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**Integrated, *In Vitro*, *In Silico* and *In Vivo* Evaluation of Essential Oils from  
Two Medicinal Plants: *Cotula cinerea* and *Origanum Majorana L*  
GC/MS Characterization, BSA Denaturation, and Molecular Approaches**

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## DEDICACE

In the name of Allah, the Most Gracious, the Most Merciful  
All praise is due to Allah, by whose grace good deeds are completed, and through whose  
guidance the fruits of hard work and perseverance are harvested.

To my dear parents,

You who have been the light of my path and the brightness of my journey,  
I offer you my deepest gratitude and appreciation, and I dedicate to you the fruit of years of  
struggle and heartfelt prayers.

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O source of tenderness, and the most beautiful gift from the Most Merciful,  
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With sincere regards,

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## Dedication

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## Dedication

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Your daughter/sister/wife/friend...

 *Nada Raihane*

**Abstract :**

This study aimed to evaluate the therapeutic potential of essential oils extracted from *Cotula cinerea* and *Origanum marjoram* in the treatment of inflammation, through a comprehensive scientific approach combining chemical analysis, *In Vitro* biological assays, and *In Silico* molecular modeling. The essential oils were extracted and their active components analyzed using gas chromatography-mass spectrometry (GC/MS), which revealed a variety of biologically active compounds. The anti-inflammatory activity was assessed by evaluating the oils' ability to inhibit the denaturation of bovine serum albumin (BSA) as an indicator of efficacy under laboratory conditions. Furthermore, computational simulations were used to explore the binding mechanisms of the main compounds with specific inflammatory receptors and target proteins. To support these findings, an experimental animal model was conducted using Wistar rats in which inflammation was induced by benzylthiouracil (BTU), followed by treatment with either *Cotula cinerea* or *Origanum marjoram* essential oils, and compared to a group treated with ibuprofen. The analyses included hematological and biochemical assessments (such as glucose, CRP, AST, urea, and creatinine), in addition to histopathological examination of target organs (liver and kidneys). The results demonstrated that the essential oils of both plants exhibited anti-inflammatory activity to varying degrees, further supported by *In Silico* findings that elucidated potential molecular mechanisms of action. These results suggest that the essential oils of *Cotula cinerea* and *Origanum marjoram* represent promising natural sources of anti-inflammatory compounds and may contribute to the development of effective and less toxic therapeutic alternatives to conventional drugs.

**Key word:** *Cotula cinerea*, *Origanum marjoram*, chromatography-mass spectrometry (GC/MS), (BSA), (BTU).

## Résumé :

Cette étude visait à évaluer le potentiel thérapeutique des huiles essentielles extraites de *Cotula cinerea* et d'*Origanum marjoram* dans le traitement de l'inflammation, à travers une approche scientifique complète combinant l'analyse chimique, les essais biologiques *In Vitro* et la modélisation moléculaire *In Silico*. Les huiles essentielles ont été extraites, puis leurs composants actifs analysés par chromatographie en phase gazeuse couplée à la spectrométrie de masse (GC/MS), révélant une diversité de composés biologiquement actifs. L'activité anti-inflammatoire a été évaluée par la capacité des huiles à inhiber la dénaturation de l'albumine sérique bovine (BSA), utilisée comme indicateur d'efficacité dans des conditions de laboratoire. De plus, des simulations informatiques ont été utilisées pour explorer les mécanismes de liaison des principaux composés avec des récepteurs inflammatoires spécifiques et des protéines cibles. Pour appuyer ces résultats, un modèle animal expérimental a été mis en œuvre sur des rats Wistar chez lesquels une inflammation a été induite par le benzylthiouracile (BTU), suivie d'un traitement soit par l'huile essentielle de *Cotula cinerea*, soit par celle d'*Origanum marjoram*, et comparée à un groupe traité avec de l'ibuprofène. Les analyses ont inclus des évaluations hématologiques et biochimiques (comme le glucose, la CRP, les transaminases AST, l'urée et la créatinine), ainsi qu'un examen histopathologique des organes cibles (foie et reins). Les résultats ont démontré que les huiles essentielles des deux plantes présentaient une activité anti-inflammatoire à des degrés divers, étayée par des données *In Silico* ayant permis d'élucider de potentiels mécanismes d'action moléculaires. Ces résultats suggèrent que les huiles essentielles de *Cotula cinerea* et d'*Origanum marjoram* représentent des sources naturelles prometteuses de composés anti-inflammatoires, susceptibles de contribuer au développement d'alternatives thérapeutiques efficaces et moins toxiques que les médicaments conventionnels.

**Mots-clés :** *Cotula cinerea*, *Origanum marjoram*, chromatographie en phase gazeuse-spectrométrie de masse (GC/MS), (BSA), (BTU).

## المخلص

هدفت هذه الدراسة إلى تقييم الإمكانيات العلاجية للزيوت الأساسية المستخلصة من نباتي *Cotula cinerea* و *Origanum marjoram* في معالجة الالتهابات، من خلال منهج علمي شامل يجمع بين التحليل الكيميائي، والاختبارات البيولوجية المخبرية، والنمذجة الجزيئية الحاسوبية. (*In Silico*) تم استخراج الزيوت الأساسية وتحليل مكوناتها الفعالة باستخدام تقنية كروماتوغرافيا الغاز المقترنة بمطيافية الكتلة (GC/MS)، والتي كشفت عن مجموعة متنوعة من المركبات الفعالة بيولوجياً. تم تقييم النشاط المضاد للالتهاب من خلال قدرة الزيوت على تثبيط تحلل ألبومين مصل الأبقار (BSA)، باعتباره مؤشراً على الفعالية في الظروف المخبرية. كما تم استخدام المحاكاة الحاسوبية لدراسة آليات ارتباط المركبات الرئيسية بالمستقبلات الالتهابية والبروتينات المستهدفة. ولتعزيز هذه النتائج، تم إجراء نموذج تجريبي على حيوانات المختبر من نوع الجرذان (Wistar)، حيث تم تحفيز الالتهاب باستخدام مركب البنزيلثيووراسيل (BTU)، تلاه العلاج بزيوت *Cotula cinerea* أو *Origanum marjoram*، وتمت المقارنة مع مجموعة عولجت بعقار الإيبوبروفين. شملت التحاليل تقييمات دموية وكيميائية حيوية) مثل الجلوكوز، البروتين التفاعلي "CRP" -C، إنزيم AST، اليوريا، والكرياتينين، بالإضافة إلى الفحص النسيجي المرضي للأعضاء المستهدفة (الكبد والكليتين). وأظهرت النتائج أن الزيوت الأساسية للنباتين أظهرت نشاطاً مضاداً للالتهاب بدرجات متفاوتة، وهو ما أكدته التحليلات الحاسوبية التي كشفت عن آليات جزيئية محتملة لعملها. تشير هذه النتائج إلى أن الزيوت الأساسية لنباتي *Cotula cinerea* و *Origanum marjoram* تُعدّ مصدراً طبيعياً واعداً لمركبات مضادة للالتهاب، وقد تسهم في تطوير بدائل علاجية فعّالة وأقل سمية من الأدوية التقليدية.

**الكلمات المفتاحية:** *Origanum marjoram*، *Cotula cinerea*، كروماتوغرافيا الغاز-مطيافية الكتلة

(GC/MS)، (BSA)، (BTU).

## List of Abbreviations:

°C: Degrees Celsius

μL: Microliter

Å<sup>2</sup>: square angstrom

AFNOR: Association Française de Normalisation

ALP: Alkaline Phosphatase

ALT: Alanine Aminotransferase

AST: Aspartate Aminotransferase

BSA: Bovine Serum Albumin

BUN: Blood Urea Nitrogen

CAT: Catalase

Crp: Creatinine

DMSO: Dimethyl Sulfoxide

eV: Electron Volt

g: Gram

GC/MS: Gas Chromatography-Mass Spectrometry

GC: Gas Chromatography

GPx: Glutathione Peroxidase

H<sub>2</sub>O: Water

HCl: Hydrochloric Acid

Ia: Acid Index

Ie: Ester Index

KOH: Potassium Hydroxide

LD<sub>50</sub>: median lethal dose

Log K<sub>p</sub>: The skin permeability coefficient

m<sub>o</sub>: Mass of the utilized plant sample

MDA: Malondialdehyde

mEO: Mass of the extracted essential oil

mL: Milliliter

MS: Mass Spectrometry

N<sub>2</sub>: Nitrogen

nm: Nanometer

Nm: Nanometer

PBS: Phosphate Buffer Solution

REO: Essential Oil Yield (Volumetric or Mass-based)

ROS:Reactive Oxygen Species

SD: Standard Deviation

SOD:Superoxide Dismutase

UV-Vis: Ultraviolet-Visible (Spectrophotometry)

VEO: Volume of the extracted essential oil

w/v: Weight/Volume

$\Delta G$ : the binding free energy

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# **Introduction**

### Introduction:

With increasing global concern regarding inflammatory disorders, the pace of research into novel therapeutic strategies has accelerated. Among these strategies, essential oils extracted from medicinal plants have garnered significant attention due to their complex chemical compositions and promising biological activities. This study addresses the urgent need for a comprehensive understanding of the anti-inflammatory potential of plant-based remedies by presenting an integrated *In Vivo*, *In Vitro*, and *In Silico* evaluation of essential oils derived from two selected medicinal plants. By combining gas chromatography-mass spectrometry (GC/MS) characterization, bovine serum albumin (BSA) denaturation assays, and molecular modeling methodologies, this study aims to elucidate the chemical profiles and underlying mechanisms of the anti-inflammatory activity of these essential oils, offering a robust and multifaceted perspective on their value. For centuries, medicinal plants have been invaluable sources of remedies for various ailments, including inflammatory conditions. Essential oils, the volatile aromatic constituents of these plants, represent a rich reservoir of bioactive compounds with potential therapeutic applications. This study delves into the anti-inflammatory properties of essential oils extracted from two carefully chosen medicinal plants, employing a synergistic approach that integrates experimental and computational methodologies. Through gas chromatography-mass spectrometry (GC/MS) analysis, *In Vitro* evaluation of bovine serum albumin (BSA) denaturation, and *In Silico* molecular modeling studies, we aim to comprehensively characterize the chemical composition and unravel the molecular mechanisms contributing to the anti-inflammatory effects of these plant-derived essential oils.

The search for effective and safe anti-inflammatory agents remains a critical area of scientific investigation. Essential oils derived from medicinal plants represent a compelling avenue for exploration due to their diverse array of bioactive molecules. To gain a comprehensive understanding of their anti-inflammatory potential, this research undertakes a multifaceted investigation encompassing the essential oils of two selected medicinal plants. This integrated approach involves gas chromatography-mass spectrometry (GC/MS) characterization to identify the major constituents, *In Vitro* bovine serum albumin (BSA) denaturation assays to evaluate anti-inflammatory activity, and *In Silico* molecular modeling studies to explore potential targets and mechanisms of action. The collective findings from these *In Vivo*, *In Vitro*, and *In Silico* analyses will provide a holistic assessment of the anti-inflammatory properties of the investigated essential oils.

**Chapter one:**  
**Medicinal Plants**

## 1. Introduction:

Among the most significant blessings granted to humanity is the natural environment, characterized by its ability to sustain diverse vegetation and agricultural crops in a wide spectrum of colors and forms. (Maathai, W., 2010) Thus, the Creator has instilled both aesthetic appeal and therapeutic value in plants, as referenced in numerous verses of the Qur'an. Since the earliest presence of humans on Earth, the use of plants and natural herbs for treatment and healing has been well established. In ancient civilizations, medicinal herbs were the primary means for addressing various ailments and health conditions. With the progression of time and the advancement of human civilization, the development of chemically synthesized pharmaceuticals began to emerge as a strong competitor to traditional herbal remedies, driven by scientific and technological progress. (Ghassan M., 1999). With the advancement of applied sciences and modern technology, societies gradually moved away from relying on herbal medicine, opting instead for chemically produced pharmaceuticals. However, in recent years, interest in herbal medicine has resurfaced, becoming a focal point of discussion among scholars, medical professionals, and patients. This resurgence has sparked both advocacy and skepticism within the scientific and medical communities. (J.Jeanfils.,1991)

## 2 . The studied plants:

### 2.1 .*Cotula cinerea* Plant:

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

*Cotula cinerea*, commonly referred to as Sheikh, is a plant species distinguished by its characteristic aroma, which closely resembles that of wormwood. Botanically, it is classified as an annual herb, marked by its densely lobed foliage and sturdy, pale-colored bristles. The plant typically blooms in the spring, producing yellow, disc-shaped flowers typical of the Asteraceae family. Sheikh is widely distributed in arid and desert environments, especially across Arab regions, and is recognized for its rich content of bioactive constituents, including flavonoids, terpenes, and volatile essential oils. (Bohlmann ,F., et al., 1973; Mahran ,G.H .,et al., 1976).

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

Taxonomical Classification and Nomenclature

**Scientific Name:** *Cotula cinerea* Del.

**Synonym:** *Brocchia cinerea* (Del.) Vis.

**Common Names:** Shihiya, Rubaita, Sheha al-Bal

**Table 1:** Vegetative Classification of *Cotula cinerea*

<b>Species</b>	Cinerea
<b>Gender</b>	Cotula
<b>Family</b>	Asteraceae (Compositae)
<b>Order</b>	Tubiflorals
<b>Class</b>	Dicotyledons
<b>Branch</b>	Angiosperms
<b>Reign</b>	Plants

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

It is a small herbaceous plant, typically ranging in height from 10 to 40 centimeters, and is characterized by a distinctive, sharp aroma. (Boning, C., 2010) The plant is adorned with fine white bristles, and its stems are either erect or slightly prostrate, exhibiting a cylindrical shape with a greenish-yellow tint. The leaves are pale green and densely covered with woolly bristles, characterized by their substantial thickness and trifoliate arrangement. Each leaf typically terminates in a three- to five-lobed apex, enhancing its distinctive morphology. The capitula are small and flattened, with a diameter ranging from 6 to 10 centimeters. (Naidu, V., 2012)The inflorescence consists entirely of tubular florets, arranged in a tetramerous and densely packed configuration, forming a compound flower head with an overlying capitulum. The buds begin as dark-colored and gradually transition to a golden yellow as they bloom. The fruits are sparse and unprotected, as illustrated in Figure (1) below. (Ahmed, A.A., et al., 1987; Malinskas, G. A. G., et al., 1987)



Figure 1: *Cotula cinerea* photograph

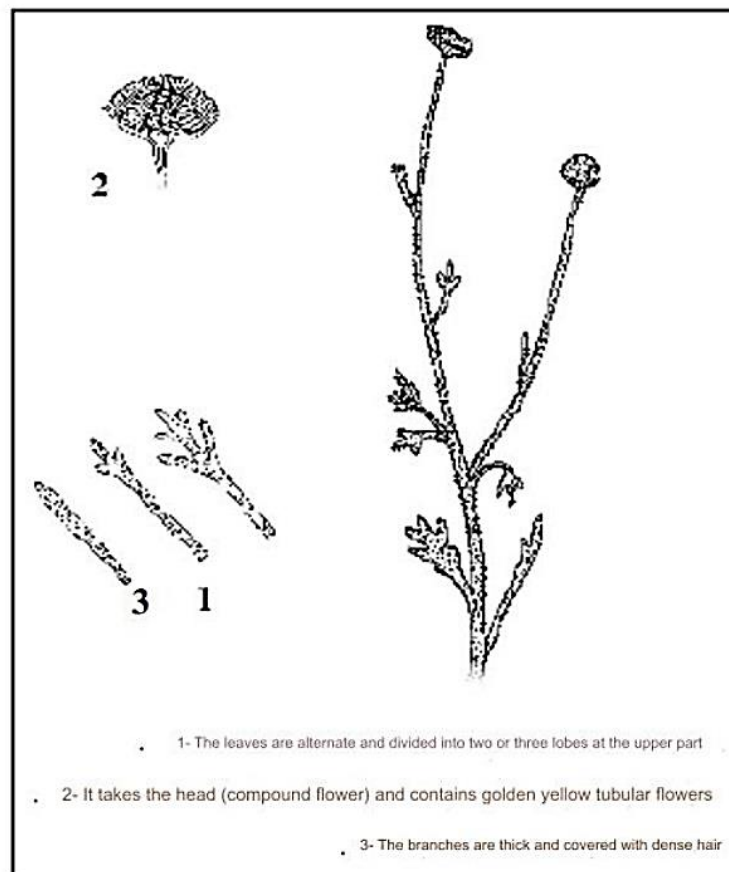
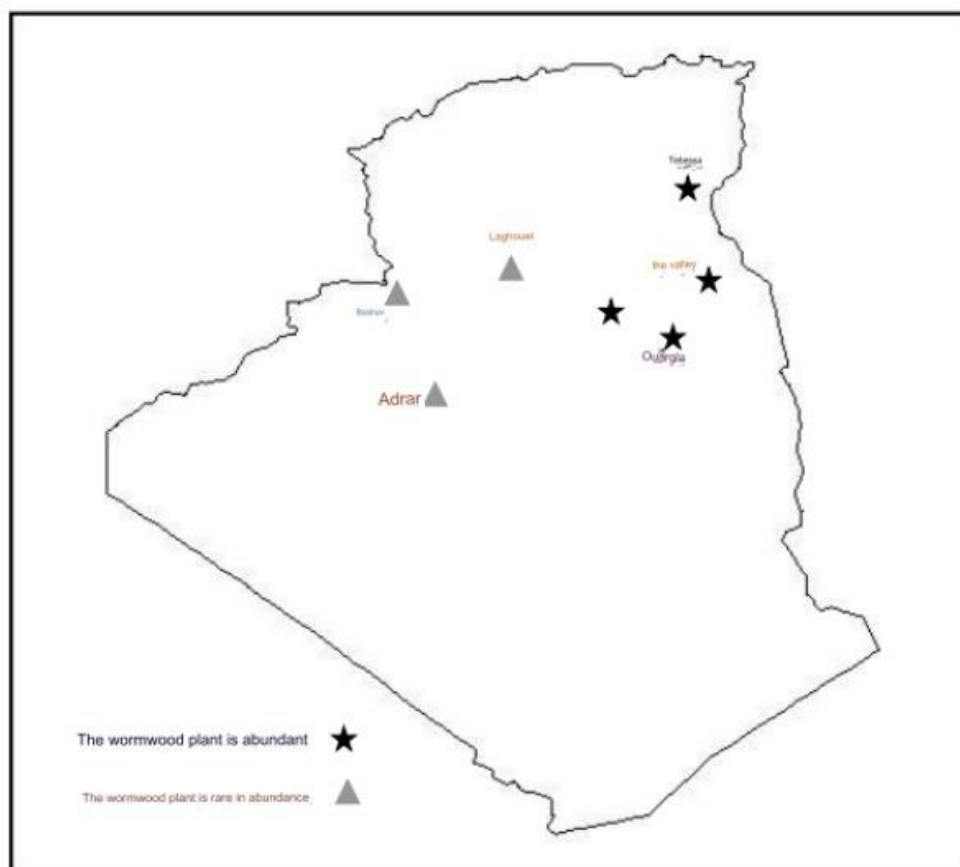


Figure 2: Diagram from of *Cotula cinerea*

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

The distribution of *Cotula cinerea* is widespread across the Southern Hemisphere, covering 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<sup>3</sup>, H., et al.). In Algeria, *Cotula cinerea* primarily occupies desert and semi-arid areas, with a particular prevalence in the southeastern regions. Figure 3 The map illustrates the geographical distribution of *Cotula cinerea* across Algeria.



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

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

In specific regions, *Cotula cinerea* is used as a flavoring agent in tea and coffee, as well as a soaking agent for beverages. Furthermore, it is utilized in traditional medicine for relieving abdominal pain and supporting digestive health. (M.Markouk, H.B et al.; Boulahbal F., et al., 2002). The efficacy of *Cotula cinerea* in treating bronchitis is linked to its cough-suppressing properties. Additionally, extracts of this plant have shown antifungal activity. (Bryskier A., et al., 1999). Flavonoids extracted from *Cotula cinerea* demonstrate sedative, anti-inflammatory,

and disinfectant properties. In some regions, the plant is also used to treat stomach and abdominal discomfort. (Larpent J.P., et al., 1989).

## 2.2 . *Origanum Majorana* L Plant:

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

The botanical term "Origanum" is etymologically derived from the Greek words "oros," meaning mountain, and "gonos," signifying joy or pleasure. This nomenclature reflects the plant's association with the aesthetically pleasing mountainous terrains and its prevalence within the Mediterranean basin's highlands (Prena et al., 2015). Taxonomically, this perennial herbaceous species was formerly classified as *Origanum Majorana* L. (Vagi et al., 2002). Exhibiting considerable morphological and chemical variation, the genus *Origanum* encompasses forty-nine recognized taxa distributed across the Mediterranean Sea region. Commonly known as sweet marjoram, this plant is native to Anatolia (Turkey) and Cyprus and is widely cultivated throughout the Mediterranean area, notably in Egypt (Novak et al., 2002). Historically, Hippocrates employed marjoram as an antiseptic, and it remains a traditional domestic remedy for respiratory ailments, including coughs and pharyngeal irritation (Bremness et al., 1994; Yazdanparast et al., 2008).

### 2.2. 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	Magnoliopsida
Branch	Magnoliophyta
Reign	Plants

### 2.2.3. Vegetative Description of *Origanum Majorana* L:

Presenting an annual growth cycle on a suffrutescent (partially woody) base, this aromatic subshrub demonstrates susceptibility to low temperatures. It typically attains a height of 30 to

60 cm and is characterized by multiple quadrangular, reddish stems exhibiting a decumbent growth pattern, forming a dense aggregation (Oakman, 1995). The stems are erect yet weak, displaying hirsute cylindrical surfaces with a green background punctuated by reddish maculations (Pimple et al., 2012).

The foliage consists of simple, soft leaves with a distinctly oblong to ovate-rectangular morphology, presenting a glaucous (green-gray) coloration and an opposite phyllotaxy. Each leaf, measuring approximately 0.5 to 1.5 cm in length and 0.2 to 0.8 cm in width, possesses an entire margin, an acute apex, a symmetrical and straight base, and pinnate venation. The adaxial and abaxial surfaces are uniformly pubescent. Small, tubular flowers, typically white or pale pink and less than 0.3 cm in length, emerge in dense spikes up to 13 cm long during the mid-to-late summer period. These flowers are bisexual (Blazovics et al., 2005).

Following anthesis, the plant produces small, dark brown, oval seeds that mature between August and September, exhibiting a cylindrical form. The subterranean root system is characterized by longitudinally oriented roots, occasionally displaying incisions and ranging from 0.2 to 0.6 mm in diameter. The root exterior is dark brown with narrow, deep fissures, which are indicative of root robustness and contribute to the plant's anchorage. These fissures enhance the absorption of water and nutrients and provide structural stability within the soil matrix. The elongated, symmetrical roots emit a subtle aromatic scent, contributing to the plant's overall characteristics and stability (Singla et al., 2014).



**Figure 4.** *Origanum Majorana* L Photograph

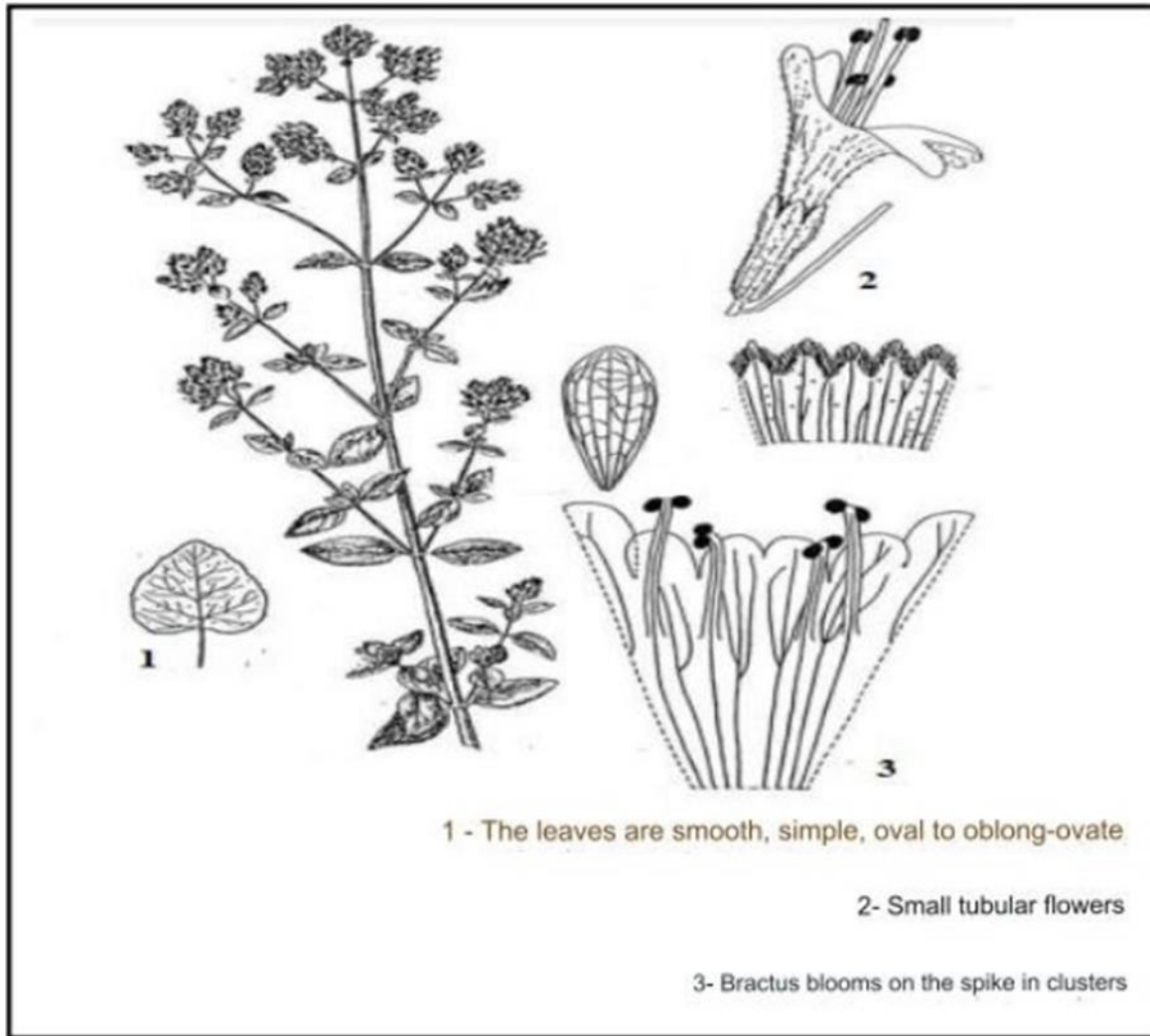
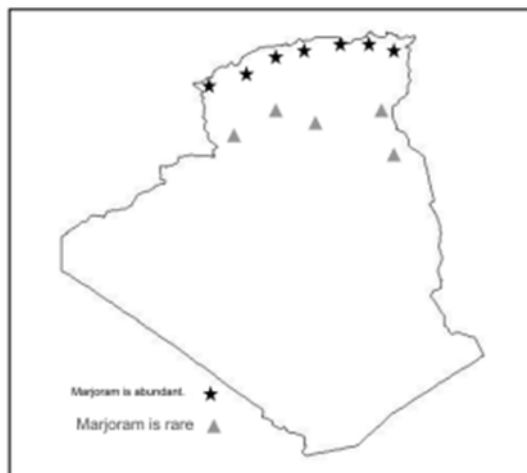


Figure 5. Diagram for *Origanum Majorana* L

#### 2.2. 4. Geographical Distribution of *Origanum Majorana* L:

*Origanum Majorana* L. exhibits a primary distribution in Turkey and Cyprus, with further occurrence in Mediterranean countries encompassing Lebanon, Iran, and North America. Its proliferation is notably evident in the Arabian Peninsula and India, particularly on sun-exposed slopes of meadows, fields, and rocky terrains within arid climates (Zahran et al., 2010). Furthermore, this species is extensively cultivated in the southern regions of Saudi Arabia.



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

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

*Origanum Majorana* L. presents a repertoire of readily applicable domestic interventions that have demonstrated efficacy in the symptomatic management of respiratory tract infections, including chest infections and coughs, as well as pharyngodynia, rheumatic discomfort, neurological sequelae, and cardiovascular ailments (Mancak et al., 2023). These easily administered at-home remedies offer palliation and symptomatic relief. Salient among these therapeutic approaches are:

1\_ Rest and Recuperation: Adequate rest and sufficient sleep are crucial for bolstering the body's immune response and facilitating an accelerated recovery trajectory (Farmawati et al., 2020).

2\_ Warm Fluid Ingestion: The consumption of warm liquids, such as herbal infusions and broths, can provide relief from nasal congestion and soothe irritation of the pharyngeal mucosa (McIntyre, 2000).

3\_ Steam Inhalation: The inhalation of steam, whether derived from heated water or facilitated by a humidifying device, can effectively alleviate respiratory congestion and provide comfort to the inflamed airways (Vathanophas et al., 2019).

4\_ Honey Application: Natural honey exhibits antimicrobial properties and can serve as a demulcent, providing relief from sore throats and mitigating cough severity (Kumar et al., 2010).

5\_Low-Impact Exercise: Engaging in gentle physical activity, such as ambulation or yoga practice, can enhance systemic blood circulation and contribute to the reduction of rheumatic pain (Govindaraj et al., 2016).

### 3. Previous Studies:

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

A review of the chemical literature on *\*Cotula cinerea\** reveals extensive investigation into various compounds, particularly lactonic sesquiterpenes. (Metwally, M., et al., 1986; Jakupovic, J., et al., 1988; Bohlmann, F., et al., 1989)A review of the chemical literature on *\*Cotula cinerea\** reveals extensive research focused on several bioactive constituents, particularly lactonic sesquiterpenes and comarenic ethereal sesquiterpenes. (Greger, H., et al., 1985),A review of the chemical literature on *\*Cotula cinerea\** reveals extensive research into a range of bioactive compounds. Notably, lactonic sesquiterpenes, comarenic ethereal sesquiterpenes, and monocyclic diterpenes have been identified as significant organic constituents. In addition, the plant has been found to contain triterpenes and distinctive acetylene compounds of the spiroketal enol ether type. (Metwally, M., et al., 1986; Bohlmann, F., et al., 1973). The study conducted by Lotfi et al. involved critical analyses of the bioactive constituents present in *\*Cotula cinerea\**, revealing a significant absence of alkaloids, in contrast to the notable presence of flavonoids and glycosides. (Lotfi B., et al.1962 ).

The comprehensive findings are presented in Table 3

**Table 3.** detailing active substances in the *Cotula cinerea* plant.

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

(+ + +): Highly abundant.

(+ +): Abundant.

(+): Weak.

(-): Absent.

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

Mortality			Clinical symptoms decrease of weight ,oedema , hair loss, convulsion /No .tested			Number of animals	Oral dose (g/Kg)
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

A research team led by Professor M. Markouk and colleagues from the University of Marrakech in Morocco investigated the anti-pathogenic properties of *Cotula cinerea*. This plant, which naturally thrives in sandy and arid desert soils, is traditionally employed in Moroccan folk medicine for its anti-inflammatory, sedative, disinfectant, and antibacterial effects. Additionally, it is used as an aromatic additive in tea preparations .

(M. Markouk et al.). A plant sample was collected from the Faculty of Science in Mashba, located in Marrakech, Morocco, and its extract was prepared for experimental purposes. The study involved a trial conducted on 21 white Swiss mice, each weighing between 20 and 22 grams. (Mehedi, N ., et al., 2013)

The mice were housed in plastic cages under controlled environmental conditions, with ambient temperatures maintained between 22 and 24 °C. They were subjected to a 14-hour fasting period prior to the commencement of the experiment. (Van de W., et al., 2002).

The experiment involved the administration of three different extracts of *Cotula cinerea*: ethyl acetate, diethyl ether, and ethanol. The injected doses of these extracts demonstrated acute

toxicity, leading to mortality in some mice and the appearance of various clinical symptoms in others. (El Kabbaoui, M., et al., 2017)

Acetate and n-butanol extracts were also employed.

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

During the toxicity evaluation of the extracts, no mortality was observed among the mice, and their behavior remained normal, even at doses as high as 6 grams per kilogram of body weight (Mukinda et al., 2007). These findings indicate that mice can tolerate concentrations up to 6 g/kg of body weight without adverse effects.

**Table 5.** Toxicity Efficacy of Cotulla Plant

Species	Strain	Ethyl acetate extract	n- Butanol extract	Novobiocine (standard)
<i>Pseudomonas fluorescens</i>	456-2	200	12	1
<i>Pseudomonas savastanoui</i>	T12-10	200	100	1
<i>Pseudomonas savastanoui</i>	73-29 88	200	50	1
<i>Bacillus sp</i>	VP5	200	25	20
<i>Bacillus brevis</i>	VP7	200	25	20
<i>Bacillus sp</i>	326	200	25	20
<i>Bacillus sphaericus</i>	324	200	200	20
<i>Bacillus sp</i>	459-1	2001	12	20

In a separate study, Dr. M. Markouk and colleagues investigated the antibacterial activity of the extract by determining the Minimum Inhibitory Concentration (MIC). Bacterial growth was assessed after a 24-hour incubation period, with each experiment performed in triplicate to ensure reliability (Mathur et al., 2006).

The results are summarized in Table 5, which presents the MIC values of *Cotula cinerea* extract, expressed in micrograms per milliliter ( $\mu\text{g/ml}$ ).

The results demonstrated that the *Cotula cinerea* extract obtained using ethyl ether exhibited no antimicrobial activity, whereas the extract obtained with ethyl acetate showed inhibitory effects against all tested pathogens (Acheuk et al., 2020). Notably, extraction using n-butanol yielded a highly potent antimicrobial effect against all pathogens examined. Furthermore, a study was conducted to evaluate the correlation between extract concentration and inhibitory activity, revealing a significant increase in the mean diameter of the inhibition zone with increasing extract concentration. A two-way ANOVA was conducted to assess the effect of *Cotula cinerea* extract on antimicrobial properties, revealing statistically significant differences in antimicrobial efficacy among the various plant extracts (Atef et al., 2015). In a study by Khallouki et al. (2015), the chemical composition of *Cotula cinerea* was analyzed to explore its medicinal potential in southeastern Morocco. Methanolic extracts were found to contain phenolic compounds amounting to  $2.5 \pm 79.23$  mg/g of dry matter (Ironi et al., 2018). High-performance liquid chromatography Coupled with electrospray ionization mass spectrometry (HPLC-ESI-MS) identified neochlorogenic acid, dicaffeoylquinic acids (3,5-, 3,4-, and 5,4- isomers), cryptochlorogenic acid, and chlorogenic acid as the predominant phenolic constituents. Antioxidant activity, as evaluated using FRAP and DPPH assays, demonstrated strong radical scavenging capabilities, which may account for the plant's traditional use in disease prevention in African ethnomedicine likely due to its high flavonoid and phenolic content (Khallouki et al., 2015). Santiuste et al. (2008) also investigated the essential oil of *Cotula cinerea*, identifying its major constituents and suggesting its application as a natural flavor enhancer in tea, potentially serving as a substitute for mint (Fatiha et al.). The plant is also traditionally used in folk medicine for the treatment of pulmonary and tracheal ailments. El-Bouzidi et al. (2011) evaluated the chemical composition and anti-candidiasis properties of *Cotula cinerea* essential oil. The oil demonstrated potent antifungal activity against all tested *Candida* strains, with inhibitory concentrations ranging from 3.2 to 4.7 mg/mL and a minimum fungicidal concentration (MFC) of 5.9 mg/mL. Gas chromatography–mass spectrometry (GC-MS) analysis revealed 24 bioactive compounds, indicating the oil's potential as a natural antifungal agent, particularly against resistant *Candida* strains (Cecchini et al., 2010).

In a related investigation, Abdenbi Asma et al. (2014) studied *Cotula cinerea* essential oil extracted by hydrodistillation from aerial parts collected near Béchar, Morocco. The oil

exhibited varying degrees of antibacterial activity up to 2% and inhibited the growth of approximately seven bacterial strains. The minimum inhibitory concentration (MIC) was defined by the lowest concentration exhibiting marked antibacterial sensitivity, with inhibition zones reaching up to 55 mm in diameter. A comprehensive review conducted in 2014 further explored the chemical composition and both antibacterial and antifungal activities of *Cotula cinerea* essential oil sourced from southern Morocco. The oil, obtained via hydrodistillation of aerial plant parts, was analyzed using GC and GC-MS techniques. Key constituents such as isothujanol-3, santolina triene, and cavor were identified, contributing to its antimicrobial potential. The study also reported antibacterial activity of *Cotula cinerea* oil against four bacterial strains, in addition to antifungal efficacy 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 subjected to analysis. Quantitative assessments revealed the following proportions: moisture (66.3%), protein (18.7%), fat (8.4%), ash (6.6%), and carbohydrates (5.7%) (Pinela et al., 2017). The concentrations of select metal elements, quantified in parts per million (ppm), were determined as follows: barium (Ba) at 0.6 ppm, iron (Fe) at 5.1 ppm, potassium (K) at 0.039 ppm, cobalt (Co) at 0.49 ppm, and sodium (Na) at 0.01 ppm. Qualitative phytochemical screening of aqueous and alcoholic extracts indicated the presence of tannins, phenols, flavonoids, saponins, carbohydrates, and alkaloids. Furthermore, an investigation into the physical and chemical characteristics, including combustion behavior and solubility, demonstrated partial solubility in polar solvents and complete solubility in non-polar solvents (Maryam et al., 2017). The efficacy of utilizing *Origanum Majorana* L. leaf powder as a preservative for beef was evaluated across a concentration range of 0.5% to 1%. Beef samples were stored at 5°C for a duration of 7 to 10 days, and alterations in meat quality were monitored through the estimation of peroxide values. The findings indicated a reduction in peroxide numbers in samples treated with *Origanum Majorana* L. leaf powder in comparison to untreated samples throughout the storage period (Sahunie et al., 2024). The study also examined the influence of the herb on the levels of coliform bacteria and Enterobacteriaceae in the meat during the specified storage period and concentrations. The results suggested that the incorporation of this herb contributes to a decrease in microbial populations in minced meat samples and exhibits antioxidant properties, consequently extending the meat's shelf life (Maryam et al., 2017).

## **4. Essential Oils:**

### **4.1. Generalities About Essential Oils:**

#### **4.1.1. Definition of Essential Oils:**

Essential oils are defined within the scientific literature as volatile oils that readily evaporate or volatilize without undergoing decomposition, a characteristic that distinguishes them from fixed oils, which are non-volatile and degrade upon exposure to evaporation or heat (Mondello et al., 1995). These compounds, also known as aromatic or ethereal oils due to their distinct aroma and lipophilicity in ether, represent complex mixtures of volatile constituents derived from plant sources. Typically exhibiting a colorless to yellowish appearance and existing in a liquid state under standard ambient conditions, essential oils are non-flammable and prone to oxidation when exposed to air. A key characteristic is their lack of fatty or greasy attributes (Guentherin, 1972; Rahal, 2004). Compositionally, they encompass a diverse array of plant-derived compounds that are commonly extracted via steam distillation and subsequently separated from the aqueous phase. The International Organization for Standardization (ISO) provides a standardized definition, characterizing essential oils as substances obtained from plant materials through either steam distillation or mechanical processing of citrus fruit peels, followed by physical separation from the resulting solution (Lemordant, 1989; Lee et al., 2004). This definition is widely recognized and employed by international regulatory bodies and within professional domains as a precise and authoritative demarcation of essential oils.

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#### **4.1.2. Storage Sites for Essential Oils:**

Essential oils are found in more than 2,000 plant species across over 60 botanical families (Nieto, 2017). These volatile compounds may be distributed throughout various plant organs or concentrated in specific tissues, with their abundance differing significantly among species. Typically, essential oils are stored in the cytoplasm of living cells, predominantly in liquid form. However, in some cases, they are bound to glycosides or resinous compounds, rendering them

non-volatile and solidified (Ogunsina, 2020). Within plant tissues, essential oils are frequently localized in specialized anatomical structures known as secretory structures.

These volatile plant oils can be found in diverse parts of the plant for example, in the leaves of mint (*Mentha* spp.), the petals of aromatic flowers such as roses and jasmine, and the fruits or peels of citrus species. The distribution of essential oils may vary not only among species but also among different organs within the same plant (Valnet, 1984; Lemordant, 1989; Anonyme, 2000; Baaliouamer, 1987; Karnich, 1994; Bauer et al., 1997; Shriner et al.).

Examples :

**Rutaceae:** In this family, essential oils are predominantly concentrated in the leaves, flowers, and seeds. A representative example is *Citrus aurantium* L. (Guzmán et al., 2021).

**Myrtaceae:** Essential oils are primarily located in the leaves, as commonly observed in species of *Eucalyptus* (Elaissi et al., 2012).

**Apiaceae:** In this family, essential oils are concentrated within the vascular channels. *Angelica archangelica* serves as a notable example (D'Amelio Sr, 1998).

**Lamiaceae:** Essential oils are mainly found in the aerial parts of the plant, including leaves and stems. This is characteristic of species such as mint (*Mentha* spp.) and thyme (*Thymus* spp.) (Abdellatif et al., 2005).

### 4.1.3. Physical Properties of Essential Oils:

Although essential oils vary in their chemical compositions, they exhibit several consistent natural characteristics when freshly extracted. These general properties are well-documented in the literature (Lemordant, 1989; Anonyme, 2000; Kennedy et al., 2002; Grayer et al., 2003).

**Color:** Most essential oils are initially colorless; however, upon exposure to air and prolonged storage, they may undergo oxidation, resulting in a darkening of color sometimes turning black (Turek & Stintzing, 2013). In rare cases, essential oils may display a greenish-blue tint, as observed in chamomile, eucalyptus, and certain mountainous plant varieties. This coloration is primarily due to the presence of azulene and chamazulene compounds.

Additionally, some essential oils may appear yellowish or pale as a result of bleaching processes (Seguin et al., 2001).

**Odor:** Essential oils are generally characterized by pleasant and distinctive aromas, rarely emitting offensive or pungent smells. These scents are attributed to specific terpenoids and other dominant volatile constituents, often detectable even before extraction (Sell, 2020). For example, citral contributes a lemon-like aroma to citrus plants, while compounds such as menthol, geraniol, and anethole provide the characteristic fragrances of mint, thyme, and anise, respectively (Arthur Riffer et al., 1969).

**Volatility:** A defining feature of essential oils is their high volatility at ambient temperatures, which sets them apart from fixed oils. When applied to a filter sheet, a drop of essential oil evaporates completely without leaving residue, whereas fixed oils remain, leaving a translucent mark. This property underscores their rapid evaporation and potent aroma release under normal atmospheric conditions (Arthur Riffer et al., 1969).

**Solubility:** Essential oils exhibit low solubility in water but are readily soluble in a wide range of organic solvents, such as petroleum ether and diethyl ether (Aziz et al., 2018). Their solubility in alcohol can reach up to 95%, with the notable exception of rose oil, which forms an emulsion due to the presence of certain paraffin-type organic compounds. The degree to which an essential oil dissolves in alcohol is commonly used as an indicator of its purity, with solubility typically tested across concentrations ranging from 95% to 35% ethanol diluted with water. The presence of fixed oils can inhibit the dissolution of essential oils in alcohol (Arthur Riffer et al., 1969; Ekundayo, 1988).

**Specific Gravity:** The specific gravity of essential oils varies according to their botanical origin, generally ranging between 0.1 and 1.7 (Schmidt, 2020). Most essential oils have a specific gravity below 1, allowing them to float on the surface of water. However, exceptions include oils such as clove oil (1.02–1.07) and cinnamon bark oil (1.03–1.04), which are denser than water and thus sink. Specific gravity provides insight into an oil's chemical composition: values below 0.9 typically indicate high concentrations of tertiary and aliphatic compounds, while values exceeding 1 suggest a predominance of compounds with chemically complex aromatic rings (Arthur Riffer et al., 1969).

**Optical Rotation:** Essential oils exhibit optical rotation, a property that serves as a valuable natural parameter for assessing oil purity and detecting adulteration. It is particularly

useful for distinguishing between naturally derived compounds and those synthesized industrially (Sheldon, 1993). Most essential oils remain in a liquid state at room temperature; however, some, such as rose oil and elemi oil, may solidify. Additionally, essential oils characteristically possess a high refractive index, further supporting their identification and quality assessment.

#### 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).

**Terpenes:** Terpenes are a class of natural products widely distributed throughout various parts of plants, where they comprise a substantial portion of the plant's secondary metabolites. These compounds are primarily responsible for the characteristic pleasant aromas associated with many plant species. The term terpenes is derived from substances originally isolated from turpentine oil (Arthur Riffer et al., 1969). Most terpenes are volatile compounds and are extracted from plants using similar methods as those used for obtaining essential oils (Ramarathnam et al., 1986). Terpenes serve essential biological and functional roles in both plants and humans. For example, vitamin (A) a compound vital for maintaining healthy vision is classified as a terpene. The diversity of terpenes is determined by the number of isoprene units ( $C_5H_8$ ) they contain. This principle was first identified in 1877 through studies conducted by Owillach at the University of Göttingen in Germany. The classification of terpenes based on their isoprene content is summarized in the table below (Seguin et al., 2001; Ghassan, 1999).

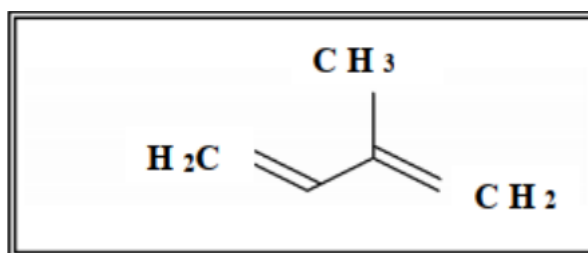
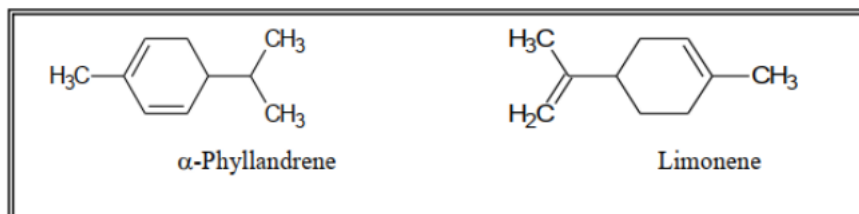


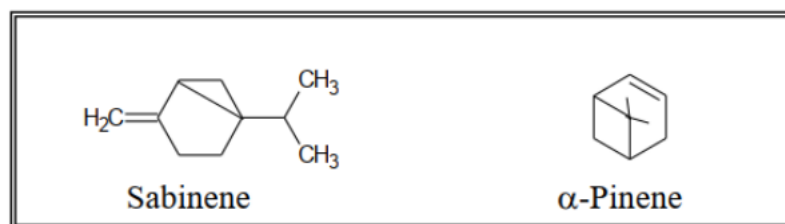
Figure 7: Isobrin unit

**Monoterpenes:** Monoterpenes are among the most abundant classes of terpenoids in the plant kingdom (Ekundayo, 1988). Composed of two isoprene units, monoterpenes typically feature a single-ring structure. Key compounds within this group include limonene and  $\alpha$ -phellandrene, both of which contribute to the characteristic aromas and biological activities of various plants.



**Figure 8.** Mono-ring Monoterpenes

Bicyclic monoterpenes, including compounds such as sabinene,  $\beta$ -pinene, and  $\alpha$ -pinene (Seguin et al., 2001; Paris, 1972), are widespread in the plant kingdom, along with their respective derivatives. These bicyclic monoterpenes are classified into four distinct groups based on the carbon structure of the hydrocarbons: the Thujane group, the Camphane group, the Bornane group, and the Fenchane group. Thujane and camphane derivatives, in particular, are commonly found in cedarwood oil, while turpentine primarily derived from the bark of pine trees serves as a significant source of these compounds.



**Figure 9.** Bicycle Monoterpenes

**Diterpenes:** Diterpenes are composed of 20 carbon atoms, equivalent to four isoprene units (Ruzicka et al., 1953). Notable examples include phytol (vitol) and vitamin A<sub>1</sub>.

**Sesquiterpenes:** Sesquiterpenes consist of over 2,500 distinct molecules, each containing three isoprene units, totaling 15 carbon atoms (Ceausescu, 2013). These compounds are typically classified based on their carbon structure. For example, cadinene represents the first type of sesquiterpene, while caryophyllene, found in celery oil, exemplifies the second type.

**Triterpenes:** Triterpenes, which include compounds such as esculetin, are significant in various biological processes. Esculetin, for instance, serves as a precursor in the biosynthesis of cholesterol.

**Tetraterpenes:** Tetraterpenes, consisting of eight isoprene units, are known as carotenoids. Three well-known carotenoids— $\alpha$ -carotene,  $\beta$ -carotene, and  $\delta$ -carotene—have been isolated and studied for their biological and nutritional properties (Riaz et al., 2021).

**Polyterpenes:** Polyterpenes, exemplified by natural rubber, are composed of long chains of repeated isoprene units. Natural rubber is found in the latex of tropical rubber trees, and its molecular structure closely resembles a series of horned structures (Long et al., 2021).

**Table 6.** Types of Terpenes

<b>Turbine Type</b>	<b>number of carbon atoms</b>	<b>Number of isoprene units</b>
<b>Monocultures</b>	<b>10</b>	<b>2</b>
<b>Half Triple Terpen</b>	<b>15</b>	<b>3</b>
<b>Bilateral terpenes</b>	<b>20</b>	<b>4</b>
<b>Triple Terpen</b>	<b>30</b>	<b>6</b>
<b>Quadruple Turbine</b>	<b>40</b>	<b>8</b>
<b>Multi-turbine</b>	<b>&lt;40</b>	<b>&lt;8</b>

#### **4.1.5. Benefits and Applications of Essential Oils:**

Thousands of years ago, aromatic oils were utilized in ancient Chinese, Egyptian, Arab, and Islamic civilizations. Over the centuries, the use of essential oils has evolved significantly and is now commonplace in cosmetic institutes and wellness centers, where they are employed as part of medical regimens aimed at promoting health and treating various diseases (Wilson, 2002). Essential oils produced by plants serve a variety of ecological and biological functions. They play a critical role in attracting insects for pollination, thus enhancing fruit production. Additionally, some essential oils act as insect repellents or toxins, protecting plants from pest infestations (Regnault-R., 1997). Beyond their role in plant protection, essential oils contribute to the elimination of metabolic waste and help fortify the body's physiological systems, including the immune and circulatory systems. For example, lavender oil has been shown to promote hair growth, improve oxygen uptake, and stimulate ATP production, which is essential for cellular energy.

The following Table 7 summarizes 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 are among the earliest methods for extracting essential oils, with a history that spans over a century (Schmidt, E., 2020). The operation of distillation apparatuses relies on the vaporization of essential oils from plant materials upon heating. The vaporized compounds are then condensed, resulting in a mixture of essential oils and water, which can be separated based on differences in solubility (Rahal, S., 2004; Abdellatif, F., 2005). Each distillation cycle produces a fraction of high-grade, high-quality essential oils.

There are three primary methods of distillation:

#### 1. Hydrodistillation:

Hydrodistillation is a widely used technique that involves immersing plant material in a distillation vessel filled with water (Lawrence, B., 1995). The plant material can be either submerged directly in the water or placed in a mesh container to prevent direct contact with the vessel's walls. The distillation apparatus, typically made of stainless steel, copper, or glass, is heated by either an open flame or an electric heater (Das, S., et al., 2013). This method is particularly suited for extracting essential oils from heat-resistant, dried plants, especially those with high oil content found in various plant parts such as roots, leaves, fruits, and certain flowers. The following Table 8 outlines the advantages and disadvantages associated with hydrodistillation.

**Table 8.** Advantages and Disadvantages of Hydrodistillation

Water distillation advantages	Water distillation defects
<ul style="list-style-type: none"> <li>- Easy to use.</li> <li>- Low-cost processing.</li> <li>- The machine is simple to make and install.</li> <li>- Do not require a designated place and no conditions Special.</li> </ul>	<ul style="list-style-type: none"> <li>- The impossibility of complete (total) debriefing.</li> <li>- Loss of some decomposable compounds in water, As in the case of oxygen compounds.</li> <li>- It takes a long time to complete the extraction process.</li> </ul>

2. Extraction with Waves Under Pressure: This method involves conducting the extraction process under pressurized conditions, utilizing microwave radiation (Jain, T., et al., 2009). The application of pressure enhances extraction efficiency by facilitating the penetration of microwave energy into the plant material, which in turn promotes the release of essential oils.

3. Extraction with Waves Under Atmospheric Pressure: In this technique, microwave radiation is applied under atmospheric pressure conditions (Yang, Y., et al., 2014). The plant material is exposed to atmospheric pressure, which allows for the efficient extraction of essential oils using microwave energy.

4. Water Distillation Extraction Using Microwave Radiation Under Vacuum: This method involves applying microwave radiation to facilitate water distillation under vacuum conditions (Li, Hong, et al., 2019). The use of vacuum reduces the boiling point of water, enhancing the efficiency of water distillation and promoting the extraction of essential oils from the plant material.

5. Extraction with Solvent Assisted by 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 solubilize the essential oils, thereby enhancing the efficiency of their extraction when exposed to microwave energy. Comparative Efficiency Species (1), (2), and (3) demonstrate superior performance compared to traditional extraction methods. However, technique (4) offers greater efficiency, particularly in terms of extraction speed, than the first three methods. Method (5) allows for the use of minimal solvent quantities and provides shorter extraction times compared to conventional techniques, such as Soxhlet extraction. One notable drawback of methods (2), (3), (4), and (5) is their significantly higher

cost. It is important to note that all these advanced extraction methods reduce extraction time and prevent damage to heat-sensitive compounds (Benkaci-A., et al., 2006; Ferhat, M.A., 2006).

### **2. Cold Pressing:**

Cold pressing is a technique specifically designed for extracting essential oils from citrus fruits, which are particularly sensitive to heat and oxidation. The process involves mechanical or manual extraction under high-pressure water streams. The essential oil is subsequently separated from the aqueous phase through centrifugation (Ferhat, M.A., 2006).

### **3. Solvent Extraction:**

An organic solvent is introduced to plant materials (e.g., jasmine blossoms or roses) at room temperature. The plant material is layered thinly to enable the solvent to effectively penetrate the cells that contain essential oils. As a result, the essential oils dissolve in the solvent, forming a mixture of oil and solvent (El Asbahani, A., et al., 2015). The separation of the solvent from the oil is then accomplished through low-pressure distillation. The resulting product, known as "concrete," solidifies due to the presence of fatty substances that are extracted by the solvent. This concrete is subsequently processed with absolute alcohol at low temperatures to eliminate the fatty components, yielding the "absolute alcohol of essential oils" (M. Vinatoru., et al., 1997; M. Toma., et al., 2001). While this method offers advantages such as reduced extraction time and the ability to recover heat-sensitive compounds, its broader use is limited by high costs and concerns regarding safety and environmental impact (G.T. Forrest., et al., 2000).

### **4/Supercritical/Liquid Carbon Dioxide Extraction:**

This technique is based on the capacity of certain gases—most notably carbon dioxide—to extract compounds such as essential oils, natural pigments, and fragrances under supercritical pressure conditions (Chakravarty, I., et al., 2021). Carbon dioxide is employed in either its liquid or gaseous form. When it reaches its critical point (31.4 °C and 73 bar), carbon dioxide enters a supercritical state, exhibiting a density comparable to that of a liquid. The efficiency of compound extraction in this state depends on variables such as temperature, pressure, and the solute's characteristics (E. Guentherin., 2000; F. Abdellatif., 2005). This approach is particularly valued for its ability to facilitate solvent separation and to operate at low temperatures, thereby

preserving the structural integrity of essential oil components. Carbon dioxide is especially favored as a solvent due to its moderate critical pressure, low operational temperature, non-flammability, non-toxicity, and lack of residual by-products, which has contributed to its widespread adoption in the perfume industry (E. Guentherin., 2000; F. Abdellatif., 2005).

#### **5/Maceration Extraction:**

This process involves immersing plant parts in a solvent for a sufficient period to enable the dissolution of essential oils (Rao, V., et al., 2007). Mechanical agitation may be employed to prevent the aggregation of plant materials and to ensure uniform exposure to the solvent. In some cases, the temperature of the solvent is raised to facilitate the melting of essential oils; however, it is crucial to maintain temperatures that do not compromise the quality of the extracted oils. The resulting solution is then subjected to distillation under low temperature and reduced pressure conditions to recover the solvent for reuse, ultimately yielding the essential oil (F. Abdellatif., 2005).

**CHAPTER TWO:**  
**Inflammation General concepts**

## 1. Definition of Inflammation :

Inflammation is a biological reaction to a disrupted tissue homeostasis (Medzhitov 2008). At its basic level, it is a tissue-destroying process that involves the recruitment of blood-derived products, such as plasma proteins, fluid, and leukocytes, into perturbed tissue. This migration is facilitated by alterations in the local vasculature that lead to vasodilation, increased vascular permeability, and increased blood flow. Infection by microbial invaders is often implicated as the major culprit that promotes inflammatory responses (Figure 1a). However, injury or trauma (in the absence of parasitic infection) and exposure to foreign particles/irritants/pollutants are also potent activators of inflammation (Medzhitov 2008), suggesting that this response likely evolved as a general adaptation for coping with damaged or malfunctioning tissue (Matzinger 2002). A common explanation for why infection and trauma might evoke similar inflammatory responses is that infection often follows wounding, which implies that it would be advantageous to respond to trauma as if infection occurred (Nathan 2002). The more parsimonious explanation is that both pathogens and wounding cause damage to cells and tissue and trigger similar responses (Bianchi 2007). The primary functions of inflammation are to rapidly destroy or isolate the underlying source of the disturbance, remove damaged tissue, and then restore tissue homeostasis (Medzhitov 2008, Soehnlein & Lindbon 2010). Inflammation, when regulated properly, is putatively adaptive. This statement is supported by the increased risk of serious infections in humans with genetic deficiencies in primary components of inflammation, such as neutropenia (abnormally low level of circulating neutrophils). Defects in the genes that encode proinflammatory cytokines and effectors of inflammation using mouse knock-out studies are also characterized by increased susceptibility to infection (Martinon et al. 2009). Conversely, there are several immune-relevant genes whose disruption leads to spontaneous inflammation, suggesting that the inflammatory response is actively suppressed by regulatory gene products to maintain health when inflammatory stimuli are not present (Nathan, 2002). When not regulated properly, excessive inflammation can have devastating effects, resulting in excessive collateral damage and pathology. On an evolutionary level, inflammation is a highly conserved phenomenon and appears to be an important first line of defense for both invertebrates and vertebrates. Many of the components associated with the inflammatory cascade, such as chemotaxis and phagocytosis, are readily employed by unicellular organisms and were later co-opted as defensive mechanisms to maintain the integrity of more complex multicellular organisms (Rowley 1996). Innate immunity in the form of phagocytosis and antimicrobial peptides is present in the earliest of invertebrates, whereas the adaptive immune

system evolved later and is unique to jawed vertebrates (Flajnik & Du Pasquier 2004). Adaptive immunity is hypothesized to have evolved to recognize and manage the complex communities of microbes that reside in the vertebrate digestive tract, which harbors a greater diversity of microbial fauna than the invertebrate gut (McFall-Ngai 2007).

## **2. Causes of Inflammation :**

Inflammation occurs when the body's white blood cells release chemicals into the blood or tissues in order to fight off invaders. As a result, the area that has been injured or infected receives an increase in blood flow. Redness and warmth can result from it. Swelling is the outcome of fluid leakage into your tissues caused by some of the chemicals. This defense mechanism could set off pain receptors. White blood cells and the substances they produce in the joints induce inflammation, joint lining swelling, and the eventual loss of cartilage (the cushions at the ends of bones). In 2000, (Berger, 2000). Microorganisms are one of the reasons that might cause inflammation.

- Real-world entities.
- Chemicals.
- Immune reactions that aren't acceptable.
- The demise of tissue

There are a number of factors that can lead to inflammation, including infectious agents like viruses and bacteria. By infiltrating and destroying cells in the body, viruses cause inflammation; bacteria create endotoxins, which can cause inflammation. Physical trauma, burns, radiation harm, and frostbite can all cause tissue damage and inflammation. Corrosive chemicals like acids, alkalis, and oxidizing agents can also cause inflammation. As previously stated, a dysfunctional immune system can lead to an inflammatory reaction that is harmful to the body. An further cause of inflammation is tissue death due to a lack of oxygen or nutrients, which is frequently brought on by a reduction in blood flow to the affected area (Pahwa et al.,2020).

## **3. Decomposition of an inflammatory reaction :**

The inflammatory reaction also occurs in the main stages: an vascular exudative phase, a cellular phase, and a degranulating phase.

### **a)Vasculo-exudative phase**

The vasculo-exudative phase corresponds to local vasoconstriction of the vessels at the site of inflammation. This reflex vasoconstriction will be quickly followed by vasodilation and an increase in blood viscosity. Vasodilation of the vessels will allow a local increase in vascular

permeability with plasma transudation (passage of plasma fluid through the vessel wall), local edema, and fibrin formation.

#### b) Cellular Phase

The cellular phase is the second phase of the classic inflammatory response. It corresponds to an extravascular influx of inflammatory cells. We will find leukocytes, neutrophils, and then macrophages. These cells will enable phagocytosis and destruction of the pathogen. The inflammatory focus will gradually be cleared by the elimination of cellular and tissue debris by macrophages.

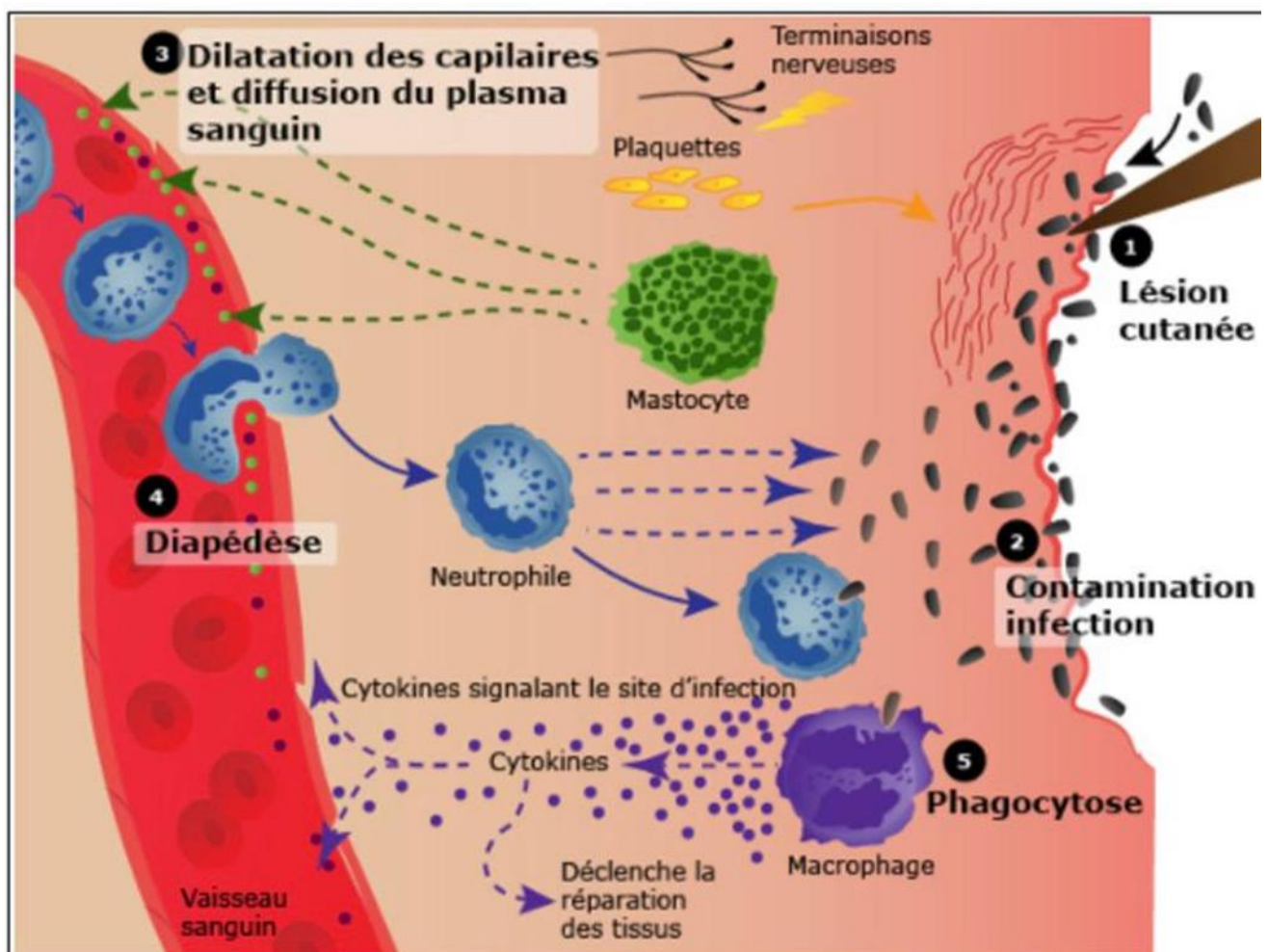
#### c) Resolution Phase

The resolution phase corresponds to the apoptosis of neutrophils.

This is the physiological death of these cells after they have fulfilled their function.

#### d) Summary of the course of the inflammatory reaction

A summary of the course of the inflammatory reaction is shown in Figure 10 below.



**Figure 10.** Décomposition of an inflammatory reaction (Maxicours.com, 2020)

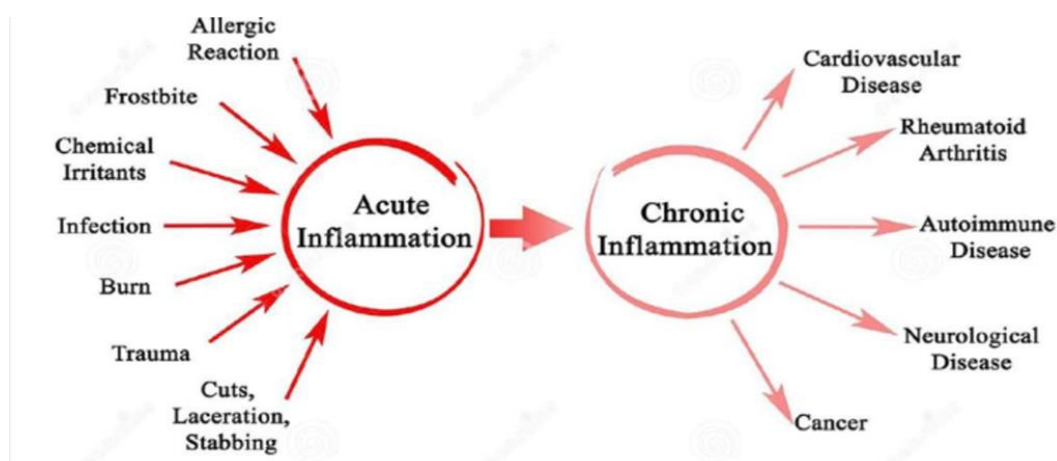
#### 4. Types of Inflammation:

There are two types of inflammation: acute and chronic (Stone WL, Basit H, Burns B. Pathology,2021).

**Acute inflammation:** This is a type of short-term inflammation that the body produces to address injuries, illnesses, and infections. When there is an injury or contract a virus, the white blood cells begin to flood the area to provide protection and healing. Symptoms of acute inflammation include redness, warmth, and swelling (Figure 11). Depending on the severity of the infliction, the inflammatory response will slowly fade away over hours or days.

**Chronic inflammation:** The body's response is the same as with acute inflammation, although there isn't always an injury that needs healing. Instead, the body wrongly signals that there is an issue of some kind, leading to inflammation that is persistent. Chronic inflammation has a more heterogeneous histological appearance than acute inflammation. In general, chronic inflammation is characterized by the presence of macrophages, monocytes, and lymphocytes, with the proliferation of blood vessels and connective tissue (Understanding acute and chronic inflammation. 2020). Chronic inflammation is typically less painful than acute inflammation and lasts for a longer amount of time. Chronic inflammation is characterised by the following symptoms that include:

- Tired Appearance
- Unexplained muscle aches and joint pain
- Stomach issues including constipation or diarrhea
- Gaining weight
- Skin rashes



**Figure 11.** Graphic representation of inputs for Acute inflammation and then acute inflammation becomes chronic inflammation what sort of diseases can develop. (J Vet

MedRes 8(4): 1219 (2021))

## 5. Mediators of inflammation:

1) Bradykinin: It is the most important mediator and most potent endogenous allopathic substance known. Bradykinin and their related kallidin are formed in the blood and tissues respectively and break down by kinases into active and inactive metabolites (Dray, A., Br. J. Anaesth. 1995).

2) Histamine: Association of histamine with H1 receptors produces numerous effects associated with the symptoms of anaphylaxis and other allergic symptoms (Simons, F.E., 2004., Bayram, H., J.L. Devalia, O.A. Khair, M.M. Abdelaziz and R.J. Sapsford et al., (1999)). Histamine leads to the production of allergic inflammatory responses by enhancing the secretion of proinflammatory cytokines such as interleukin 1 $\alpha$ , interleukin 1 $\beta$ , interleukin 6 or interleukin 8 in different types of cells and tissues. Endothelial cells express functional histamine receptors H1 and H2 (Jeannin, P., Y. Delneste, P. Gosset, S. Molet and P. Lassalle et al., (1994), Moncada, S., R.M.J. Palmer, E.A. Higgs, (1991)).

3) Nitric oxide and mast cells: Nitric oxide is generated due to the inflammation (Coleman, J.W., (2001), Ross, R. and A.B. Reske-Kunz, (2001)) Nitric oxide is a proinflammatory at the low concentration by inducing vasodilation and the recruitment of neutrophils but at the high concentration there is downregulation of adhesion molecules (Myint, K.M., Y.K. Kim, (2003)). Nitric acid is formed from L-arginine by nitric oxide synthetase. There are two isoenzymes:

- c-NOS
- i-NOS

c-NOS is calcium dependent and i-NOS is calcium independent and is induced by inflammation in macrophages and microglia.

4) Neuropeptide: Neuropeptide Y is a cotransmitter of sympathetic nervous innervations and it causes the potentiation of actions of NE. NPY promotes smooth muscle proliferation in the vasculature which results in vascular hypertrophy. It causes the increase in leukocyte adhesion and together with CA, platelet aggregation and macrophage activation (Dinarello, C.A., (1996)). The neuropeptide CRF is located within the PSGN as well as in sensory nerves. Substance P is a sensory neuropeptide which is present in autonomic nerves and ganglia.

5) Cytokines: Cytokines are different group of proteins and is called as hormone of the immune system. Interaction between the proinflammatory cytokines (IL-1 $\beta$ , IL-6, IL-8, TNF $\alpha$ ) results in synergistic activities in cytokine production and cytokine activities (Sabio G, Davis RJ. TNF. 2014). There is other family of cytokine called as antiinflammatory cytokines (IL-1Ra,

IL-4, IL-10, and TGF $\beta$ 1) which antagonize the action of proinflammatory cytokines. There are two components for cytokine balance. First is the IL-1 which increases the synthesis and secretion of IL-1Ra upregulation which is purposed to attenuate the delirious effect of IL-1 by blocking the IL-1 (Freidin, M., M.V. Bennett, J.A. Kessler, (1992), Adams, D.O., (1994)) The second is balance between different kinds of cytokine system like TGF $\beta$ 1 which inhibit IL-1 and TNF- $\alpha$  activity 114,115. Astrocytes and microglial secrete cytokines in the brain, neuron can also produce cytokines but under certain conditions (Williams, R.B., (1994)).

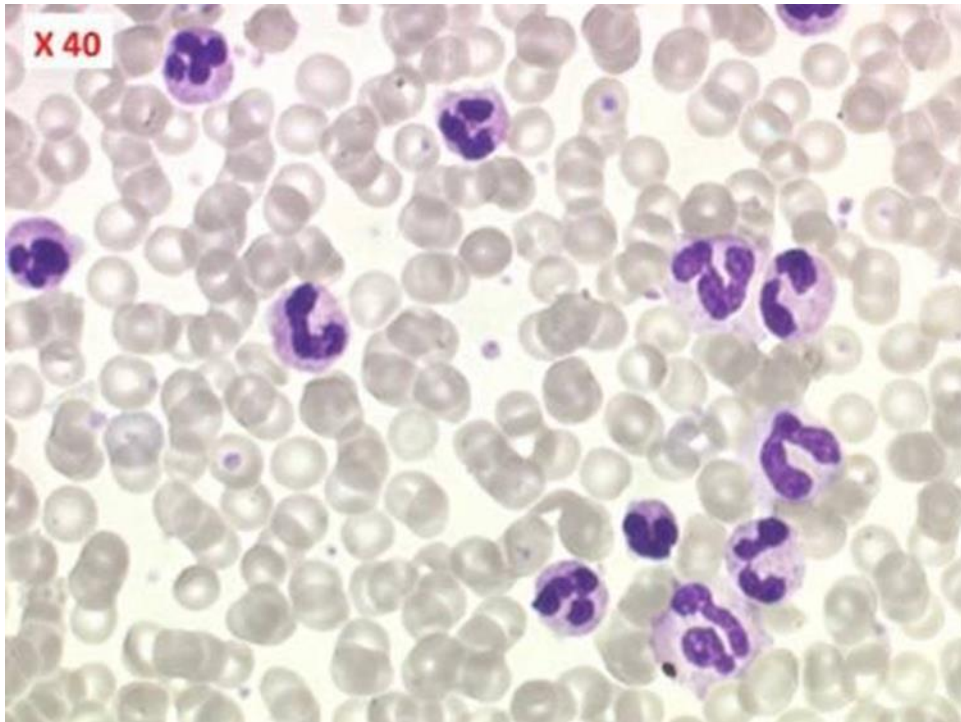
**6) Macrophages:** Macrophages are involved in the inflammation. Macrophages cause the release of cytokines at a faster rate when they are activated 76,118. Macrophage can also get activated by increase in cholesterol caused by stress with the SNS agonist 118. Presence of cholesterol results in upregulation of  $\beta$  adrenergic receptors ultimately results in amplification of catechols and hence macrophage activation. Oxidized LDL caused by stress, binds to the scavenger receptor results in macrophage activation (J Cell Sci.2005).

## **6. Inflammatory Cells:**

Inflammatory cells can be divided into two main cell types: circulating blood cells and tissue-resident cells. Circulating blood cells include neutrophils, monocytes, eosinophils, basophils, platelets, lymphocytes, and plasma cells. Tissue-resident cells include macrophages, histiocytes, mast cells, fibroblasts, and endothelial cells. To help understand the rest of this article, we will describe some cell types involved in the inflammatory response.

**a) Neutrophilic Molecules (NMP):** (Dumas A.2009) NMP, seen under a microscope in Figure 12, are leukocytes (white blood cells). Their maturation and proliferation are

controlled by growth factors. As we saw previously, they will be recruited in the cellular phase of the acute inflammatory reaction. Their action will be mediated by chemotactic receptors. This involves the migration of neutrophils to the site of infection through the secretion of molecules by inflammatory cells already present (e.g., monocytes). Neutrophils will specifically recognize patterns found on the surface of pathogenic microorganisms; this recognition will activate the phagocytosis process. This involves the ingestion of pathogens and their destruction.



**Figure 12.** Microscopic view of neutrophils (Cytologie-sanguine,2020)

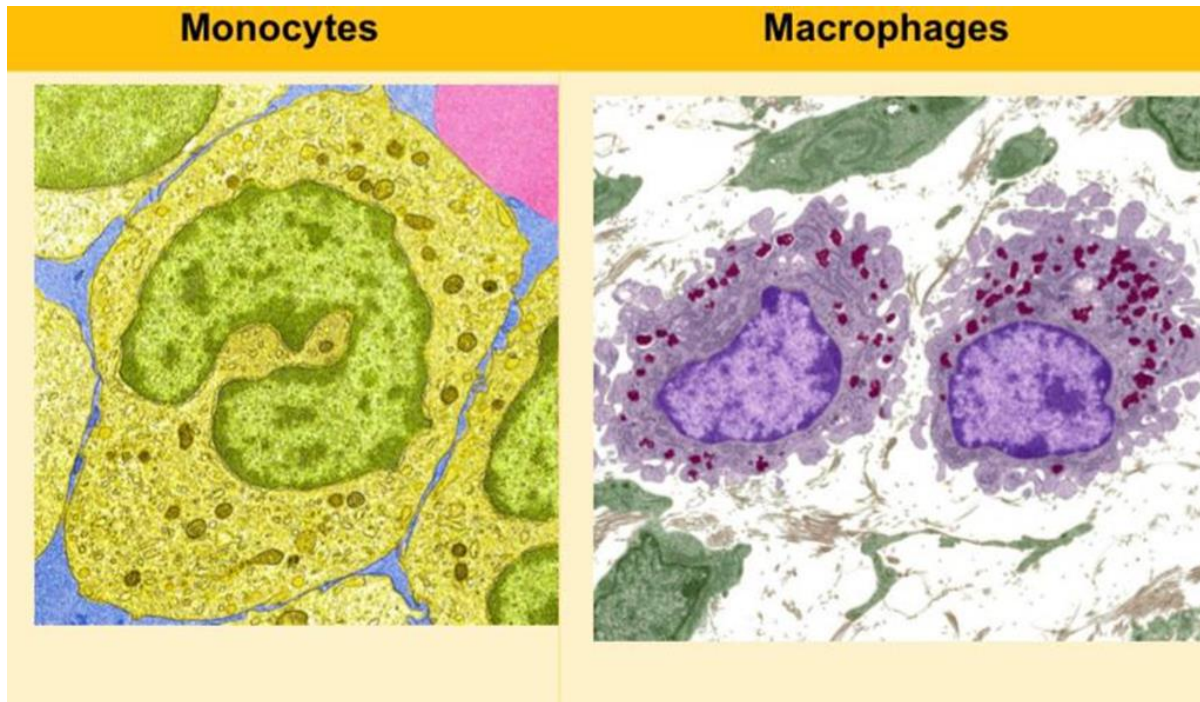
**b) Monocytes and macrophages:**

(CHUPS JUSSIEU,2020) Macrophages are the differentiated form of monocytes. The latter transform into macrophages when they migrate into tissues due to inflammation. Monocytes and macrophages are phagocytes; therefore, like the previously discussed PNNs, they can phagocytose pathogenic microorganisms.

Macrophages can be activated after encountering a pathogen, a tissue degradation product, or after binding to a natural ligand for one of their membrane receptors. Macrophage activation has two consequences:

- First, slow and incomplete phagocytosis. Peptides will be stored in vacuoles in the macrophage's cytoplasm, called phagosomes. Phagocytosis ends with the presentation of these peptides to T lymphocytes.

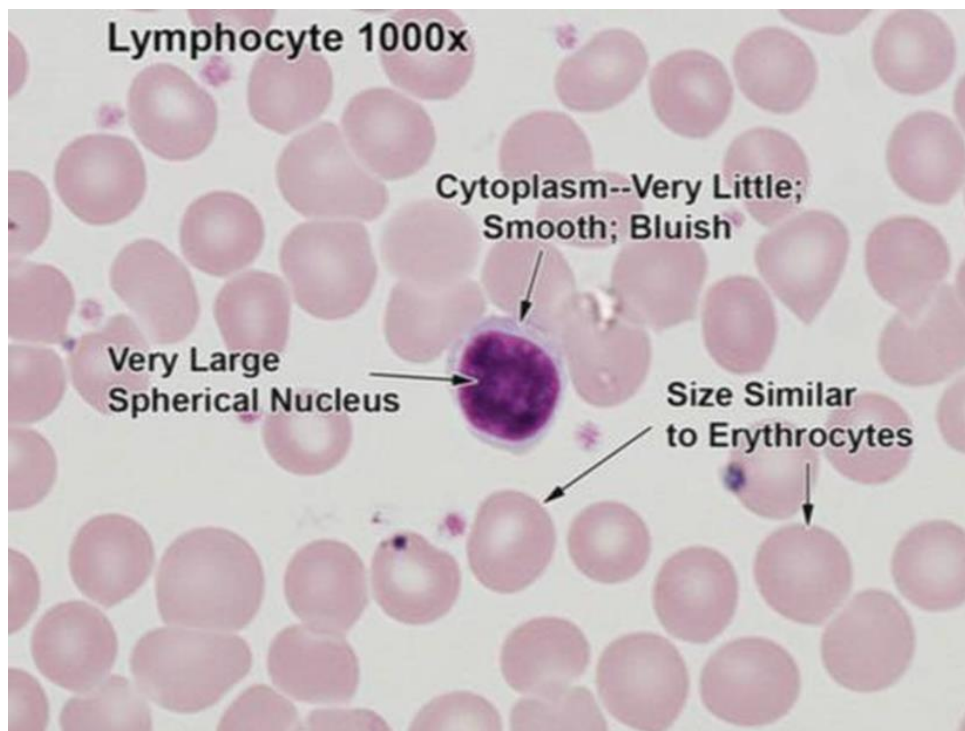
- The second action of macrophages after their activation is the release of secretory products that will intervene in the inflammatory process (complement fractions, enzymes, cytokines, etc.). Cytokines (e.g., interleukin-6, TNF-alpha) are soluble glycoproteins that act as intercellular mediators. They react with specific membrane receptors located on the surface of target cells, enabling the activation of immune cells and their recruitment to the inflammatory site.



**Figure 13-** Microscopic views of monocytes and macrophages

**c) T Lymphocyte:**

(Weill B. 2020) Lymphocytes are immune cells that are part of the leukocyte (white blood cell) family. Two main families are distinguished based on the receptors located on their surface: B lymphocytes and T lymphocytes. Figure 14 shows a lymphocyte under microscopic view.

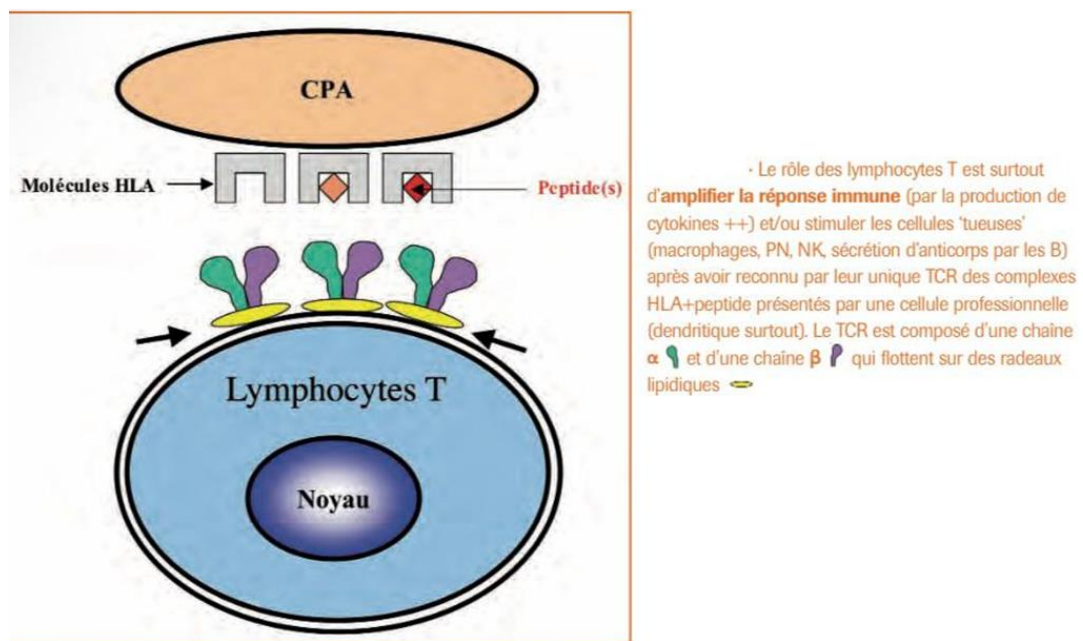


**Figure 14-** Microscopic view of T lymphocytes (Montgomery College,2020)

T lymphocytes are cellular immune cells involved in the adaptive immune response. Most of the time, in the case of infection, the pathogen is phagocytosed and destroyed non-specifically. This is referred to as innate, non-adaptive immunity. In contrast, when the immune response involves T lymphocytes, it is considered adaptive. These lymphocytes are activated by the binding of antigenic peptides, presented by antigen-presenting cells (APCs), to the antigen receptors located on their surface. This binding not only triggers a specific response depending on the recognized antigenic peptide, but also provides the organism with protection against future infections by the same pathogen through immune memory developed during the first encounter. There are various types of antigen-presenting cells (APCs) such as dendritic cells, B lymphocytes, and macrophages. As previously mentioned, the macrophage is activated after encountering a pathogen, leading to incomplete phagocytosis. The antigenic peptides or degradation products from this process are presented to T lymphocytes to complete the destruction of the pathogen.

Recognition of the peptide by the antigen receptors on the surface of the T lymphocyte, as shown in Figure 15, leads to an increased immune response through:

- Activation and production of factors that enhance the organism's bactericidal and phagocytic properties by stimulating phagocytic cells (macrophages, neutrophil granulocytes, etc.)
- Production of a significant number of cytokines that amplify the immune response (by activating immune cells and recruiting them to the site of inflammation)



**Figure 15** - Activation of T lymphocytes by antigen-presenting cells

## 7. Indicators of inflammation :

### 7.1. Sedimentation rate (ESR) :

The erythrocyte sedimentation rate (ESR) remains a simple and commonly used tool for detecting inflammatory syndromes. It is easy to perform, inexpensive, and fairly sensitive; however, it lacks high specificity. The ESR reflects the extent of red blood cell sedimentation in plasma after placing the sample vertically in a tube containing an anticoagulant.

It is important to note that certain physiological factors or non-inflammatory conditions can lead to an elevated ESR. Additionally, normal values may sometimes give a false sense of reassurance. It is also worth noting that isolated ESR elevation is not uncommon, occurring in approximately 20% of cases.

When an elevated ESR is observed, it is not advisable to hastily attribute it to an inflammatory condition without conducting additional tests such as a complete blood count (CBC), serum protein electrophoresis, and C-reactive protein (CRP), in order to accurately determine the cause.

### 7.2. C-reactive protein (CRP) :

In healthy adults, the average concentration of C-reactive protein (CRP) is approximately 0.8 mg/L. However, following an acute inflammatory response, CRP levels can rise significantly, sometimes exceeding 500 mg/L. The plasma half-life of CRP is about 19 hours and remains constant regardless of the individual's health status (Forcada et al., 2001). CRP has several important functions, including:

- Enhancing immune clearance independently of the complement system
- Binding to components of bacterial membranes
- Increasing neutrophil chemotaxis and promoting phagocytosis
- Inhibiting fibrinolysis resulting from coagulation

## 8. Anti-inflammatories :

### 8.1. Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) :

Like acetylsalicylic acid, NSAIDs primarily act by inhibiting the synthesis of prostaglandins through the blockage of an enzyme called cyclooxygenase (COX), a discovery for which Sir John Vane was awarded the Nobel Prize in Physiology or Medicine in 1982. Various stimuli (mechanical, chemical, etc.) activate phospholipase A2 enzymes within cell membranes, leading to the transformation of membrane phospholipids into arachidonic acid. This acid is then metabolized into prostaglandins and thromboxane A2 by COX. The inactivation of this enzyme by aspirin and NSAIDs inhibits the synthesis of pro-inflammatory

prostaglandins, but also that of cytoprotective prostaglandins (such as prostacyclin), particularly in the stomach and kidneys, as well as thromboxane A<sub>2</sub> in platelets. This dual action explains both the anti-inflammatory effects and certain gastrointestinal and renal adverse effects (discussed further below). In the early 1990s, advances in molecular biology revealed two isoforms of COX: COX-1, known as constitutive, is involved in the synthesis of protective physiological prostaglandins in the gastric mucosa and kidneys; COX-2, known as inducible, emerges in inflammatory sites and stimulates the formation of pro-inflammatory prostaglandins with harmful effects. Following this discovery, the pharmaceutical industry rapidly developed NSAIDs with preferential or specific COX-2 activity, hoping to reduce the side effects associated with COX-1 inhibition. Unfortunately, while gastric toxicity was significantly reduced, renal toxicity was not avoided. Moreover, the first marketed selective COX-2 NSAID (rofecoxib) had to be withdrawn due to the occurrence of cardiovascular incidents.

Over the years, the pharmaceutical industry has developed a wide range of NSAIDs from different chemical families, with varying durations of action. Some have an action time of less than 6 hours, requiring multiple doses per day, while others act for more than 12 hours, allowing for once-daily dosing. Most NSAIDs act non-selectively on both COX isoforms. Generally, the more powerful the anti-inflammatory effect (as with oxicams), the higher the gastric and renal toxicity. Conversely, aryl carboxylic acid derivatives (such as ibuprofen) are better tolerated but less potent. Therefore, the choice among these different classes should be guided by the severity of the underlying condition and the desired therapeutic effect. Meloxicam has preferential COX-2 activity, and two other NSAIDs (celecoxib and etoricoxib) have specific COX-2 action. These latter drugs have faced usage restrictions in clinical practice following the withdrawal of rofecoxib due to potential cardiovascular toxicity. The safety and tolerance profile of NSAIDs is well established, particularly regarding their digestive and renal toxicity. Nevertheless, adverse effects remain common in clinical practice, especially since some NSAIDs are available over the counter and may be taken without medical supervision even by patients who are at risk for complications. Side effects can lead to hospitalizations for gastroduodenal ulcers with a risk of bleeding (as the prostaglandins inhibited by NSAIDs are gastroprotective, notably by increasing mucus secretion), or for acute renal failure, which is exacerbated by any condition that leads to dehydration (since the prostaglandins inhibited by NSAIDs act as vasodilators in the kidneys) (table 9) (Becker G, Monassier L. 2018)

**Table 9-** Classification of nonsteroidal anti-inflammatory drugs

Chemical families	Molecules	COX selectivity	Half-life
Salicylates	Acetylsalicylic acid	Non-specific (*)	< 6 h (low dose)
	Lysine acetylsalicylate	Non-specific	> 12 h (high dose)
Pyrazoles	Phenylbutazone	Non-specific	< 6 h (low dose)
Indolesque	Indometacine	Non-specific	> 12 h (high dose)
	Sulindac	Non-specific	> 12 h
Aryl carboxylic acids	Diclofenac	Non-specific	< 6h
	Aceclofenac	Non-specific	< 6h
	Ibuprofen	Non-specific	< 6h
	Naproxen	Non-specific	< 6h
	Furbiprofen	Non-specific	< 6h
Fenamates	Mefenamic acid	Non-specific	< 6h
	Niflumic acid	Non-specific	< 6h
Oxicams	Piroxicam	Non-specific	> 12 h
	Tenoxicam	Non-specific	> 12 h
	Meloxicam	COX 2 preferential	> 12 h
Coxibs	Celecoxib	COX 2 specific	> 12 h
	Etoricoxib	COX 2 specific	> 12 h

(\*) COX 1 selective at low dose (100-300 mg, antiplatelet dose)

## 8.2. Steroidal Anti-Inflammatory Drugs (Glucocorticoids) :

Corticosteroid therapy was born in the late 1940s. It was the result of a successful collaboration between three complementary disciplines: extractive chemistry (led by T. Reichstein), synthetic chemistry (E.C. Kendall), and clinical research (P. Hench). This synergy led to the discovery of cortisone in 1948, and the three scientists were awarded the Nobel Prize in Physiology or Medicine in 1950 for demonstrating its remarkable and unprecedented efficacy in the treatment of rheumatoid arthritis. Cortisone is the prototype of a series of related compounds belonging to the glucocorticoid family. The addition of a fluorine group to the base steroid structure allowed for the development of more active and better-tolerated molecules. The anti-inflammatory potency varies between different compounds, as do their pharmacodynamic and pharmacokinetic properties. Corticosteroids have become a mainstay in the treatment of a wide range of conditions, including allergies, transplant rejection, and many rheumatologic, dermatologic, respiratory, gastrointestinal, ophthalmologic, and ENT diseases—all of which have a predominant inflammatory component. The anti-inflammatory mechanisms of glucocorticoids are complex and still not fully understood. Corticosteroids act on specific cytosolic receptors and exert their effects primarily through genomic mechanisms—

affecting the transcription of DNA into RNA and the post-transcriptional regulation of messenger RNA. This process explains the delay of several hours before the biological effect begins, though this effect lasts much longer than the plasma half-life of the drugs in this pharmacological class. Glucocorticoids act on numerous targets, both humoral and cellular. At the humoral level, they interact with cytokines, chemokines, adhesion molecules, enzymes, and molecules involved in cell proliferation and apoptosis. At the cellular level, they affect a wide range of immune cells, both innate and adaptive, including macrophages, neutrophils, mast cells, T and B lymphocytes, dendritic cells, fibroblasts, and also other cells such as epithelial, endothelial, mesangial, and smooth muscle cells. This broad spectrum of effects explains both their remarkable efficacy in many inflammatory and autoimmune diseases, and their delicate safety profile, which can be problematic in clinical practice. Glucocorticoids are associated with numerous side effects that can affect nearly every system. Some appear quite rapidly, within days or weeks (e.g., the onset or worsening of diabetes), while others emerge after years of use (e.g., osteoporosis). In general, adverse effects are dose- and exposure-dependent, prompting clinicians to use the lowest effective daily doses for the shortest possible duration. In addition to carefully selecting indications, dose, and treatment duration, two other strategies are commonly used in clinical practice to improve tolerance. The first is combining glucocorticoids with other drugs that also have anti-inflammatory or immunomodulatory effects, allowing for a significant reduction in corticosteroid dosage. This approach is used, for example, in the treatment of rheumatoid arthritis (combined with methotrexate) or to prevent transplant rejection (combined with calcineurin inhibitors). The second strategy is to avoid systemic administration (oral, intravenous, or intramuscular) whenever possible by favoring localized delivery targeting the inflamed tissues or organs. Examples include inhaled corticosteroids for pulmonary inflammatory diseases (asthma being the archetype), local sprays or drops for ENT or eye inflammations, topical applications for dermatological conditions (such as psoriasis), intra- or peri-articular injections in certain rheumatic or musculoskeletal disorders, and enemas for inflammatory rectal conditions. In many inflammatory and autoimmune diseases, glucocorticoids remain highly valuable, with a favorable benefit-risk ratio—provided contraindications are respected and use is carefully managed to limit both dosage and duration of exposure. In allergology, glucocorticoids are also very effective, especially when H1 antihistamines fail. They are widely used in oncology not only for their anti-inflammatory effects but also, for example, as antiemetics or to prevent cytokine release reactions during certain chemotherapy regimens. During the COVID-19 pandemic, glucocorticoids came into the spotlight after dexamethasone was shown to reduce the need for assisted ventilation and

mortality one of the few drugs to demonstrate clear efficacy. Finally, some patients may develop “corticosteroid resistance,” which necessitates turning to alternative therapies, particularly biologics. (table 10) ( Burns CM. The history of cortisone discovery )

**Table 10.** Relative anti-inflammatory potency of the main glucocorticoids, compared to other properties of this pharmacological class

Molecules	Molecules Anti-inflammatory activity	Sodium retention power	Duration of biological action (a)	Equivalent dose (b)
<b>Cortisol(c)</b>	1	1	8-12h	20
<b>(Hydro)Cortisone</b>	0.8	0.8	8-12h	25
<b>Fludrocortisone</b>	10	125	8-12h	0.025-0.100(d)
<b>Prednisone</b>	4	0.8	12-36h	5
<b>Methylprednisolone(e)</b>	5	0.5	12-36h	4
<b>Triamcinolone</b>	5	0	12-36h	4
<b>Betamethasone</b>	25	0	36-72h	0.75
<b>Dexamethasone (f)</b>	25	0	36-72h	0.75

## 9. Mechanisms of Inflammation :

Inflammation involves a series of immunological, physiological, and behavioral processes that are regulated by immune signaling molecules called cytokines. The first step of this cascade is the recognition of infection or damage through the detection of PAMPs (pathogen-associated molecular patterns) and DAMPs (damage-associated molecular patterns), which alert the innate immune system. TLR and NLR receptors then recognize these patterns, activating signaling pathways that culminate in the activation of NF- $\kappa$ B, which acts as a transcription factor to upregulate genes responsible for the inflammatory response. This activation leads to the expression of proinflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$ , which help recruit immune cells like neutrophils and monocytes to the site of inflammation. Neutrophils release toxic substances to destroy pathogens, which causes tissue damage in the surrounding area.

The typical signs of inflammation, including heat, swelling, redness, pain, and loss of function, appear as a result of this process. The adaptive immune system further regulates these responses. In the final phase, inflammation is resolved in a coordinated manner by macrophages, which switch from producing proinflammatory molecules to those that reduce neutrophil recruitment and promote monocyte infiltration, aiding tissue repair. ( Janeway et al. 2005)

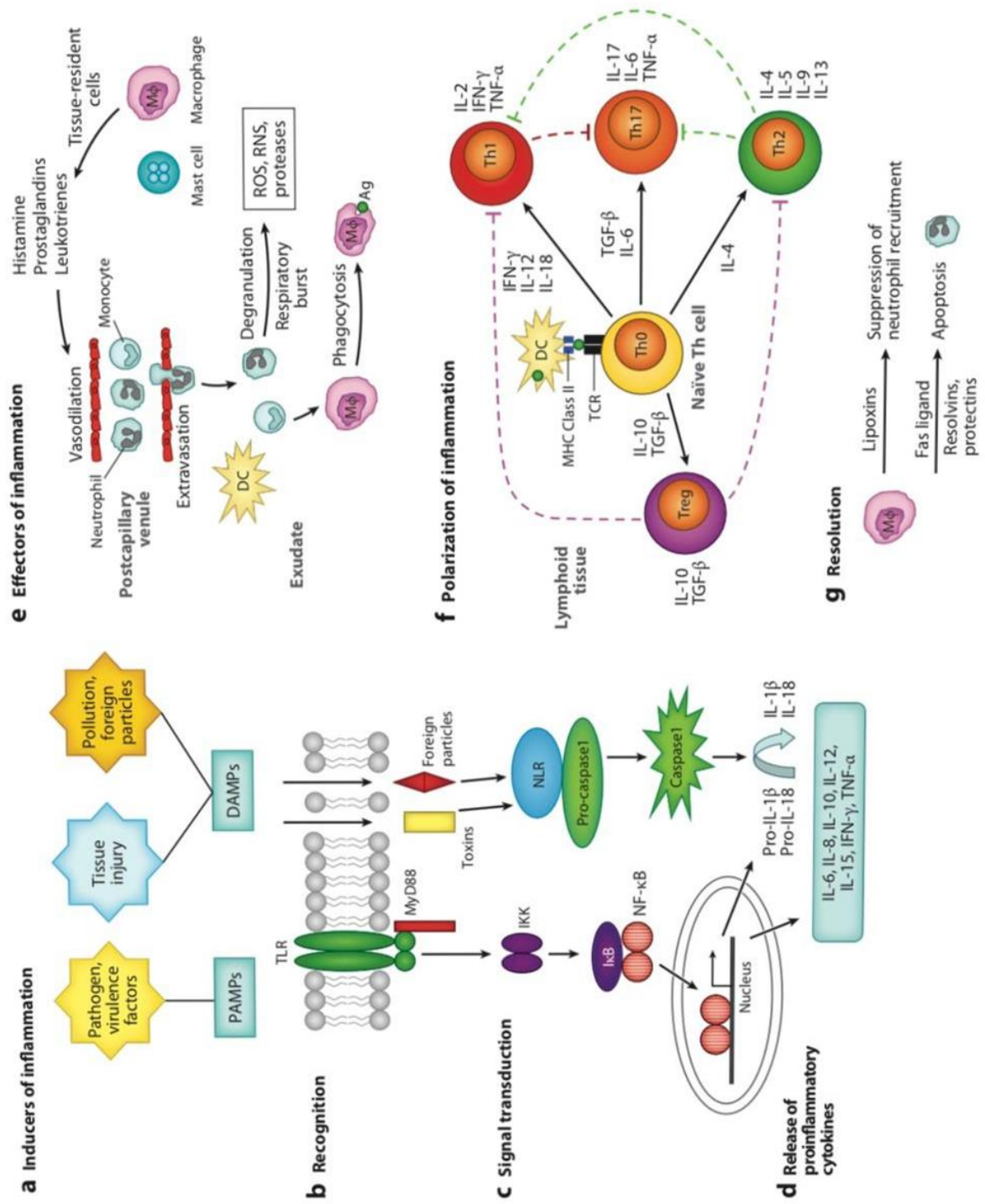
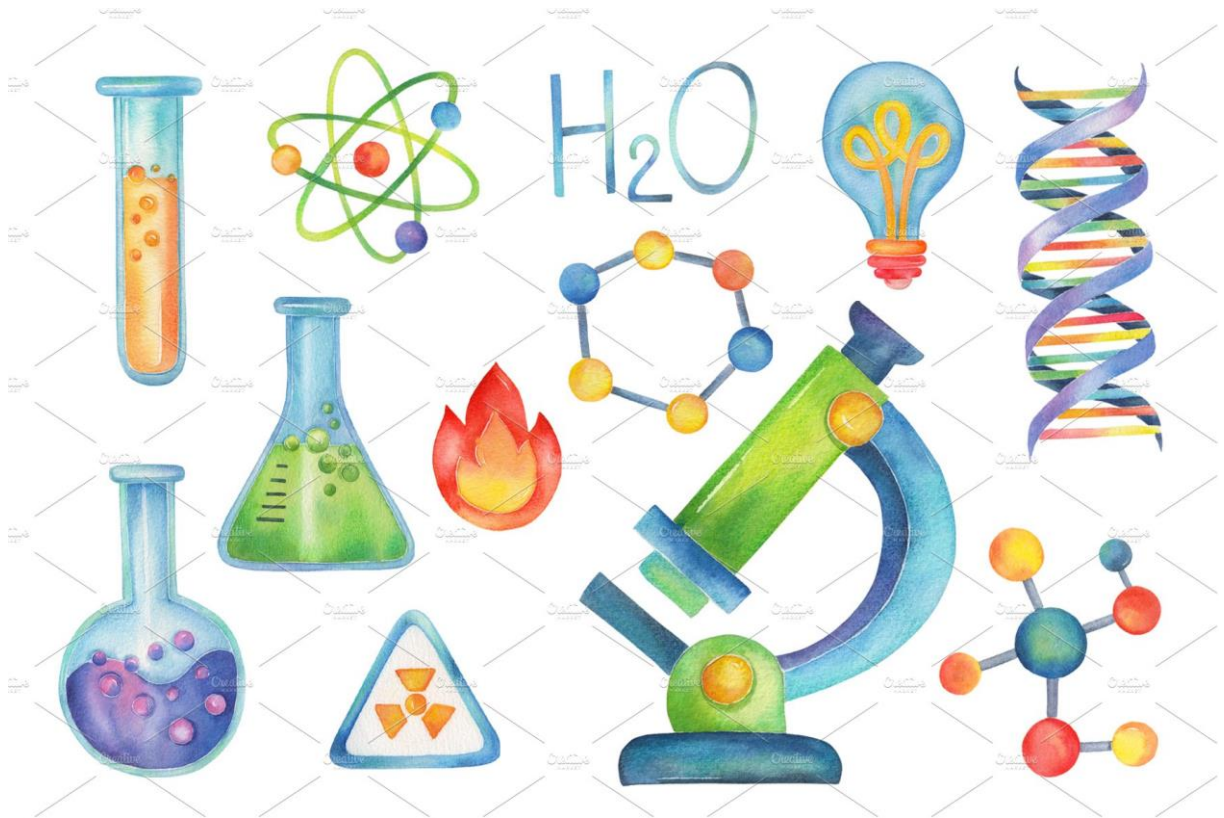


Figure 16- Mechanisms of Inflammation

# 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:

### 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 [Figure17](#)

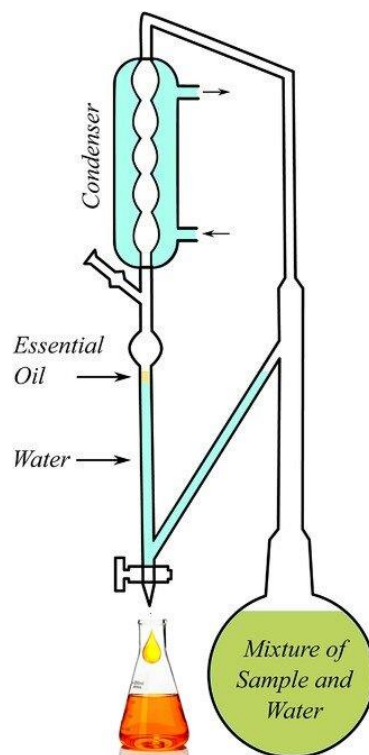


Figure 17. Schematic representation of the Clevenger apparatus used in the essential oil extraction process ([Biswa et al., 2023](#))

#### 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](#):

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

(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](#):

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

(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<sup>2</sup> 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):

$$d = \frac{m_{EO}}{m_{H_2O}}$$

(3)

Where<sup>2</sup>:  $m_{EO}$ : 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.

$$I_a = \frac{56.11 \times V \times C}{m}$$

(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:

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

(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<sup>[7]</sup> 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 stationary 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,<sup>[7]</sup> 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<sub>2</sub> 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<sub>2</sub>) 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.

## **5. *In Vivo* study**

**1-Care of Animals:** The experimental group consisted of twenty-five (25) Wistar mice, initially weighing between 150 and 200 grams. All animals were of uniform age and in good physiological condition. These rats were obtained from the Pasteur Institute in Algiers and housed in a dedicated animal facility within the Faculty of Natural and Life Sciences at the University of El Oued. They were kept under standardized environmental conditions, including a temperature of  $25 \pm 2$  °C and a 12-hour light/dark cycle. The rats were housed in plastic cages, each accommodating five rats. The cages were furnished with substrates composed of sawdust, bran, and water, which were replenished daily throughout the experimental period. Regular assessments of the rats body weights were also conducted.

### 1. Hypersensitivity Reaction:

BTU may act as a hapten, binding to liver proteins and forming a foreign complex that the immune system recognizes as a foreign substance.

This leads to T cells being activated and attacking liver cells, causing autoimmune hepatitis.

This type is characterized by hypereosinophilia in some cases and a marked elevation in liver enzymes (ALT, AST).

### 2. Direct hepatotoxicity:

Metabolites resulting from BTU metabolism in the liver may be directly toxic to liver cells.

These products may cause oxidative stress within the cell, leading to mitochondrial degeneration and liver cell dysfunction.

### 3. Drug-induced autoimmunity:

In some cases, prolonged use of BTU can trigger autoimmunity against liver tissue.

**3- Experimental Description:** After inducing inflammation, the experimental groups were maintained under identical conditions. The animals were then randomly divided into five groups, each consisting of five male rats Adaptation period 15 days:

**Group 1 (Control):** We have, as a control group, healthy rats that were fed bran and water throughout the duration of the experiment.

**Group 2 (BTU):** All members of this group were fed only standard food and given drinking water cloudy with benzylthiouracil at a rate of 2mg/kg for 21 days.

**Group 3 (BTU+CC):** All members of this group were fed only standard food and given drinking water cloudy with benzylthiouracil at a rate of 2mg/kg for 21 days with 100 µL/rat/day essential oil of *Cotula cinerea* Plant for 7days .

**Group 4 (BTU+OM):** All members of this group were fed only standard food and given drinking water cloudy with benzylthiouracil at a rate of 2mg/kg for 21 days with 100 µL/rat/day essential oil of *Origanum marjoram* L Plant for 7 days .

**Group 5 (BTU+IBU):** All members of this group were fed only standard food and given drinking water cloudy with benzylthiouracil at a rate of 2mg/kg for 21 days with 2mg /kg ibuprofen for 7days.

### **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. Following euthanasia, the liver, 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. 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.

### 5-Organ weight index :

The scientific index used for calculating organ weights is employed to assess changes in organ size relative to total body weight, particularly in toxicological and nutritional studies. It is commonly used for comparisons between experimental groups and serves as an important tool for monitoring pathological or therapeutic effects on organ size. The formula for calculating the organ weight index is:

$$\text{OWT} = \left( \frac{\text{organ weight}(g)}{\text{body weight}(g)} \right) \times 100$$

### 6-Methods of blood analysis:

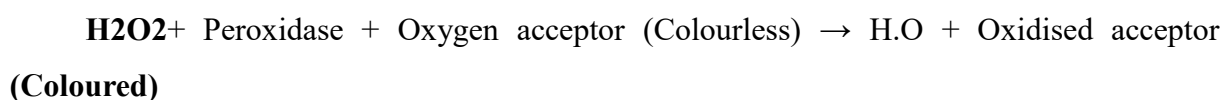
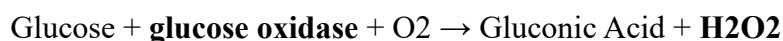
#### 1. Hematological parameters assay :

The hematological parameters including leukocytes (WBC), lymphocytes (LYM), granulocytes (GRA), erythrocytes (RBC), hemoglobin (HGB), mean corpuscular volume (MCV), and platelets (PLT) are determined using the Coulter method with a Medonic-type automated hematology analyzer, specifically designed for Complete Blood Count (CBC) analysis.

#### 2. Biochemical parameters assay:

##### Blood glucose

Beta-D-glucose in plasma is oxidized by the enzyme glucose oxidase to D-glucono-1,5-lactone, with the simultaneous production of hydrogen peroxide. The lactone is then slowly hydrolyzed to D-gluconic acid. The hydrogen peroxide produced is subsequently broken down by the enzyme peroxidase into oxygen and water. The released oxygen then reacts with an oxygen acceptor, forming a colored compound whose concentration can be measured colorimetrically. (Trinder, 1969).

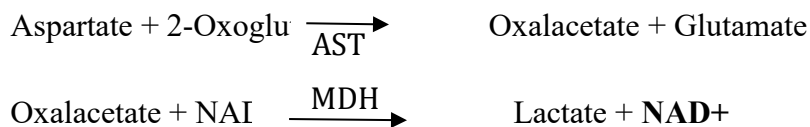


##### C- reactive protein

The concentration of C-reactive protein (CRP) in serum was determined using the turbidimetric method on a COBAS INTEGRA 400 analyzer. This method employs an efficient monospecific antibody-based equilibrium turbidimetric immunoassay. Polyethylene glycol-6000 is used to accelerate and enhance the immunoprecipitation reaction, while the surfactant Tween-20 is added to reduce and stabilize sample blank values. (Otsuji et al., 1982).

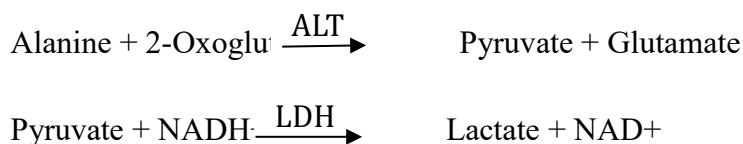
#### Aspartate transaminase

Aspartate transaminase (AST), also known as glutamate oxaloacetate transaminase (GOT), activity was measured spectrophotometrically using an enzymatic kinetic method. AST catalyzes the transfer of an amino group from aspartate to  $\alpha$ -ketoglutarate, producing glutamate and oxaloacetate. Subsequently, malate dehydrogenase (MDH) catalyzes the conversion of oxaloacetate to malate, utilizing NADH in the process. The rate of NADH oxidation, indicated by a decrease in absorbance, is directly proportional to the AST activity in the sample. Absorbance is measured at a wavelength of 340 nm. (Schumann et al., 2010).



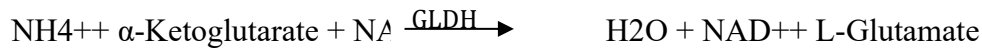
#### Alanine transaminase

The activity of alanine aminotransferase (ALT) was measured spectrophotometrically using an enzymatic kinetic method. ALT, also known as glutamate-pyruvate transaminase (GPT), is a transaminase enzyme that catalyzes the transfer of an amino group from L-alanine to  $\alpha$ -ketoglutarate, resulting in the formation of L-glutamate and pyruvate. Subsequently, pyruvate is reduced to lactate by lactate dehydrogenase (LDH) in the presence of NADH. The enzymatic activity of ALT in the sample is directly proportional to the consumption of NADH. The reaction is monitored by measuring the decrease in absorbance at 340 nm using a spectrophotometer (Schumann et al., 2010).



#### Urea

Urea concentration in the sample is determined enzymatically. Urease catalyzes the hydrolysis of urea into ammonia ( $\text{NH}_4^+$ ) and carbon dioxide ( $\text{CO}_2$ ). The ammonia produced is then incorporated into  $\alpha$ -ketoglutarate by the enzyme glutamate dehydrogenase (GLDH), accompanied by the oxidation of NADH to  $\text{NAD}^+$ . The decrease in NADH concentration is directly proportional to the amount of urea present in the sample. (Kaplan, 1984)



### Creatinine

Creatinine concentration is determined based on its reaction with sodium picrate. In an alkaline medium, creatinine reacts with picrate to form a red-colored complex. The measurement is carried out within a defined time interval to minimize interference from other serum components. The intensity of the resulting color is directly proportional to the creatinine concentration in the sample. (Peake and Whiting, 2006).

### Histopathological Study

For histological examination, tissue fragments from the organs of all experimental groups were collected promptly to prevent autolysis, which begins shortly after the animal's death. The samples were first rinsed with distilled water followed by 0.9% NaCl solution, then immediately immersed in 10% formaldehyde as a fixative (Leclerc-Mercier et al., 2016).

The histological processing involved several steps. First, the tissue samples were placed into specialized cassettes with perforated walls to allow fluid exchange. Dehydration was then carried out using an automatic tissue processor that gradually immersed the samples in ethanol baths of increasing concentrations (70%, 95%, and 100%).

Following infiltration, the embedding step was carried out by enclosing the paraffin-impregnated tissues in paraffin blocks. This process used embedding devices equipped with a heated paraffin reservoir, a dispensing tap, and a refrigerated metal plate to allow rapid solidification. Once solidified, thin sections averaging 5  $\mu\text{m}$  in thickness were obtained using a microtome. These sections were mounted on microscope slides, smoothed, and adhered to the slide using heated gelatinous water.

For staining, the Hematoxylin-Eosin (or Hematein-Eosin) technique was employed (Elmalti et al., 2007). The reagents used included:

Acid alcohol: 100 mL of 70% ethyl alcohol + 50 mL hydrochloric acid

Ammoniacal water: 100 mL distilled water + 2 mL ammonia Eosin solution: 100 mL of 3% aqueous eosin + 125 mL of 95% ethanol + 375 mL distilled water + 2 drops of acetic acid

**The staining protocol involved:**

1. Deparaffinization and hydration of the slides under tap water, followed by rinsing with distilled water.
2. Immersion in Harris Hematoxylin for 15 minutes to stain basophilic structures (nuclei) purplish-blue.
3. Differentiation using acid alcohol (1–2 dips), followed by rinsing with tap water and microscopic verification.
4. Immersion in ammoniacal water, then staining with eosin for 15 seconds to 2 minutes to stain acidophilic structures (cytoplasm) pink.
5. Tap water washes were used between all staining steps.

Finally, the prepared slides were dried and examined under an optical microscope. Photographs of representative histological sections were captured using a digital camera (BremondGignac et al., 2004).

## 6. *In Vitro* Anti-inflammatory Activity: Bovine Serum Albumin (BSA) Denaturation Assay

### 6.1. Overview

Protein denaturation is a key process in inflammatory disorders (Farooq et al., 2025), making BSA denaturation inhibition an effective model for assessing anti-inflammatory potential (Smati et al., 2025). This assay evaluates the ability of *Cotula cinerea* and *Origanum Majorana L.* extracts and synthesized nanoparticles to prevent protein denaturation, which may indicate their therapeutic potential for inflammation-related diseases.

### 6.2. Chemicals and Reagents

- Bovine serum albumin (BSA, 5% w/v) from Sigma-Aldrich
- deionized water Plant extracts (0.1–1 mg/mL)
- Diclofenac sodium (positive control, anti-inflammatory drug)

### 6.3. Procedure

In the present study, the assay was conducted following established methodologies (Gangadharan et al., 2025) with slight modifications to optimize experimental conditions. The reaction mixture was prepared by combining 500 $\mu$ L of a 5% BSA solution with 250  $\mu$ L of the test sample at varying concentrations (100 – 1000  $\mu$ g/mL). Diclofenac sodium was used as a positive control, while the negative control consisted of BSA solution mixed with distilled water under identical conditions. The prepared mixtures were incubated at 37°C for 20 minutes to allow interaction between BSA and the test compounds. Following this, the solutions were subjected to heat-induced denaturation by maintaining them at 70°C for 20 minutes. After the heating phase, the samples were cooled to room temperature and diluted with 500 $\mu$ L with deionized water before measuring their absorbance at 660 nm using a UV-Vis spectrophotometer. Blank was prepared with mixing: 1mL water + 250  $\mu$ L DMSO. The percentage inhibition of protein denaturation was calculated using the Equation 4:

$$\% \text{ Inhibition} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100 \quad (6)$$

Where  $A_{\text{control}}$  corresponds to the absorbance of the negative control, and  $A_{\text{sample}}$  represents the absorbance of the test sample or positive control. All experiments were performed in triplicate, and the results were expressed as mean  $\pm$  standard deviation (SD).

## 7. *In-Silico* analysis:

### 7.1. 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.

### 7.2. 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/>).

### 7.3. 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  $7.0 \pm 2.00$ , 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 Cyclooxygenase-1 and Cyclooxygenase-2 (Table 11) (PDB ID: 5wbe and 3ln1), respectively (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  $7.0 \pm 2.00$ . 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 11.** Target receptor information chosen for docking studies

Cyclooxygenase-1 (COX-1)	Detaille's		
	PDB ID	5WBE	
	Mutation	No	
	Resolution (Å)	2.75	
	R-Value Free	0.229	
	R-Value Observed	0.197	
	Organism	Ovis aries	
	Space Groupe	P 6 <sub>5</sub>	
	Sequence Length	600	
	Cyclooxygenase-2 (COX-2)	Detaille's	



?

PDB ID	3LN1
Mutation	No
Resolution (Å)	2.75
R-Value Free	0.229
R-Value Observed	0.197
Organism	Ovis aries
Space Group	P 6 <sub>5</sub>
Sequence Length	600

#### 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  $\Delta G$  (MM-GBSA  $\Delta G$ ) 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



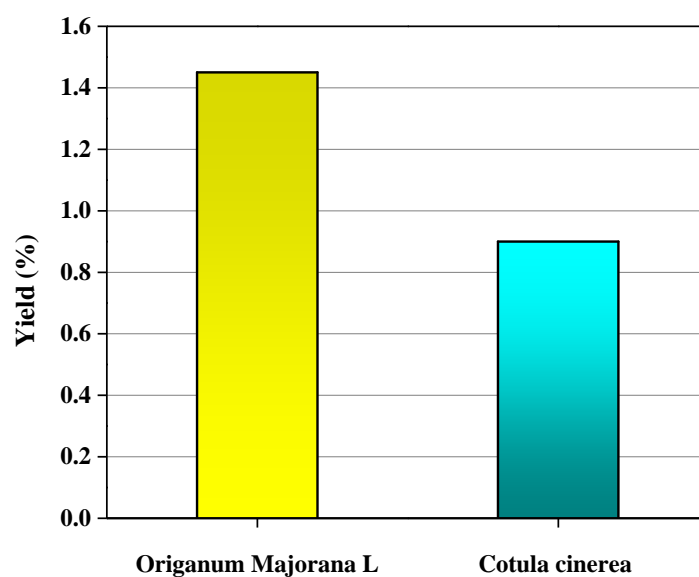
## 1. Introduction:

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



**Figure 18.** 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 12) organoleptic properties:

**Table 11.** The organoleptic characteristics of essential oils

Plant	Smell	Aspect	Colour
<i>Cotula cinerea</i>	A distinctive aroma	liquid at room temperature	Dark- yellow
<i>Origanum Majorana L</i>	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 13.

Table 12. The physicochemical properties of essential oils

Plant	Relative Density	Refractive Index	Acidity Index	Ester Index
<i>Cotula cinerea</i>	0.958	1.4720	5.01	43.72
<i>Origanum Majorana L</i>	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:  $0.834 \pm 0.02$ ,  $0.835 \pm 0.02$ , refractive index, at 25°C  $1.4596 \pm 0.03$ ,  $1.4622 \pm 0.04$ ), 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 Wiley 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 19 and Figure 20 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:

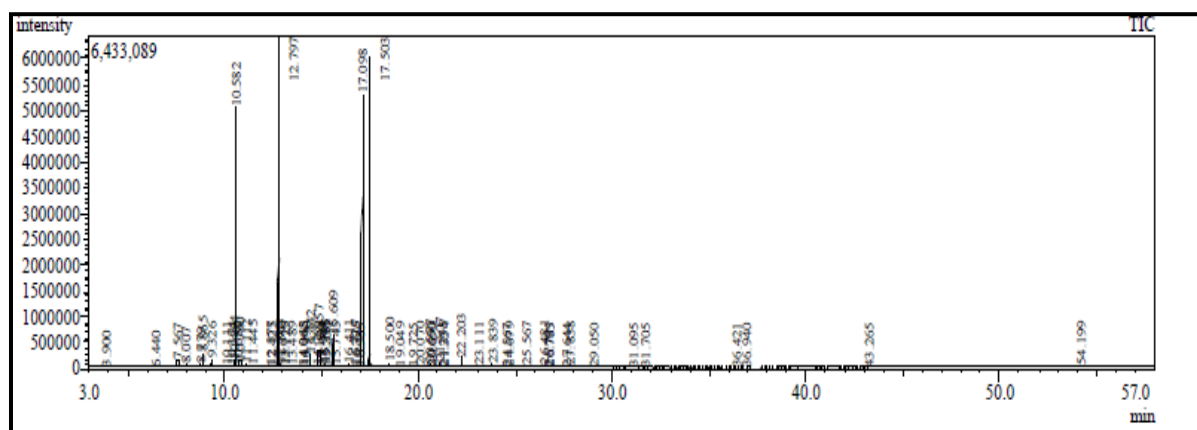
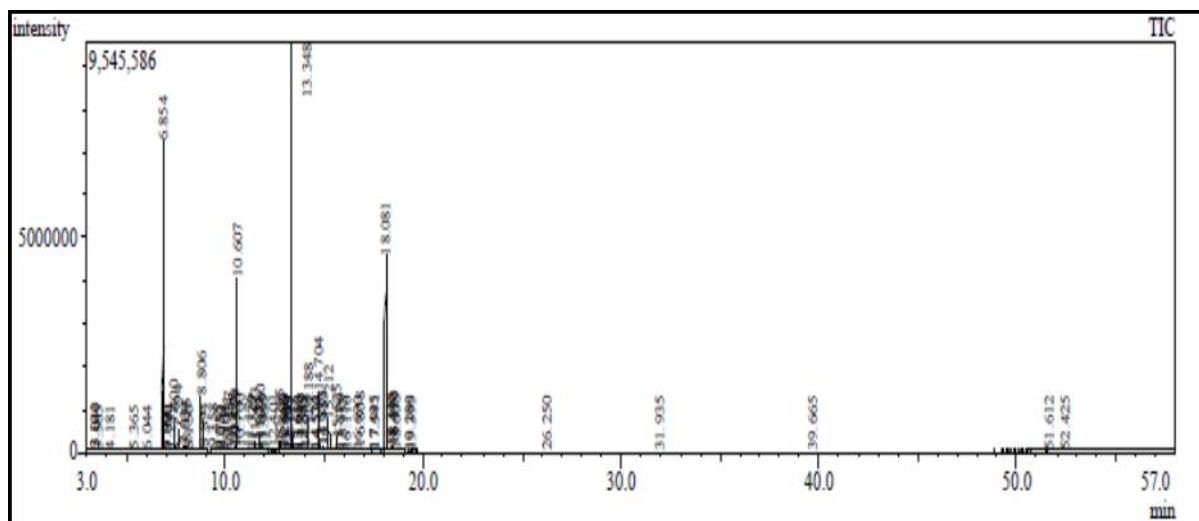


Figure 19. Chromatogram of the essential oil of *Cotula cinerea* plant obtained by GC/MS



**Figure 20.** 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 14](#)). 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 14.** Essential oil constituents of *Cotula cinerea* identified by GC/MS

No	Compounds	IR <sub>Exp</sub>	IR <sub>Ref</sub>	(%)
<b>01</b>	<b>Santolina triene</b>	<b>908</b>	<b>914</b>	<b>10.6</b>
02	Alpha-thujene	931	935	0.88
<b>03</b>	<b>Alpha-pinene</b>	<b>939</b>	<b>943</b>	<b>2.02</b>
04	camphene	953	956	0.85
<b>05</b>	<b>Sabinene</b>	<b>976</b>	<b>976</b>	<b>6.17</b>
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
<b>13</b>	<b>1.8-cineole</b>	<b>1033</b>	<b>1033</b>	<b>5.34</b>
<b>14</b>	<b>Delta-terpinene</b>	<b>1062</b>	<b>1057</b>	<b>1.57</b>
15	Cis sabinene hydrate	1068	1069	0.46
16	Terpinolene	1088	1088	0.36
17	Cis thujone	1102	1100	0.52
<b>18</b>	<b>Trans thujone</b>	<b>1114</b>	<b>1117</b>	<b>51.86</b>
<b>19</b>	<b>Camphor</b>	<b>1143</b>	<b>1140</b>	<b>2.63</b>
<b>20</b>	<b>Beta-terpineol</b>	<b>1163</b>	<b>1160</b>	<b>1.39</b>
<b>21</b>	<b>Terpin-4-ol</b>	<b>1177</b>	<b>1178</b>	<b>1.73</b>
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
<b>26</b>	<b>Cis verbenyl acetate</b>	<b>1262</b>	<b>1264</b>	<b>5.07</b>

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
<b>Total</b>			<b>95.64</b>	

Our analysis unveiled ten compounds with concentrations surpassing 1% (Table 14), 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  $\alpha$ -pinene, terpin-4-ol,  $\beta$ -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 Ekhilil et al.'s (Ekhilil 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 21, which depicts the chemical structures and IUPAC names of the predominant constituents identified in the essential oil of *Cotula cinerea*.

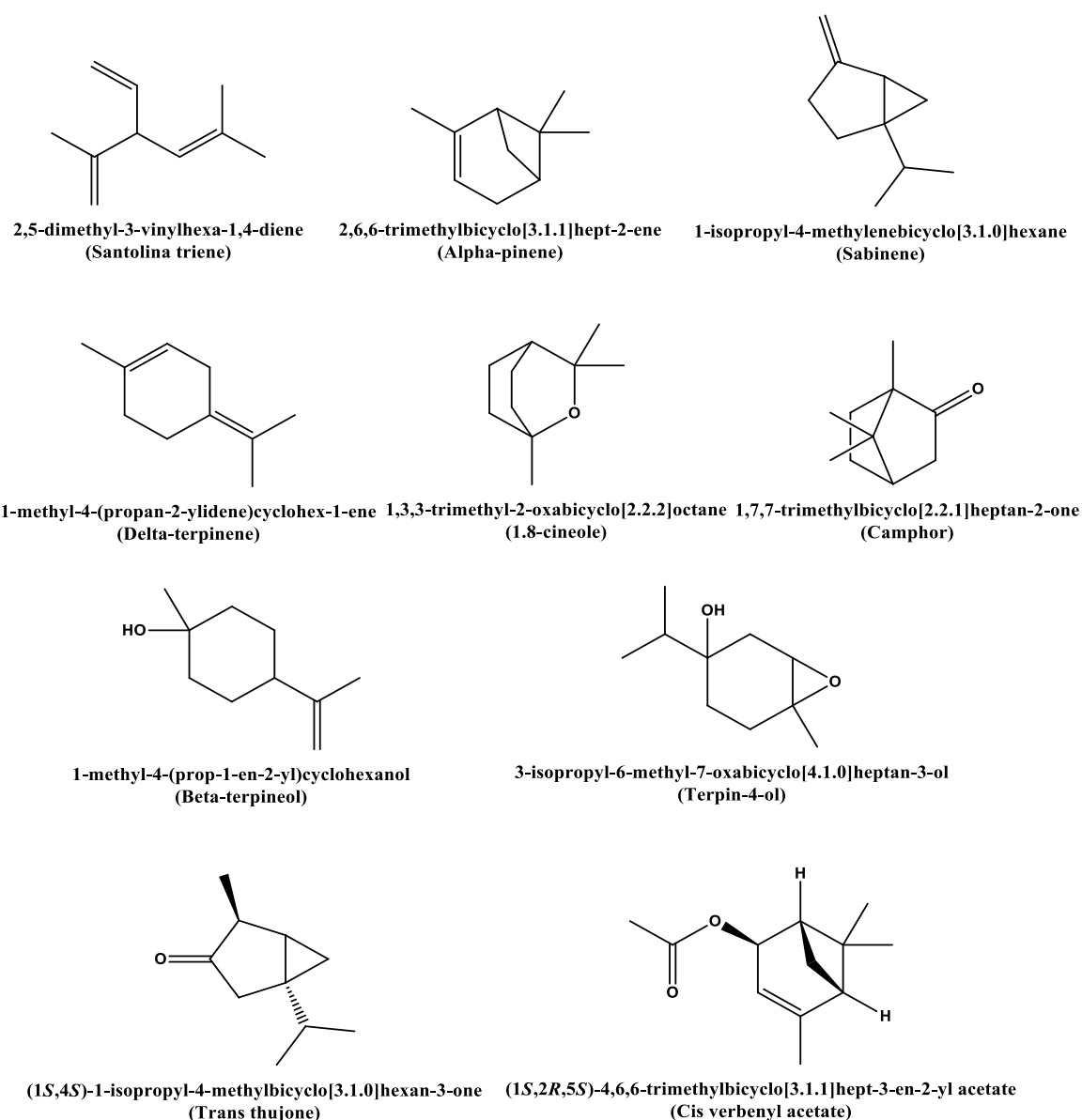
### 3.3.2. *Origanum Majorana L.*:

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 15). 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 absent 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 absent 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  $\alpha$ -Thujene (1.44%),  $\alpha$ -Pinene (1.19%), and  $\beta$ -Pinene oxide (4.42%).



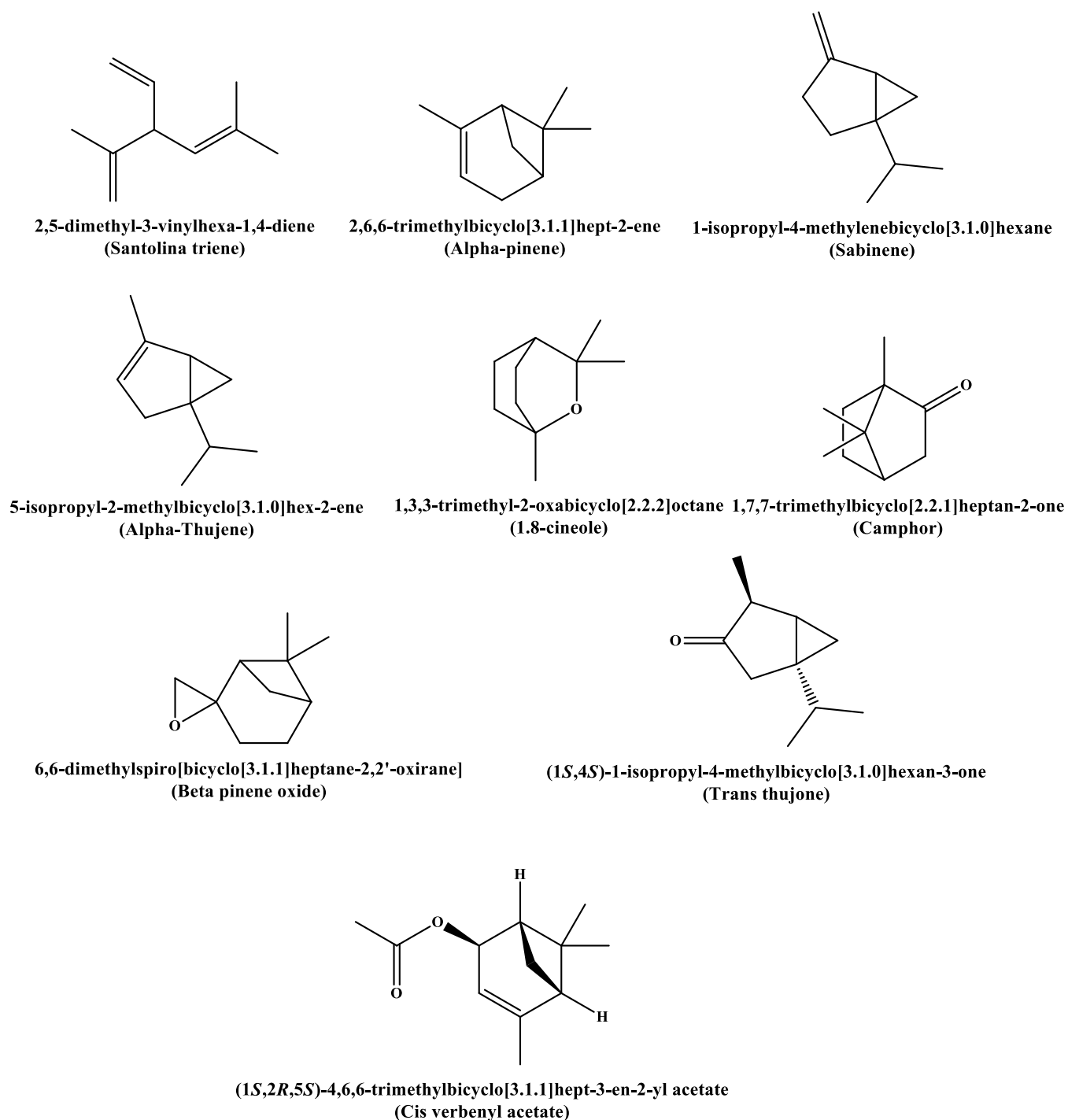
**Figure 21.** Chemical structures and IUPAC names of the top major compounds identified in the essential oil of *Cotula cinerea*

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 22](#), displaying the structures and IUPAC nomenclature of these constituents.

**Table 13.** Essential oil constituents of *Origanum Majorana L* identified by GC/MS

No	Compounds	IR <sub>Exp</sub>	IR <sub>Ref</sub>	(%)
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	<b>Santolina triene</b>	<b>898</b>	<b>906</b>	<b>16.42</b>
07	Tricyclene	912	921	0.02
08	<b>Alpha-Thujene</b>	<b>918</b>	<b>924</b>	<b>1.44</b>
09	<b>Alpha-Pinene</b>	<b>925</b>	<b>932</b>	<b>1.19</b>
10	Camphene	941	946	0.43
11	<b>Sabinene</b>	<b>969</b>	<b>969</b>	<b>3.12</b>
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	<b>1,8-Cineole</b>	<b>1031</b>	<b>1026</b>	<b>10.71</b>
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	<b>trans-Thujone</b>	<b>1119</b>	<b>1112</b>	<b>33.30</b>
29	iso-3-Thujanol	1136	1134	0.04
30	<b>Camphor</b>	<b>1146</b>	<b>1141</b>	<b>2.85</b>
31	<b>Beta pinene oxide</b>	<b>1163</b>	<b>1154</b>	<b>4.42</b>
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	<b>cis Verbenyl acetate</b>	<b>1277</b>	<b>1280</b>	<b>15.05</b>
40	neiso-3Thujanol acetate	1287	1281	0.09
41	Isobornyl acetate	1290	1283	0.21
42	Lavandulyl acetate	1293	1288	0.32
<b>Total</b>			<b>95.64</b>	



**Figure 22.** Chemical structures and IUPAC names of the top major compounds identified in the essential oil of *Origanum Majorana L*

#### 4. *In Vivo* Anti-Inflammatory activities:

##### 1. Organ Weight Index:

Compared to the control group, exposure to benzylthiouracil (BTU) resulted in a significant increase in the liver weight index and a highly significant increase in the kidney

weight index. The treatment with *Cotula cinerea* (BTU + CC) led to a significant reduction in liver weight index ( $p < 0.05$ ) and a highly significant reduction in kidney weight index ( $p < 0.01$ ) compared to the BTU group. Similarly, treatment with *Origanum Majorana* L (BTU + OM) produced a highly significant decrease in liver weight index ( $p < 0.01$ ), while the kidney weight index was reduced in a non-significant manner compared to the BTU group. Furthermore, treatment with ibuprofen (IBU) significantly decreased both liver and kidney weight indices relative to the BTU-exposed group.

**Table 16:** Organ weight Index of different experimental groups

	Organ Weight Index %	
	Liver	Kidneys
<b>Control</b>	3.0±0.03	0.53±0.02
<b>BTU</b>	4.36±0.18 <sup>a</sup>	0.65±0.01 <sup>b</sup>
<b>BTU + CC</b>	2.7 ±0.25 <sup>NS *</sup>	0.52±0.005 <sup>NS**</sup>
<b>BTU + OM</b>	2.8±0.09 <sup>NS**</sup>	0.54±0.02 <sup>NS</sup>
<b>BTU + IBU</b>	3.0±0.05 <sup>NS*</sup>	0.54±0.02 <sup>NS**</sup>

NS: Non-significant differences; Comparison with the control group:  $p < 0.05$  (a),  $p < 0.01$  (b),  $p < 0.001$  (c); Comparison with BTU group:  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*)

The table presents the "Organ Weight Index %" for the liver and kidneys of mice in different experimental groups, aiming to evaluate the effects resulting from the administration of substance BTU alone or with potential therapeutic agents (CC, OM, IBU). The following is a discussion of the results:

#### First: BTU Group Compared to the Control Group

**Liver:** The liver weight index significantly increased in the BTU group ( $4.36 \pm 0.18$ ) compared to the control group ( $3.0 \pm 0.03$ ), with strong statistical significance ( $p < 0.001$ ); this indicates the occurrence of hepatomegaly, possibly due to a toxic effect of the BTU substance.

**Kidneys:** Similarly, an increase in the kidney weight index was observed in the BTU group ( $0.65 \pm 0.01$ ) compared to the control group ( $0.53 \pm 0.02$ ) with statistical significance ( $p < 0.01$ ). This may indicate a negative effect of BTU on the kidneys as well, such as inflammation or fluid retention.

#### Second: Effect of Therapeutic Agents (CC, OM, IBU) Co-administered with BTU

##### BTU + CC:

**Liver:** A significant decrease in liver weight ( $2.7 \pm 0.25$ ) was observed compared to the BTU group, with strong statistical significance ( $p < 0.001$ ). This suggests a protective effect of substance CC against BTU-induced hepatotoxicity.

Kidneys: The weight decreased ( $0.52 \pm 0.005$ ), which is very close to the control group. This also indicates potential renal protection from CC.

BTU + OM:

Liver: This group also showed a significant decrease ( $2.8 \pm 0.09$ ) with statistical significance ( $p < 0.001$ ) compared to BTU, demonstrating a protective effect of OM.

Kidneys: The kidney weight ( $0.54 \pm 0.02$ ) is close to the control group, supporting the hypothesis of renal protection.

BTU + IBU:

Liver: The liver index ( $3.0 \pm 0.05$ ) returned to the level of the control group, which is strong evidence of a potent protective effect of IBU against the effects of BTU.

Kidneys: The value ( $0.54 \pm 0.02$ ) also suggests potential kidney protection.

General Conclusion:

The substance BTU caused an increase in the weight of the liver and kidneys, indicating potential organ toxicity.

The co-administered substances (CC, OM, IBU) all showed the ability to reduce this increase, suggesting their protective properties, especially against hepatic and renal effects.

## 2. Hematological parameters:

Table 16 presents the plasma concentrations of various hematological parameters across the experimental groups. In the control group, baseline values were within normal physiological ranges for all measured indices.

Exposure to benzylthiouracil (BTU) caused marked hematological alterations. A significant increase in white blood cell count (WBC) ( $4.23 \pm 0.18$ ;  $p < 0.01$ ), lymphocytes (LYM) ( $3.1 \pm 0.08$ ;  $p < 0.05$ ), and platelets (PLT) ( $676 \pm 11.5$ ;  $p < 0.001$ ) was observed compared to the control group. In contrast, hemoglobin (HGB) and red blood cell count (RBC) were significantly reduced ( $9.4 \pm 0.21$  g/dL and  $4.9 \pm 0.14 \times 10^{12}/L$  respectively;  $p < 0.05$ ), indicating BTU-induced anemia. Granulocyte (GRA) levels showed a non-significant decrease.

The treatment with *Cotula cinerea* (BTU + CC) effectively improved several hematological parameters. While WBC and LYM levels decreased compared to BTU (though not significantly), PLT levels were significantly reduced ( $456.3 \pm 44$ ;  $p < 0.05$  vs. BTU). Moreover, HGB and RBC levels significantly increased compared to BTU ( $15.4 \pm 0.12$  g/dL and  $7.17 \pm 0.11 \times 10^{12}/L$ ;  $p < 0.01$ ), reflecting a protective or restorative effect.

*Origanum Majorana* (BTU + OM) treatment further modulated hematological profiles. WBC and LYM were elevated compared to control and BTU ( $5.1 \pm 0.05$  and  $3.6 \pm 0.26$  respectively;  $p < 0.01$ ), suggesting an immune-stimulatory response. HGB and RBC levels

improved significantly ( $p < 0.05$  vs. BTU), and PLT levels increased substantially ( $800.67 \pm 5.4$ ;  $p < 0.01$  vs. control and  $p < 0.05$  vs. BTU).

In the BTU + ibuprofen (IBU) group, hematological values showed improvement over the BTU group. HGB and RBC levels were significantly elevated ( $15.16 \pm 0.2$  g/dL and  $6.3 \pm 0.19 \times 10^{12}/L$ ;  $p < 0.001$  and  $p < 0.05$  vs. BTU, respectively). However, WBC, LYM, and PLT remained elevated and were not significantly different from the BTU group.

**Table 17** : Plasma concentration of hematological parameters of different experimental groups

	WBC( $\times 10^9/L$ )	LYM( $\times 10^9/L$ )	GRA( $\times 10^9/L$ )	HGB (g/dL)	RBC( $\times 10^{12}/L$ )	PLT ( $\times 10^9/L$ )
<b>Control</b>	1.46 $\pm$ 0.29	1.4 $\pm$ 0.26	1.1 $\pm$ 0.4	14.46 $\pm$ 0.78	7.8 $\pm$ 0.47	516 $\pm$ 9.23
<b>BTU</b>	4.23 $\pm$ 0.18 <sup>b</sup>	3.1 $\pm$ 0.08 <sup>a</sup>	0.16 $\pm$ 0.08 <sup>NS</sup>	9.4 $\pm$ 0.21 <sup>a</sup>	4.9 $\pm$ 0.14 <sup>a</sup>	676 $\pm$ 11.5 <sup>c</sup>
<b>BTU + CC</b>	2.63 $\pm$ 0.31 <sup>NS</sup>	2.23 $\pm$ 0.12 <sup>NS*</sup>	1.7 $\pm$ 0.49 <sup>NS</sup>	15.4 $\pm$ 0.12 <sup>NS**</sup>	7.17 $\pm$ 0.11 <sup>NS**</sup>	456.3 $\pm$ 44 <sup>NS*</sup>
<b>BTU + OM</b>	5.1 $\pm$ 0.05 <sup>b NS</sup>	3.6 $\pm$ 0.26 <sup>b NS</sup>	1.45 $\pm$ 0.55 <sup>NS</sup>	15.26 $\pm$ 0.76 <sup>NS*</sup>	6.77 $\pm$ 0.57 <sup>a NS</sup>	800.67 $\pm$ 5.4 <sup>b*</sup>
<b>BTU + IBU</b>	4.67 $\pm$ 0.88 <sup>a NS</sup>	3.0 $\pm$ 0.45 <sup>a NS</sup>	1.06 $\pm$ 0.14 <sup>NS*</sup>	15.16 $\pm$ 0.2 <sup>NS***</sup>	6.3 $\pm$ 0.19 <sup>NS*</sup>	761.3 $\pm$ 18 <sup>b NS</sup>

NS: Non-significant differences; Comparison with the control group:  $p < 0.05$  (a),  $p < 0.01$  (b),  $p < 0.001$  (c); Comparison with BTU group:  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*)

#### Discussion of Results

##### 1\_WBC (White Blood Cell Count):

Control:  $1.46 \pm 0.29$

BTU: Significantly increased to  $4.23 \pm 0.18$  ( $p < 0.001$  compared to the control group), indicating an acute inflammatory response due to BTU exposure.

BTU + CC: The count decreased to  $2.63 \pm 0.31$ , with statistical significance ( $p < 0.01$ ), demonstrating an anti-inflammatory effect of CC.

BTU + OM and BTU + IBU: Both groups showed a further increase in WBC, particularly OM ( $5.1 \pm 0.05$ ), which might suggest a persistent immune response or a stimulatory effect on the immune system.

##### 2\_LYM (Lymphocytes):

A significant increase was observed in the BTU group ( $3.1 \pm 0.08$ ) compared to the control ( $1.4 \pm 0.26$ ), reflecting an immune response.

The different treatments reduced this increase, especially CC and IBU, suggesting an anti-inflammatory or immunomodulatory effect.

##### 3\_GRA (Granulocytes):

Significantly decreased in the BTU group ( $0.16 \pm 0.08$ ), indicating potential immunosuppression or an acute deficiency.

This indicator improved with the treatments, particularly with CC and OM, suggesting their role in restoring immune balance.

#### 4\_HGB (Hemoglobin):

Decreased in the BTU group to  $9.4 \pm 0.21$  ( $p < 0.001$ ), suggesting potential anemia induced by toxicity.

All three treatments showed a significant improvement in HGB, especially with OM and IBU, indicating their protective effect against anemia.

#### 5\_RBC (Red Blood Cells):

A decrease was observed in the BTU group ( $4.9 \pm 0.14$ ), associated with a direct toxic effect on the bone marrow.

Values improved with CC and IBU, but did not fully return to normal, indicating only partial improvement.

#### 6\_PLT (Platelets):

Decreased in the BTU group ( $676 \pm 11.5$ ) compared to the control, indicating a toxic effect on platelet production.

Treatment with OM showed a significant increase ( $800.67 \pm 5.4$ ), suggesting high effectiveness in protecting/restoring platelet function.

#### 6\_Overall Conclusion:

BTU causes acute hematological changes including anemia, inflammation, and immunosuppression. The three compounds CC, OM, and IBU showed protective effects to varying degrees, with OM appearing to be the most effective in restoring normal blood parameters, followed by IBU and then CC.

### 3.Glycemia, liver and kidneys function parameters:

Table 18 summarizes the effects of different treatments on fasting blood sugar (FBS), liver enzymes (AST and ALT), kidney function (urea), and creatinine (CT) levels in the experimental groups.

In the control group, all measured parameters remained within normal physiological ranges.

Exposure to benzylthiouracil (BTU) significantly disrupted metabolic and organ function markers. FBS levels increased markedly to  $0.93 \pm 0.01$  g/L ( $p < 0.05$ ), indicating hyperglycemia. Similarly, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were significantly elevated ( $226.06 \pm 8.84$  U/L and  $116 \pm 2.63$  U/L, respectively;

$p < 0.05$  and  $p < 0.01$ ), suggesting hepatocellular injury. Kidney function was also impaired, as reflected by a significant rise in urea ( $0.66 \pm 0.011$  g/L;  $p < 0.01$ ) and creatinine (CT) ( $5.56 \pm 0.033$  mg/L;  $p < 0.05$ ) compared to control values.

Treatment with *Cotula cinerea* (BTU + CC) led to notable improvements. FBS significantly decreased to  $0.76 \pm 0.01$  g/L ( $p < 0.05$  vs. BTU), while AST and ALT activities declined to  $111.1 \pm 18.7$  U/L and  $71.56 \pm 2.17$  U/L, respectively ( $p < 0.01$  vs. BTU), approaching control levels. Urea levels normalized ( $0.43 \pm 0.015$  g/L;  $p < 0.01$  vs. BTU), and CT also decreased to  $4.63 \pm 0.27$  mg/L, though not significantly.

In the *Origanum Majorana* (BTU + OM) group, FBS values were moderately reduced ( $0.64 \pm 0.05$  g/L), though the change was not statistically significant compared to BTU. Liver enzyme levels (AST:  $99.6 \pm 0.31$  U/L; ALT:  $76.11 \pm 1.15$  U/L) improved significantly

( $p < 0.01$ ), suggesting strong hepatoprotective effects. Urea and CT levels also improved, with urea significantly reduced ( $0.47 \pm 0.03$  g/L;  $p < 0.05$  vs. BTU) and CT showing a non-significant decrease ( $4.86 \pm 0.38$  mg/L).

Administration of ibuprofen (BTU + IBU) also demonstrated protective effects. FBS decreased significantly ( $0.63 \pm 0.032$  g/L;  $p < 0.01$  vs. BTU), and both AST and ALT levels were significantly reduced ( $102.3 \pm 9$  and  $78.0 \pm 5.7$  U/L, respectively;  $p < 0.001$  and  $p < 0.05$  vs. BTU), nearing normal levels. Kidney markers (urea and CT) also improved, with urea significantly reduced ( $0.47 \pm 0.02$  g/L;  $p < 0.05$ ), and CT lowered to  $4.63 \pm 0.24$  mg/L, though not significantly different from BTU.

**Table 18:** Glycemia, liver and kidneys function parameters of different experimental groups

	FBS (g/L)	AST (U/L)	ALT (U/L)	Urea (g/L)	CT (mg/L)
<b>Control</b>	0.58±0.04	126.13±6.48	79.23±2.9	0.43±0.017	5.06±0.066
<b>BTU</b>	0.93±0.01 <sup>a</sup>	226.06±8.84 <sup>a</sup>	116±2.63 <sup>b</sup>	0.66 ±0.011 <sup>b</sup>	5.56±0.033 <sup>a</sup>
<b>BTU + CC</b>	0.76±0.01 <sup>a*</sup>	111.1±18.7 <sup>NS**</sup>	71.56±2.17 <sup>NS**</sup>	0.43±0.015 <sup>NS**</sup>	4.63±0.27 <sup>NS</sup>
<b>BTU + OM</b>	0.64±0.05 <sup>NS</sup>	99.6±0.31 <sup>NS**</sup>	76.11±1.15 <sup>NS**</sup>	0.47±0.03 <sup>NS*</sup>	4.86±0.38 <sup>NS</sup>
<b>BTU + IBU</b>	0.63±0.032 <sup>NS**</sup>	102.3±9 <sup>NS**</sup>	78.0±5.7 <sup>NS*</sup>	0.47±0.02 <sup>NS*</sup>	4.63±0.24 <sup>NS</sup>

The table compares five experimental groups:

- Control
- BTU (treated with BTU substance)
- BTU + CC (co-treatment with BTU and compound CC)
- BTU + OM (BTU with compound OM)

- BTU + IBU (BTU with ibuprofen)

#### 1-FBS (Fasting Blood Sugar)

• A significant increase was observed in the BTU group ( $0.93 \pm 0.01$ ) compared to the control group ( $0.58 \pm 0.04$ ), indicating that BTU causes hyperglycemia.

• Co-treatment with CC, OM, and IBU led to a decrease in sugar levels, suggesting their protective or therapeutic role.

Scientific Interpretation: BTU may cause dysregulation of glucose metabolism or insulin resistance, while CC, OM, and IBU may possess antioxidant or anti-inflammatory effects that mitigate this impact.

#### 2-AST and ALT (Liver Enzymes)

• BTU significantly elevated AST (226.06) and ALT (116.2) levels, indicating liver damage.

• In the treated groups (BTU + CC, OM, IBU), a significant reduction in enzyme levels was observed, approaching the levels of the control group.

Scientific Interpretation: This suggests that CC, OM, and IBU may contribute to protecting the liver from BTU-induced damage, possibly by reducing inflammation or oxidative stress.

#### 3-Urea (Kidney Function Indicator)

• A noticeable increase in urea was observed in the BTU group ( $0.66 \pm 0.011$ ), suggesting a potential impact on kidney function.

• The return of values to levels close to the control in the other groups indicates a protective effect of the co-treatments.

Scientific Interpretation: This suggests that BTU may affect the renal excretion of waste products, while the accompanying compounds have a protective effect on the kidneys.

#### 4-CT (Total Cholesterol Level)

• There was a slight increase in the BTU group ( $5.56 \pm 0.033$ ), indicating a negative effect on lipids.

• The co-treatments (CC, OM, IBU) lowered this level, indicating a potential anti-hyperlipidemic effect.

Scientific Interpretation: BTU may cause metabolic changes leading to increased cholesterol, while the accompanying compounds improve lipid metabolism.

General Conclusion: It appears that BTU has a toxic effect on the liver and kidneys and affects blood sugar and lipid levels. In contrast, co-treatment with CC, OM, or IBU showed a

protective role to varying degrees, with the BTU + OM and BTU + IBU groups showing a clear advantage in reducing the damage.

The results shown in the figure 23 indicate that the BTU group (treated with the inflammation inducer only) recorded the highest concentration of C-reactive protein (CRP), reaching 21 mg/L, which confirms the induction of an acute inflammatory state. In contrast, the groups treated with *Cotula cinerea* (CC) oil and *Origanum Majorana* (OM) oil showed a significant decrease in CRP concentration, with values of 5 mg/L and 5.16 mg/L, respectively—close to that of the control group (4.53 mg/L). Similarly, the ibuprofen (IBU) group showed a comparable decrease (4.5 mg/L).

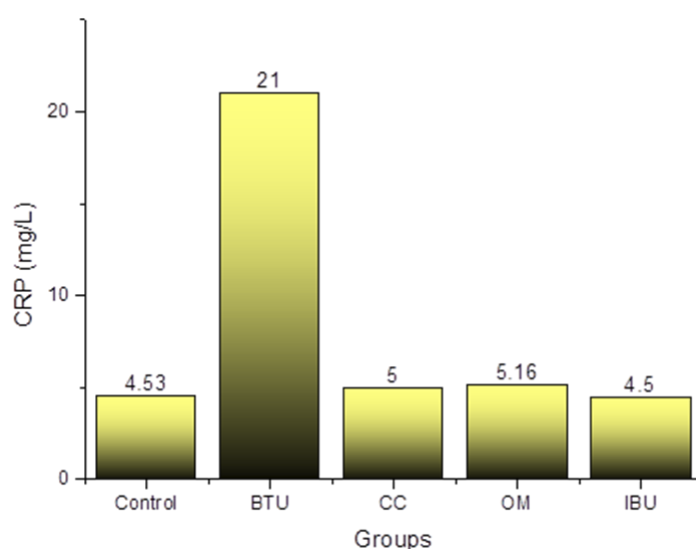
#### 4.C-reactive protein:

C-reactive protein (CRP) levels, an important marker of systemic inflammation, are presented in the table for all experimental groups.

In the control group, CRP levels were within normal physiological range ( $4.53 \pm 0.31$  mg/L). Exposure to benzylthiouracil (BTU) caused a highly significant increase in CRP levels ( $21 \pm 0.57$  mg/L;  $p < 0.01$ ), indicating a pronounced inflammatory response.

The treatment with *Cotula cinerea* (BTU + CC) and *Origanum Majorana* (BTU + OM) significantly reduced CRP concentrations to  $5.0 \pm 0.97$  mg/L and  $5.16 \pm 0.08$  mg/L, respectively ( $p < 0.01$  vs. BTU), restoring values close to control levels. These findings suggest strong anti-inflammatory potential of both herbal treatments.

Similarly, administration of ibuprofen (BTU + IBU) markedly decreased CRP levels to  $4.5 \pm 0.05$  mg/L ( $p < 0.001$  vs. BTU), fully restoring them to control levels, confirming its potent anti-inflammatory action.



**Figure 23:** Plasma concentration of C-reactive protein of different experimental groups

These results reflect the anti-inflammatory effect demonstrated by the essential oils of the studied plants, an effect comparable in efficacy to the well-known non-steroidal anti-inflammatory drug ibuprofen. This suggests the potential of these oils as effective natural alternatives in reducing inflammatory responses and lowering plasma CRP levels.

### **5. Histopathological studies:**

#### 5.1. Liver:

This histopathological study investigates the effects of benzylthiouracil (BTU)-induced liver inflammation and evaluates the protective role of different treatments. The control group (C) displays normal hepatic architecture, with well-organized hepatocytes and no signs of inflammatory infiltration or cellular damage, indicating healthy liver tissue.

In contrast, liver sections from the BTU group exhibit marked pathological changes. There is significant inflammatory infiltration, where immune cells accumulate in the tissue, accompanied by structural disorganization and hepatocellular damage. This confirms the hepatotoxic effect of BTU. When BTU is combined with ibuprofen (BTU + IBU), there is still evidence of inflammatory infiltration, although it appears slightly reduced compared to the BTU group. This suggests that ibuprofen has a moderate anti-inflammatory effect but does not completely restore normal liver architecture.

A more substantial improvement is observed in the BTU + *Cotula cinerea* (BTU + CC) group. The liver tissue shows a more preserved structure with reduced inflammation, indicating the potential hepatoprotective effect of *Cotula cinerea*. Similarly, the BTU + *Origanum Majorana* (BTU + OM) group exhibits near-normal liver histology. Hepatocytes appear healthy, and inflammatory cell infiltration is minimal, suggesting a strong protective effect of *Origanum Majorana* against BTU-induced liver damage.

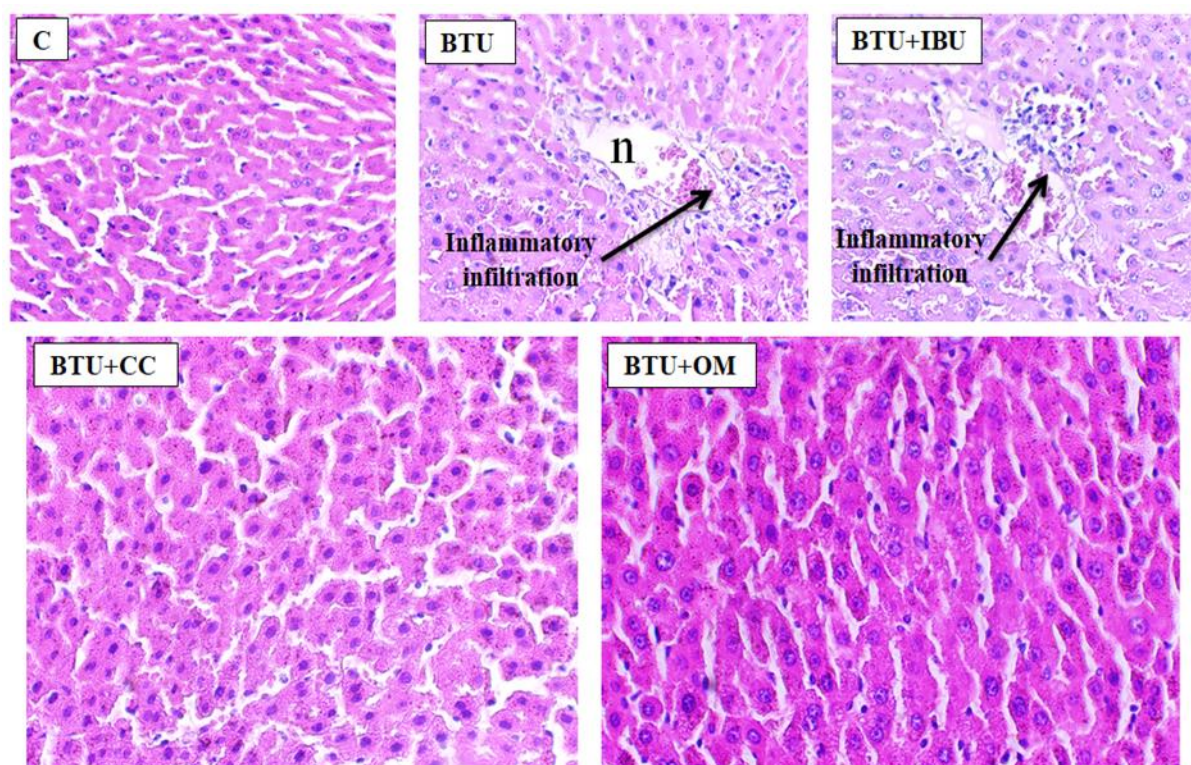


Figure 24: Microscopic observation of Liver histological sections from different experimental groups, (C) Control group, (BTU) Benzylthiouracil group, (BTU+Levo) Group treated with levothyroxine, (BTU+CC) Group treated with *Cotula cinerea*, and (BTU+OM) Group treated with *Origanum Majorana*, (V) Indicates thyroid follicles, (FC) Indicate follicular cells, (BV) Indicate blood vessel, Magnification  $\times 40$ .

Based on the histological sections of the liver under a microscope at  $\times 40$  magnification, as shown in Figure 24, the following discussion and interpretation of the liver histology results can be presented: The study demonstrated a clear effect of benzylthiouracil (BTU) on the liver's histological structure, while the different treatments contributed to the mitigation of these effects to varying degrees:

#### 1-Control Group (C):

The liver tissue exhibits a normal pattern, with hepatocytes arranged in radiating cords around the central veins, and clear intracellular vacuoles and cell boundaries.

There are no signs of inflammatory infiltration or pathological changes.

#### 2-BTU Group (Group treated with Benzylthiouracil only):

Clear pathological changes are evident, including severe inflammatory infiltration and hepatocyte degeneration.

Vascular changes and congestion were also observed, indicating the toxic effect of BTU on the liver, which could be attributed to oxidative stress or immune reaction.

### 3-BTU+Levo Group (Treated with Levothyroxine):

Despite observing partial improvement compared to the BTU group alone, inflammatory infiltration is still present, albeit to a lesser extent.

This indicates that levothyroxine alleviated some histological symptoms but was not sufficient to fully restore the normal liver structure.

### 4-BTU+CC Group (Treated with *Cotula cinerea*):

The histological sections showed a significant improvement in hepatocyte structure with a clear reduction in signs of inflammation. This suggests the effectiveness of *Cotula cinerea* in protecting the liver, likely due to its antioxidant and anti-inflammatory properties.

### 5-BTU+OM Group (Treated with *Origanum Majorana*):

This group was the closest to the normal tissue, as the liver showed an almost complete regularity in cell structure and the disappearance of most inflammatory manifestations.

This reflects the strong protective effect of *Origanum Majorana*, likely attributed to its rich content of phenolic compounds and flavonoids.

### Scientific Conclusion:

Exposure to benzylthiouracil leads to pathological histological changes in the liver, including inflammation and cellular damage.

Treatment with levothyroxine showed partial improvement.

However, *Cotula cinerea* and *Origanum Majorana* demonstrated a significant protective effect, with *Origanum Majorana* particularly restoring the histological structure to near normalcy .

### 5-2-Kidneys:

Histopathological examination of hematoxylin and eosin (H&E)-stained kidney sections was performed to assess the nephrotoxic effects of benzylthiouracil (BTU) and to evaluate the protective efficacy of ibuprofen, *Cotula cinerea*, and *Origanum Majorana* L. in mitigating BTU-induced renal damage. In the control group (C), renal tissue exhibited normal histological features, including well-defined glomeruli and intact tubular structures with no evidence of inflammatory infiltration or cellular degeneration, indicating physiological renal morphology. In stark contrast, kidney sections from BTU-treated animals revealed severe histopathological alterations, characterized by pronounced infiltration of inflammatory cells (indicated by “I”), structural disarray, and loss of normal renal architecture, thereby confirming BTU-induced nephrotoxicity and tissue injury. Treatment with ibuprofen (BTU + IBU) markedly attenuated these pathological changes, as evidenced by the substantial restoration of renal histoarchitecture

and a significant reduction in inflammatory infiltration, reflecting its potent anti-inflammatory and nephroprotective effects. The treatment with *Cotula cinerea* (BTU + CC) led to moderate histological improvement, with partial preservation of renal tissue and a noticeable reduction in inflammatory cells, indicating a mild to moderate protective influence likely attributable to its phytochemical constituents with anti-inflammatory potential. Similarly, administration of *Origanum Majorana* (BTU + OM) resulted in considerable amelioration of BTU-induced renal damage, as demonstrated by a decreased presence of inflammatory infiltrates and relatively preserved renal structures, suggesting a more pronounced protective effect.

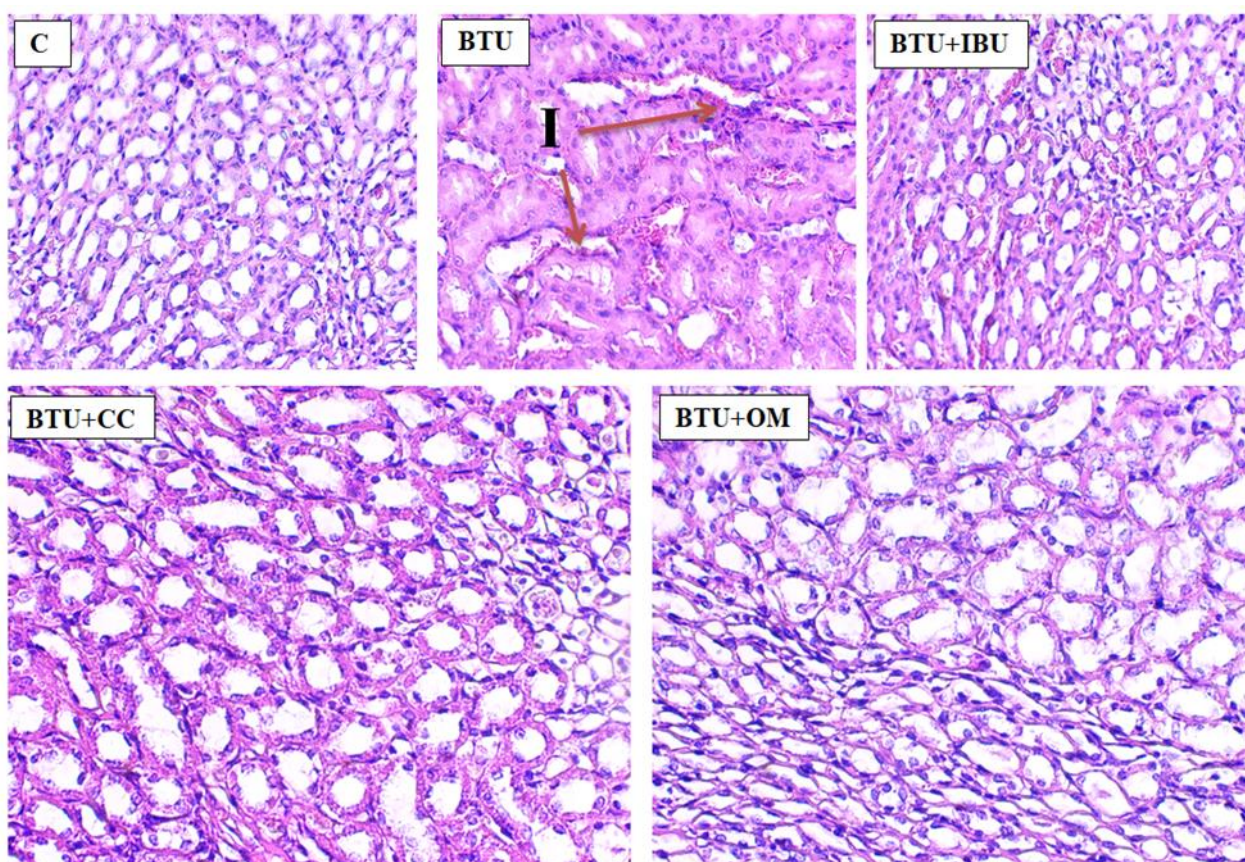


Figure 25 : Microscopic observation of Kidney histological sections from different experimental groups, (C) Control group, (BTU) Benzylthiouracil group, (BTU+Levo) Group treated with levothyroxine, (BTU+CC) Group treated with *Cotula cinerea*, and (BTU+OM) Group treated with *Origanum Majorana* L., (V) Indicates thyroid follicles, (FC) Indicate follicular cells, (BV) Indicate blood vessel, Magnification  $\times 40$ .

Discussion and Interpretation of Results:

The microscopic images illustrate the effect of Benzylthiouracil (BTU) on kidney tissue and the effectiveness of different treatments (Levothyroxine, *Cotula cinerea*, and *Origanum Majorana*) in mitigating the damage.

Control Group (C): The kidney tissues appear normal, with regular arrangement of renal tubules and intercellular spaces, indicating the integrity of the tissue structure and the absence of any inflammation or damage.

BTU Group: This group shows clear histological changes such as: Congestion in blood vessels (BV) Presence of inflammation (I) Damage to renal cells

These changes indicate the toxic effect of BTU on kidney tissue, which is often attributed to the inhibition of thyroid hormone secretion, leading to an imbalance in metabolism that causes renal damage.

BTU+Levo Group: There appears to be a noticeable improvement in the tissue structure compared to the BTU group, suggesting that Levothyroxine treatment helped reduce the damage and restore some renal function.

BTU+CC (*Cotula cinerea*) Group: The image shows a significant improvement in the kidney tissue structure, with a decrease in signs of inflammation and congestion, indicating the anti-inflammatory and antioxidant effectiveness of the *Cotula cinerea* plant.

BTU+OM (*Origanum Majorana*) Group: This group also shows a similar improvement to the *Cotula cinerea* group, with better regularity in the renal tubules and a clear reduction in indicators of damage, supporting the hypothesis that the *Origanum Majorana* plant has a protective effect due to its antioxidant properties.

General Conclusion: The substance BTU causes clear damage to kidney tissue.

All three treatments (Levo, CC, OM) showed a relative improvement in the histological picture of the kidney. The medicinal plants (*Cotula cinerea* and *Origanum Majorana*) demonstrated significant efficacy in reducing tissue damage, making them promising options as antioxidants and anti-inflammatories in cases of drug-induced hypothyroidism.

## **5. In Vitro Anti-Inflammatory Activity:**

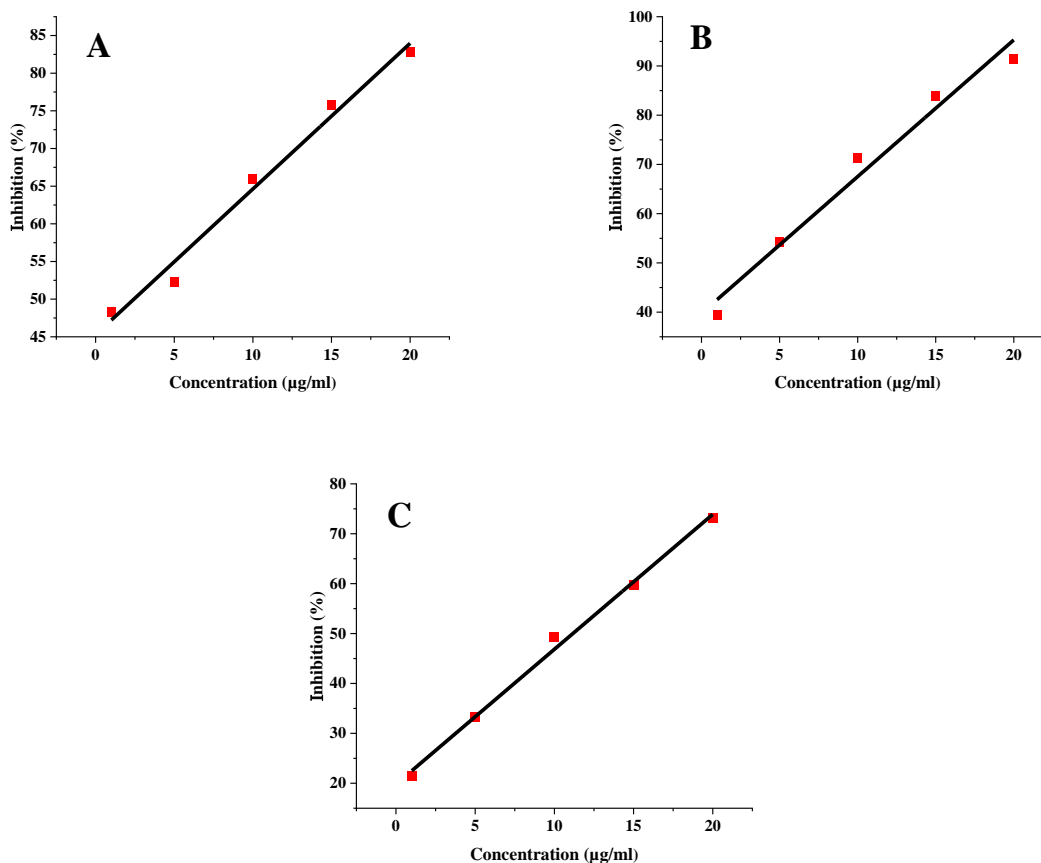
### **5.1. BSA inhibitory activities (IC<sub>50</sub>):**

The in-vitro anti-inflammatory potential of the two essential oils obtained from *Cotula cinerea* and *Origanum Majorana* L was investigated using the BSA (bovine serum albumin) denaturation inhibition model, and the results are presented in [Table 19](#). From the linear plots of *Cotula cinerea* and *Origanum Majorana* L concentration ( $\mu\text{g/mL}$ ) against percentage inhibition of BSA denaturation ([Figure 26](#)), the studied EOs significantly increased inhibition

(%) with corresponding increases in EO concentration. The effective inhibitory concentration (IC<sub>50</sub> value) at which 50% of protein denaturation was inhibited by the *Cotula cinerea* and *Origanum Majorana* L essential oils was calculated to be 2.434 and 3.684 µg/mL, respectively, indicating strong anti-inflammatory potential through protein stabilization mechanisms.

The inhibitory potential of diclofenac sodium, a standard non-steroidal anti-inflammatory drug (NSAID), on BSA denaturation was also evaluated in this study to serve as a positive control. The result obtained showed that the two essential oils inhibited BSA denaturation to a greater extent (about 8.82-fold higher), with *Cotula cinerea* essential oil showing stronger activity compared to diclofenac (IC<sub>50</sub> = 11.166 µg/mL) (Figure 26).

Inflammation is often associated with protein denaturation, where inflammatory conditions can cause structural changes in proteins such as albumin, leading to loss of function (Anigboro et al., 2018). Agents that can prevent or reduce protein denaturation are considered potential anti-inflammatory compounds. The significant protein-stabilizing (anti-denaturation) effects of the studied essential oils, particularly *Cotula cinerea*, indicate promising anti-inflammatory action. Previous studies have also reported that essential oils extracted from medicinal plants exhibit anti-inflammatory properties by inhibiting protein denaturation and reducing inflammatory mediators (Adefegha et al., 2017; Ibrahim et al., 2019; Sahin Basak & Candan, 2010).



**Figure 26.** Linear regression of the inhibition of BSA activity by the essential oils of: *Cotula cinerea* (A), *Origanum Majorana L* (B) and Diclofenac (C)

**Table 19.** *In Vitro* Anti-inflammatory activity of the essential oils extracted from *Cotula cinerea* and *Origanum Majorana L* by BSA denaturation inhibitory assay

Concentration (µg/mL)	Percentage of BSA Inhibition		
	<i>Cotula cinerea</i>	<i>Origanum Majorana L</i>	Diclofenac
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
<b>IC<sub>50</sub></b>	<b>2.434</b>		<b>11.166</b>
<b>Values</b>	<b>µg/mL</b>	<b>3.684 µg/mL</b>	<b>µg/mL</b>

## 5.2. BSA 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 BSA solution by increasing essential oils and diclofenac 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 BSA and its complexes with the studied essential oils respectively, while  $\varepsilon_0$  and  $\varepsilon$  are respectively their extinction coefficients, and  $C$  represents the concentration of EOs and acarbose ( $\text{mmol.L}^{-1}$ ),  $K_b$  refers to the binding constant ( $\text{L.mol}^{-1}$ ).

A plot of  $A/(A_0 - A)$  versus  $1/C$  (Figure 27) 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.

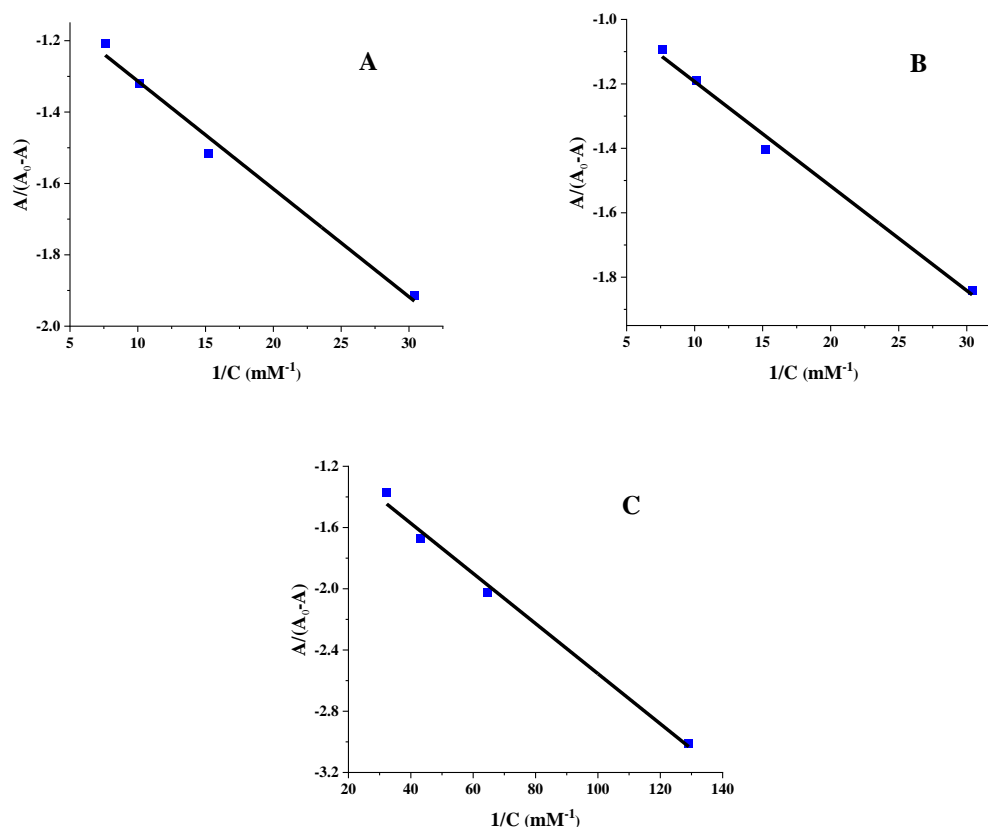
### 5.2.2. Binding free energy:

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  $\Delta G$  is the binding free energy in  $\text{KJ.mol}^{-1}$ ,  $R$  is the gas constant,  $8.32 \text{ J.mol}^{-1}\text{K}^{-1}$  and  $T$  is the absolute temperature,  $298\text{K}$ .

The negative values of  $\Delta G$  indicate the spontaneity of the BSA 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 20.



**Figure 27.** Plots of  $A/(A_0 - A)$  versus  $1/[Cotula\ cinerea]$  (A),  $1/[Origanum\ Majorana\ L]$  (B) and  $1/[Diclofenac]$  (C) used to calculate the binding constants

**Table 20.** Binding constant and binding free energy values for *Cotula cinerea*, *Origanum Majorana L* and acarbose with BSA

Adduct	$K_b$ ( $M^{-1}$ )	$-\Delta G$ ( $\text{KJ.mol}^{-1}$ )
BSA_ <i>Cotula cinerea</i>	5.61 $\times 10^4$	27.11
BSA_ <i>Origanum Majorana L</i>	3.35 $\times 10^4$	25.83
BSA_ Diclofenac	2.68 $\times 10^4$	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 19), Physicochemical properties (Table 14), lipophilicity and water solubility characteristics (Table 15 & 16), pharmacokinetic parameters (Table 17), drug likeness rule and bioavailability score (Table 18) and medicinal chemistry properties (Table 19), 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 21.** General characteristics of the phytoconstituents of essential oils

I. No	Compounds	Molecular formula	Canonical SMILES	Molecular weight (g/mol)
	Santolina triene	C <sub>10</sub> H <sub>16</sub>	<chem>CC(=CC(C=C)C(=C)C)C</chem>	136.23
	trans-Thujone	C <sub>10</sub> H <sub>16</sub> O	<chem>CC1C2CC2(CC1=O)C(C)C</chem>	152.53
	1,8-Cineole	C <sub>10</sub> H <sub>18</sub> O	<chem>CC1(C2CCC(O1)(CC2)C)C</chem>	154.25
	cis-Verbenyl acetate	C <sub>12</sub> H <sub>18</sub> O <sub>2</sub>	<chem>CC1=CC(C2CC1C2(C)C)OC(=O)C</chem>	194.27

**Table 22.** Physicochemical properties of the phytoconstituents of essential oils

Properties	Santolina triene	trans-Thujone	1,8-Cineole	cis-Verbenyl acetate
Num. heavy atoms	10	11	11	14
Num. arom. heavy atoms	0	0	0	0
Fraction Csp3	0.40	0.90	1.00	0.75
Num. rotatable bonds	3	1	0	2
Num. H-bond acceptors	0	1	1	2
Num. H-bond donors	0	0	0	0
Molar refractivity	48.76	45.90	47.12	56.12
TPSA (Å <sup>2</sup> )	0.00	17.07	9.23	26.30

**Table 23.** Lipophilicity characteristics of the phytoconstituents of essential oils

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

**Table 24.** 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	
<b>Santolina triene</b>	-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
<b>trans-Thujone</b>	-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
<b>1,8-Cineole</b>	-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
<b>cis-Verbenyl acetate</b>	-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

**Table 25.** Pharmacokinetics parameters of the phytoconstituents of essential oils

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

**Table 26.** Druglikeness rule and bioavailability score of the phytoconstituents of essential oils

Proprieties	Santolina triene	trans-Thujone	1,8-Cineole	cis-Verbenyl acetate
<b>Lipinski</b>	Yes; 0 violations	Yes; 0 violations	Yes; 0 violations	Yes; 0 violations
<b>Ghose</b>	No; 1 violation: MW<160	No; 1 violation: MW<160	No; 1 violation: MW<160	Yes
<b>Veber</b>	Yes	Yes	Yes	Yes
<b>Egan</b>	Yes	Yes	Yes	Yes
<b>Muegge</b>	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,
<b>Bioavailability score</b>	0.55	0.55	0.55	0.55

**Table 27.** Medicinal Chemistry properties of the Phytoconstituents of essential oils

Proprieties	Santolina triene	trans-Thujone	1,8-Cineole	cis-Verbenyl acetate
<b>PAINS</b>	0 alert	0 alert	0 alert	0 alert
<b>Brenk</b>	1 alert: isolated_alkene	0 alert	0 alert	1 alert: isolated_alkene
<b>Leadlikeness</b>	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
<b>Synthetic accessibility</b>	3.22	2.79	3.65	4.50

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  $\log P_{o/w}$ , 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), KOWWIN (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 27), 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 K<sub>p</sub>), a multiple linear regression, the more negative the log K<sub>p</sub> (with K<sub>p</sub> 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 (LD<sub>50</sub>), and toxicity class (TC).

According to *In Silico* toxicity profiles presented in Table 27, 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 28. *In Silico* toxicity profiles of the studied compounds

Molecule	Dili	Carcino	Immuno	Mutagen	Cyto	<i>LD</i> <sub>50</sub> (mg.Kg <sup>-1</sup> )	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

### 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 COX-1 and COX-2 was further investigated by carrying out a molecular docking analysis of the binding interaction between BSA 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 COX-1 and COX-2 enzymes to elucidate their potential interactions at the active site. Diclofenac, a well-known non-steroidal anti-inflammatory drug (NSAID), was likewise docked with both COX-1 and COX-2 to serve as a positive control. Among the major constituents of *Cotula cinerea* and *Origanum Majorana L* essential oils, the binding affinities toward COX enzymes were comparable to that of diclofenac (IFD score = – 5.259 kcal/mol) (Table 28). This suggests that these essential oils are rich in bioactive compounds which, either individually or through synergistic effects, could effectively inhibit COX enzyme activity, supporting the anti-inflammatory effects observed *In Vitro*.

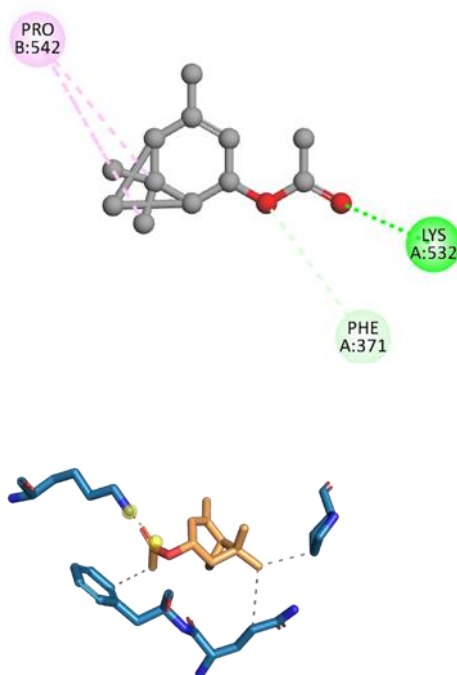
Moreover, the interactions were energetically favorable, as indicated by the negative values of Gibbs free energy ( $\Delta G$ ), confirming the spontaneous nature of the binding process (Avwioroko et al., 2020). Structural analysis of COX enzymes reveals that their active sites include key amino acid residues such as ARG-120, TYR-355, and SER-530 in COX-1 and HIS-90, VAL-349, and TYR-385 in COX-2 that are critical for catalytic activity and substrate binding (Anigboro et al., 2021). The docking poses demonstrated favorable hydrogen bonding and hydrophobic interactions with these residues, suggesting a potential inhibitory mechanism similar to standard NSAIDs.

**Table 29.** Docking score of the studied compounds

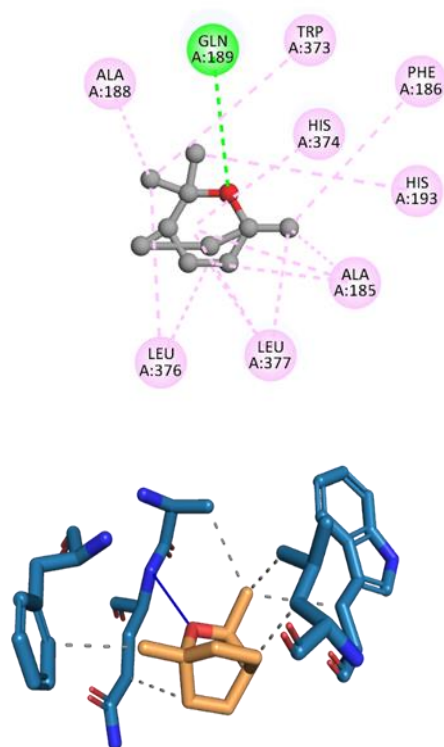
Compound	COX-1	COX-2
trans thujone	-6.1259	-12.35615
alpha-Terpineol	-5.5572	-11.3997
beta-Terpineol	-5.5363	-11.36455
cis Verbenyl acetate	-5.4384	-11.1999
Camphor	-4.9555	-10.38775
Sabinene	-4.8015	-10.12875
gamma terpinene	-4.6035	-9.79575
alpha-Pinene	-4.2724	-9.2389
1,8-Cineole	-4.0766	-8.9096
Santolina triene	-3.2846	-7.5776
alpha-Thujene	-4.851	-10.2157
Beta Pinene oxide	-4.5265	-9.66625
Diclofenac	-3.5343	-9.72915

Each compound was docked with COX-1 and COX-2 enzymes to elucidate their potential interactions at the active site. Diclofenac, a well-known non-steroidal anti-inflammatory drug (NSAID), was likewise docked with both COX-1 and COX-2 to serve as a positive control. Among the major constituents of *Cotula cinerea* and *Origanum Majorana* L essential oils, the binding affinities toward COX enzymes were comparable to that of diclofenac (IFD score = – 5.259 kcal/mol) (Table 29). This suggests that these essential oils are rich in bioactive compounds which, either individually or through synergistic effects, could effectively inhibit COX enzyme activity, supporting the anti-inflammatory effects observed *In Vitro*.

Moreover, the interactions were energetically favorable, as indicated by the negative values of Gibbs free energy ( $\Delta G$ ), confirming the spontaneous nature of the binding process (Avwioroko et al., 2020). Structural analysis of COX enzymes reveals that their active sites include key amino acid residues—such as ARG-120, TYR-355, and SER-530 in COX-1 and HIS-90, VAL-349, and TYR-385 in COX-2—that are critical for catalytic activity and substrate binding. The docking poses demonstrated favorable hydrogen bonding and hydrophobic interactions with these residues, suggesting a potential inhibitory mechanism similar to standard NSAIDs.



*Docking complex and interaction plot for compound trans- thujone with COX-2*



*Docking complex and interaction plot for compound alpha-Terpineol with COX-2*

Figure 28. Molecular interactions of studied compounds with inflammatory enzymes

# CONCLUSION



## Conclusion

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### Conclusion:

At the conclusion of this study, the anti-inflammatory properties of essential oils extracted from *Cotula cinerea* (Shihya) and *Origanum Majorana* (Marjoram) were evaluated, within the framework of the search for natural and safe therapeutic alternatives to combat inflammation. The obtained results demonstrated promising efficacy of these two oils, reflecting their richness in biologically active compounds, particularly those known for their anti-inflammatory properties, such as terpenes and phenolics.

These findings support the importance of valorizing local medicinal plants and utilizing them in the development of complementary treatments that could reduce reliance on synthetic drugs with associated side effects. However, these results remain preliminary and require further studies to determine mechanisms of action, safety profiles, and efficacy in various biological and clinical models.

Advancing research in this field would enhance the integration of traditional knowledge with modern scientific approaches and open new avenues for the use of essential oils as sustainable therapeutic alternatives.



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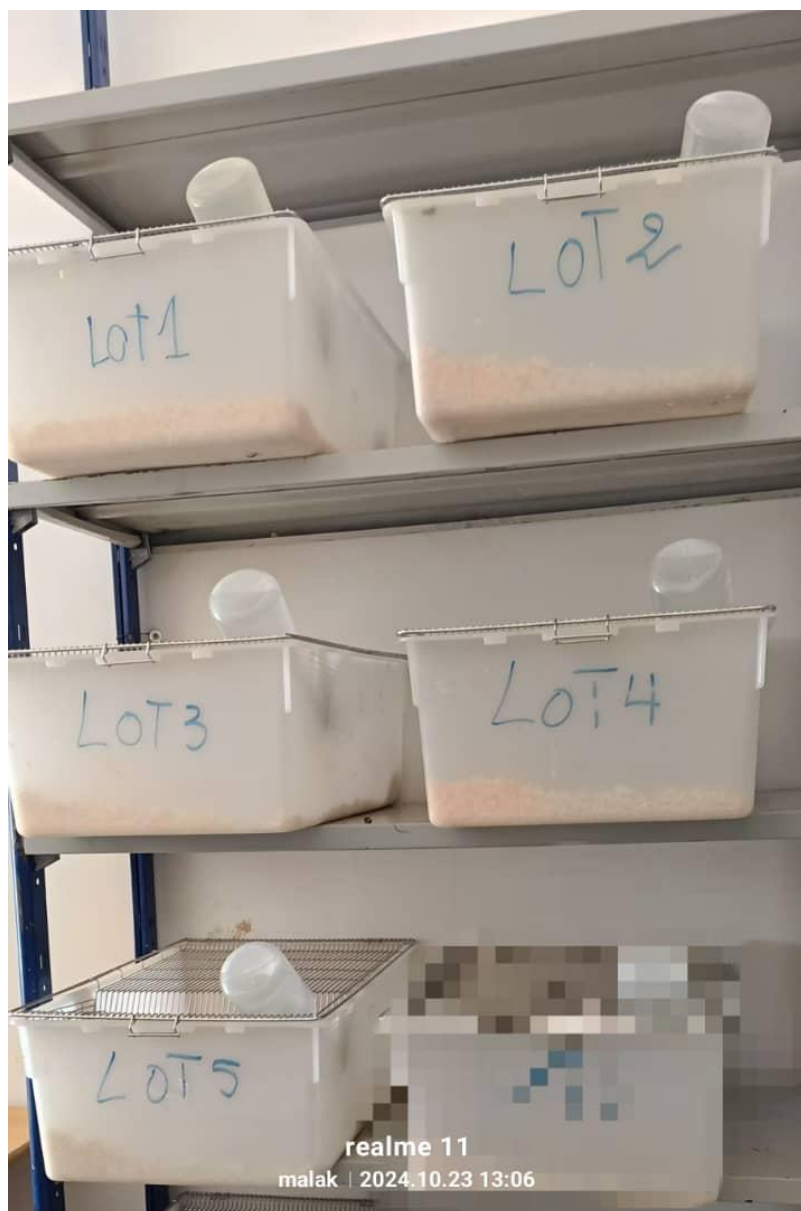
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# **Annex**



1. Experimental cages for study rat: Evaluation of the anti-inflammatory effects of essential oils (LOT1–LOT5)



2.Gavage



3.Dissection and Organ Collection of Experimental rat for Biochemical and Histological Analysis

