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Development of a Marrubium Vulgare-Based Herbal Ointment: Biological Evaluation and Safety Assessment

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Abstract:

The aim of this work is to develop an herbal ointment based on the extract of the plant Marrubium vulgare and to evaluate its expectorant efficacy in addition to the study of some of its biological properties and safety. This plant was selected due to its traditional uses and its content of natural compounds of biological significance. Ointments were prepared at different concentrations (0.5%, 1%, 3%, 5% and 8%), and their efficacy was assessed. The results of the expectorant efficacy test showed an improvement in the expectorant activity with increasing concentration, while the ointments showed antioxidant, anti-inflammatory and antimicrobial activities to different degrees. The results of the HET-CAM test also confirmed that the low and medium concentrations had an acceptable level of safety. Based on the balance between efficacy and safety, a concentration of 3% was proposed as the optimal concentration. The results obtained demonstrate that Marrubium vulgare can be used in the development of promising natural herbal preparations.

Keywords: Marrubium vulgare, herbal ointment, expectorant, antioxidant activity, anti-inflammatory activity, antimicrobial activity, HET-CAM test.

الملخص:

يهدف هذا العمل إلى تطوير مرهم عشبي يعتمد على مستخلص نبات المريوث (Marrubium vulgare) وتقييم فعاليته الطاردة للبلغم بالإضافة إلى دراسة بعض خصائصه البيولوجية وسلامته. تم اختيار هذا النبات نظراً لاستخداماته التقليدية واحتوائه على مركبات طبيعية ذات أهمية بيولوجية. تم تحضير المراهم بتراكيز مختلفة (0.5%، 1%، 3%، 5% و8%)، وتم تقييم فعاليتها. أظهرت نتائج اختبار الفعالية الطاردة للبلغم تحسناً في النشاط الطارد للبلغم مع زيادة التركيز، في حين أظهرت المراهم نشاطات مضادة للأكسدة ومضادة للالتهابات ومضادة للميكروبات بدرجات متفاوتة. كما أكدت نتائج اختبار HET-CAM أن التراكيز المنخفضة والمتوسطة كانت بمستوى مقبول من السلامة. وبناءً على التوازن بين الفعالية والسلامة، تم اقتراح تركيز 3% كتركيز مثالي. تظهر النتائج التي تم الحصول عليها أن نبات Marrubium vulgare يمكن استخدامه في تطوير مستحضرات عشبية طبيعية واعدة.

الكلمات المفتاحية: المريوث (Marrubium vulgare)، مرهم عشبي، طارد للبلغم، النشاط المضاد للأكسدة، النشاط المضاد للالتهاب، النشاط المضاد للميكروبات، اختبار HET-CAM.

Dedication

بسم الله الرحمن الرحيم

Praise be to Allah, by whose grace and favour all matters are completed, who

illuminated my path with patience and hope, and who was with me every step until I reached this position.

I dedicate this work

To the one whose efforts were silent and whose giving knew no bounds, **my dear father**. I ask Allah to grant your health and wellbeing, and to reward you generously on my behalf.

To the heart that granted me love, prayers, and strength, to the one who was my support at every step, **my beloved mother**. I pray that Allah protects you and grants you a long life.

To those who share my days, in both joy and hardship, whose presence has always been a beautiful reward in my life, **my dear sisters.**

To **my dear brother**, I pray that Allah protects you and fills your days with happiness and success.

To the joy of my heart**, Roufane and Djaser**, you are the purity that lightens the weight of my days, and the happiness born from the simplest moments.

To my **beloved grandmother**, who still showers me with her sincere prayers, her blessings have accompanied me in every success.

To my extended family.

To my **friends and companions** on my journey, who have always been a support and encouragement at every step.

And to everyone who has left a good mark on my life.

I also dedicate it to my heart, to myself, to every part of me that worked hard, was patient, and endured, for every effort and every dream that became reality.

This work is the fruit of your love, your patience, and my faith in myself. It is a piece of my heart that I dedicate to everyone who was a reason for me to reach this point.

AYA

Dedication

Praise be to Allah, and blessings and peace be upon our master Muhammad,
the chosen and trustworthy.
We thank Allah who has granted us success in completing this work

I dedicate this success to

The one for whom Paradise was placed beneath her feet,
my dear mother.
The one whose name I carry with pride,
my dear father.
My support, my strength, and my refuge,
my siblings.
The one whom fate brought me together with, and with whom I shall
continue the journey of life, God willing,
my fiancé.

Praise be to Allah out of love, gratitude, and thankfulness for
the beginning and the end.

Rania

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List of Abbreviations	
Ab	Absorbance of blank
Ac	Absorbance of control
As	Absorbance of sample
C	Concentration
CLSI	Clinical and Laboratory Standards Institute
DPPH	2,2-Diphenyl-1-picrylhydrazyl
I (%)	Inhibition percentage
IC_{50}	Inhibitory Concentration 50%
MHA	Mueller–Hinton Agar
MHB	Mueller–Hinton Broth
Me	Mass of extract
Mp	Mass of powder
NI	No Inhibition
PBS	Phosphate Buffered Saline
PI	Percentage Inhibition
R^2	Coefficient of determination
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
(V/V)	Volume/Volume
(W/V)	Weight/Volume

General Introduction

General Introduction:

Medicinal plants have been used for decades to treat numerous diseases, with various civilisations relying on them in traditional therapy due to their containing a wide spectrum of active compounds such as phenolic compounds, essential oils, flavonoids, and alkaloids, which confer a range of pharmacological activities. This has given them great importance in the pharmaceutical field, as they are a primary source for the discovery and development of many modern medicines, in addition to their use in the preparation of various natural therapeutic products (Dar et al., 2023). In recent years, an increased interest in medicinal plants has been observed, within the context of seeking safer, more natural alternatives with fewer side effects compared with some synthetic chemical compounds. From this standpoint, the topic of this study was chosen, which centres on developing a plant-derived pharmaceutical preparation in the form of an ointment prepared from the plant Marrubium Vulgare, which is known in traditional medicine for uses related to the respiratory system, as well as for its anti-inflammatory and antioxidant properties, especially as an aid in expectoration.

The widespread use of medicinal plants in traditional practice has not obscured the fact that there are limitations in converting this plant material into scientifically developed pharmaceutical forms that combine efficacy and safety, particularly with regard to topical preparations with therapeutic effects. Although some studies have addressed this topic, they have mainly focused on applications such as wound healing, burns, or certain biological activities, whereas studies aiming to develop herbal ointments from Marrubium vulgare with a comprehensive evaluation of their biological efficacy remain limited. Accordingly, the need for this study emerges, with the aim of filling this scientific gap by developing an herbal ointment from Marrubium vulgare with an expectorant property while evaluating its efficacy and investigating certain biological properties that support its safety and bioactivity.

Thus, this study has scientific and practical significance in contributing to the development and utilisation of medicinal plants and converting them into natural and effective pharmaceutical preparations to reduce reliance on chemical substances, in addition to developing low-cost natural products with potential efficacy that contribute to enhancing the value and use of local plant resources while strengthening research in the field of herbal pharmaceutical preparations. We have divided our work as follows:

- Introduction.

- Chapter 1: falls under the title of the bibliographical study.
- Chapter 2: Tools and methods.
- Chapter 3: Results and discussion.
- Conclusion.

Theoretical aspect

Chapter I: Bibliographic study

Chapter Introduction:

This chapter conducted a bibliographic study on the plant Marrubium vulgare (horehound), covering its identification, classification, botanical and chemical qualities, and therapeutic uses. It also gave a general review of medicinal plants and their importance. In addition, the phytochemical studies and extraction techniques, especially maceration extraction, are the methods used in the practical aspect. Further pharmaceutical forms were discussed, and ointment has been selected as the pharmaceutical form. This section offered an overview of phlegm, the respiratory system, the anti-inflammatory and antioxidant activities of plant extracts the uses of plant extracts, and previous research conducted on the plant under study.

I.1. Medicinal plants and their uses:

I.1.1. Concept of Medicinal Plants:

Medicinal plants are plants that have healing properties and can help people with certain diseases. A medicinal plant may contain one or more active compounds that have physiological (functional) effects in treating diseases. These compounds may be found in their natural form (fresh, dried, or partially extracted) or in their pure form after extraction. Medicinal plants, also known as herbal medicines, have been used in traditional medicine since prehistoric times. About 80% of the people in the world still use medicinal plants as traditional alternative medicine to treat a wide range of illnesses. Medicinal plants have served as a principal therapeutic approach for numerous ailments across various cultures globally.(الأغواني, 2024)

I.1.2. The Importance of Medicinal Plants in Traditional and Modern Medicine:

I.1.2.1. Use of medicinal plants in traditional medicine:

The treatment and cure of illness have been the concern of humans since prehistoric times. Local practitioners all over the world for generations have used indigenous plants and herbs to cure a wide range of illnesses, which is a clear indication of their medical efficacy. Traditional medicine is practiced in all five continents, based on centuries-old customs and beliefs. It is better to use compound herbal medicines than single plants because some elements are useful only when combined with other compounds. Traditional medicines have been developed through thousands of years of human experience and knowledge. Theories have been developed on the use of plant materials, and the positive and negative impacts of these materials have been studied.(حسن, 2021)

I.1.2.2. The focus of modern medicine on pharmaceutical preparations:

The prevalence of fatal illnesses like diabetes and the negative side effects of produced medications have caused emphasis to move from conventional medicine to alternative medical systems. The commerce in herbal medicines has had a global renaissance as a result of consumer reaction to plant-derived medications. According to estimates from the World Health Organization, 4 billion people utilize herbal remedies. These herbal remedies range from standardized plant extracts (including two or more components) and phytochemicals made in contemporary pharmaceutical laboratories to medicinal teas, skincare products, anti-wrinkle agents, anti-aging lotions, and raw tablets used in traditional medicine.(حسن, 2021)

I.2. presentation of the selected plant (Marrubium Herb):

I.2.1. Introduction to the Marrubium Herb:

I.2.1.1. Scientific name:

Marrubium vulgare L. is the scientific name for the horehound herb. The Hebrew term marrob, which signifies bitter juice, is the source of the Latin name Marrubium, whilst vulgare means common or well known.(A´cimovi´c et al., 2020)

I.2.1.2. Botanical classification:

Marrubium vulgare is a medicinal plant that includes more than 30 types of flowers and belongs to the Lamiaceae family.(Bahammou et al., 2019)

I.2.1.3. Common names:

The two old English terms har and hune, which refer to a plant covered with fuzz, are the origin of the English word horehound, which is used for the herb Marrubium vulgare. Since the tea of this herb was traditionally used as a bitter medicine for infertile women, its most common popular name in Serbian is ocajnica, meaning the desperate woman. It is often referred to as Marrubia in Tunisia and white horehound in Europe.(A´cimovi´c et al., 2020; Lodhi et al., 2017)

I.2.2. Botanical Description of the Marrubium Herb:

I.2.2.1. External appearance:

Figure I.1: Marrubium Herb

Stiff, woody, branched taproot or many fibrous lateral roots and many stems that are erect, quadrangular, extremely downy, and between 20 and 100 cm high. Roundish, ovate, petiolate, veined, and typically serrated leaves. They are grouped in opposing pairs on a long stem and have a hoary surface. White flowers in densely packed axillary whorls form inflorescences in the axils of higher leaves. Each of the ten teeth on the tubular, lobed calyx has a tiny hooked spine or bristle. The corolla is tubular, bilabiate, and white to pale lavender; the lower lip is three-lobed with a larger central lobe, while the upper lip is two-lobed, bifid, and erect. Style, stamens, and anthers with diverging sacs make up the corolla tube. The vegetative and generative organs of M. vulgare have a thick layer of glandular and non-glandular trichomes on their surface.(A´cimovi´c et al., 2020)

I.2.2.2. Places of growth and distribution:

The plant is found in the northern region of India, including the Union Territory of Jammu & Kashmir's Himalayan foothills. native to Central Asia, North Africa, Southwest Africa, and Europe. It is common in Morocco's northeast.(Jaadan et al., 2020; P. Kumar et al., 2024)

I.2.3. Chemical composition of Marrubium herb:

I.2.3.1. Volatile oils:

The essential oil of Marrubium vulgare L. from Algeria contains several main chemicals, including eugenol and β-bisabolene. The amounts of these compounds vary depending on the growth stage of the plant. β-bisabolene was higher in the vegetative stage and eugenol was higher in the oil extracted from flowering plants. Previous studies have shown less amount of germacrene D in M. vulgare essential oil than this study with concentrations of about 9.37% in samples from Tunisia and 4.71% in samples from Lithuania. The chemical composition of the oil is dependent on its geographic source. Lithuanian oils were rich in (Z)-β-farnesene, β-caryophyllene, (E)-hex-2-enal, α-humulene, and germacrene D, while Tunisian oils were rich in γ-eudesmol, β-citronellal, citronellyl formate, and germacrene D.(ZAWIŚLAK, 2012)

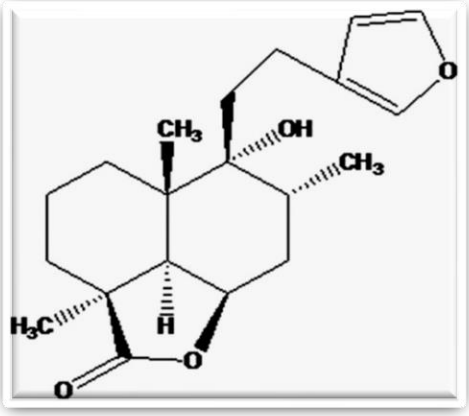


Figure I.2: Chemical structure of marrubiin

I.2.3.2. Active compounds:

The horehound herb (Marrubium vulgare L.) is distinguished by containing a variety of active compounds with diverse chemical natures, which will be discussed in the following sections.

I.2.3.2.1. Diterpene compounds:

I.2.3.2.1.1. Marrubiin:

Up to 3 mg/g of fresh weight, M. vulgare accumulates labdane-type diterpenes as the main bitter components, with marrubiin being the most prevalent (0.12–1%). The methanol extract of M. vulgare is estimated to contain 156 mg/g of marrubiin.(A´cimovi´c et al., 2020)

I.2.3.2.1.2. Other diterpenes identified in Marrubium vulgare:

- Pre-marrubiin.
- 12(S)-hydroxymarrubiin.
- 11-oxomarrubiin.
- 3-deoxo-15(S)-methoxyvelutine.
- Marrubenol.
- Marruliba-acetal.
- Cyllenil A.
- Polyodonine.
- Preleosibirin.(A´cimovi´c et al., 2020)

I.2.3.2.2. Other bioactive compounds:

The phytochemical screening of Marrubium vulgare L. revealed a high concentration of phenolic substances such as polyphenols, flavonoids and catechin tannins especially in organic extracts. Their concentration varied depending on the solvent used to extract them, as well as on the climate and the place of origin. In general, the amount of organic extracts, particularly methanolic and ethanolic extracts, was significantly higher than that of aqueous extracts. This high content of phenolic chemicals showed a strong antioxidant activity and suggests their involvement in the biological processes of the plant.(Jaadan et al., 2020)

I.2.4. Therapeutic Properties of the Marrubium Herb:

I.2.4.1. Expectorant properties:

M. vulgare was included in the Pharmacopoeias and Merck's Phytotherapy Index (1910). In 1927, researchers stated that M. vulgare has been applied to pulmonary illnesses. It was reported in 1941 that M. vulgare is the most widely used herbal treatment for pectoral disorders and utilized as an expectorant and bitter tonic. It generates possible consequences for colds, coughs, and lung infections.

Belgian literature, the Materia Medica Vegetabilis, was published in 1954. demonstrated how to use M. vulgare as a honey-based decoction syrup to treat coughs and bronchitis.(Lodhi et al., 2017)

I.2.4.2. Anti-inflammatory properties:

Experimental studies have shown the presence of anti-inflammatory properties against prostaglandin E2 and carrageenan-induced inflammation and analgesic properties in p-benzoquinone-induced. Methanolic extracts of M. vulgare have effects comparable to those of the common medications' acetylsalicylic acid and indomethacin, according to an abdominal constriction test.

M. vulgare contains six compounds (luteolin-7-O-βglucopyranoside, apigenin-7-O-β-glucopyranoside, oleanolic acid, β-sitosterol, luteolin-7-O-rutionside, and rosmarinic acid) that prevent the production of hormones like prostaglandins and peroxasalandine, which are involved in the production of inflammatory mediators as determined by in vitro anti-inflammatory activity.(Babyvanitha et al., 2021)

I.2.4.3. Traditional uses:

Marrubium vulgare a was used traditionally in the treatment of dyspeptic complaints, lung infections, cough, rheumatoid arthritis, night blindness, loss of appetite, as cholagogue, purgative, diuretic, bitter tonic, carminative and appetizer.(Al-Snafi et al., 2021)

I.3. Phytochemical study of the plant extract:

I.3.1. Qualitative analysis:

It is a series of chemical tests carried out on a plant extract to find out the presence or absence of certain chemical compounds using approved protocols.(Velavan, 2015a)

I.3.2. Quantitative analysis:

Quantitative analysis is considered as one of the most important steps of phytochemical study and it is performed after the qualitative analysis stage, as it aims to determine the amounts and concentrations of compounds present in the plant sample. This type of analysis is thought to be more accurate and complete in determining proportions.(EGBUNA et al., 2019)

I.3.3. Phytochemical compounds:

I.3.3.1. Flavonoids: They are widespread secondary natural compounds in plants, found in various parts of the plant and classified among low molecular weight phenolic compounds, possessing a polyphenolic chemical structure. This type of compound has antioxidant activity and anti-inflammatory effects, in addition to anti-mutagenic and anti-cancer properties. They also play an important role in the plant, being responsible for giving colour to flowers, as well as contributing to the growth and development of the plant and protecting it from external factors. Phenolic compounds are divided into several subgroups such as: Flavonols, isoflavones, flavones and chalcones.(Panche et al., 2016)

I.3.3.2. Phytosterols: They are plant steroid compounds belonging to the lipid class, naturally found in all parts of plants. Their structure is similar to cholesterol, with a slight difference in the side chain or bonds. They may contribute to improving human and animal health when consumed, and also help to reduce cholesterol levels in the blood. Phytosterols are divided into two main types: sterols, which are unsaturated compounds (with a double bond in the sterol ring) such as sitosterols and campesterols; and the second type is stanols, which are saturated compounds with a double bond in the sterol ring.(Ogbe et al., 2015)

I.3.3.3. Tannins: They are high molecular weight polyphenolic compounds, soluble in water. They are found in both flowering and non-flowering plants, in most parts of the plant, specifically within the cells, particularly in the vacuoles. Tannins play an important role in defending plants against living organisms. They are also responsible for imparting an astringent taste due to their ability to bind with proteins and form complexes, and they also bind with starch, cellulose, and minerals. Tannins are divided into two types: hydrolysable tannins, which contain a sugar core linked to phenolic acids, and condensed tannins, which are composed of flavonoid units linked together.(Hassanpour et al., 2011)

I.3.3.4. Carbohydrates: They are stable organic compounds, derived from the primary metabolites, and are synthesized in plants from carbon dioxide and water, using light energy. Carbohydrates contain carbon, hydrogen and oxygen. They are classified as:

- Sugars: composed of monosaccharides and disaccharides.
- Oligosaccharides: consisting of 3 to 15 sugar units, are found in plant seeds.
- Polysaccharides: These are also called complex carbohydrates and are subdivided into starch and non-starch polysaccharides.(Lunn & Buttriss, 2007)

I.3.3.5. Alkaloids: The largest groups of natural compounds, which belong to plant secondary metabolites, are of a basic nature. They are characterised by great diversity in their chemical structure and contain a nitrogen atom. They possess numerous properties such as defending the plant against living organisms, in addition to their biological activities and effective pharmacological effects.(Bribi, 2018)

I.3.3.6. Coumarins: The largest groups of natural compounds, which belong to plant secondary metabolites, are of a basic nature. They are characterised by great diversity in their chemical structure and contain a nitrogen atom. They possess numerous properties such as defending the plant against living organisms, in addition to their biological activities and effective pharmacological effects.(EGAN et al., 1990)

I.4. Methods of extraction for active compounds:

I.4.1. Definition of extraction:

Extraction is a process used to separate active compounds from plant materials such as polyphenols, carotenoids, flavonoids, and alkaloids. These compounds are used in various fields such as food, pharmaceutical, and cosmetic industries. The process involves the transfer of the compounds from the plant to the solvent that is used. The extraction process is influenced by a number of factors like concentration gradient, diffusion factor and the characteristics of the plant material. Classical extraction methods include Soxhlet extraction, solvent extraction, boiling extraction and steam distillation.(Babaei et al., 2026)

I.4.2. Extraction methods:

I.4.2.1. Decoction:

Decoction extraction is considered one of the traditional methods used to extract active compounds from medicinal plants. This technique is often employed with tough plant parts such as peels, fibres, and roots, as well as plants containing chemical compounds that are water-soluble. It relies on the use of water as a solvent in the traditional method known as Quanta. The plant, after being ground, is placed in a copper or tin-coated ceramic vessel, then water is added and the mixture is heated over a low flame until the volume reduces to a quarter of the original size in the case of soft medicines, or to an eighth in the case of medicines that are moderately or highly tough. As for the amount of water, it varies depending on the hardness of the plant material. Usually, four times the amount of water is used for soft medicines, eight times for moderately tough medicines, and sixteen times for highly tough medicines.(Tandon & Rane, 2008)

I.4.2.2. Steam distillation:

Steam distillation is one of the soporific methods traditionally used for the extraction of volatile compounds and essential oils from plants. Several types of distillation have been used including water distillation, water and steam distillation and direct steam distillation. The technique involves boiling the plant in water or steaming it and releasing the essential oils which vaporize with the steam. The latter, which contains the essential oils, goes into the condenser where it is cooled and becomes a liquid. Therefore, the oil can be easily separated from the water because it has lower density and stays on the top. Yield is usually between 0.005 and 10% and is affected by many

factors, such as the temperature, distillation time, quality and type of plant and pressure during the process.(Chema & Boutekedjiret, 2015)

I.4.2.3. Extraction using a Soxhlet apparatus:

The Soxhlet apparatus was invented in 1879 by von Soxhlet, with the aim of extracting fats from milk. It subsequently became one of the most widely used traditional techniques for extracting compounds from solid materials. The conventional Soxhlet extraction process involves placing the sample in a special holder, which is gradually filled, after the device is started, by the condensed solvent present in the distillation flask. Once full, the solvent carrying the compounds is drawn through the siphon tube back into the flask, and the cycle is repeated several times until the compounds are completely extracted. These techniques are characterised by the ability to repeatedly expose the sample to the solvent and the lack of need for continuous filtration, as well as the possibility of controlling the temperature. However, they may take a long time and consume large quantities of solvent. Consequently, and due to these drawbacks, modern methods have been developed, such as automated Soxhlet extraction, high-pressure Soxhlet extraction, microwave-assisted Soxhlet extraction, and ultrasound-assisted Soxhlet extraction.(Luque de Castro & Priego-Capote, 2010; Luque-García & Luque de Castro, 2004)

I.4.2.4. Maceration:

Maceration extraction is one of the classic methods for extraction of active compounds from plants. This process involves taking the plant after it has been ground up and placing it in a solvent for a certain amount of time with intermittent stirring to increase the contact between the solvent and the plant material and to improve transfer. The process is carried out at room temperature so as to retain the heat labile compounds.(PALMA et al., 2013)

I.4.3. Extraction by maceration:

I.4.3.1. Principle of extraction by maceration:

- The dried and ground plant material is soaked in a suitable solvent.
- The process is usually carried out at room temperature.
- The soaking period lasts from several hours to several days.
- Soaking facilitates the transfer of soluble compounds from the plant to the solvent due to diffusion and the difference in concentration. (Abd Alelah et al., 2026)

I.4.3.2. Factors affecting extraction by maceration:

- Temperature: It affects the rate of extraction and the transfer of compounds.
- Extraction duration: A longer time allows for the extraction of a greater quantity of compounds.
- Type and concentration of solvent: It should be suitable for the nature of the compounds to be extracted.
- Size of plant particles: Grinding increases the surface area and facilitates the transfer of compounds.
- Stirring: It helps improve mass transfer and prevents saturation.
- Amount of solvent relative to plant material: It affects the quantity of compounds extracted. (Abd Alelah et al., 2026)

I.4.3.3. Advantages and disadvantages of extraction by maceration:

Table I.1: Advantages and disadvantages of maceration.(PALMA et al., 2013)

Advantages	Disadvantages
▪ Simple and easy to apply. ▪ Does not require complex equipment. ▪ Can be carried out at room temperature, which preserves heat-sensitive compounds.	▪ It takes a long time. ▪ You may need large amounts of solvent.

I.5. Pharmaceutical dosage forms:

I.5.1. Oral dosage forms:

Oral dose forms are given orally for either a systemic effect in the body following absorption from the mouth or gastrointestinal tract, or for a local effect in the mouth, throat, or gastrointestinal tract.

Depending on the dosage's physical state, oral dosage forms can be separated into two major categories. liquid oral dosage forms such as solutions, syrups, emulsions, and powders for suspensions, and solid oral dosage forms such as tablets, capsules, or powders.(de Villiers, 2004)

I.5.2. Parenteral dosage forms:

Sterile, non-pyrogenic liquid dosage forms (solutions, emulsions, suspensions) or solids with one or more active agents that are packaged in single-dose or multi-dose containers are known as injectable preparations. They are designed to be administered into the body by injection, infusion, or implantation. Injectable preparations come in four primary forms: injections, intravenous infusions, injection powders, and implants.(World Health Organization, n.d.)

I.5.3. Topical pharmaceutical forms:

The majority of dose formulations used topically are semi-solid. They mix the characteristics of liquids and solids and are thought of as a physical body. They act more like a solid when at rest, allowing for stability and a longer residence period at the application site. Under compression, they liquify and can be applied gently and evenly on the skin surface. Being among the earliest dosage forms, they continue to be crucial in medicine, particularly in dermatology, as well as for nasal or ocular delivery and wound care.

Topical distribution is desirable since it allows to transport the active directly to a localized portion of the body without exposing the whole organism systemically, therefore lowering issues of toxicity and side effects.(Herbig et al., 2023)

I.5.3.1. Topical routes of effect:

I.5.3.1.1. Inhalation route:

In this case, the medication enters the respiratory system through the nose or mouth, and from there it passes to other parts such as the pharynx, trachea, bronchi, bronchioles, and pulmonary alveoli.(ديوب, 2019)

I.5.3.1.2. Vaginal and urethral route:

Pharmaceutical forms specifically designed for administration of medication via the vaginal route are used, such as suppositories, tablets, foams, gels, and solutions. Suppositories and solutions are used exclusively for the urethral route.(ديوب, 2019)

I.5.3.1.3. Ophthalmic, otic, and nasal route:

- **Ophthalmic preparations:** Includes solutions, suspensions, ophthalmic ointments, gels, and ocular implants, in addition to preparations used with surgical implantation such as intraocular lenses.(Chowhan et al., 2013)

- **Otic preparations:** They are viscous preparations; this viscosity contributes to prolonging the contact time of the medication with the affected area. Otic preparations are used for various purposes, including the removal of cerumen, relief of ear pain, as well as the treatment of ear infections. (ديوب, 2019)
- **Nasal preparations:** Nasal preparations are used to produce a local effect at the level of the nasal mucosa, or to produce a systemic effect. Among these preparations are nasal drops, nasal gel pump, aqueous nasal sprays, pressurised metered dose inhalers, and dry powder inhalers.(Alagusundaram et al., 2010)

I.5.3.1.4. Dermal route:

Dermatological preparations are available in various pharmaceutical forms, including:

- Semi-solid forms such as creams and ointments.
- Liquid forms such as solutions and lotions.
- Solid forms in the shape of powders, as is the case with some antifungals.(ديوب, 2019)

I.5.4. Classification of the semi-solid topical dermatological pharmaceutical forms:

I.5.4.1. Gels:

The word gel comes from gelatin, and both gel and jelly have roots in the Latin Gelu, which means frost, and gel an, which means freeze or congeal. This genesis highlights the fundamental concept of a liquid turning into a solid-like substance that is elastic and has certain liquid properties but does not flow. When chemists tried to categorize semisolid materials based on their phenomenological traits rather than their molecular compositions in the late 1800s, the term gel was first used.(Rathod & Mehta, 2015)

Figure I.3: Gel form.

I.5.4.2. Creams:

Topical medicines that can be applied to the skin are called creams. Creams are defined as viscous liquid or semi-solid emulsions of either the oil-in-water or water-in-oil type dosage forms whose consistency varies by oil and water. Because they are made using methods developed in the pharmaceutical industry, creams—both medicated and unmedicated—are widely used to treat a variety of skin conditions or dermatoses. Ayurvedic, herbal, or allopathic creams are utilized by individuals based on their skin issues. They include one or more drug ingredients that have been dissolved or scattered in an appropriate base. Based on phases, creams can be categorized as either an o/w or w/o type of emulsion.(Lalita & Shalini, 2020)

Figure I.4: Cream form.

I.5.4.3. Ointments:

I.5.4.3.1. Definition of ointments:

Ointments are homogeneous, viscous semisolid preparation, most typically a greasy, oily Oil-80%, Water-20% with high viscosity that is designed for external application to skin or mucous membranes. They are employed as emollients or for the administration of active ingredients to the skin for protective, medicinal, or preventive purposes.(Bhaskar et al., 2016)

Figure I.5: Ointment form.

I.5.4.3.2. General composition of ointment:

According to this definition, an ointment consists of two components: the medicinal or active ingredient and the base. "The base may be said to be the ointment without a medicament" because it serves as a vehicle or carrier for the active ingredient or ingredients.(BILLUPS, 1964)

I.5.4.3.3. Ointment bases:

The vehicle or carrier of an ointment is known as ointment base. The type of medication, ointment stability, and therapeutic indication all influence the choice of ointment base.(Bhaskar et al., 2016)

- **Hydrophobic Ointments:** Ointments that are hydrophobic (lipophilic) typically absorb very little water and are anhydrous. Water-insoluble hydrocarbons such hard, soft, and liquid paraffin, vegetable oil, animal fats, waxes, synthetic glycerides, and polyalkylsiloxanes are common bases utilized in their production.(Bora et al., 2014)

- **Water-Emulsifying Ointments:** Large volumes of water can be absorbed by water-emulsifying ointments. They usually consist of a hydrophobic fatty base that can be made hydrophilic by adding a w/o agent, such as wool fat, wool alcohols, sorbitan esters, monoglycerides, or fatty alcohols. They might also be without emulsions that permit the addition of more watery solutions. These ointments are particularly useful when creating aqueous solutions or liquids.(Bora et al., 2014)

- **Hydrophilic Ointments:** Water is miscible with hydrophilic ointment bases. Typically, the bases consist of a blend of solid and liquid polyethylene glycols (macrogols).(Bora et al., 2014)

I.5.4.3.4. Properties of ointments:

- The ointment should be smooth and grit-free.
- It should be physically and chemically stable.
- The ointment's basis shouldn't have any medicinal properties.
- The finely divided active ingredients should be evenly distributed throughout the ointment base.(Bhaskar et al., 2016)

I.5.4.3.5. Advantages and disadvantages of ointments:

Ointments are easier to handle than large liquid dose forms. Compared to liquid dose forms, they are more chemically stable. They extend the duration of the drug's contact with the affected area. They are appropriate for individuals who have trouble taking the medications orally and parenterally, and in exchange Compared to solid dosage forms, they are thicker. It is challenging to calculate the precise amount of ointment that has to be applied to the affected area. Compared to solid dose forms, they are less stable.(Naik B, n.d.)

I.6. Phlegm and Respiratory Diseases:

I.6.1. Definition of Phlegm:

Phlegm, which comes from the Greek word for inflammation, is the term we use to describe the purulent fluid that results from airway inflammation. This includes breakdown products. comprising epithelial and inflammatory cells, including microorganisms, cell debris, co-polymers of DNA and filamentous (F-) actin, and varying levels of mucus.(Rubin, 2009)

Phlegm is also known as the abnormal substance produced by the respiratory system, and it results from an increase in natural secretions (the accumulation of mucus).(Kim, 1997)

I.6.2. Physiological and chemical composition of phlegm:

I.6.2.1. Components:

- Mucins (MUC5AC and MUC5B).
- Inflammatory cells.
- Epithelial cells.
- DNA.
- F-actin.
- Bacteria.
- Cell debris.(Rubin, 2009)

I.6.2.2. Effect of composition on viscosity and difficulty of expulsion:

There are two major types of mucins in the airways: MUC5AC and MUC5B, which determine the elastic and viscous properties of mucus. Both MUC5AC and MUC5B are secreted by goblet cells but MUC5B is also secreted by the submucosal glands. These mucins create a flexible gel due to disulphide bonds. Therefore, even a small change in the concentration or bonding of mucins could have a large effect on the viscosity of phlegm and therefore the difficulty of its clearance.(Izadifar et al., 2022)

I.6.3. Causes of phlegm formation:

- Inhalation of irritating substances.
- Acute respiratory tract infection.
- Inhalation of harmful fumes.(Kim, 1997)

I.6.4. Methods for Lowering the Viscosity of Phlegm:

I.6.4.1. Mucolytic pharmacological agents:

I.6.4.1.1. Hyperosmolar Aerosols:

Despite being referred to as mucolytic agents, hyperosmolar drugs like mannitol and hypertonic saline do not fit the real definition of a mucolytic due to their little impact on mucus viscoelasticity. According to research by Suriya and associates, dornase alfa is more effective than hypertonic saline for enhancing pulmonary function in CF patients. Since mannitol is given as a dry powder inhaler, patients may find it more enticing.(Rubin, 2009)

I.6.4.1.2. Classic Mucolytics:

Mucolytics are drugs that, make mucus less viscous. These fall into one of two categories: peptide mucolytics or traditional mucolytics. By cutting the disulfide connections that bind mucin monomers across terminal cysteine residues, classical mucolytics disassemble mucin oligomers into smaller fragments. Although N-acetyl L-cysteine (NAC) is the archetypal classic mucolytic, additional thiol compounds have also been investigated for possible mucolytic properties. There is no established clinical advantage to using them, and none have been demonstrated to be efficient as mucolytics when inhaled as aerosols.(Rubin, 2009)

I.6.4.2. Methods for clearing phlegm that are both physical and physicochemical:

the effect of treatment modalities was assessed, including reduction of cohesion and adhesion with a surfactant, lysis of the mucin polymer with a reducing agent to cleave disulfide bonds, and hydration of the mucus with saline solution. All these techniques were found useful when used independently but the combination of mucin lysis and hydration proved to be particularly effective.(Dickey, 2018)

I.7. Therapeutic applications of plant extracts:

I.7.1. Antioxidant activity:

Plant extracts are rich in polyphenols and flavonoids, both of which are highly effective phenolic compounds in neutralising free radicals. The antioxidant efficacy of these extracts is attributed to their ability to capture free radicals and reduce oxidative stress. The quantity of these active compounds is directly linked to antioxidant activity. However, studies indicate that the effects within the body (in vivo) are not identical to those observed in the laboratory (in vitro) due to factors such as absorption and the bioavailability of substances.(Pietta et al., 1998)

I.7.2. Anti-inflammatory activity:

Inflammations are among the most common health disorders, and medicinal plants have been used since ancient times for their treatment. The presence of bioactive compounds in plant extracts has been scientifically proven in many studies to have anti-inflammatory activity. The most important compounds are flavonoids, which are considered important anti-inflammatory agents, and polyphenols, terpenoids, and alkaloids. These compounds have different mechanisms of action, such as inhibition of enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX) and reduction of the production of inflammatory mediators such as TNF-α. Studies from scientific journals have confirmed that these properties make plant extracts promising alternatives in the treatment of inflammations.(S. Kumar et al., 2013)

I.7.3. Antibacterial activity:

Several studies have indicated that plant extracts possess a strong antibacterial activity mainly due to their chemical constitution, in particular the content of phenolic compounds. The efficacy of these extracts was influenced by the type of bacteria. Gram-positive bacteria were found more susceptible than Gram-negative bacteria. This is because Gram-Negative bacteria have an outer membrane which blocks the active substances from entering the cell and thus diminishes their

effects. The results indicate the importance of the chemical composition of plant extracts for antibacterial activity.(Klančnik et al., 2010)

I.7.4. Wound healing:

The use of plant extracts for the treatment of wounds is traditional because of the active compounds present in them that help accelerate the healing process. Their efficiency is due to their antibacterial and antioxidant properties, which make them useful for the protection of the wound site from infection and the reduction of tissue damage caused by free radicals. Certain plant compounds such as flavonoids and polyphenols also help in stimulating cell proliferation, increasing collagen production, and improving blood vessel formation, which are all important factors in tissue regeneration and acceleration of wound healing. Thus, plant extracts have become a major focus in the development of topical therapeutic preparations, with ointments used in the treatment of wounds and skin inflammations.(Ghosh & Gaba, 2013)

I.8. Previous studies:

I.8.1. Studies on the traditional uses of the Marrubium vulgare plant:

Numerous previous studies have shown that the plant Marrubium vulgare has been used for generations in traditional medicine in many countries around the world, with the aim of treating a range of health disorders. Among these studies is one conducted by Yabrir(2019), which stated that Marrubium vulgare was used to treat cough, asthma, colds, and various respiratory system disorders. It was also used to treat common colds as an anti-inflammatory, an expectorant, and a phlegm suppressant. Other studies have confirmed all these uses, such as the study by Ashkorfo et al(2024a), in addition to Losacco et al(2025), who mentioned other uses for this plant. It has also been used in traditional veterinary medicine to treat diarrhoea and digestive disorders in rabbits, sheep, and cows, as well as being used as an antimicrobial and an anthelmintic. Yabrir(2019) also mentioned that it was used as an antidiabetic and an anticonvulsant in cases of acute and chronic bronchitis. These widespread folk uses indicate the great therapeutic importance of this plant in traditional medicine across several regions of the world.

I.8.2. Studies on the pharmacological activities of the Marrubium vulgare plant:

With the aim of verifying the effectiveness of the plant Marrubium vulgare in treating a number of diseases, Bardai et al. (2001) conducted an experimental study on the pharmacological activity of the aqueous extract of this plant; this experiment was carried out on rats with hypertension. The results of this study showed that the plant's aqueous extract led to a reduction in blood pressure in the rats, and it also had a vasodilatory effect. In another study, and to demonstrate the effectiveness of the same plant in protecting and improving liver function, Bahar Ahmed et al. (2010) were able to isolate a new compound called marrubic acid, which showed notable efficacy in protecting the liver from toxicity caused by CCL4 when tested on Wistar rats. This compound reduced the levels of liver enzymes (SGOT, SGPT and ALP) and improved blood protein levels. Histological examination of the liver also showed a clear improvement in hepatocytes compared with untreated rats.

In another study on the essential oil of Marrubium vulgare, Yabrir (2019) demonstrated that this plant has several biological activities, including antibacterial and antifungal activities and antioxidant activity. It also showed efficacy against antibiotic-resistant bacterial strains such as Staphylococcus aureus and Klebsielle pneumoniae. In addition to its ability to inhibit the growth of some cancer cells, it also has pesticidal activity against certain insects and snails.

As for the study carried out by Ashkorfo et al. (2024)and Losacco et al. (2025) on the same plant, it confirmed that it possesses antioxidant activity by improving antioxidant markers and reducing oxidative stress, in addition to its antibacterial effect by reducing the growth of certain harmful microbes and increasing beneficial bacteria.

I.8.3. Studies on preparations containing Marrubium vulgare plant:

In a study conducted by Lazaza-Attig. (2016), the aim was to evaluate the effectiveness of the plant Marrubium vulgare in accelerating wound healing using an animal model via the skin excision method. A topical pharmaceutical preparation was developed in the form of an ointment using a base consisting of hard paraffin, liquid paraffin, and glycerine; an acetone extract of the plant was then added at a concentration of 1%. After applying the preparation to mice, a clear improvement was observed in wound contraction and the speed of the healing process, and these results were comparable with the reference ointment Madecassol.

In a study carried out by Mssillou et al. (2022), a topical ointment containing 10% of the hydroethanolic extract of the same plant was also prepared. Its effectiveness in accelerating burn healing was evaluated by applying it to experimentally induced burns in rats, where these ointments showed a notable efficacy in accelerating burn healing compared with the untreated group. It contributed to a reduction in burn area and improved the healing process over 21 days of treatment.

In the same study, Marrubium vulgare was used in a combined herbal formulation with Dittrichia viscosa, which gave a better therapeutic result, especially in the early stages.

As for the study conducted by Gonzales (2018), in which a topical gel of the plant Marrubium vulgare was developed within a Carbomer gel base at different concentrations (1%, 2%, 3%), this study showed that the gel containing the extract retained its antibacterial activity, especially against Staphylococcus aureus and Staphylococcus epidermidis.

These studies indicate the potential use of this plant in the preparation of topical formulations with various therapeutic applications.

Chapter Conclusion:

The bibliographic study has also made it possible to gather theoretical knowledge about medicinal plants and their therapeutic importance. In addition, it has highlighted the botanical and chemical characteristics of Marrubium vulgare and the active substances it contains, which may explain its many traditional and medicinal uses. Together with the different pharmaceutical forms, with an emphasis on ointments as the pharmaceutical form used in this study, it also allowed to understand the basics of phytochemical research, extraction techniques and the variables that influence them. However, the information about phlegm, the respiratory system, the use of plant extracts, and earlier research helped to provide a scientific foundation for the study's practical component, which will involve the preparation of the plant extract, the development of the pharmaceutical form, and an assessment of its characteristics and efficacy.

Practical aspect

Chapter II: Materials and Methods

Chapter Introduction:

The theoretical aspects of the studied plant, its characteristics and medicinal benefits were presented in the previous chapter. This chapter presents the practical side of the study by describing the materials, instruments and experimental techniques used to complete the task. This chapter contains the preparation of the plant material and extraction of active compounds from it, qualitative and quantitative phytochemical analysis of the extract and preparation of the selected pharmaceutical formulation. In order to evaluate the therapeutic potential of the prepared formulations, this chapter also describes the methods used to evaluate their safety using the HET-CAM test, their expectorant activity using artificial mucus, and their biological characteristics using a variety of microbiological and biological tests.

II.1. Materials used:

II.1.1. Plant material: The plant material used in this study is the dried and ground leaves of Marrubium vulgare.

II.1.2. Chemicals used:

Table II.1: Chemicals.

Material	Chemical Formula
Distilled water	H_2O
Ethanol	C_2H_5OH
Sodium carbonate	Na_2CO_3
Sodium hydroxide	$NaOH$
Ferric chloride	$FeCl_3$
Aluminium chloride	$AlCl_3$
Hydrochloric acid	HCl
Sodium nitrite	$NaNO_2$
Magnesium oxide	MgO
Sodium sulfate	Na_2SO_4
Chloroform	$CHCl_3$
Acetic anhydride	$(CH_3CO)_2O$
Sulfuric acid	H_2SO_4
Gallic acid	$C_7H_6O_5$
Rutin	$C_{27}H_{30}O_{16}$
Folin–Ciocalteu reagent	–
Mayer's reagent	–
Fehling's solution A	–
Fehling's solution B	–
White beeswax	–
Cetearyl alcohol	–
White Vaseline	–
Sodium chloride	NaCl
Glycerol	$C_3H_8O_3$
Xanthan gum	–
DPPH	$C_{18}H_{12}N_5O_6$

Ascorbic acid	$C_6H_8O_6$
Diclofenac sodium	$C_{14}H_{10}Cl_2NNaO_2$
PBS	–

II.2. Methods:

Various practical tasks for this study were carried out at the Biotechnology, Biomaterials and Condensed Matter Laboratory located within the faculty. The different experimental stages related to the preparation of plant material, extraction, phytochemical study, preparation of the pharmaceutical formulation, and the completion of various tests were carried out.

The antimicrobial activity test was also conducted at the Al-Majd Medical Analysis Laboratory.

II.2.1. Extraction procedure:

II.2.1.1. Preparation of plant material:

The Marrubium vulgare plant used in this study was collected from the Wilaya of Bordj Bou Arreridj (Algeria) and was submitted to the Head of the Preparatory Department at the Higher School of Saharan Agriculture (El Oued) for the determination of its identity and scientific classification. The green leaves of the plant were used. They were collected manually and cleaned by sieving to remove impurities and unwanted parts and dried in the shade at room temperature. This was done to preserve the active substances that it contained. The plant was dried and then ground with an electric grinder, then sifted and stored in tightly sealed glass containers away from light.

Figure II.1: The geographical location of Bordj Bou Arreridj (Algeria)

Figure II.2: Steps for preparing the plant powder.

II.2.1.2. Solvent selection:

In the extraction process, an aqueous alcoholic solvent composed of ethanol and distilled water was chosen, due to its ability to dissolve and extract a large number of active compounds present in the plant material, especially polar and semi-polar compounds.(Tourabi et al., 2023)

II.2.1.3. Extraction method:

At this stage, the maceration extraction method was chosen, using a mixture of ethanol and water as the solvent in a ratio of 70:30 (v/v)(Tourabi et al., 2023), and plant powder at a ratio of 1:20 (w/v), meaning that for every 1 gram of solute, 20 ml of solvent was used.(Isnindar et al., 2025)

To prepare 20 g of plant powder, it was soaked in 400 ml of solvent (280 ml ethanol and 120 ml water). The mixture was shaken well and kept in a dark place at room temperature (20 - 25°C) for 24 hours, with stirring every 6 hours.

II.2.1.4. Preparation of extract:

The extract obtained after 24 hours of maceration was filtered through a vacuum filtration apparatus to remove the plant material residues. The filtrate was then placed in a rotary evaporator, and the solvent evaporated to give a concentrated extract.

- The extraction yield was calculated using the following equation:

$$Yield(\%) = \left(\frac{m_e}{m_p}\right) \times 100$$

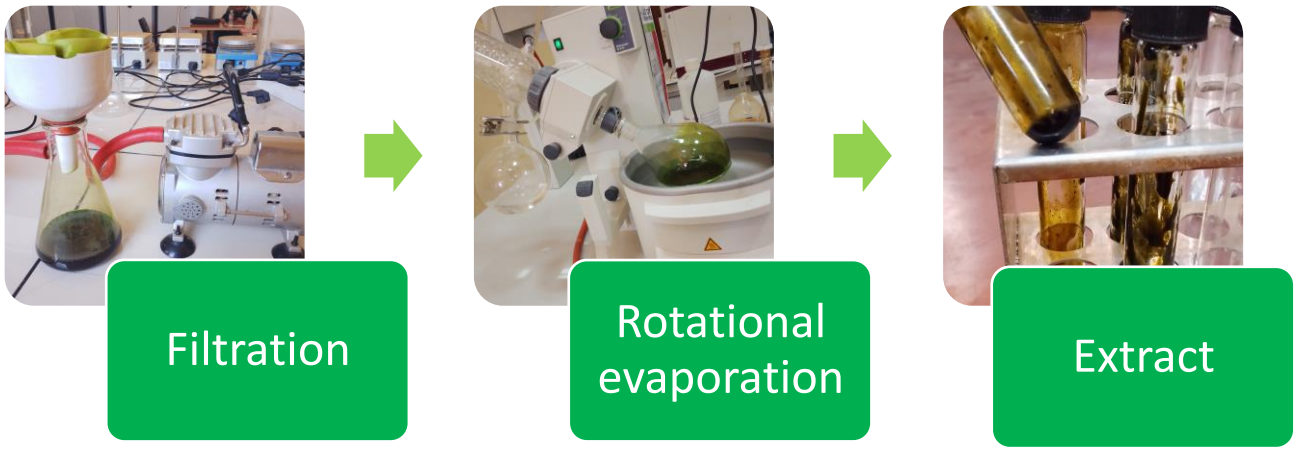


Figure II.3: Stages of Preparing the Extract.

II.2.2. Phytochemical study:

II.2.2.1. Qualitative analysis:

II.2.2.1.1. Detection of flavonoids: The alkaline reagent test was used to detect flavonoids, whereby drops of 10% sodium hydroxide solution were added to the extract. The appearance of a yellow colour that turns colourless upon the addition of 1N diluted HCl acid indicates the presence of flavonoids. (Audu et al., 2007)

II.2.2.1.2. Detection of phytosterols: Phytosterols were detected using the Salkowski test, whereby the extract was dissolved in chloroform and a few drops of concentrated sulphuric acid were added. The mixture is stirred well and left for some time. The formation of a red lower layer indicates the presence of sterols, while the appearance of a yellow colour in the lower layer indicates the presence of triterpenoids.(Ibrahim et al., 2020)

II.2.2.1.3. Detection of tannins: In a test tube, 0.5 g of the extract was mixed with 10 ml of distilled water, and the mixture was heated to boiling. After that, it was filtered. A few drops of 0.1% ferric chloride were added to the filtrate. In the presence of tannins, a greenish-brown or bluish-black colour appears.(Suliman & Alnass, 2025)

II.2.2.1.4. Detection of alkaloids: Mayer's reagent was used to detect alkaloids; in the presence of these compounds, and upon treating the filtrate with Mayer's reagent, a yellow precipitate forms.(Meharie & Tunta, 2020)

II.2.2.1.5. Detection of coumarins: 2 ml of the aqueous extract was taken, and 3 ml of 10% sodium hydroxide solution was added to it. In the presence of coumarins, a yellow colour is formed.(Velavan, 2015b)

II.2.2.1.6. Detection of carbohydrates: The cold was treated with dilute hydrochloric acid for hydrolysis, then the medium was adjusted by adding sodium hydroxide solution, and Fehling's solutions (A) and (B) were added. A red precipitate is observed in the presence of reducing sugars. (Audu et al., 2007; Obasi et al., 2010)

II.2.2.2. Quantitative analysis:

II.2.2.2.1. Total phenolic content (TPC): 0.5 ml of Folin-Ciocalteu reagent, diluted tenfold, was taken with 100 µl of the diluted extract, and 2 ml of 20% sodium nitrate solution was added. The mixture was incubated for 30 minutes in the dark, then the absorbance was measured at a wavelength of 760 nm against a blank sample. Gallic acid was used as a reference standard, and the results were expressed as gallic acid equivalents per gram of extract. (Singleton et al., 1999)

II.2.2.2.2. Total flavonoid content (TFC): Mix 1 ml of the extract with 2 ml of pure water and 0.25 ml of sodium nitrate solution at a concentration of 5%. Leave the mixture for 6 minutes to react. Afterwards, add 0.15 ml of Aluminium chloride solution at 10% and mix well, then also leave for 6 minutes. Next, add 2 ml of sodium hydroxide solution at a concentration of 4% and leave for 15 minutes. The absorbance of the mixture is measured at a wavelength of 510 nm against a blank sample. Rutin was used as a standard compound, and the results were expressed in mg of rutin equivalents per gram of extract.(Zou et al., 2004)

II.2.3. Preparation of pharmaceutical formulation:

II.2.3.1. Preparation of the ointment base:

In a beaker, 8% it in pe of cetearyl alcohol, 12% of beeswax, and 80% of white petrolatum were placed. These ingredients were put in a water bath to melt at a temperature of 70°C with stirring to ensure their homogeneity.

II.2.3.2. Preparation of the ointment:

25g of ointment was prepared at several different concentrations by gradually adding the ointment base, after cooling, to the extract with thorough stirring in order to achieve a homogeneous distribution. Finally, the ointment was filled into sterile containers.

The mass of extract used for each concentration was determined based on the following equation: $m_e = \frac{c \times 25}{100}$

Where: m_e: mass of extract (g), c: concentration (%).

All concentrations and masses used are summarised in the following table:

Table II.2: Concentrations and quantities used in the ointment.

concentration (%)	Extract mass (g)	Base mass (g)
0.5	0.125	24.875
1	0.25	24.75
3	0.75	24.25
5	1.25	23.75
8	2	23

II.3. Evaluation methods:

II.3.1. Evaluation of expectorant efficacy:

❖ **Preparation of artificial mucus:**

With the aim of evaluating the effectiveness of the expectorant ointment, we prepared 250 mL of artificial mucus by adding 0.9% (w/v) of NaCl to 85% (v/v) of water in a beaker and placing it on a hot plate magnetic stirrer at a temperature of 70°C. Gradually, we added 10% (v/v) of glycerol. Then, 0.5% (w/v) of xanthan gum was added very slowly while continuing to stir to avoid clumping of the mixture. After half an hour of stirring, the mixture acquired a homogeneous gel-like consistency and was then left to cool gradually to be used afterwards in testing the effectiveness of the ointment according to the following steps:

- Six beakers were prepared, five of which were allocated for the ointments prepared at different concentrations, while the sixth beaker was allocated for the ointment base as a control.
- 5 g of artificial mucus was placed in each beaker.
- 0.5 g of each ointment was added to its designated beaker.
- The ointment was mixed well with the mucus and left for an hour.
- The samples were put into tubes, which were placed in an inclined position (30°).
- The distance travelled was measured using a graduated ruler, then the transit time of each sample was recorded.
- The mucus transport velocity of the samples was calculated using the following equation: $\boldsymbol{V = d/t.}$

Where:

V: Mucus transport velocity (m/s).

d: Distance travelled (m).

t: Time taken (s).

II.3.2. HET -CAM test:

The HAT-COM test is one of the methods used to assess the irritant potential of topical preparations on the chorioallantoic membranes of fertilised chicken eggs. The test is based on the observation of vascular changes (haemorrhage, coagulation and hyperaemia) occurring after the application of the tested preparations to the chorioallantoic membrane.

II.3.2.1. Materials:

- Fertilised chicken eggs on the ninth day of incubation.
- Sterilised scissors.
- Tweezers.
- Stopwatch.
- Water bath set at 37°C.
- Sodium chloride solution (0.9%).

- Sodium hydroxide solution.
- Cellophane paper.
- Prepared ointments and ointment base.

II.3.2.2. Experimental work:

- Various stages of the test were conducted under suitable lighting. The eggs were placed vertically with the air gap facing upwards, then a hole was carefully made at this area to avoid damaging the chorioallantoic membrane (CAM). After that, part of the shell and the inner membrane were removed using tweezers in order to expose the chorioallantoic membrane.

The inner membrane was moistened using a preheated NaCl solution at 37°C, then the solution was removed by tilting the egg. Any egg that showed bleeding or damage at the level of the chorioallantoic membrane was excluded.

Figure II.4: Egg sorting process.

- Two eggs were used as controls, negative control with NaCl solution and positive control with NaOH solution.

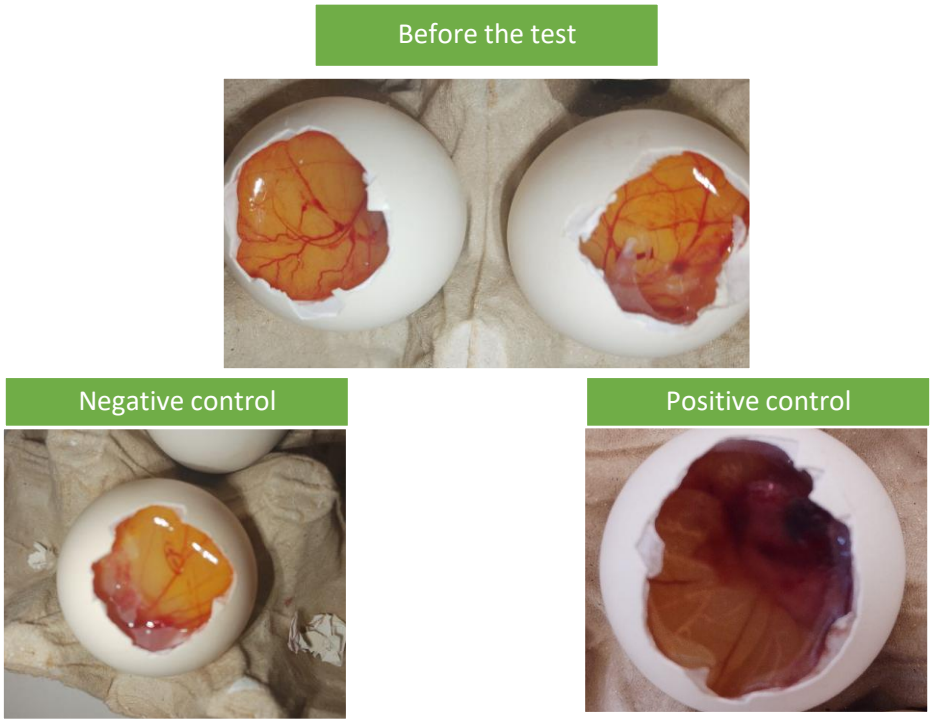


Figure II.5: control.

- Concerning the ointment, each ointment was deposited on cellophane paper and then carefully applied to the chorioallantoic membrane ensuring they were in direct contact for 5 minutes.

Figure II.6: Applying the ointment.

- The stop clock was started immediately after removal of the samples and vascular irritation sign appearance was observed, the time of appearance of each phenomenon was recorded.

II.3.2.3. Reading procedures:

The product rating is based on observations that must be made with the unaided eye. When each phenomenon first appears, the time is noted.

❖ **Hyperaemia:**

Observed phenomenon: visible capillaries dilate and turn redder, while capillaries that were invisible before to the product's inclusion become visible. This occurrence may also affect larger-diameter vessels.

❖ **Haemorrhage:**

The phenomena of blood escaping from veins and/or capillaries have been seen. It can manifest in a variety of ways, most notably as a "cauliflower," a sheet, a diffuse veil, or speckling (blood escaping in isolated areas at distinct places of the membrane). It should be mentioned that, although bleeding may be temporary, it still needs to be considered. Masked hyperaemia must be taken into account if significant bleeding is seen within the first 30 seconds.

❖ **Coagulation (thrombosis and/or opacity):**

a-Opacity:

The phenomena that have been seen are the emergence of either direct opacity or an opalescent veil that may later turn into opacity on all or part of the membrane. Make sure the phenomena have nothing to do with the product's physico-chemical behaviour in an aqueous environment, such as the creation of a colloid or a precipitate.

b-Thrombosis:

characterised by a segmented appearance (alternating constrictions and more or less dark, turgid patches) due to the disruption of blood flow in the vessels. It should be mentioned that observations should not account for changes that take place at the capillary level.

II.4. Microbiological study:

II.4.1. Antimicrobial test:

The antimicrobial and antifungal activity of the five prepared ointments (0.5%, 1%, 3%, 5%, and 8%) in addition to the base was evaluated by the agar well diffusion method. The efficacy of the ointments was evaluated against two reference strains of microorganisms, Escherichia coli ATCC 25922 (Gram-negative bacteria) and Staphylococcus aureus ATCC 25932 (reference fungal strain). This enables us to assess the comparative evaluation of the dose-dependent effect of the active substance introduced into the formulation and to estimate the minimum effective concentration based on the observed zones of inhibition in a semi-solid pharmaceutical system. The test was performed in the following steps:

- Culture Media: Sabouraud Dextrose Agar (SDA) was used for antifungal testing against Staphylococcus aureus, and Mueller-Hinton Agar (MHA) was used as the standard medium for antibacterial testing against E. coli. The media were prepared according to manufacturer instructions, sterilized by autoclaving at 121°C for 15 min, and aseptically

transferred onto sterile Petri dishes of 90 mm diameter to a uniform thickness of approximately 4 mm.

Figure II.7: Preparation of culture media.

- Preparation of inocula: Fresh cultures of E. coli, aged 18 to 24 hours in Mueller-Hinton broth (MHB), and of S. aureus, obtained after 24 to 48 hours in Sabouraud Dextrose broth (SDB), were individually adjusted to a turbidity equivalent to the 0.5 McFarland standard in a sterile physiological saline solution (NaCl 0.9%), corresponding to a density of 1.5×10^8 CFU/mL for E. coli and $1–5 \times 10^6$ CFU/mL for S. aureus.

Figure II.8: Preparation of inocula.

- Plating of the Petri dishes: Each Petri dish was uniformly inoculated using a sterile swab by three successive streaks in perpendicular directions in order to obtain a homogeneous and reproducible microbial lawn over the entire agar surface. The dishes were then left to dry under aseptic conditions for 3 to 5 minutes before making the wells.

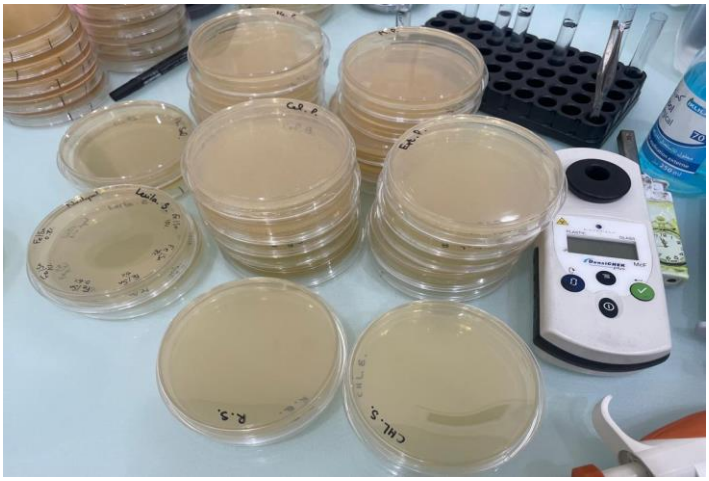


Figure II.9: Plating of the Petri dishes.

• Preparation of wells and application of ointment: Wells of 6 mm diameter were punched aseptically with a sterile punch into solidified agar, and the agar plugs were removed carefully without disturbing the surrounding microbial lawn. Each plate was arranged according to a pre-defined spatial arrangement to accommodate the six wells corresponding to the six formulations: base, 0.5%, 1%, 3%, 5% and 8%. A volume of 50 µL of each homogenised ointment formulation was introduced into the corresponding well using a calibrated micropipette. The base ointment, devoid of active ingredient, was included as a negative control to exclude any inhibitory effect attributable to the excipients in the formulation, ensuring that the observed zones of inhibition are exclusively due to the incorporated active ingredient.

Figure II.10: application of ointment.

• Incubation: The plates inoculated with E. coli were incubated at 37 °C for 24 hours, whilst the plates containing S. aureus were incubated at 35–37 °C for 48 hours, in accordance with CLSI recommendations for antimicrobial and antifungal susceptibility testing.

Figure II.11: Incubation.

- At the end of the incubation, the antimicrobial and antifungal activity was determined by measuring the total diameter of the zones of inhibition (mm) surrounding each well, including the diameter of the well itself, using a digital calliper. Zones of inhibition with a total diameter exceeding 6 mm were considered indicative of genuine inhibitory activity, while values equal to or below this threshold reflected the absence of active diffusion of the active principle (Bonev et al., 2008; Kiehlbauch et al., 2000).

II.4.2. Anti-inflammatory test:

The anti-inflammatory activity of the ointments was evaluated by the modified egg-albumin denaturation method of A.S et al. (2014). The test was performed following the subsequent procedures:

- Samples were prepared with a 2.5 ml reaction mixture containing 0.1 ml of fresh chicken egg albumin, 1.4 ml of phosphate-buffered saline (PBS, pH 6.4) and 1 ml from the stock solution in different doses (The stock solution for the ointments was prepared in advance by dissolving 4 mg of the ointment in 4 ml of distilled water).
- The mixtures were incubated at 37 °C for 15 min and then heated at 70 °C for 5 min to denature the proteins.
- The samples were cooled and the absorbance was measured at 660 nm using a UV-visible spectrophotometer. A blank sample (PBS + egg albumin without ointment) and an untreated control were used for comparison. Sodium diclofenac was used as the reference drug.
- The formula given by Akinyemi et al (2021) was used to calculate the percentage inhibition of protein denaturation (PI), which indicates anti-inflammatory activity. This formula accounts for baseline absorbance and provides an indication of the ointment's ability to prevent protein denaturation.

$$PI=(1-((As-Ab)/(Ac-Ab))) \times 100$$

where:

- ✓ As = absorbance of the sample.
- ✓ Ac = absorbance of the untreated control.
- ✓ Ab = absorbance of the blank.

Table II.3: Preparation of ointment sample dilutions for the egg albumin denaturation test.

Stock solution (µl)	100	200	300	500	700	1000
Distilled water (µl)	900	800	700	500	300	0

II.4.3. Antioxidant test:

The antioxidant activity of the ointments was determined by the DPPH free radical scavenging method using the methods described by Baliyan et al. (2022).

- For the preparation of ointment solutions, 4 mg of each ointment were dissolved in 4 ml of methanol. Then, 1 mL of the ointment solution was added to 1 ml of the methanol solution containing 0.1 mM 1,1-diphenyl-2-picryl-hydrazyl (DPPH•). The samples were kept in the dark at room temperature for 15 minutes.
- The absorbance was measured at 517 nm using UV-Vis spectrophotometer.
- Ascorbic acid in concentrations from 0.005 to 0.5 mg/mL was used for comparison as a reference.
- The percentage of inhibition of free radicals (%I) was determined by the following formula:

$$\%I = Ac - AsAc \times 100$$

where:

Ac: absorbance of the control.

As: absorbance of the sample.

Determination of IC50 value: The IC50 value was determined by linear regression by plotting the percentage of antioxidant activity against the ointment concentration, and this value represents the concentration required to inhibit 50% of DPPH• free radical.

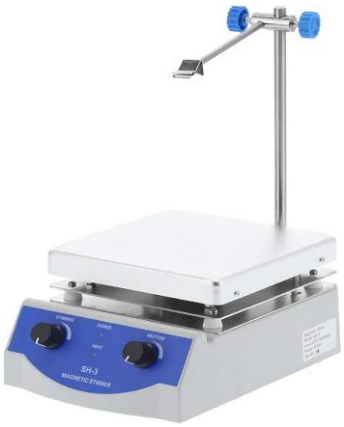
Table II.4: Preparation of ointment sample dilutions for DPPH testing.

Ointment solutions (µl)	100	250	500	1000
Methanol (µl)	900	750	500	0

II.4. Equipment Used:

Figures II.12: Analytical balance.

Figures II.13: Vacuum filtration apparatus.



Figures II.14: Hot plate magnetic stirrer.

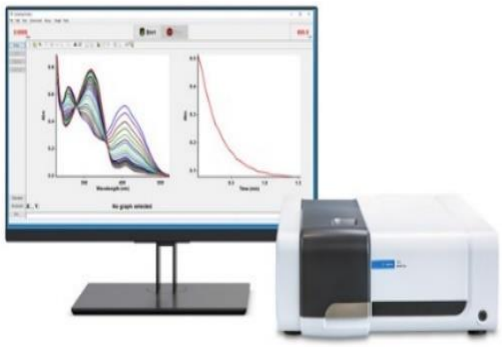


Figure II.15: UV-Visible spectrophotometer.

Figures II.16: Rotary evaporator.

Chapter Conclusion:

In this chapter, an overview of the experimental methodology used throughout the study was presented, clarifying the various steps that were adopted from the preparation of the plant material and the obtaining of the extract, passing through the development of the pharmaceutical formulation and the conduct of various tests to assess its properties and effectiveness. The procedures and techniques described in this chapter constitute a practical basis to guarantee the reliability of the obtained results. The techniques and procedures described in this chapter also constitute a fundamental reference to understand and analyse the results that will be presented and discussed in the chapter dedicated to results and discussion.

Chapter III: Results and Discussion

Chapter Introduction:

After the presentation of the experimental technique and the approaches used during the practical phase, this chapter is dedicated to presenting and discussing the results obtained during the many stages of the study. It also includes the results of phytochemical analysis and extraction, production of ointments, evaluation of their biological safety and expectorant efficacy, and results of other biological tests. To evaluate the efficacy of the generated formulations and the degree of accomplishment of the desired goal of the research; The discussion of these results attempts to interpret the data gathered and relate them to the chemical and biological properties of the plant under study.

III.1. Presentation of results:

III.1.1. Plant extract:

Table III.1: Results of the plant extract.

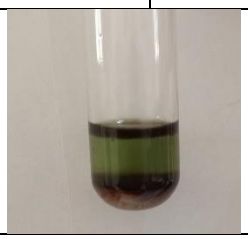
Feature	Extract colour	Consistency / Texture	The scent	m_p(g)	m_e (g)	Yield (%)
Description /Value	Dark green	Viscous with an oily appearance.	Strong characteristic odor.	20	5.362	26.81

III.1.2. Phytochemical screening:

III.1.2.1. Qualitative analysis:

This study revealed the presence of all the studied compounds in varying degrees, confirming the existence of tannins, alkaloids, coumarins, with a strong presence of flavonoids, phytosterols, and carbohydrates.

Table III.2: Phytochemical analysis of the extract.

Test	Flavonoids	Phytosterols		Tannins	Alkaloids	Coumarins	Carbohydrates
		triterpenoids	Sterols				
Results	++	++	++	+	+	+	++
Photos							

+: Present, ++: Strongly present.

III.1.2.2. Quantitative analysis:

III.1.2.2.1. Total phenolic content (TPC):

III.1.2.2.1.1. Calibration curve:

Standard solutions of gallic acid were prepared at different concentrations for the determination of total phenolic content, with the aim of assessing the linearity of the regression line for the calibration curve. This was given by the following equation: $y = 3.14x + 0.046$ with a correlation coefficient: $R^2 = 0.990$.

Where y refers to the absorbance, and x to the concentration of gallic acid.

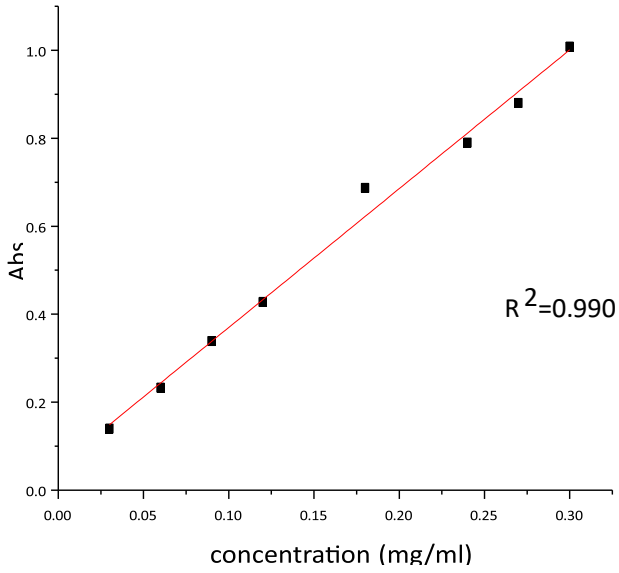


Figure III.1: Calibration curve for gallic acid.

After preparing the calibration curve for gallic acid, the absorbance of the plant extract was measured at different concentrations using the same protocol. This allowed for the representation of the relationship between absorbance and extract concentration, as shown in the following figure:

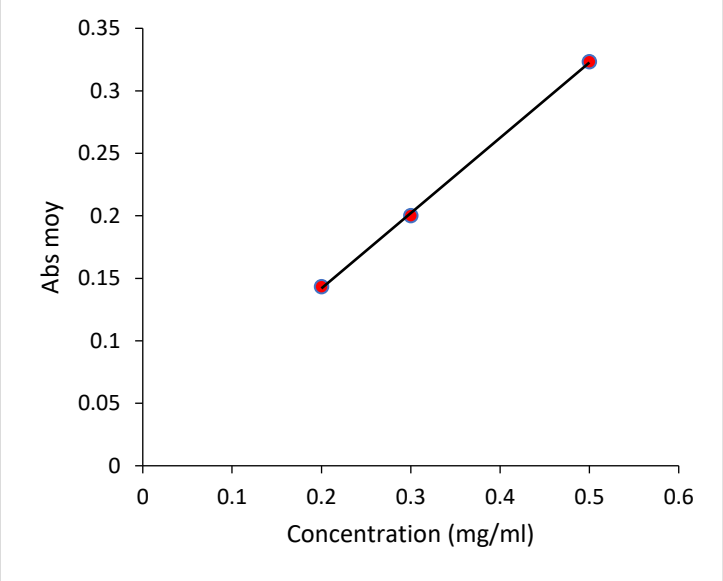


Figure III.2: Change in absorbance as a function of extract concentration (phenolics).

III.1.2.2.1.2. Determination of total phenolic content:

- The average absorbance was calculated according to the following equation:

$$y = \frac{\sum \text{absorbance}}{n}$$

- The concentration of phenols for each sample was calculated according to the following equation**:** $x = \frac{y-0.046}{3.14}$ which is derived from the calibration curve equation of gallic acid.
- The phenolic content for each sample was calculated according to the following equation: $content = \frac{x \times 1000}{c}$

Whereas: y: absorbance, x: concentration of phenols(mg/mL), c: extract concentration(mg/mL), n: number of measurements.

All the results have been summarised in the following table:

Table III.3: Calculation of the Total Phenolic Content.

Y	x(mg/mL)	Content (mg gallic acid / g extract)
0.143333	0.030998	154.9894
0.2	0.049045	163.482
0.323333	0.088323	176.6454

Finally, the total phenolic content was calculated based on the average, according to the following equation: $TPC = \frac{\sum \text{Content}}{3}$

The total phenolic content was found to be 165.03893 mg gallic acid / g extract.

III.1.2.2.2. Total flavonoid content (TFC):

III.1.2.2.2.1. Calibration curve:

Standard solutions of rutin with different concentrations were prepared for the total flavonoid content, in order to assess the linearity of the calibration curve, which yielded the following equation: Y=1.92X-0.00567 and a correlation coefficient of $R^2 = 0.997$.

Where y refers to the absorbance, and x to the concentration of rutin.

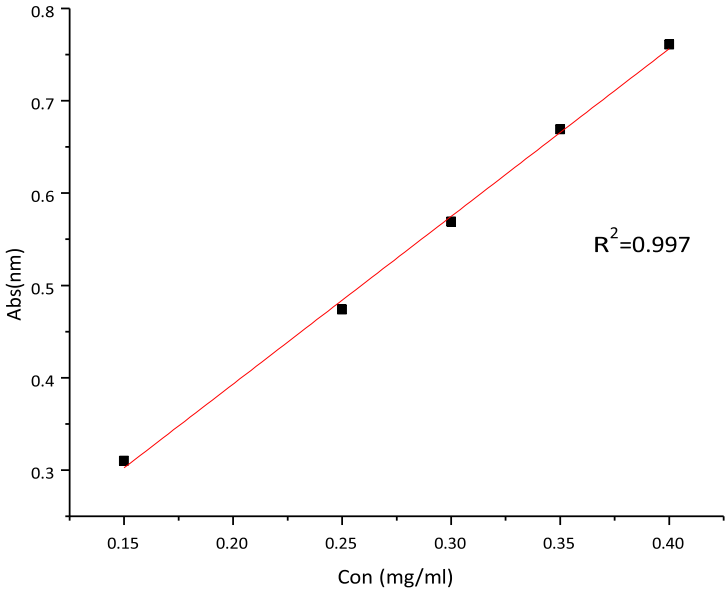


Figure III.3: Calibration curve for rutin.

After preparing the calibration curve for rutin, the absorbance of the plant extract at various concentrations was measured using the same protocol. This allowed for the representation of the relationship between absorbance and extract concentration as shown in the following figure:

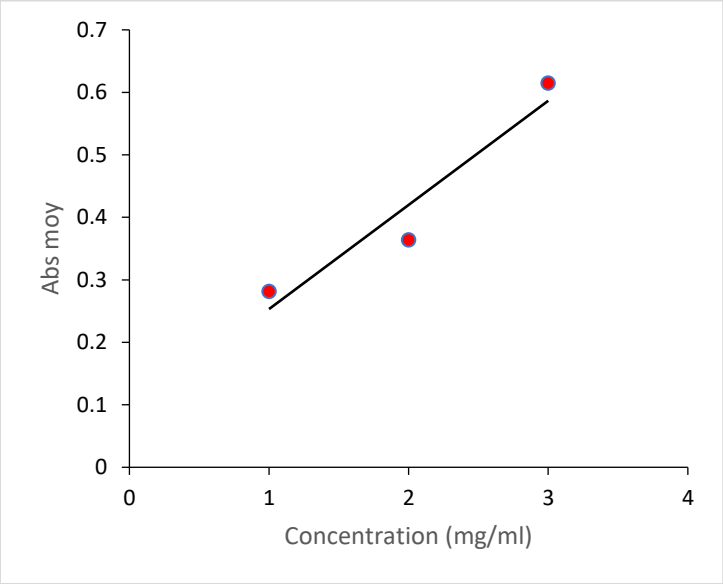


Figure III.4: Change in absorbance as a function of extract concentration (flavonoids).

III.1.2.2.2.2. Determination of total flavonoid content:

- The average absorbance was calculated according to the following equation: $y = \frac{\sum \text{absorbance}}{n}$
- The concentration of flavonoids for each sample was calculated according to the following equation**:** $x = \frac{y+0.00567}{1.92}$ which is derived from the calibration curve equation of rutin.
- The flavonoid content for each sample was calculated according to the following equation: $content = \frac{x \times 1000}{c}$

Whereas: y: absorbance, x: concentration of flavonoids (mg/mL), c: extract concentration(mg/mL), n: number of measurements.

All the results have been summarised in the following table:

Table III.4: Calculation of the Total flavonoid Content.

Y	x(mg/mL)	Content (mg rutin / g extract)
0.281333	0.149481	149.4809
0.363667	0.192363	96.18142
0.614667	0.323092	107.6973

Finally, the total flavonoids content was calculated based on the average, according to the following equation: $TFC = \frac{\sum \text{Content}}{3}$

The total phenolic content was found to be 117.78654 mg rutin / g extract.

III.2. Discussion:

III.2.1 Discussion of the result of the plant extract:

This type of hydroalcoholic solvent used in the extraction process, known for its ability to extract a large number of active compounds and both volatile and non-volatile molecules, has made the extraction process effective. This is evident from the calculated extraction yield, as well as the strong and distinctive aroma and the dark green colour. Additionally, the nature and concentration of the extracted compounds are reflected in the viscous and oily texture of the extract.

III.2.2. Discussion of the qualitative analysis results:

The presence of these compounds indicates that the plant extract is rich in active substances. This suggests that it may possess various biological activities, such as antioxidant and antimicrobial properties, which could explain its traditional use.

III.2.3. Discussion of the quantitative analysis results:

The results obtained showed richness of the extract in phenolic compounds with TPC value of 165.03 mg gallic acid/g extract. On the other hand, the content of flavonoids was significant (TFC) with 117.79 mg rutin/g extract, which indicates a considerable proportion of these compounds. These results are also related to the nature of the hydroalcoholic solvent used (ethanol/water), which is known to have a high capacity to extract polar and semi-polar compounds. Also, we can relate this significant rise in TPC and TFC to the possible biological activities of this plant, especially its antioxidant activity, as a result of the ability of these compounds to activate free radicals and reduce oxidation.

III.3. Evaluating the pharmaceutical formulation:

III.3.1. Results of pharmaceutical formulation preparation:

After combining the plant extract with the ointment base, a homogeneous ointment with a semi-solid consistency was obtained. The ointments also showed differences in colour depending on the varying concentrations of the plant extract in each ointment.

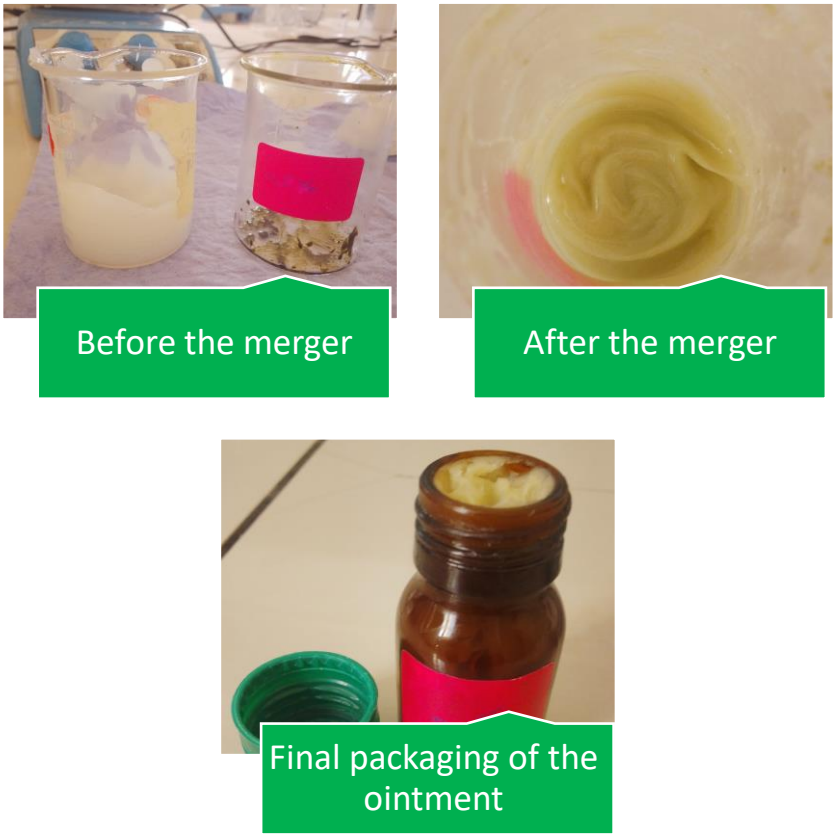


Figure III.5: Stages of ointment preparation.

Table III.5: Sensory characteristics of ointments.

Specifications	Ointment	Standards
Appearance	Base	Semi-solid and homogeneous
	0.5	Semi-solid and homogeneous
	1	Semi-solid and homogeneous
	3	Semi-solid and homogeneous
	5	Semi-solid and homogeneous
	8	Semi-solid and homogeneous
Colour	Base	White
	0.5	Light yellowish green
	1	Light green
	3	Green
	5	Dark green
	8	Very dark green
odour	Base	The distinctive smell of the ointment base
	0.5	A light herbal scent
	1	Relatively mild vegetal scent
	3	Moderate herbal aroma

	5	Strong herbal scent
	8	A very strong vegetal smell

❖ **Stability of the prepared ointment:**

The prepared ointments were stable during the storage period. They had semi-solid consistency and uniformity without apparent separation of components or significant changes of external appearance for one month after preparation.

III.3.2. Results of the Evaluation of Expectorant Efficacy:

By assessing the transit speed of artificial mucus treated with various ointment concentrations and comparing it to the ointment base used as a control, the expectorant efficacy of the produced ointments was assessed. The outcomes are displayed below.

Table III.6: Results of mucus transport velocity of the ointment formulations.

Ointment	**Base**	**0.5**	**1**	**3**	**5**	**8**
Distance (m)	0.05	0.05	0.05	0.05	0.05	0.05
Time (s)	371	277	156	150	145	131
Flow (m/s)	0.00013	0.00018	0.00032	0.00033	0.00034	0.00038

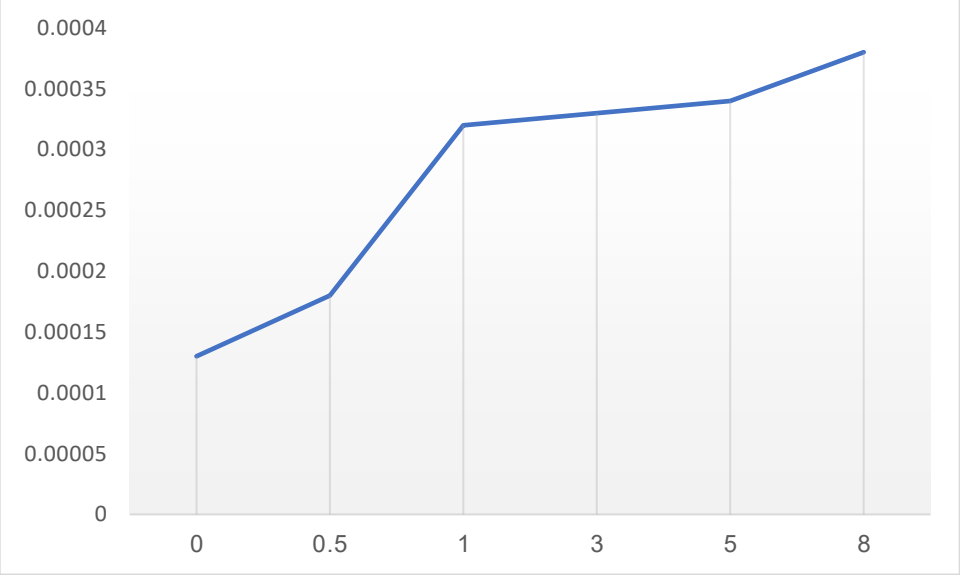


Figure III.6: The effect of ointment concentration on mucus transport velocity.

These findings demonstrated that the extract-containing formulations (ointments) had higher mucus transport speed values than the control, suggesting enhanced mucus movement and maybe an

expectorant impact. The extract's capacity to lessen the mucus's density or viscosity, which makes it easier for it to move, is responsible for the improvement in transport speed.

III.3.3. HET-CAM Test Results:

III.3.3.1. Analysis of results:

The data are evaluated using the Irritation Index (IS) based on the time of onset of vascular changes at the level of the chorioallantoic membrane, represented by hyperaemia, haemorrhage and coagulation. The IS value was calculated according to the following formula:

$$IS = \left(\frac{301 - H}{300} \times 5\right) + \left(\frac{301 - L}{300} \times 7\right) + \left(\frac{301 - C}{300} \times 9\right)$$

where:

H = Haemorrhage.

L = Hyperaemia.

C = Coagulation**.**

Table III.7: Standard and Irritability Index.

IS	**Classification**
0 – 0.9	Non-irritant.
1 – 4.9	Mild irritant.
5 – 8.9	Moderate irritant.
≥ 9	Severe irritant.

❖ **Phenomena and Classification Tables:**

Table III.8: HET-CAM results of control.

Control	Observation	Classification
Negative control	No vascular changes were observed.	Non-irritant.
Positive control	Immediate clotting observed after application	Severe irritant.

Table III.9: Phenomena and Classification of base Ointment.

Ointment (Base)	
Hyperaemia	/
Haemorrhage	/
Coagulation	/
IS	0
Classification	Non-irritant.

Table III.10: Phenomena and Classification of Ointment 0.5%

Ointment (0.5%)	
Hyperaemia	/
Haemorrhage	/
Coagulation	/
IS	0
Classification	Non-irritant.

Table III.11: Phenomena and Classification of Ointment 1%

Ointment (1%)	
Hyperaemia	300 seconds
Haemorrhage	/
Coagulation	/
IS	0.023
Classification	Non-irritant.

Table III.12: Phenomena and Classification of Ointment 3%

Ointment (3%)	
Hyperaemia	267 seconds
Haemorrhage	/
Coagulation	/
IS	0.79
Classification	Non-irritant.

Table III.13: Phenomena and Classification of Ointment 5%

Ointment (5%)	
Hyperaemia	80 seconds
Haemorrhage	280 seconds
Coagulation	/
IS	5.50
Classification	Moderate irritant.

Table III.14: Phenomena and Classification of Ointment 8%

Ointment (8%)	
Hyperaemia	17 seconds
Haemorrhage	80 seconds
Coagulation	/
IS	10.31
Classification	Severe irritant.

III.3.3.2. Interpretation and Discussion:

The negative control (NaCl) did not reveal any vascular changes at the level of the chorioallantoic membrane during the whole test period, indicating that it does not have any irritant effect.

On the other hand, the positive control (NaOH) caused immediate coagulation, indicating that it is a strong irritant, which confirms the HET-CAM test efficiency and the correct application of the protocol.

The results of the ointment test showed that they cause varying degrees of irritation, depending on the concentration used. The ointment base and the 0.5% concentration did not show any vascular changes, and the IS value was 0 and they were classified as non-irritant preparations. The 1% and 3% concentrations also showed very low IS values (0.023 and 0.79) with a delay in the appearance of vascular changes, indicating no significant irritant effect at these concentrations. However, higher values of the irritation index were obtained when the concentration was increased to 5% and 8%, 5.50 and 10.31, respectively. This was due to the rapid development of haemorrhage and hyperaemia, thus indicating an increased irritant potential.

These results confirm the relation of the concentration of the active substance with the possible irritant of the ointments. Increases in concentrations were associated with higher IS values and a faster onset of changes.

Moreover, the lower concentrations (0.5%, 1%, and 3%) showed mild to no irritation index values, indicating good compatibility with the chorioallantoic membrane and, consequently, local safety. Higher concentrations (5% and 8%) resulted in increased vascular responses, suggesting that the higher concentration of the active substance can influence the tolerability of the preparation and increase its irritant potential.

III.4. Therapeutic applications of the extract:

III.4.1. Microbiological test results:

III.4.1.1. Antimicrobial activity:

The comparison of diameters among the six formulations, with increasing concentration, allowed for the assessment of the dose-response relationship of the active substance and the determination of the minimum concentration at which a noticeable antimicrobial activity appears in this pharmaceutical system.

Table III.15: Results of Antimicrobial Test.

	Microbial inhibition (mm)						Negative control
Ointments	B	0.5%	1%	3%	5%	8%	
Escherichia coli ATCC 25922	**NI**	**NI**	**NI**	**10**	**9**	**11**	**NI**
Staphylococcus aureus ATCC 25932	**NI**	**NI**	**NI**	**7**	**13**	**14**	**NI**

NI= No Inhibition

The results of the antimicrobial activity test indicated that no significant antimicrobial activity was found at concentrations of 0.5% and 1% for the tested strains, and no inhibition zones were found in the negative control. Inhibitory zones, on the other hand, started to form at 3% concentration, and their diameters increased gradually at 5% and 8% concentrations. The highest inhibitory zones were recorded at 8% for Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 25932, indicating an increasing antibacterial activity with increasing extract concentration in the ointment formulation.

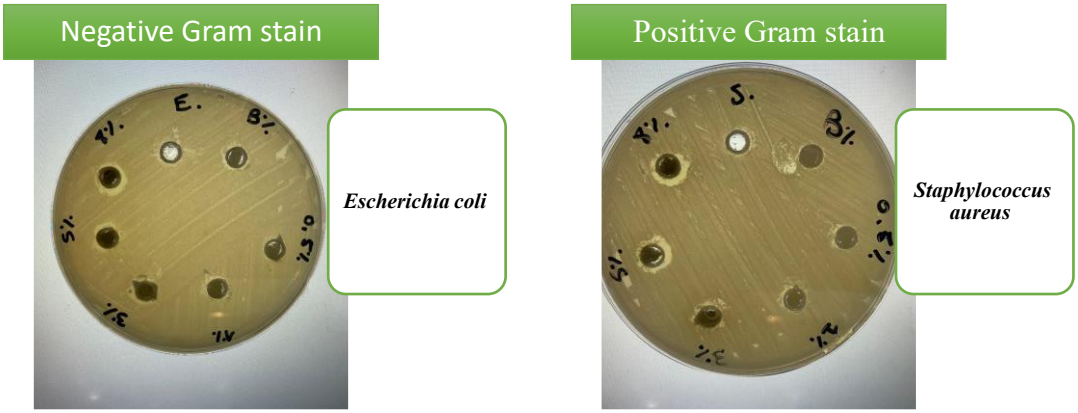


Figure III.7: Antimicrobial activity.

III.4.1.2. Anti-inflammatory activity:

The anti-inflammatory properties of the produced ointments were evaluated by the egg-albumin denaturation assay. Table X displays the determined IC50 values for the reference ingredient (diclofenac sodium) and the different formulations.

Table III.16: IC50 values for the anti-inflammatory activity of the ointments.

Sample	IC50 (µg/µl)
Reference (sodium diclofenac)	263.5 ± 6.6
Ointment (0.5%)	519 ± 0.006
Ointment (1%)	204.39 ± 0.5
Ointment (3%)	990.7 ± 0.12
Ointment (5%)	817.7 ± 0.2
Ointment (8%)	445.08 ± 0.4

The egg albumin denaturation test showed the different levels of anti-inflammatory activity of the prepared ointments as indicated by the obtained IC50 values. The reference chemical sodium diclofenac gave a value of 263.5±6.6 µg/µl. The ointment at a concentration of 1% showed the strongest anti-inflammatory action amongst the investigated ointments and recorded the lowest value (204.39±0.2 µg/µl), Interestingly, it also demonstrated a higher anti-inflammatory effect compared to the reference drug, indicating superior inhibitory under the experimental conditions. Other concentrations also showed different levels of anti-inflammatory activity; the 8% concentration of ointment showed a value of 445.08±0.006 µg/µl, followed by the 0.5% concentration of ointment (519±0.4 µg/µl), the 5% concentration of ointment (817.7±0.5 µg/µl) and the 3% concentration of ointment (990.7±0.12 µg/µl).

III.4.1.3. Antioxidant activity:

The DPPH free radical scavenging assay was used to evaluate the produced ointments' antioxidant properties. Table III.7 displays the IC50 values for the reference ingredient (ascorbic acid) and the different formulations.

Table III.17: IC50 values for the antioxidant activity of the ointments.

Sample	IC50 (µg/µl)
Reference (ascorbic acid)	18 ± 0.3
Ointment (0.5%)	30 ± 0.5
Ointment (1%)	28 ± 0.3
Ointment (3%)	22 ± 0.2
Ointment (5%)	23 ± 0.3
Ointment (8%)	20 ± 0.05

Based on the DPPH test results, the obtained IC50 proved that all of the produced ointments had antioxidant activity with different levels of activity. The best antioxidant activity was the ointment with a concentration of 8% which had the lowest IC50 value of 20±0.01 µg/µl. Followed by the ointment with a concentration of 3% (22±0.2 µg/µl) and a concentration of 5% (23±0.3 µg/µl). The lower concentrations of 1% and 0.5% showed less activity with values of 28±0.3 µg/µl and 30±0.5 µg/µl, respectively. Ascorbic acid used as a reference substance also showed the highest antioxidant activity with an IC50 value of 18±0.3 µg/µl.

III.4.2. Discussion of the microbiological results:

III.4.2.1. Antimicrobial:

The antibacterial activity of the prepared ointments was concentration-dependent, as the widths of the inhibition zones increased progressively with an increase in extract concentration from 3% to 8%. These results show a dose-effect relationship, where the biological efficacy of the active ingredient is increased with the increase in its concentration. Also, the absence of inhibition zones in the negative control confirms that the activity measured is due only to the added active component and that the base used in the ointment preparation has no inhibitory effect.

However, the inhibition zone diameters against the fungal strain indicated that Staphylococcus aureus was more sensitive than Escherichia coli. In addition to the effect of the diffusion properties of the gel medium on the efficacy of the ointment, this could indicate a variation in the response of microorganisms to the active substances of the plant extract.

III.4.2.2. Anti-inflammatory:

The anti-inflammatory action of the produced ointments depended on the results of the egg-albumin denaturation test, which assesses the effectiveness of inhibition by the ability of the active compounds to inhibit protein denaturation. One of the mechanisms associated with anti-inflammatory activity is the suppression of protein denaturation as protein denaturation may induce inflammatory response. Lower IC50 values indicate higher ability to show anti-inflammatory activity, which is represented by higher efficacy to prevent protein denaturation. Differences in activity between the various concentrations can be attributed to fluctuations in the amount of active chemicals released from the ointment and their availability in the reaction medium.

III.4.2.3. Antioxidant:

The DPPH test is based on determining the ability of active compounds to inhibit free radicals. It is the result of their ability to donate electrons or hydrogen atoms to the DPPH free radical, thus showing antioxidant efficiency. The results of this test on the prepared ointments indicated that they have antioxidant activity with different degrees. The lower the IC50, the higher the efficacy of free radical inhibition. Thus, the active compounds present in the ointments, like phenols and flavonoids, are known for their ability to neutralise free radicals and reduce oxidative stress and have conferred antioxidant properties to the ointment. The IC50 values vary from one ointment to another due to the variation in the concentrations of these compounds in each ointment.

Chapter Conclusion:

The presentation and discussion of the results permitted the evaluation of the different aspects of the plant extract and the formulations prepared and the interpretation of the data obtained and their correlation with the chemical composition of the plant and the possible biological properties. Moreover, the results were used to show the effectiveness of the generated formulations and to

determine their characteristics based on the tests performed.
The results obtained generally supported the aim of the study and showed that the plant extract under investigation could be valued and used to prepare formulations of therapeutic interest. But further, more rigorous studies are needed to confirm the findings.

General Conclusion

General Conclusion:

In the context of the valorisation of medicinal plants and the development of their applications by transforming them into pharmaceutical forms with therapeutic value, this study aimed to prepare a herbal ointment based on Marrubium vulgare and to evaluate its expectorant efficacy in addition to the study of some biological properties that support its safety and bioactivity. The phytochemical analysis of the plant extract showed the presence of secondary compounds of biological importance, which validates the traditional uses of the plant and explains its possible therapeutic properties. Several concentrations of the herbal ointment (0.5%, 1%, 3%, 5% and 8%) were prepared to select the most appropriate concentration in terms of efficacy and safety.

The results of the expectorant activity test showed that the expectorant activity improved with increasing concentrations. The findings showed that the flow rate of artificial mucus increased with increasing ointment concentration. The biological tests also demonstrated that the activities studied were concentration dependent with some concentrations showing higher activity in some tests than others which shows the effect of concentration on the biological properties of the ointment. The results of the antioxidant activity test showed different abilities of the different concentrations to inhibit free radicals and the results of the anti-inflammatory activity test showed differences in the effectiveness of the concentrations studied. The results of the antimicrobial activity test verified the inhibitory capacity of the ointment against the tested microorganisms to different extents depending on the concentration used.

In terms of safety, the HET-CAM test findings indicated that, in contrast to the higher concentrations, which displayed an increase in the irritation index, the low and medium concentrations recorded an acceptable degree of safety. A 3% concentration was suggested as the ideal concentration based on balancing expectorant efficacy, biological characteristics, and safety level since it showed a suitable balance between efficacy and a tolerable degree of discomfort.

Based on the findings, it can be concluded that the primary goal of the study was accomplished by creating a herbal expectorant ointment based on Marrubium vulgare. The best concentration was found, and its safety and biological qualities were assessed, increasing its potential for use as a promising natural preparation.

As part of completing this work, some future prospects can be proposed, including:

- Performing additional research to isolate and characterise the active compounds with biological activity and expectorant effects.
- Physicochemical property and stability study of ointment at different storage periods.
- Advanced studies to confirm effectiveness and safety Improvement of the pharmaceutical formulation of the ointment and development of other dosage forms derived from the plant studied.
- Valorisation of local plant resources in the development of low-cost natural products: an assessment of their potential for industrial use.

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