MASTER’S DEGREE

Master’s degree in cell and molecular biology

A study of the anti-inflammatory and anti-microbial activities of phenolic compounds from Marrubium vulgare

Presented by
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MAY GOD BLESS YOU ALL
Dedication

I dedicate my dissertation to the supervisors and my family

Special dedications to:

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Uncle Mr. Patrick Musenda, for your generosity, courage and all sorts of help

Friends. Dr. Zziwa Emmanuel, your guidance, generosity and constant helps
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<table>
<thead>
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibition Concentration</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>IC</td>
<td>Inhibition Concentration</td>
</tr>
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Abstract

The species of *Marrubium vulgare* belongs to *Lamiaceae* family considered one of the most valuable components of herbal treatments for a variety of illness. It is widely distributed in Algeria. This study aimed to determine the antimicrobial and anti-inflammation activities of polyphenols contained in *Marrubium vulgare*. Fresh leaves harvested from the province of Ain Defla, Algeria, have been dried and ground to powder. The powder has been macerated in a solvent of ethanol and water (7:3). The obtained hydro alcoholic extract has total polyphenols content ranging between 64.72 and 66.87 mg/g of dry extract and flavonoid content ranging between 77.55 to 82.2 mg/g of dry extract (Boularas et al., 2019), *Marrubium vulgare* leaves are rich in both total phenolic and flavonoid compounds.

The anti-microbial activity had to be determined using a disc diffusion method against bacteria: *Bacillus subtilis*, *staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aureginosa* and fungi: *Umbelopsis ramanniana*, *Fusarium culmorum*. The extract is more effective against *Bacillus subtilis*, *Staphylococcus aureus*, moderately effective against *Escherichia coli* and ineffective against *Pseudomonas aueroginosa*. The extract exhibits a moderate to significant antimicrobial activity (Amessis-Ouchemoukh et al., 2014).

The anti-inflammation activity had to be determined using carrageenan induced paw edema using 5 groups including test groups 1, 2, 3, reference group and the control group. We suggest that the reduction of edema was dose dependent, it increased from application of a gel low, medium to high concentrations on test groups 1, 2, 3 in reference to group massaged with 1% of diclofenac. The hypothesis is that the percentage reduction in edema induced by carrageenan is directly proportional to the extract doses contained in the gels applied. *Marrubium vulgare* has an anti-inflammatory effect depending on the dose of the polyphenols contained in the gel. The antimicrobial and anti-inflammation activities of *M. vulgare* cannot be attributed on single compound rather to different combinations of compounds with the same effect or synergetic effect. Further research, analysis and purification of *Marrubium vulgare* extracts are needed to identify the exact bioactive molecules responsible for the pharmacological activities. More research is needed to investigate also the antifungal activities of polyphenols extracts from *M. vulgare*.

Keywords: *Marrubium vulgare*, antimicrobial, anti-inflammation, polyphenols
Résumé

L’espèce de *Marrubium vulgare* appartient à la famille des Lamiacées considérée comme l’un des composants les plus précieux des traitements à base de plantes pour une variété de maladies. Il est largement distribué en Algérie. Cette étude vise à déterminer les activités antimicrobiennes et anti-inflammatoires des polyphénols contenus dans *Marrubium vulgare*. Des feuilles fraîches récoltées dans la province d’Ain Defla, Algérie, ont été séchées et broyées en poudre. La poudre a été macérée dans un solvant d’éthanol et d’eau (7: 3). L’extrait hydroalcoolique obtenu a une teneur en acide phénolique comprise entre 64,72 et 66,87 mg / g d'extrait sec et une teneur en flavonoïdes comprise entre 77,55 à 82,2 mg / g d'extrait sec (Boularas et al., 2019). Les feuilles de *Marrubium vulgare* sont riches en acide phénolique et flavonoïde.

L’activité antimicrobienne a été déterminée à l’aide d’une méthode de diffusion sur disque contre les bactéries: *Bacillus substilus*, *staphylococcus aureus*, *Escherichia coli* et *Pseudomonas auriginosa* et champignons: *Umbelopsis ramanniana* et *Fusarium culmorum*. L'extrait est plus efficace contre *Bacillus substilis* et *Staphylococcus aureus*, modérément efficace contre *Escherichia coli* et inefficace contre *Pseudomonas auruginosa*. L'extrait présente une activité antimicrobienne modérée à significative (Amessis-Ouchemoukh et al., 2014). L’activité anti-inflammatoire a été déterminée en utilisant un œdème de patte induit par la carragénine utilise 5 groups par exemple les groupes de test 1, 2, 3, un groupe référence et un groupe témoin. Nous suggérons que la réduction de l'œdème dépendra de la dose, elle augmentera après l'application d'un gel à des concentrations faibles, moyennes à élevées sur les groupes d'essai 1, 2, 3 en comparant au groupe référence massé avec 1% de diclofénac. L'hypothèse est que le pourcentage de réduction de l'œdème induit par la carragénine est directement proportionnel aux doses contenues de l’extrait dans les gels appliqués. *Marrubium vulgare* possède un effet anti-inflammatoire dépendant de la dose des polyphénols contenus dans le gel. Les activités antimicrobiennes et anti-inflammatoires de *M. vulgare* ne peuvent pas être attribuées à un seul composé plutôt à différentes combinaisons de composés ayant le même effet ou des effets synergiques. De plus amples recherches, analyses et purification des extraits de *M. vulgare* sont nécessaires pour identifier les molécules bioactives responsables des activités pharmacologiques. Des recherches supplémentaires sont nécessaires pour étudier également les activités antifongiques des extraits de polyphénols de *Marrubium vulgare*.

Mot clés : *Marrubium vulgare*, polyphénols, antimicrobienne, antiinflammatoire
وتتمي نبتة Lamiaceae إلى عائلة Marrubium vulgare P.ゅٍٔٞ ٔجزخ M. vulgare إٌٝ ػبئٍخ Lamiacea. ماربوب blanc أ٠عًب ثبسُ Marrube blanc فٟ أٚسٚثب ٚاٌّش٠ٛد فٟ اٌجضائش، ح١ش رزٛصع ػٍٝ ٔطبق ٚاسغ فٟ ججبي اٌّس١ٍخ ثبٌجضائش. رّذف ٘زٖ اٌذساسخ إٌٝ رحذ٠ذ الأٔشطخ اٌّعبدح، اٌطج١خ اٌزٟ رسزخذَ فٟ ِؼبٌجخ ِجّٛػخ ِزٕٛػخ ِٓ الأِشاض. رؼشف ٔجزخ M. vulgare أ٠عًب ثبسُ ماربوب blanc فٟ أٚسٚثب ٚاٌّش٠ٛد فٟ اٌجضائش، ح١ش رزٛصع ػٍٝ ٔطبق ٚاسغ فٟ ججبي اٌّس١ٍخ ثبٌجضائش. رّذف ٘زٖ اٌذساسخ إٌٝ رحذ٠ذ الأٔشطخ اٌّعبدح.

تمت عملية قطف لأوراق طازجة نبتة المربوت من ولاية عين الدفلى ثم تم تجفيفها وطحنها إلى مسحوق الذي أدب ذاً بمطا في مذيب الإيثانول والماء (7: 3). يحتوي المستخلص الكحولي المائي الذي تم الحصول عليه على مستوى من حمض الفينوليك ما بين 64.72 و 66.87 مغ / غ من المستخلص الجاف و مستوى من فلافونويد يتراوح بين 77.55 إلى 82.2 مغ / غ من المستخلص الجاف (et al Boularas, 2019). من هذا يتبين أن أوراق المربوت غنية بالفينول والفلافونويد.


تم تحديد النشاط المضاد للالتهابات باستخدام وذمة القدم الناجمة عن استعمال مادة الكاراجينين على مجموعات الاختبار 1 و 2 و 3 ومجموعة مرجعية ومجموعة شاهدة. حيث توقع أن انخفاض الودم يعتمد على الجرعة، ويزداد بعد تطبيق الهايم بتركيزات منخفضة ومتواضعة إلى عالية على مجموعات الاختبار 1 و 2 و 3 مقارنة بالمجموعة المرجعية المكونة من 1% ديكلافونيك. الفرضية هي أن انخفاض النسبة المئوية للودم التي يسببها الكاراجينين يقترب طريقاً مع الجرعة الموجودة في المستخلص في المواد الهالامية المطابقة. تأثير المربوت كمضاد للالتهابات يعتمد على جرعة البوليفينولي الموجود في الهلام. لا يمكن أن تنسب أنشطة المربوت المضادة للالتهابات على المربوت واحد، بل هي راجعة إلى مجموعة مختلفة من المركبات التي لها نفس التأثير أو تأثيرات متكاملة. من الضروري إجراء المزيد من البحوث حول تحليل وتنقيح مستخلصات المربوت لتحقيق الجزيئات الفيسيولوجية والفعالة في المجال المضادات. كما هناك حاجة لدراسة المزيد من النشاط المضاد للالتهابات للبوليفينولي المستخلص من نبات المربوت.

مربوت، بوليفينولي، مضاد للميكروبات، مضاد للالتهابات: الكلمات المفتاحية
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Introduction

In the recent years as a result of side effects and resistance that pathogenic micro-organisms build against antibiotics, many scientists have paid attention to extracts and biological active compounds isolated from plant species used in herbal medicines.

Herbal medicines are the most ancient form of medicinal remedies known to humans. Moreover, at least 120 distinct chemical substances extracted from plants are considered important drugs currently in use, other drugs are simple synthetic modifications of natural products (Ghosh, 2016). According to the world health organization (WHO), traditional herbal medicine is used by 80% of the population in developing countries and herbal products are valued at worldwide annual market value of US dollars 60b. There are many hopes that traditional herbal medicine research will play a critical role in the global health.

In Algeria, many species belonging to Lamiaceae family are strongly used in traditional medicines to treat several pathologies among them, Marrubium vulgare. Marrubium vulgare commonly known as Marriott is widely distributed in Algeria (Mehalaine et al., 2017). This plant is used in folk medicine for the treatment of a variety of diseases. M. vulgare is currently used by traditional healers alone or combined with other herbs to treat bronchitis, coughs, and colds. It is also used traditionally for its antioxidant, antibacterial, analgesic, and hypoglycemic effects. Marrubium vulgare is essentially rich in phenolic compounds among other phytochemicals and is widely used in traditional medicine. The healing activities of the Marrubium vulgare have been known since the ancient Egypt. The Greek physician Hippocrates and other ancients valued horehound as a panacea (Haratym and Weryszko-Chmielewska, 2017).

Current study determines the anti-inflammatory and anti-microbial activities of polyphenols extracts from Marrubium vulgare. On addition to the introduction and conclusion, this study is subdivided into three parts.

First part: it is focalized to a general description about medicinal plants, Marrubium vulgare, polyphenols and the biological activities (anti-microbial and anti-inflammation).

Second part: it is reserved for the materials and methods.

Last part: it presents the hypothesis and discussion
CHAPTER I
LITERATURE REVIEW
I. Generalities

I.1. Generalities on *Marrubium vulgare*

I.1.1. Botanic description

It is a tall robust herbaceous perennial, growing at 1500-2400 m altitude. *M. vulgare* has fibrous roots and numerous stems, which are quadrangular, erect, very downy and from 12 to 18 inches high. The leaves are roundish ovate, dentate or profoundly serrate, wrinkled, veined hoary on surface and supported in pairs. The flowers are white and in crowded axillary whorls. The calyx is tubular and divided into 10 narrow segments at the margin, which are hooked at the end. The corolla is also tubular, with a labiates margin, of which the upper lip is bifid, the under reflected and three cleft, with the middle segment slightly scalloped. Seeds are lying in the bottom of calyx (Lodhi et al., 2017).

I.1.2. Taxonomy of *Marrubium vulgare*

Scientific name: *Marrubium vulgare* L.

English : white hore hound (Haratym and Weryszko-Chmielewska, 2017).

French : Marrube blanc (Bouterfas et al., 2016).

Algeria : Marriout (Mehalaine et al., 2017).

Table I: taxonomic hierarchy of *Marrubium vulgare*

(https://plants.usda.gov/core/profile?symbol=MAVU)

<table>
<thead>
<tr>
<th>Rank</th>
<th>Scientific name and common names</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kingdom</td>
<td><em>Plantae</em> - plants</td>
</tr>
<tr>
<td>Sub kingdom</td>
<td><em>Tracheobionta</em> – vascular plants</td>
</tr>
<tr>
<td>Super Division</td>
<td><em>Spermatophyta</em> – seed plants</td>
</tr>
<tr>
<td>Division</td>
<td><em>Magnoliopyta</em> – flowering plants</td>
</tr>
<tr>
<td>Class</td>
<td><em>Magnoliopsida</em> – dicotyledons</td>
</tr>
<tr>
<td>Subclass</td>
<td><em>Asteridae</em></td>
</tr>
<tr>
<td>Order</td>
<td><em>Lamiales</em></td>
</tr>
<tr>
<td>Family</td>
<td><em>Lamiaceae / Labiatae</em> – mint family</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Marrubium L.</em> -horehound</td>
</tr>
<tr>
<td>Species</td>
<td><em>Marrubium vulgare L.</em> – horehound</td>
</tr>
</tbody>
</table>
Figure 1: A photo of *Marrubium vulgare* in its natural habitat (https://davesgarden.com/guides/pf/showimage/307945).
I.1.3. Phytochemical components of Marrubium vulgare

Previous phytochemical studies have showed the presence of alkaloids, lactones, steroids, flavonoids, tannins, phenylpropanoid esters, vitamin C and diterpenoids in *M. vulgare*.

The genus is known to produce many diterpenoids such as marrubiina, identified in 1842, which was the first diterpenoid and major compound to be isolated and characterized from *M. vulgare* leaves (Ahvazi et al. 2016).

I.1.4. Geographic distribution

*M. vulgare* has natural habitats in Western Asia, North Africa, and Europe. Moreover, this plant is cultivated worldwide as a source for food flavoring and medicinal purposes (Haratym and Weryszko-Chmielewska, 2017). In Algeria, it is widely distributed in areas of M’Sila (Boudjelal et al., 2012).

1.1.4.1 Biological activities of Marrubium vulgare

*M. vulgare* was also reported to possess hypoglycemic, vasorelaxant, antihypertensive, analgesic, anti-inflammatory, and antioedematogenic activities (Bouterfas et al., 2016).

I.1.5. Secondary metabolites

I.1.5.1. Definition

Secondary metabolites are compounds that are not required for a cell (or organism) to survive, but that play a role in the interaction of the cell (or organism) with its environment. These compounds are often involved in plants protection against biotic or abiotic stresses (Pagare et al., 2015).

I.1.5.2. Classification of secondary metabolites

Secondary plant metabolites are classified according to their chemical structures into several classes. The classes include (A. Hussein and A. El-Ansary, 2019):

1- Phenolic
2- Alkaloids
3- Saponins
4- Terpenes
5- Polyketide
Figure 2: General schema of biosynthetic pathways and precursors for major classes of secondary metabolites (Gutzeit and Ludwig-Müller, 2014).
I.1.5.2.1. Phenolic compounds

Phenolic compounds are own defined as compounds that possess an aromatic ring with at least one hydroxyl group and their structure can vary from simple molecule to complex polymer with high molecular weight (Durazzo et al. 2019).

Polyphenol is a class of secondary metabolites, ubiquitous, essential and located throughout most plant tissues that contribute to the plants physiology. More than 8000 phenolic structures are currently known, and among them over 4000 flavonoids have been identified.

Polyphenols can be classified in many classes, but the main classes in the polyphenols are phenolic acids, flavonoids, stiblins, phenolic alcohols, and lignans (Abbas et al., 2017).

❖ Phenolic acids

Phenolic acids are non-flavonoid polyphenol compounds present in foodstuffs and are characterized by a carboxyl group linked to benzene ring (Lafay & Gil-Izquierdo, 2008). They are derived from two main phenolic compounds, benzoic and cinnamic acids. Examples of hydroxybenzoic derivatives are Gallic, p-hydroxybenzoic, vanillic, and syringic acids, whereas caffeic, ferulic, sinapic, and p-coumaric acids belong to hydroxycinnamic acids (Durazzo et al., 2019).

❖ Flavonoids

Flavonoids are an essential group of polyphenol compounds, and their flavan nucleus is the main structural characteristic. They are one of the most widely found classes of compounds in vegetables and fruits. The chemical structure of flavonoids is based on a fifteen-carbon skeleton consisting of two benzene rings connected through a heterocyclic pyrane ring. The flavonoids can be divided into an assortment of classes, for example, flavones (e.g., flavone, apigenin, and luteolin), flavonols (e.g., quercetin, kaempferol, myricetin, and fisetin), and flavanones (e.g., flavanone, hesperetin, and naringenin) (Ferraz et al., 2020).
Figure 3: The general structural formular of phenolic acids (Abbas et al., 2017)

Figure 4: The basic structure of flavonoid groups (Kumar and Pandey, 2013)
 entra in plants, foods and beverages, and are of great economic
and ecological interest. They are water soluble and with molecular weights ranging between
500 and 3000 Daltons. They also form complexes with water-insoluble proteins, alkaloids and
gelatin. They are responsible for the astringent taste of many fruits and vegetables, causing
precipitation of salivary glycol-proteins and reducing oral lubrication (de Jesus et al., 2012).

Tannins are grouped into 2 groups: hydrolysable tannin and condensed tannin. Hydrolysable
tannin can be further divided into gallotannin and ellagitannin while condensed tannin are
oligomers of flavan-3-ol and flavan-3,4-diol (Smeriglio et al., 2017).

lignans are a group of phenolic compounds derived from a combination of two
phenylpropanoid C6–C3 units at the β and β’ carbon. They can also be linked to additional
ether, lactone, or carbon bonds (Durazzo et al., 2018).

![Figure 5: The chemical structure of main dietary lignan](Durazzo et al., 2018)
I.2. Bacterial Strains

I.2.1. *Escherichia coli*

*Escherichia coli* are typically Gram-negative, rod shaped (2.0–6.0 mm in length and 1.1–1.5 mm wide bacilli) bacteria with rounded end. *Escherichia coli* is of faecal origin and almost exclusively found in the digestive tract of warm-blooded animals, particularly humans. Several *E. coli* serogroups are known and the majority are non-pathogenic. However, some groups can cause severe diarrheal disease, occasionally with fatal outcome.

I.2.2. *Bacillus Subtilis*

*Bacillus subtilis* is a Gram-positive, rod-shaped bacterium that forms heat-resistant spores. It is commonly found in the soil. It is nonpathogenic. It demonstrates an ability to form spores that are heat-resistant. It produces several commercially important products (Du et al., 2019).

I.2.3. *Staphylococcus aureus*

*Staphylococcus aureus* is a Gram-positive bacterium and causative agent of wide range of infectious diseases such as skin infections, bacteremia, endocarditis, pneumonia and food poisoning. The organism was originally a leading nosocomial pathogen and afterwards epidemiologically distinct clones emerged in community settings. *S. aureus* expresses number of virulence factors which help to establish infection by facilitating tissue attachment, tissue invasion and evading from host immune response. The ability to acquire resistance to multiple antibiotics classes makes *S. aureus*, a challenging pathogen to treat (Gnanamani et al. 2017).

I.2.4. *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a Gram-negative, aerobic rod bacterium of the *Pseudomonadaceae* family. *P. aeruginosa* is commonly found in soil and water as well as in plants and humans. *Pseudomonas* bacteria are believed to be one of only a few true pathogens for plants. *P. aeruginosa* exploits weaknesses in host defense to mount an infection. Indeed, *P. aeruginosa* is the epitome of an opportunistic pathogen of humans. The bacterium hardly infects uncompromised tissues, but it can invade any tissue beleaguered by immunodeficiency.
*P. aeruginosa* causes infection in the urinary tract, respiratory system, dermis, soft tissue, bacteraemia, bone and blood, particularly in patients with severe burns, tuberculosis, cancer and AIDS (Wu and Li, 2015).

### I.3. Fungi strains

#### I.3.1. *Umbelopsis ramanniana*

*Umbelopsis ramanniana* is a common and abundant soil fungus, a representative of a unique group of zygomycete fungi. *Umbelopsis ramanniana* is important from a biochemistry and biotechnology perspective because it is highly tolerant to fungicides of benomyl group and it is oleaginous (it regularly produces oils) (Kartali et al., 2019).

#### I.3.2. *Fusarium culmorum*

*Fusarium culmorum* is a ubiquitous soil-borne fungus able to cause foot and root rot and Fusarium head blight on different small-grain cereals, in particular wheat and barley. It belongs to Ascomycota fungi. *F. culmorum* has been reported as one of the main pathogens of wheat worldwide (Scherm et al. 2013).

### I.4. Biological activities

The consumption of diets rich in polyphenol have usually been associated with beneficial effects to human health. These benefits have usually been associated with their diverse biological activities, such as anti-oxidation, anti-inflammation, anti-bacteria, enzyme inhibition, glycation inhibition, immunomodulation and miRNA interference (Xie et al., 2017).

The present study is focused in anti-microbial and anti-inflammatory activities of polyphenols extracted from leaves of *Marrubium vulgare*.

#### I.4.1. Anti-microbial activity

The antibacterial activities of polyphenol have attracted much interest due to the potential in dealing with the drug-resistant bacteria that are insensitive to conventional antibiotics, especially flavonoids, have been suggested to exert their antibacterial effects in three ways;
namely, direct killing of bacteria, synergistic activation of antibiotics and attenuation of bacterial pathogenicity (Xie et al., 2017).

I.4.1.1. Mechanisms of action of polyphenols

Several authors explained this activity by

- Modification of the cell wall rigidity with integrity losses due to different interactions with the cell membrane.

- The elevation of the lipophilic character of phenolic compounds enhances their antimicrobial activity by favoring their interaction with the cell membrane. This may induce irreversible damages of the cytoplasmic membrane and coagulation of the cell content that can also lead to the inhibition of intracellular enzyme (Bouarab-Chibane et al., 2019).

- Phenolic acids have been shown to disrupt membrane integrity, as they cause consequent leakage of essential intracellular constituents (Borges et al., 2013).

- Flavonoids may link to soluble proteins located outside the cells and with bacteria cell walls thus promoting the formation of complexes.

- Flavonoids also may act through inhibiting both energy metabolism and DNA synthesis thus affecting protein and RNA syntheses. In the case of Gram-positive bacteria, intracellular pH modification as well as interference with the energy (ATP) generating system were reported (Bouarab-Chibane et al., 2019). Phenolic acids have been shown to disrupt membrane integrity, as they cause consequent leakage of essential intracellular constituents (Borges et al., 2013).

- Flavonoids may link to soluble proteins located outside the cells and with bacteria cell walls thus promoting the formation of complexes.

Flavonoids also may act through inhibiting both energy metabolism and DNA synthesis thus affecting protein and RNA syntheses. In the case of Gram-positive bacteria, intracellular pH modification as well as interference with the energy (ATP) generating system were reported (Bouarab-Chibane et al., 2019).
I.4.1.2. Major cellular targets

The mechanism of action of phenolic compounds on the microbial cells demonstrate that the major cellular targets include

a. The cell membrane.

b. Proteins (cell receptors, ion channels, enzymes, structure proteins, transcription factors, transport systems).

c. Nucleic acids (DNA and RNA)

Figure 6: Different sites of action of antimicrobial polyphenols at cellular levels *(Bouarab Chibane et al., 2019)*

I.4.2. Anti-inflammatory activity

Chronic inflammation is known to be a major cause linked to different human disorders involving cancer, diabetes type II, obesity, arthritis, neurodegenerative diseases, and cardiovascular diseases. Polyphenols derived from botanic origin have shown anti-inflammatory activity *in vitro* and *in vivo* highlighting their beneficial role as therapeutic tools in multiple acute and chronic disorders (Yahfoufi et al., 2018).
I.4.2.1. Inflammation

❖ Definition
Inflammation may be defined as the normal response of living tissue to injury or infection, characterized by redness, heat, swelling, pain and loss of various functions.

Mechanical injury, chemical toxins, and invasion of microorganisms, and hypersensitivity reactions may trigger the inflammatory response. The major goal of the inflammatory response is to localize and remove the causative agent or substance and repair the surrounding tissue (Rankin, 2004).

❖ Classification of inflammation
Stages of inflammation depend on the duration of the process as well as various immune factors. The inflammation has been classified into two distinct classes, which are acute and chronic processes:

a. Acute Inflammation

Acute inflammation is a short procedure, lasting from minutes to a few days, characterized by plasma proteins or fluid leakage and the migration of leukocytes into an extravascular area (Arulselvan et al., 2016).

b. Chronic Inflammation

Chronic inflammation in tissue usually happens when inflammatory responses are in the absence of an actual stimulus, occurs through infections that are not resolved either within endogenous protection mechanisms or via some other resistance mechanism from host defenses. They can also happen from physical or chemical agents, which cannot be broken down, as well as from some kind of genetic susceptibility. Persistence of foreign bodies, continuous chemical exposures, recurrent acute inflammation, or specific pathogens are all crucial reasons for chronic inflammation (Arulselvan et al., 2016).

❖ Actors involved inflammation process
Inflammatory responses are governed by innate immune cells, such as macrophages, dendritic cells, or neutrophils involved in the recognition of pathogen-associated molecular patterns (PAMPs) through a panel of conserved pattern-recognition receptors (PRRs) and toll-like
receptors (TLRs). Toll-like receptor 4 (TLR4), a member of the TLR family, is recognized and activated by a major component of Gram-negative bacteria, i.e., lipopolysaccharide (LPS). Myeloid differentiation primary response gene (88) (MyD88) is involved in independent and dependent pathways stimulated by the signaling cascades activated by LPS featuring nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPKs) pathway activation. Activated macrophages produce pro-inflammatory mediators, such as NO produced by inducible nitric oxide synthase (iNOS) and PGE2 produced by cyclooxygenase-2 (COX-2) proteins and cytokines, like TNF-α, interleukin (IL)-6, and IL-1β (Su et al., 2019).

Figure 7: Cells and molecules involved in acute inflammation

(https://www.creative-diagnostics.com/acute-inflammation.htm)

1.4.2.2 Mechanism of Anti-Inflammatory Effects of Polyphenols

Polyphenols may exert anti-inflammatory effects notably through radical scavenging activities, regulation of cellular activities in inflammatory cells, and modulation of the activities of enzymes involved in arachidonic acid metabolism (phospholipase A2, COX) and arginine metabolism (NOS), as well as the modulation of the production of other
proinflammatory molecules. Molecular mechanisms of polyphenol anti-inflammatory activities include inhibition of enzymes associated with proinflammatory properties such as COX-2, LOX, and iNOS, inhibition of NF-κB and the activating protein-1 (AP-1), activation of phase-II antioxidant detoxifying enzymes, and activation of mitogen activated protein kinase (MAPK), protein kinase-C, and nuclear factor erythroid 2-related factor (Hussain et al., 2016).
Figure 8: Molecular mechanism of anti-inflammatory action of phytoconstituents (phenols/flavonoids) (Yatoo et al., 2017)
Chapter II

Materials and methods
II. Materials and methods

The aim of our study was to determine the anti-microbial and anti-inflammatory activities of hydroalcoholic extract from *Marrubium vulgare*.

This study had to be realized from three different laboratories, experimentale station (Natural and Life Sciences Faculty, Blida 1 University), Microbial Systems Laboratorys (Kouba, Algiers), and Cytology Laboratory (CHU H-DEY Hospital, Algiers) for a period of three months.

II.1. Materials

II.1.1. Biological materials

II.1.1.1. Plant materials

Green leaves of *Marrubium vulgare* plant were carefully harvested from plants found in the province of Ain Defla, Algeria. These leaves were air dried under the shade for about 15 days. The dried leaves were ground into a powder and stored in a container at room temperatures.

II.1.1.2. Animal materials

The animals and the pet shop operating conditions are represented in the table below

Table II : table representing the animals type , pet shop and operating conditions

<table>
<thead>
<tr>
<th>RACE</th>
<th>Albinos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scientific name</td>
<td><em>Mus musculus</em></td>
</tr>
<tr>
<td>Breed</td>
<td>N.M.R.I (Naval Medical Research Institute).</td>
</tr>
<tr>
<td>Line</td>
<td>Swiss</td>
</tr>
<tr>
<td>Breeding place</td>
<td>Pasteur institute of Algeria</td>
</tr>
<tr>
<td>number</td>
<td>5 groups of 6 mice</td>
</tr>
<tr>
<td>Weight( g)</td>
<td>22+- 2</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
</tr>
<tr>
<td>Food</td>
<td>Feeds provided by national office of livestock feeds of Algeria ( l'O.N.A.B)</td>
</tr>
<tr>
<td>Water</td>
<td>Tap water</td>
</tr>
<tr>
<td>Temperature</td>
<td>20 -24 °C</td>
</tr>
<tr>
<td>humidity</td>
<td>50 %</td>
</tr>
<tr>
<td>lighting</td>
<td>10 hrs. par day</td>
</tr>
</tbody>
</table>
II.1.1.3. Microbial strains

The microbial strains had to be supplied by to us by Microbial Systems Laboratorys (Kouba, Algiers).

II.1.1.3.1. Bacteria strains

- *Bacillus subtilis* ATCC 6633
- *Escherichia coli* ATCC 10536
- *Pseudomonas aeruginosa* CIP A22
- *Staphylococcus aureus*

II.1.1.3.2. Fungi strains

- *Umbelopsis ramanniana* NRRL
- *Fusarium culmorum* NRRL. 1829

II.1.1.4. The culture mediums

- *Potato Dextrose Agar (PDA)*: 1000 ml of Irish potato filtrate, 20 g of agar, 20 g of glucose, 1000 ml of distilled water, pH = 6.5. Irish potato filtrate was prepared from boiling 200-250 g of peeled potato in 1000 ml of distilled water.

- *International Streptomyces Project 2 (ISP2)*: 4 g of glucose, 4 g of yeast extract, 10 g of malt extract, 1000 ml of distilled water, 20 g of agar, pH = 7.2.

II.1.2. Non biological material

The apparatus and chemical reagents used are presented in the annexes

II.2. Methods

II.2.1. extraction of the total phenolic compounds

- 10 g of *Marrubium vulgare* leaves powder was macerated in 100 ml of the extraction solvent (v/v) ethanol – H₂O (70/30) for 72 hrs.

- The mixture was filtered using a filter paper then centrifuged at 3000g for 10 minutes. The extract was dried, dissolved in 15 ml of distilled water and stored at 4°C for further experiments. Three extracts have been prepared.
II.2.2. Standardization of a hydro-alcoholic extract of *Marrubium vulgare*

II.2.2.1. total Phenolic compound assay

The determination of total phenolic compounds had to be carried out using the Folin Ciocalteu reagent, according to the method described by (Singleton et al., 1999).

- 1ml of Folin ciocalteu solution had to be added to 200 µl of each extract (1 mg/ ml) for 5 minutes.

- 800 µl of Sodium Carbonate solution (Na$_2$CO$_3$) added and the final mixture incubated for 2 hrs. at room temperature in darkness.

- The absorbance measured at $\lambda$= 760 nm against a blank. The blank is a solution containing the same reagents mentioned above, with the extract replaced by the solvent (ethanol- H$_2$O ) used during extraction.

- The same procedure had to be repeated with a standard solution of Gallic acid prepared at different concentrations to establish a calibration line.

For each analysis, the samples were to be prepared in triplicate and the mean value of absorbance calculated.

Based on the measured absorbance, the concentration of phenolic determined (mg/ml) using the calibration line; then the content of phenolic in extract were expressed in terms of Gallic acid equivalent (mg of GA/ g of extract).

II.2.2. 2. Flavonoid assay

The flavonoids contents had to be determined using Aluminium Chloride (AlCl$_3$) method described by Chris and Muller 1960.

- 1 ml of Aluminium Chloride (Al Cl$_3$) was to be added to 1 ml of the extract (1mg / ml). The mixture incubated for 10 minutes, at room temperature in darkness.

- The absorbance measured at $\lambda$= 430 nm against the blank. The blank is a solution containing the same reagents mentioned above, with the extract replaced by the solvent (ethanol- H$_2$O ) used during extraction.
For each analysis, the samples had to be prepared in triplicate and the mean value of absorbance was calculated.

The same procedure repeated for the standard solution of quecertain and the calibration line traced. Based on the measured absorbance, the concentration of flavonoids determined (mg/ml) using the calibration line; then, the content of flavonoids in extracts expressed in terms of quecertain equivalent (mg of QN /g of extract).

II.2.3. Anti-microbial activity

The study of anti-microbial activity had to be determined using the disc diffusion method (Bauer et al., 1966), against 4 bacteria and 2 fungi provided by the Microbial systems of Biology Laboratory, Algeria.

II.2.3.1. Disc diffusion

II.2.3.1.1. Protocol

a. Inoculum preparation

A new culture had to be prepared from nutritive medium with bacteria and fungi for 24 hrs. And 48 hrs. respectively.

With aid of a sterile loop, 3-5 identical, isolated colonies were to be transferred to the saline solution to form a suspension. The suspension vortexed for some seconds. The suspension calibration is done by spectrophotometric.

The bacteria suspension (Bacillus subtilis and Staphylococcus aureus) absorbance reading at wavelength of 625 nm having a value reading between 0.08 – 0.13 corresponding to a standard calibration of 0.5 Mc Farland, approximately 1 \(\times\) \(10^8\) CFU / mL.

The fungi suspension were to be calibrated to 0.5 Mc Farland at wavelength of 530 nm, the value reading was between 0.15-0.17 which corresponds to 0.4-5 \(\times\) \(10^6\) CFU/ mL.

a. Inoculation

Medium (semi–solid) had to be prepared prior in petri dish forming 4 mm thickness per dish (ISP2 medium and PDA medium) for the bacteria and fungi respectively. The medium cultures inoculated with test strain of 200 \(\mu\)L / 100 ml of medium culture and then left to solidify at laboratory temperature.
b. Disk disposition

After solidification, sterile paper filter disks impregnated with different concentrations of hydro-alcoholic extract or with reference antibiotics were to be manually deposited on the surface of the medium with the sterile forceps. The dishes placed at 4°C for 2 hrs to allow complete diffusion of the extract and stop the growth of the germs which are then incubated for 24 hrs (bacteria) and 48 hrs (fungi) at 30°C. Results were to be determined by measuring with a calibrated ruler the diameter of the inhibition zone.

II.2.4. Evaluation of anti-inflammation activity

Carrageenan –induced paw edema essay was to be used to evaluate the anti-inflammatory activity of a hydro-alcoholic gel based on a *Marrubium vulgare* extract.

II.2.4. 1. Principle

Carrageenan induces an acute inflammation, well researched and highly reproducible. Cardinal signs – edema, hyper-algesia, and erythema develop immediately following a subcutaneous carrageenan injection resulting from action of pro-inflammatory agents like bradykinin, histamine, complements, reactive oxygen and nitrogen species. Such agents can be generated *in situ* at site of insult or by infiltrating cell; neutrophils readily migrate to the site of inflammation and can generate pro-inflammatory reactive oxygen species and reactive nitrogen species. The response is usually quantified by increase in paw size (edema) which is maximal around 3- 5 hrs. Post- carrageenan injection.

II.2.4. 2. Choice of doses

− The gel doses had to be considered low, medium and high concentration dose of *Marrubium vulgare*.
− Gel 1% - 1.16 g diclofenac diethylamine (voltaren) was to be used as an anti-inflammatory reference gel.

II.2.4. 3. Experimental protocol

- Animals had to be weighed, randomized into 5 groups (n=6), and kept for 1 week to acclimatize to the laboratory conditions. This would help to keep stress levels low, which is important for the development of a good inflammatory response.
- Preparation of 20 mg of 1% carrageenan diluted in 2 ml of saline solution
At $T_0$

- 50 µl of 1% carrageenan solution was to be injected subcutaneously into the plantar of left hind paw of all mice groups

- 50 µL of saline solution also had to be injected subcutaneously into the plantar regions of right hind paw of all mice groups

- The mice marked on the tail with an indelible pen for identification and returned to the cage.

After 30 minutes

- **Reference group** had to be cutaneous massaged with a gel of 1% diclofenac diethylamine (voltaren) on plantar region of the left hind paw.

- **Control group** cutaneous massaged with saline solution on the plantar region of the left hind paw

- **Experimental test group 1** cutaneous massaged with gel containing low concentrations of *M. vulgare* extract on the plantar regions of the left hind paws.

- **Experimental test group 2** cutaneous massaged with a gel containing medium concentration of *M. vulgare* extract on the plantar region of the left hind paw

- **Experimental test group 3** cutaneous massaged with a gel containing a high concentration of of *M. vulgare* extract on the plantar region of the left hind paw.

After 3 hours

After three hours, the mice had to be sacrificed and their hind paws are weighed for the determination of the percentage of edema using the following formula

$$\text{% of edema} = \left[\frac{\text{weight of left hind paw} - \text{weight of right hind paw}}{\text{weight of the right hind paw}}\right] \times 100$$

The anti-inflammation activity of *M. vulgare* had to be determined using the percentage of reduction of the edema calculated.
% reduction of odema = (reference % of odema - test group % of odema) / Reference % of odema
*Table III.* Table summarizing the mice groups and models of administration used in anti-inflammatory activity assay.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tests</th>
<th>Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reference</strong></td>
<td>1 % Carrageenan solution</td>
<td>LHP</td>
</tr>
<tr>
<td></td>
<td>saline solution</td>
<td>RHP</td>
</tr>
<tr>
<td></td>
<td><strong>Gel of 1% <em>diclofenac diethylamine</em></strong></td>
<td>LHP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Subcutaneous injection in the plantar</td>
</tr>
<tr>
<td>Control</td>
<td>saline solution</td>
<td>RHP</td>
</tr>
<tr>
<td></td>
<td>1 % carrageenan solution</td>
<td>LHP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>subcutaneous injection in the plantar</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Test 1</strong></td>
<td>saline solution</td>
<td>RHP</td>
</tr>
<tr>
<td></td>
<td>1% carrageenan solution</td>
<td>LHP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Subcutaneous injection in the plantar</td>
</tr>
<tr>
<td></td>
<td>gel with low concentration of the extract</td>
<td>LHP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cutaneous massage</td>
</tr>
<tr>
<td></td>
<td>saline solution</td>
<td>RHP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cutaneous massage</td>
</tr>
<tr>
<td>Test 2</td>
<td>1% carrageenan solution</td>
<td>LHP</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>- gel with medium concentration of the extract</td>
<td>LHP</td>
<td>Cutaneous massage</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test 3</th>
<th>saline solution</th>
<th>RHP</th>
<th>Subcutaneous injection in the plantar</th>
</tr>
</thead>
<tbody>
<tr>
<td>- 1% carrageenan solution</td>
<td>LHP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gel with high concentration of the extract</td>
<td>LHP</td>
<td>Cutaneous massage</td>
<td></td>
</tr>
</tbody>
</table>

**RHP – Right hind paw**

**LHP - Left hind paw**
Chapter III
Hypothesis, discussion and conclusion
III. Hypothesis and discussion

III. 1. Hypothesis

Phenolic compounds constitute one of the major groups of compounds known to inhibit some molecular targets of pro-inflammatory mediators in inflammatory responses, act as primary antioxidants or free radical terminators and antimicrobial agents (Amessis-Ouchemoukh et al., 2014). For these reasons, we had to try to determine the total phenolic content and to evaluate the biological activities of *Marrubium vulgare* extract.

III.1.1. *Marrubium vulgare* extract are rich in phenolic acid and flavonoids contents

The determination of total phenolic content had to be carried out according to the colorimetric method of Singleton and Rossi (1965) while the determination of flavonoids had to be according to the method of Chris and Muller (1960). Based on Amessis-Ouchemoukh and collaborators results, the methanolic extracts of *Marrubium vulgar* are rich on the phenolic acid and flavonoids and their contents are 40.57 mg of Gallic acid extract /g of dry weight leaves and 10.249 mg of quercetin extract /g of dry weight leaves (Amessis-Ouchemoukh et al., 2014). And according to the results of Boularas et al., (2019), which showed that the *Marrubium vulgar* hydroalcoholic extract has a high phenolic acid content of between 64.72 and 66.87 mg / g of dry extract. In addition, the hydroalcoholic extract of *Marrubium vulgar* also has high flavonoid contents with levels ranging from 77.55 to 82.2 mg / g of dry extract. By extrapolation, we suggest, that the *Marrubium vulgar* leaves are rich in phenolic acid and flavonoid.

III.1.2. Anti-microbial activity

*In vitro* anti-bacterial activity of different extracts of *Marrubium vulgar* at 50, 100, 200, 400 and 600 mg/mL has been studied against *Bacillus substilis*, *Staphylococcus aureus* (gram +) and *Escherichia coli*, *Pseudomonas auregonus* (gram -) compared to the standard antibiotic “ciprofloxacin” (10µg/ml).

Based on the results published by a number of authors about hydro-alcoholic extract of *Marrubium vulgar*, this last exhibits a moderate to significant antibacterial activity against the test microorganisms as compared to the standard antibiotic (Amessis-Ouchemoukh et al., 2014).

Below are two tables of results from different studies illustrating the antibacterial activity of *Marrubium vulgar* extracts on the different bacteria against standard ciprofloxacin.
antibacterial and antifungal activity of methanolic extract of *M. vulgare* (Kanyonga et al., 2011)

Table IV antibacterial and antifungal activity of methanolic extract of *M. vulgare* (Kanyonga et al., 2011).

<table>
<thead>
<tr>
<th>Germs</th>
<th>Diameter of inhibition of <em>M. vulgare</em> methanolic extract (mm) (mg/ml)</th>
<th>Diameter of inhibition (mm) of Ciprofloxacin (10µg/mL)</th>
<th>MIC Mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 100 200 400 600</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>0 14 16 20 26</td>
<td>32</td>
<td>100</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0 0 0 13 17</td>
<td>25</td>
<td>400</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>0 15 17 20 25</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>0 10 13 19 23</td>
<td>28</td>
<td>100</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>0 0 0 0 24</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>0 10 16 21 27</td>
<td>-</td>
<td>100</td>
</tr>
</tbody>
</table>

Table V: antimicrobial activity of methanolic extract of whole plant of *M. vulgare*. (Masoodi et al., 2007.)

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Diameter of zones of inhibition (mm) (mg/ml)</th>
<th>MIC (mg/ml)</th>
<th>Ciprofloxacin (10µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 100 200 400 600</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>0 10 13 17 24</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>0 09 11 15 20</td>
<td>100</td>
<td>22</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0 0 10 15 15</td>
<td>400</td>
<td>25</td>
</tr>
<tr>
<td><em>P. vulgaris</em></td>
<td>0 0 11 16 16</td>
<td>400</td>
<td>22</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>0 0 0 0 24</td>
<td>0</td>
<td>23</td>
</tr>
</tbody>
</table>

*Diameter of inhibition zone (mm) are average of triplicate experiments. Disc diameter = 6 mm*

In comparison to the results of the above tables, *M. vulgare* extract are;

- Very much effective against *bacillus subtilus*, *staphylococcus aureus* (gram+) with M.I.C of 100 mg /ml.
Moderate against Escherichia coli (gram -) at 400 mg/ml.

ineffective against Pseudomonas aeruginosa at all concentrations (Kanyonga et al., 2011).

In vivo, we suggest that hydroalcoholic gel based on Marrubium vulgare leaves extract present a more potent antimicrobial activity than in vitro. Since in vivo experiments involves the use of a gel containing; in addition; to leaves extract; an ethanolic solution which allow us to suggest additional antimicrobial effects of leaves extract and of the ethanolic solution that can improve the antimicrobial activity of the gel. knowing that the in vitro test requires use of paper discs impregnated with leaves extract and the evaporation of the hydroalcoholic solution

III.1.3. Anti-inflammation activity

The carrageenan induced paw edema is the most used essay to evaluate the capacity to reduce local edema in the hind paws of the mice by novel anti-inflammatory agents.

The anti-inflammation effect of hydro-alcoholic extract of M. vulgare had to be evaluated using a gel containing three doses classified as low, medium and high depending on the concentrations massaged on the left hind paws with edema induced by carrageenan. We had to calculate the percentage of edema for each mouse from the weights of both hind paws, obtain the average of the mice in every group then calculate the percentage of reduction of the edema in comparison to the reference group that was massaged with a gel 1% of diclofenac diethylamine (voltaren).

Carrageenan induced inflammatory responses consist of three phases; the primary phase mediated by both histamine and 5 hydroxytryptamine. The secondary phases mediated by kinin notably an endogenous none peptide bradykinin and the last phase attributed to local production of prostaglandins (PG) most especially those of E series. the precursors of both PG’s and thromboxane is prostaglandins H₂ (PGH₂) derived from arachidonic acids by the action of cyclooxygenase (cox) enzyme (Morris, 2003).

According to the previous studies, that reveal the hydro-alcoholic extract of Marrubium vulgare inhibits carrageenan induced inflammation by:

- inhibition of cyclooxygenase enzymes (Cox) (Amessis-Ouchemoukh et al., 2014)
- Decrease of pro-inflammatory mediators; tumor necrosis factor α (TNFα), IL-1, myeloperoxidase levels and peripheral neutrophil count (Namoune et al., 2018).

Results from (Amessis-Ouchemoukh et al., 2014) demonstrate that, hydro-alcoholic extract of Marrubium vulgare leaves induced 68.15% inhibition percentage of cyclooxygenase (Cox) at 0.033 mg/ml and it presented a potent activity with IC₅₀ of 0.082 mg/ml which statistically not different from a standard chemical indomethacin. The anti-inflammation activity of this extract increased dose dependently.

Cyclooxygenase (Cox) is a bi-functional enzyme that first catalyzes the addition of two molecules of oxygen to arachidonic acid to form the hydro peroxide prostaglandin G2 (PGG2), and then reduces the hydro peroxide to the alcohol, PGH2, by a peroxidase activity. Prostaglandins (PGs) are important biological mediators of inflammation (Amessis-Ouchemoukh et al., 2014).

![Figure 9: Cyclooxygenase inhibitory activity of the different plants extract (concentration of plant extract is 33µg/ml (Amessis-Ouchemoukh et al., 2014)]](image)

Results from (Namoune et al., 2018) demonstrate that, hydro-alcoholic extracts of Marrubium vulgare are efficacy in the decreasing the release of TNFα, IL-1 β and IL -8 cytokines levels in PBMCs with a dose-dependent effect.
Table VI Effect of methanolic (MeOH) extract of *M. vulgare* and standards on the plantar edema induced by carrageenan in rats

<table>
<thead>
<tr>
<th>Groups &amp; Doses (mg/Kg)</th>
<th>Time (hours) and plantar diameter (mm)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1h</td>
<td>2h</td>
<td>3h</td>
<td>4h</td>
</tr>
<tr>
<td>Control group</td>
<td></td>
<td>6.40 ± 0.5</td>
<td>6.87 ± 0.7</td>
<td>6.99 ± 0.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.87 ± 0.41&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MeOH extract</td>
<td></td>
<td>6.39 ± 0.2</td>
<td>6.42 ± 0.5</td>
<td>4.80 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.41 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diclofenac</td>
<td></td>
<td>6.37 ± 0.9</td>
<td>5.95 ± 0.5</td>
<td>4.73 ± 0.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.71 ± 0.62</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td></td>
<td>6.41 ± 0.00</td>
<td>5.23 ± 0.71</td>
<td>4.16 ± 0.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.98 ± 0.86&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values represented as mean ± SD (n = 3). Significant differences in the same row are shown by letters<sup>a,b</sup> varieties (P<0.05).

Anti-inflammatory potential of MeOH extract and standards was assessed in terms of inhibition of plantar diameter. The results illustrated in Table VII demonstrated that the administration of *M. vulgare* methanol extract at a dose of 200 mg/kg b.w prevents significantly (P< 0.05), the plantar edema in rats from the third hour of treatment which is close to that of diclofenac and aspirin. The highest value of inhibition estimated by 87.3 ± 0.25% compared to diclofenac and aspirin (85.52 ± 0.47% and 90%) respectively (Ghedadba et al., 2016.).

In agreement with these previous study results, we suggest that the gel with low, medium, high concentrations of hydro-alcoholic extracts massaged on the hind paw edema induced by carrageenan reduces the edema thus it exhibits dose dependent anti-inflammatory effect. We suggest that the hydro-alcoholic extract *M. vulgare* exhibits an inhibitory effect of carrageenan induced inflammation. The gel containing *M. vulgare* extract had to decreases the edema induced by carrageenan with a high dose gel having the highest decrease in the edema followed by medium and lastly low dose gel.

The percentage of reduction of edema had to be dose dependent, the percentage increase with increase in low, medium and high doses. Therefore, there exists a proportionality relationship between the percentage of reduction of edema and the doses of hydro-alcoholic extract of *M. vulgare* in the gel, its effect to reduce the edema and the anti-inflammatory activity.

**III.2. Discussion**

The main objective of this study was to determine the anti-microbial and anti-inflammatory activities of polyphenol compounds from *Marrubium vulgare*. 
Several studies reported the presence of phenolic compounds and flavonoids in *Marrubium vulgare* extract (Lodhi et al., 2017). The amount of polyphenols can vary depending on the extraction methods and indeed methanolic extracts seem to be richer than extracts obtained by other solvents but it is very toxic. The amount of phenolic extract can also be affected by the genotype, the conditions of development and growth, maturity and storage of the plants (Yahiaoui et al., 2018).

The literature results confirm the effectiveness of the medicinal plants extracts and their antiseptic power. Many studies emphasize the antibacterial effect of natural products. It was reported that the aqueous extract of the leaves of the species *M. vulgare* exerts a strong inhibitory activity on some bacterial strains (Masoodi et al., 2007.).

Despite the large data have now been existing about antibacterial and antimicrobial effects of medicinal plants, little has been conducted in the antifungal areas especially *M. vulgare* (Rezgui et al., 2020). However, basing on the few studies report that the effect of plant extracts on fungal pathogen may be attributed to their content on secondary metabolites like alkaloids, phenolic and terpenoid compounds.

The antimicrobial activity of plant extracts is due to the various chemical substances present in these extracts. Thus, the optimal antibacterial activity cannot be attributed only to a single compound but different combination of compounds with the same effect and / or synergistic effects on the microorganism. Therefore the anti-microbial activity of *Marrubium vulgare* occurs due to the combined action of compounds present in the plant not the activity of a single compound (Bouterfas et al., 2018).

According to the different results of literatures in agreement with (Poole, 2001), gram negative bacteria are typically more resistant to the extract of *Marrubium vulgare* than gram positive bacteria. This has been long explained by the presence of outer membrane which acts as a permeability barrier in gram negative limiting access of the anti-microbial agents to their targets in the bacterial cells.

According to research, there is an association between the phenolic compounds, flavonoids and antibacterial activity, the extract compounds of *Marrubium vulgare* have moderate to significant antimicrobial activity against the some bacterial strains.
Inflammation is immune response to infection, injuries and it plays a causative role in the development of various diseases including asthma, atherosclerosis. *Marrubium vulgare* has been traditionally used for treatment of inflammatory related symptoms such as colds, fevers and sore throats. Results obtained with (Namoune et al., 2018) show the efficacy of some *Marrubium vulgare* extracts for the release of TNF-α, IL-1β and IL-8 cytokines levels in PBMC’s with a dose-dependent effect.

In agreement with several previous studies, we suggest that, *Marrubium vulgare* possesses anti-inflammation effect against many inflammatory mediators. Some studies indicated that this plant also has an inhibitory effect on cyclooxygenase enzyme (Cox) (Amessis-Ouchemoukh et al., 2014). Several phytochemical studies indicated the presence of diterpenes, phenylethanoids, flavonoids, and monoterpenes in *Marrubium vulgare* (Lodhi et al., 2017). These studies confirmed that this variety of molecules could be responsible for the inhibition of some molecular targets of pro-inflammatory mediators such as TNF-α, MPO, IL-1β, and cyclooxygenase enzyme in the inflammatory response (Namoune et al., 2018).
Conclusion

This study reveals that the hydro-alcoholic extract of *Marrubium vulgare* is rich in the phenolic and flavonoid content. Bacteria: *Bacillus subtilis, Staphylococcus aureus* (gram +) are susceptible, *Escherichia coli* is intermediate and *Pseudomonas aeruginosa* is resistant to the extract of *M. vulgare*. That confirms that *M. vulgare* possesses a moderate to significant antimicrobial activity. The direct proportionality between the percentage of reduction of edema and the gel with high, medium and low doses confirms that *M. vulgare* exhibits an inhibition of inflammation dose dependent. Therefore, the polyphenol extract of *Marrubium vulgare* exhibit both the antimicrobial and anti-inflammation activities. The hypothesis of this study justifies the traditional uses of *Marrubium vulgare* in folk medicine.

Polyphenols contain important properties with numerous biological activities thus they can be exploited for therapeutically applications for example to provide an alternative to antibiotics and non-steroid anti-inflammatory drugs (NSAID)

Further research, analysis and purification of *Marrubium vulgare* extracts are needed to identify the exact bioactive molecules responsible for the pharmacological activities also how to improve the safety of herbal medicines and the right dosing of herbal medicines. More research is needed to investigate also the antifungal activities of polyphenols extracts from *Marrubium vulgare*. 

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PlantFiles Pictures: Marrubium Species, Horehound (Marrubium vulgare) by Xenomorf [WWW Document], n.d. Dave’s Garden. URL

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