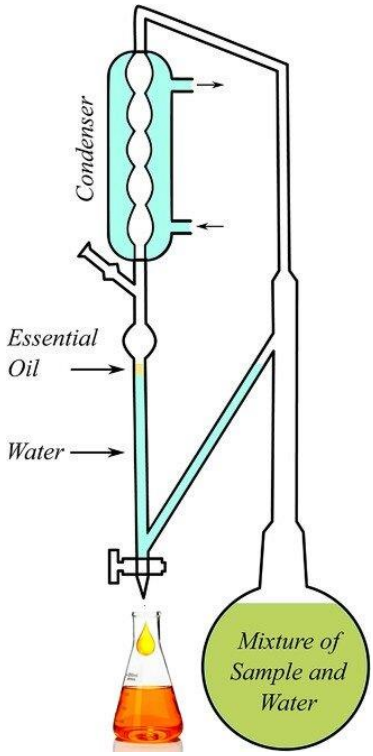


Figure 16. Schematic representation of the Clevenger apparatus used in the essential oil extraction process (Biswa et al., 2023)

4.1.2. Procedure:

The procedure for essential oil extraction involved a series of meticulous steps to ensure optimal results. The extraction process was conducted as follows:

- ✓ Cleaning of the plant sample to remove any impurities.
- ✓ Weighing the plant sample to a quantity of 100 g using a sensitive balance.
- ✓ Washing the plant sample with water to prevent burning, followed by placement in a heating flask.
- ✓ Addition of small pieces of boiling regulator to the flask.
- ✓ Direct heating of the mixture at a temperature of 100 degrees Celsius for three and a half hours.
- ✓ Conducting the steam distillation process and subsequent separation of oil and water phases using liquid-liquid separation with diethyl ether.
- ✓ Drying of the organic oily phase with anhydrous sodium sulphate.
- ✓ Filtration of the dried oil phase through filter paper to remove diethyl ether particles.

- ✓ Evaporation of the filtered oil phase using a rotary evaporator to remove any remaining organic solvent.
- ✓ Storage of the obtained essential oil in small brown bottles and refrigeration at a temperature of 5°C.

4.2. Yield of Essential Oil Extraction:

Two distinct methodologies are commonly employed to ascertain the yield of essential oil extraction, each offering unique perspectives on the efficiency of the process.

4.2.1. Volumetric Yield - Mass-based: (Naima et al., 2019)

This method entails an assessment of the mass of the utilized plant material intended for essential oil extraction, juxtaposed against the volume of the resultant oil. The yield is then computed utilizing the following Equation 1:

$$R_{EO} = \frac{V_{EO}(ml)}{m_0(g)} \times 100 \quad (1)$$

Where: R_{EO}: Essential oil yield, m_0: Mass of the utilized plant sample, V_{EO}: Volume of the extracted essential oil

4.2.2. Mass-based Yield - Mass-based: (Larbi et al., 2018)

Alternatively, the essential oil extraction yield is defined as the quotient of the mass of the extracted essential oil and the mass of the plant material utilized. This yield is calculated using Equation 2:

$$R_{EO} = \frac{m_{EO}(g)}{m_0(g)} \times 100 \quad (2)$$

Where: R_{EO}: Essential oil yield, m_0: Mass of the utilized plant sample, m_{EO}: Mass of the extracted essential oil

These methodologies have been systematically applied to derive essential oil yields for all examined plant specimens.

4.3. Characterization of Essential Oils:

The characterization of essential oils comprises two fundamental components: physicochemical properties and Gas Chromatography-Mass Spectrometry (GC/MS) analysis.

4.3.1. Physicochemical Properties:

Here, we delve into the fundamental traits of essential oils, including their relative density, acidity, ester content, and refractive index. These properties offer valuable insights into the composition and behavior of essential oils, helping us understand their chemical makeup and how they interact with their environment (Atti-Santos et al., 2005).

➢ *Relative Density (AFNOR NF T75-111 Standard):*

At 20°C, 1 mL of the essential oil is measured using a pipette, and its mass is then determined. The procedure is repeated for distilled water, and density is calculated using the following Equation 3 (Valarezo et al., 2015):

$$d = \frac{m_{EO}}{m_{H_2O}} \quad (3)$$

Where: m_{EO}: Mass of the extracted essential oil, m_{H_2O}: Mass of the distilled water

➢ *Acidity Index (AFNOR NF T75-111 Standard):*

To determine the acid value, representing the concentration of free acids in 1 g of the essential oil, a titration method with potassium hydroxide (KOH) solution is employed (Sahoo et al., 2007).

Initially, a small aliquot (0.5 mL) of the essential oil is mixed with 2 to 3 drops of phenolphthalein indicator in a small vessel. Subsequently, titration is performed with 0.5 N KOH solution until the appearance of a faint pink colour, indicating complete neutralization of the acids. The acid value is then calculated using the following Equation 4.

$$I_a = \frac{56.11 \times V \times C}{m} \quad (4)$$

Where: V: the volume of the KOH solution, C: Concentration of KOH, m: the mass of the essential oil

➢ *Ester Index (AFNOR NF T75-111 Standard):*

The determination of free acids resulting from ester hydrolysis within the essential oil involves a titration process utilizing 0.5 N potassium hydroxide (KOH) solution (Alajtal et al., 2018).

- Begin by placing 0.5 mL of the essential oil into a small vessel.
- Add 1 mL of 0.5 N potassium hydroxide (KOH) solution to the vessel to initiate the titration process.

- Place the mixture in a gas-evacuated water bath for a specified duration to facilitate reaction.
- After cooling, introduce 0.5 mL of distilled water and add 3 drops of phenolphthalein indicator to the mixture.
- Titrate the excess potassium hydroxide (KOH) using 0.5 N hydrochloric acid (HCl) until a colour change is observed, indicating neutralization.
- Quantify the volume of hydrochloric acid (HCl) required to neutralize the excess potassium hydroxide (KOH).
- Calculate the ester content using the following Equation 5:

$$I_e = \frac{2805}{m}(V_0 - V_1) - I_a \quad (5)$$

Where: V_0 (ml): Volume of hydrochloric acid (HCl) without essential oil, V_1 (ml): Volume of hydrochloric acid (HCl) in the presence of essential oil, I_a: Acid value, I_e: Ester value and m: Mass of the essential oil sample.

➢ *Refractive Index (AFNOR NF T75-111 Standard):*

The refractive index of an essential oil is defined as the ratio between the sine of the angle of incidence and the sine of the angle of refraction of a light ray, with a specific wavelength, transitioning from air into the essential oil, while the latter is maintained at a constant temperature (Singh, 2002)

The refractive index of the essential oil is directly measured using a refractometer at a reference temperature 20°C.

4.3.2. Gas Chromatography-Mass Spectrometry (GC/MS) analysis:

The coupling (GC/MS) technique stands as the most frequently employed method within the field of essential oils, facilitating the concurrent separation, identification, and quantitative measurement of the various constituents present in extracted oils.

➢ *Principle:*

The principle is founded on the varying affinities of compounds within the mixture towards two phases: a stationary phase and a mobile phase. This technique relies on the distribution of constituents between a stationary phase and a gas phase. The stationary phase comprises a silicone-based liquid that permeates an inert and granular solid material, housed within a typically coiled steel or glass column measuring 1 to 3 meters in length and 2 to 4 millimetres in diameter. The mobile phase consists of an inert carrier gas such as nitrogen, helium, or argon.

The column is maintained at a high temperature via a furnace. Under the influence of temperature, constituents vaporize and become separable. The basis of separation lies in the discrepancy of partition coefficients of volatile compounds between the stationery and gas phases. A detection system generates a signal at the exit of each molecule from the column, manifesting as the recording of peaks corresponding to each constituent.

Gas chromatography is coupled with a mass spectrometer (MS); this coupling relies on computerized comparison of the spectrum of an unknown peak with one or more reference libraries, enabling its identification.

➢ *Apparatus:*

The identification of the chemical constituents of our essential oils was performed using a gas chromatographic system (HP 5890-SERIE II) equipped with an HP5 MS capillary column (30 meters in length, 0.25 mm internal diameter, and 0.25 µm film thickness) coupled with a mass spectrometer (HP-MSD 5972).

N_2 was employed as the carrier gas for the analysis of the two essential oils. Spectra were recorded at an emission energy of 70 eV, and spectral analysis of the compounds was conducted by comparison with their counterparts using the WILEY275 (Chiu et al., 1982; Guinaudeau et al., 1975; Kiryakov, 1968; Shamma, 1972) spectral libraries.

➢ *Procedure:*

The carrier gas (N_2) is introduced at a flow rate of 1 mL/min, with the injected volume of the essential oil being 1 µL in split injection. The injector and detector temperatures are set at 250°C and 320°C, respectively. The oven temperature is programmed to initially reach 60°C and held for 8 minutes, then gradually increased to 250°C at a rate of 2°C/min, maintained isothermally at 250°C for 15 minutes, and finally elevated to 300°C at a rate of 10°C/min.

4.4. *In vivo* antidiabetic activities:

4.4.1. Animals Care:

The experimental cohort comprised Thirty-five (35) Wistar Mise rats, initially weighing between 100 and 150 grams and of uniform age, with favourable physiological status. Procured from the Institute Pasteur in Algiers, these animals were domiciled in a dedicated animal facility within the Faculty of Natural Sciences and Life at the University of Eloued, where they were subjected to standardized environmental conditions: a temperature regimen of 25 ± 2 °C and a light-dark cycle of 12 hours/24 hours. Housing arrangements involved plastic cages,

accommodating five rats each and furnished with sawdust substrates, replenished biweekly throughout the experimental duration. Regular assessments of body weight and blood glucose levels were conducted weekly to monitor the animals' physiological parameters.

4.4.2. Induction of Experimental Diabetes in Rats:

Diabetes induction in the rat model was achieved through intraperitoneal insulin administration in conjunction with a freshly prepared solution of Alloxan monohydrate (Sigma) at a dosage of 150 mg/kg body weight, following an overnight fasting period. Alloxan was dissolved in a physiological saline solution (NaCl 0.9%). Conversely, healthy control rats received an equivalent volume of intraperitoneal buffer (NaCl 0.9%). Subsequently, to mitigate the risk of hypoglycaemia induced by Alloxan, the experimental rats were provided with glucose-enriched water, initially comprising a 20% glucose solution on the first day, followed by a 5% glucose solution on the subsequent two days. This precautionary measure was essential to avert potential fatality resulting from Alloxan-induced hypoglycaemia in rats. Diabetes status confirmation was attained 72 hours post-injection through blood glucose measurement using a glucometer, with only rats exhibiting blood glucose levels exceeding 14 mmol/l deemed diabetic and thus included in the experimental cohort.

4.4.3. Experimental Design:

Following diabetes induction, the experimental groups were maintained under identical conditions. Subsequently, the animals were randomly allocated into seven groups, each comprising five rats: Group 01 (control), consisting of rats fed a standard diet; Group 2(Diabetic group), comprising diabetic rats fed a standard diet; Group 3(Diabetic+ Acarbose), consisting of diabetic rats administered 10 µL of essential oil from Origanum Majorana L Plant alongside a standard diet; Group 4(Diabetic+EOOm), comprising diabetic rats administered 10 µL of essential oil from Cotula cinerea Plant alongside a standard diet; and Group 5(Diabetic+ EOCc), comprising diabetic rats administered Acarbose alongside a standard diet. Treatment initiation commenced 72 hours post-diabetes induction and persisted for a total duration of 15 days. and Group 6(EOOm),we gave the Essential oil from Origanum Majorana L Plant to the mise without causing diabetes , which means the mice were healthy and not infected ,Finally Group 7(EOCc), also we gave the Essential oil from Cotula cinerea Plant to the mice without causing diabetes.

4.4.4. Sacrifice and Collecting Blood and Organs:

At the conclusion of the treatment period, the animals underwent a fasting period of 16 hours before being anesthetized with 94% chloroform inhalation and subsequently euthanized by decapitation. Blood glucose levels were measured during the sacrifice process. Blood samples were collected into EDTA tubes for hematological analysis and into tubes without anticoagulants for biochemical analysis. The latter samples were centrifuged at 3000 rpm for 10 minutes to obtain serum, which was then stored at -4 °C for subsequent biochemical analysis, including assessments of glycemia, urea, creatinine, triglycerides, cholesterol, TGO, and TGP levels.

Following euthanasia, the liver, pancreas, and kidneys were meticulously dissected, and adipose tissue was carefully stripped from these organs. The isolated organs were then weighed and rinsed in a 0.9% NaCl solution. Subsequently, organ specimens were prepared for the detection of oxidative stress factors, including Malondialdehyde (MDA) and Reduced Glutathione (GSH). Notably, The slices of pancreas, liver, and kidney from each mouse were immersed in formalin solution within each experimental group to facilitate tissue preservation and subsequent histological analysis.

4.5. *In Vitro* alpha-amylase inhibition assay:

Inhibition of α-amylase activity was carried out as described by E. Deveci et al (Deveci et al., 2020), with nuanced adjustments. Briefly, A starch solution (1% w/v) was prepared by stirring 1g starch in 100 ml of 20 mM of phosphate buffer (pH 6.9) containing 6.7mM of sodium chloride. The enzyme solution was prepared by mixing 50 mg of α-amylase in 100 ml of 20 mM of phosphate buffer (pH 6.9 with potassium chloride) to 200 µl of appropriate dilutions (1 - 20 µg/mL) of each essential oil ,500 µl of alpha amylase solution was added and the mixture was incubated at 37 ºc for 10 min. To the reaction mixture 100 µl (1%) starch solution was added and incubated at 37ºC for 10 min. The reaction was stopped by addition of 25 µL HCl (0.1 M) and 100 µL Lugol solutions and kept it in a boiling water bath for 5 minutes and cooled to room temperature. The reaction mixture was then diluted to 2 ml with distilled water, and absorbance measured at 545 nm with UV–Vis spectrometer, (Shimadzu 1800, Japan) using a cell of 1 ml with an optical path length of 1 mm. For each concentration, blank tubes were prepared by replacing the enzyme solution with 500 µL in distilled water. The experiment was repeated using acarbose as a standard antidiabetic drug (positive control). The experiments were repeated thrice using the same protocol. The alpha-amylase inhibitory activity was expressed

as percentage inhibition. The percentage inhibition was calculated using the following Equation 6:

$$Percentage\ inhibition\ (\%) = \left(1 - \frac{A}{B}\right) \times 100 \quad (6)$$

Where, A represents the absorbance of negative control (enzyme activity in the absence of the standard inhibitor or extract). B is the absorbance of test assay (enzyme activity in the presence of only the extract or only the standard inhibitor).

4.6. *In-Silico* analysis:

4.6.1. Software

Computational simulations, including Induced Fit Docking, molecular dynamics studies, and MM-GBSA calculations, were conducted employing the Glide module, Induced Fit Docking module, Prime module, and the Desmond module within the Maestro version 11.7 user interface of the Schrödinger suite (Small-Molecule Drug Discovery Suite 2021-4, Schrödinger, LLC, New York, NY, 2021) (Schrödinger, 2015). The simulations were executed on a DELL Intel(R) Core(TM) i9-13900HX CPU @ 2.20 GHz processor, equipped with 32.0 GB RAM, and operated on a 64-bit Linux Ubuntu 18,04.1 LTS operating system.

4.6.2. ADMET and drug-likeness evaluation:

Drug candidates should possess favourable ADMET properties and ideally non-toxic. Therefore, the major identified compounds from the essential oils extract were evaluated of their ADME profile, including physicochemical, lipophilicity, water solubility, pharmacokinetics, drug-like nature, medicinal chemistry, and several other parameters using SwissADME (Daina et al., 2017; Riyadi et al., 2021) module provided in SIB (Swiss Institute of Bioinformatics) webserver (https://www.sib.swiss). Furthermore, the toxicity aspect of designed compound was also predicted using ProTox (Banerjee et al., 2018) webserver (https://comptox.charite.de/protox3/).

4.6.3. Docking setup:

- *Ligands preparation:*

The three-dimensional configurations of the major compounds isolated from *Cotula cinerea* and *Origanum Majorana L* essential oils were obtained from the National Library of Medicine (NCBI) database (Kim et al., 2016), accessible through the NCBI website (https://pubchem.ncbi.nlm.nih.gov/).

Ligand preparation involved an energy optimization process to derive the most energetically favourable conformations for each compound. Utilizing LigPrep module within the Schrödinger suite (Schrödinger, 2024), this optimization procedure ensured the attainment of the lowest energy state for the studied drugs, including Montbretin A (a co-crystallized ligand). The ionization states were established at a pH of 7.0 ± 2.00, as computed by the Epik classic module, while maintaining specified chirality and generating relevant tautomeric forms. Furthermore, partial atomic charges were computed using Optimized Potentials for Liquid Simulations OPLS4 force field (Lu et al., 2021).

➢ *Receptor Preparation:*

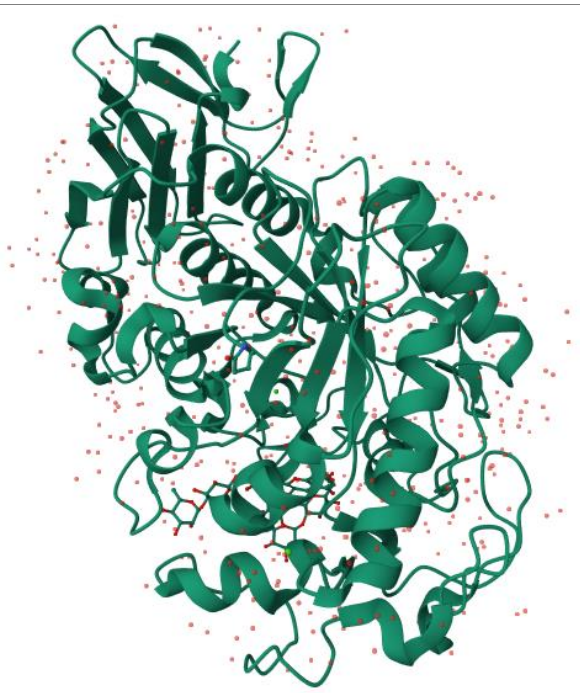
The crystallographic data for Human pancreatic alpha-amylase (Table 1) (PDB ID: 4W93) (Williams et al., 2015) was retrieved from the Protein Data Bank (http://www.rcsb.org) (Rose et al., 2017), adhering to specific parameters such as a resolution of 1.35Å and R value-free of 0.211. Processing of the protein structure was executed through the "protein preparation Workflow" module within the Schrödinger suite (Madhavi Sastry et al., 2013), involving consecutive stages of import and processing, review and modification, and refinement.

In the initial stage, the Prime tool was employed to address missing residues and side chains, maintaining the pH of PROPKA at 7.0 ± 2.00. Subsequent steps included the optimization and assignment of hydrogen bonds, along with the removal of water molecules beyond 8 Å. Restrain minimization utilizing the Optimized Potentials for Liquid Simulations (OPLS4) force field was performed to achieve a low-energy state for the protein (Lu et al., 2021). This phase of protein preparation signifies an energy optimization methodology, presenting the protein in its energetically favourable state for subsequent *in-silico* studies.

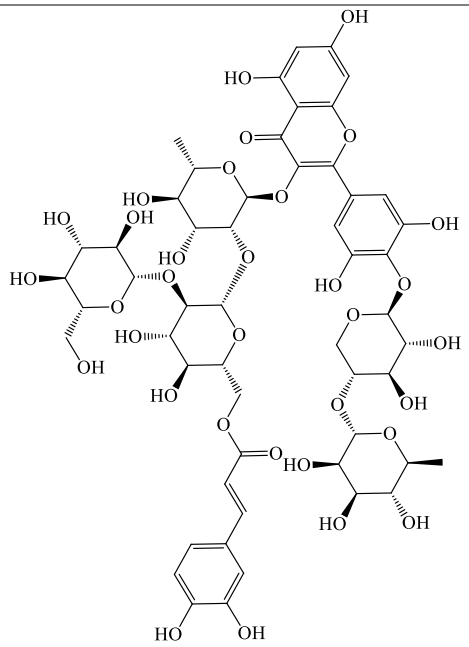
The "Receptor grid generation" panel facilitated the creation of a grid encompassing the active site of the protein, delimited by the co-crystallized ligand Montbretin A. Default parameters were maintained, and the grid centre was generated at the coordinates X = -6.65; Y = 6.99; Z = -20.65.

Table 10. Target receptor information chosen for docking studies

Human pancreatic alpha-amylase	**Detaille's**	
	PDB ID	4W93
	Mutation	No
	Resolution (Å)	1,35
	R-Value Free	0,211



R-Value Work	0,198
R-Value Observed	0,198
Organism	Homo sapiens
Expression System	Komagataella pastoris
Space Groupe	P 2_1 2_1 2_1
Sequence Length	496

Co-Crystallized Ligand	**Detaille's**	
	Name	Montbretin A
	Formula	$C_{53}H_{64}O_{33}$

➢ *Molecular Docking:*

Computational simulations, including Induced Fit Docking, molecular dynamics studies, and MM-GBSA calculations, were conducted employing the Glide module, Induced Fit Docking module, Prime module, and the Desmond module within the Maestro version 11.7 user interface of the Schrödinger suite (Small-Molecule Drug Discovery Suite 2021-4, Schrödinger, LLC, New York, NY, 2021) (Schrödinger, 2015). The simulations were executed on a DELL Intel(R) Core(TM) i9-13900HX CPU @ 2.20 GHz processor, equipped with 32,0 GB RAM, and operated on a 64-bit Linux Ubuntu 18,04.1 LTS operating system.

The molecular docking tool employed for all docking studies was Glide (Grid-based Ligand docking with Energetics), a module within the Schrödinger suite (Yang et al., 2021). The prepared ligands underwent docking onto the specified protein site utilizing the Glide module, in Standard Precision (SP) modes (Friesner et al., 2006).

➢ *Induced Fit Docking (IFD):*

The Induced Fit docking (IFD) module of the Maestro molecular modelling suite has been noted as a reliable and effective docking approach to consider flexibility in both ligands and the binding pocket residues in the binding pocket of target receptors (Khelil et al., 2020). During the IFD process, Glide/SP (Standard Precision) was performed for each ligand, the Prime refinement step specifically addressed the side chains of residues within a 5 Å radius of the ligand. Noteworthy is the retention of a maximum of 20 poses for each ligand.

➢ *Molecular Dynamics Simulation (MDS):*

The best compound in each plant exhibiting the highest IFD docking score was chosen for Molecular Dynamics Simulation (MDS). Recognizing the limitations of ligand docking in representing the biological system under aqueous conditions (Korb et al., 2012), a 100 ns simulation time for MDS was executed using the Desmond module within the Schrödinger suite (Bowers et al., 2006).

The MDS protocol comprised three essential steps: system builder, minimization, and MDS. In the system builder phase, the protein and ligand complex were selected and immersed in a biological environment. The transferable intermolecular potential with 3 points (TIP3P) solvent model, with the boundary condition maintained in an orthorhombic box form throughout the process with dimensions of 10 x 10 x 10 Å (Akbar et al., 2022). The OPLS-3e force field was consistently applied (Roos et al., 2019). The neutralization of model was conducted by addition of counter ions when needed and 0.15 M of NaCl salt was included to mimic the physiological state.

Subsequently, the NPT ensemble was utilized for energy minimization, maintaining pressure and temperature at 1.0132 bar and 300 K, respectively. Finally, MDS was conducted for the minimized protein-ligand complex (Halder et al., 2023).

➢ *Free Energy (MM-GBSA) Calculation:*

Upon completion of the dynamic simulation, an assessment of the free binding energy between the protein and the ligand was systematically undertaken utilizing the Prime MM-GBSA module within the Maestro molecular modelling suite (E. Wang et al., 2021). The calculation

of ligand binding affinities was accomplished through the Molecular Mechanics/Generalized Born Surface Area ΔG (MM-GBSA ΔG) metric applied to the optimized Receptor-Ligand Complex. This computation, facilitated by the VSGB solvation model and the OPLS4 force field, stands as a methodologically rigorous approach for the comprehensive evaluation of molecular interactions and binding strengths (Gorla et al., 2021).

CHAPTER TWO: RESULTS AND DISCUSSION

1. Introduction:

This chapter delves into the findings regarding the extraction yield, characterization, and comprehensive exploration of the anti-diabetic properties inherent in two essential oils derived from the aerial constituents of *Cotula cinerea* and *Origanum Majorana L*. The overarching aim of this investigation was to assess the potential anti-diabetic efficacy exhibited by essential oils as significant agents in the domain of novel pharmaceutical development.

The elucidations provided herein furnish intricate insights into the chemical constitution of the essential oils, thereby enabling their utilization in the *in-silico* study. This involved the application of advanced computational techniques such as Induced Fit Docking (IFD) and Molecular Dynamics Simulation (MDS) for each compound, capabilities not feasible in traditional *in vitro* and *in vivo* studies.

2. Extraction Yield:

Figure *17* illustrates the yields of essential oils obtained through hydrodistillation of the aerial parts of *Cotula cinerea* and *Origanum Majorana L*. variety of Eloued.

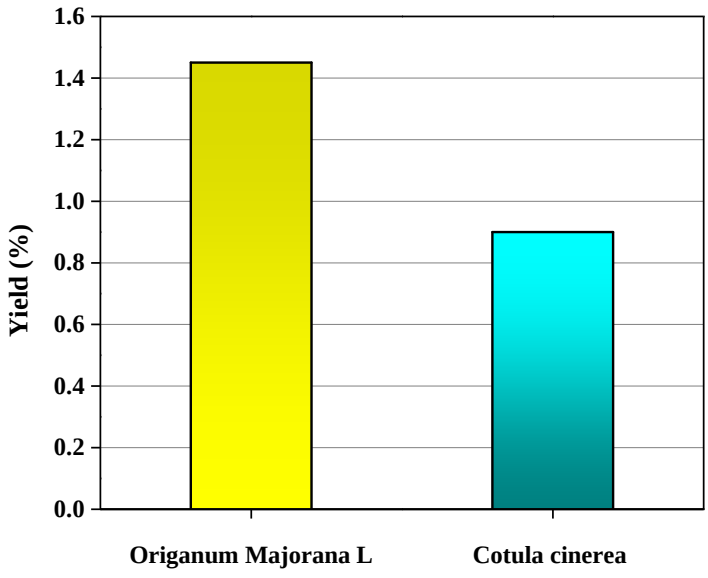


Figure17. The yields of extracted essential oils

We observe that *Origanum Majorana L* exhibits a higher essential oil yield than *Cotula cinerea* (1.45% and 0.90% respectively). However, the difference between the two yields is non-significant.

Nevertheless, findings reported by (Alimi et al., 2022) indicate lower yields of essential oils extracted by hydrodistillation from *Origanum Majorana L* and *Cotula cinerea* at ambient temperature.

Similar results to ours were, however, obtained by (Naima et al., 2019) for the essential oil extracted from the same variety using the same method.

Furthermore, significantly higher yields were achieved by (Vera & Chane-Ming, 1999) during the hydrodistillation of *Origanum Majorana L*, Indian variety.

These variations in results can be explained by the fact that essential oil yields are influenced by several factors during extraction: either factors related to the plant (species, variety, chemical composition, etc.) or factors associated with experimental conditions (extraction process, extraction duration, etc).

3. Chemical composition of essential oils:

3.1. Organoleptic characteristics:

Through the conducted work, it has been revealed that the essential oil extracted from the studied plants exhibits the following (Table 11) organoleptic properties:

Table 11. The organoleptic characteristics of essential oils

Plant	Smell	Aspect	Colour
Cotula cinerea	A distinctive aroma	liquid at room temperature	Dark-yellow
Origanum Majorana L	A pleasant fragrance	liquid at room temperature	Light-yellow

3.2. Physicochemical Properties:

The physicochemical properties were meticulously determined according to the standards of the French Association for Standardization (AFNOR) using established methodologies to measure relative density, refractive index, acidity index, and ester index, as depicted in the following Table 12.

Table 12. The physicochemical properties of essential oils

Plant	Relative Density	Refractive Index	Acidity Index	Ester Index
Cotula cinerea	0.958	1.4720	5.01	43.72
Origanum Majorana L	0.908	1.4512	4.39	41.23

By comparing these results to those obtained by (Naima et al., 2019) (density 0.953, refractive index 1.474, acidity index 4.93 and ester index 45.96) and (Alimi et al., 2022) (density: 0.834

± 0.02, 0.835 ± 0.02, refractive index, at 25°C 1.4596 ± 0.03, 1.4622 ± 0.04), and considering that the refractive index was measured at a temperature of 23°C, we can conclude that these values are in accordance with the standards described by AFNOR (Afnor, 1982).

3.3. Gas Chromatography-Mass Spectrometry (GC/MS) analysis:

The analysis focused on the essential oil constituents extracted from two specific plant species using gas chromatography-mass spectrometry (GC/MS). Mass spectra corresponding to each chromatographic peak were juxtaposed with spectra from relevant scientific literature and the Weily electronic database for mass spectra (Horai et al., 2010). Retention indices were employed to ascertain compound identities. Moreover, utilizing the identical non-polar HP5 stationary phase in the gas chromatography column facilitated the preservation of consistent peak numbers and elution sequences for the compounds under investigation.

Figure 18 and Figure 19 depict the chromatograms illustrating the essential oil compositions of *Cotula cinerea* and *Origanum Majorana L*, respectively, as obtained through gas chromatography-mass spectrometry (GC/MS) analysis:

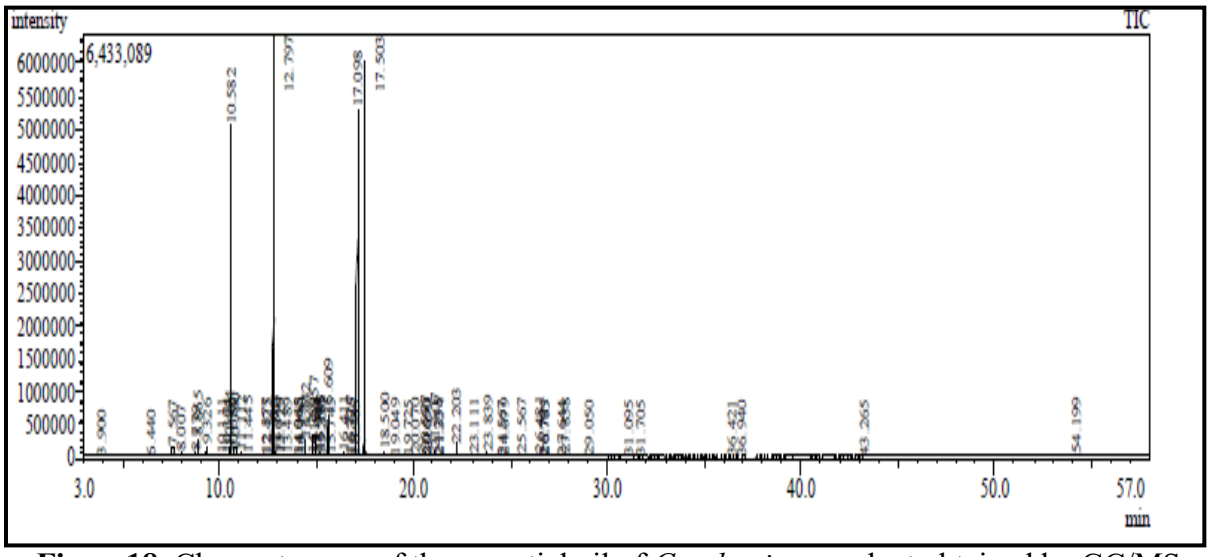


Figure18. Chromatogram of the essential oil of *Cotula cinerea* plant obtained by GC/MS

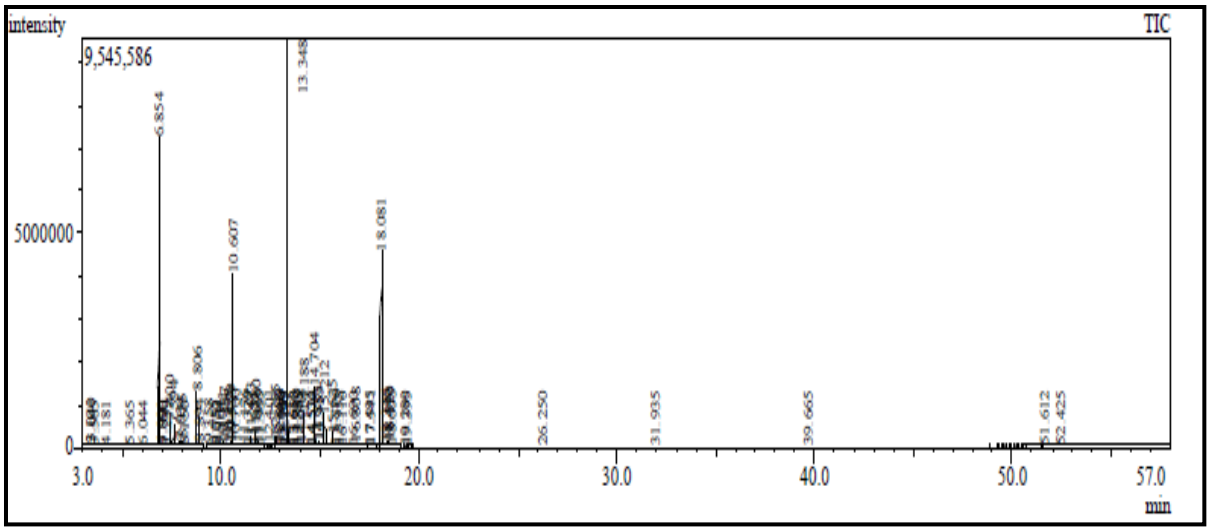


Figure 19. Chromatogram of the essential oil of *Origanum Majorana L* plant obtained by GC/MS

Upon scrutinizing the two chromatograms, notable resemblances are evident, with discernible bands interspersed with overlapping ones. Generally, distinct regions can be identified, delineating three segments:

1. The initial segment, delimited within the time frame of (4 min to 12.4 min), hosts several bands denoting the presence of hydrocarbon monoterpenes.
2. The succeeding segment, spanning from (12.4 min to 24 min), encompasses the predominant bands, indicative of oxygenated monoterpenes prevalent in the plant's essential oil.
3. The final segment, spanning (24 min to 32.5 min), exhibits minimal banding, suggesting a negligible presence of hydrocarbon sesquiterpenes in the plant's essential oil.

The pertinent compounds in each essential oil, extracted from *Cotula cinerea* and *Origanum majorana L*, have been identified and collated as follows

3.3.1. *Cotula cinerea:*

The hydrodistillation extraction of *Cotula cinerea* yielded a dark yellow oil with a yield of 0.91%. Gas chromatography-mass spectrometry (GC/MS) analysis identified 31 compounds, collectively constituting 95.64% of the oil's composition (as presented in Table 13). Predominantly, oxygenated monoterpenes accounted for 68.16%, followed by hydrocarbon monoterpenes at 23.27%, while the percentage of oxygenated sesquiterpenes was minimal at 0.15%. The remaining 4.06% comprised various other compounds.

Table 13. Essential oil constituents of *Cotula cinerea* identified by GC/MS

No	Compounds	IR_{Exp}	IR_{Ref}	(%)

01	**Santolina triene**	**908**	**914**	**10.6**
02	Alpha-thujene	931	935	0.88
03	**Alpha-pinene**	**939**	**943**	**2.02**
04	camphene	953	956	0.85
05	**Sabinene**	**976**	**976**	**6.17**
06	Beta- pinene	980	981	0.58
07	Dehydro-1.8-cineole	991	988	0.64
08	Myrcene	991	993	0.07
09	Meta mentha-1(7)-8dien	999	997	0.06
10	Alpha-terpinene	1018	1017	0.83
11	Ortho cymene	1022	1020	0.43
12	Para cymene	1026	1029	0.6
13	**1.8-cineole**	**1033**	**1033**	**5.34**
14	**Delta-terpinene**	**1062**	**1057**	**1.57**
15	Cis sabinene hydrate	1068	1069	0.46
16	Terpinolene	1088	1088	0.36
17	Cis thujone	1102	1100	0.52
18	**Trans thujone**	**1114**	**1117**	**51.86**
19	**Camphor**	**1143**	**1140**	**2.63**
20	**Beta-terpineol**	**1163**	**1160**	**1.39**
21	**Terpin-4-ol**	**1177**	**1178**	**1.73**
22	Alpha-terpineol	1189	1190	0.58
23	Myrtenol	1194	1195	0.13
24	Neo iso dihydro carveol	1226	1224	0.53
25	Carvotanacetone	1246	1247	0.9
26	**Cis verbenyl acetate**	**1262**	**1264**	**5.07**
27	Iso pulegol acetate	1273	1274	0.06
28	Para cymen-7-ol	1287	1285	0.08
29	Neryl acetate	1365	1367	--
30	Cis jasmone	1394	1390	0.15
31	Germacrene D	1480	1481	0.06
Total		**95.64**		

Our analysis unveiled ten compounds with concentrations surpassing 1% (Table 13), with trans-thujone emerging as the predominant compound at 51.86%, followed by santolina triene at

10.69%, and sabinene at 6.17%. Additionally, 1,8-cineole constituted 5.34%, while four compounds α-pinene, terpin-4-ol, β-terpineol, and camphor appeared in lesser proportions at 2.02%, 1.73%, 1.39%, and 2.63%, respectively.

Comparison with other studies revealed some congruence, particularly with the percentage of santolina triene in Ekhlil et al.'s (Ekhlil et al., 2016) investigation (11.67%). However, disparities arose in the primary compound, with iso-thujanol predominating at 47.38% in their study. Conversely, El bouzidi et al.'s (Bouzidi et al., 2011) findings aligned closely with ours, particularly regarding trans-thujone (41.4%), 1,8-cineole (8.2%), and santolina triene (7.2%), while reporting camphor at 5.5%. Notably, Fournier et al.'s (Fournier et al., 1989) study diverged, identifying camphor as the principal compound at 50%.

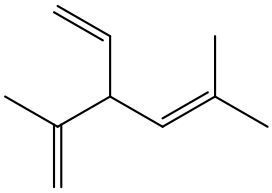
For a visual representation of the top major compounds (more than 1%) and their structures, refer to Figure 20 , which depicts the chemical structures and IUPAC names of the predominant constituents identified in the essential oil of *Cotula cinerea*.

3.3.2. Origanum Majorana L:

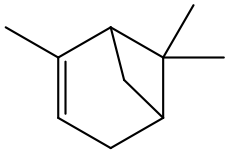
The hydrodistillation extraction method was employed to obtain the essential oil from *Origanum Majorana L.*, yielding a noteworthy 1.5% output. Analysis revealed the identification of 98.84% of the constituents, comprising a total of 42 compounds (as presented in Table 14). Oxygenated monoterpenes dominated the composition, constituting over 57%, followed by hydrocarbon monoterpenes at 25.14%.

Of particular interest is the prominent presence of trans-Thujone, constituting 33.3% of the essential oil, a compound conspicuously absents in the majority of prior studies. For instance, J. Chane et al.'s (Vera & Chane-Ming, 1999) investigation reported Terpinen-4-ol as the primary compound at 38.4%, a constituent entirely absents in our study. Similarly, Santolina triene emerged as the second major compound in our analysis at 16.40%, a finding not corroborated in extant literature.

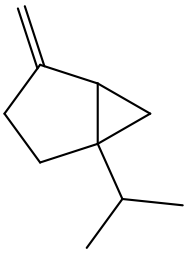
Despite these disparities, concordance exists with previous studies regarding the occurrence of Sabinene, which manifested at 3.12% in our study, compared to 15% in J. Chane et al.'s (Vera & Chane-Ming, 1999) study and 17% in K.H.C Baser et al.'s study (Baser et al., 1993). Additionally, Sellami et al (Sellami et al., 2009) documented Sabinene at 2.14%. Supplementary compounds identified include α-Thujene (1.44%), α-Pinene (1.19%), and β-Pinene oxide (4.42%).



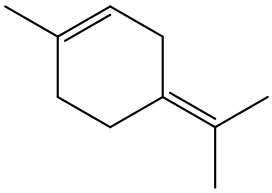
2,5-dimethyl-3-vinylhexa-1,4-diene
(Santolina triene)



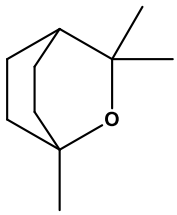
2,6,6-trimethylbicyclo[3.1.1]hept-2-ene
(Alpha-pinene)



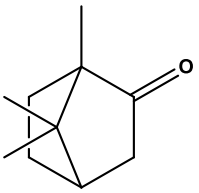
1-isopropyl-4-methylenebicyclo[3.1.0]hexane
(Sabinene)



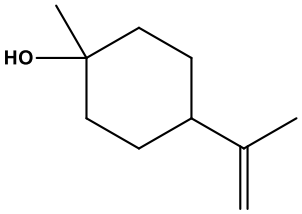
1-methyl-4-(propan-2-ylidene)cyclohex-1-ene
(Delta-terpinene)



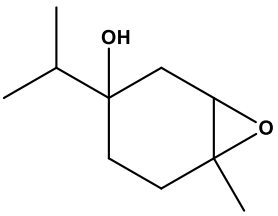
1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane
(1.8-cineole)



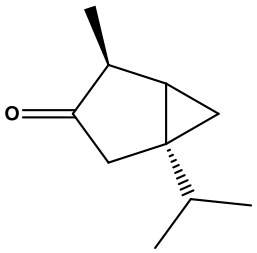
1,7,7-trimethylbicyclo[2.2.1]heptan-2-one
(Camphor)



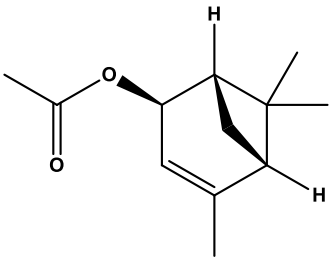
1-methyl-4-(prop-1-en-2-yl)cyclohexanol
(Beta-terpineol)



3-isopropyl-6-methyl-7-oxabicyclo[4.1.0]heptan-3-ol
(Terpin-4-ol)



(1*S*,4*S*)-1-isopropyl-4-methylbicyclo[3.1.0]hexan-3-one
(Trans thujone)



(1*S*,2*R*,5*S*)-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-yl acetate
(Cis verbenyl acetate)

Figure 20. Chemical structures and IUPAC names of the top major compounds identified in the essential oil of *Cotula cinerea*

In summary, the essential oil derived from *Origanum Majorana L.* exhibits distinctive chemical compositions in Eloued region compared to oils from other geographical locales, characterized notably by the prevalence of trans-Thujone and Santolina triene as major constituents. This variance likely stems from multifactorial influences such as soil quality, climatic conditions,

and harvest timing. For an illustrative overview of the major compounds identified, Figure 20, displaying the structures and IUPAC nomenclature of these constituents.

Table 14. Essential oil constituents of *Origanum Majorana L* identified by GC/MS

No	Compounds	IR_{Exp}	IR_{Ref}	(%)
01	Pentanol	759	762	0.12
02	Cis-2-Penten-1-ol	762	765	0.03
03	Hexanal	801	801	0.02
04	(Z)-Salvene	844	847	0.02
05	Isopentyl acetate	869	869	0.02
06	**Santolina triene**	**898**	**906**	**16.42**
07	Tricyclene	912	921	0.02
08	**Alpha-Thujene**	**918**	**924**	**1.44**
09	**Alpha-Pinene**	**925**	**932**	**1.19**
10	Camphene	941	946	0.43
11	**Sabinene**	**969**	**969**	**3.12**
12	Beta-Pinene	972	974	0.22
13	Beta-Myrcene	989	988	0.14
14	Alpha-Phellandrene	1003	1002	0.03
15	Propanoic acid, 2-methyl-, 3-methylbutyl ester	1012	1007	0.02
16	Alpha-Terpinene	1016	1014	0.36
17	Para cymene	1024	1020	0.20
18	Ortho Cymene	1026	1022	0.45
19	Sylvestrene	1028	1025	0.20
20	**1,8-Cineole**	**1031**	**1026**	**10.71**
21	Trans-decahydroNaphthalene	1054	1053	0.03
22	Terpinene	1059	1054	0.76
23	Cis Sabinene hydrate	1068	1065	0.89
24	Para-Mentha-2'4(8)-diene	1088	1085	0.11
25	Trans-Sabinene hydrate	1100	1198	0.86
26	Isopentyl-2-methylbutanoate	1104	1100	0.14
27	cis-Thujone	1107	1101	0.24
28	**trans-Thujone**	**1119**	**1112**	**33.30**
29	iso-3-Thujanol	1136	1134	0.04
30	**Camphor**	**1146**	**1141**	**2.85**
31	**Beta pinene oxide**	**1163**	**1154**	**4.42**

32	3-Thujanol	1168	1164	0.48
33	Alpha Terpineol	1192	1186	0.88
34	Dihydrocarveol	1197	1192	0.06
35	Safranal	1203	1197	0.01
36	Cis-3-Hexenyl 2-methyl butanoate	1233	1229	0.25
37	trans-Myrtanol	1257	1258	0.04
38	2-(1E)-propenyl Phenol	1258	1264	0.09
39	**cis Verbenyl acetate**	**1277**	**1280**	**15.05**
40	neoiso-3Thujanol acetate	1287	1281	0.09
41	Isobornyl acetate	1290	1283	0.21
42	Lavandulyl acetate	1293	1288	0.32
Total		**95.64**		

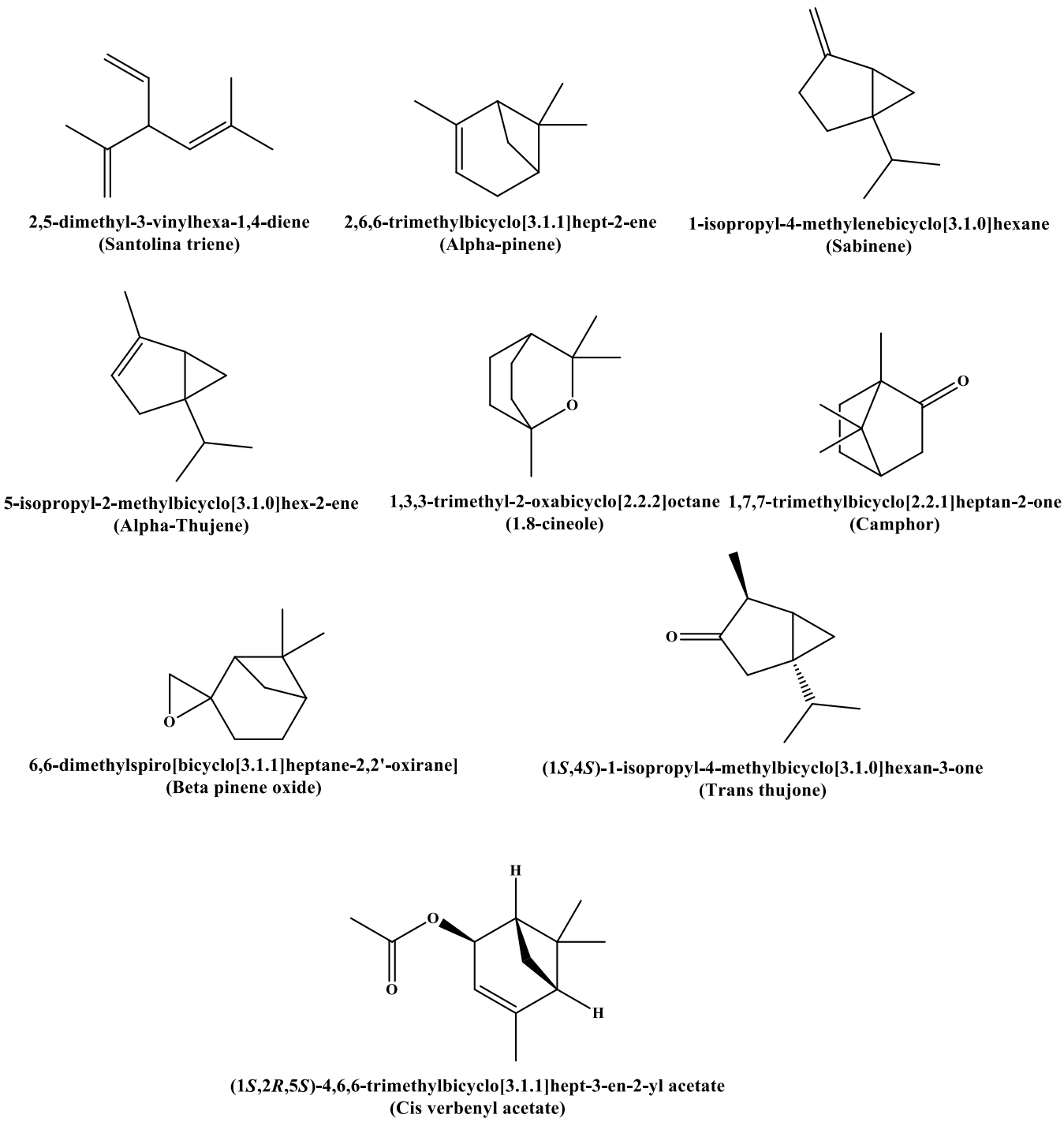


Figure 21. Chemical structures and IUPAC names of the top major compounds identified in the essential oil of *Origanum Majorana L*

4. *In vivo* antidiabetic activities:

4.1. Hematological parameters

The table below displays the hematological parameters results for all the experimental groups. The results indicate that the diabetic group's exposure to alloxan resulted in a very highly

significant decrease ($p<0.001$) in lymphocytes (LYM), a significant decrease ($p<0.05$) in granulocytes (GRA), and a highly significant decrease ($p<0.01$) in platelets (PLT). On the other hand, the results show no significant variation in the number of leucocytes (WBC), erythrocytes (RBC), and hemoglobin (HGB) level when compared to the control group. When compared to the diabetic group, therapy with the essential oils of *Origanum Majorana L* (Diabetic+EOOm group) and *Cotula cinerea* (Diabetic+EOCc group) plants considerably restored the platelet count.

Table 15: Plasma concentration of hematological parameters of different experimental groups

	WBC ($\times10^9$/L)	**LYM** ($\times10^9$/L)	**GRA** ($\times10^9$/L)	**HGB** (g/dL)	**RBC** ($\times10^{12}$/L)	**PLT** ($\times10^9$/L)
Control	6.81±0.73	4.43±0.51	3.9±0.1	11.93±0.37	7.19±0.06	919.6±26.2
Diabetic	4.06±1^{NS}	2.46±0.5^{c}	1.5±0.5^{a}	12.46±0.45^{NS}	7.52±0.05^{b}	475.0±25^{b}
Diabetic+EOOm	4.5±0.41^{NS}	3.36±0.85^{NS}	2.03±0.95^{NS}	12.56±1.34^{NS}	7.7±0.56^{NS}	912.6±11.3^{NS***}
Diabetic+ EOCc	6.5±0.5^{NS}	3.56±0.51^{NS}	2.43±0.51^{NS}	12.13±0.45^{NS**}	7.36±0.23^{NS}	800.6±9.5^{a**}
EOOm	7.6±0.52^{NS}	4.8±0.72^{NS}	2.26±0.64^{NS}	10.96±0.4^{NS}	6.75±0.25^{NS}	772.3±4.5^{b}
EOCc	7±1^{NS}	7.83±0.76^{b} ^{NS}	3.9±0.2^{NS}	11.03±0.2^{NS}	6.45±0.06^{b} ^{NS}	778.0±60.3^{a}

Comparison with diabetic group: * $p<0.05$; ** $p<0.01$; *** $p<0.001$, comparison with control group: a $p<0.05$; b $p<0.01$; c $p<0.001$, (NS) non significatif.

4.2. Biochemical parameters

Glycemia and parameters of liver function

The glucose concentration and liver function parameters for all of the experimental groups are shown in the following table. Whereas, in comparison to the control group, alloxan significantly increased the glycemia (FBS) in the diabetic group ($p<0.01$), and when compared to the diabetic group, there is a highly significant decrease in the (Diabetic+ Acarbose) and (Diabetic+EOOm) groups, and a significant decrease in the group (Diabetic+ EOCc).

Alloxan caused a significant increase in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) compared to the control group, and when compared to the diabetic group, the aspartate aminotransferase value changed non-significantly in the (Diabetic+ Acarbose) group, and decreased significantly in the (Diabetic+EOOm) and (Diabetic+EOCc) groups. Meanwhile, alanine aminotransferase decreased highly significantly in the (Diabetic+ Acarbose) and (Diabetic+EOCc) groups and significantly in the (Diabetic+EOOm) group.

Alkaline phosphatase (ALP) was affected by a very highly significant increase in the diabetic and (Diabetic+EOOm) groups, a highly significant increase in (Diabetic+EOCc) group compared to the control group, and compared to the diabetic group, its value decreased highly significantly in the (Diabetic+ Acarbose) group, very highly significantly in the (Diabetic+EOCc) group.

Table 16: Concentration of Glucose and parameters of liver function of different experimental groups

	FBS (g/L)	**AST** (U/L)	**ALT** (U/L)	**ALP** (IU/L)
Control	0.65±0.05	242.3±0.7	85.3±3.05	370.6±11
Diabetic	1.47±0.04^{c}	310.3±21^{a}	127.0±7^{a}	843.3±3.5^{c}
Diabetic+ Acarbose	0.74±0.04^{NS*}	315.6±14.2$^{a\ NS}$	88±2 ^{NS**}	319.6±70.5^{NS**}
Diabetic+EOOm	0.94±0.03^{a**}	242.0±2^{NS*}	76.0±11.53 ^{NS*}	842.6±2.51$^{c\ NS}$
Diabetic+ EOCc	1.09±0.13^{a*}	254.0±4^{a*}	84.1±21.1 ^{NS**}	549.3±9$^{b\ ***}$
EOOm	0.96±0.01^{b}	216.0±6^{a}	71.0±4^{a}	241±1^{b}
EOCc	1.05±0.06^{a}	211.6±11.5^{a}	74.0±14.01^{NS}	429±1^{a}

Comparisonwith diabetic group: * p <0.05; ** p <0.01; *** p <0.001, comparison with control group: a p <0.05; b p <0.01; c p <0.001, (NS) non significatif.

Parameters of kidney function and triglycerides

The values of urea, creatinine (Crea) and Uric Acid (UA) increased significantly in the diabetic group compared to the control group, while, when compared with diabetic group the urea value decreased highly significantly in (Diabetic+EOOm) group and significantly in (Diabetic+EOCc) group. As for triglycerides (TG) value increased very highly significantly in the diabetic group compared to the control group, and decreased highly significantly in the (Diabetic+ Acarbose) group, decreased very highly significantly in the (Diabetic+EOOm) and (Diabetic+EOCc) groups compared to the diabetic group.

Table 17: Parameters of kidney function and triglycerides of different experimental groups

	Urea (g/L)	**Crea** (mg/L)	**UA** (mg/L)	**TG** (g/L)
Control	0.2±0.04	3±0.0	14.67±1.52	0.37±0.02
Diabetic	0.39±0.03^{a}	4±0.0^{a}	21±1^{a}	1.03±0.03^{c}
Diabetic+ Acarbose	0.39±0.027^{NS}	3±0.0^{NS}	16.67±5.5 ^{NS}	0.54±0.07^{NS**}
Diabetic+EOOm	0.27±0.02^{NS**}	3±0.0^{NS}	18±0.0 ^{NS*}	0.54±0.04^{b***}
Diabetic+ EOCc	0.19±0.01^{NS*}	4±0.0$^{a\ NS}$	21±1.73 $^{a\ NS}$	0.40±0.0$^{NS\ ***}$
EOOm	0.27±0.02^{NS}	3±0.0 ^{NS}	18.0±1^{a}	0.57±0.02^{NS}
EOCc	0.26±0.01^{NS}	3±0.0 ^{NS}	15.3±0.57^{NS}	0.44±0.03^{NS}

Comparison with diabetic group: * p <0.05; ** p <0.01; *** p <0.001, comparison with control group: a p <0.05; b p <0.01; c p <0.001, (NS) non significatif.

4.3. Oxidative stress parameters

Pancreas

Alloxan caused in the pancreas a very highly significant increase in the level of MDA and a very highly significant decrease in the concentration of GSH compared to the control group. Acarbose treatment affected a very highly significant decrease in the concentration of GSH compared to the control group, and compared to the diabetic group, the treatment with the essential oils of *Origanum Majorana L* and *Cotula cinerea* contributed to the decrease of MDA and the increase of GSH concentration.

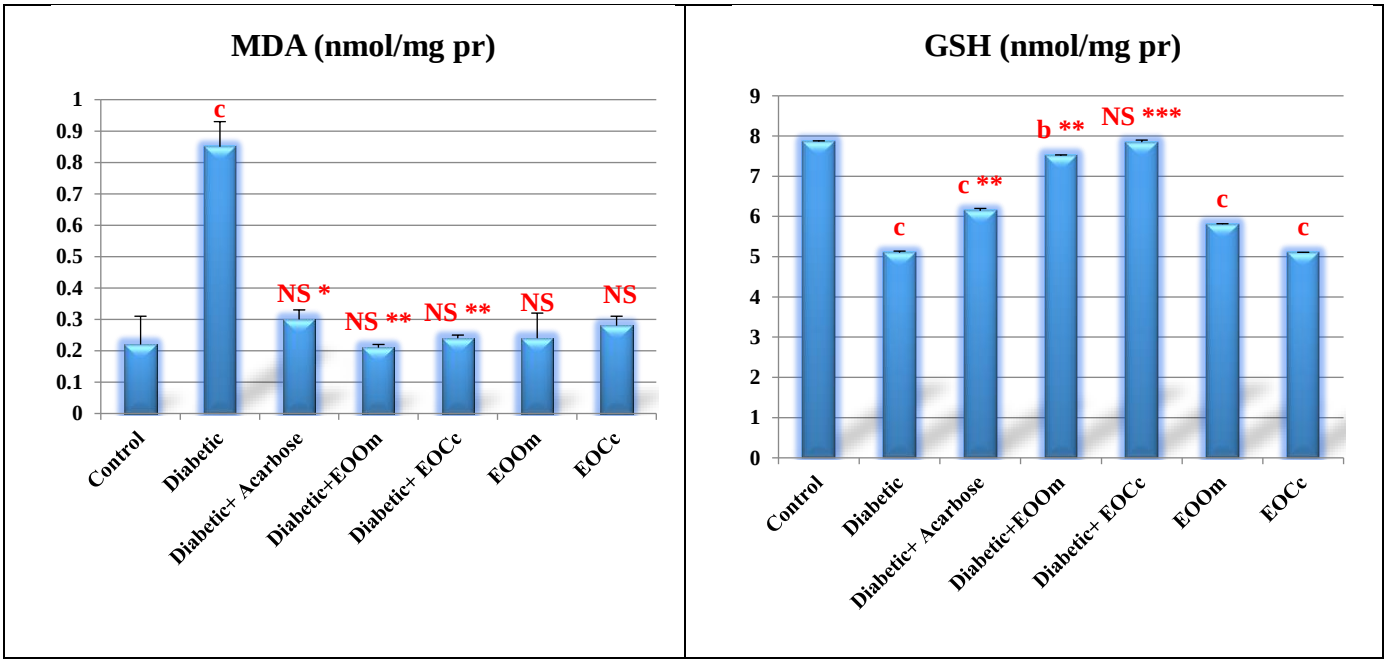


Figure 22: Oxidative stress parameters in the pancreas of control and experimental groups.

Comparison with diabetic group: * p <0.05; ** p <0.01; *** p <0.001, comparison with control group: a p <0.05; b p <0.01; c p <0.001, (NS) non significatif.

Liver

Oxidative stress of liver results indicated that alloxan caused a significant increase in MDA level and a very highly significant decrease in GSH concentration compared to the control group. However, acarbose contributed to a significant decrease in the level of MDA a very highly significant increase the concentration of GSH compared to the diabetic group. The treatment with the essential oils of *Origanum Majorana L* and *Cotula cinerea* significantly improved MDA level, GSH concentration compared to the diabetic group.

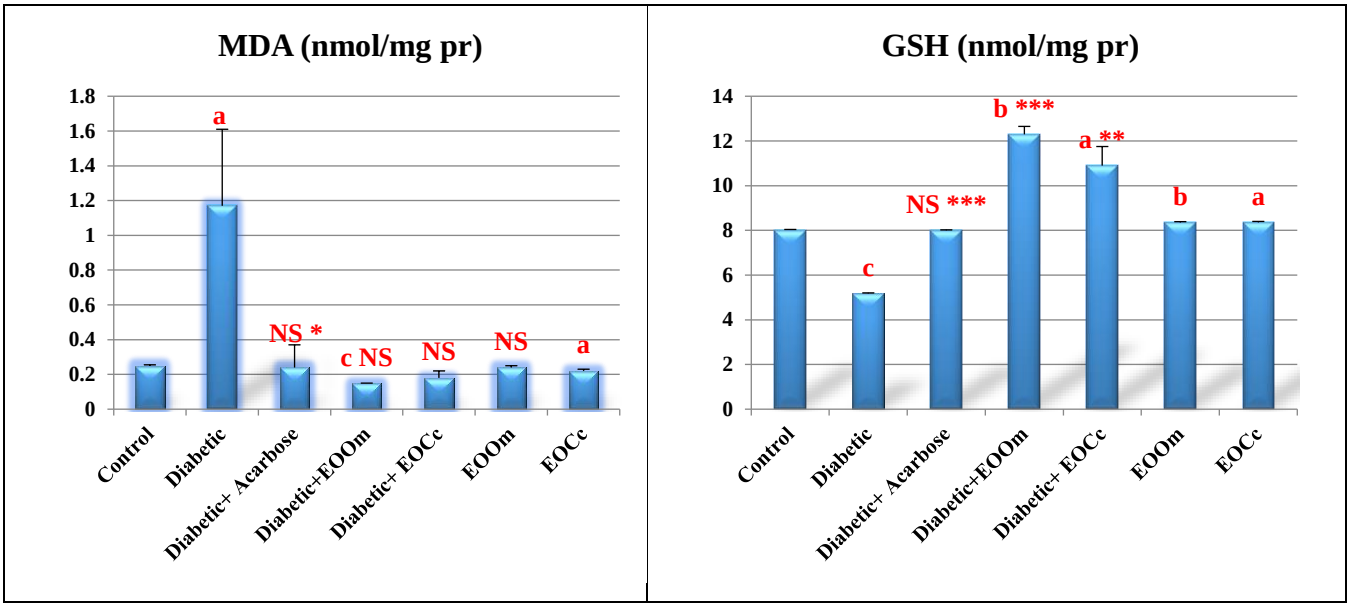


Figure 23: Oxidative stress parameters in the liver of control and experimental groups. Comparison with diabetic group: * p <0.05; ** p <0.01; *** p <0.001, comparison with control group: a p <0.05; b p <0.01; c p <0.001, (NS) non significatif.

Kidneys

In the kidneys, alloxan did not affect the level of MDA, but it caused a significant decrease in the concentration of GSH compared to the control group. Acarbose treatment caused a no significant increase in MDA level, a significant decrease in GSH concentration compared to the control group. And compared to the diabetic group, the essential oils contributed to a highly significant increase in the concentration of GSH.

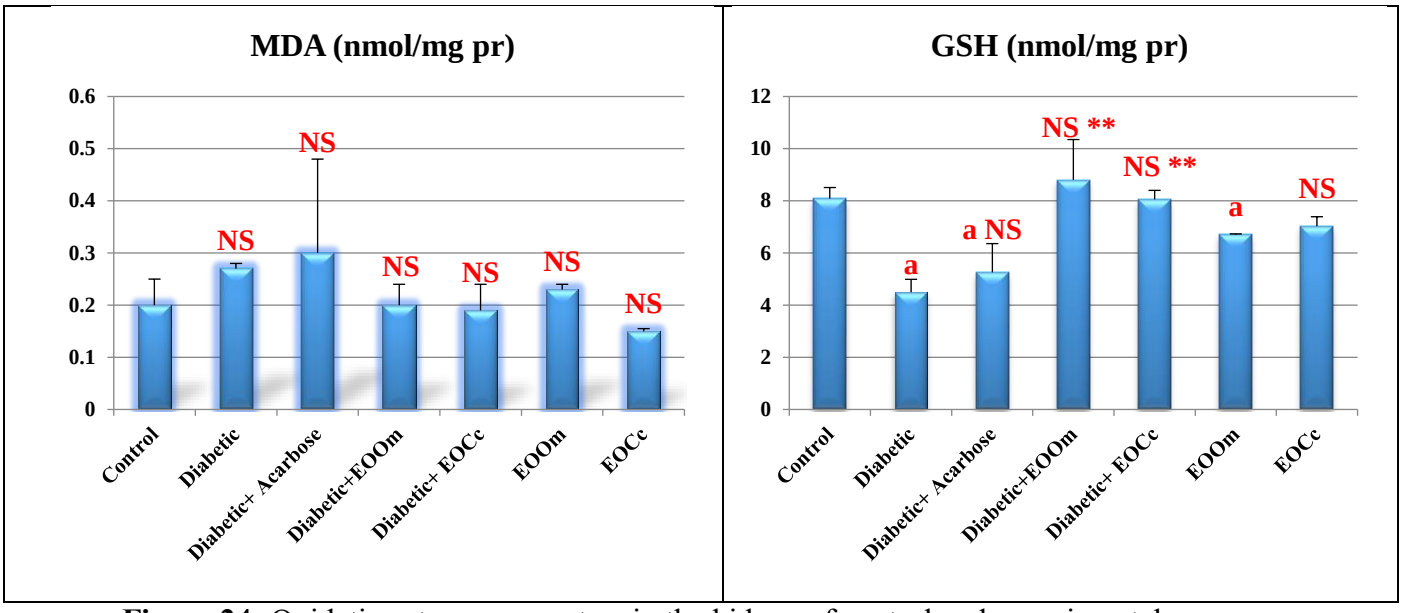


Figure 24: Oxidative stress parameters in the kidney of control and experimental groups. Comparison with diabetic group: * p <0.05; ** p <0.01; *** p <0.001, comparison with control group: a p <0.05; b p <0.01; c p <0.001, (NS) non significatif.

4.4. Histopathological studies

Pancreas

Microscopic photos of pancreatic tissue showed normal pancreatic tissue in the control group. Where, formed of pancreatic acini and islet of Langerhan's showing islet cells arranged in trabecular and acinar pattern with abundant eosinophilic cytoplasm and central small nucleus, separated by thin loose connective tissue. Islets have regular shape with a large number of β-cells which have a normal round shape with will distinct round nuclei. The pancreatic tissues of the diabetic group displayed irregularly shaped islets with a degraded outer layer of connective tissue, as well as reduced islet size and number of β-cells. The tissue of the group treated with acarbose showed a similar appearance to the tissue of the diabetic group. The pancreatic tissues in the essential oil-treated groups showed a significant improvement, including the restoration of the islets of Langerhans' size, regular and increased number of islets cells, islets form, and the surround connective tissue sheet.

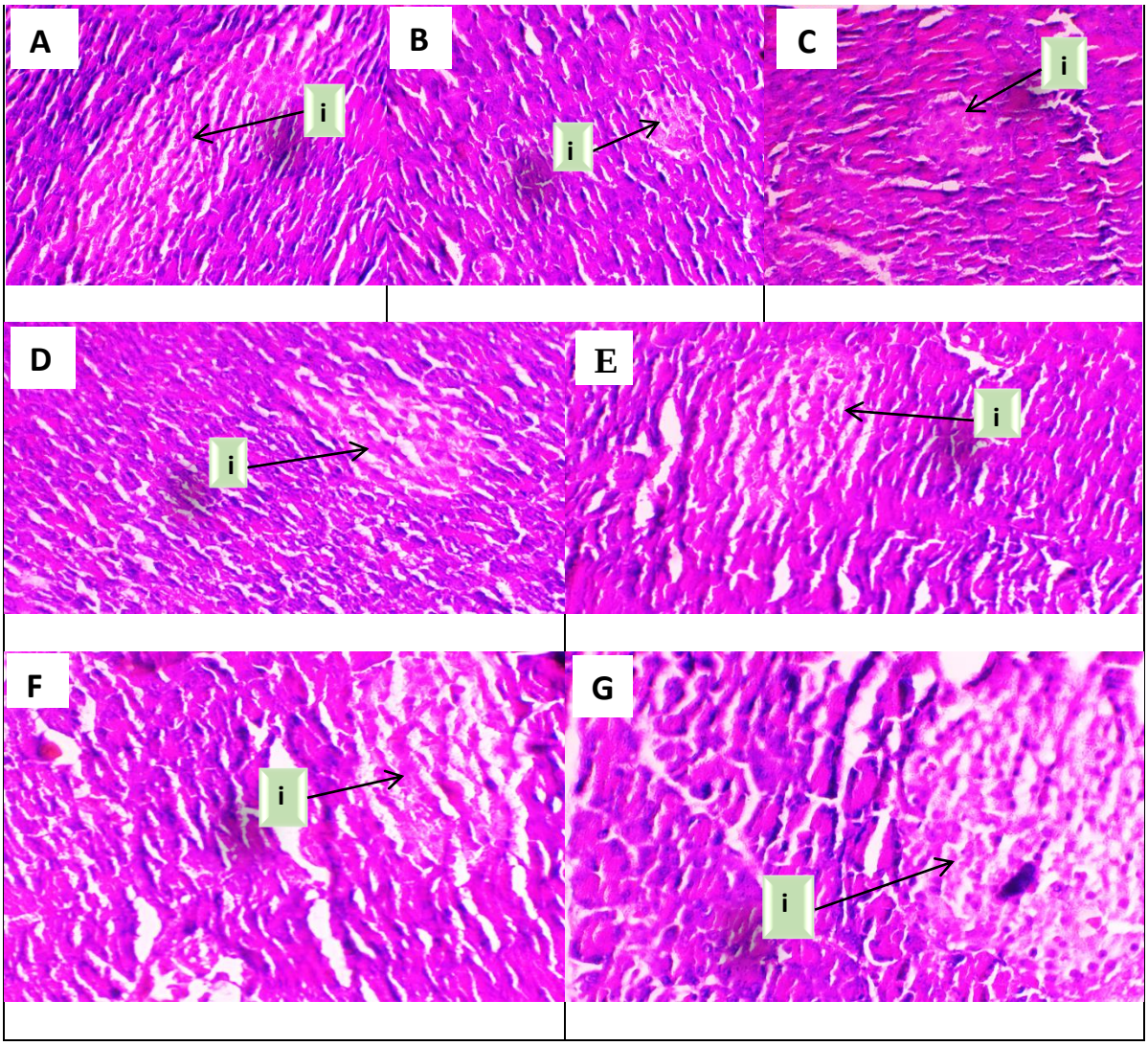


Figure 25 : Microscopic observation of a histological section of pancreas in different experimental groups, Control group (A), diabetic group (B), Diabetic+ Acarbose group (C), Diabetic+EOOm group (D), Diabetic+ EOCc group (E), EOOm group (F) and EOCc (G), (i) islets of Langerhans, magnification x40.

Liver

Microscopic photos of liver tissue showed a normal structure of hepatocytes with a normal appearance in the control group, while the diabetic group revealed pathological changes manifested in the appearance of immune cells (lymphocytes, polynuclear) with mild inflammatory infiltration in addition to focal lobular necrosis. The liver tissues of rats treated with acarbose and essential oils had a healthy architecture and a normal appearance similar to the liver of the control rats.

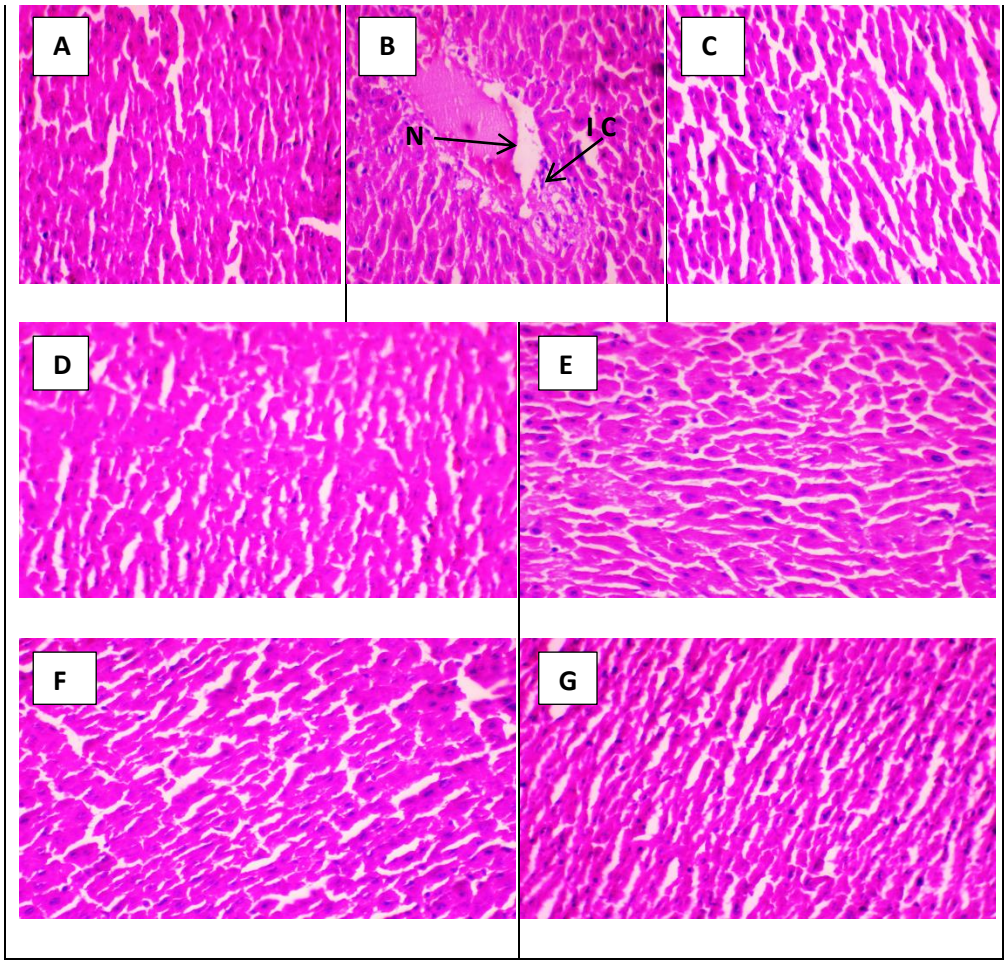


Figure26 . Microscopic observation of a histological section of liver in different experimental groups, Control group (A), diabetic group (B), Diabetic+ Acarbose group (C), Diabetic+EOOm group (D), Diabetic+ EOCc group (E), EOOm group (F) and EOCc (G), (I C) immune cells, (N) necrosis, magnification x40.

Kidneys

Microscopic images of rat kidneys show a normal architecture and normal appearance, in addition to the absence of immune cells, no inflammatory infiltration, and no cell necrosis in the tissues of all groups of rats studied.

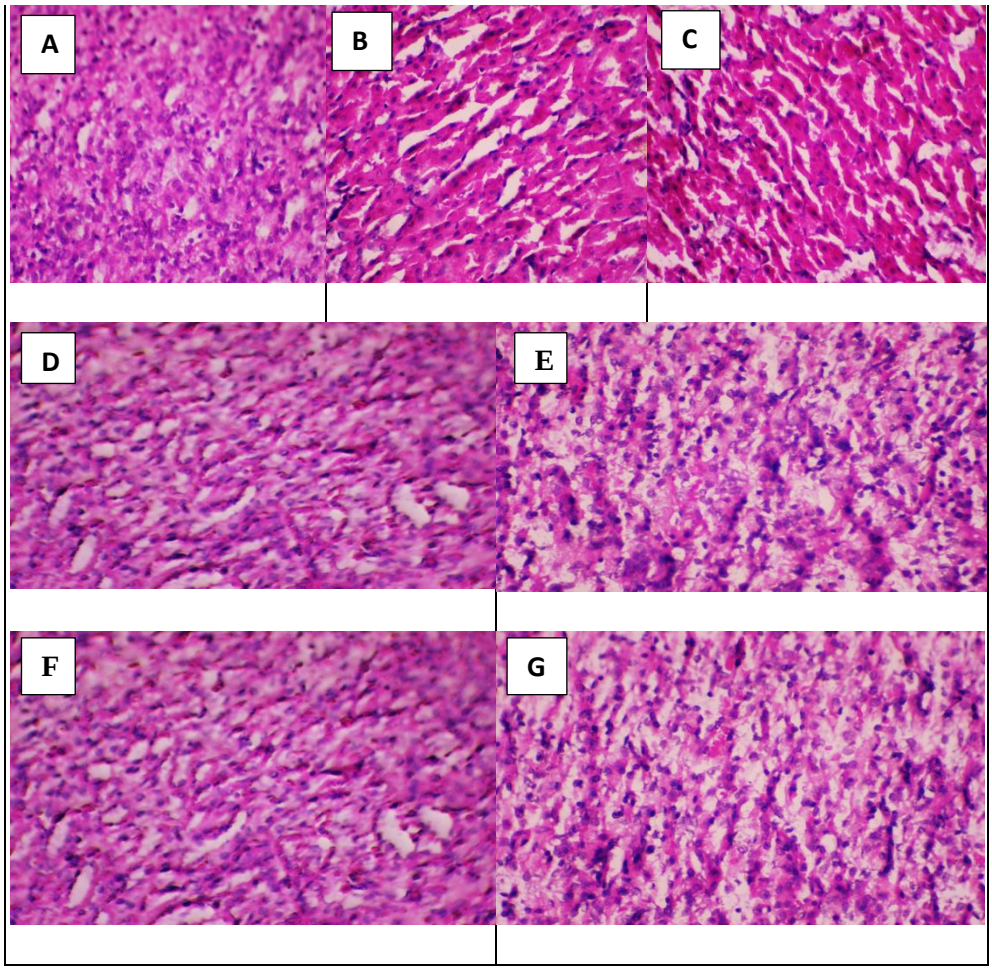


Figure 27: Microscopic observation of a histological section of kidney in different experimental groups, Control group (A), diabetic group (B), Diabetic+ Acarbose group (C), Diabetic+EOOm group (D), Diabetic+ EOCc group (E), EOOm group (F) and EOCc (G), magnification x40.

Alloxan is the major agent utilized for creating experimental diabetes. The death of the pancreatic islet beta cell caused by alloxan leads to the development of persistent diabetes mellitus in several animals. Alloxan specifically targets the islet beta cell while having limited impact on other components **(Furman, 2017)**.

In our study, alloxan caused a decrease in lymphocytes and granulocytes, as well as a decrease in platelets. Lymphocytes and granulocytes function as phagocytes that eliminate foreign material. The presence of hyperglycemia in uncontrolled diabetes mellitus leads to the oxidation of membrane proteins, resulting in a decrease in white blood cells. This oxidation process promotes the generation of lipid peroxides, which in turn causes leukolysis **(Mahmoud et al, 2013).**

An abnormally low or high platelet count can result in excessive bleeding or the production of blood clots, respectively. The latter can impede or block blood vessels that provide blood to the brain and/or heart, resulting in a stroke or heart attack, respectively. Insulin can directly regulate platelet function via a functional insulin receptor found on platelets and the absence of insulin due to alloxan in the diabetic rat group led to a decrease in platelets **(Kakouros et al, 2011)**.

Alloxan is a toxic chemical that affects pancreatic β cells and the liver. It works by attacking sulfhydryl groups, chelating, altering enzyme and metabolic processes, altering the way electrolyte membranes are transported, increasing lipoperoxidation, and reducing antioxidant defenses **(Gargouri et al, 2016)**.

The results show an increase in blood sugar and liver enzymes in the diabetic group.The observed disruptions in alloxan-treated rats validated that diabetes mellitus is a metabolic ailment defined by elevated blood sugar levels caused by deficiencies in insulin synthesis and efficacy. The main process of hyperglycemia in diabetes mellitus involves excess synthesis and reduced use of glucose by the tissues **(Adesokanet al. 2010)**.

Whereas ALT and AST are cytosolic enzymes, ALP is an enzyme that is membrane bound. These enzymes are concentrated in the liver and are only detected in considerable amounts in the serum when the cell membrane ruptures or becomes leaky. An indicator of liver cell injury is an increase in the blood level of certain intracellular enzymes or a reduction in their tissue level **(Gargouri et al, 2016)**.

Elevations in plasma levels of urea, creatinine, and uric acid are caused by diabetic hyperglycemia and are thought to be important indicators of kidney damage.The current study's results indicate that the diabetic group's serum urea, creatinine, and uric acid levels have significantly increased **(Ikewuchi et al, 2017)**.

High glucose (blood sugar) levels are a symptom of diabetes. Insulin facilitates the conversion of glucose into glycogen, a form that can be stored. It also aids in the liver's glycogen storage. The body will utilise glucose to produce fatty acids if the liver contains an excessive amount of glycogen. Triglycerides are produced by the acids. They can accumulate in fat cells and contribute to body fat when they are released into the circulation. However, because the body is producing more triglycerides and is unable to eliminate fat from the blood, the danger of renal failure has made it difficult to control blood fat levels. These issues eventually result in elevated levels of triglycerides **(Alexopoulos et al, 2019)**.

Acarbose acts as an inhibitor of some complex glycolytic enzymes found in the intestine, as it inhibits pancreatic alpha-amylase and membrane-bound alpha-glucosidase - including

intestinal glucoamylase, sucrase, maltase, and isomaltase, thus preventing the breakdown of complex starches, oligos, trisaccharides, and disaccharides into simple absorbable sugars. Acarbose reduces the absorption of dietary carbohydrates and the subsequent increase in blood glucose levels postprandial **(Mao et al. 2014)**. In this study, acarbose contribute to reducing blood sugar and all the problems associated with it, such as decreases in lymphocytes and granulocytes, liver and kidney function alterations.

The principal processes responsible for the antidiabetic effect of the Essentail Oils were found to be the inhibition of alpha-glucosidase and alpha-amylase and the decrease in gluconeogenesis. EOs work through a variety of mechanisms, such as scavenging free radicals, regulating antioxidant enzymes, and reducing lipid peroxidation, to exert their hypoglycemic properties, which are linked to their capacity to increase glucose uptake, hinder glucose production, and have antioxidant effects. Owing to the varied chemical makeup of phytocompounds present in specific plants, these outcomes highlight the potential of EOs as adjuvant therapy in the treatment of diabetes mellitus **(Bungau et al, 2023)**.

Due to the remarkable effect of *Origanum Majorana L* and *Cotula cinerea*, which both had hypoglycemic results of 0.94 g/l and 1.09 g/l, the essential oils of the studied plant played a major role in the significant reduction of blood sugar levels. Adjusting the liver and kidneys' functions, low triglycerides levels, and the pancreas, liver, and kidneys' state of stress are all part of this. Results indicating that most parameters were similar to the control group and that the treatment was very safe were also obtained from the groups that were given essential oils alone.

5. *In Vitro* Anti-Diabetic Activity:

5.1. Alpha-amylase inhibitory activities (IC_{50}):

The *in-vitro* antidiabetic potential of the two essential oils obtained from *Cotula cinerea* and *Origanum Majorana L* was investigated using α-amylase inhibition model and the results are as shown in Table 6. From the linear plots of *Cotula cinerea* and *Origanum Majorana L* concentration (µg/mL) against percentage α-amylase inhibition (Figure 7), the studied EOs significantly increased α-amylase inhibition (%) with corresponding increase in EO concentration. The effective inhibitory concentration (IC_{50} value) at which 50% of α-amylase activity was inhibited by the *Cotula cinerea* and *Origanum Majorana L* essential oils was obtained to be 2.434 and 3.684 and 85.36 ± 0.12 µg/mL respectively, indicating a powerful potential to slowdown starch digestion via α-amylase inhibition.

The inhibitory potential of acarbose, a standard type 2 antidiabetic drug, on α-amylase activity was also analysed in the present study to serve as positive control. The result obtained showed that the two essential oils inhibited α-amylase with more degree (about 8.82-fold more), with *Cotula cinerea* essential oil show a more degree, compared to acarbose (IC_{50} = 11.166 μg/mL) (Figure 7D).

Metabolism of carbohydrate (complex sugar or polysaccharide) by hydrolases (such as α-amylase, α-glucosidase, maltase etc.) leads to release of simple sugars such as glucose and thus boosts blood sugar level rise immediately after consumption of a carbohydrate-rich food (Anigboro et al., 2018); inhibitors of α-amylase, amongst others, are thus very useful in preventing sudden rise in blood glucose level especially in type 2 diabetic patients, following their consumption of carbohydrate-rich food substances (Adefegha & Oboh, 2012; Avwioroko et al., 2020). The relatively inhibitory prowess of the two studied compounds as α-amylase inhibitor compared to acarbose is a desirable property in the present study. This is because previous studies had reported that the extremely high inhibitory effect of acarbose against α-amylase eventually leads to excessive delay of starch hydrolysis in gastrointestinal tract thereby causing abdominal discomfort and flatulence in diabetic patients who take the drug (Malekaneh & Zarban, 2011). Hence, it is believed that the moderately high α-amylase inhibitory potential of *Cotula cinerea* and *Origanum Majorana L* essential oils could help in control of diabetes by diminishing the absorption of glucose, in the case of *Cotula cinerea* would help to prevent sudden rise in blood glucose level in diabetic patients following consumption of a carbohydrate diet. Some researchers also suggested that essential oils extract from plant materials displayed the anti-diabetic activity against amylase (Adefegha et al., 2017; Ibrahim et al., 2019; Sahin Basak & Candan, 2010).

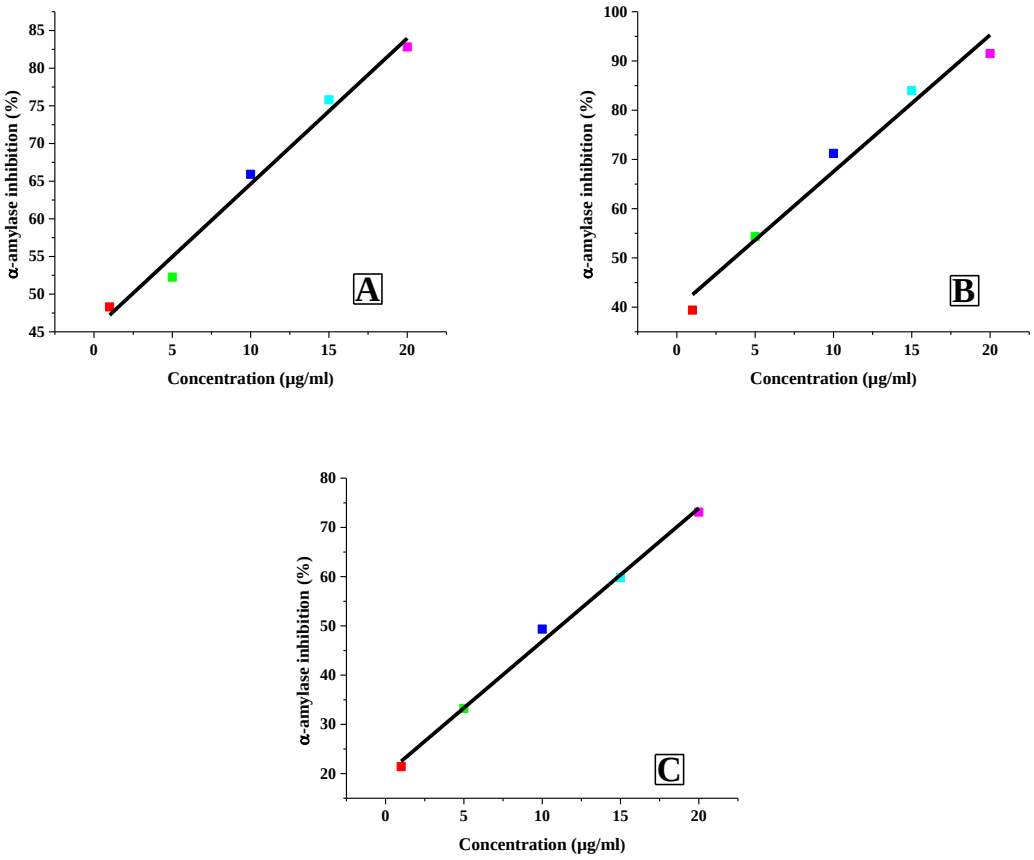


Figure 28. Linear regression of the inhibition of α-amylase activity by the essential oils of: *Cotula cinerea* (A), *Origanum Majorana L* (B) and Acarbose (C)

Table 18. *In vitro* Anti-diabetic activity of the essential oils extracted from *Cotula cinerea* and *Origanum Majorana L* by α-amylase inhibitory assay

Concentration (µg/mL)	**Percentage of α-Amylase Inhibition**		
	Cotula cinerea	*Origanum Majorana L*	Acarbose
1	48.293	39.407	21.405
5	52.252	54.313	33.239
10	65.916	71.227	49.353
15	75.799	83.987	59.829
20	82.820	91.493	73.112
IC_{50} Values	**2.434 µg/mL**	**3.684 µg/mL**	**11.166 µg/mL**

5.2. Alpha-amylase molecular binding interaction:

One of the effective techniques for monitoring alterations in protein conformation during its interaction with ligands is absorption spectroscopy (Benesi & Hildebrand, 1949; B. L. Wang et al., 2020). Usually, when measured at the same wavelength range, the absorption peak(s) of a

free protein and that of the protein-ligand complex are expected to differ; such difference could be attributed to the alteration in the native structure of the protein due to its binding interaction with the ligand.

5.2.1. Binding constants:

The gradual decrease in the absorption values of the α-amylase solution by increasing essential oils and acarbose concentrations can be exploited to calculate the binding constant by applying the following Equation 7 (Benesi & Hildebrand, 1949):

$$\frac{A}{A_0 - A} = \frac{\varepsilon}{\varepsilon_0 - \varepsilon} + \frac{\varepsilon}{\varepsilon_0 - \varepsilon}\frac{1}{K_b \times C} \quad (7)$$

Where A_0 and A are the absorbencies of the α-amylase and its complexes with the studied essential oils respectively, while ε_0 and ε are respectively their extinction coefficients, and C represents the concentration of EOs and acarbose (mmol.L^{-1}), K_b refers to the binding constant (L.mol^{-1}).

A plot of $A/(A_0 - A)$ versus $1/C$ (Figure 8) gave a slope of $\varepsilon/(\varepsilon_0 - \varepsilon)K_b$ and a 'y' intercept equal to $\varepsilon/(\varepsilon_0 - \varepsilon)$, where K_b is the ratio of the slope to the y intercept.

5.2.2. Binding free energy:

The binding free energy change was calculated using the following Equation 8: (Laraoui et al., 2023)

$$\Delta G = -nRT \ln K_b \quad (8)$$

Where ΔG is the binding free energy in KJ.mol^{-1}, R is the gas constant, 8.32 J.mol^{-1}K^{-1} and T is the absolute temperature, 298K.

The negative values of ΔG indicate the spontaneity of the α-amylase and the essential oils interaction, whereas its magnitude indicates the strong binding between the protein and the studied compounds (Gil et al., 2002). The obtained values of binding constants and their corresponding free binding energies are summarized in Table 7.

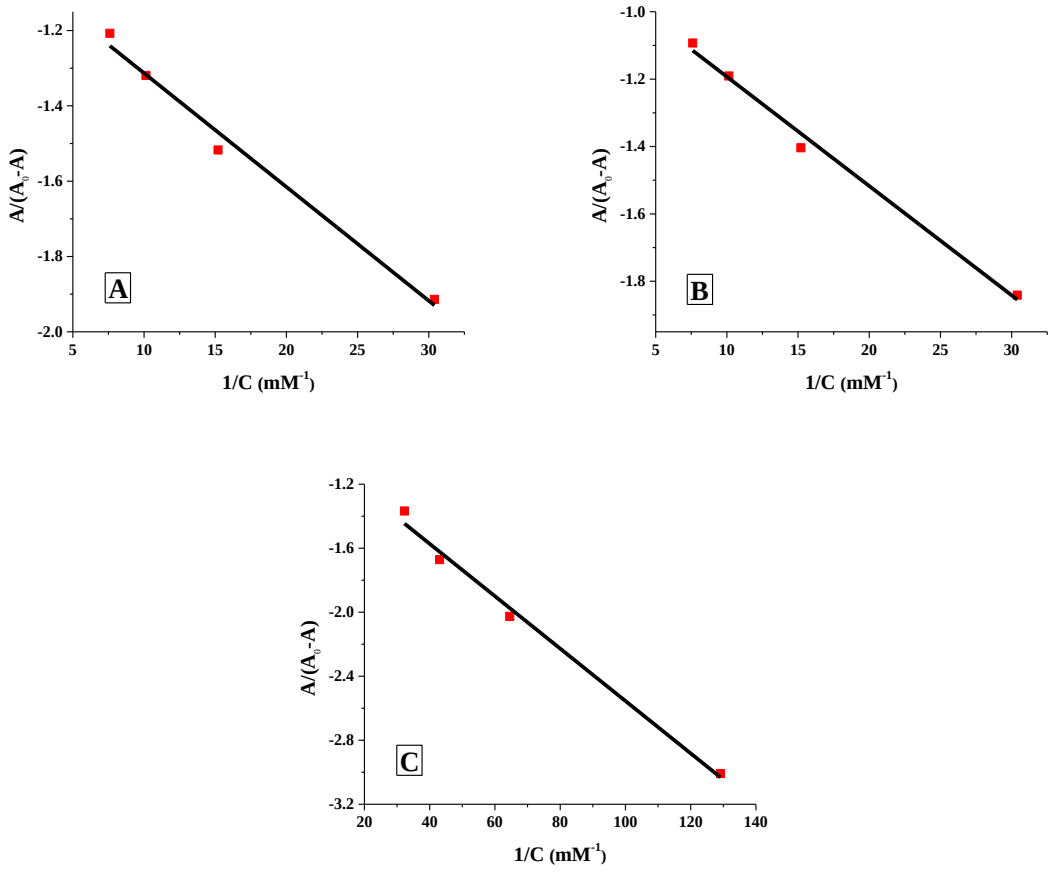


Figure 29. Plots of A/(A_0 - A) versus 1/[*Cotula cinerea*] (A), 1/[*Origanum Majorana L*] (B) and 1/[Acarbose] (C) used to calculate the binding constants

Table 19. Binding constant and binding free energy values for *Cotula cinerea*, *Origanum Majorana L* and acarbose with alpha-amylase

Adduct	K_b (M^{-1})	-ΔG (KJ.mol^{-1})
α-amylase_*Cotula cinerea*	5.61×10^4	27.11
α-amylase_*Origanum Majorana L*	3.35×10^4	25.83
α-amylase_Acarbose	2.68×10^4	25.28

6. In-Silico analysis:

6.1. ADMET and drug-likeness prediction:

Modern drug discovery involves assessment of competence of the dynamic molecules and their strength to reach target site in bioactive form, which involves cellular, animal and human clinical trials which are highly priced and encumbered with risks (Ranjith & Ravikumar, 2019; Ranjith D & Viswanath S, 2019). Presently computer aided drug development encouraged the estimate of absorption, distribution, metabolism and excretion of drugs (ADME), they postulate anticipatory and dependable data very quickly and compliment for experimental approaches (Ranjith & Ravikumar, 2019; Sliwoski et al., 2014). It has been determined that the initial appraisal of ADME properties in the discovery period diminishes remarkably the fraction of

pharmacokinetics related failures in the clinical phase (Hay et al., 2014; Ranjith & Ravikumar, 2019).

In the present study we evaluated the ADME properties of the major compounds (more than 10%) present in both essential oils using SwissADME web tool. A total of 4 potent phytoconstituents were analysed to study general characteristics (Table 8), Physicochemical properties (Table 3), lipophilicity and water solubility characteristics (Table 4 & 5), pharmacokinetic parameters (Table 6), drug likeness rule and bioavailability score (Table 7) and medicinal chemistry properties (Table 8), respectively, the six compounds are compared to the standard drug (Acarbose). General characteristics of the studied compounds revealed all the compounds having molecular weight less than 500 Da, which is a good prime property to be called as drug likeness of the small molecules and they still have a low molecular weight than the Acarbose.

Table 20. General characteristics of the phytoconstituents of essential oils

Sl. No	Compounds	Molecular formula	Canonical SMILES	Molecular weight (g/mol)
1	Santolina triene	$C_{10}H_{16}$	CC(=CC(C=C)C(=C)C)C	136.23
2	trans-Thujone	$C_{10}H_{16}O$	CC1C2CC2(CC1=O)C(C)C	152.53
3	1,8-Cineole	$C_{10}H_{18}O$	CC1(C2CCC(O1)(CC2)C)C	154.25
4	cis-Verbenyl acetate	$C_{12}H_{18}O_2$	CC1=CC(C2CC1C2(C)C)OC(=O)C	194.27
5	Acarbose	$C_{25}H_{43}NO_{18}$	CC1C(C(C(C(O1)OC2C(OC(C(C2O)O)OC3C(OC(C(C3O)O)O)CO)CO)O)O)NC4C=C(C(C(C4O)O)O)CO	645.60

Table 21. Physicochemical properties of the phytoconstituents of essential oils

Properties	Santolina triene	trans-Thujone	1,8-Cineole	cis-Verbenyl acetate	Acarbose
Num. heavy atoms	10	11	11	14	44
Num. arom. heavy atoms	0	0	0	0	0
Fraction Csp3	0.40	0.90	1.00	0.75	0.92
Num. rotatable bonds	3	1	0	2	9
Num. H-bond acceptors	0	1	1	2	19
Num. H-bond donors	0	0	0	0	14
Molar refractivity	48.76	45.90	47.12	56.12	136.69
TPSA (Å²)	0.00	17.07	9.23	26.30	321.17

Table 22. Lipophilicity characteristics of the phytoconstituents of essential oils

Properties	Santolina triene	trans-Thujone	1,8-Cineole	cis-Verbenyl acetate	Acarbose
iLOGP	2.90	2.27	2.58	2.53	0.63
XLOGP3	4.22	2.27	2.74	3.73	-8.53
WLOGP	3.33	2.26	2.74	2.54	-8.56
MLOGP	3.56	2.30	2.45	2.65	-6.94
SILICOS-IT	2.88	2.63	2.86	2.26	-7.69
Consensus Log Po/w	3.38	2.35	2.67	2.74	-6.22

Table 23. Water Solubility characteristics of the phytoconstituents of essential oils

Small molecules	ESOL				Ali				SILICOS-IT			
	Log S (ESOL)	Solubility		Class	Log S (Ali)	Solubility		Class	Log S (SILICOS-IT)	Solubility		Class
		mg/ml	mol/L			mg/ml	mol/L			mg/ml	mol/L	

Santolina triene	-3.15	9.75e-2	7.16e-4	S	-3.93	1.60e-2	1.17e-4	S	-2.04	1.24e-0	9.10e-3	S
trans-Thujone	-2.15	1.08e-0	7.11e-3	S	-2.27	8.27e-1	5.43e-3	S	-2.15	1.08e-0	7.10e-3	S
1,8-Cineole	-2.52	4.63e-1	3.00e-3	S	-2.59	3.98e-1	2.58e-3	S	-2.45	5.45e-1	3.53e-3	S
cis-Verbenyl acetate	-3.26	1.06e-1	5.47e-4	S	-3.97	2.06e-2	1.06e-4	S	-2.11	1.51e-0	7.78e-3	S
Acarbose	2.13	8.61e+4	1.33e+2	HS	2.56	2.32e+5	3.60e+2	HS	6.40	1.62e+9	2.51e+6	S

Table 24. Pharmacokinetics parameters of the phytoconstituents of essential oils

Proprieties	Santolina triene	trans-Thujone	1,8-Cineole	cis-Verbenyl acetate	Acarbose
GI absorption	Low	High	High	Low	Low
BBB permeant	Yes	Yes	Yes	Yes	No
P-gp substrate	No	No	No	No	Yes
CYP1A2 inhibitor	No	No	No	No	No
CYP2C19 inhibitor	No	No	No	No	No
CYP2C9 inhibitor	No	No	No	Yes	No
CYP2D6 inhibitor	No	No	No	No	No
CYP3A4 inhibitor	No	No	No	No	No
Log Kp (Skin Permeation) (cm/s)	-4.13	-5.62	-5.30	-4.84	-16.29

Table 25. Druglikeness rule and bioavailability score of the phytoconstituents of essential oils

Proprieties	Santolina triene	trans-Thujone	1,8-Cineole	cis-Verbenyl acetate	Acarbose
Lipinski	Yes; 0 violations	Yes; 0 violations	Yes; 0 violations	Yes; 0 violations	No; 3 violations: MW>500, NorO>10, NHorOH>5
Ghose	No; 1 violation: MW<160	No; 1 violation: MW<160	No; 1 violation: MW<160	Yes	No; 4 violations: MW>480, WLOGP<-0.4, MR>130, #atoms>70
Veber	Yes	Yes	Yes	Yes	No; 1 violation: TPSA>140
Egan	Yes	Yes	Yes	Yes	No; 1 violation: TPSA>131.6
Muegge	No; 2 violations: MW<200, XLOGP3>3.5	No; 2 violations: MW<200, Heteroatoms<2	No; 2 violations: MW<200, Heteroatoms<2	No; 1 violation: MW<200,	No; 5 violations: MW>600, XLOGP3<-2, TPSA>150, H-acc>10, H-don>5
Bioavailability score	0.55	0.55	0.55	0.55	0.17

Table 26. Medicinal Chemistry properties of the Phytoconstituents of essential oils

Proprieties	Santolina triene	trans-Thujone	1,8-Cineole	cis-Verbenyl acetate	Acarbose
PAINS	0 alert	0 alert	0 alert	0 alert	0 alert
Brenk	1 alert: isolated_alkene	0 alert	0 alert	1 alert: isolated_alkene	1 alert: isolated_alkene
Leadlikeness	No; 2 violations: MW<250, XLOGP3>3.5	No; 1 violation: MW<250	No; 1 violation: MW<250	No; 2 violations: MW<250, XLOGP3>3.5	No; 2 violations: MW>350, Rotors>7
Synthetic accessibility	3.22	2.79	3.65	4.50	7.34

Lipophilicity property of the compounds portrays an important role for molecular discovery activities in multifarious domains. The quantitative descriptor of the lipophilicity is the partition coefficient P of a given molecule between *n*-octanol and water system (Daina et al., 2014). Because of its amphiphilic nature, *n*-octanol is considered a good mimic of phospholipid membrane characteristics (Liu et al., 2011). Multifarious algorithms are accessible to compute log Po/w, which rely on factual methodologies. The classic log P predictors branched in to two division, first ones split molecular structures into molecular fragments includes fragmental approach e.g. KLOGP (Klopman et al., 1993), KOWWNIN (Meylan & Howard, 2000) or atomic approach e.g. ALOGP (Ghose et al., 1998; R. Wang et al., 1997), XLOGP (Cheng et al., 2007; Moriguchi et al., 1994). The second division gathers the topological methods in which, the molecules description is related to its topology being as count or flags for specific atoms, groups or structural properties e.g. MLOGP (Brenk et al., 2008; Moriguchi et al., 1992), the prediction attained by manifold linear regression trained on large molecular data sets. The SILICOS-IT is a hybrid technique which combines both molecular fragments and topological parameters, which confide on 27 fragments and 7 topological descriptors, it was disciplined on 23,455 molecules with experimental *n*-octanol/water partition values (Daina et al., 2014). The version three of the XLOGP atomic model is established on a system of 87 fragments and two corrective factors. If the input structures are similar to a reference compound, the fragments differentiating them are treated and the corresponding log P contributions added to the reference structure log P value (Cheng et al., 2007). Lipophilicity estimated as consensus Log P, which is the average value of all Log P evaluated with various lipophilicity criteria, determined acarbose as most lipophilic whereas santolina triene as least lipophilic and water solubility of the small molecules ranged from highly water soluble (Acarbose) to water soluble.

The SwissADME model returns “Yes” or “No” if the compound under examination has greater probability to be a substrate or non-substrate of P-gp or inhibitor or non-inhibitor of Cytochrome P450 isoenzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4).

The pharmacokinetics and drug likeness performed using SwissADME showed a low level of GI absorption and BBB permeant with santolina triene and cis-Verbenyl acetate while a high absorption detected with trans-Thujone and 1,8-Cineole. All the compounds present in the essential oils are not the substrates for P-gp except contrarily to the Acarbose (Table 12), so they are not susceptible to the efflux mechanism performed by this transporter which is used by many tumours cell lines as a drug-resistance mechanism (Ranjith & Ravikumar, 2019)

All of the small molecules returned as non-inhibitors for inactivation for CYP isoenzymes. The skin permeability coefficient (Log Kp), a multiple linear regression, the more negative the log Kp (with Kp in cm/s), the less skin permeant is the molecule. Among the phytoconstituents, trans-Thujone (-5.62) is the least permeant and santolina triene (-4.13) is highly permeant respectively. This SwissADME section gives access to five different rule-based filters, with diverse ranges of properties inside of which the molecule is defined as drug-like. The Lipinski (Pfizer) filter is the pioneer rule-of-five implemented and with the Ghose (Amgen), Veber (GSK), Egan (Pharmacia) and Muegge (Bayer) methods. Multiple estimations allow consensus views or selection of methods best fitting the end-user's specific needs in terms of chemical space or project-related demands. Any violation of any rule described here appears explicitly in the output panel. All the four compounds followed the filtered rule invoked in the SwissADME; the violation shown by the molecules are minimal.

SwissADME interpretation posts 0 PAINS alert of the 4 studied compounds. Brenk considered compounds that are smaller and less hydrophobic and not those defined by "Lipinski's rule of 5" to widen opportunities for lead optimization. This was after exclusion of compounds with potentially mutagenic, reactive and unfavourable groups such as nitro groups, sulphates, phosphates, 2-halopyridines and thiols (Brenk et al., 2008). All the compounds examined flouted Brenk's rule with only one alert, all the compounds failed Lead-likeness criteria due to their molar weight.

In silico toxicity study aims to help in optimizing compounds regarding their toxicity proprieties. The study could offer an important improvement to the awareness of the full perspective of virtual screening for the identification of target compounds with negligible or no toxicity, which may open a path for the selection of novel nontoxic phytoconstituents present in *Cotula cinerea* and *Origanum Majorana L* essential oils with high antidiabetic activity. In silico toxicity study of the chosen compounds was performed using the ProTox-II web server (Drwal et al., 2014). It aims to predict hepatotoxicity (Dili), carcinogenicity (Carcino), immunotoxicity (Immuno), mutagenicity (Mutagen), cytotoxicity (Cyto), median lethal dose (LD50), and toxicity class (TC).

According to in silico toxicity profiles presented in Table 15, the toxicity class of all the phytoconstituents was detected to be equal to 5 except the trans-Thujone which predicted to be 4. Santolina triene, trans-Thujone**,** 1,8-Cineole and cis-Verbenyl acetate were predicted to be nontoxic except in immunotoxicity for the last wrote compound. Acarbose the standard of type 2 diabetes was predicted to be immunotoxin with a toxicity class of 6.

Table 27. In silico toxicity profiles of the studied compounds

Molecule	Dili	Carcino	Immuno	Mutagen	Cyto	LD_{50} ($mg.Kg^{-1}$)	TC
Santolina triene	Inactive	Active	Inactive	Inactive	Inactive	2610	5
trans-Thujone	Inactive	Inactive	Inactive	Inactive	Inactive	500	4
1,8-Cineole	Inactive	Inactive	Inactive	Inactive	Inactive	2480	5
cis-Verbenyl acetate	Inactive	Inactive	Active	Inactive	Inactive	2600	5
Acarbose	Active	Inactive	Active	Inactive	Inactive	24000	6

6.2. Molecular Docking Study:

In the current study , the binding interaction of all the major compounds presented in *Cotula cinerea* and *Origanum Majorana L* essential oils compounds with α-amylase was further investigated by carrying out a molecular docking analysis of the binding interaction between human pancreatic α-amylase with compounds: trans thujone, alpha-Terpineol, beta-Terpineol, cis Verbenyl acetate, Camphor, Sabinene, gamma terpinene, alpha-Pinene, 1,8-Cineole, Santolina triene, alpha-Thujene and Beta Pinene oxide **(**the major phytochemicals present in the two plants with yield > 1) using Maestro version 11.7 user interface of the Schrödinger suite (Small-Molecule Drug Discovery Suite 2021-4, Schrödinger, LLC, New York, NY, 2021) (Schrödinger, 2015).

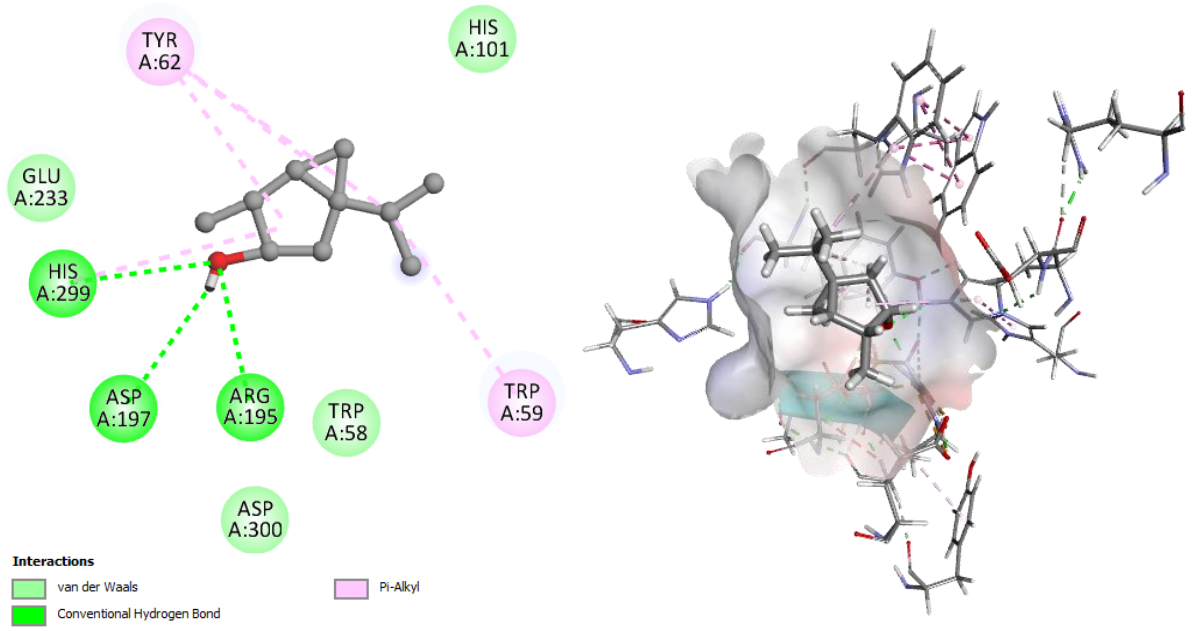
Each compound was docked with human pancreatic α-amylase (HPA) to understand their interactions at the enzyme's active site. Acarbose, a standard type 2 antidiabetic drug and HPA inhibitor, was likewise docked with HPA to serve as positive control. Of all the major compounds present in *Cotula cinerea* and *Origanum Majorana L* essential oils, the binding affinities of them for α-amylase were comparable to that of acarbose (IFD score = □□.259 kcal/mol) (Table 16). This result indicates that the plant is a rich source of compounds which, either individually or synergistically, resulted in the decrease of α-amylase activity as observed in the *in vitro* and *in vivo* experimental α-amylase inhibition study. Also, the interactions were spontaneous as observed from the negative Gibb's free energy values (Avwioroko et al., 2020).

From studies on the X-ray crystal structure and enzyme kinetics of HPA, it was found that HPA active site is characterized by the existence of three special amino acid residues namely ASP-197, GLU-233 and ASP-300 which contribute to the overall catalytic action of the enzyme towards starch hydrolysis (Anigboro et al., 2021). ASP-197 acts as a catalytic nucleophile during starch hydrolysis,GLU-233 residue acts as acid–base catalyst, while ASP-300 plays the role of optimizing the orientation of the substrate (Anigboro et al., 2021).

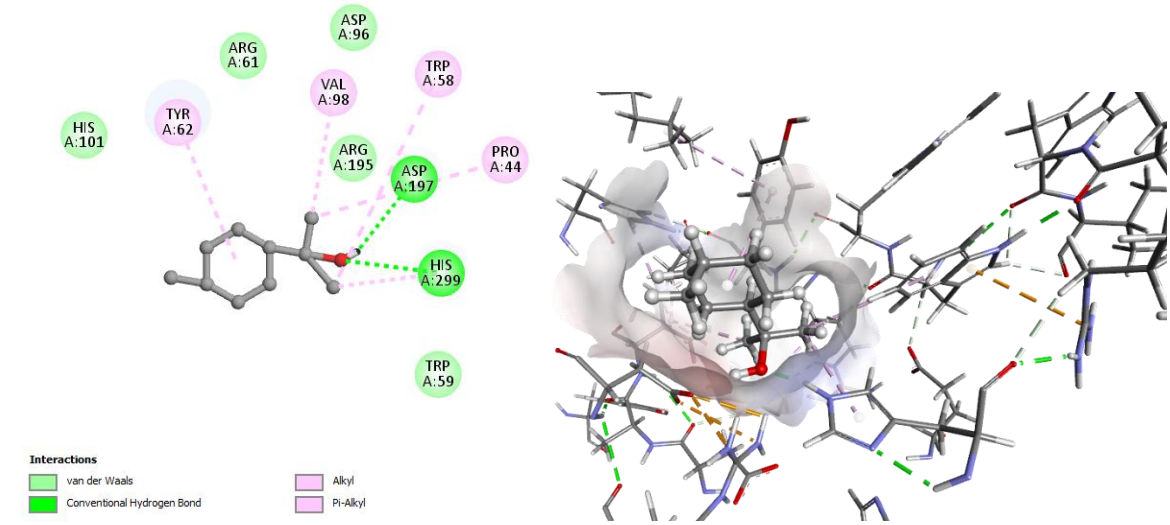
Table 28. Docking score of the studied compounds

Compound	Rigid Docking Score (Kcal/mol)	IFD scores (Kcal/mol)
trans thujone	-5.569	-6.679
alpha-Terpineol	-5.052	-6.162
beta-Terpineol	-5.033	-6.143
cis Verbenyl acetate	-4.944	-6.054
Camphor	-4.505	-5.615
Sabinene	-4.365	-5.475
gamma terpinene	-4.185	-5.295
alpha-Pinene	-3.884	-4.994
1,8-Cineole	-3.706	-4.816
Santolina triene	-2.986	-4.096
alpha-Thujene	-4.41	-5.522
Beta Pinene oxide	-4.115	-5.225
Acarbose	-3.213	-5.259

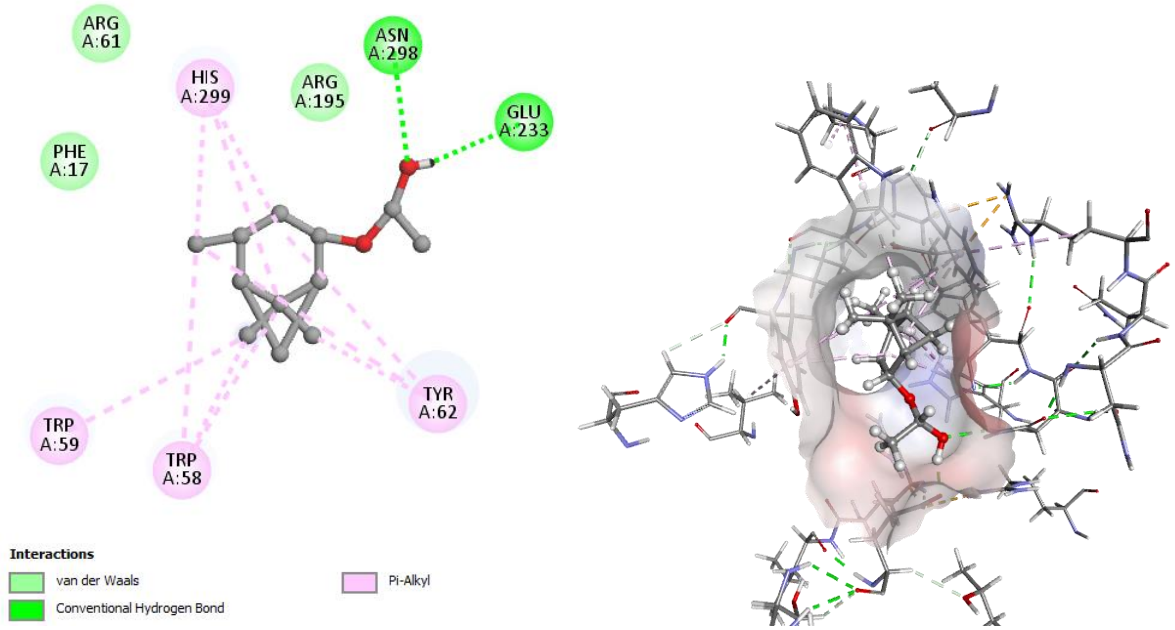
The results in Figure 9 revealed that the best two compounds in each plant interacted with a similar set of amino acid residues at the active site of the protein just as the standard HPA inhibitor did. It is noticed in their interaction with the enzyme's key catalytic residues (ASP-197, GLU-233 and ASP-300). compound trans thujone in addition to the standard (acarbose) showed interaction with the three key amino acids involved in catalysis at HPA active site, whereas alpha-Terpineol showed interaction with two of the catalytic triads (GLU-197 and ASP-197). It is quite surprising to note that cis Verbenyl acetate show interaction with only one of the three key α-amylase catalytic residues even though it had the second highest binding affinity to α-amylase compared to the other compounds. This perhaps denotes that the inhibitory effect contributed by cis Verbenyl acetate to the overall inhibitory effect of the other compounds may be that of non-competitive inhibition whereas that of other compounds including trans thujone (the compound with the best binding affinity) may be that of competitive mode of inhibition like that of acarbose. Overall, the *in-silico* analysis of the ligand protein interaction of these compounds with amino acid residues present in the α-amylase active site domain (such as TRP-58, TRP-59, TYR-62, GLN-63, ASP-197, GLU-233, ASP-300 etc.) gave credence to the inhibitory effect of the compounds reported in this study. Furthermore, it also confirmed the possibility of the binding interaction between α-amylase and the phytoconstituents of *Cotula cinerea* and *Origanum Majorana L* essential oils investigated and observed using spectroscopic methods reported. The best ligand-binding poses in the catalytic domain of HPA obtained after docking as well as the amino acid residues involved in the interaction are as illustrated in Figure 9.



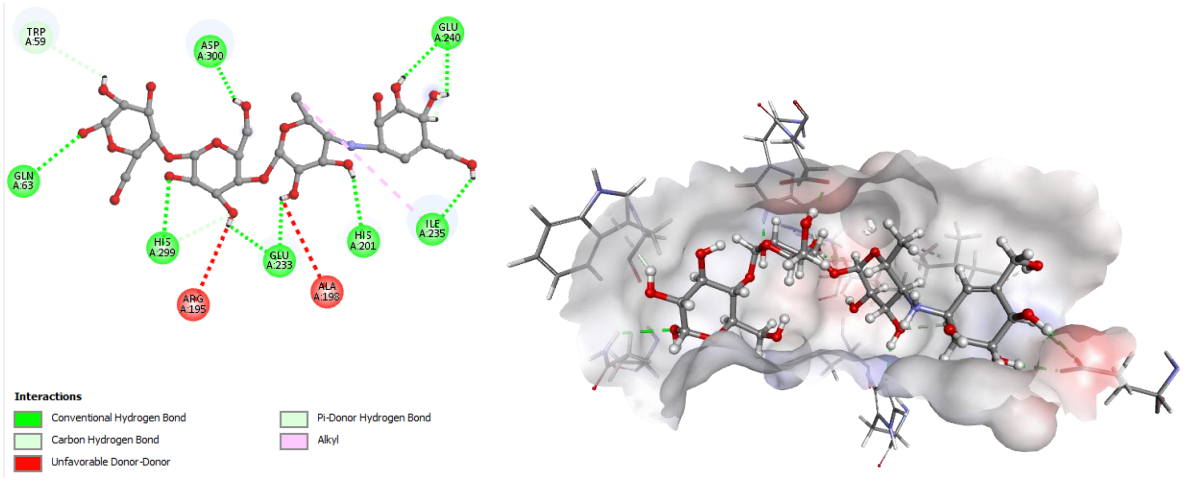
Docking complex and interaction plot for compound trans- thujone with HPA



Docking complex and interaction plot for compound alpha-Terpineol with HPA



Docking complex and interaction plot for compound cis Verbenyl acetate with HPA



Docking complex and interaction plot for Acarbose with HPA

Figure 30. Molecular interactions of studied compounds & Acarbose with humane pancreatic α-amylase

6.3. Molecular Dynamics Simulation:

Molecular Dynamics Simulation (MDS) investigations were conducted for the premier compound show the best IFD score, trans- thujone, in complex with α-amylase. The principal objective of these MDS endeavours was to subject the ligand-receptor complex to physiological conditions, a feat unattainable through the confines of molecular docking (Dumitrică & James, 2007). Throughout the MDS protocol, a trajectory frame was generated at intervals of 100 picoseconds (Tu et al., 2008), accumulating a total of 1000 frames over the course of a 100-nanosecond simulation. Analysis encompassed metrics such as 'Root Mean Square Deviation

(RMSD), and 'Root Mean Square Fluctuation (RMSF). Specifically, RMSD values were computed by aligning the frames of the protein and ligand-protein complex with the reference frame, respectively. These analytical approaches provide a detailed understanding of the structural stability and dynamic fluctuations exhibited by the Acarbose-α-amylase complex throughout the simulation period (Schreiner et al., 2012).

The Root Mean Square Deviation (RMSD) values for the complex consistently fall within the acceptable range of 0 – 2 Å. Examination of the analysed complex revealed no significant conformational alterations; however, an initial minor deviation was discerned in the vicinity of 8 – 20 nanoseconds, and a subtle drift was observed approximately between 45 – 55 nanoseconds. Beyond these periods, the complex demonstrated marked stability throughout the entirety of the simulation process, with no notable occurrence of major conformational changes. (Figure 10).

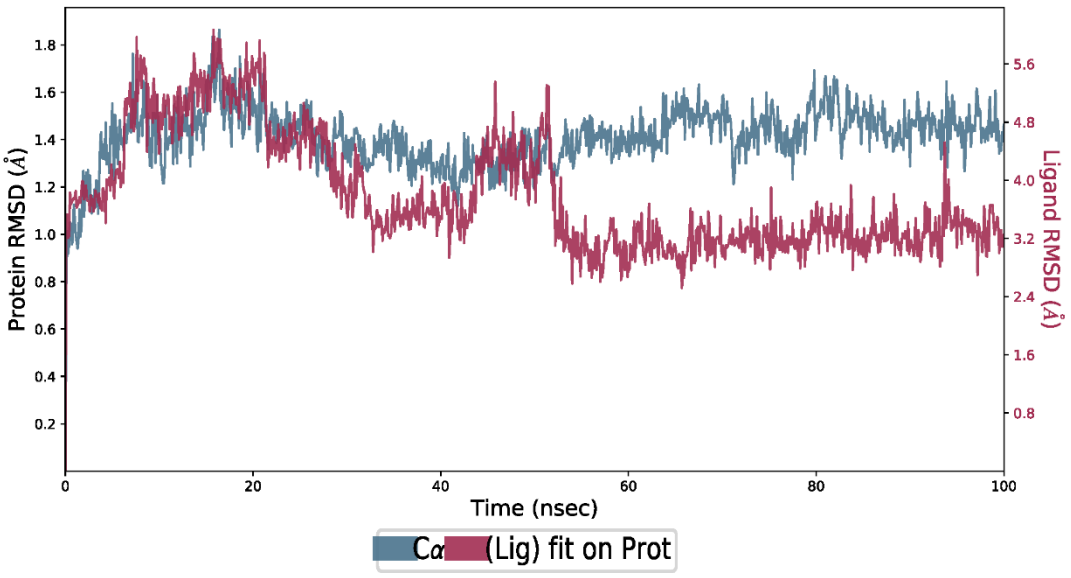


Figure 31. RMSD plot of trans- thujone and α_amylase complex

The Root Mean Square Fluctuation (RMSF) analysis provides insights into local variations along the protein chain, facilitating the identification of residues contributing to structural fluctuations within the complex (Dey et al., 2021). In the examined complex, the observed fluctuation variation remained below 3 Å. Major fluctuations (2.20-2.63 and 1.81-2.96Å) were observed in the C-terminal and loop regions (223-225 and 363-366), respectively, which are positioned away from the binding pocket of alpha amylase. Except for the loop regions, the RMSF values of most residues are less than 1.5 Å, indicating that the residue conformation is relatively stable during the simulation. A graphical representation of the RMSF plot depicting

the specific ligand interactions with amino acid residues in the protein is presented in Figure 32.

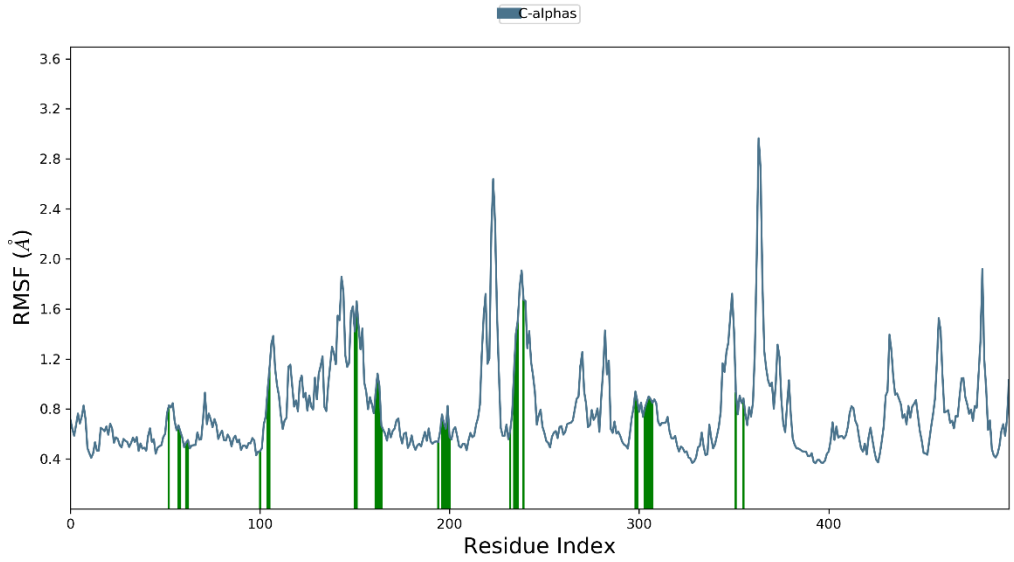


Figure 32. RMSF plot of trans- thujone and α_amylase complex

Within the investigated complex, notable hydrogen bond interactions were observed involving HIS299, ASP197, and TRP58. Additionally, water-mediated hydrogen bond interactions were formed, encompassing the same residues, as visually depicted in Figure 33.

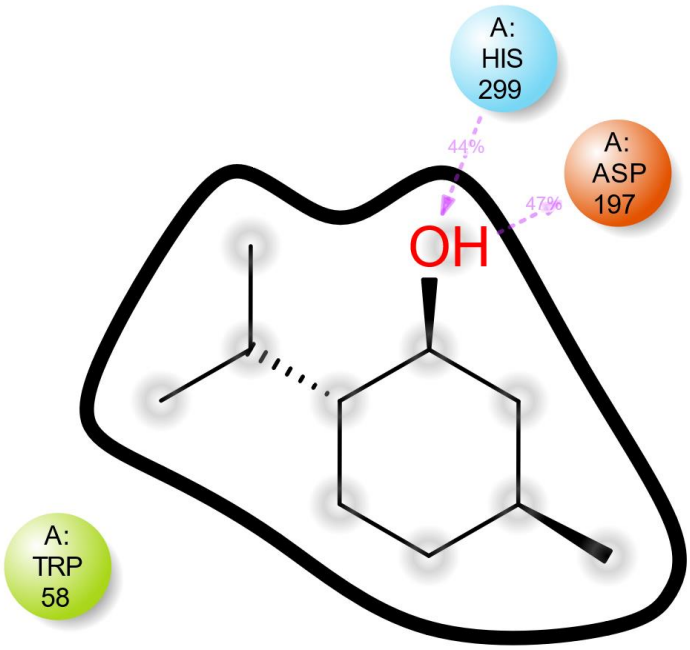


Figure 33. Interaction diagram of protein-ligand after MDS

The analysis of Protein-ligand contact serves to elucidate the temporal continuity of interactions between the ligand and protein amino acids throughout the simulation. A numerical representation is employed, where a value of 0.5 denotes that a specific interaction was sustained for 50% of the simulation duration. Conversely, a value exceeding 1 suggests that protein amino acids may establish multiple contacts of the same subtype with the ligand (Hatmal & Taha, 2017).

In the context of the investigated complex, the interaction values for the residues GLU240, HIS201, ASP197, GLN63, GLU233, and THR163 are recorded as 1.25, 1, 1.75, 1, 0.60, and 1.5, respectively. These values signify varying degrees of sustained interactions between the ligand and corresponding amino acid residues. The graphical representation of the protein-ligand contact histograms for the complex is depicted in Figure 34 for visual elucidation.

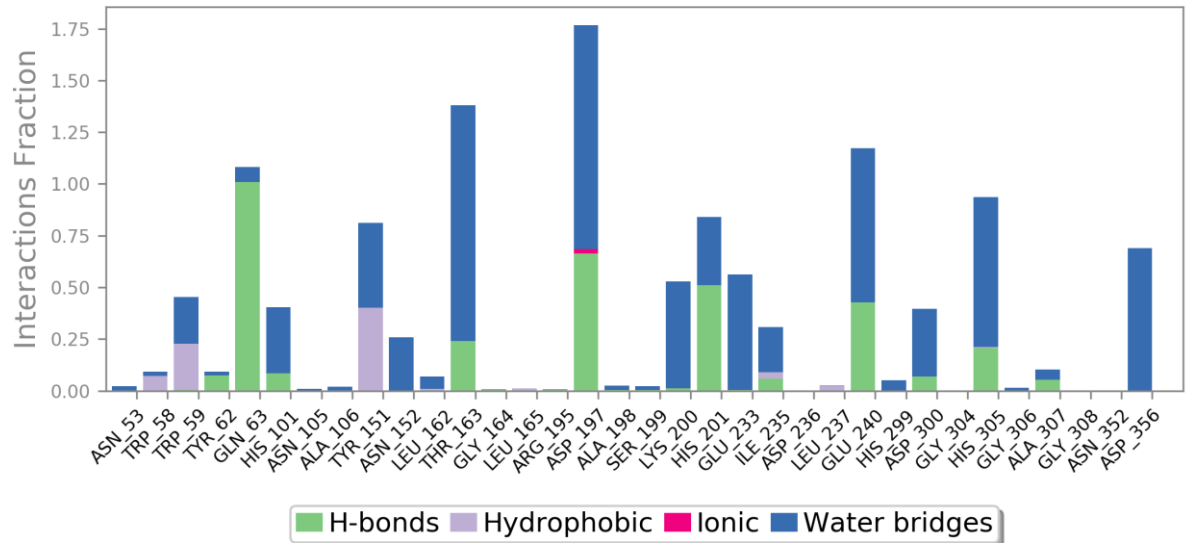


Figure 34. Histogram of protein-ligand complex

To comprehensively investigate the interactions between the ligand and alpha-amylase receptor, two supplementary panels were introduced by Schrödinger post Molecular Dynamics Simulation (MDS), as depicted in Figure 35. The upper panel provides insights into the total number of specific contacts established by the protein with the ligand throughout the trajectory. Conversely, the lower panel delineates the residues engaged in interactions with the ligand in each trajectory frame.

Notably, certain residues exhibit multiple specific contacts with the ligand, visualized through varying shades of orange on the plot, in accordance with the scale positioned to the right of the diagram. This nuanced representation captures the dynamic nature of the interactions, offering a detailed portrayal of the residues that consistently contribute to the ligand-receptor interface.

The incorporation of these supplementary panels enhances the granularity of our understanding of the molecular dynamics and specific contacts governing the ligand-receptor interaction over the course of the simulation.

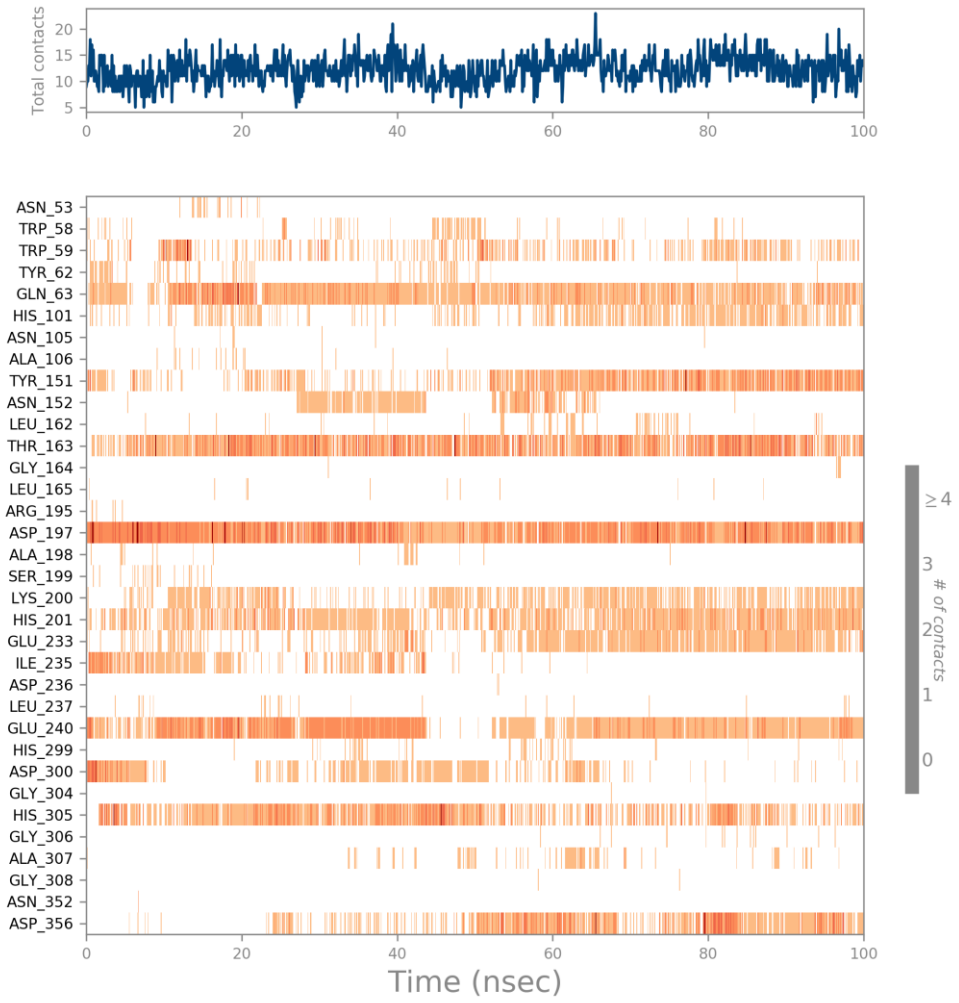


Figure 35. Details of the protein ligand contact

6.4. Free Energy (MM-GBSA) Calculation:

To evaluate the binding efficacy of the preeminent compound, trans- thujone, with the alpha-amylase protein, the Prime-MM-GBSA method was applied to compute the binding free energies. The establishment of stable complexes is evidenced by favourable binding interactions between the compound and the protein. The determination of binding free energy entails the consideration of various energy components, encompassing van der Waals (vdW), lipophilic (lipo), Generalized Born electrostatic solvation (Solv GB), Coulomb, and hydrogen-bonding (hbond) energies. The discrete contributions of these energy components to the overall binding free energy of the trans- thujone -alpha-amylase complex are delineated in Table 35.

Table 29. Energy components of the studied complex

Component	Energy (kcal/mol)
ΔG Bind	-61.14
ΔG Bind Coulomb	-30.03
ΔG Bind Solv GB	48.10
ΔG Bind vdW	-54.91
ΔG Bind lipo	-20.51
ΔG Bind hbond	-3.79

Notably, ΔG Bind is calculated at -61.14 kcal/mol, indicating a robust favourable binding interaction. Individual positive contributions include Coulombic energy at -30.03 kcal/mol, van der Waals interactions at -54.91 kcal/mol, lipophilic energy at -20.51 kcal/mol, and hydrogen-bonding energy at -3.79 kcal/mol. However, solvation energy through Generalized Born model contributed negatively at 48.10 kcal/mol. This collective interplay of energy components underscores the strong binding affinity observed in the complex.

CONCLUSION

Conclusion:

Phytotherapy has experienced significant growth in recent years. This interest in phytotherapy is explained by the need to return to nature and the significant benefits associated with reducing adverse effects. In this subject, we focused on the study of two plants. Cotula cinerea, Origanum majorana L, We have extracted the essential oil from plants. The components of these essential oils have been identified.. Study in vitro and in vivo and in silico on the inhibitory effect of essential oils on the enzymatic activity of α-amylase, partially responsible for the increase in blood sugar levels and the onset of diabetes

The results obtained in both laboratories have shown that plant essential oils Cotula cinerea Origanum majorana L have an effective effect on diabetes activity by inhibiting amylase activity

. In vivo results on experimental mice have shown that essential oils have a significant effect on adjusting blood sugar levels.

n silico tests confirmed the results using SwissADME and molecular dynamics simulation Protox Docking. The compounds present in the essential oil have good medicinal properties.

. The entirety of this study work has allowed for a better understanding of the interest of local medicinal plants in the regulation of blood sugar levels. However, it remains preliminary and opens up numerous perspectives.

Perspective :

- The results of this study have opened up new research horizons on the uses of essential oils from plants to treat diseases such as diabetes. Further studies can be conducted to improve, for example, by studying other biological activities such as antimicrobial, antibacterial, antioxidant, anti-inflammatory, and anticancer activities.
- Using other techniques in chemical analysis, such as (GC-FTR , LC-MS, RMN…..)
- It would also be interesting to conduct in vivo tests on different biological models to find a therapeutic application of the isolated active molecules and to study the synergistic effect of the essential oil to improve the therapeutic index.

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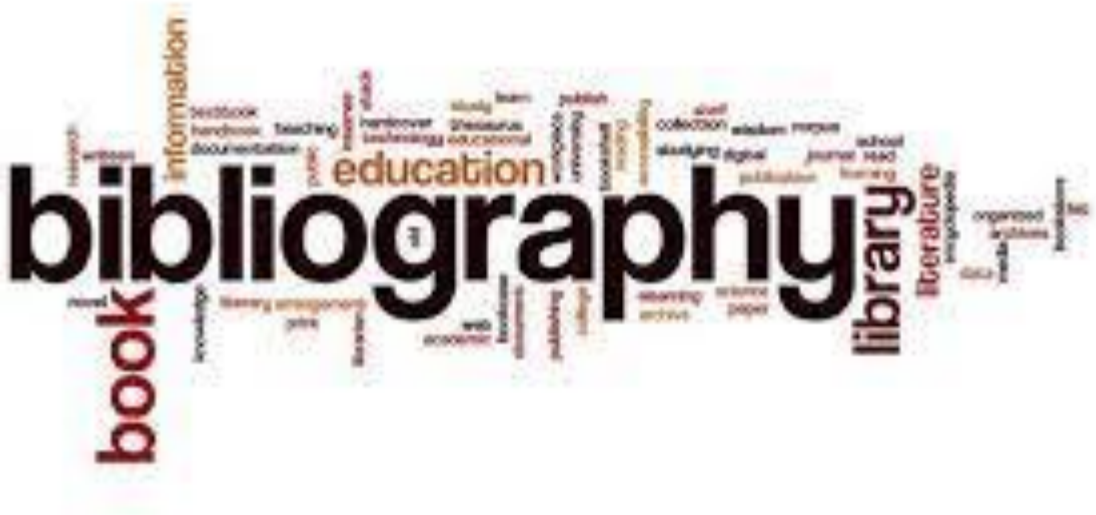
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Appendices

Figure 36 : Rat cages **(Original photo, 2024).**

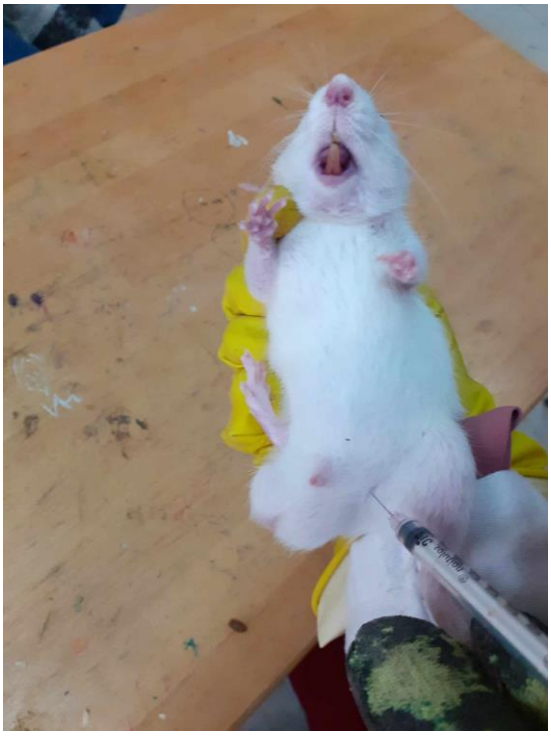

Figure 37 : Injection intrapéritonéal **(Photo originale, 2024).**

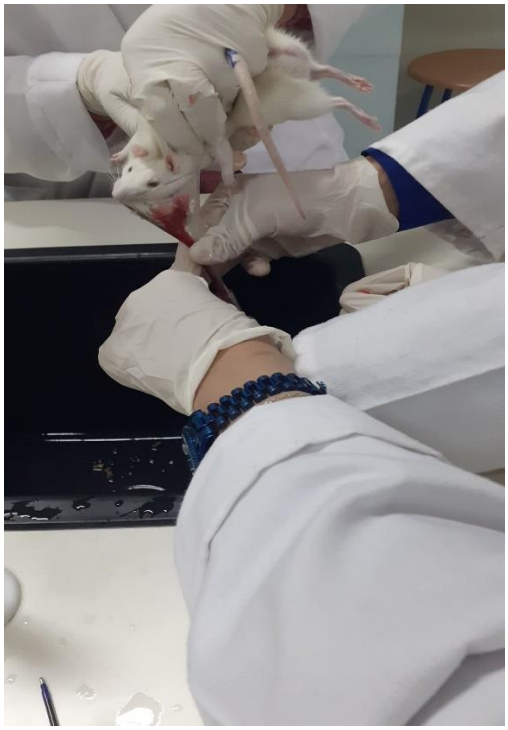

Figure 38 . Sacrifice, blood drawing **(Original photo, 2024).**

Figure 39. Dissection et prélèvement des organes **(Photo originale, 2024).**

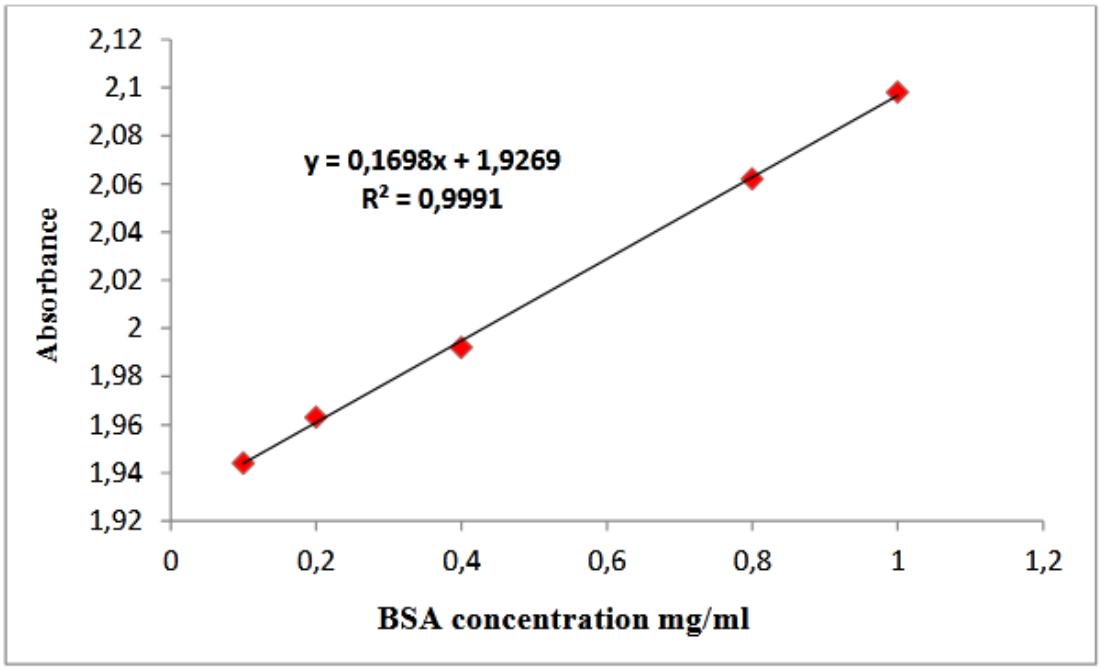

Figure 40: Calibration curve of BSA for determination of protein level