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DOTTORATO DI RICERCA IN
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COORDINATORE Prof.Dr. Stefano MANFREDINI

Identificazione, Quantificazione, Formulazione e Valutazione di attività biologiche di oli essenziali dall'Albania

Settore Scientifico Disciplinare Chem/08

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Identification, Quantification, Formulation and Evaluation of Biological Activities of Essential Oils from Albania

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To my doughters
Ana and Sofia

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CONTENTS

List of Figures

List of Tabels

Glossary of Terms

MAP's – Medicinal aromatic plants

GC/FID – Gas chromatograph with Fid dedector

GC – Gas chromatograph

S.montana – Satureja montana

PCL method – Photochemiluminescence

ACL - Antioxidant Capacity of Liposoluble substance

DMS - Dymethilsulfoxide

CHAPTER 1: INTRODUCTION

1.Introduction

1.1 Medicial Plants

Currently there is a revival of interest in the use of plants as source of food and medicine. It is well known that plants are the richest source of bioactive phytochemicals and antioxidant nutrients (Elless et al., 2000). It is now broadly accepted that certain classes of plant-based compounds such as dietary fiber, phenolic acids, flavonoids, vitamins, and antimicrobial agents and neuropharmacological agents play preventive role against the incidence of some common diseases like cancer, cardiovascular and neurodegenerative disorders (Siddhuraju and Backer, 2007; Fan et al., 2007; Liu et al., 2008).

The increasing uses of herbal products demand extra attention with particular focus on their safety, effectiveness and drug interactions. Over the last few decades, a substantial body of scientific evidence is available demonstrating wide range of pharmacological and nutraceutical activities of medicinal herbs (Burt, 2004; Celiktas et al., 2007; Edris, 2007). These include antioxidant, anticancer, anti-inflammatory activities. (Abdullah Ijaz Hussain, Characterization And Biological Activities Of Essential Oils Of Some Species Of Lamiaceae, 2009)

The infectious diseases mainly caused due to microbial contamination of foods are becoming a major problem in the world, particularly in the developing societies (Burt, 2004; Sokmen et al., 2004; Sokovic and Van Griensven, 2006; Hussain et al., 2008). The consumption of microbes-infected foods is a serious challenge and threat for the health of the consumers (Hussain et el., 2008).The microbial growth in foods not only leads to decrease the nutritive and organoleptic value of food commodities, nevertheless it generates several toxins that are harmful for the health of humans (Celiktas et al., 2007). Recently, the essential oils and herbs-derived extracts are gaining much recognition as a potential source of natural and safer antioxidants and bioactives (Burt et al., 2003; Burt, 2004; Cantore et al., 2004; Bozin et al., 2006; Celiktas et al., 2007; Edris, 2007;Dastmalchi et al., 2008; Hussain et al., 2008; Kelen and Tepe, 2008).

In addition countries there are rich of herbal drug usually aromatic one MAPs are supported for their export as a very important income for the country and for the individuals also. Albania is one of these countries. A USAID lately raports that Albania is very important exporte for aromatic plants and their essential oils.

1.2 Essential oil

An essential oil is a concentrated hydrophobic liquid containing volatile aromatic compounds from plants. Essential oils are also known as volatile oils, ethereal oils or aetherolea, or simply as the "oil of" the plant from which they were extracted, such as oil of clove. Volatile oils are the odorous and volatile products of various plant and animal species. As they have a tendency to undergo evaporation on being exposed to the air even at an environment temperature, they are invariably termed as volatile oils, essential oils or ethereal oils. They mostly contribute to the odoriferous constituents or “essences” of the aromatic plants that are used abundantly in enhancing the scent by seasoning of eatables. Oil is "essential" in the sense that it carries a distinctive scent, or essence, of the plant.

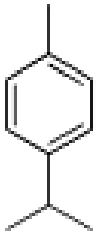
Arabs were the first who developed the techniques for obtaining essential oil from the naturally occurring organic materials (Saeed, 1989). Arab physician, Avicenna, designed the protocol to extract the essential oil from the flowers by distillation in the tenth century (Poucher, 1959; Pouchers, 1974). He isolated the perfume in the form of oil or attar from the rose flowers and produced rose water. Therefore, the first description of rose water had been reported by an Arab historian, Ibn-e-Khulduae.

Chemically, the essential oils are a complex and highly variable mixture of constituents that belong to two groups: terpenoids and aromatic compounds.(Figure1) The name terpene is derived from the English word “Turpentine” (Guenther, 1952; Guenther, 1985). The terpenes are the unsaturated hydrocarbons which have a distinct architectural and chemical relation to the simple isoprene molecule (CH2==C(CH3)—CH==CH2). These having molecular formula $C_{10}H_{16}$, are thus constituted by two isoprene units combining by head to tail union (Gunther, 1960; Pinder, 1960). The essential oils in 17 additions to the terpenes $C_{10}H_{16}$ often contain more completed hydrocarbons of the same composition but of higher molecular weight. Their composition can be expressed by the general formula $(C_5H_8)_n$. For monoterpene n=2; for diterpene ($C_{20}H_{32}$) and sesquiterpenes ($C_{15}H_{24}$) n is greater than 2 (Figure 1.1). Although essential oils are comprised of many types of compounds, the major ones are monoterpenes (Seigler 1998)

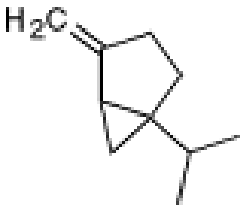
These essential oils can be produced in almost all plant organs such as flowers, buds, stems, leaves, fruits, seeds and roots etc. These are accumulated in secretary cells, cavities, channels, and epidermic cells (Burt, 2004; Chalchat and Ozcan, 2008; Hussain et al., 2008; Anwar et al., 2009a). The extracted oils can vary in quality, quantity and in the chemical composition depending upon the agro climate, plant organ, age and vegetative cycle stage (Masotti *et al.*, 2003; Angioni *et al.*, 2006)

Monoterpenes

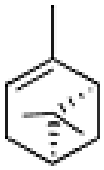
Cymene ("y") or p.cymene



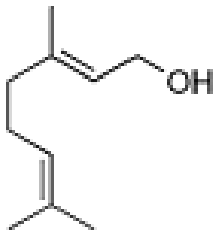
Sabinene



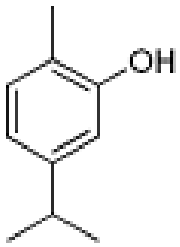
Alpha-pinene



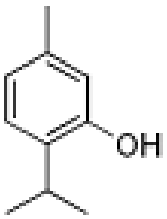
Geraniol



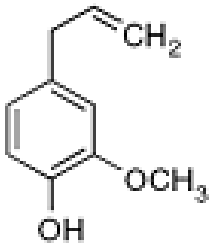
Carvacrol



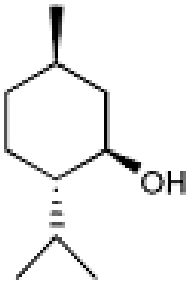
Thymol



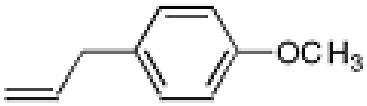
Eugenol



Menthol

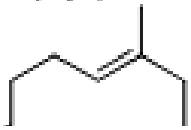


Estragole



Sesquerpitenes

Caryophyllene



Farnesol

Figure 1.Chemical strucure of some major components

1.3 Isolation of Essential Oil

(Extraction Methods)

Essential oils are generally extracted by distillation. Steam distillation is often used. Other processes include expression or solvent extraction. They are used in perfumes, cosmetics, soaps and other products, for flavouring food and drink, and for adding scents to incense and household cleaning products. In general, it has been observed that a single volatile oil invariably comprises even more than 200 different chemical components, and mostly the trace constituents are solely responsible for attributing its characteristics flavor and odor.

Another type of aromatic product available on the market is CO_2 extracts, referred to simply as that, CO_2 extracts. They differ in chemistry from their related distilled essential oils but are becoming increasing available on the market

Distillation appears to have been practiced throughout ancient times. Based upon the current interpretation Paolo Rovesti's discovery of an earthenware distillation apparatus, the production or extraction of aromatic oils by means of steam distillation has been known for 5000 years. During the fifth century AD, the famed writer, Zosimus of Panopolis, refers to the distilling of a divine water and panacea. Throughout the early Middle Ages and beyond, a crude form of distillation was known and was used primarily to prepare floral waters or distilled aromatic waters. These appear to have been used in perfumery, as digestive tonics, in cooking, and for trading.

In 900 AD, Avicenna, the famous child prodigy from Arabia who wrote many documents on plants and their uses and also instructions for massage, was accredited with refining the process of distillation by improving the cooling system.

Today distillation is still the most common process of extracting essential oils from plants. The advantage of distillation is that the volatile components can be distilled at temperatures lower than the boiling points of their individual constituents and are easily separated from the condensed water. During distillation the plant material is placed upon a grid inside the still. Once inside, the still is sealed, and, depending upon the above methods, steam or water/steam slowly breaks through the plant material to remove its volatile constituents. These volatile constituents rise upward through a connecting pipe that leads them into a

condenser. The condenser cools the rising vapor back into liquid form. The liquid is then collected in a vehicle below the condenser. Since water and essential oil do not mix, the essential oil will be found on the surface of the water where it is siphoned off. Occasionally an essential oil is heavier than water and is found on the bottom rather than the top, such as with clove essential oil. In this study we have used hydro distillation by Clevenger apparatus.

1.4 Uses of essential oils

Essential oils have been used medicinally in history. Medical applications proposed by those who sell medicinal oils range from skin treatments to remedies for cancer and often are based solely on historical accounts of use of essential oils for these purposes. Claims for the efficacy of medical treatments, and treatment of cancers in particular, are now subject to regulation in most countries. Interest in essential oils has revived in recent decades. They are used in in aromatherapy as they are believed to exhibit certain medicinal benefits for curing organ dysfunction or systemic disorder (Perry et al., 1999; Silva et al., 2000; Hajhashemi et al., 2003).Recent scientific reports have also focused on the antioxidant principles and biological activities of essential oils (Skocibusic et al., 2006; Yuenyongsawad and Tewtrakul, 2005; Tepe et al., 2007; Hussain et al., 2008; Anwar et al., 2009b).The essential oils have shown potential as anti-bacterial agents, disinfectants, anti-fungal agents, insecticides and as herbicides (Skocibusic *et al*., 2006; Bozin *et al*., 2006; Maksimovic *et al.,* 2007; Van Vuuren *et al.,* 2007)Essential oils of some spices and herbs such as sage, oregano, thyme, and Satureja etc. have shown their antioxidant potential (Ruberto and Baratta, 2000; Rota *et al.*, 2004; Rota *et al.*, 2008) and thus can be used as natural antioxidants for the protection of fats/oils and related products (Burt, 2004; Sacchetti et al., 2005; Bozin et al., 200

Recently, the uses of natural antioxidants are becoming very popular in food and preventive medicine due to the claims that they are safer and have disease–preventing and health promoting attributes. Research is now in progress to exploring the applications of some essential oils for therapeutic uses and management of infectious diseases as an alternative to standard drugs remedies (Bozin *et al*., 2006; Celiktas *et al*., 2007; Kelen & Tepe, 2008; Politeo *et al.*, 2007; Sokovic and Van Griensven, 2006). In Figure 2 is shown the application of Phytotherapy in Albania.(Figure2). It clear that Albanian people are ancient consumers of medicinal plants which are endemic in our country. Lamiace family has a traditional use in Albania expecially *Origanum vulgaris*, *Salvia officinalis, Thymus vulgaris*, *Satureja Montana*, *Myrtus communis* and *Rossmarinus officinalis.*

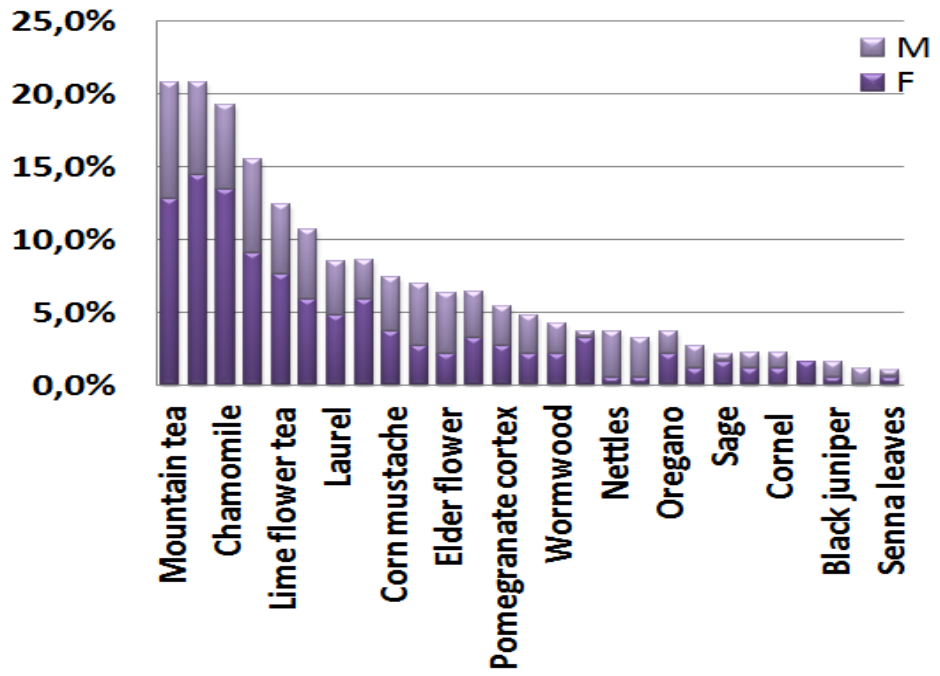


Figure 2. % of MAP's in Albania. (Pasho I. Papajani V., "Study of the aplication of Phytopherapy in Albania (Tirana area)", Conference of Bio-Medical Sciences, 2013, Tirana, Albania.

Albania is one of the most important exporters of medicinal and aromatic plants in Europe (Asllani, 2004). *Satureja montana* L.(winter savory), is an important medicinal and aromatic plant in Albania. It is a perennial shrub which grows wild throughout Albania (Paparisto et al., 1996) and as a medicinal and aromatic plant plays an important role in everyday life. It is consumed fresh and dried as seasonings, stews, meat dishes, poultry, sausages, vegetables phytomedicines, herbal tea, etc. (Paparisto and Balza, 2003). Its flowers are known to attract honeybee and the honey is a famous folk remedy for bronchitis (Paparisto and Balza, 2003). In addition, *S.montana* is being used as a stimulant, stomachic, carminative, expectorant, anti-diarrheic, and aphrodisiac in Albanian folkloric

medicine (Asllani, 2004).The plant of *S.montana* contains various biologically active constituents such as essential oil and triterpenes (Escudero et al., 1985), flavonoids, and rosmarinic acid (Reschke, 1983). The tea and extracts of this plant among many groups of natural compounds contain free and glycoconjugated aroma compounds. The essential oil is high in carvacrol and thymol (Lawrence, 1979). Further, the content of thymol and carvacrol is variable involving mostly on the origin and vegetative stage of the plant (Kustrak et al., 1996). The essential oil is used in the food industry as a flavoring agent in liqueurs and perfumery.

1.5 Medicinal Aromatic Plants in Albania Economy

Harvesting of medicinal and aromatic plants from the wild is an important economic factor in rural areas of Albania. Albania is in terms of quantities, the two leading exporters of MAPs in Southeast Europe. USAID studies lately studies showed out that Aromatic and Medicinal Plants (MAPs) is the main agro-forestry business in Albania, and is generating more than 16 m Euro per year and involving, mostly as a part time activity, more than 100,000 rural dwellers. The sector was already an important source of revenue during the planned economy.The value chain is mainly export-oriented: about 60% of MAPs are shipped to Germany and USA. Exports of MAPs account for more than half of the timber

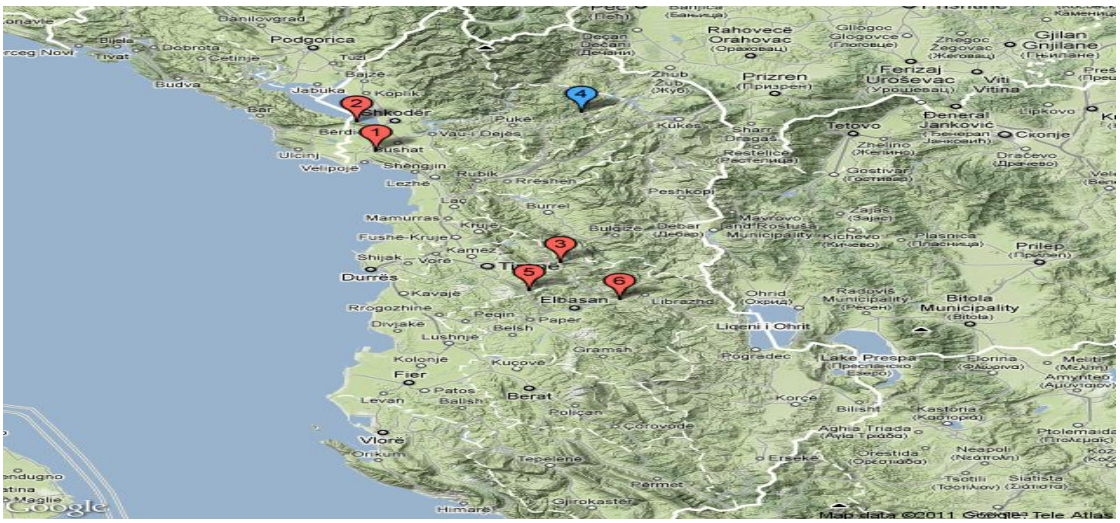


Figure 3. Geographical zone of MAP's plants

and non-timber forestry products exports and 25% of all agro-food exports. Albania is a major international player for some products, such as sage, thyme, oregano and winter savoury (*Satureja Montana*). In some specific markets and market segment, Albanian products are market leaders, such in the case of sage in USA and wild thyme in Germany. Most of the MAPs business is made by wild products. Increasing procurement cost,

competition between wholesalers and difficulty to match the market potential only with wild products are increasing the interest of the operators for cultivating some MAPs, such as sage, oregano, thyme lavender and cornflower.

At present, it is estimated that the total cultivated surface area, should not exceed 500 ha. The efforts of the main operators for expanding their business are now concentrated on widening the range of wild MAPs offered and on investing in MAPs processing for the production of essential oils. MAPs are found all over the country, but collection is more organized in some districts: Malesia e Madhe, Shkoder, Skrapar, Elbasan, Korce, Berat, Permet, and Durres. Sage from Northern districts is generally considered better, while the best oregano and thyme are mainly coming from Central and Southern Albania.

The structure of the food chain is relatively simple: rural families collect and dry the MAPs, which are purchased by 30-40 regional collectors all around the country. Collectors are selling to exporters. Some of the exporters have also started to process MAPs to produce essential oil. There are still a few farmers cultivating MAPs. During the last ten years, the sector is grown in size and efficiency. Ten years ago, exports were almost exclusively made by a single company, a subsidiary of a multi-national group which is now facing hard competition (USAID– Albania Agriculture Competitiveness (Aac) Program, prepared by DAI)

1.6 At present, the main issues to be addressed are:

- The improvement of quality, the adoption of standards and quality controls along the whole value chain. At present, the average export price of Albanian MAP's is generally lower than that one of main competitors, in spite of high organoleptic qualities of Albanian wild MAP's. Such a situation is mainly due to the lack of officinal and accredited laboratory and standard methods of analyzing essential oils.
- The essential oil of MAP-s are very popular in Albania due to their antibacterial and antifungal properties, so it's very important to evaluate the proper therapeutic uses.
- Essential oils are lipid soluble well-known ingredient often applied to the skin for their important properties that ranges from antimicrobial to anti-inflammatory and skin whitening. Current applications of these volatile compounds turn out to be complicated because of chemical and physical properties. This is one of the major problems for their uses; therefore, microencapsulation could be the solution to problems of stability, evaporation and controlled release.

1.7 Aims and Objectives

General aim of the present study was, in particular, the re-investigation of traditional use of food and medicinal plants from the Albania region on the light of the most update technologies and the new role assumed by medicinal food, food supplements and dermo-cosmetics in general health politics. Costs of health care are growing with the increasing of population age, thus Governments are taking into account other strategies than medicinal treatment. Prevention of diseases and health promotion are unavoidable instruments of a modern heath care program through Pharmacists and Physicians counseling. In this latter case, when the diseases are only in the maintainement or prevention state, keeping the physiological natural equilibrium is an aim that can be easily reached by the use of natural, tradition inspired, products. Obviously, natural and traditional, are not synonymous of SAFE, especially on the light of the most recent scientific acquisition. Thus some side effects can be easily avoided by checking the purity, using technology devices, providing good practice suggestions. Moreover, once re-investigated, newer use my be discovered. In detail the research plans were divided as follow:

- Preparation and study of Chemical composition of essential oil for quality assessment (QA) in the service of the collectors, cultivators, exporters of aromatic-medicinal species
- Evaluation of Antibacterial and Antifungal Activity of Albanian essential oils
- Formulation and Preparation of Cyclodextrine/essential oil complexes and evaluation their antibacterial properties.(*Satureja Montana*)
- Exploitation of new biological propertie/applications of essential oils/cyclodextrine complexes in particular, antibacterial and antifungal properties

1.8 Our Study Design

No earlier study is done as far as we know of complexing in β-cyclodextrine of these Albanian essential oils. We have studied especially *Satureja Montana* essential oil for GC-FID analyses, antibacterial, and complexes of β-cyclodextrine with and *Myrtus Communis*, *Mentha Piperita*, *Origanum Vulgaris* for antifungal activity. The work is organized as below.

1. Collection of plants from different area of north Albania
2. Extraction of essential oils form collected herbal drug
3. Identification and quantification of essential oil by GC-FID
4. GC-FID method validation for *Satureja Montana* essential oil
5. Evaluation of antibacterial and antifungal properties of essential oils
6. Formulation of β-cyclodextrine essential oil complexes
7. Study of essential oil complexes with β-cyclodextrine in vertical diffusion cell (future advise)

CHAPTER 2: LITERATURE REVIEW

2.1. Essential Oils

The fragrant mixture of liquids, obtained through distillation of aromatic plant materials, is known as an essential oil (Burt, 2004). Essential oils are mixtures of fragrant substances or mixtures of fragrant and odorless substances. A fragrant substance is a chemically pure compound, which is volatile under normal conditions and which owing to its odor can be useful for the society (Gunther, 1952).

2.2 Sources of Essential Oils

The occurrence of essential oils is restricted to over 2000 plant species from about 60 different families, however only about 100 species are the basis for the economically important production of essential oils in the world (Van de Braak and Leijten, 1999). The ability of plants to accumulate essential oils is quite high in both Gymnosperms and Angiosperms, although the most commercially important essential oil plant sources are related to the Angiosperms. Most of the aromatic plants and essential oil commodities in terms of world trade belong to the families of *Lamiaceae*, *Umbelliferae* and *Compositae* (Burt, 2004; Teixeira da Silva, 2004; Hammer *et al*., 2006; Celiktas *et al*., 2007; Hussain *et al*., 2008; Anwar *et al*., 2009a).

Essential oils are isolated from various parts of the plant, such as leaves (basil, patchouli, cedar), fruits (mandarin, fennel), bark (cinnamon), root (ginger), grass (citronella), gum (myrrh and balsam oils), berries (pimenta), seed (caraway), flowers (rose and jasmine), twigs (clove stem), wood (amyris), heartwood (cedar), and saw dust (cedar oil) (Dang *et al*., 2001; Burt 2004; Sood *et al*., 2006; Cava *et al.,* 2007; Hussain *etal.,* 2008).

2.3. Lamiaceae Essential Oils

Lamiaceae (syn. Labiatae) herb family consists of more than 252 genus and 7000 species (Hedge, 1992). Lamiaceae family is known for the wealth of species with medicinal properties, which have been used since early times and many of these species are common in Mediterranean region (Ali *et al*., 2000). The Lamiaceae plants are 24 generally aromatic in all parts including a number of widely used culinary herbs, such as sage, thyme, rosemary, oregano, basil, mint lavender, marjoram, savory, and perilla (Wink, 2003; Celiktas *et al*., 2007; Hussain *et al*., 2008). Some of them are shrubs, and a very few are vines or trees. The aromatic essential oils are contained in leaves which emerge oppositely with each pair positioned at right angles to the previous one (called *decussate*). The cross section of stems is square in shape. The flowers are symmetrical with 5 united sepals and 5 united petals. Such plants are mostly bisexual and verticillastrate (a flower cluster that

looks like a whorl of flowers but actually consists of two crowded clusters) (Cantino *et al*., 1992; Heywood *et al*., 2007). Albania is considerably productive with regards to the cultivated growth of Lamiaceae plants, while the different regions of the country possess a variety of the wild growing species of this family. Many species belonging different genera of the family Lamiaceae have been reported to occur in different parts of the country. Among these species *Mentha arvensis, M. piperita, Ocimum basilicum, O.sanctum, Thymus vulgaris, T. linearis* are cultivated as crops (Wazir *et al*., 2004; Hussain *et al*., 2008; Hussain *et al*., 2010). The rest of the species grow wild, frequently in mountainous terrains at different heights.

2.4. Taxonomy Hierarchy (ITIS REPORT)

Table 1. Taxonomic Hierarchy of Lamiace family

Kingdom	Plantae – plantes, Planta, Vegetal, plants
Subkingdom	Viridaeplantae – green plants
Infrakingdom	Streptophyta – land plants
Division	Tracheophyta – vascular plants, tracheophytes
Subdivision	Spermatophytina – spermatophytes, seed plants, phanérogames
Infradivision	Angiospermae – flowering plants, angiosperms, plantas com flor, angiosperma, plantes à fleurs, angiospermes, plantes à fruits
Class	Magnoliopsida
Superorder	Asteranae
Order	Lamiales
Family	Lamiaceae – mints, menthes

2.5 . The Genus Rosmarinus

Rosmarinus officinalis L. (Rosemary) is a very important medicinal and aromatic plant, which belongs to the genus *Rosmarinus* of the Lamiaceae family. Rosemary, a perennial herb, has fragrant evergreen needle-like leaves (Bousbia *et al*., 2009). Anthropologists and archaeologists have found evidence that rosemary herbs were widely used in folk

medicine, culinary and cosmetic virtues in the ancient Egypt, Mesopotamia, China, India and Pakistan and for the flavouring of food products (Pintore *et al*., 2002). *Rosmarinus officinalis* essential oil is of immense medicinal worth for its powerful antimutagenic, antiphlogistic, antioxidant, chemopreventive and antibacterial properties (Daferera *et al*., 2000; Koschier and Sedy, 2003; Ohno *et al*., 2003; Oluwatuyi *et al*., 2004; Celiktas *et al*., 2007). The Rosemary is good for memory, concentration and helps being focused at. Modern science attributes much of rosemary's action on the central nervous system to it`s potent antioxidant, rosmarinic acid.

2.6 The Genus Origanum

The genus *Origanum* (oregano) is significant in the family Lamiaceae and comprises of around 900 species of annual, perennial and shrubby herbs, widespread throughout the world (Bayder *et al*., 2004; Kordali *et al*., 2008). The genus includes some important culinary herbs, including Turkish wild oregano (*O. vulgare*) and sweet marjoram (*O majorana* L), commercially available and exportable plants with appreciable market values (Baytop, 1999; Esen *et al*., 2007). *Origanum* plants are extensively used for the flavoring of alcoholic beverages, food products and in perfumery due to their spicy fragrance (Olivier, 1994; Filippo-D-Antuono *et al*., 2000). Besides their commercial importance, such plants have been used, for long, as condiments and spices for foods like salads, soups, sausages and meats (Baydar *et al*., 2004; Sagdic and Ozcan, 2004). Their use for the treatment of various diseases was also in practice, being sudorific, expectorant, stomachic, antiseptic, stimulant, and emmenagogic (Ozcan, 1998). Both academia and the food industry have been interested in the biological properties of *Origanum* extracts and essential oils due to their antimicrobial and antioxidant potential (Dorman and Deans, 2000; Aligiannis *et al*., 2001; Ozcan and Erkmen, 2001; Sagdic and Ozcan, 2004).

2.7 The Genus Salvia

In the Lamiaceae family, *Salvia* is the biggest genus which consists of about 900 species. The *Salvia officinalis*, one of the common species of this genus is now widely cultivated in various parts of the world and is popularly used as a culinary herb for flavoring and seasoning. *Salvia officinalis* has a variety of medicinal uses such as astringent, antiseptic and spasmolytic (Perry *et al*., 1999). Salvia species have been employed locally as traditional medicine to treat a variety of diseases such as wounds, malaria, microbial infections and cancer (Kamatou *et al*., 2008). Salvia species also exhibited various in-vitro pharmacological properties (Kamatou *et al*., 2005; Kamatou *et al*., 2006). Essential oil

associated with *Salvia officinalis* was 28 characterized by high concentrations of camphor, 1,8-cineole and thujone (Dean and Ritchie, 1987; Piccaglia and Marottu, 1993). Various phenolic compounds in plants related to this genus have shown excellent antioxidant capacity as well as antimicrobial activity (Jalsenjak *et al*., 1987; Sivropoulou *et al*., 1997; Tepe *et al*., 2004;).

2.8 The genius Satureja

Summer savory: *Satureja hortensis* and L., Winter savory: *Satureja montana* L., syn. The etymology of the Latin word 'satureia' is unclear. Speculation that it is related to *saturare*, to *satyr*, or to za'atar is not well supported. The ancient Hebrew name is ṣathrá צתרה.(Wikipedia) *Flowers:* Spikes of dainty white or lilac, with purple spotting on the lower lip, *leaves are* Semi-evergreen, narrow, dark-green and glossy, Light well drained soil in full sun, *Flowering Season:* July - September. You can harvest fresh leaves as needed, *Distribution:* natives of the Mediterranean region, grown worldwide in temperate zones. *Satureja Montana* is used widely for its antibacterial properties and spice ones. The dominant components in the oil of S. montana were reported to be caryophyllene and geraniol (Sevarda et al., 1986) and carvacrol (Palić and Gisic, 1993; Chalchat et al., 1999). In spite of S. montana being an important medicinal and aromatic plant in Albania and its being included in Albanian National Plants Red Data

2.9. Factors Affecting Essential Oil Accumulation

Factors that determine the composition and yield of the essential oil obtained are numerous. These variables may include seasonal and maturity variation, geographical origin, genetic variation, growth stages, part of plant utilized and postharvest drying and storage (Marotti *et al.* 1994; Anwar *et al.*, 2009b).

Other factors which affect the growing plants thus leading to variations in oil yield and composition include part of plant used, length of exposure to sunlight (Burbott and Loomis, 1957; Clark and Menary, 1979), availability of water, height above sea level (Galambosi and Peura, 1996), time of sowing (Galambosi and Peura, 1996). The oil composition and yield may also change as a result of the harvesting methods used (Bonnardeaux, 1992), the isolation techniques employed (Weston, 1984; Charles and Simon, 1990; Moates and Reynolds, 1991), the moisture content of the plants at the time of harvest (Burbott and Loomis, 1957) and the prevailing steam distillation conditions.

2.10 Chemistry of Essential Oils

Essential oils are made up of three elements almost exclusively carbon, hydrogen, and oxygen.(Figure 3) The most common component class is the terpenes. Terpenes are made from combinations of several 5-carbon- base (C5) units called isoprene (Gunther, 1952). Terpenes can form building blocks by joining together in a "head-to-tail" configuration to

form monoterpene, sesquiterpenes, diterpene and larger sequences (Pinder, 1960).

The chief terpenes are the monoterpenes (C10) and sesquiterpenes (C15) and in some cases hemiterpenes (C5), diterpenes (C20), triterpenes (C30) and tetraterpenes (C40) also exist

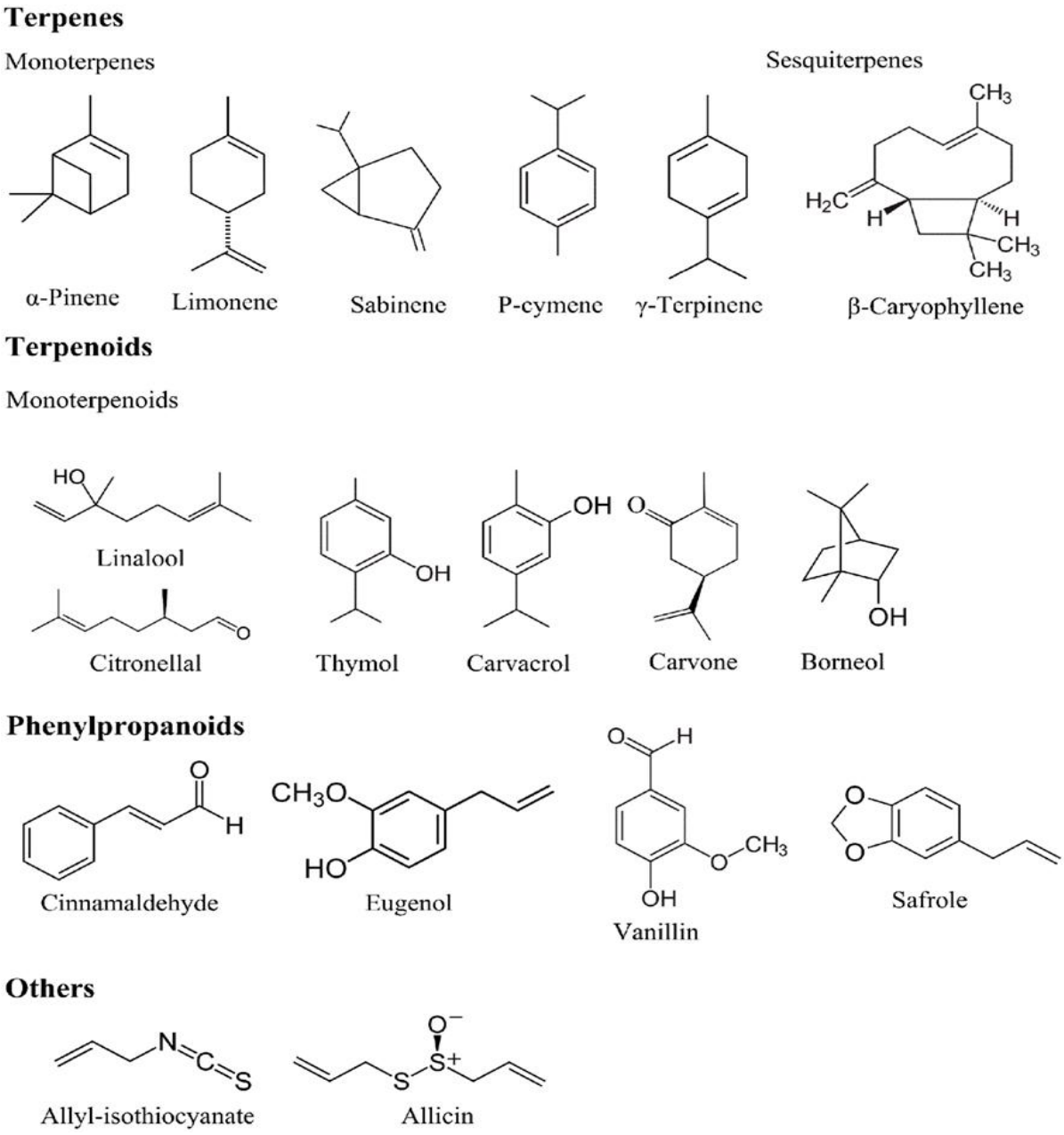


Figure 4. Chemical composition of MAP's

A terpene containing oxygen is called a terpenoid. The monoterpenes are generally formed by the combination of two isoprene units. They are the mainly representative molecules constituting 80-90% of the essential oils and allow a vast variety of structures. They also contain several functional groups like carbures (ocimene, myrcene, terpinenes, phellandrenes, pinenes, etc.), aldehydes (geranial, citronellal, etc.), ketone (menthones, pulegone, carvone, fenchone, pinocarvone, etc.), alcohols (geraniol, citronellol, nerol, menthol, carveol, etc.), esters (linalyl acetate, citronellyl acetate, isobornyl acetate, etc.), ethers (1,8-cineole, menthofurane, etc.) (Burt, 2004).

There is plenty of literature on the characterization of essential oils. Capillary gas chromatography (GC) with flame ionisation detection (FID), are, in most cases, the method of choice for quantitative determinations. Capillary columns selected, in most cases, are HP- 5ms, DB-5 (cross-linked 5% diphenyl/95% dimethyl siloxane) or DB-1, also known as SE-30, (polydimethyl siloxane) stationary phases. Essential oils are very complicated mixtures of natural compounds at quite different concentrations (Burt, 2004; Bakkali, 2008). They are characterized by two or three major components at fairly high concentrations (20–70%) compared to others components present in trace amounts (Bauer *et al*., 2001; Burt, 2004). For example, carvacrol (30%) and thymol (27%) are the major components of the *Satureja montana* essential oil. In addition we have analysed the y-terpinene, borneol and p-cymen. The concentrations of Carvacrol varied from 21.07 to 77.79%; Thymol from 0.72 to 39.9%; y-Terpinene from 4 to 13.8% and p-Cymene from 0.74 to 17.4%. (Ibraliu at al 2010)

2.11 Methods of Isolation of Essential Oils

Methods to isolate essential oils may be categorized into enfleurage, steam distillation, solvent extraction, hydrodistilation, and supercritical fluid extraction. Hydrodistillation or steam distillation is the most widely utilized physical method for isolating essential oils from the botanical material (Whish, 1996; Masango, 2004).

Although steam distillation is much popular for the isolation of essential oils oncommercial scale and 93% of the oils are produced by this process, but it is not apreferred method in research laboratories (Masango, 2004).

This is probably due tounavailability of steam generators and suitable distillation vessels. Most studies whichfocus on the essential oil of herbs have made use of hydrodistillation in Clevenger-type apparatus (Kulisic *et al*., 2004; Sokovic and Griensven, 2006; Hussain *et al*., 2008). In hydrodistillation procedure, the material is immersed in water, which is heated to boiling point using an external heat source. In both hydro-, and steam- distillation

techniques, the vapors are allowed to condense and the oil is then separated from the aqueous phase (Houghton and Raman, 1998). Care must be taken to ensure efficient condensation of steam, thereby preventing the loss of the more volatile oil components

2.12 Physico-chemical properties of essential oils

Although volatile oils differ greatly in their chemical constitution, they have a number of physical properties in common. They possess characteristic odors, they are characterized by high refractive indices, most of them are optically active, and their specific rotation is often a valuable diagnostic property. As a rule, volatile oils are immiscible with water; however, they are soluble in ether, alcohol, and most organic solvents.

Several points of differentiation exist between volatile oils and fixed oils. Volatile oils can be distilled from their natural sources; they do not consist of glyceryl esters of fatty acids. Hence, they do not leave a permanent grease spot on paper and cannot be saponified with alkalies. Volatile oils do not become rancid, as do the fixed oils, but instead, on exposure to light and air, they oxidize and resinify.

Practically all volatile oils consist of chemical mixtures that are often quite complex; they vary widely in chemical composition. Almost any type of organic compound may be found in volatile oils (hydrocarbons, alcohols, ketones, aldehydes, ethers, oxides, esters, and others), and only a few possess a single component in a high percentage (clove oil contains not less than 85% of phenolic substances, chiefly eugenol).. The absence of even one component may change the aroma. Plants of the same species grown in different parts of the world usually have the same components, but the percentages that are present may differ.

*2.*13 Biological Effects of Essential Oils

Essential oils from different plants have gained much interest due to their antioxidant, antitumor, antibacterial, antifungal and insecticidal properties (Burt, 2004). Since to ancient times were known the anifungal and antibacterial of some plants and later their respective essential oils. There are many publications that confirm this fact. In recent years (1987-2001), a large number of essential oils and their constituents have been investigated for their antimicrobial properties against some bacteria and fungi in more than 500 reports. This paper reviews the classical methods commonly used for the evaluation of essential oils antibacterial and antifungal activities. The agar diffusion method (paper disc and well) and the dilution method (agar and liquid broth) as well as turbidimetric and impedimetric

monitoring of microorganism growth in the presence of tested essential oils are described. Factors influencing the in vitro antimicrobial activity of essential oils and the mechanisms of essential oils action on microorganisms are reported. This paper gives an overview on the susceptibility of human and food-borne bacteria and fungi towards different essential oils and their constituents. Essential oils of spices and herbs (thyme, origanum, mint, cinnamon, salvia and clove) were found to possess the strongest antimicrobial properties among many tested.

2.13.1 Antioxidant activities

What are antioxidants?

From a biological point of view, antioxidants have been defined as substances that when present in concentrations lower than the oxidation substrate are capable of delaying or inhibiting oxidative processes.

2.13.2 Measurement of antioxidant activity

Natural antioxidant compounds exhibit their antioxidant activity by various mechanisms like:; (1) chain breaking by donation of hydrogen atoms or electrons that convert free radicals in to more stable species, (2) chelating metal ions which are involved in the generation of reactive oxygen species, (3) decomposing lipid peroxides into stable final products, and (4) inhibiting the deleterious action of prooxidant enzymes.

Due to complexity of the composition of plants and plant based foods, separation of each antioxidant compound and studying it individually is difficult.

Researchers are searching innovative methods, to measure the antioxidant activity of the foods and other biological systems which are yet in the development stages (Natella *et al.*, 1999; Wright *et al.*, 2001; Cai *et al.*, 2006; Siquet *et al.*, 2006).

2.13.3 In Vitro assays for antioxidant activities of essential oils

The antioxidant potential of essential oils and extracts has been known in a number of in-vitro studies. Most commonly used methods for the determination of antioxidant activity of plant essential oils and extracts are;

- 2,2-di(4-*tert*-octaphenyl)-1-picrylhydrazyl (DPPH.) radical scavenging assay
- Inhibition of linoleic acid peroxidation
- Bleaching of β- carotene in linoleic acid system assays

Based on the latest literature (Morris, at al) developments we have taken into consideration the first method to evaluate the antioxidant properties of our selected essential oils.

2.13.4 Antioxidant potential of essential oils

Synthetic antioxidants may cause liver swelling and influence liver system activities and cerebro-vascular diseases (Choi *et al*., 2007; Fan *et al*., 2007**)**. There is a strong need for effective and safer antioxidants based on natural sources, as alternatives, to prevent the deterioration of foods. The literature shows many reports of extracts from the natural sources that have demonstrated strong antioxidant activity (Paradiso *et al*., 2008; Descalzo and Sancho 2008). Many sources of antioxidants have been explored and still research is going on. Essential oils and extracts from botanical materials are known to have varying degrees of antioxidant activities (Descalzo and Sancho 2008; Tabata *et al*., 2008) Some recent publications (Bendini *et al*., 2002; Cervato *et al*., 2000; Damechki *et al*., 2001; Martinez-Tome *et al*., 2001; Vichi *et al*., 2001) showed antioxidative activities of essential oils. Some of these essentials oils and extracts have been reported to be more effective than some synthetic antioxidants (Mimica-Dukic, 2004; Hussain *et al*., 2008).

Literature reported the antioxidant activities of the *Mentha* essential oils (Kofidis*et al*., 2004; Pandey *et al*., 2003;) Recently,many studies have focused on the biological and antioxidant activities of the Origanum and Rosemary essential oils (Daferera *et al*., 2000; Faleiro *et al*., 1999; Koschier & Sedy, 2003; Ohno *et al*., 2003; Sacchetti et al. 2005; Sokmen *et al*., 2004). Rosemary's antioxidant extracts are still used to extend the shelf-life of prepared foods (Cuvelier, Richard, & Berest, 1996; Ibanez *et al*., 2003). *Myrtus Communis* essential oils and extracts also exhibited good antioxidant potential (Hohman *et al*., 1999; Ivanova *et al*., 2005; The antioxidant effects of plant essential oils and extracts are mainly due to the presence of hydroxyl groups in their chemistry).

2.14. Antimicrobial activities

2.14.1 Antimicrobial agents

There are two groups of antimicrobial agents used in the treatment of infectious diseases.

1) Antibiotics, that are natural substances produced by certain groups of microorganisms
2) Chemotherapeutic agents, who are chemically synthesized (Davidson & Harrison, 2002).

The range of bacteria or other microorganisms that is affected by a certain antibiotic is expressed as its spectrum of action (Burt, 2004). One the other hand the inhibition zone is the diameter of zones where there is no growth of bacterial colony. Higher is the inhibition zone higher is the antibacterial activity of essential oil.A number of methods used for

evaluation of antibacterial activity of essential oils have been reported in literature (Bozin *et al*., 2006; Celektas *et al*., 2006; Kelen and Tepe, 2008).

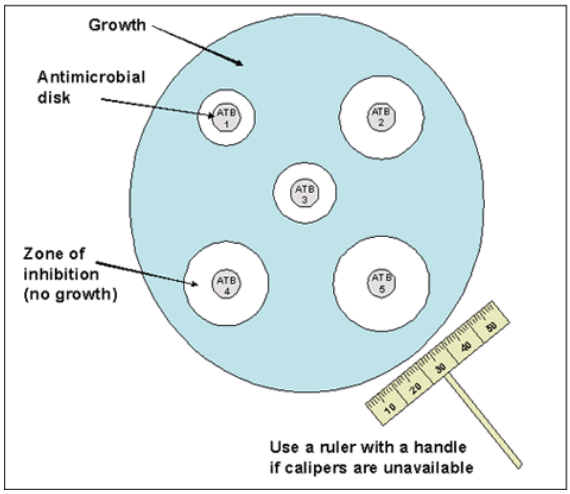


Figure 5. Inhibition zone

Different assays like disc diffusion assay, well diffusion assay, microdilution assay, measurement of minimum inhibitory concentration are often used for measuring the antimicrobial activity of essential oils and plants based constituents (Juliano *et al*., 2000; Lambert *et al*., 2001; Burt, 2004; Holley and Patel, 2005; Bakkali *et al*., 2008).

There is no standardised method are developed for assessing the antimicrobial activity of plant based compounds against food-spoiling and pathogenic microorganisms (Davidson and Parish, 1989). The old NCCLS method for antibacterial susceptibility testing has been modified for testing essential oils and extracts (Hammer *et al*., 1999; NCCLS, 2000).

Researchers adapt different experimental protocols to better represent future applications in their particular field. On the other hand, researchers adapt different experimental protocolsto better represent future applications. Screening of essential oils for antibacterial activity is often done by the disk diffusion assay, in which a paper disk soaked with known concentration of essential oil is laid on top of an inoculated agar plate. This is generally used as a preliminary check for antibacterial activity prior to more detailed studies. A number of factors such as the amount of essential oil placed on the paper discs and the thickness of the agar layer vary considerably between studies.This method is mostly sued as a screening method when large numbers of essential oils and/or large numbers of bacterial isolates are to be screened (Deans *et al*., 1993; Dorman and Deans, 2000).

2.14.2 Essential oils as a natural antimicrobial agents

Essential oils and other naturally occurring antimicrobials are attractive to the food industry for the following reasons:

1. It is highly unlikely that new synthetic compounds will be approved for use as food antimicrobials due to the expense of toxicological testing
2. There exists a significant need for expanded antimicrobial activity both in terms of spectrum of activity and of broad food applications
3. Food processors are interested in producing "green" labels, i.e., ones without chemical names that apparently confuse consumers, and (d) there are potential health benefits that come with the consumption of some naturally occurring antimicrobials.

Recently, essential oils and extracts of certain plants have been shown to have antimicrobial effects, as well as imparting flavour to foods (Burt, 2004). Some essential oils have shown promise as potential food safety interventions when added to processed and raw foods. Some of the most effective natural antimicrobials are extracted from spices and herbs and essential oils and isolates of the different plant families (Juliano *et al*., 2000; Lambert *et al*., 2001; Burt, 2004; Holley and Patel, 2005; Bakkali *et al*., 2008). Extracts/essential oils from dietary herbal species belonging to the family Lamiaceae, including thyme, have been used as sources of medicine and food preservatives for over 4000 years (Burt, 2004; Rota *et al*., 2008).),

There are many reports in literature regarding the antimicrobial activity of essential oils (Kofidis *et al*., 2004; Pandey *et al*., 2003; Singh *et al*., 2005; Kaur and Kapoor, 2002). The antifungal and antibacterial activity exhibited by *Satureja, Rosmarinus* and *Origanum* essential oil has been demonstrated by several researchers (Burt, 2004; Rota *et al*., 2004; Sokmen *et al*., 2004; Skovia and Griensven, 2006).

In Albania there are not many studies for antibacterial properties of essential oils extracted from Albanian origin herbals. Although a huge number of plant species have been investigated for their essential oil potential and biological activities, however, to the best of our knowledge there are no earlier reports yet available regarding the detailed chemical characterization and evaluation of biological and antioxidant principles of essential oils from plants of Lamiaceae family, native to Albania country (Figure 5)

2.14.3 Antifungal agents and essential oils

Fungal infection is very often occurring on these days. They are getting more and more resistant to antifungal agents, which are very expensive one and associated with a dozen side effects. On the other hand traditional medicine usually is cheaper and more effective than modern medicine. It is necessary to evaluate, in a scientific base, the potential use of folk medicine for the treatment of antifungal disease. We chose some herbal from Lamiace

family used in folk medicine to determine their antifungal activity against clinical pathogens i.e. A survey of literature reveals that there are many essential oils which possesses antifungal activity especially dermatophytes (Kishore N1, Mishra AK, Chansouria JP). For these reasons, we investigated Albanian essential oils in some Dermatophytes**.**

2.15 Essential oils problems

Satureja Montana oil can suffer oxidation and volatilisation or react with other formulation component that may cause skin irritation. However, some of researcher reported that encapsulation is a feasible alternative way to increase the stability of this compound.

2.16 Mechanism of Action

The antibacterial and antiviral mechanism of action of the major compound in savories essential oils, lipophilic terpenes (volatile mono- and sesquiterpenes), is most likely based on their solubility in biomembranes. At their higher concentration, they influence the environment of membrane proteins (ion channels, transporters, receptors) and thus change their conformation and bioactivity. The mechanism of action of savory essential oils against cell membranes and walls of bacteria was confirmed by measurements of the intracellular pH, ATP concentration and the electronic microscopy observations of the bacterial cells treated with essential oils.The sites or structures of the bacterial cell that are considered targets for action by the components of natural products are illustrated in Fig.5. The action mechanisms of natural compounds are related to disintegration of cytoplasmic membrane, destabilization of the proton motive force (PMF), electron flow, active transport and coagulation of the cell content. Not all action mechanisms work on specific targets, and some sites may be affected due to other mechanisms

Important characteristics responsible for the antimicrobial action of essential oils include hydrophobic components that allow the participation of lipids from the bacterial cell membrane, which disturbs cell structures and make them more permeable.

Chemical compounds from essential oils also act on cytoplasmic membrane proteins. Cyclic hydrocarbons act on ATPases, enzymes known to be located at the cytoplasmic membrane and surrounded by lipid molecules. In addition, lipid hydrocarbons may distort the lipid-protein interaction, and the direct interaction of lipophilic compounds with hydrophobic parts of the protein is also possible . Some essential oils stimulate the growth

of pseudo-mycelia, evidencing that they may act on enzymes involved in the synthesis of bacterium structural components Several compounds and their mechanisms of action on microorganisms are listed below.

Carvacrol and thymol

The structure of thymol is similar to that of carvacrol; however, they differ as to the location of the hydroxyl group in the phenolic ring. Both substances seem to make the membrane permeable.Their structure disintegrates the external membrane of gram-negative bacteria, releasing lipopolysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane to ATP. The presence of magnesium chloride does not influence this action, suggesting a chelating mechanism of different cations on the external membrane .

Eugenol

Different concentrations of eugenol may inhibit the production of amylase and protease by *B. cereus*. Furthermore, cell wall degradation and cell lysis were also reported .

p-Cymene

A precursor of carvacrol, this hydrophobic compound provokes greater swelling of the cytoplasmic membrane compared to carvacrol.

Carvone

When tested at concentrations higher than its minimum inhibitory concentration, carvone dissipates gradient pH and cell membrane potential. The growth of *E. coli*, *Streptococcus thermophilus* and *Lactococcus lactis* may decrease according to the concentrations of carvone, suggesting that it acts by disturbing the general metabolic status of the cell (56).

Cinnamaldehyde

Cinnamaldehyde is known to inhibit *E. coli* and *Salmonella Typhimurium* growth at concentrations similar to those of carvacrol and thymol. However, it neither disintegrates the outer membrane nor weakens the intracellular ATP (53). Its carbonyl group has affinity for proteins, preventing the action of decarboxylase amino acids on *E. aerogenes* (57).

2.17 Essential oil cyclodextrin complexes.

2.17.1 Cyclodextrins

Cyclodextrin molecules are cyclic oligosaccharides made up of six to twelve α-D-glucopyranose monomers, which are connected at 1 and 4 carbon atoms. Cyclodextrins with six to eight α-D-glucopyranose units are denoted as α-, β- and γ-Cyclodextrins respectively. Among these various types of cyclodextrins, α-cyclodextrin is not suitable for many drugs and γ-cyclodextrin is expensive. β-cyclodextrin is widely used because it is readily available, and its cavity size is suitable for a wide range of guest molecules. In general, the special characteristic of cyclodextrins is the ability to form an inclusion complex with various organic molecules through host-guest interaction with the interior cavity that provides hydrophobic environment to trap an apolar pollutant.

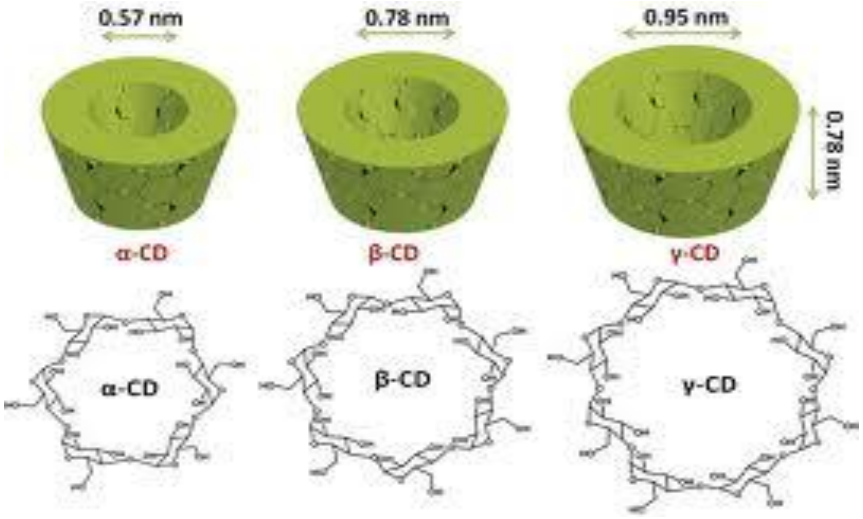


Figure 6. Cyclodextrine structure

The inclusion complex of these host–guest systems occurs through various interactions, such as hydrogen bonding, van der Waals interaction, hydrophobic interactions and also electrostatic attraction where the described types of bonding would alter the photochemical and photophysical properties of the guest molecules. Thus, the physical, chemical and biochemical properties of guest molecules will be modified and the application criteria of those guest molecules also can be improved. So far, various kinds of guest molecules such as drugs, steroids, ionic liquids and dyes were used as host-guest interaction to change the properties of the guest molecules into the desired form. Schiff bases are compound with a functional group that contains a carbon nitrogen double bond with the nitrogen atom

connected to an aryl or alkyl group. For example inclusion of Satureja Montana is one of these cases

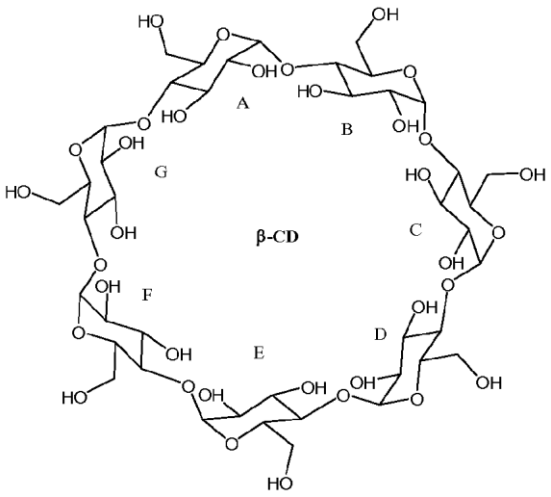


Figure 7. β-cyclodextrine structure

Because of this arrangement, the interior of the CD is not hydrophobic, but considerably less hydrophilic than the aqueous environment and thus able to host other hydrophobic molecules. In contrast, the exterior is sufficiently hydrophilic to impart CDs (or their complexes) water solubility. β-cyclodextrin (β-CD) has been since 1998, as a flavour carrier and protector, at a level 2% in numerous food products. Based on the previous researches they

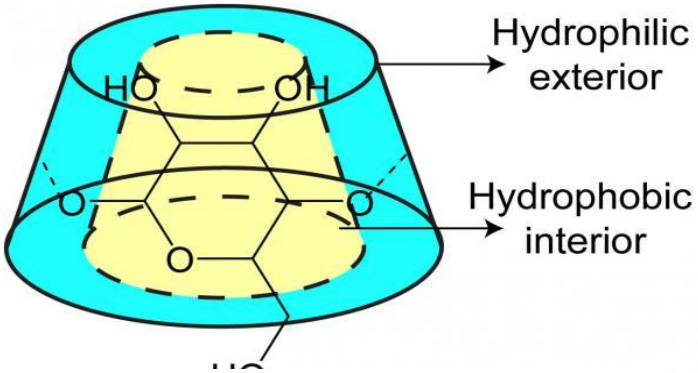


Figure 8. β-cyclodextrine structure

commonly used β-CD and its derivatives to form a complex with other compounds due to its ability to produce a complex with comparable quality as aroma, colour and appearance. Usually, β-CD been used as an encapsulation agent. Several researchers had encapsulated complex materials like oleoresin, essential oil (*Salvia sclarea* L. essential oil, *Lippia sidoides* oil and lemon oil) and fatty acid compounds (lineoleic acid and cholesterol) with CD. This study significantly endeavors in microencapsulating of *Satureja Montana*

essential oil. It can be useful, especially in food industry but also any other field including pharmaceutical and cosmetic areas. Besides this, it can be used as a model study for future research on inclusion complex of any plant materials that contain carvacrol.

CHAPTER 3: MATERIALS AND METHODS

The research work presented in this thesis was conducted in the laboratories of the Department of Pharmacy Univeristy of Tirana, Department of Pharmacy University of Ferrara, National Laboratory of Drug Controll of Albania, Department of Pharmacy Univeristy of Aldent.

3.1 Materials

3.1.1 Chemical and standard compounds

Reagents: Hexan, methanol, ether, 2, 2,-diphenyl-1-picrylhydrazyl, anhydrous sodium, DMS (dymethilsulfoxide)

Reference chemicals: (*p*-cymene, γ-terpinene, borneol, thymol, carvacrol) used to identify the constituents were obtained from Sigma-Aldrich Chemie Gmbh Munich, Germany, β-cyclodextrine was purchased by Titolchimicha Italy. All culture media and standard antibiotic discs were purchased from Biochek Comp.

3.1.2 Instruments

The instruments used for different analyses during the study along with their company identification are listed in Table below.

Table 2. Instruments and apparatus used

APPARATUS	*MODEL*	*COMPANY*
Gas/Fid	**Seria 3800**	**Varian , England**
Electric Balance	**Ohaus Corporation, Usa. Sn 8732351170**	**Ohaus , China**
Magnetic Stirrer	**Hj-3. No 981121**	**China**
Petri Plates	**Biochek Lab.**	**Greece**
Water Bath	**Shp 02036140a**	**China**
Clevenger Apparatius	**L.Assany**	**China**

3.2 Collection of Herbal samples *(Figure 5, Table 3)*

Herbal plants of *Satureja montana*, *Myrtis Communis*, *Origanum vulgaris*, *Rosmarinus Officinalis* and *Salvia Officinalis* were collected from north of Albania (Malësia e Madhe) due to their high concentrations of carvarol and thymol, at the full blooming period, end of July and air-dried in a room (under shade) and were identified from our botanist Skerdilaid Xhulaj in Botanic Department, Faculty of Natural University of Tirana, Albania

Table 3. Herbal samples samples from north of Albania	
	***Rosmarinus officinalis***
	***Satureja montana***
	***Origanum vulgaris***
	***Salvia Officinalis***
	***Myrtus Communis***

3.3 Humidity

(BP 2008) Place about 2 gr of samples in dried and wighted dishes. After that place them in thermostat for about 4 hours.Then cool and weight the dishes.

Humidity values ranges from 4.89 % - 7.83 %

Figure 9. Humidity

3.4 Ash assessment

(BP 2008). Place about 1 g of the sample material, accurately weighed, in a suitable tarred dish of silica, previously ignited, cooled and weighed. Incinerate the material by gradually increasing the heat, not exceeding 800 °C, until free from carbon; cool, and weight.

Total ash ranges from 5.74 % - 75.8%

Figure 10. Muffel oven

3.5 Strains of microorganisms utilized to access the antimicrobial and antifungal activity of essential oils

3.5.1 Bacterial Strains

(i) *Staphylococcus aureus* (S.Aerus) ATCC 29737 Lot 58312397

(ii) *Proteus Vulgaris* (P.Vulgaris) ATCC 1978 Lot 0876523C.

(iii) *Escherichia coli* (E. coli) E.Coli ATTC 8456 LOT 6543109

3.5.2 Fungal Strains

Candida Albicans (C.Albicans) ATCC 2091 Lot 7051869 *Epidermophyton floccosum* CBS 358.93 strain; *Trichophyton violaceum* CBS 459.61 strain; *Trichophyton tonsurans* CBS 483.76 strain, *Trichophyton mentagrophytes* CBS 160.66 strain, *Microsporum canis* CBS 131110 strain; *Trichophyton rubrum* CBS 132252 strain, *Microsporum gypseum* CBS 130948 strain; *Arthroderma cajetani* CBS 49570; *Botrytis cinerea* CBS 179.71*; Pyricularia oryzae* CBS 433.70

3.5.3 Positive Controls

Cefuroxime 30ug lot 1A3208 Biorad

Tetracyclini 30ug lot OD3313 Biorad

Cyprofloxacini 5 ug lot OM3189 Biorad

3.5.4 Negative Control

Dimethylsulfoxide (DMSO)

Medium Mueller--Hinton agar (Lot 685C2S, Code 060098),

Bacterial and Candida albicans colocny are obtained in National Laboratory of Drug Control of Albania.

Dermatophytes and Phytopatogens colonies are obtained from obtained from CBS-KNAW Fungal Biodiversity Centre, an Institute of the Royal Netherlands Academy of Arts and Sciences, Uthrecht, Netherlands

3.6 Essential oil extraction

3.6.1 Isolation of the essential oil

The hydrodistillation was carried out with a Clevenger-type apparatus according to the Hungarian Pharmacopiea VII. (1986). Drug quantity of 20 g was used; it was distilled with 500 ml of water for 3 hours. The resulting essential oil was dried over anhydrous sodium sulphate and stored at 4°C.(Figure 12, 13,14)

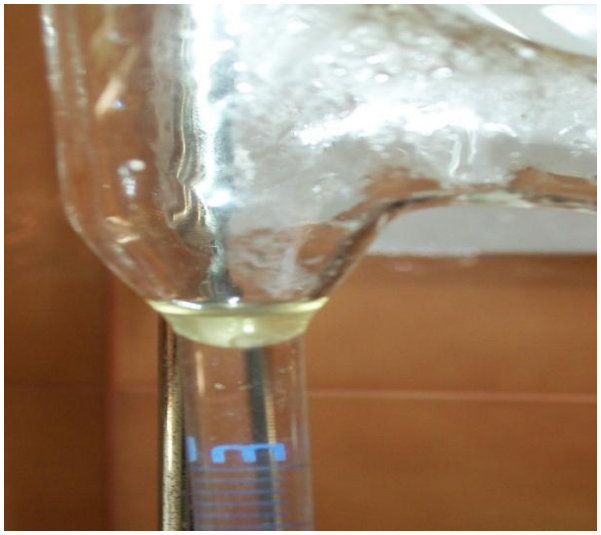


Figure 12. Essential oil extration by Cleveneger

Figure 11. Clevenger Apparatius

…..

Figure 13. *Satureja Montana* essential oil

3.7 Analysis of Essential Oil

3.7.1 Physical analyses

The refractive index (25^0C) of Lamiaceae essential oils were determined following standard methods (Guenther, 1960). An **ABE** refractometer was used for the determination of refractive index of theessential oils tested.

3.7.2 Chromatographic analysis

Gas/Fid chromatography analysis of S.montana essential oil

GC/FID conditions GC analysis of the essential oil was performed using a Varian CP-3800 instrument equipped with a capillary column. Helium was used as the carrier gas at the constant flow of 1.2 ml/min and split ratio 1:30. The oven temperature was held at 50 °C for 1 min, and then programmed to 280°C at a rate of 5°C /min. Helium flux is 30ml/min and air flux is 300ml/min. The injector temperature is 280 and detector (FID) temperature is 300°C. Injection volume is 1µl. [3

Figure 14 .Gas/Fid Varian 3800

Compounds identification

The identification of the main components was based on comparison of their spectra with those of authentic standards and and those described by Adam (2001),as well as literature values (Mimica-Dukic et al., 2003; Adam, 2001;Vagionas et al., 2007).

3.7.3 Method Validation

3.7.3.1 Standard and sample Stock Solutions

Satureja montana L. essential oil stock solution was prepared dissolving 5 mg essential oil in 5 ml hexane and was stored in refrigerator (-4°C) for stability. Six samples were prepared and each one was injected three times. The standards stock solution were prepared in following concentration p-cimen 2mg/ml carvacrol 2mg/ml, γ-terpinen 2mg/ml, thymol 8mg/ml, borneol 0,5mg/ml.

3.7.3.2 Linearity – Calibration Curbes

We prepared serial dilutions of each standard. The calibration lines were constructed by plotting the areas of p-cymen, borneol, carvacrol, γ-terpinen and thymol against their corresponding concentration . The concentration studies ranges between 0.5-5mg/ml for borneol, 1.0-8.0 mg/ml for γ-terpinen, 0.1-2.0mg/ml for carvacrol, 0.4-2mg/ml for p-cymen and 2.0-10 mg/ml for thymol. The statistical parameters slope, intercept, residual standard

on deviation response correlation co-efficient and p- values were calculated by GraphPad 6.02 version. . Their correspondative graph is shown in results chapter.

3.7.3.3 Optimization of GC Condition:

First Method applied

Reference *: University of Split, Faculty of Science, Department of Biology, Teslina 12, 21000 Split, Croatia 2 University of Zagreb, Faculty of Science, Division of Biology, Department of Molecular Biology, Horvatovac 102A, Zagreb 10000, Croatia*)

GC analysis of the essential oil was performed using a Varian CP-3800 instrument equipped with a capillary column. Helium was used as the carrier gas at the constant flow of 1.2 ml/min and split ratio 1:30. The oven temperature was held at 50 • °C for 1 min, then programmed to 280 • °C at at ate of 5 • °C / min. Helim flux is 30ml/min and air flux is 300ml/min The injector temperature is 280 and detector (FID) temperature is 300°C. Injection volume is 1µl

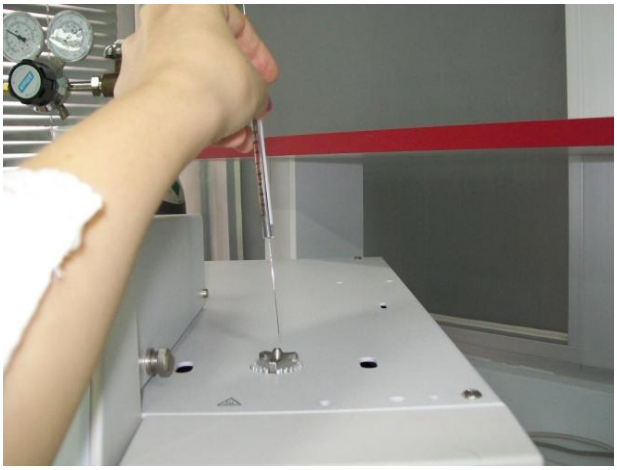


Figure 15. Injection essential oil in GC-FID

Second Method

We changed the temperature of injection to 250°C and the temperature of dedector to 280°C, the other parameters were identical to the first method

Third Method

We changed the temperature of injection to 250 C and the temperature of dedection to 300 C with three scale gradient, the split ratio and the speed of helium. We obtained better chromatogram symmetric one. The temperature three scale gradient was as follow $50^{o}C$ – $100^{0}c$ for 2 min, $100^{0}c$- $200^{o}C$ for 5 min and $200^{0}C$– $280^{0}c$ for 7 min

3.7.3.4 Precision and Accuracy study

The results of precision and accuracy determination were obtained from the recoveries of the ratios of found quantities to the injected quantities. The precision of the proposed method was verified by calculation of their repeatability's RSD preparations done successively during one day and the following 3 consecutive days. The accuracy was determined by calculation of the mean recoveries ± SD of five levels of concentrations

3.7.3.5 Robustness

1- **Change oven temperature**. We changed the oven temperature from 280 °C to 290 °C

2- **Change the flow rate** from 30 ml/min to 25 ml/min

In both two cases we didn't have statistically differences in results obtained conducting recovery at different level of thymol and the average percentage and recovery was found to be in the range

3.7.3.6 Limit of Dedection –Lowering injection volume

The limit of detection (LOD) and limit of quantitation (LOQ) were evaluated by serial dilutions of five standards stock solutions in order to obtain signal to noise ratios of 3:1 for LOD and 10:1 for LOQ. The LOD values for analyte were found to be as in (Tab.__).

3.8 Biological Activities of Essential oils

3.8.1 Evaluation of antioxidant activity:Photochemiluminescence (PCL) Method (S.montana,R.officinalis,O.vulgaris,M.communis,S.officinalis)

PCL assay, based on the methodology of Popov and Lewin [Lewin, G.; Popov, at al), was used to measure the antioxidant activity of extracts with a Photochem® apparatus (Analytik Jena, Leipzig, Germany) against superoxide anion radicals generated from luminol, a photo-sensitizer, when exposed to UV light (Double Bore® phosphor lamp, output 351 nm, 3 mWatt/cm^2). The antioxidant activity was measured using both ACW (Antioxidant Capacity of Water soluble substance) and ACL (Antioxidant Capacity of Liposoluble substance) kits provided by the manufacturer designed to measure the antioxidant activity of hydrophilic and lipophilic compounds, respectively [Popov, I.; Lewin, G. at al). For ACW studies, the luminol reagent and Trolox work solution were freshly prepared according to the ACW protocol. The presence of Trolox (or any other antioxidants from the extracts) retarded luminescence for a period: hence, a lag time was noted before a signal was measured. The duration of the lag, which is calculated by the computer software from the first derivative of the detector signal at its turning point and intersection with the x-axis, was plotted against the concentration of Trolox added to the assay medium. The concentration of the added extract solution was such that the generated

luminescence fell within the limits of the standard curve. Therefore, the lag time (seconds) for the ACW assay was used as the radical scavenging activity and the antioxidant capacity calculated by comparison with a Trolox standard curve and then expressed as micromoles of Trolox per gram of dry matter of red fibre. In ACL studies, the kinetic light emission curve, which exhibits no lag phase, was monitored for 180 s and expressed as micromoles of Trolox per gram of dry matter. The areas under the curves were calculated using the PCLsoft control and analysis software. As greater concentrations of Trolox working solutions were added to the assay medium, a marked reduction in the magnitude of the PCL signal and hence the area calculated from the integral was observed. This inhibition was used as a parameter for quantification and related to the decrease in the integral of PCL intensities caused by varying concentrations of Trolox. The observed inhibition of the signal was plotted against the concentration of Trolox added to the assay medium. The concentration of the added extract solution was such that the generated luminescence during the 180 s sampling interval fell within the limits of the standard curve. The extracts for ACW and ACL measurements were centrifuged (5 min at 16000 g) prior to analysis. The antioxidant assay was carried out in triplicate for each sample, and 20 μL of the diluted extract (1:40, v/v) in HPLC-grade water (ACW) or HPLC-grade methanol (ACL) was sufficient to correspond to the standard curve.

3.8.2 Evaluation of antimicrobial activities of essential oil of Satureja montana

Satureja Montana also provides of interesting antimicrobial properties and is used for topical treatment against incipient baldness and to treat arthritic joints. GC-FID spectrometry analysis of the isolated oil resulted in the identification of twentyone compounds in the oil of S. montana. Carvacrol is the major constituent of the S. montana oil (around 60 %). Other important compounds are the monoterpenic hydrocarbons p-cymene, y- terpinene and the oxygenated compounds borneol and thymol. The screening of the antimicrobial activities of essential oil were individually evaluated against three microorganisms Escherichia coli, Staphylococcus aureus and Proteus Vulgaris, using a disc diffusion method. The present study is done to evaluate the antibacterial properties of Satureja Montana essential oil of Albanian origin related to high percentage of carvacrol and thymol of its essential oil. Essential oils rich in phenoliccompounds are widely reported to possess high levels of antimicrobial activity (Panizi Et Al., 1993; Sivropoulou Et Al., 1996).

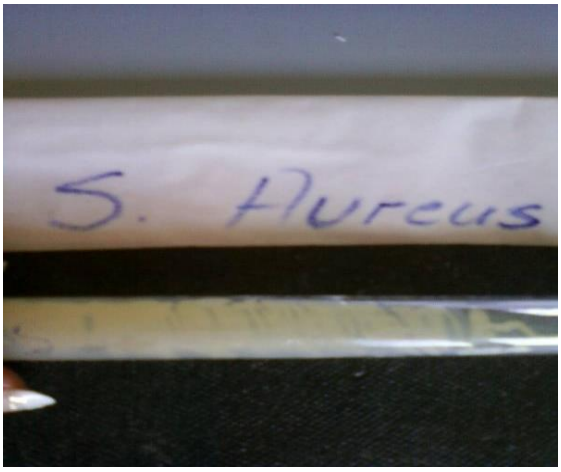


Figure 16. ***S.Aureus*** **colony**

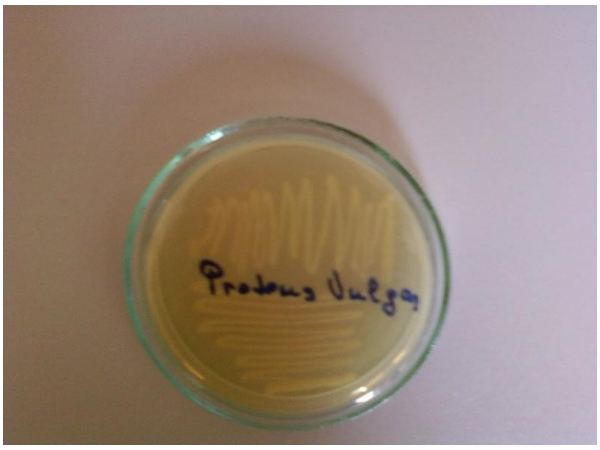


Figure 17. ***P.vulgaris*** **colony**

3.8.2.1 Evaluation of Antibacterial activity

The essential oil samples were tested for antibacterial activity by the disc diffusion method using 100µL of suspension of the tested microorganisms, containing 2.0 x 106 colony forming units (cfu mL–1) for bacteria and 2.0x105 spore mL–1 for fungal strains. Mueller--Hinton agar and dextrose agar were distributed to sterilized Petri dishes with a diameter of 9 cm. Figure 16,17,18. The filter paper discs (6 mm in diameter) were individually impregnated with 10µL and 30µL of the essential oils dissolved in dimethylsulfoxide (DMSO). The Petri dishes were kept at 4°C for 2 h. The plates inoculated with bacteria incubated at 37°C for 24 h .The diameters of the inhibition zones were measured in millimetres. Controls were set up with equivalent quantities of DMSO. Studies were performed in triplicate. In addition, positive contorols antibiotic discs such as Cefuroxime, ciprofloxacine, tetracycline

Figure 18. Petri plates of *S.montana* essential oil

3.8.3 Evaluation of Antifungal properties of essential oils.

(S.montana,R.officinalis,O.vulgaris,M.communis,S.officinalis)

3.8.3.1. Microorganisms

The essential oil samples before and after encapsulation were tested on fungal species, pathogenic for animals and humans, such as some dermatophytes and pathogenic for plants, such as some phytopatogens.

The dermatophytes used were *Epidermophyton floccosum* CBS 358.93 strain; *Trichophyton violaceum* CBS 459.61 strain; *Trichophyton tonsurans* CBS 483.76 strain, *Trichophyton mentagrophytes* CBS 160.66 strain, *Microsporum canis* CBS 131110 strain; *Trichophyton rubrum* CBS 132252 strain, *Microsporum gypseum* CBS 130948 strain; *Arthroderma cajetani* CBS 49570; *Botrytis cinerea* CBS 179.71*; Pyricularia oryzae* CBS 433.70 obtained from CBS-KNAW Fungal Biodiversity Centre, an Institute of the Royal Netherlands Academy of Arts and Sciences, Uthrecht, Netherlands.

The cultures were maintained in the laboratory as agar slants on a suitable culture medium, that is, on Sabouraud dextrose agar (SDA; Difco), for the dermatophytes or Potato dextrose agar (PDA) for phytopatogens.

3.8.3.2.Evaluation of Antifungal Activity

To evaluate antifungal activity, cultures of each fungus were obtained by transplanting mycelium disks, 10 mm in diameter, from a single culture in stationary phase. These were incubated at 26 ± 1 °C on the medium suitable for each organism (SDA or PDA), on thin sterile sheets of cellophane, until the logarithmic phase of growth was reached. Then the fungi were transferred to Petri dishes containing the medium supplemented with the compound to be tested. Each compound was dissolved into dimethyl sulfoxide (DMSO), and a proper dilution was aseptically added to the medium at 45 °C to obtain a final concentration of 20, or 100 μg/mL. The DMSO concentration in the final solution was adjusted to 0.1%. Controls were set up with equivalent quantities (0.1%) of DMSO. The growth rate was determined by measuring daily colony diameter for 7 days after the transport of the fungus onto dishes containing the substance to be tested. At this time the percentage growth inhibition in comparison with the control was evaluated for each fungus. Three replicates were used for each concentration. The percentage of growth inhibition was expressed as the mean of values obtained in three independent experiments.

The relative inhibition rate of the circle mycelium compared to blank assay was calculated via the following equation:

$$\text{Relative inhibition rate (\%)} = [(\text{dex} - \text{dex}')/\text{dex}] \times 100\%$$

where dex is the extended diameter of the circle mycelium during the blank assay; and dex' is the extended diameter of the circle mycelium during testing.

Statistical Analysis

Three samples of each plant material were assayed. Each sample was analyzed individually in triplicate for its antioxidant, antimicrobial andantifungal activities and data is reported as mean (n = 3 x 3 x 1) ± standard deviation (n = 3 x 3 x 1). Data were analyzed by analysis of variancce GraphPad Prisem

3.9 Complexation of *Satureja montana* essential oils in β-cyclodextrine

Complexes of β-cyclodextrine and essential oils were prepared by co-precipitation method with the four ratios oil: β-cyclodextrine as follows 5:95, 10:90, 15:85 and 20:80 (w/w) in order to determine the effect of the ratio on the inclusion efficiency of β-cyclodextrin for encapsulating oil. A precipitation method was used to prepare the β-cyclodextrin complex (Reineccius, 1989). Five grams of β-cyclodextrin was dissolved in 50 mL of an ethanol/water (1:2) mixture at 55°C (±2°). A predetermined quantity of essential oil dissolved in ethanol (10% w/v) was then slowly added to the warm β-cyclodextrin solution. The following starting ratios of essential oil to β-cyclodextrin were used: 5:95, 10:90, 15:85 and 20:80 (w/w). The mixture was continuously stirred on the magnetic stirrer and the temperature maintained at 55°C. The mixture was stirred for another 4 h, without heating, while its temperature decreased spontaneously to 25°C. The final solution was refrigerated overnight at 7°C. The cold precipitated material was recovered by vacuum filtration. The precipitate was dried in a convection oven at 50°C for 24 h. The powder was then allowed to air-dry at 25°C for an additional 24 h in order for the powder to reach its equilibrium moisture content.The obtained complex was stored in airtight glass containers, at room temperature, prior to further analysis.

3.9.1 GC-FID Analysis of Essential oil after complextion

The standard oil and the concentrated oils extracted from the complexes (total oil and surface oil) were analyzed by method, analysis conditions of the essential oil was performed using a Varian CP-3800 instrument equipped with a capillary column. Helium was used as the carrier gas at the constant flow of 1.2 ml/min and split ratio 1:30. The oven temperature was held at was as follow $50^{o}C - 100^{0}c$ for 2 min, $100^{0}c$- $200^{o}C$ for 5 min and

200^0C– 280^0c for 7 min.Helium flux is 30ml/min and air flux is 300ml/min The injector temperature is 280 and detector (FID) temperature is 300°C. Injection volum is 1µl.

The characterization of the complex involved the analysis of the initial essential oil, surface and total extracted oils. The difference between total oil extracted and surface oil absorbed is the amount of essential oil complexed by the cavity of b-cyclodextrine. Total oil contents in the complex were determined by using extraction method with hexane and by its results obtained. The method applied by analyses is the one we have standardized in our previous research work.

For the quantitative determination of essential oil components (present in the initial oil, total oil extracted from the powder and surface oil), a calibration curve with initial *S.Montana* oil was set up. Quantities of initial oil were weighed and dissolved in hexane, to obtain the concentration in the range of 1 - 20 mg/mL. Statistical analyses were performed by Graph Pad program.The identification of the compounds was made by their correspondive standartds obtained by Sigma Aldrich company.

3.9.2 Total oil extraction

The total oil content in the complex powder was determined using a solvent (hexane) extraction method, followed by analysis of the concentrated extract. Distilled water (20 mL), hexane (10 mL) and 0.5 g of the sample powder were put in a glass container. The solution was then kept in an ultrasonic bath at 85°C for 20 min. The organic phase containing the volatile compounds was decanted, and the aqueous phase was exhaustively extracted with hexane 3 times using the above method. The combined hexane extract was dried over anhydrous sodium sulphate and decanted. The final extract was evaporated was weighed and stored at 7°C till the analysis. The total oil corresponds to the amount of the complexed molecules in the β-cyclodextrin cavity plus the surface adsorbed oil.

3.9.3 Surface oil extraction

The volatile compounds adsorbed on the surface of the β-cyclodextrin were determined by washing a sample of powder (3 g) with hexane(20 mL) which was gently shaken manually for 20 min (Bhandari et al., 1998). The suspension was then filtered and the residue was further washed with hexane (10 mL). The obtained extract was treated as it was described above. The difference between the total oil and the surface adsorbed oil is the amount complexed in the β-cyclodextrin cavity

3.9.4 Quantitative analysis of oil volatiles .

For the quantitative determination of essential oil components (present in the initial oil, total oil extracted from the powder and surface oil), a calibration curve with initial *S.Montana* oil was set up. Precise quantitiesof initial oil were weighed and dissolved in hexane, to obtain the concentration in the range of 1 – 20 mg/mL.

Figure 19 . **Magnetic stirring of mixture essential : β-cyclodextrine**

3.10 Evaluation of antibacterial activity of Satureja Montana essential oil after microencapsulation (after two weeks)

The essential oil complexes with β-cyclodextrine samples were tested for antibacterial activity by the disc diffusion method using 100µL of suspension of the tested microorganisms, containing 2.0 x 106 colony forming units (cfu mL–1) for bacteria and 2.0x105 spore mL–1 for fungal strains. Mueller--Hinton agar and dextrose agar were distributed to sterilized Petri dishes with a diameter of 9 cm. Figure 16,17,18. First were weighted the complex powder that have correspondive amount of 10µL and 30µL of the essential oils and later dissolved in DMSO.The filter paper discs (6 mm in diameter) were individually impregnated with 10µL and 30µL of the essential oils dissolved in dimethylsulfoxide (DMSO). The Petri dishes were kept at 4°C for 2 h. The plates inoculated with bacteria incubated at 37°C for 24 h .The diameters of the inhibition zones were measured in millimetres. Controls were set up with equivalent quantities of DMSO. Studies were performed in triplicate. In addition, positive contorols antibiotic discs such as Cefuroxime, ciprofloxacine, tetracycline

3.11 Evaluation of Antifungal Activity of essential oils after encapsulation

(S.montana,R.officinalis,O.vulgaris,M.communis,S.officinalis)

To evaluate antifungal activity of essential oils after encapsulation the same method described in paragraph 3.8.2.2 was developed.

Chapter 4: Results

4.1 Humidity, ash of Samples

Humidity and ash results are shown in table 4. Values ranges for humidity 4.989 to 8.087 % and for Ash from 3.112 to 7.526. Both values are below 8 % which is the upper limits by Britsh pharmacopeia of humidity and Ash values. *Satureja montana* (M_1) has the minimum humidity and *Satureja montana* (M_3) has the maximum humidity %.

Samples	Zone of collection	Month/year	Period of Collection	Humidity %	Assh %
***Satureja Montana* M_1**	Lezhe, Albania	May - July 2010	Full Blooming	**4.989±0.02**	**5.747±0.07**
***Satureja Montana* M_2**	Kruja, Albania	May - July 2010	Full Blooming	**6.464±0.21**	**7.004±0.01**
***Satureja Montana* M_3**	Malesi e Madhe, Albania	May - July 2010	Full Blooming	**8.087±0.36**	**7.526±0.03**
***Satureja Montana* M_4**	Lezhe, Albania	May - July 2010	Full Blooming	**7.675±0.25**	**6.858±0.01**
***Satureja Montana* M_5**	Mone Negro	May - July 2010	Full Blooming	**7.922±0.12**	**4.810±0.01**
Rosmarinus officinalis	Lezhe, Albania	May - July 2010	Full Blooming	**6.544±0.33**	**5.145±0.05**
Myrtus Communis	Lac, Albania	May - July 2010	Full Blooming	**7.009±0.11**	**3.112±0.03**
Origanum Vulgaris	Malesie madhe, Albania	May - August 2010	Full Blooming	**6.996±0.09**	**4.336±0.01**
Salvia officinalis	Lezhe, Albania	August - September 2010	Full Blooming	**7.521±0.22**	**5.009±0.02**

Table 4. Zone of collection, period of collection , Humidity and Ash values of *S.montana, R.officinalis, M.communis, O.vulgaris, S.officinalis.*

4.2 Refractive index and Oil yield (%)

Table 5. Physical parameters of essential oils extracted

SAMPLE	OIL YIELD (%)G 100G-1	REFRACTIVE INDEX (25^0C)
***Satureja Montana* M_1**	0.97±0.11	1.4767 ± 0.002a
***Satureja Montana* M_2**	1.11±0.32	1.4766 ± 0.001a
***Satureja Montana* M_3**	1.31±0.15	1.4785 ± 0.003a
***Satureja Montana* M_4**	1.23±0.24	1.4769 ± 0.002a
***Satureja Montana* M_5**	1.56±0.52	1.4755 ± 0.002a
Rosmarinus officinalis	1.04±0.20	1.4770 ± 0.001a
Myrtus Communis	0.83±0.13	1.4778 ± 0.005a
Origanum Vulgaris	1.47±0.03	1.4768 ± 0.001a
Salvia Officinalis	1.19±0.38	1.4766 ± 0.002a

Figure 20. ABE Refractometer

Refractive index

Refractive index our essential oils ranges from 1.4785 to 4755 (Table 5). *Satureja montana* essential oil (M_3) has the higher value of refractive index and lower value has *Satureja Montana* essential oils (M_5). Meanwhile between essential oils taken into consideration in this study *Satureja Montana* essntial oil has the highest one and *Salvia Officinalis* has the lowest. The refractive index is an important physical constant often used for identification of the purity of essential oils. BP vales for refractive index are 1.490-1.510. All our essential oils samples are between accepted limits.

Oil yield %

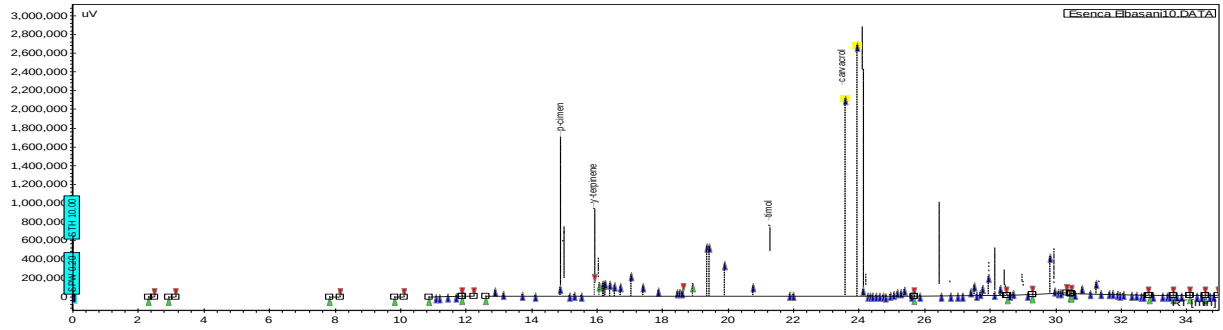
Oil yield (Table 5) ranges from 1.56 for *Satureja montana* (M_5) to 0.83 for *Myrtus communis*. For Albanian plants *Origanum vulgaris* has the highst yield % of essential oil, followed by *Satureja Montana* M_3 (1.31 g 100g-1), *Salvia Officinalis* (1.19 g 100g-1), *Rosmarinus officinalis* (1.04 g 100g-1) and *Myrtus communis* has the lowest one (0.83 g 100g-1).

4.3 GC-FID Chromatogram of *Satureja Montana* essential oil

Satureja Montana Optimization method

Gas/Fid analyses of *Satureja Montana* is not widly studied We adapted a method published by *University of Split, Faculty of Science, Department of Biology, Teslina 12, 21000 Split, Croatia 2 University of Zagreb, Faculty of Science, Division of Biology, Department of Molecular Biology, Horvatovac 102A, Zagreb 10000, Croatia*) This method didn't gave a very good separation of peaks of all *Satureja montana* samples (Figure 22 , Figure 23, Figure 24, Figure 25), so we tried to change and develop a new method.The second method gave better separation and more distinguish peaks but still not very well separated (Figure 27, Figure 28) Since the separation was fairy good we thought to change temperature by three scale gradient giving more time to the components to travel separately according to thieir volatile temperature. At this point the results were satisfied, the peaks obtained were well separated and the noises were belw the report 1:10 which gives the possibility to do quantify analyses of essential oils contents (Figure 29, 30)

First- Method



Peak results :

	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
	p-cimen	14.88	9.61	1707717.0	557429.9	9.614
	y-terpinene	15.91	2.86	953871.9	166085.5	2.865
	timol	21.24	4.76	807439.7	276182.3	4.763
	carvacrol	23.49	25.78	2249223.9	1494516.1	25.776
Total			100.00	24096893.6	5798021.8	100.000

Figure 21. GC-FID of *Satureja montana* M_1

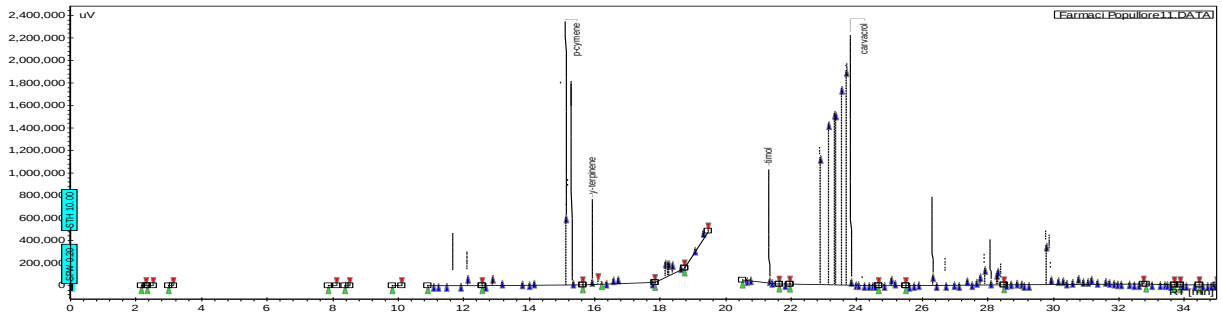


Figure 22 . GC-FID of *Satureja montana* M_2

Peak results :

	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
	p-cymene	15.10	20.86	2354763.0	968451.9	20.862
	y-terpinene	15.92	2.05	770767.7	94961.1	2.046
	timol	21.30	6.20	1027526.2	287804.3	6.200
	carvacrol	23.80	6.11	2242225.6	283856.6	6.115
Total			100.00	27336859.1	4642103.8	100.000

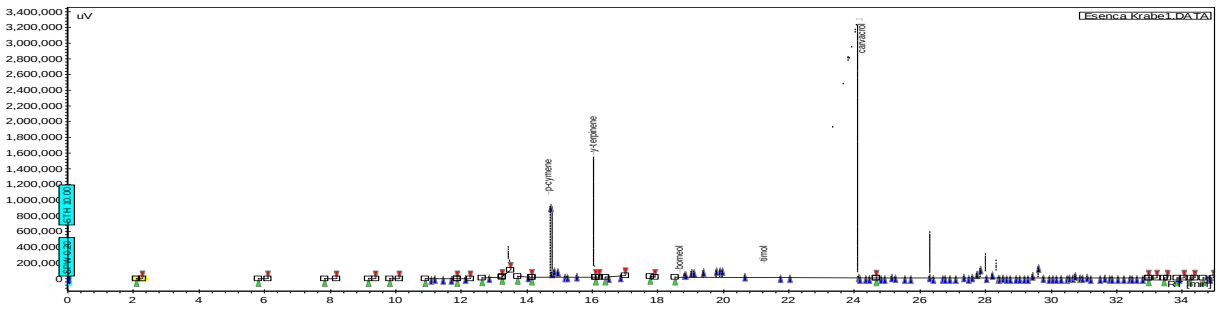
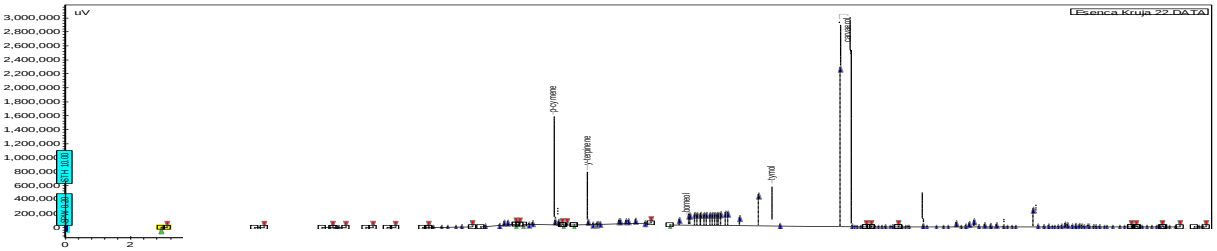


Figure 23 . Gas/Fid of *Satureja montana* M_3

Peak results :

	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
	p-cymene	14.67	5.90	1036189.6	272884.2	5.901
	y-terpinene	16.04	8.45	1551380.0	390550.1	8.446
	borneol	18.69	0.20	54884.8	9442.7	0.204
	timol	21.22	1.82	177972.4	84069.9	1.818
	carvacrol	24.05	67.68	3287314.5	3129986.2	67.685
Total			100.00	13910572.0	4624356.4	100.000

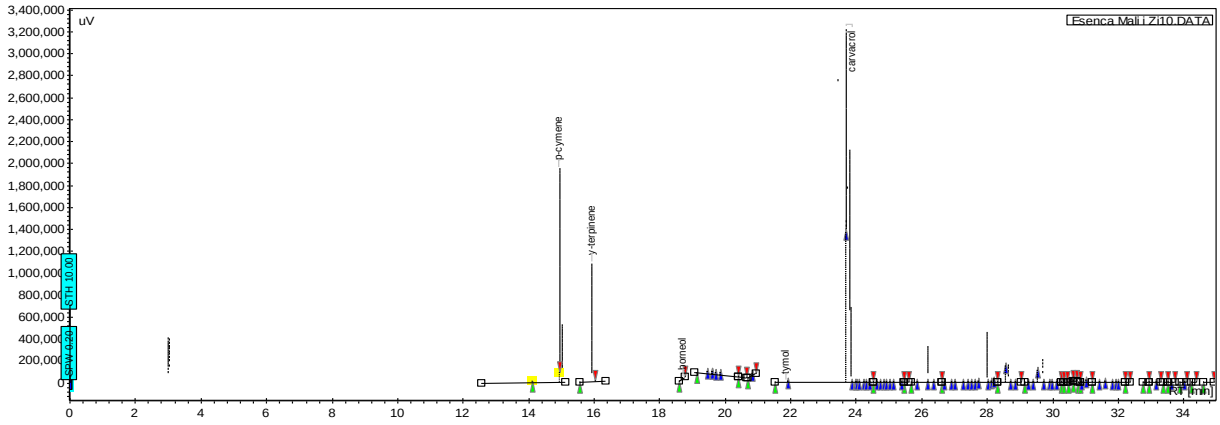
M 4 – Sample



Peak results :

	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
	p-cymene	14.90	11.04	1578112.5	589977.3	11.037
	y-terpinene	15.93	3.07	777371.0	164060.4	3.069
	borneol	18.97	0.67	151848.9	35804.7	0.670
	tymol	21.55	3.94	604550.8	210344.3	3.935
	carvacrol	23.64	46.18	2970927.4	2468448.7	46.179
Total			100.00	18036066.4	5345431.6	100.000

Figure 24. Gas/Fid of *Satureja montana* M_4

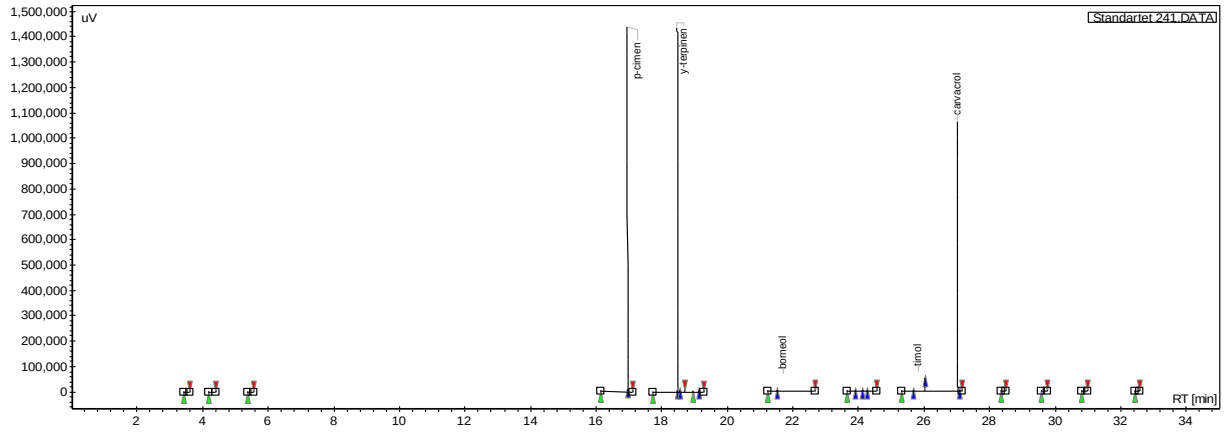


Peak results :

	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
	p-cymene	14.93	16.29	1956180.4	682346.6	16.290
	y-terpinene	15.93	4.72	1090068.9	197563.7	4.716
	borneol	18.69	0.05	25389.8	2064.7	0.049
	tymol	21.85	0.11	21809.3	4720.9	0.113
	carvacrol	23.68	66.96	3241140.2	2804925.2	66.962
Total			100.00	11454977.7	4188830.5	100.000

Figure 25 . GC-FID of *Satureja montana* M_5

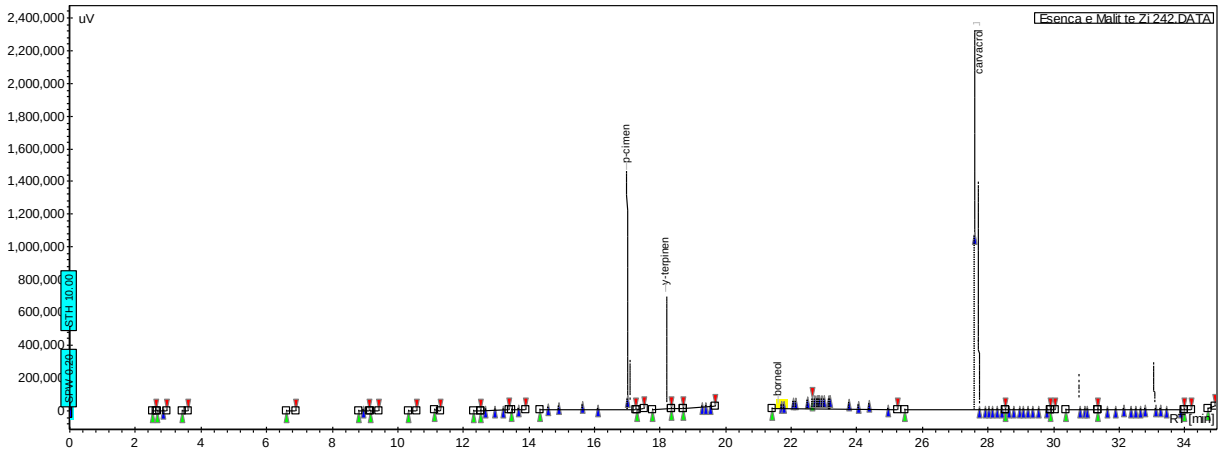
Second Method



Peak results :

	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
	p-cimen	16.95	32.78	1443857.7	502785.9	32.776
	y-terpinen	18.47	30.87	1432663.1	473493.6	30.867
	borneol	21.70	0.90	68430.7	13760.2	0.897
	timol	25.84	1.14	77418.6	17536.4	1.143
	carvacrol	27.02	33.58	1066013.6	515184.0	33.585
Total			100.00	4219382.0	1533984.2	100.000

Figure 26. GC-FID Chromatograms of standards with second method

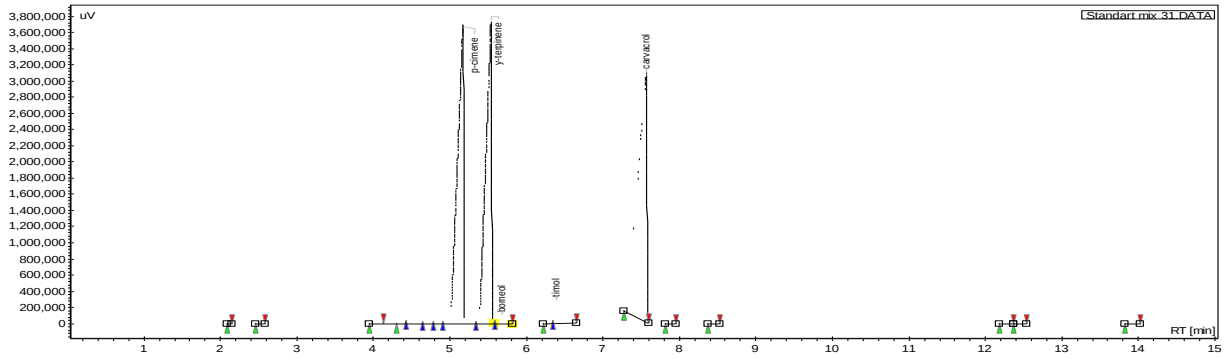


Peak results :

	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
	p-cimen	16.99	15.63	1463469.4	553811.3	15.632
	y-terpinen	18.18	4.26	713417.6	150777.5	4.256
	borneol	21.61	0.16	29479.7	5546.4	0.157
	carvacrol	27.57	64.83	2351888.6	2296784.2	64.831
Total			100.00	9019636.6	3542738.6	100.000

Figure 27 . GC-FID Chromatograms of *Satureja Montana* M_4 essential oils with second method

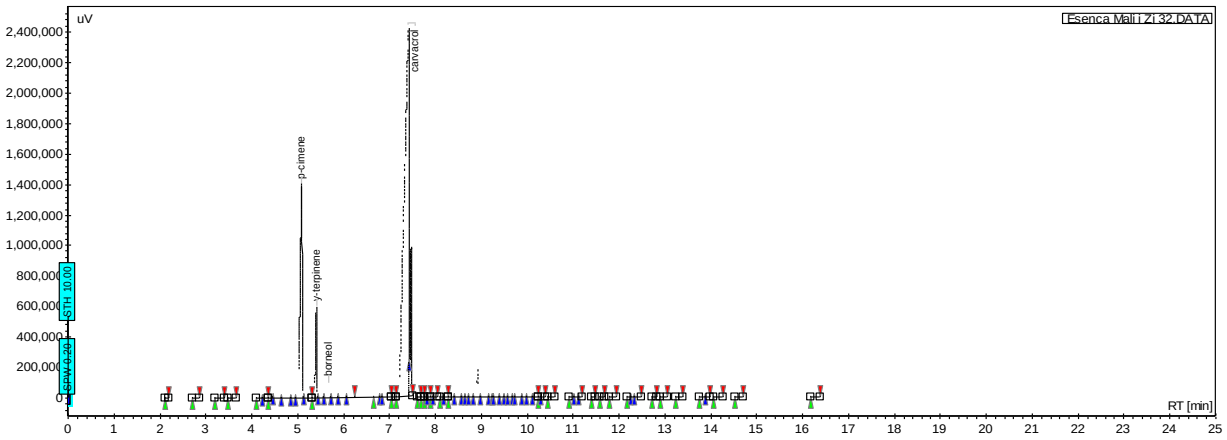
Third Method



Peak results :

	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
	p-cimene	5.17	30.44	3696089.7	335666.9	30.436
	y-terpinene	5.54	29.22	3746505.7	322289.4	29.223
	borneol	5.68	0.24	94135.5	2621.2	0.238
	timol	6.40	1.08	284537.3	11897.8	1.079
	carvacrol	7.56	38.77	3080362.0	427577.3	38.770
Total			100.00	10950572.4	1102853.3	100.000

Figure 28 . GC-FID results of standards



Peak results :

	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
	p-cimene	5.09	14.69	1403968.9	68072.2	14.687
	y-terpinene	5.41	3.93	600081.9	18200.2	3.927
	borneol	5.67	0.76	92925.5	3513.4	0.758
	carvacrol	7.42	62.97	2434205.2	291830.0	62.966
Total			100.00	6955483.4	463470.9	100.000

Figure 29 . GC-FID of *Satureja Montana* M_4 essential oil

Chemical composition of S.montana, R.officinalis, M.communis, O.vulgaris, S.officinalis essential oil is done by my Albanian research group and datas are taken from these studies in order to compare their chemical contents with biological properties.

Table 6. Chemical composition of *Origanum vulgaris* GC-MAS (my group studies)

Component	RI	Origanum Vulgaris
D - thujene	931	0.15
D - pinene	939	0.90
camphene	953	0.12
1- octen-3-ol	973	-
E - pinene	980	0.20
myrcene	991	1.37
D - phellandrene	1005	0.65
G – 3 - carene	1011	0.02
D - terpinene	1018	1.21
p - cymene	1026	6.74
limonene	1030	0.44
1.8 - cineole	1033	0.25
E - ocimene	1043	0.12
γ - terpinene	1062	3.75
terpinolene	1088	0.12
linalool	1098	2.55
borneol	1165	0.35
terpinen – 4 - ol	1177	0.55
D - terpineol	1189	0.95
methyl eugenol	1235	0.23
bornyl acetate	1285	0.01
geraniol	1255	1.22
thymol	1290	5.20
carvacrol	1298	54.35
eugenol	1356	0.15
geranyl acetate	1362	0.12

Table 7. Chemical composition of *Rosmarinus Officinalis* Gas/Mas (my group studies)

Compound	Rosmarinus Officinalis
α-pinene	17.19
camphene	2.90
β-pinene	0.75
myrcene	1.26
felandrene	0.74
α-terpinene	0.34
p-cymene	0.38
limonene	2.94
1,8- cineole	16.67
γ-terpinene	0.72
terpinolene	1.17
linalool	3.08
camphor	12.92
borneol	8.06
terpinen-4-ol	2.38
α-terpineol	1.94
verbenone	8.29
thymol	9.52
carvacrol	0.59
acetate borneili	3.15

Table 8. Chemical composition of *Salvia Officinalis* GC-MAS (my group studies))

Component	RI	Salvia Officinalis
α- thujone	931	34.5
β - thujone	939	5.8
camphor	953	37.2
sabiene	976	0.8
b - pinene	980	3.41
myrcene	991	1.31
b-cariophilen	1022	1.10
a - terpinene	1018	1.16
α-humulen	1025	2.75
d-limonene	1030	1.14
1.8 - cineole	1033	25.6
E - ocimene	1043	0.12
β-myrcen	1055	0.52
terpinolene	1088	0.06
linalool	1098	2.55
borneol	1165	1.72
terpinen – 4 - ol	1177	0.55
geranyl acetate	1362	0.12

Table 9. Chemical composition of *Satureja montana* Gas/Mas (another project)

Compound	Satureja montana
α-pinene	1.56
camphene	1.44
β-pinene	0.75
myrcene	0.94
felandrene	7.91
α-terpinene	0.34
p-cymene	0.38
limonene	2.94
1,8- cineole	16.67
γ-terpinene	0.72
terpinolene	1.17
linalool	0.71
camphor	12.92
borneol	8.06
terpinen-4-ol	2.38
α-terpineol	1.94
verbenone	8.29
thymol	9.52
carvacrol	59.0
acetate borneili	3.15

Genius *Origanum Vulgaris*

The main components of *Origanum vulgaris* were p-cymen (6.74), y-terpiene (3.75), borneol (0.35), thymol (5.2), carvacrol (54.35), (Tabel 6, Figure 31). Terpenoids have more higher levels as terpens.

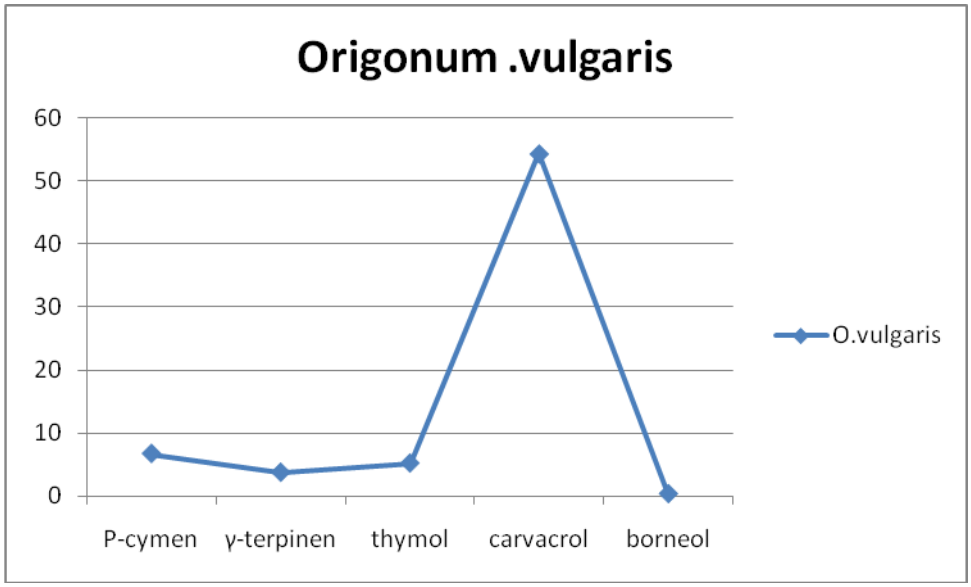


Figure 30 . *O.Vulgaris* main components composition

Genius *Rosmarinus Officinalis*

The main components of *Rosmarinus officinalis* were p-cymen (0.38), y-terpiene (0.72), borneol (8.06), thymol (9.52), carvacrol (0.59), (Tabel 6, Figure 31). This essential oil is more rich with campor (12.92), cineol (16.67), α-pinene (17.19). Compare to *Origanum vulgaris* essential oil, *Rosmarinus officinalis* has lower concentration of carvacrol and thymol. (Table 7, Figure 32) so is less richer with terpenoids.

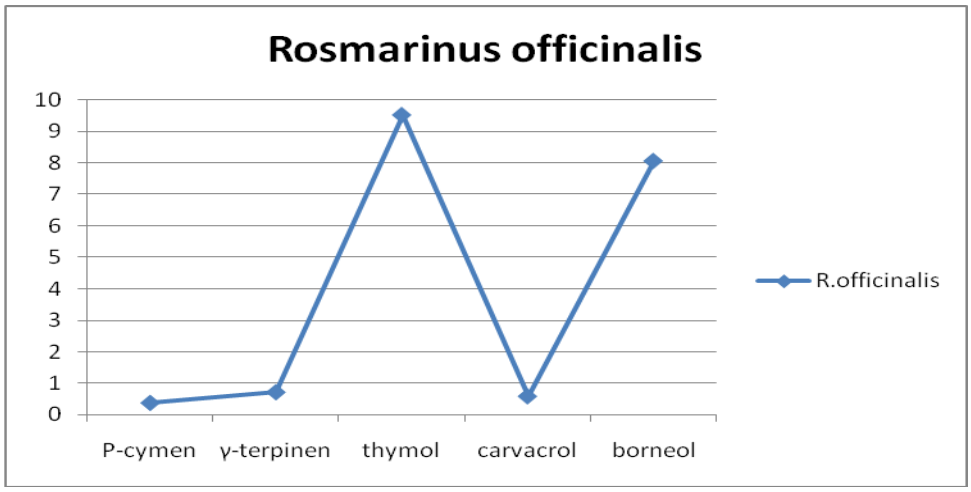


Figure 31. *R.officinalis* main components composition

Genius *Salvia Officinalis*

The main components of *Salvia officianlis* were α-thujone (12.2–49.3%), β-thujone (3.1–10.5%) camphor (13.7–37.8%) and 1,8-cineole (3.9–23-4%). Compare to *other* essential oil, it has no evidence concentration of carvacrol and thymol, p-cymen, y-terpiene and p-cymen (Table 8, Figure 33).

Genius *Satureja montana*

Satureja montana chimica contents is studied by GC-FID analytical method. Main components of *Satureja motanana* essential oil are shown in Table 9, p-cymen, y-terpienen, borneol, thymol, carvacrol. All the sample are rich with carvacrol M_1 (61.1), M_2 (67.68), M_3 (46.18), and M_4 (64.22). Carvacrol is followed by p-cymen, thymol , y-terpinen and borneol.The sample M_2 has has higher carvacrol and thymol level the other samples followed by M_4. Satureja montana essential oil has high level of carvacrol and thymol, besides this it the richest with terpenoids then other plants studied here.

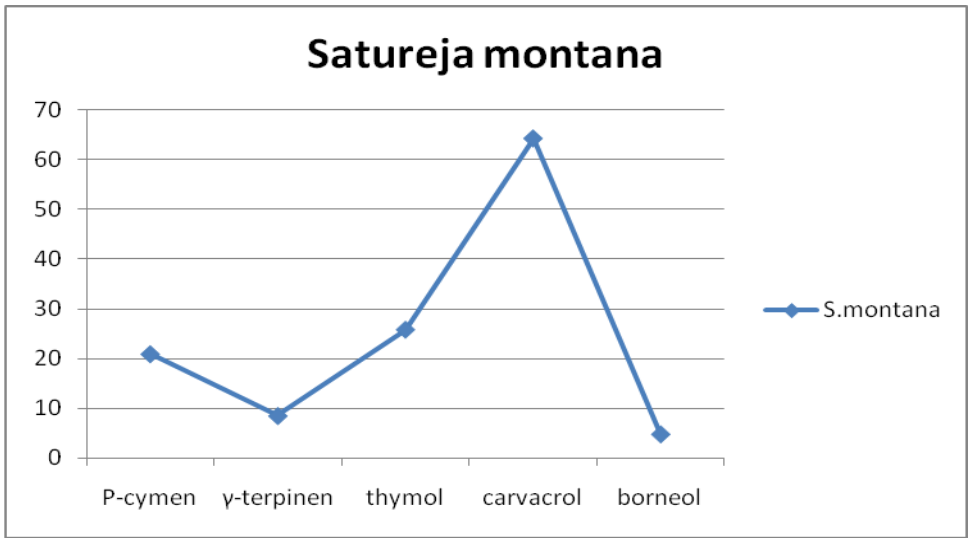


Figure 32 *S.montana* main components composition

Variation of *Satureja montana* main components

Table 10. Main componentns of essential oils of *Satureja Montana*

	(M_1)	(M_2)	(M_3)	(M_4)
p-cymen	20.86	5.9	11.04	9.61
y-terpienen	2.05	8.45	3.07	2.86
borneol	0	0.2	0.67	4.76
thymol	6.2	1.82	3.94	25.78
carvacrol	6.11	67.68	46.18	64.22

Genius *Myrtus communis*

Myrtus communis essential oil is not very rich with terpens and terpenoids. It has not considerable levels of carvarol, thymol, borneol y-terpiene. In the oil obtained from plants harvested in the wild the fraction of monoterpene hydrocarbons represented 28.9%, oxygenated monoterpenes represented 61.6% of die whole oil, whereas die sesquiterpene fraction represented 1.8% and other compounds 0.2% of the total oil composition.(Katarina P. Savikin-Fodulovic[a], Vanja M. Bulatovic[a], Nebojsa R. Menkovic[a] & Dragoljub V. Grubisic[b], Journal of essential oil, pages 75-78)

4.3 Method validation

4.3.1 Linearity

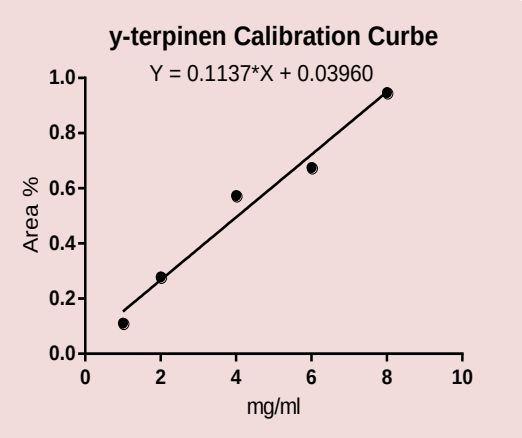


Figure 33. Calibration curve for γ-terpinene

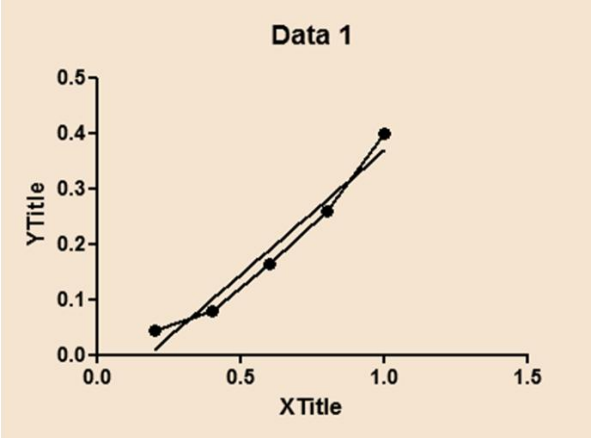


Figure 34 .Calibration curve for carvacrol

γ-terpineni	
Concetration	**Area**
1mg/ml	**0,111**
2mg/ml	**0,279**
4mg/ml	**0,574**
6mg/ml	**0,675**
8mg/ml	**0,947**
Correlation Coeficent	**r=0,9929,r2=0,9759,p=0.02**

Figure 35 .Serial dilution of γ-terpinene

Carvacrol		
concentration	***Area***	***Time***
1mg/ml	***0.400***	***26.25***
0.8 mg/ml	***0.260***	***26.11***
0.6 mg/ml	***0.164***	***26.12***
0.4 mg/ml	***0.078***	***26.12***
0.2 mg/ml	**0.043**	26.12
Correlation coefficent	**r= 0.9880, p=0.02**	

Figure 36 .Serial dilution of carvarol

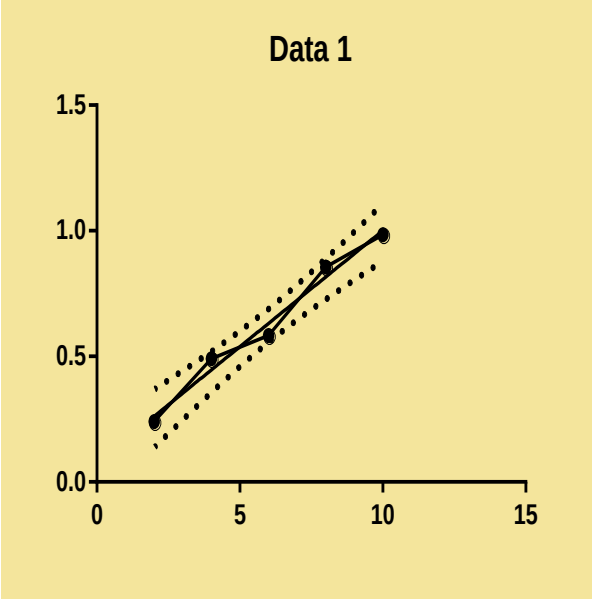


Figure 38 . Calibration curve for thymoFigure

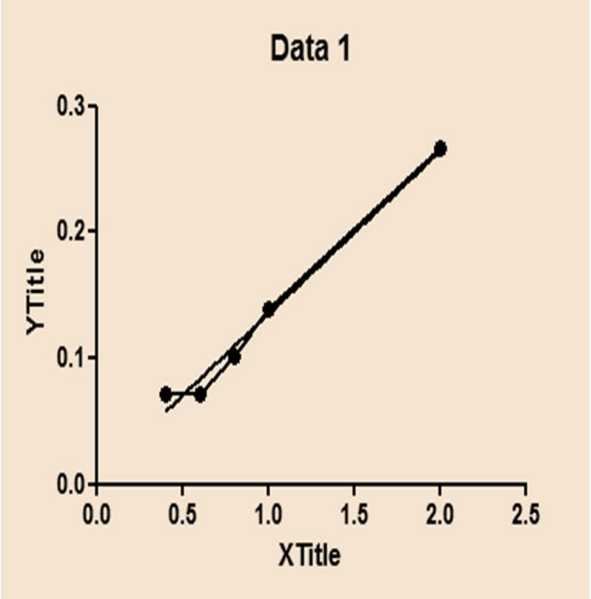


Figure 37. Calibration curve for p-cymen

Thymol	
Concentration	Area
10mg/ml	0.984
8 mg/ml	0.855
6 mg/ml	0.583
4 mg/ml	0.491
2 mg/ml	0.241
Correlation coeficent	0,9963 p.=0.001

Figure 39. Serial dilution of thymol

p-Cymen	
concentration	Area
2mg/ml	0.266
1mg/ml	0.139
0.8 mg/ml	0.084
0.6 mg/ml	0.071
0.4 mg/ml	0.072
Correlation coeficent	r=9825, r2=9914 p=0.010

Figure 40. Serial dilution of p-cymen

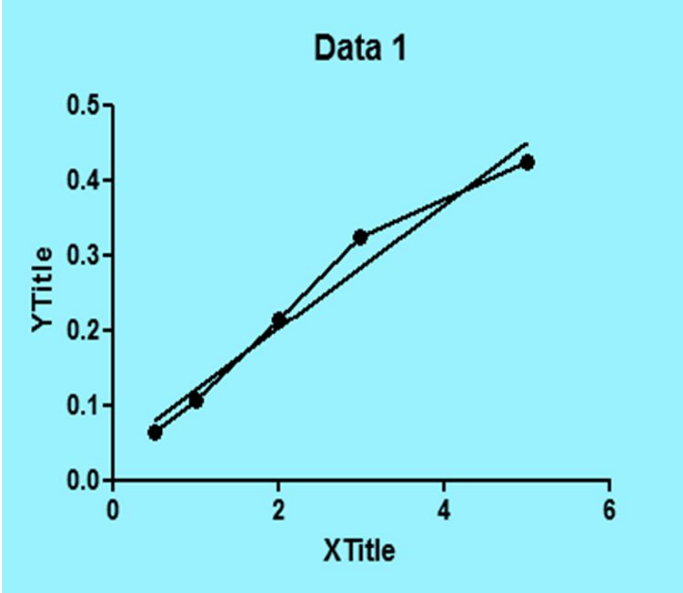


Figure 41. Calibration curbe for borneol

Borneol	
concentration	**Area**
0.5mg/ml	**0.065**
1mg/ml	**0.107**
2 mg/ml	**0.215**
3 mg/ml	**0.374**
5 mg/ml	**0.423**
Correlation coeficent	**r=9559, r2=9843 , p=0.024**

Figure 42. Seria dilution of Borneol

4.3.2 Statistical analyses

Table 11. It shows slope, residual standard and intercept

Component	Slope	r	R^2	p
Borneol	0.08463 ± 0.015	0.9559	0.9138	0.0110
y-terpineni	0.1137 ± 0.0103	0,9929	0,9759	0.0016
Carvacrol	0.4205 ± 0.0975	0.9880	0.9885	0.0200
p-cymen	0.1307 ± 0.0143	0.9825	0.9653	0.0028
Thymol	0.0925 ± 0.0073	0.9963	0.9813	0.0011

4.3.3 Precision and Accuracy study

Table 12. RI and %Area of main components of *S.montana* essential oil injected 5 time within day.

Then we injected 1 µl five times in the same day.

P-cymen		
Time (min)	**Area (%)**	**SD of Area**
16,42	0,050	SD. ±0.04960
16.30	0.123	
16.48	0.087	
16.30	0.164	
16.34	0.164	

z/terpinen		
Time (min)	**Area (%)**	**SD**
17,90	0,182	SD. ± 0.03355
17.78	0.469	
17.93	0.322	
17.79	0.594	
17.88	0.611	

Borneol		
Time (min)	**Area (%)**	**SD**
21.62	0.089	SD. ± 0.03001
21.60	0.133	
21.69	0.053	
21.65	0.089	
21.63	0.069	

Thymol		
Time (min)	**Area (%)**	**SD**
25,88	0,418	SD. ± 0.31018
25.78	0.114	
25.87	0.790	
25.78	0.475	
25.97	0.289	

Carvacrol		
Time (min)	**Area (%)**	**SD**
26,13	0,055	SD. ± 0.09955
26.05	0.297	
26.13	0.099	
26.05	0.058	
26.16	0.124	

Injection in three consecutive days 2 Mars. 3 Mars and 4 Mars 2011

Table 13. RI and %Area of main components of *S.montana* essential oil injected 3 consecutive days.

P-cymen		
Time (min)	**Area (%)**	**SD**
16.29	0.069	SD. ± 0.01007
16.23	0.061	
16.36	0.081	
z-terpinen		
Time (min)	**Area (%)**	**SD**
17.76	0.244	SD. ± 0.03821
17.71	0.213	
17.86	0.289	
borneol		
Time (min)	**Area (%)**	**SD**
21.59	0.051	SD. ± 0.03208
21.54	0.054	
21.68	0.108	
thymol		
Time (min)	**Area (%)**	**SD**
25.75	0.901	SD. ± 0.24077
25.71	0.995	
25.96	0.539	
carvarol		
Time (min)	**Area (%)**	**SD**
26.03	0.157	SD. ± 0.02829
25.99	0.213	
26.17	0.178	

Table 14. Precision nd Accuracy s total results of *Satureja montana* essential oils.

		In Day **N=5**			**Between day** **3 days, n=5**		
	Sample mg/ml	Found	Recovery	R.S.D %	Found	Recovery	R.S.D %
Carvacrol	0.41	0.42±0.02	102 %	2.4	0.41±0.02	100%	2.3
Thymol	1.60	1.59 ±0.02	99.3 %	0.62	1.58 ±0.04	96.6%	0.62
Borneol	0.10	0.09 ±0.03	92%	0.85	0.1 ±0.03	100%	0.86
Y-terpinen	0.45	0.43±0.04	89 %	2.2	0.42 ±0.01	93.3%	2.1
P-cymen	0.45	0.44 ±0.01	97.7%	2.1	0.43±0.05	97.7%	2.2

4.3.4 Robustness

1- **Change oven temperature**. We changed the oven temperature from 280 °C to 290 °C

2- **Change the flow rate** from 30 ml/min to 25 ml/min

In both two cases we didn't have statistically differences in results obtained conducting recovery at different level of thymol and the average percentage and recovery was found to be in the range

4.2.5 Limit of quantitation

Table 15 .It shows limit of quantitation

Compound	LOD
Carvacrol	0.6 mg/ml
Thymol	1.2 mg/ml
Borneol	0.5 mg/ml
γ-Terpinen	0.6 mg/ml
p-Cymen	0.3 mg/ml
Carvacrol	0.2 mg/ml

GC-FID Method validation for *Satureja Montana* essential oil

During the method validation process we found that Calibration curbes of p-cymen, borneol, carvacrol, γ-terpinen and thymol against their corresponding concentration were linear, (Figure 35, 36, 37, 38, 39, 40, 41, 42, 43, 44) . Statistical analyses are done by Gaph Pad version 6. Their correspondative slope, r, R^2 and p value are shown in table 10 were signicative(0.99-0.95). In addition Presision were and Accuracy study results are shown Table 11 , Table 12, Table 13). Recovery and RSD were respectively for carvacrol 102% and 2.3, thymol 99.3 % and 0.62 , Borneol 92 % 0.86, y-terpienen 89% and 2.1 , P-cymen 97.7 % and 2.2. These value are statistically accepted. The robustness test showed that even small changes of temperature or smoll changes in flow rate dosent effect the GC-FID results. Limit of quantification was found for carvarol 0.6mg/ml, thymol 1.2 mg/ml, borneol 0.5mg/ml, y-terpiene 0.6 mg/ml, p-cymen 0.3 mg/ml, carvacrol 0.2 mg/ml . .(Tabel 12, 13,14).

4.4 Biological Activities of Essential oils

Biological effects

Antibacterial activity – *Satureja montana* essential oil

The antibacterial activity were studied about the *Satureja montana* in different samples. essential oil versus *Proteus vulgaris* colony, *Escherichia coli* colony and *Staphylococcus aureus* colony disc diffusion method. Essential oils are applied in two different concentrations **10** µL and **30** µL. The results of inhibition zone in (mm) are shown in Table 15. The results showed up high inhibition zones of all samples of essential oil comperable to inhibition zone of positive controls (cefuroxime, cyprofloxacini and tetracicline) even in low concetrations (**10** µl) . It was observed that the essential oils with high levels terpenoids like M_1 and M_4 have higher antibacterial activity.

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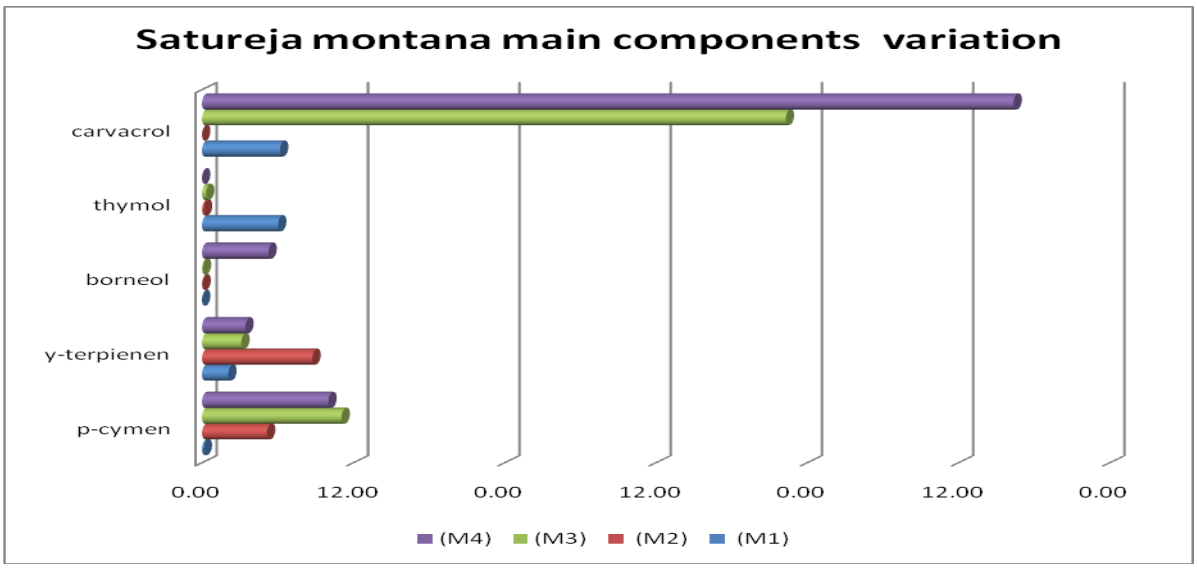
Variation of *Satureja montana* main components

Table 16. Main components of essential oils of *Satureja Montana*

	(M_1)	**(M_2)**	**(M_3)**	**(M_4)**
p-cymen	20.86	5.9	11.04	9.61
y-terpienen	2.05	8.45	3.07	2.86
borneol	0	0.2	0.67	4.76
thymol	6.2	1.82	3.94	25.78
carvacrol	6.11	67.68	46.18	64.22



4.4.1 Antibacterial activity

Table 17. Antimicrobial activity of the *Satureja montana* L. essential oil different samples (M_1, M_2, M_3, M_4).Diameter of disc (6 mm). nt – non tested; Inactive (–); moderately active (7–12mm); highly active Antibiotics-Positive control (>13mm).

Essential oils samples	Bacterias	*Satureja montana*		Positive Control		
		10 μL	**30** μL	**Cyrofloxacini**	**Cefuroxim**	**Tetracyclini**
M_1						
	Proteus Vulgaris	32.0±0.05	34.2±0.07	27.0±0.14	29.0±0.04	23.0±0.08
	Escherichia coli	16.3±0.30	17.8±0.11	16.0±0.22	18.0±0.07	nt
	Staphylococcus aureus	24.8±0.61	26.4±0.15	29.5±0.25	28.0±0.11	17.1±0.14
M_2						
	Proteus Vulgaris	30.0±0.06	31.0±0.06	27.2±0.05	27.5±0.09	22.7±0.07
	Escherichia coli	19.0±0.07	22.8±0.17	16.9±0.03	18.6±0.06	nt
	Staphylococcus aureus	25.5±0.01	27.0±0.04	26.4±0.07	27.6±0.05	17.5±0.03
M_3						
	Proteus Vulgaris	39.1±0.06	41.3±0.13	28.5±0.15	27.9±0.06	21.9±0.04
	Escherichia coli	23.0±0.04	25.5±0.04	17.3±0.12	17.9±0.09	nt
	Staphylococcus aureus	25.2±0.01	26.2±0.05	27.0±0.55	26.9±0.19	19.0±0.08
M_4						
	Proteus Vulgaris	40.0±0.07	42.0±0.09	29.0±0.32	28.1±0.07	21.6±0.01
	Escherichia coli	21.5±0.02	24.3±0.05	16.5±0.08	19.0±0.04	nt
	Staphylococcus aureus	26.1±0.02	28.0±0.09	27.8±0.01	27.3±0.02	18.0±0.11

4.3.1.2 *Satureja Montana* essential oils inhibition zones vs *S. Aureus*

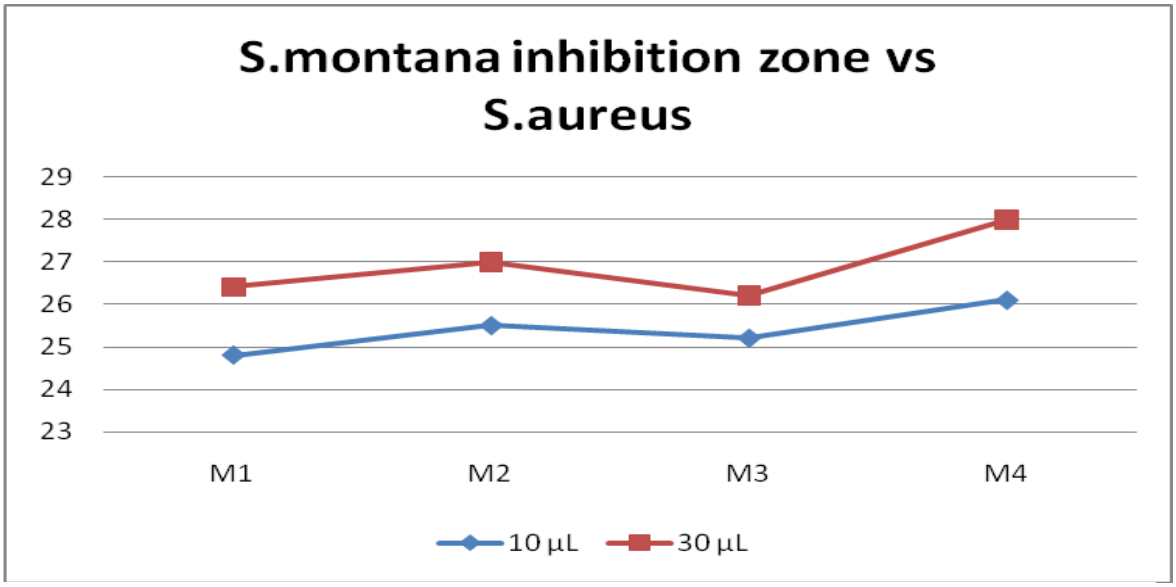


Figure 43 .*Satureja Montana* essential oils inhibition zone vs *S.Aureus*

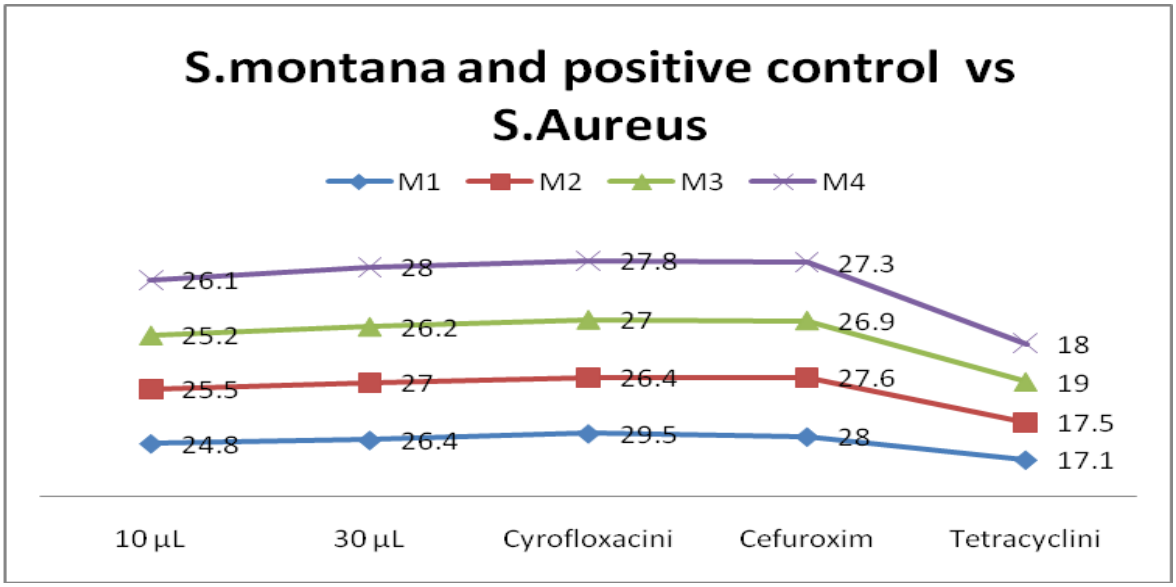


Figure 44. *Satureja montana* and postitive control /*S.Aureus*

Figure 45. Disc difussion method

***Satureja montana* essential oil versus *S.aureus* (** Figure 44, Figure 45, Figure 46**.)**

The antibacterial activity of *Satureja montana* essential oil agains *S.aureus* was high in all our the geographical samples in both concentrations a) **10** µl (M_1= 24.8±0.61, M2 = 25.5±0.01, M_3=25.2±0.01 and M_4=26.1±0.02) b) **30** µL (M_1= 26.4±0.15, M2 = 27.0±0.04, M_3=26.2±0.05, M_4= 28.0±0.09) compare to positive controls (cyprofloxacini=26.4 – 29.5) cefuroxim (26.9 – 28.0) and tetracycline (17.1 – 19.0). Sample M_4 has larger inhibition zone then others followed by sample M_2 maby because of their high levels of carvacrol and thymol (table 17).

***Satureja montana* essential oil versus *P.vulgaris* (Figure 48, Figure 49.)**

The antibacterial activity of *Satureja montana* essential oil agains *P.vulgaris* was high in all our the geographical samples in both concentrations a) **10** µl (M1= 32.0±0.05, M2 = 30.0±0.06, M3=39.1±0.06 and M4= 40.0±0.07) b) **30** µL (M1= 34.2±0.07, M2 = 31.0±0.06 M3=41.3±0.13, M4= 42.0±0.09) compare to positive controls (cyprofolxacini = 29.0 – 27.0), cefuroxime (26.9 – 28.0) and tetracycline (21.6 – 23.0). Sample M4 has larger inhibition zone then others followed by sample M3 maby because of their high levels of carvacrol and thymol.

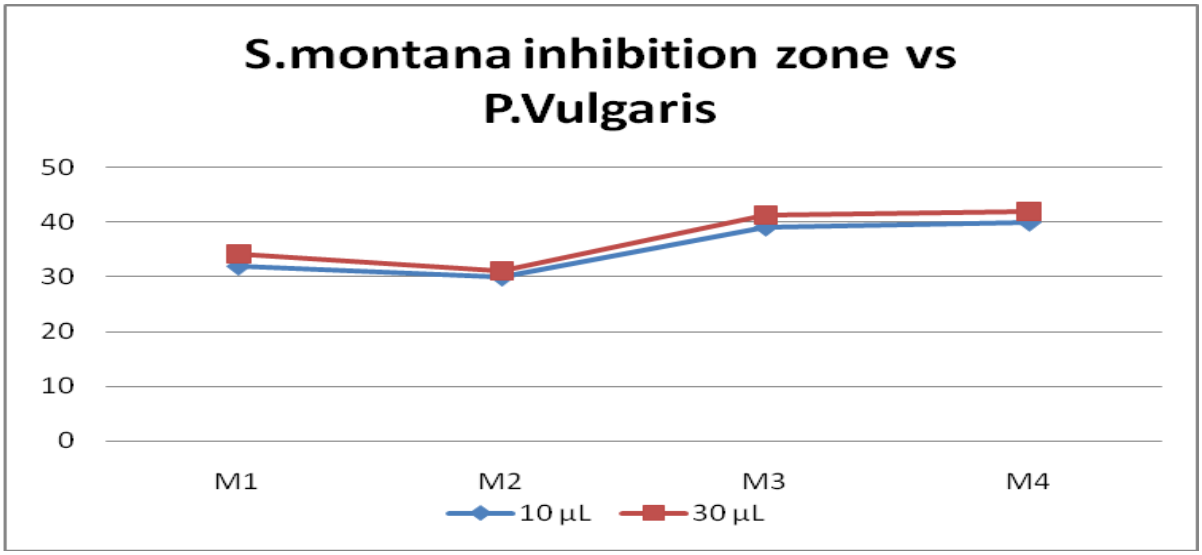


Figure 46. *Satureja Montana* essential oils inhibition zone / *P.vulgar*

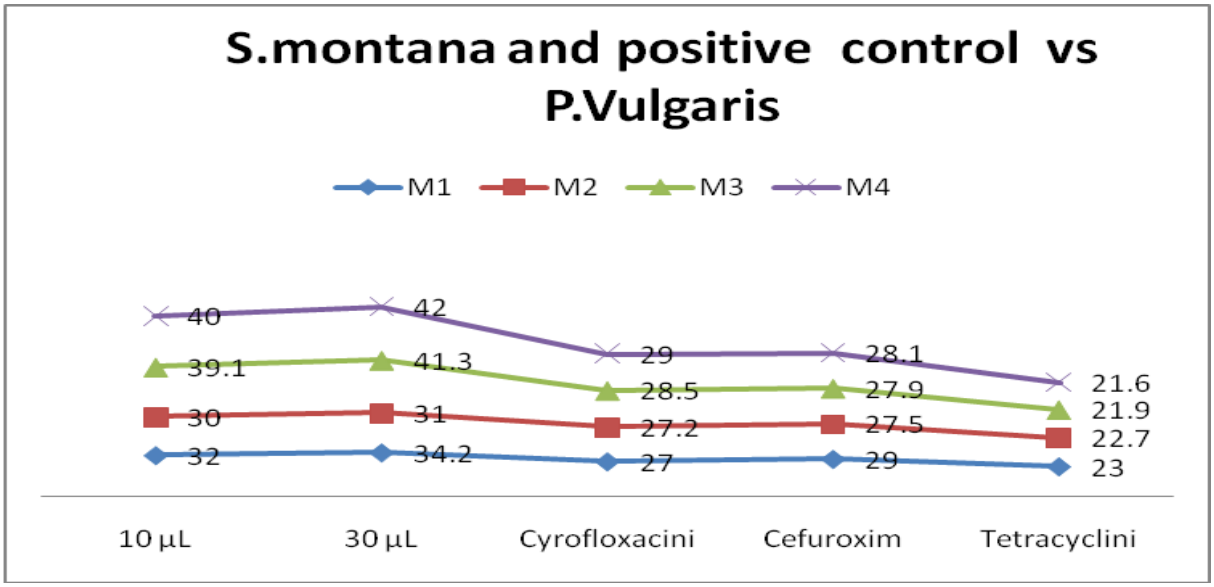


Figure 47. *S.montana* and Positive control-*P.Vulgaris*

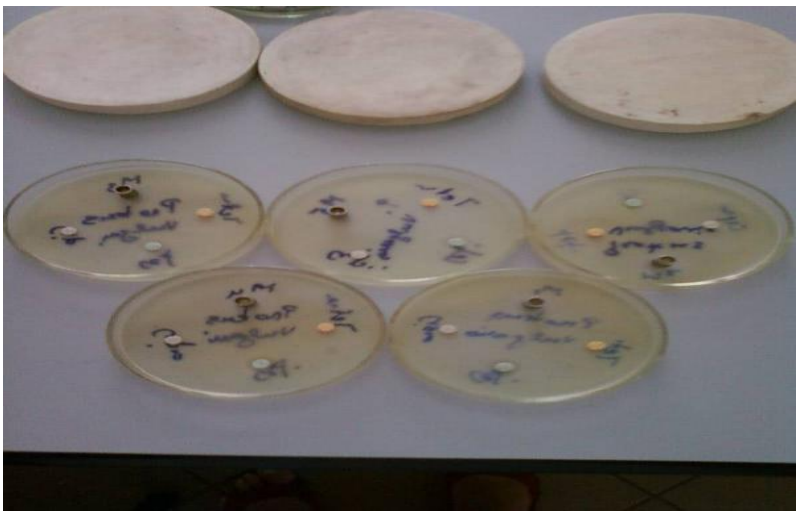


Figure 48. Petri dishes with *P.vulgaris*

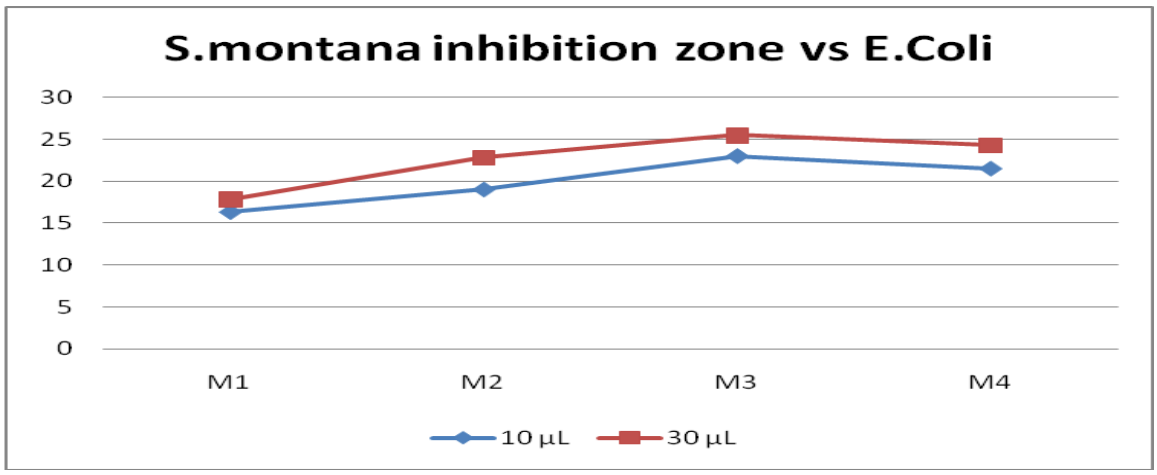


Figure 49. ***Satureja Montana*** **inhibition zone vs** ***E.Coli***

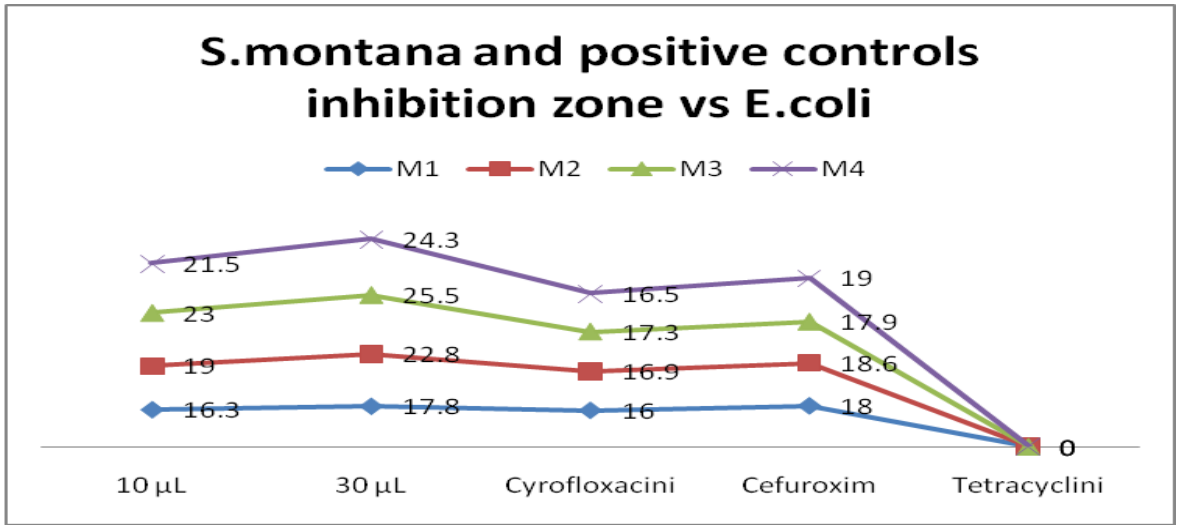


Figure 50. ***Satureja Montana*** **and positive control inhibition zone vs** ***E.Coli***

Satureja montana **essential oil versus** ***E.Coli*** **(Figure 51, Figure 52.)**

The antibacterial activity of *Satureja montana* essential oil agains *E.Coli* was high in all our the geographical samples in both concentrations a) **10** µl (M1= 16.3±0.30, M2 = 19.0±0.07, M3=23.0±0.04 and M4= 21.5±0.02) b) **30** µL (M1= 17.8±0.11, M2 = 22.8±0.17, M3=25.5±0.04, M4= 24.3±0.05) compare to positive controls (cyprofloxacini = 16.0 – 17.31), cefuroxime (17.9 – 19.0) and tetracycline (nt). Sample M4 has larger inhibition zone then others followed by sample M3 maby because of their high levels of carvacrol and thymol.

Satureja montana essential oils showed a high antibacterial activity **(>13mm)** especially was more sensible again *Proteus vulgaris* colony compare to positive control . Also it exhibited appreciable antimicrobial activities to *E.Coli* followed by *S.aureus* colony.

4.5 Antifungal Activity of essential oils

Antifungal activity *Origanum vulgaris, Rosmarinus Officinalis, Satureja Montana, Myrtus communis, Salvia officinalis* were tested again 8 colony Dermatophytes *M. Gypseum, M. canis, A. cajetani T.violaceum, T.mentagrophytes, E. floccosum, T. rubrum, T. tonsurans* and 2 colonies of phytopatogens *B.cinere,P.oryzae*

4.5.1 Antifungal activity of *Origanum vulgaris*

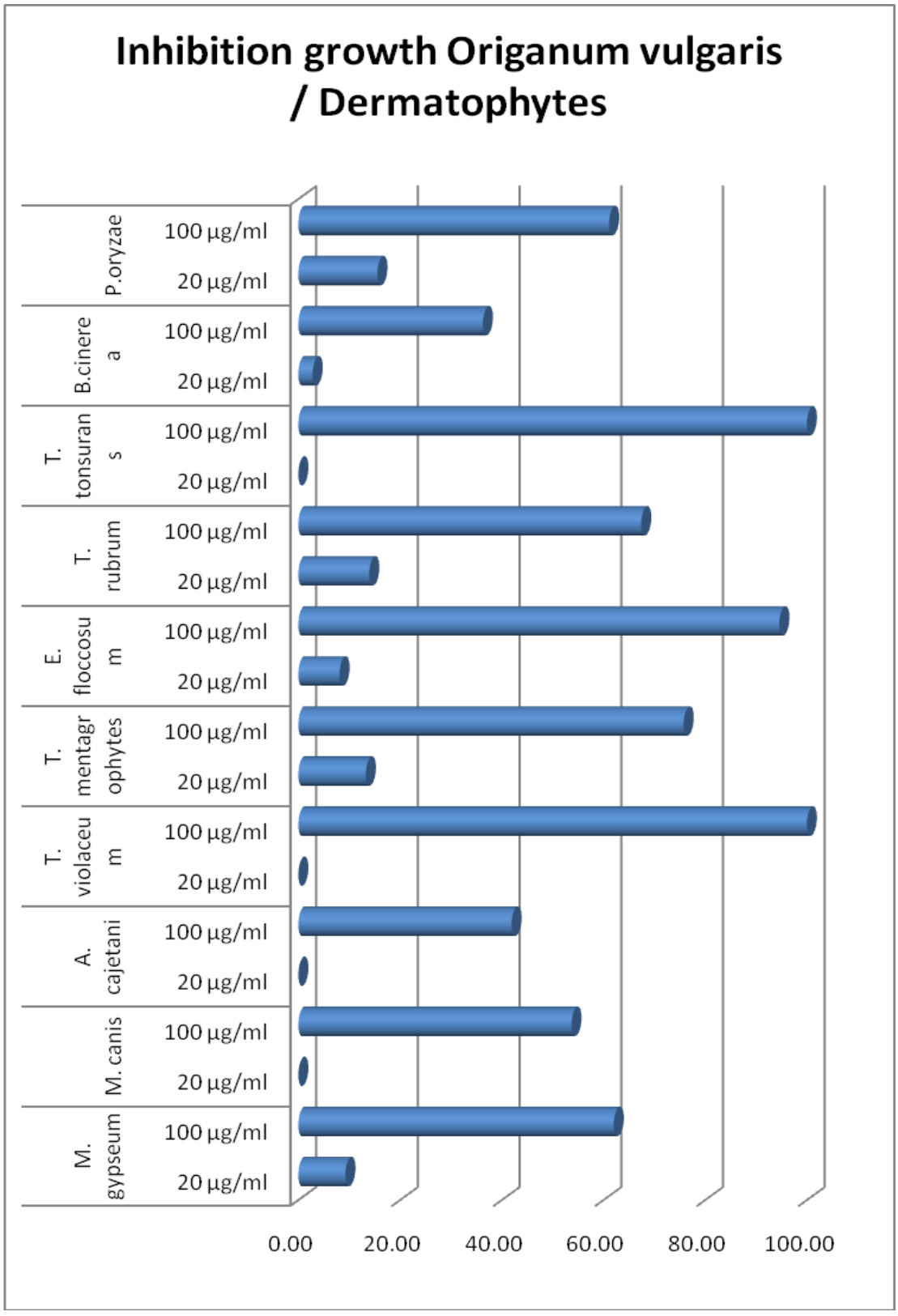


Figure 51. Inhibition groth (%) of *Origanum vulgaris* vs Dermatophytes (7 days) and Phytopatogens (5days)

***Origanum vulgaris* versus Dermatophytes (Figure 54)**

O.vulgaris essential oils showed a good performance again Dermatophytes and Phytopatogens colonies. It was more sensible again *T.violaceum*, follwed by *T.tonsuran*, *E.floccosum*, *T.mentagrophytes*, *M.gypseum*. On the other hand *B.cinere* colony was resistant to essential oil. It is evident that the MIC concentration should be around 20 µg/ ml as in many cases below this concentration the antifungal activity is not considerable. Meanwhile the 100 µl/ml concentration of essential oil is very sensible against all the above mentioned microorganisms.

Origanum vulgaris essential has high carvacrol and thymol amounts which can lead to the increases of antifungal activity of this essential oil. (Figure30)

4.5.2 Antifungal activity of *Salvia Officinalis*

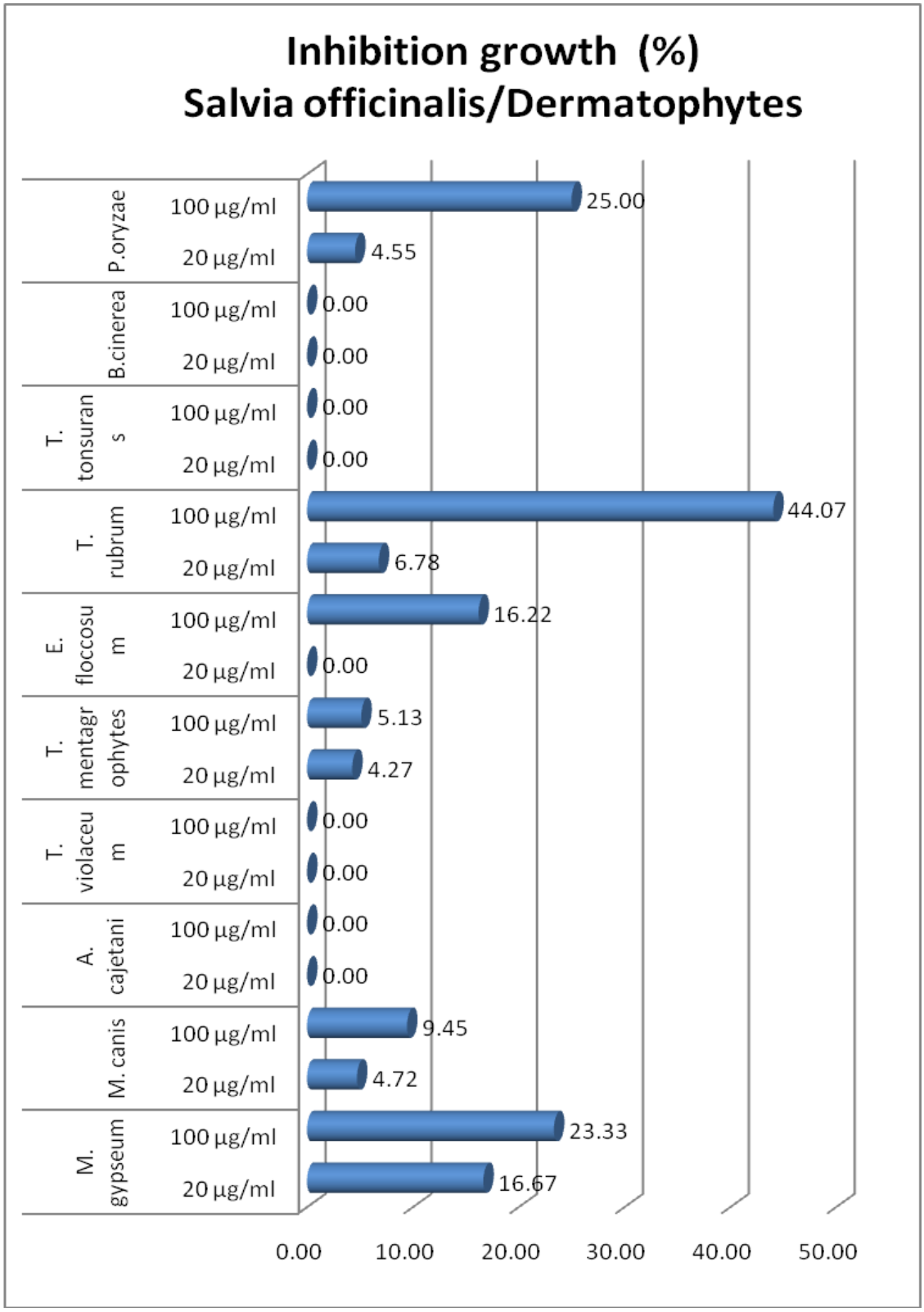


Figure 52. Inhibition groth (%) of *Salvia officinalis* vs Dermatophytes (7 days) and Phytopatogens (5days)

***Salvia officinalis* versus Dermatophytes (Figure 53)**

S.officinalis essential oils showed a weak performance again Dermatophytes and Phytopatogens colonies *T.rubrum*, followed *M.gypseum* and *E.Floccosum* colonies were sensible to essential oil and it's evident that concentration 100 µl/ml is more effective then 20 µl/ml. All other colonies were resistant to essential oil of *Salvia officinalis*. This resistance maby is caused from lower concentrations of carvacrol and thymol.(Table 53)

***Rosmarinuss officinalis* versus Dermatophytes (Figure 54)**

R.officinalis essential oils showed a low performance again Dermatophytes and Phytopatogens. The highest antifungal activity was again colony of *T.rubrum* (28.21 %) *T.vialceum* (20.45%). It's evident that concentration 100 µl/ml is more effective then 20 µl/ml. *M.Canis* (1.8 %) and *E.floccosum* (0.0 %) colonies were resistant to *R.officinalis* essential oil. The resistent colonies to this essential oil is explained by low levels of carvacrol and thymol

***Satureja montana* versus Dermatophytes (Figure 55)**

S.montana essential oils showed a very good performance again Dermatophytes and Phytopatogens. The highest antifungal activity was again colony of *T.vialceum* (100 %), followed by *T.rubrum* (93.55 %), *T.tonsurans* (83.33%), *M.metnagrophytes* (77 %). It's evident that concentration 100 µl/ml is more effective then 20 µl/ml. *M.Canis* (1.8 %) and *E.floccosum* (0.0 %) colonies were resistant to *S.montana* essential oil. The wide inhibition zone of essential oil is explained by high levels of carvacrol and thymol.

***Myrtus communis* versus Dermatophytes (Figure 56)**

M.communis essential oils showed a moderate performance again Dermatophytes and Phytopatogens. The highest antifungal activity was again colony of *M.mentagrophytes* (82 %), followed by *M.canis* (68.5 %). It's evident that concentration 100 µl/ml is more effective then 20 µl/ml. *T.violaceum*, *A.cajetani*, *T.rubrum*, *T.tonsurans* colonies were resistent to *M.communis* essential oil.

4.5.3 Antifungal activity of *Rosmarinus Officinalis*

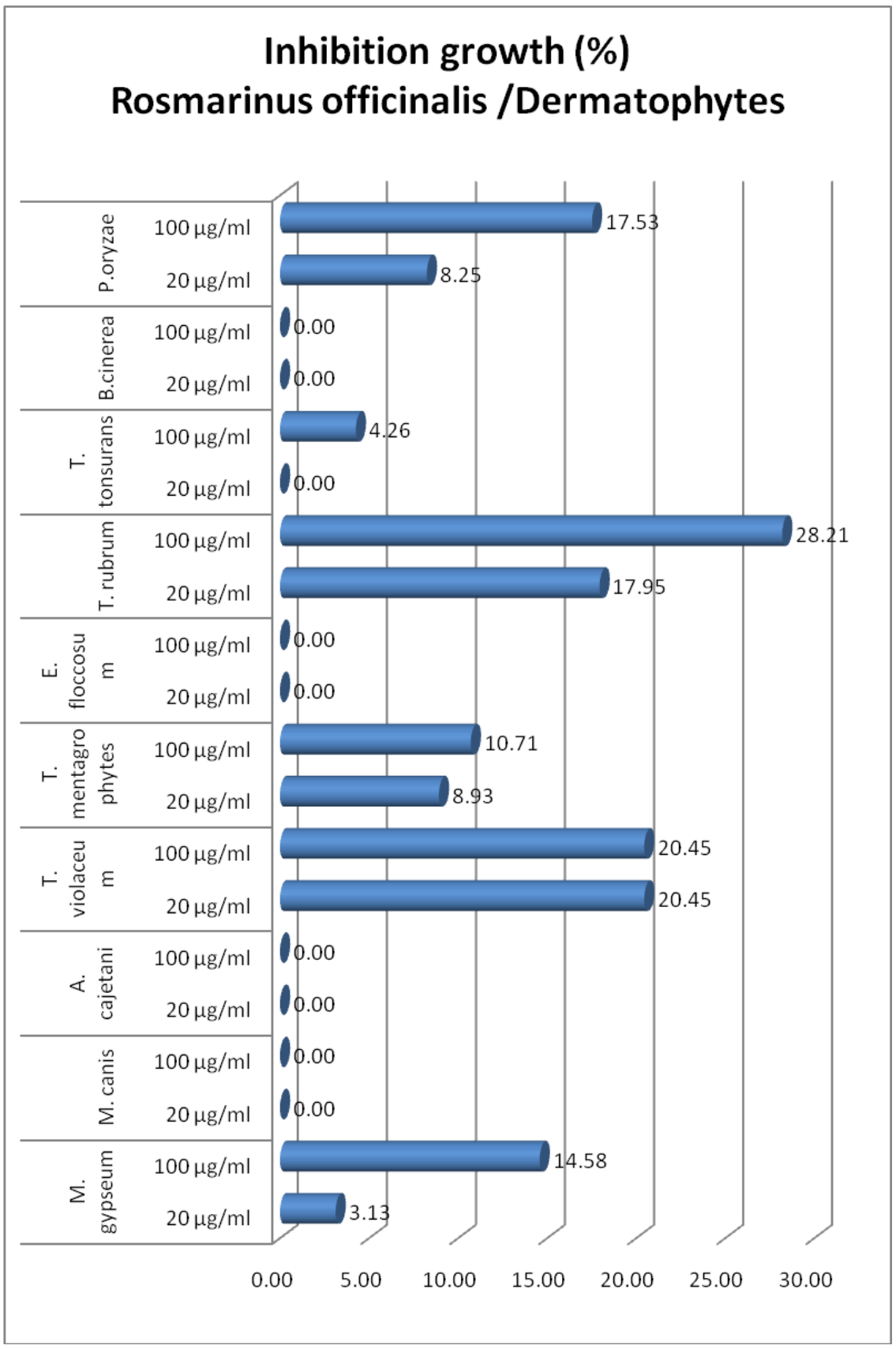


Figure 53. Inhibition growth (%) of *Rosmarinus officinalis* vs Dermatophytes and Phytopatogens (5days)

4.5.4 Antifungal activity of *Satureja montana*

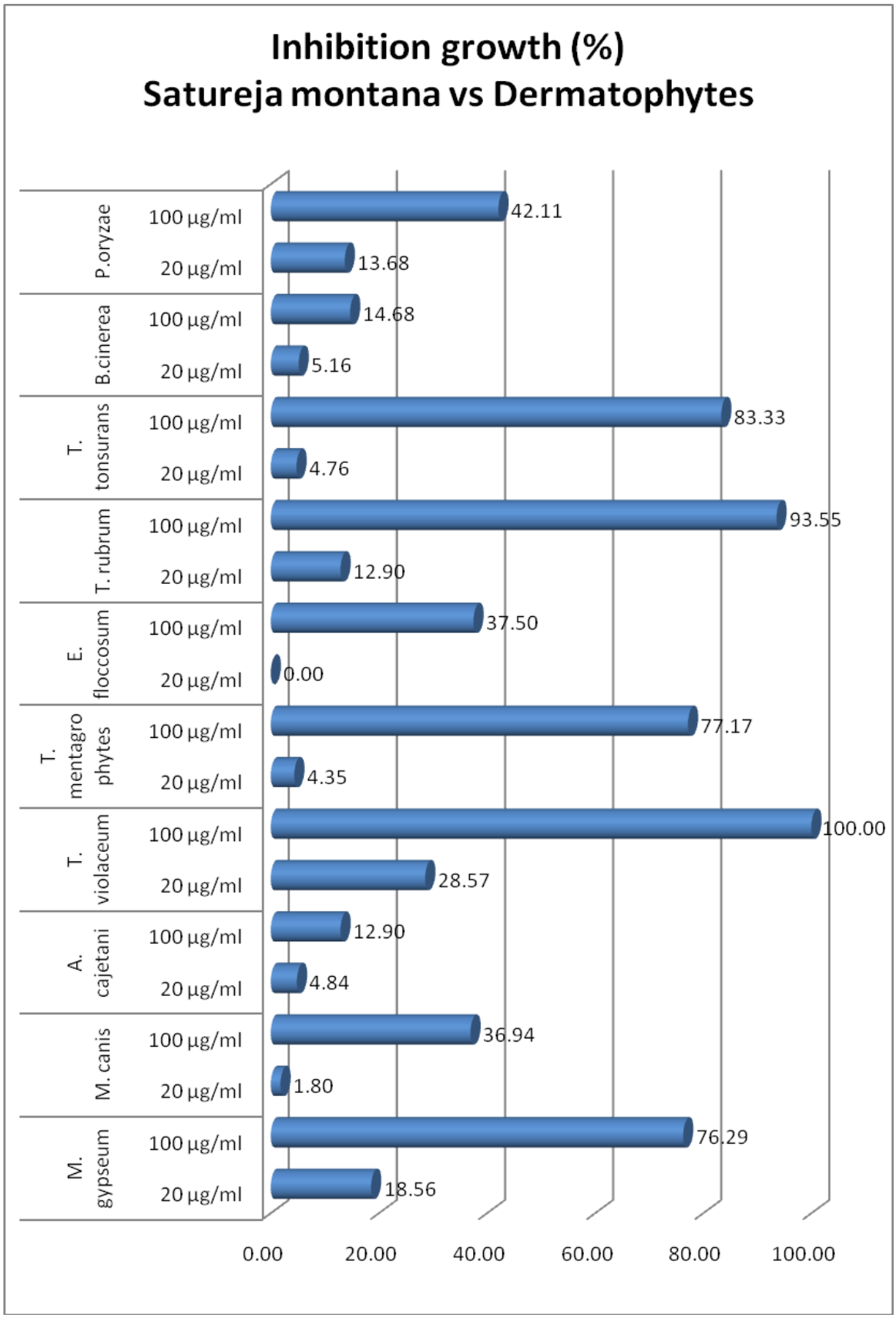


Figure 54. Inhibition growth (%) of *Satureja montana* essential oil vs Dermatophytes and Phytopatogens (5days)

4.5.5 Antifungal activity of *Myrtus communis*

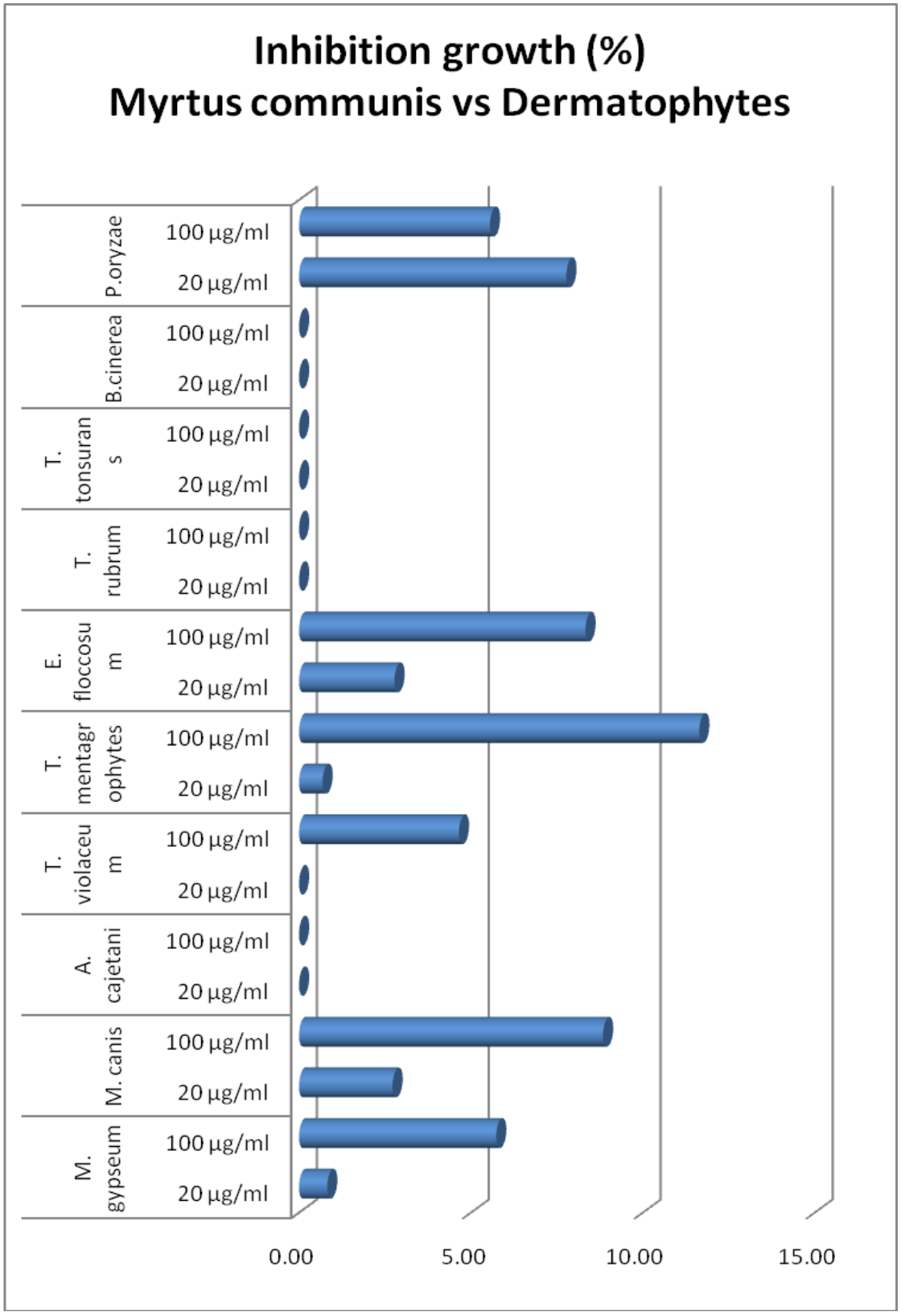


Figure 55. Inhibition growth (%) of *Myrtus communis* essential oil vs Dermatophytes (7 days) and Phytopatogens (5days)

4.5.6 Essential oil and Dermatophytes

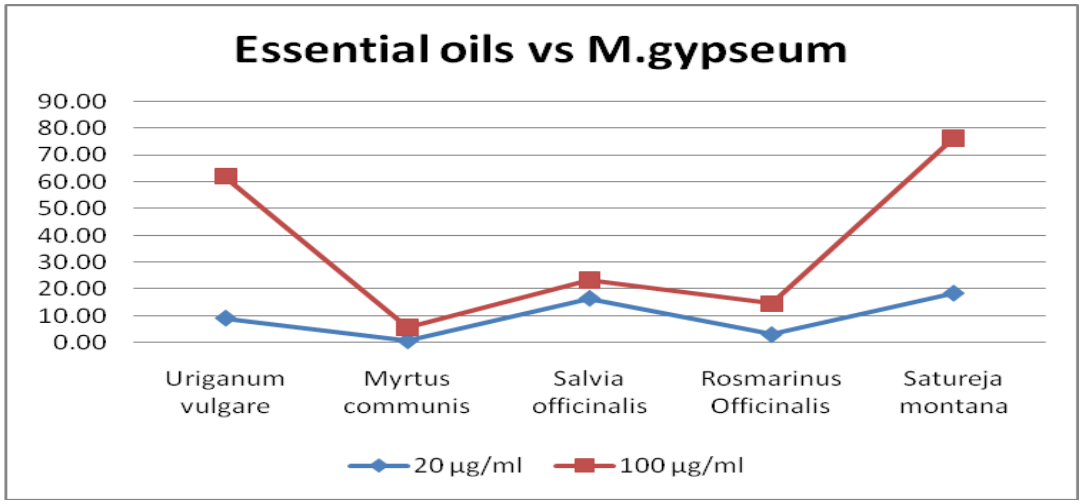


Figure 56. Variation of Inhibition growth (%) of *M.gypseum* colony versus *Origanum Vulgaris, Myrtus Communis , Salvia officinalis, Rosmarinus Officinalis*

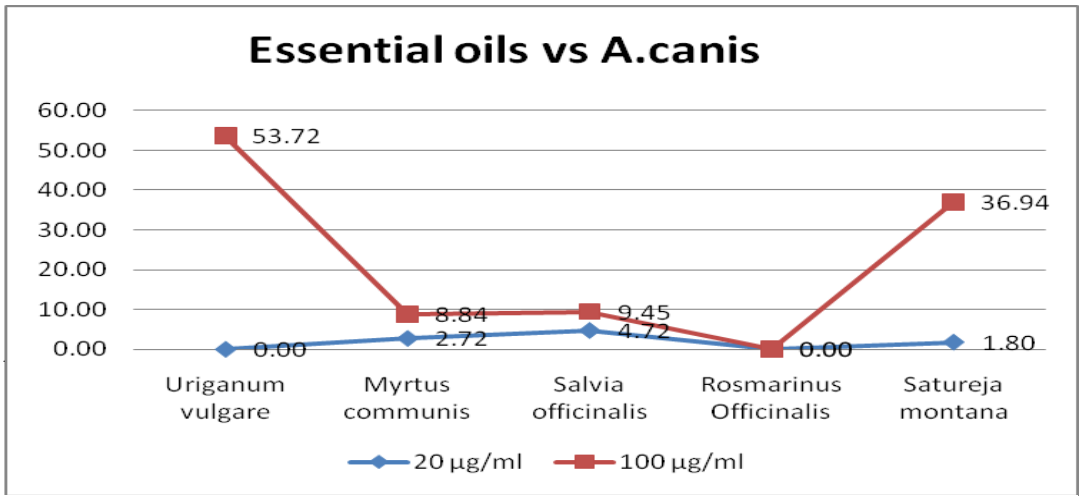


Figure 57. Variation of Inhibition growth (%) of *A.canis* colony versus *Origanum Vulgaris, Myrtus Communis , Salvia officinalis, Rosmarinus Officinalis*

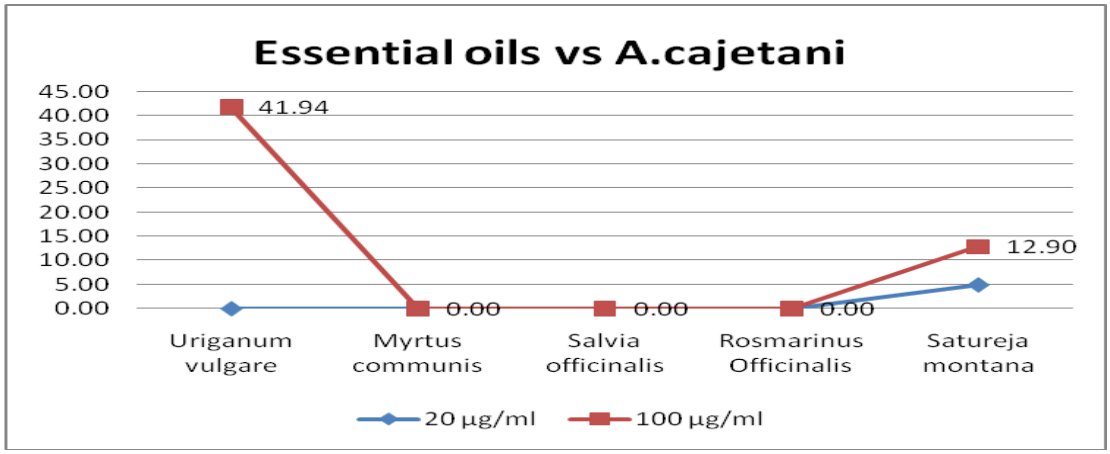


Figure 58. Variation of Inhibition growth (%) of *A.cajetani* colony versus *Origanum Vulgaris, Myrtus Communis , Salvia officinalis, Rosmarinus Officinalis*

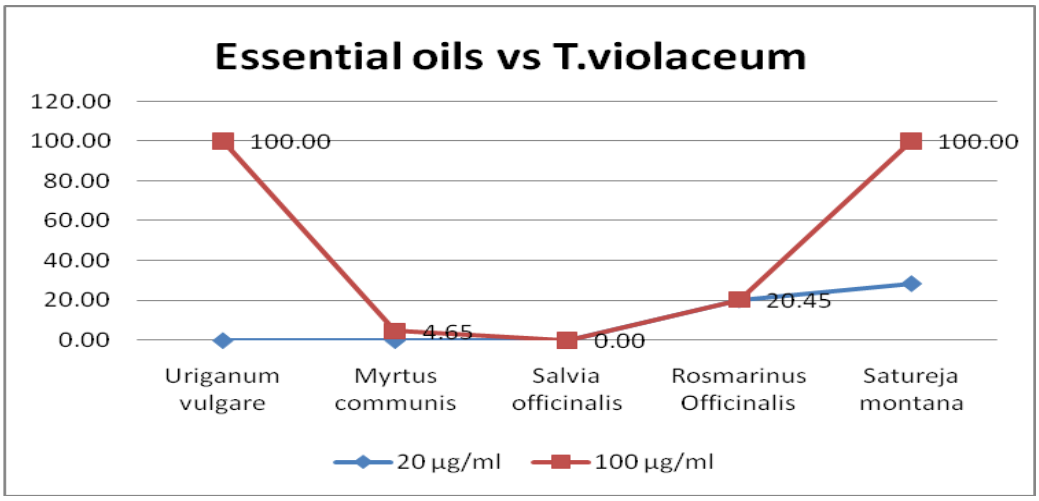


Figure 59. Variation of Inhibition growth (%) of *T.violaceum* colony versus *Origanum Vulgaris, Myrtus Communis, Salvia officinalis, Rosmarinus Officinalis*

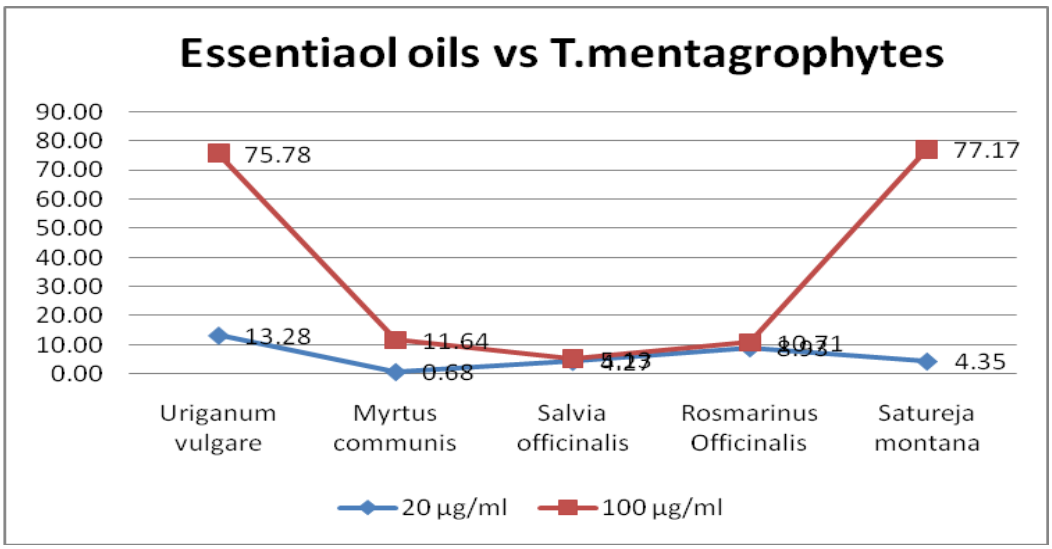


Figure 60. Variation of Inhibition growth (%) of *mentagrophytes* colony versus *Origanum Vulgaris, Myrtus Communis , Salvia officinalis, Rosmarinus Officinalis*

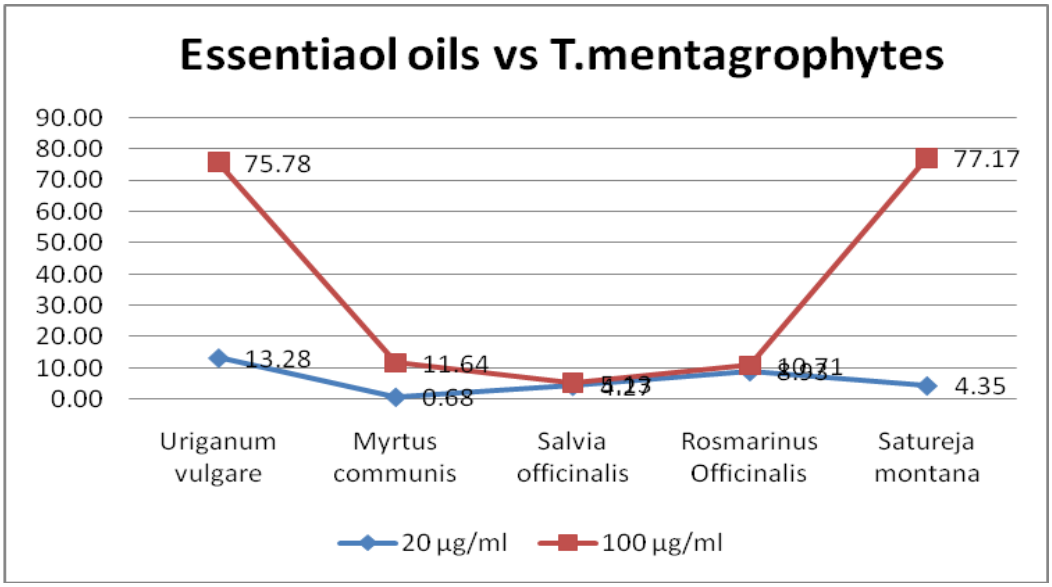


Figure 61. Variation of Inhibition growth (%) of *T.mentogrophytes* colony versus *Origanum Vulgaris, Myrtus Communis , Salvia officinalis, Rosmarinus Officinalis*

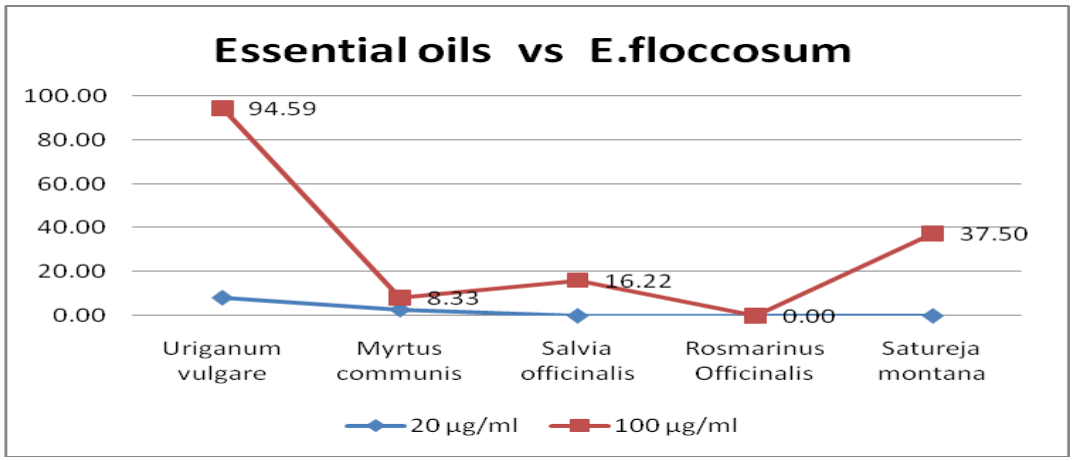


Figure 62. Variation of Inhibition growth (%) of *T.floccosum* colony versus *Origanum Vulgaris, Myrtus Communis , Salvia officinalis, Rosmarinus Officinalis*

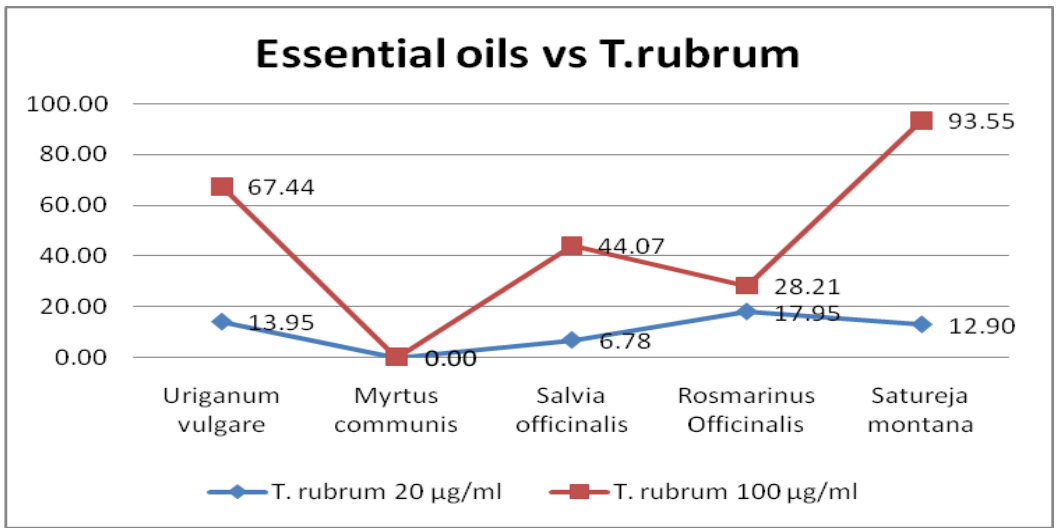


Figure 63. Variation of Inhibition growth (%) of *T.rubrum* colony versus *Origanum Vulgaris, Myrtus Communis , Salvia officinalis, Rosmarinus Officinalis*

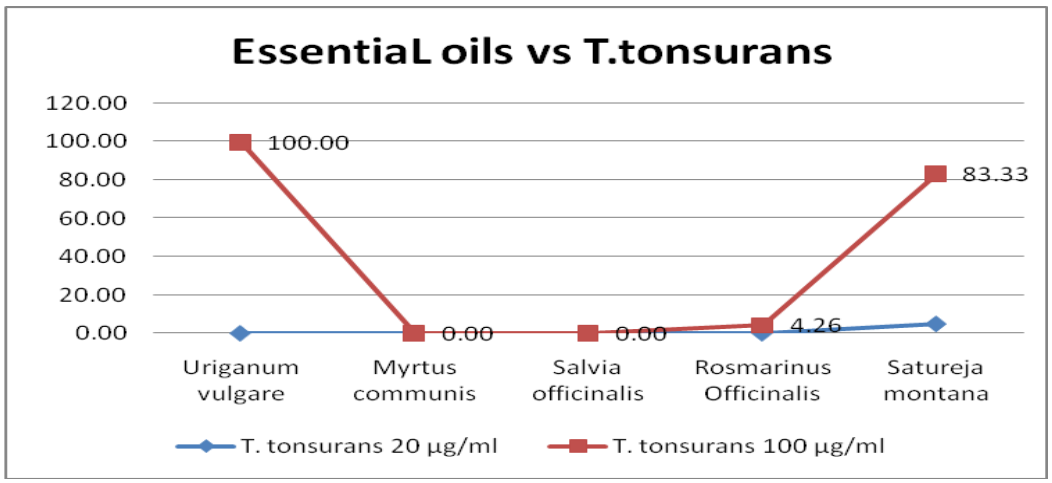


Figure 64. Variation of Inhibition growth (%) of *T.tonsuras* colony versus *Origanum Vulgaris, Myrtus Communis , Salvia officinalis, Rosmarinus Officinalis*

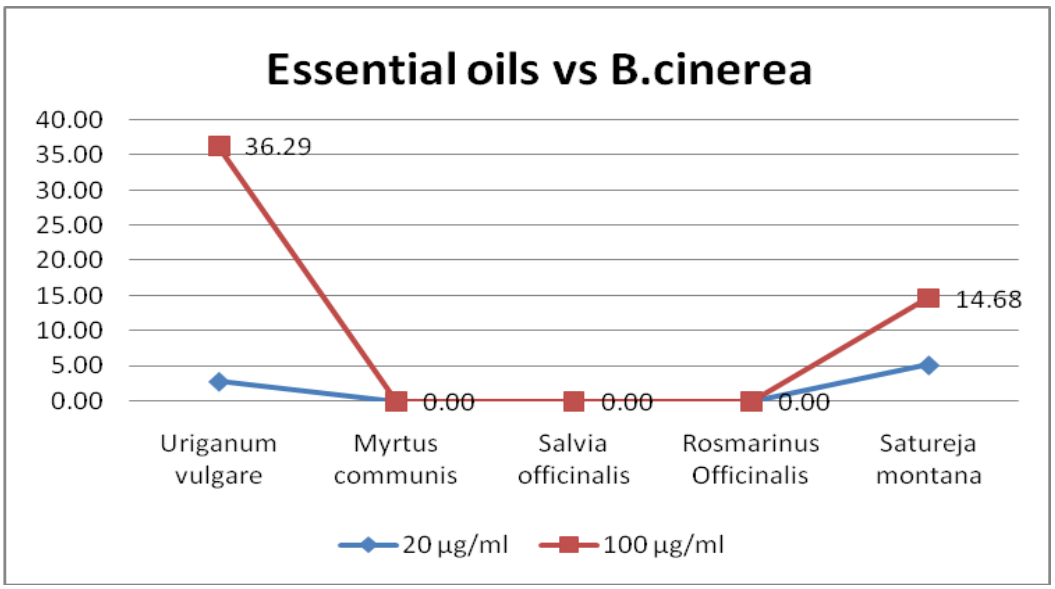


Figure 65. Variation of Inhibition growth (%) of *B.cinerea* colony versus *Origanum Vulgaris, Myrtus Communis , Salvia officinalis, Rosmarinus Officinalis*

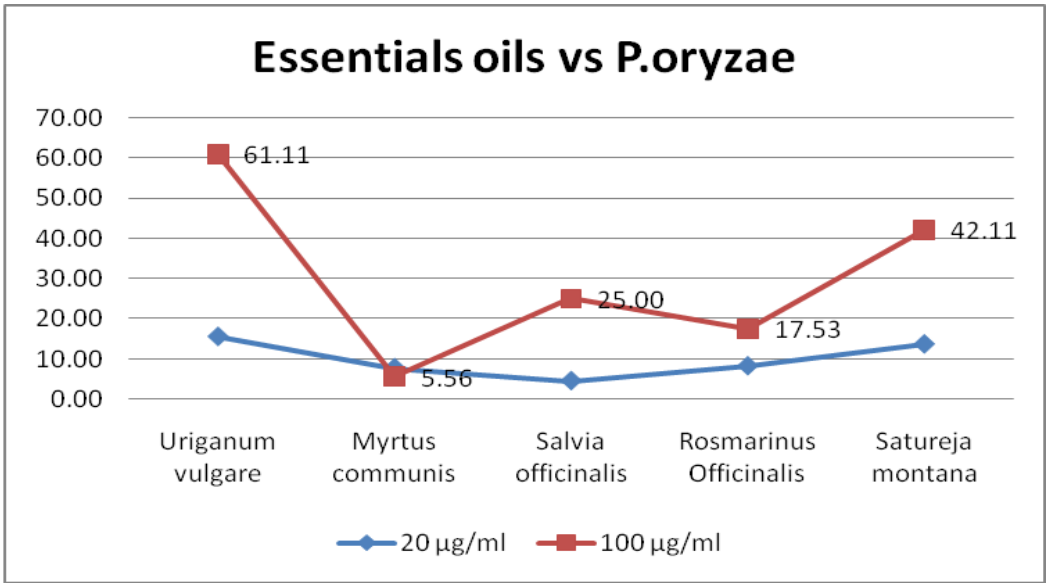


Figure 66. Variation of Inhibition growth (%) of *B.cinerea* colony versus *Origanum Vulgaris, Myrtus Communis , Salvia officinalis, Rosmarinus Officinalis*

Variation of antifungal activity versus Dermatophytes

All the Dermatophytes colony are more sensible related to *Origanum vulgaris* and *Satureja Montana* essential oil. (Figure 57, 60, 61, 62, 63, 64, 65, 67, 66) They give around 65 – 82 % inhibition growth of this colony. We think that these two essential oils confirm again the fact that due to their high concentration of carvacrol and thymol inhibited all the Dermatophytes colonies. This fact brings us to the conclusion that high concentrations of carvacrol and thymol are the responsible components of aromatic plants for the antibacterial and antifungal properties.

4.5.7 *Satureja Montana* essential oil *Candida albicans*

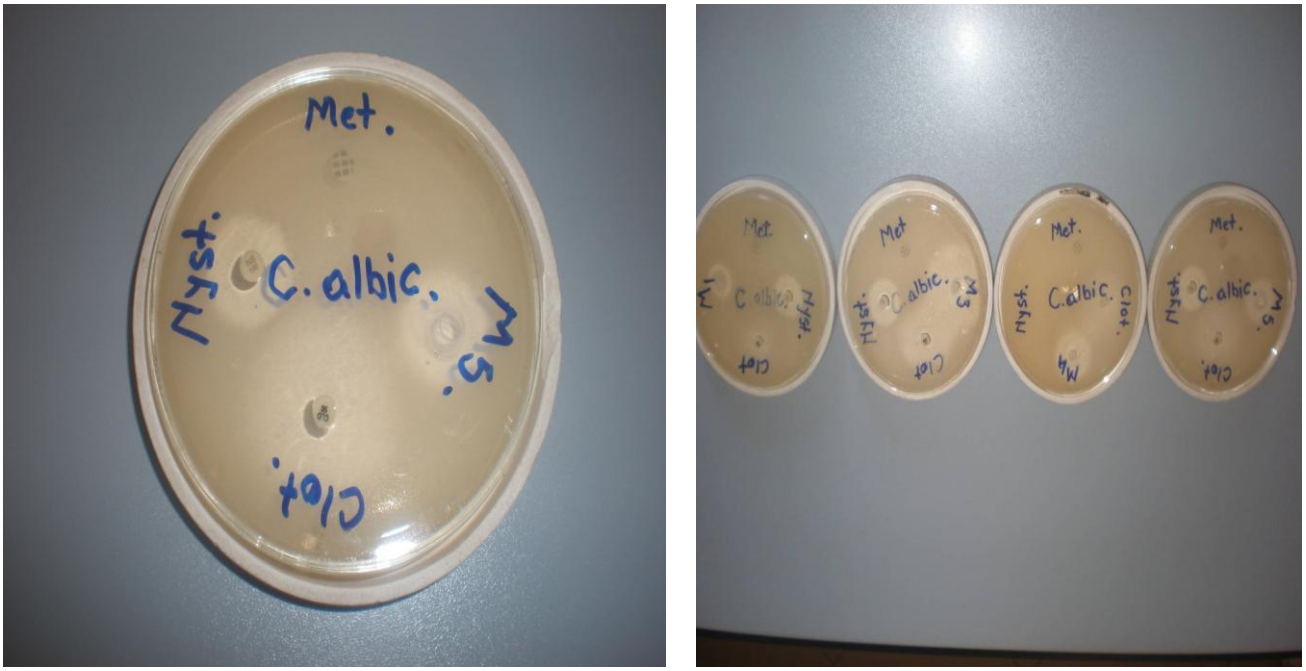


Figure 67. Inhibition growth of *Candida albicans* vs *Satureja Montana* essential oil

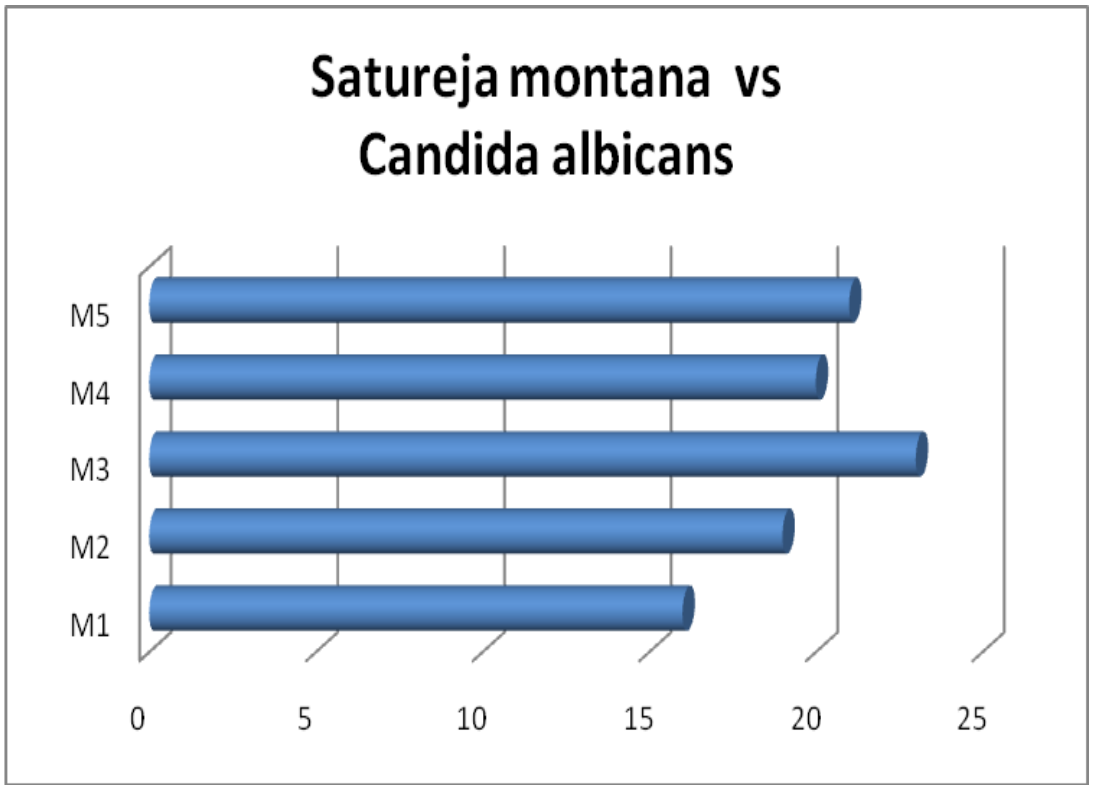


Figure 68. Inhibition growth of *Candida albicans* vs *Satureja Montana* essential oil

Satureja montana* versus *Candida albicans

Satureja Montana M_3 and *Satureja Montana* M_5 has shown higher inhibition zones (Figure 68, 69) versus *Candida albicans*. M_3 is more rich with carvacrol and thymol which give us the idea that they are the responsible also for antifungal activity.

4.6 Antioxidant activity

Satureja Montana (0.62) and *Origanum vulgaris* (0.49) essential oil has shown higher antioxidant activity then *R.officinalis*, *S. officinalis* and *M.Communis*. Even the antioxidant activity is related as seen in Figure 70 to the higher carvacrol and thymol concentrations of essential oils.

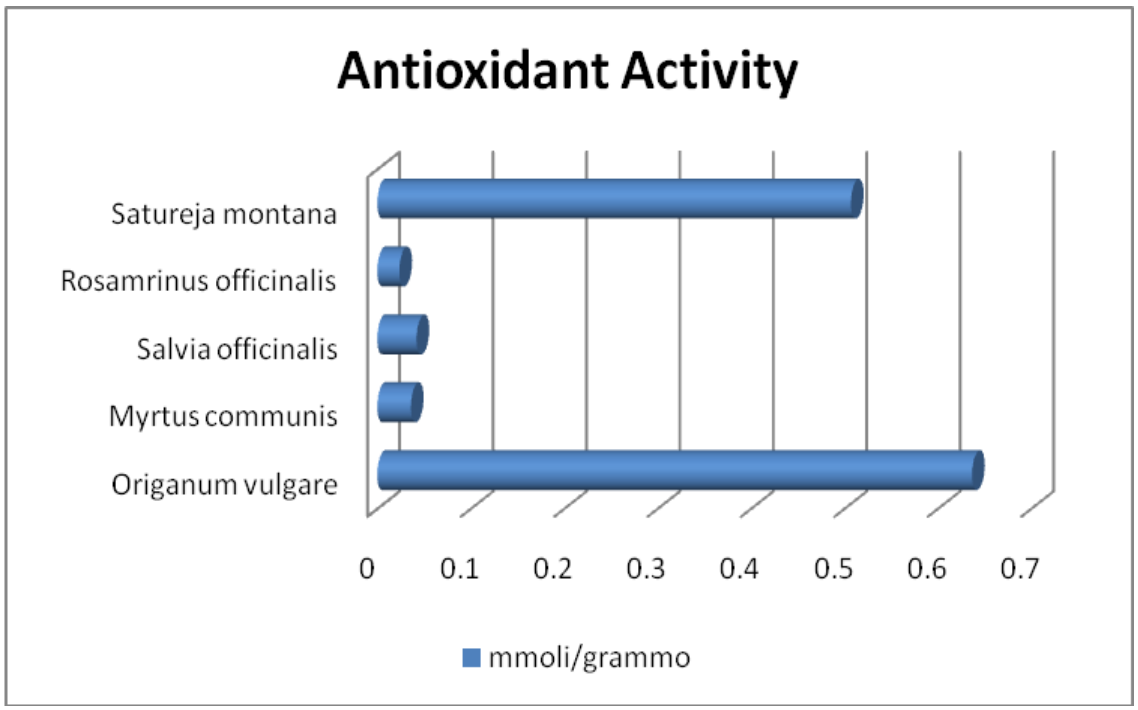


Figure 69. Antioxidant activity of different essential oils of Lamiace family

4.7 Encapsulation of *Satureja Montana* essential oil

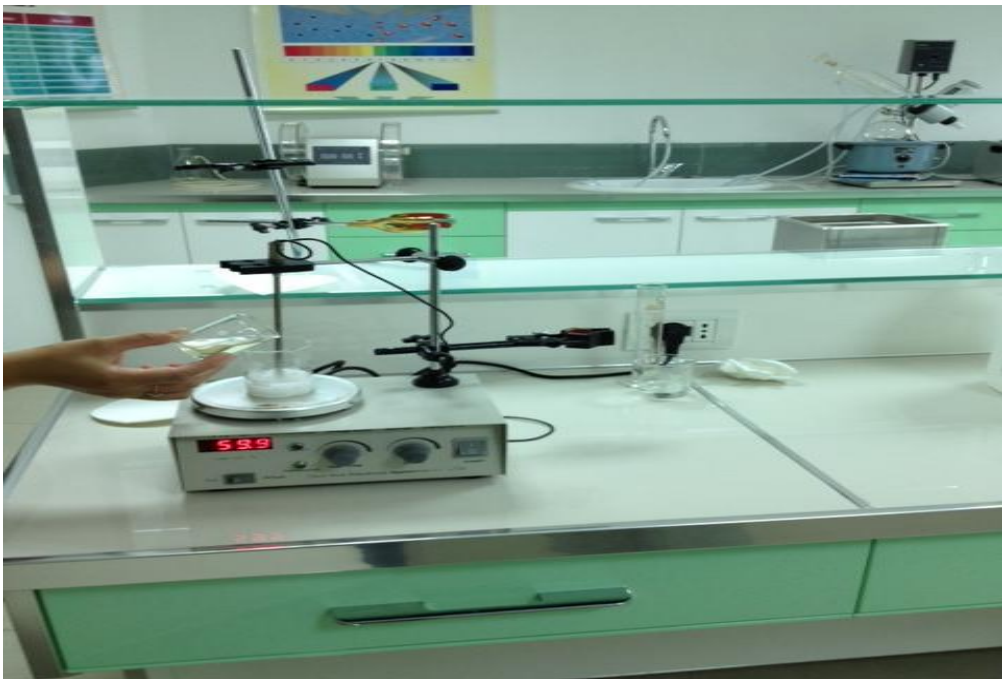


Figure 70. Encapsulation of *Satureja Montana* essential oil

4.7.1 Powder recovery

Table 18. Recovery of the powder (complex) at various *S.Montana* essential oil to β - cyclodextrin ratios.

S.Montana oil: b-CD ratio	Starting material (g, db*)	Recovered powder (g, db*)	Recovery (%)
5:95	5.004 ±0.02	4.116 ± 0.14	82.25
10:90	5.121 ±0.01	4.311 ± 0.19	84.18
15:85	5.058 ± 0.01	4.395 ± 0.22	86.89
20:80	5.568 ± 0.03	5.238 ± 0.32	94.07

[a]Total amount of dry β-CD plus *S.montana* oil used. db* - dry weight basis.

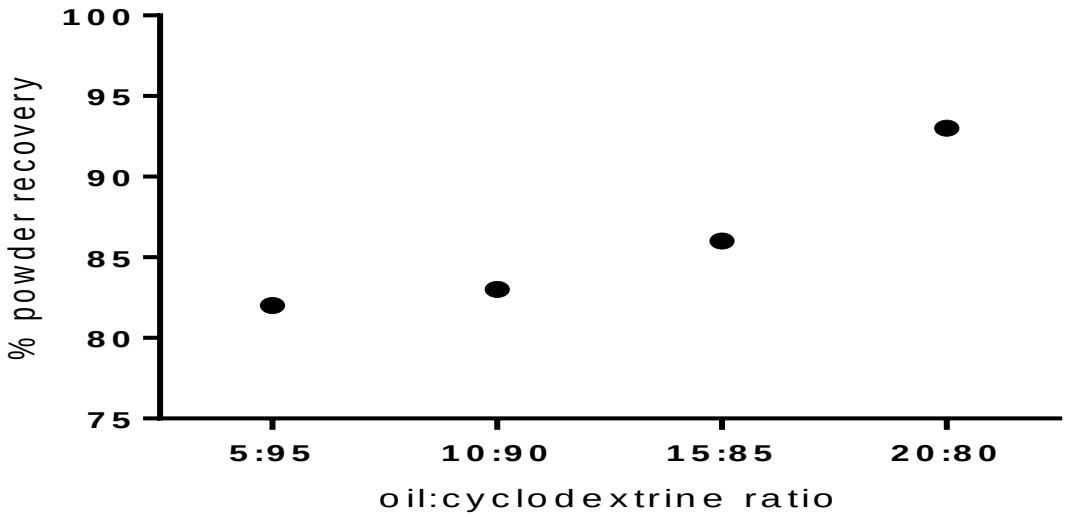


Figure 71. Recovery of the powder (complex) at various *S.Montana* essential oil to β - cyclodextrin ratios

4.7.2 Surface oil

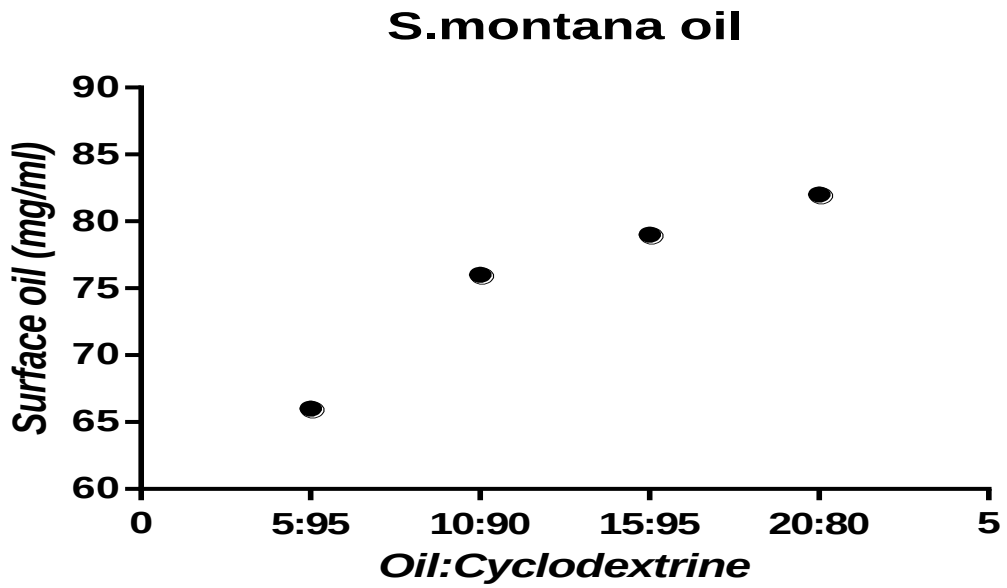


Figure 72. Surface oil (%) (complex) at various ratios of *S.Montana* essential oil to β - cyclodextrin

4.7.3 Retention

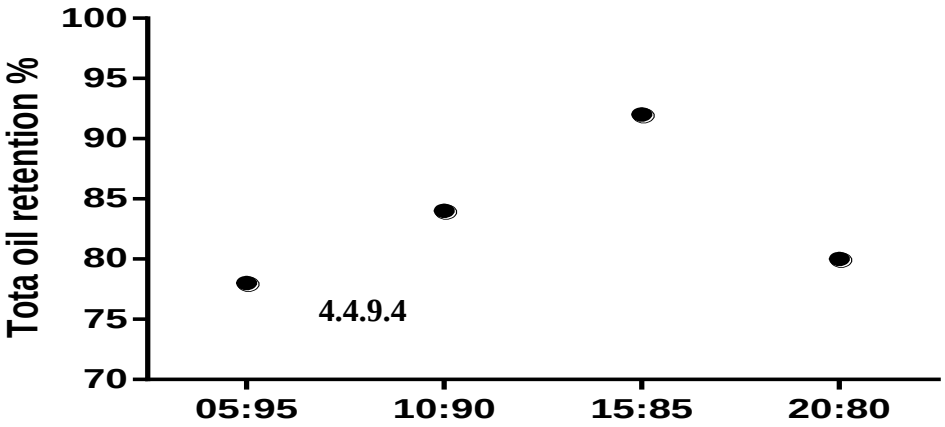
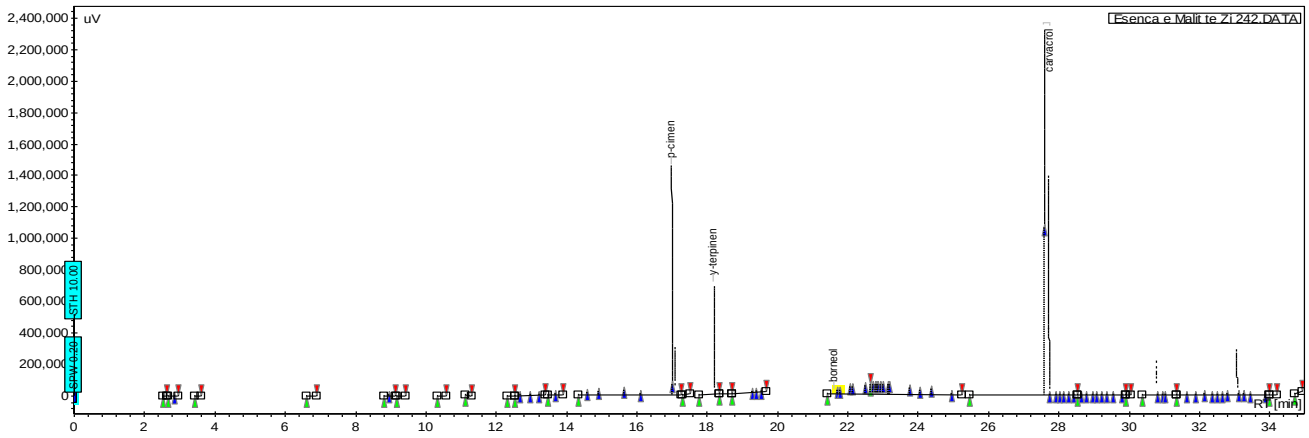


Figure 73. Total retention of flavor volatiles as a function of the initial essential oil to β-cyclodextrin ratio

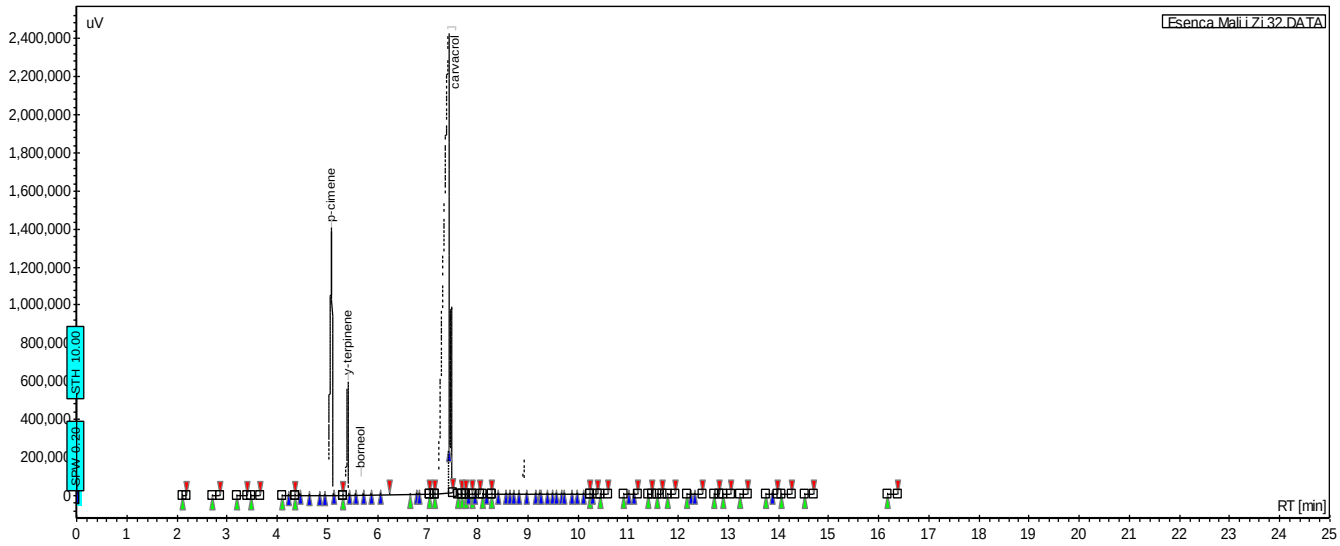
4.7.4 GC-FID chromatograms of initial oil (figure) and of total oil extracted form powder complex



Peak results :

	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
	p-cimene	5.17	30.44	3696089.7	335666.9	30.436
	y-terpinene	5.54	29.22	3746505.7	322289.4	29.223
	borneol	5.68	0.24	94135.5	2621.2	0.238
	timol	6.40	1.08	284537.3	11897.8	1.079
	carvacrol	7.56	38.77	3080362.0	427577.3	38.770
Total			100.00	10950572.4	1102853.3	100.000

Figure 74. Gas/Fid chromatograms of initial oil



Peak results :

	Name	Time [Min]	Quantity [% Area]	Height [uV]	Area [uV.Min]	Area % [%]
	p-cimen	16.99	15.63	1463469.4	553811.3	15.632
	y-terpinen	18.18	4.26	713417.6	150777.5	4.256
	borneol	21.61	0.16	29479.7	5546.4	0.157
	carvacrol	27.57	64.83	2351888.6	2296784.2	64.831
Total			100.00	9019636.6	3542738.6	100.000

Figure 75. Gas/Fid chromatograms of total essential oil extracted

Table 19. Main componentns of essential oils of *Satureja Montana*

	(M1)	**(M2)**	**(M3)**	**(M4)**
p-cymen	20.86	5.9	11.04	9.61
y-terpienen	2.05	8.45	3.07	2.86
borneol	0	0.2	0.67	4.76
thymol	6.2	1.82	3.94	25.78
carvacrol	6.11	67.68	46.18	64.22

The maximum inclusion efficiency of β-cyclodextrin was achieved at the ratio of 20:80.The qualitative and quantitative composition of the volatiles in the total oil extracts was similar to the starting ones. In the ratios of the 15:85 and 20:80 , before the filtration of the powder, one or two droplets of oil were noticed on the surface of the solutions. This observation suggested that some of the essential oil was not included into the β-cyclodextrin molecules.

Table 16 shows the recovery of the powder at various *S.montana* essential oil to β-cyclodextrin ratios. As can be seen, the amount of the powder that was recovered is less than the amount of essential oil and β-cyclodextrin originally used. The material loss can be attributed to the oil, β-cyclodextrin and complex dissipation. There are several factors which may contribute to the loss of *Satureja montana* oil: retention of the oil in the solution after forming the complex; equilibrium of flavors between the liquid and the complexed state; evaporation of surface oil during the long complexation process and evaporation during the drying step. The loss of the β-cyclodextrin and complex powder is mainly assigned to their solubility in water.

It appears that high starting ratios of the *S.montana* essential oil to β-cyclodextrin produce the maximum recovery of the oil powder, maximum inclusion of essential oil and minimum noncomplexed β-cyclodextrin. An optimum ratio of essential oil to β-cyclodextrin during complexation existed at around 20:80. The retention of total oil volatiles is determined as a percentage of total extracted volatiles to the volatile content of the essential oil used (as determined by GC). The retention of essential oil reached a maximum of 92.8 % at the oil to β-cyclodextrin ratio of 15 : 85. (Figure 75). GC-FID chromatograms for oil after extraction from the complex were tidentical to the starting ones. This means that the complexing process dosent affect the quality of essential oil. (Figure 76, 77).

4.8 Antibacterial activity of *Satureja montana* after microencapsulation

Satureja Montana essential oil had considerable inhibitory effects on following bacterias *Proteus Vulgaris* , *Escherichia coli*, *Staphylococcus aureus*. The inhibition zone of essential oil after encapsulation (Figure 79, 80, 81,82) are considerably near and sometime higher to those of antibiotics taken in consideration in this study (cyprofloxacini, cefuroxime, tetracycline).All our samples M_1, M_2, M_3, M_4 collected from different geographical zones has shown that have high inhibiton zone for bacterial culture and light differences between them because of their different % of main components such as carvacrol, p-cymen, borneol, thymol, y-terpienen. It resulted that sample M_3 and M_4 that have the highest levels of carvacrol and thymol and also higher inhibition zone than other samples.

On the other hand the inhibition zone of essential oil after encapsulation are very closed to those before encapsulation which means that the microencapsulation process seems that it dosent damage the antibacterial properties of *Satureja montana* essential oil. Further in some cases they have higher inhibition zone, maybe due to the modified releasing from b-cyclodextrine. The antibacterial and the physic-chemical benefits from microencaspultaion of essential oil in b-cyclodextrine has shown in this study considerable data and results. (Figure 85, 86, 87).

Table 20. Antimicrobial activity of the ecapsulated *Satureja montana* L. essential oil different samples (M1, M2, M3, M4).Diameter of disc (6 mm). nt – non tested; Inactive (–); moderately active (7–12mm); highly active Antibiotics-Positive control (>13mm).

			Antibiotics-Positive control		
Sample	**Bacteria**	**10** μL	Cyrofloxacini	Cefuroxim	Tetracyclini
M1	*Proteus Vulgaris*	29.5±0.02	27.0±0.14	29.0±0.04	23.0±0.08
	Escherichia coli	14.1±0.15	16.0±0.22	18.0±0.07	nt
	Staphylococcus aureus	25.2±0.03	29.5±0.25	28.0±0.11	17.1±0.14
M2	*Proteus Vulgaris*	28.0±0.07	27.2±0.05	27.5±0.09	22.7±0.07
	Escherichia coli	18.7±0.01	16.9±0.03	18.6±0.06	nt
	Staphylococcus aureus	24.9±0.04	26.4±0.07	27.6±0.05	17.5±0.03
M3	*Proteus Vulgaris*	40.5±0.02	28.5±0.15	27.9±0.06	21.9±0.04
	Escherichia coli	28.2±0.011	17.3±0.12	17.9±0.09	nt
	Staphylococcus aureus	26.6±0.06	27.0±0.55	26.9±0.19	19.0±0.08
M4	*Proteus Vulgaris*	44.2±0.07	29.0±0.32	28.1±0.07	21.6±0.01
	Escherichia coli	22.8±0.01	16.5±0.08	19.0±0.04	nt
	Staphylococcus aureus	25.8±0.08	27.8±0.01	27.3±0.02	18.0±0.11

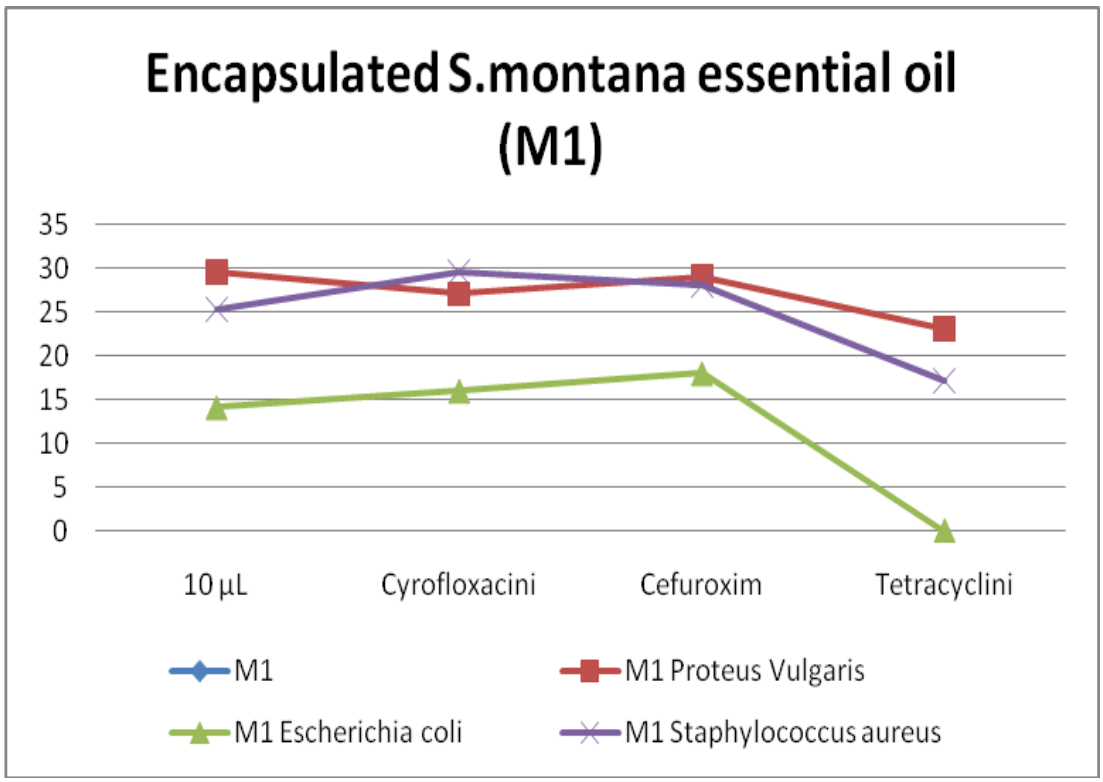


Figure 76. Antibacterial activity (inhibition zone mm) of *Satureja montana* essential oil (M1) versus *E.Coli, P.Vulgaris, S. Aureus*

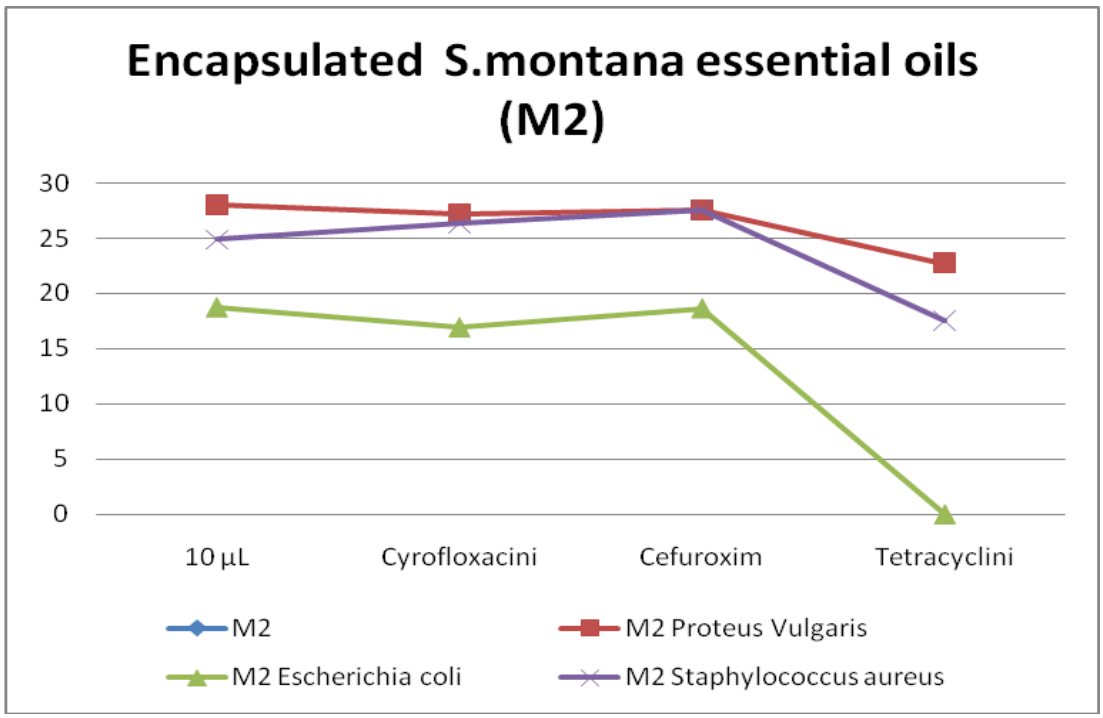


Figure 77. Antibacterial activity (inhibition zone mm) of *Satureja montana* essential oil (M2) versus *E.Coli, P.Vulgaris, S. Aureus*

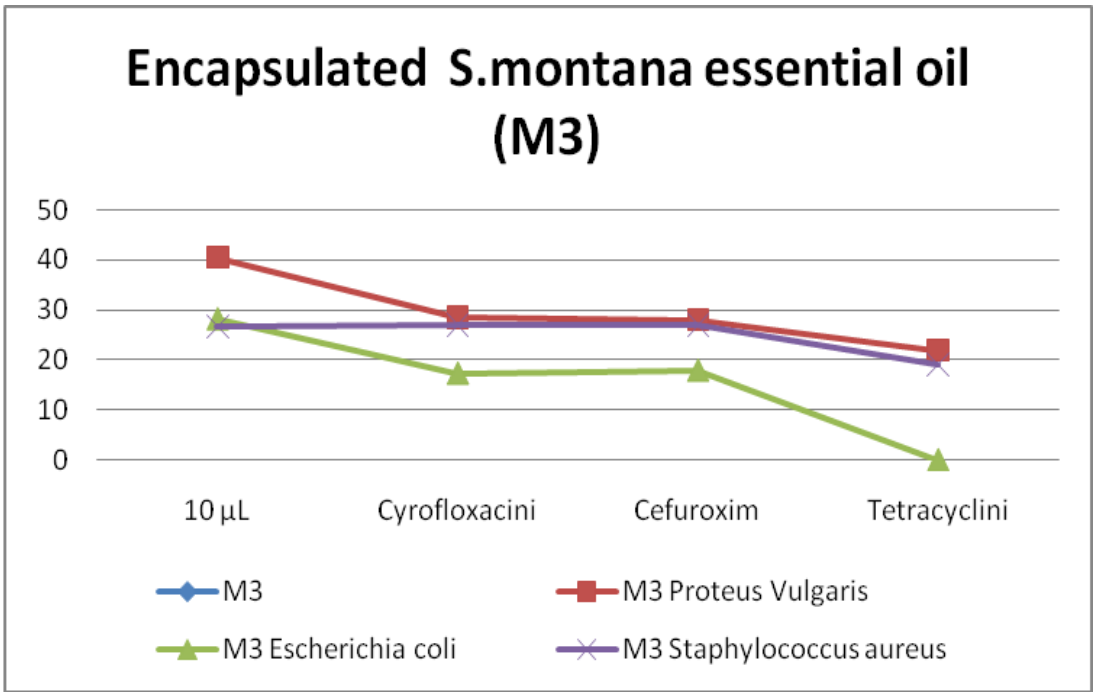


Figure 78. Antibacterial activity (inhibition zone mm) of *Satureja montana* essential oil (M3) versus *E.Coli, P.Vulgaris, S. Aureus*

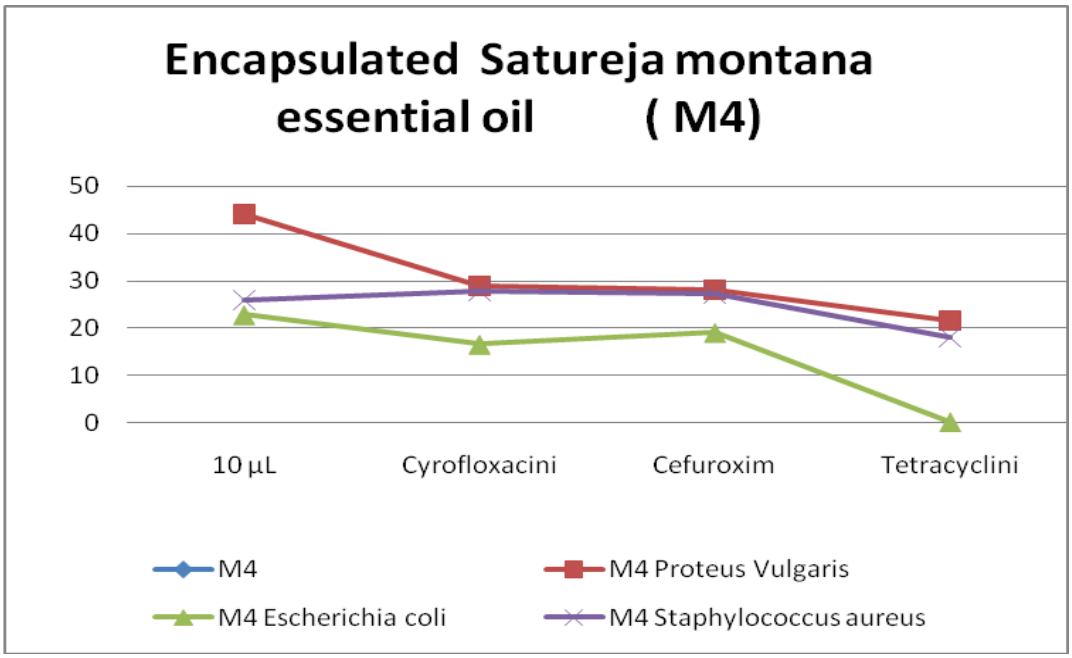


Figure 79. Antibacterial activity (inhibition zone mm) of *Satureja montana* essential oil (M4) versus *E.Coli, P.Vulgaris, S. Aureus*

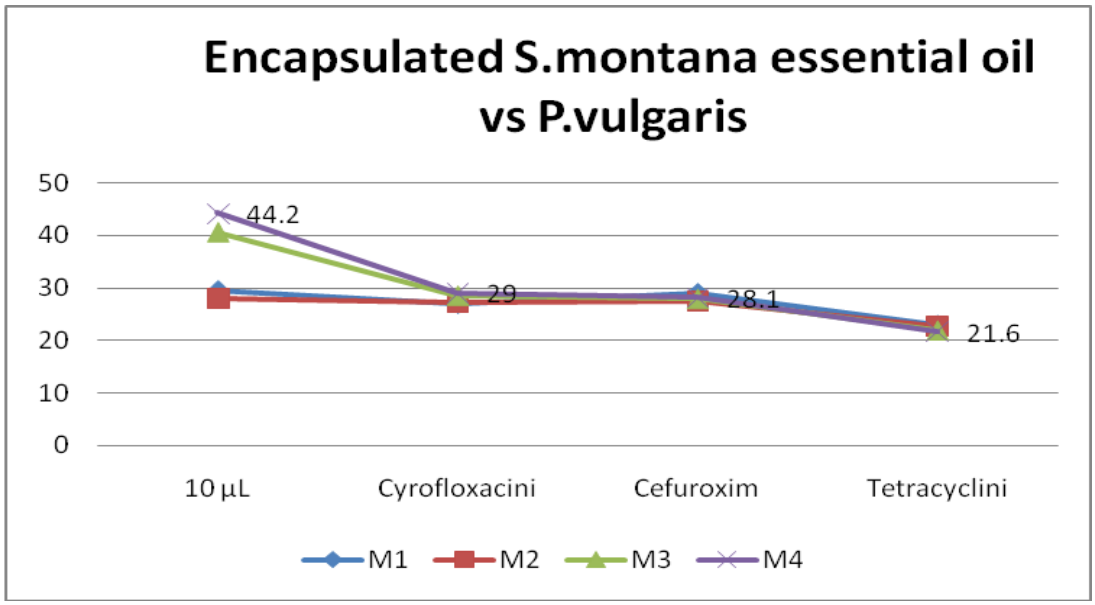


Figure 80. Variation of Antibacterial activity (inhibition zone-mm) of encapsulated *Satureja montana* essential oil versus *P. Vulgaris*

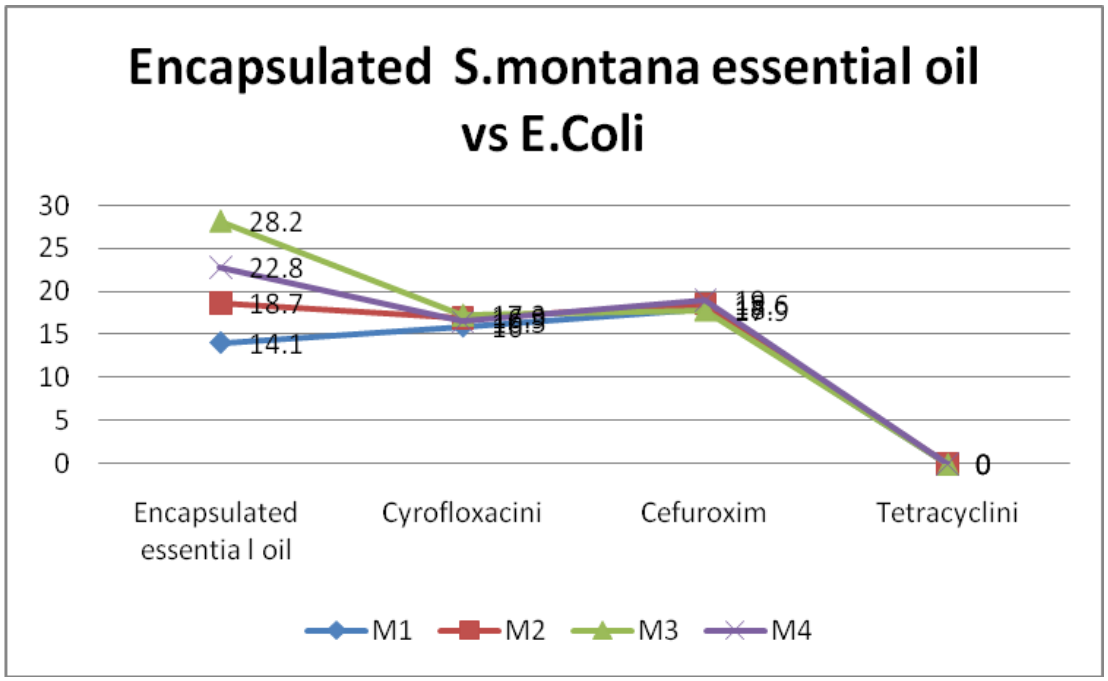


Figure 81. Variation of Antibacterial activity (inhibition zone-mm) of encapsulated *Satureja montana* essential oil versus *E.Coli*

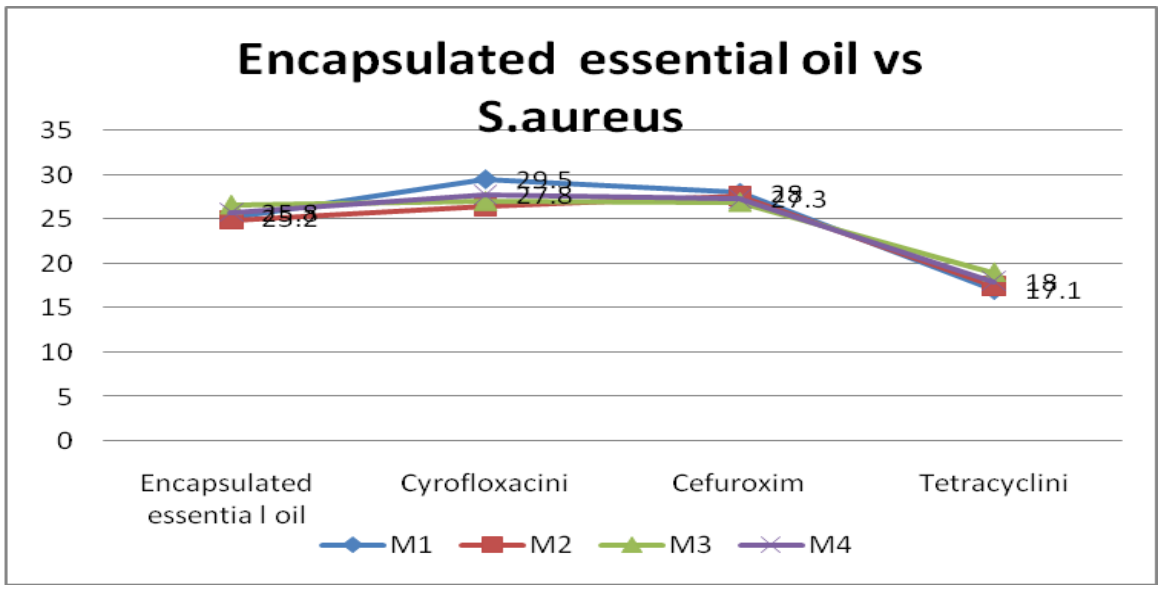


Figure 82. Variation of Antibacterial activity (inhibition zone-mm) of encapsulated Satureja montana essential oil versus S.aureus

4.9 Comparing of antibacterial activity before and after encapsulation

4.9.1 *S.montana* essential oil vs *P.vulgaris*

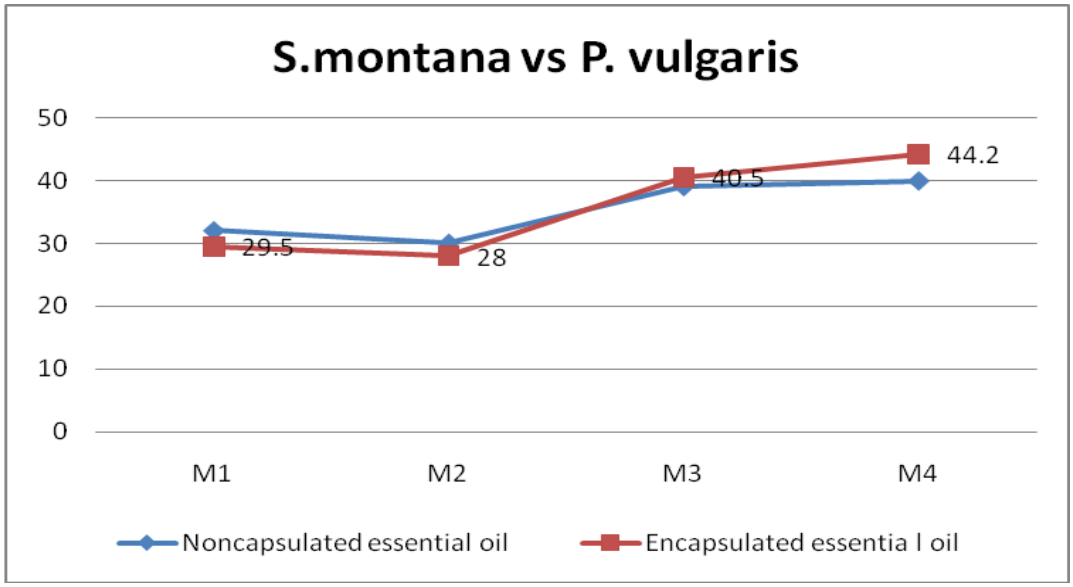


Figure 83. Variation of Antibacterial activity (inhibition zone mm) of encapsulated and noncapsulated *Satureja montana* essential oil versus *P. Vulgaris*

4.9.2 *S.montana* essential oil vs *E.Coli*

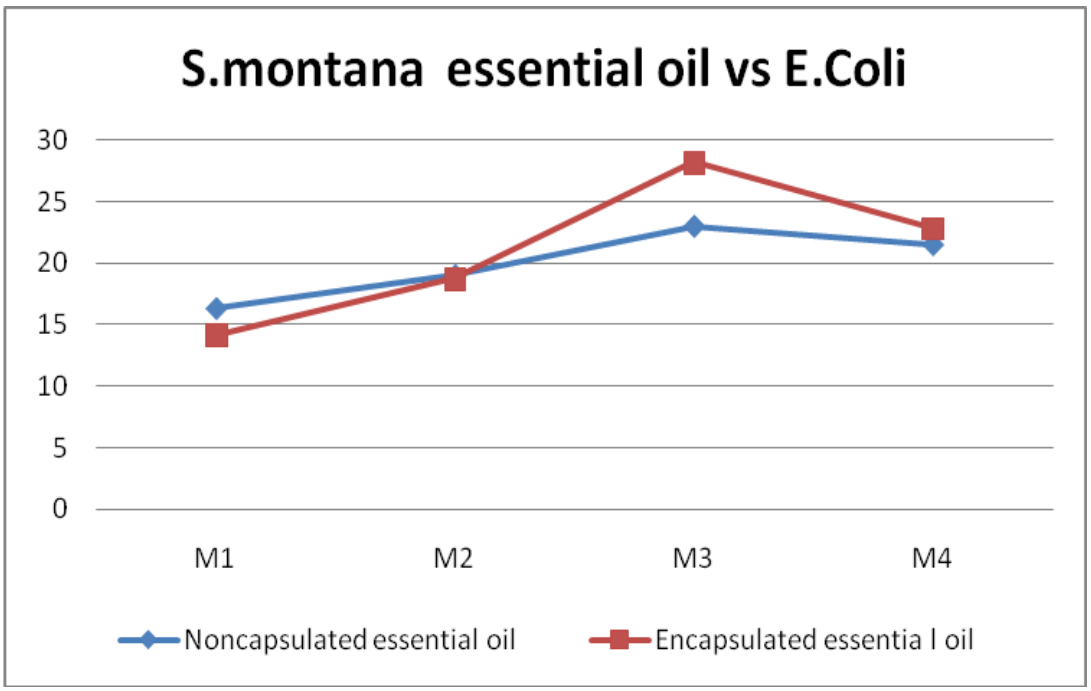


Figure 84. Variation of Antibacterial activity (inhibition zone mm) of encapsulated and noncapsulated *Satureja montana* essential oil versus *E.Coli*

4.9.3 *S.montana* essential oil vs *S,aureus*

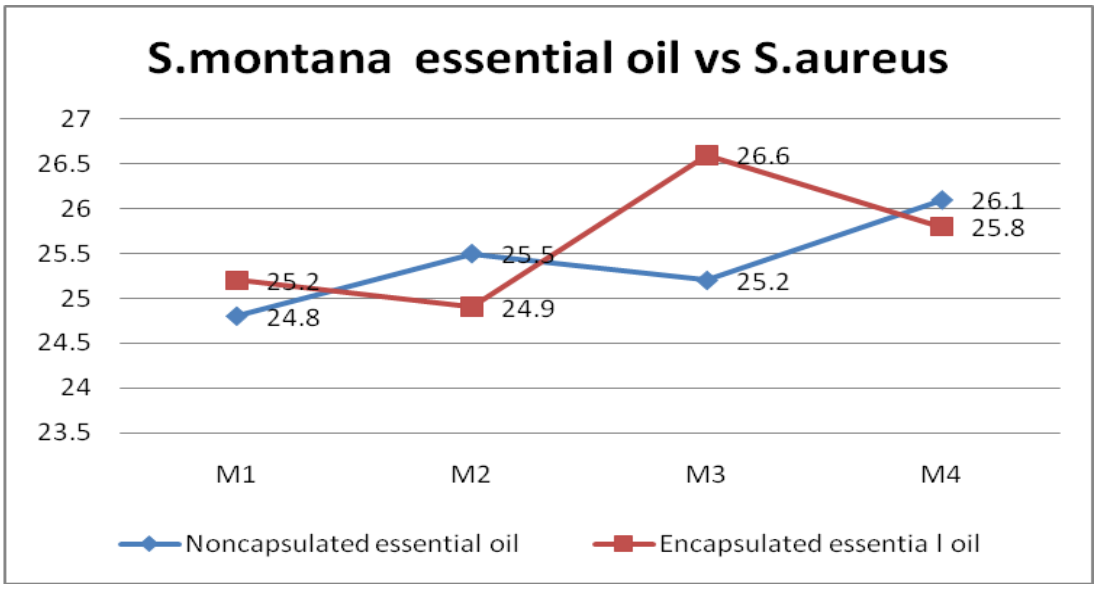


Figure 85. Variation of Antibacterial activity (inhibition zone mm) of encapsulated and noncapsulated *Satureja montana* essential oil versus *E.Coli*

4.10 Antifungal activity of *S.montana* encapsulated essential oil

Satureja Montana essential oil encapsulated showed high % of growth inhibition of Dermatophytes and Phytopatogens colonies. (Figure 88). *T.violaceum* was sensible against encapsulated essential oil in ratio oil : β-cyclodextrine 5 : 95. The ratio complex 10 : 90 was found to be more effective for antifungal activity then other ratios. This complex seems to be successful against *T.rubrum* (100 %), *T.mentagrophytes* (100 %), *T violaceum* (100 %), *M.Canis* (93.5 %), *T.tonsurans* (92.5 %), *E.flocossum* (89.0 %. On the other side the raio complex 20:80 was not so sucssefull against dermatophytes in only *T.rubrum* (41.67 %), *T.violaceum* (34.44%) and *M.Gypseum* (37.25 %).

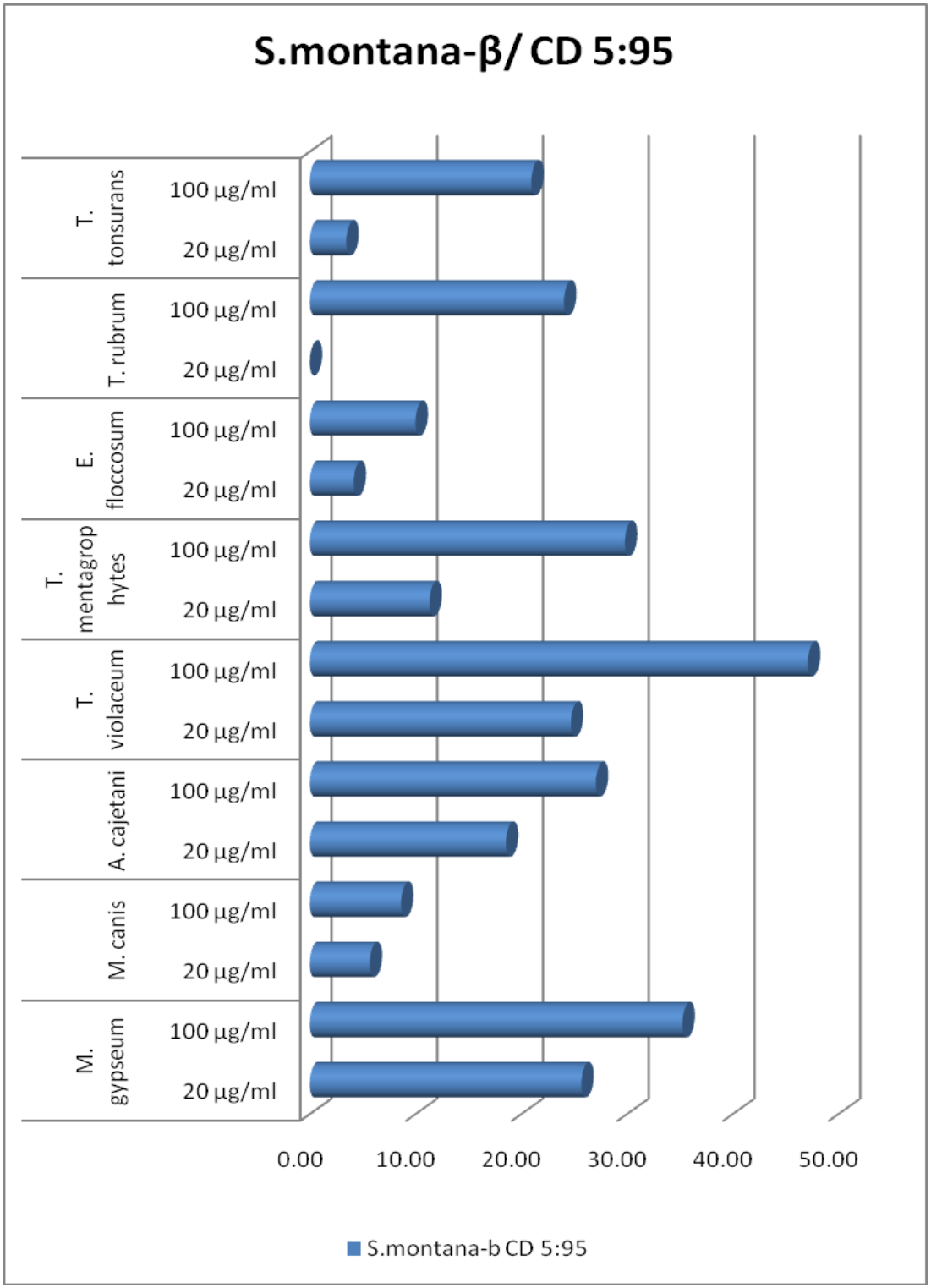


Figure 86. Inhibition growth (%) of *Satureja montana* encapsulated essential oil (oil :β-cyclodextrine 5:95) vs Dermatophytes

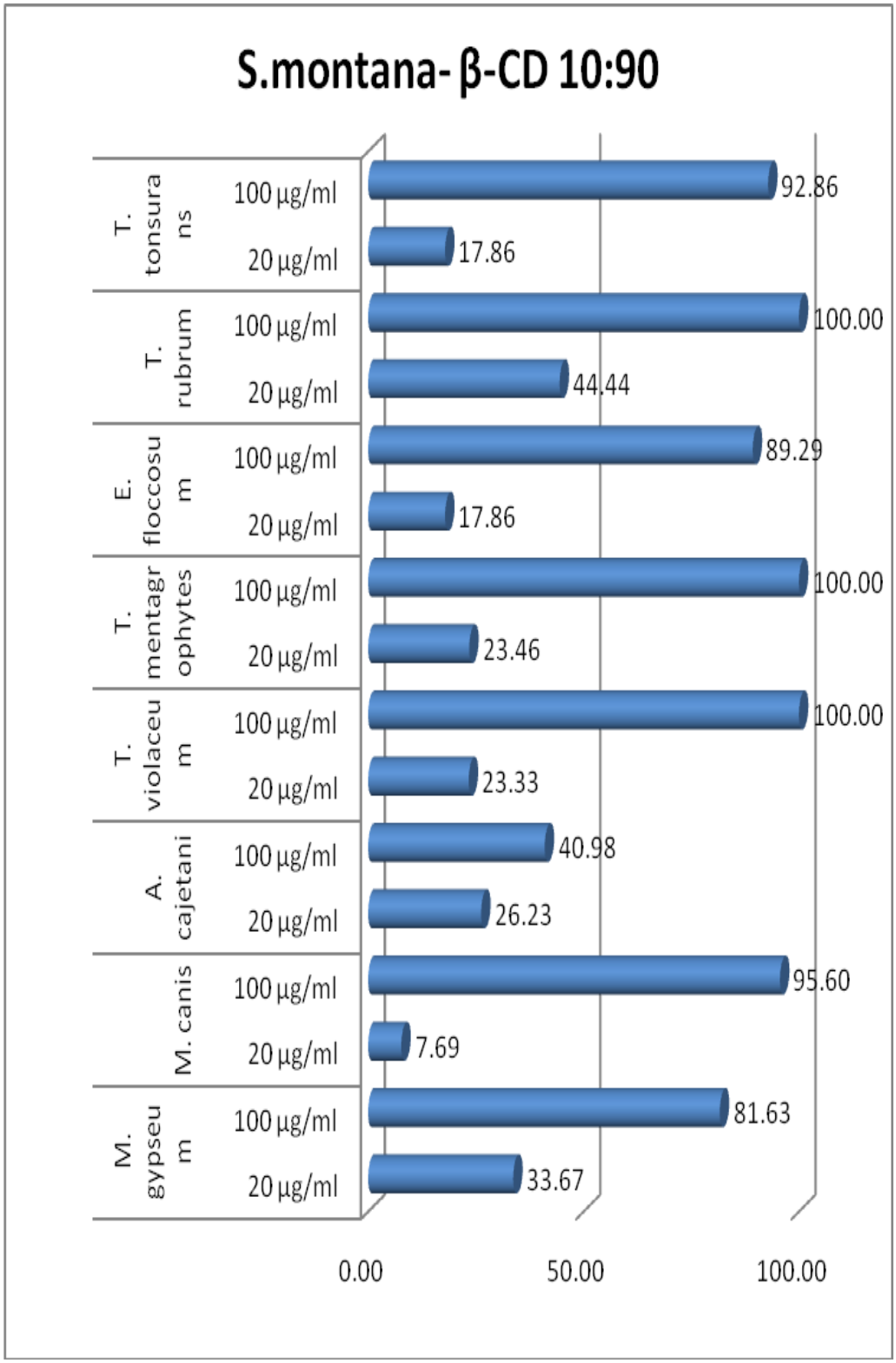


Figure 87. Inhibition growth (%) of *Satureja montana* encapsulated essential oil (oil :β-cyclodextrine 10:90) vs Dermatophytes

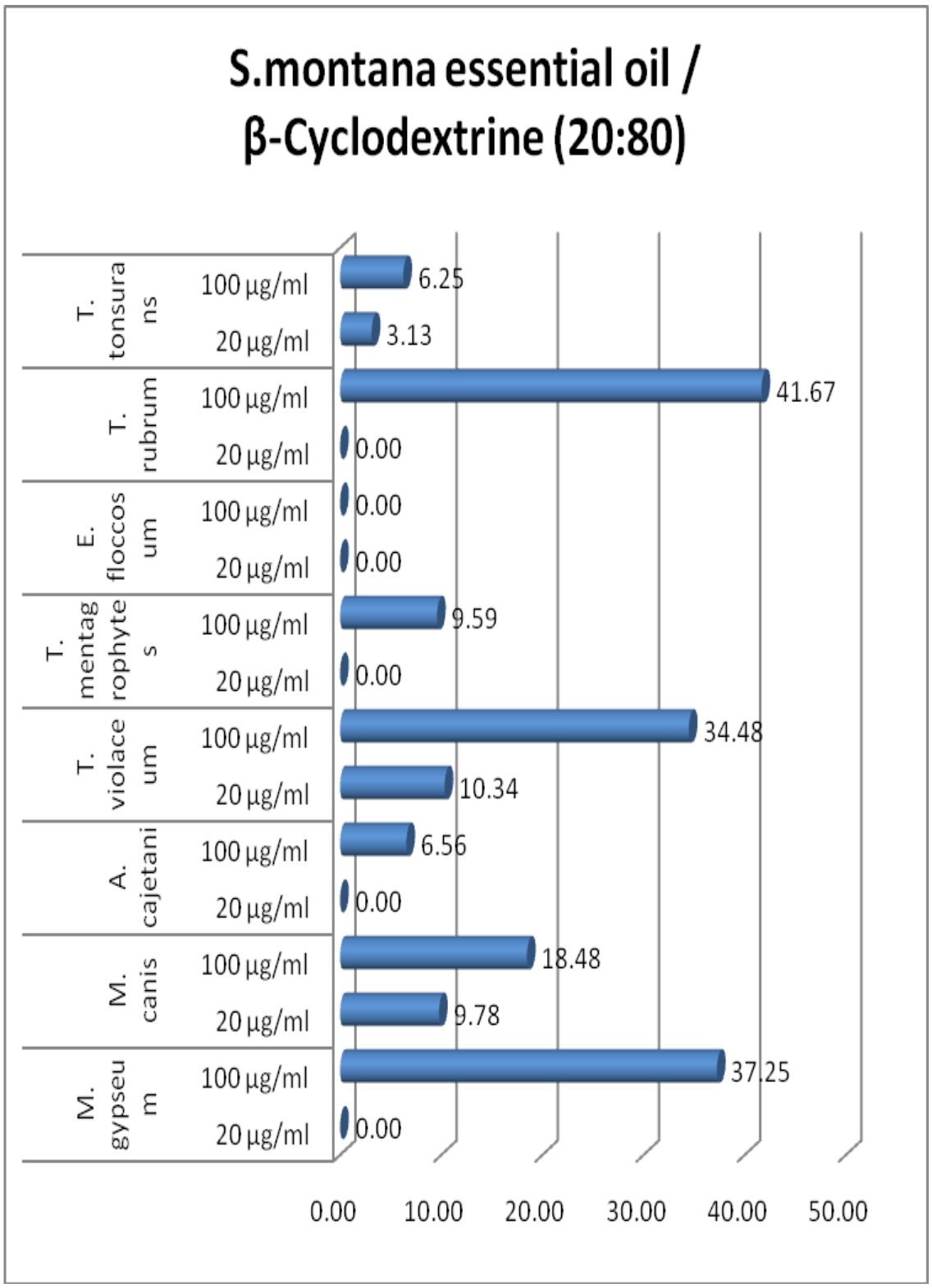


Figure 88. Inhibition growth (%) of *Satureja montana* encapsulated essential oil (oil :β-cyclodextrine 20:80) vs Dermatophytes (7 days)

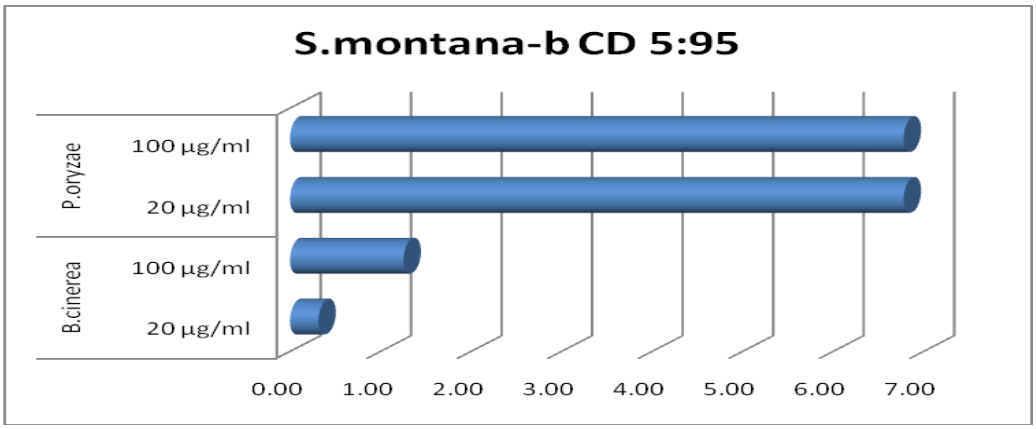


Figure 89. Inhibition growth (%) of *Satureja montana* encapsulated essential oil (oil :β-cyclodextrine 5:95) vs Phytopatogens (5 days)

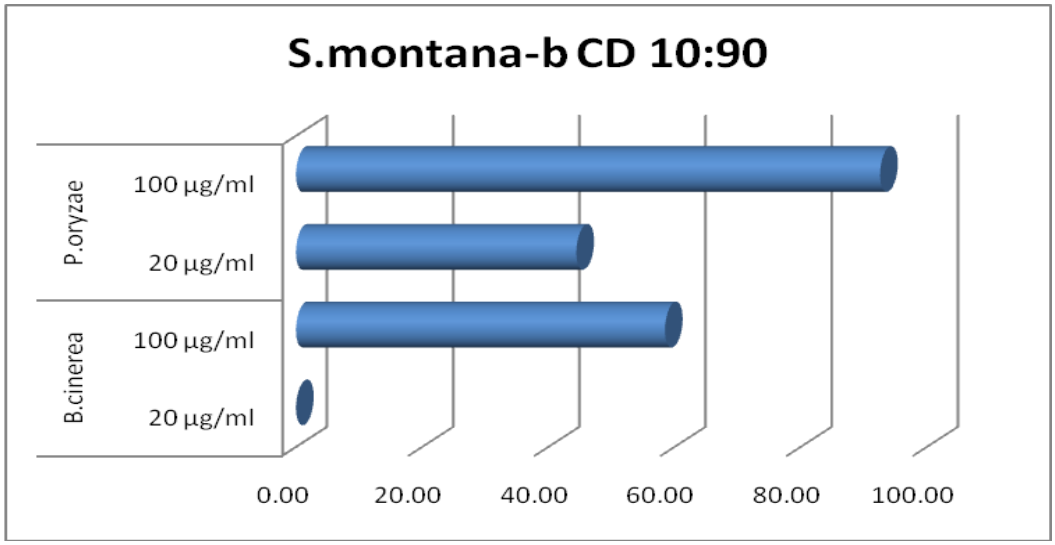


Figure 90. Inhibition growth (%) of *Satureja montana* encapsulated essential oil (oil :β-cyclodextrine 10:90) vs Phytopatogens (5 days)

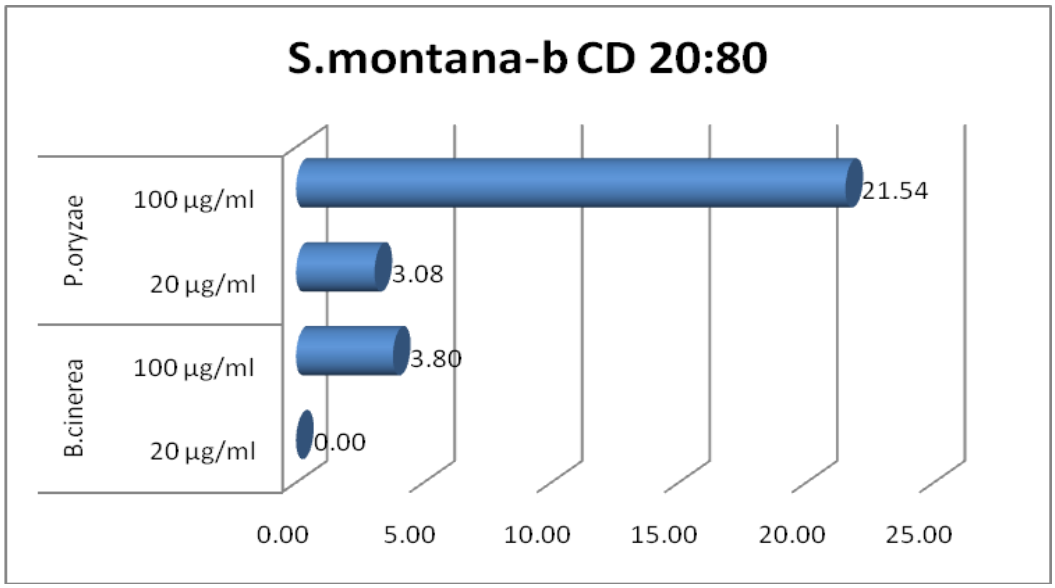


Figure 91. Inhibition growth (%) of *Satureja montana* encapsulated essential oil (oil :β-cyclodextrine 10:90) vs Phytopatogens (5 days)

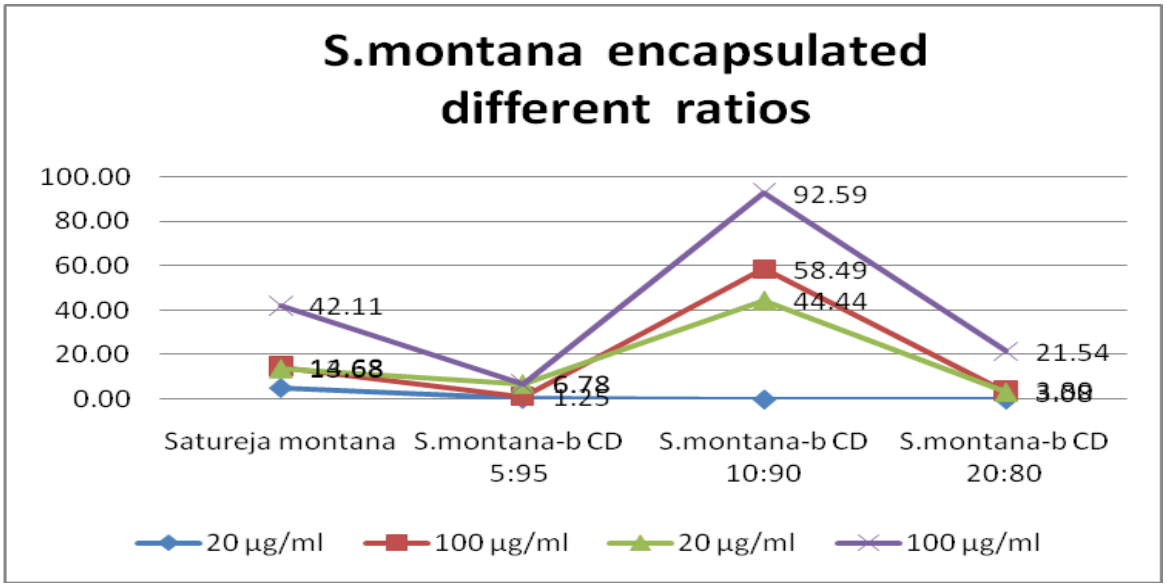


Figure 92. Comperative analyses of Inhibition growth (%) of *Satureja montana* encapsulated essential oil and non capsulated in β-CD versus phytopatogens

There is a clear evidence for phytopatogens inhibition results (Figure 91, 92, 93) that confirm again that the successful report that releases easily the essential oil from the complex is 10:90. This is shown in Figure 94. As conclusion this report is the most effective one which resolve the physical problems of essential oil and keeps and increase the antibacterial and antifungal properties.β- cyclodextrine improve the quality of essential applications and also their antibacterial and antifungal properties.

Chapter 5 Conclusions

1- Method Validation

The method proposed (third one) of GC-FID analyses for Satureja montana essential oil were considered validated and it is proposed in further studies for improvement of quality, the adoption of standards and quality controls along the whole value chain. This method if it will be used in quality control analyses of Satureja montana essential loil in Albania it will increase the average export price of Albanian MAP's and conceguently it will be reclected in albanian economy chain.

2- Main components

Main componenents of essential oils considerd in this study were y-terpienen, carvacrol, thymol, p-cymen,borneol. Origanum vulgaris and Satureja motana essential oils were rich of carvacrol, thymol and y-terpinen. The three others Rosamrinus officinalis, Myrtus communis, Salvia officinalis has lower carvacrol amounts bur were rich in y-terpiene, α-pinen, borneol, p-cymen and thymol.

3- Antibacterial activities

Samples of Satureja montana essential oil with high amounts of carvacrol and thymol showed up high activity against S.aureus, P.vulgaris and E.coli . In this study we evaluate the antibacterial activity of Satureja montana essential oil and its variation related to carvacrol and thymol contents. Satureja montana essential oils showed a high antibacterial activity (>13mm) especially was more sensible again Proteus vulgaris colony compare to positive control . Also it exhibited appreciable antimicrobial activities to E.Coli followed by S.aureus colony. As results this essential oil is more sensible against gram positive bacterias then gram negative ones.

4- Antifungal activities

Evaluation of antibacterial activity were studied not only for Satureja montana essential oil but for R.officinalis, O.vulgaris, S.officinalis and M.communis also. All the respevtive essential oils showed up an high activity against Dermatophytes *M. Gypseum, M. canis, A. cajetani T.violaceum, T.mentagrophytes, E. floccosum, T. rubrum, T. tonsurans* and 2 colonies of phytopatogens *B.cinere,P.oryzae.*

Origanum vulgaris and Satureja montana essential oil was found to be more sensible to Dermatophytes colonies. Even the anifugal activity is related to concentrations of carvacrol and thymol, maby this components reacts with te same mechanisem to bacteria and fungi- memebrane disintegration

5- Antioxidant effect

Essential oil showed up a considerable antioxidant activity, expecially the essential oil of Satureja montana and Origanum vulgaris showed a higer antioxidant activity.

6- Encpasulation of Satureja montana was found to be more efficient to ratio oil-β-cyclodextrine 20:80 and the retention oil in ratio 15:85. Evaluation of biological activity after encapsulation lead to the conclusion that the antibacterial and antifungal activity are at the same level even higher because of the slow releasing of essential oil from the complex. This fact was observed in ratio 10:90. In conclusion these essential can be complexed in β-cyclodextrine in optmal ratios

can be applied in dermatological formulations due to their low risk of skin sentisizing and high antibacterial and antifungal activity they have after encapsulation. On the other hand we tested the biological effects of ancapsulated essential oil ofter two weeks of their preparation which means that this complexes are stable because of the same inhibition zone they have before encapsulation

7- Suggestions

A-It is necessary to continue to study the new formulations of this essential oils with b-cyclodextrine in vivo and to consider the formulation of this eesntial oils and antibacterial and antifungal agents due tio their synergist effect and their lower side effets.
B- Study of essential oil complexes with β-cyclodextrine in vertical diffusion cell (future advise)
C- Study the stability of the complexes oil – β-cyclodextrine

Chapter 6
List of Pubblications

PUBBLICATION RELATED TO THE PHD THESIS

1- ***SCREENING THE ANTIFUNGAL ACTIVITY OF SATUREJA MONTANA ESSENTIAL OIL BEFORE AND AFTER INCLUSION IN B-CYCLODEXTRINE-*** **International Journal of Pharmacy and Pharmaceutical Sciences. Impact Factor 1.59 , on process February 2014 – original Article**

2- ***DEVELOPMENT AND VALIDATION OF A GAS/FID METHOD FOR IDENTIFICATION AND QUANTIFICATION OF MAIN COMPONENTS OF SATUREJA MONTANA L. ESSENTIAL OIL,*** **International Journal of Pharmacy and Pharmaceutical Sciences. Impact Factor1.59, accepted November 2013 – Published Article**

3- ***EVALUATION OF ANTIBACTERIAL ACTIVITY OF SATUREJA MONTANA ESSENTIAL OIL BEFORE AND AFTER INCLUSION IN b-CYCLODEXTRINE*** **, International Journal of Pharmaceutical Analyses Impact Factor 1.23, accepted November 2013 – Published Article**

4- ***ENCAPSULATION OF ESSENTIAL OILS IN B-CYCLODEXTRINE*** **, Journal of Phenomenia and Mycrocyclic Chemistry Impact Factor 1.39, accepted with revision November 2013 – Article**

5- ***MOLECULAR DYNAMICS OF THYMOSIN A- 1: STRUCTURE STABILIZATION BY PEO- DIAMINE STAPLING-*** **Albanian Journal of Pharmaceutical Sciences October 2013 – Article**

6- ***ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY ASSESMENT OF NIGELLA SATIVA ESSENTIAL OILS*** **- World Academy of Science, Engineering and Technology Volume.66 2012 ISSN** Print ISSN 2010-376X Electronic ISSN 2010-3778

7- ***COMPLEMENTARY AND ALTERNATIVE MEDICINE (CAM) FOR PAIN, HERBAL ANTI-INFLAMMATORY DRUGS*** **- Europian Scientific Journal May 2013 -** ISSN:1857-7881(Print)ISSN: 1857 - 7431 (Online) **Article , Vol.9 Nr.9 . pg 90 -105**

EXTENDED POSTER

1- ***ENCAPASULATION OF SATUREJA MONTANA ESSENTIAL OIL-*** **extented poster in European Journal of Pharmaceutical Sciences. Athens September 2013** Imprint: ELSEVIER, ISSN 0928-0987; Impact Factor 2.987; Vol.50, Supplement 1, 30 September 2013

2- CHARACTERIZATION, ANTIBACTERIAL ACTIVITY ASSESMENT AND INCLUSION IN CD OF SATUREJA MONTANA L. ESSENTIAL OILS, extended poster Europian Journal of Pharmaceutical sciences pg 168, ISSN 0928-0987, Impact Factor 2.987**. *lovenia 2011***

POSTERS

1- ASSESMENT OF SUSTAINABILITY OF PRIMULACID BY HPTLC-SCANNER METHOD IN SEVERAL ALCOLOHIC EXTRACTS FROM RADIX OF PRIMULA VERIS COLLECTED IN ALBANIA **- extented poster in European Journal of Pharmaceutical Sciences. Athens September 2013**

2- NOVEL NUTRACEUTICALS FROM ETNOPHARMACY: NIGELLA SATIVA SEEDS OILS AGAINST RECURRENT BLADDER INFECTIONS,**Meeting ; Nuove Prospettive in Chimica Farmaceutica" Maj 2013 – poster**

3- PHARMACEUTICAL CONTROL ANALYSES OF SOME PARACETAMOL SYRUPS, **International Congress of Biomedical Sciences, Albania, Maj 2013**

4- ASSESMENT OF SUSTAINABILITY OF PRIMULACID BY HPTLC-SCANNER METHOD IN SEVERAL ALCOLOHIC EXTRACTS FROM RADIX OF PRIMULA VERIS COLLECTED IN ALBANIA. **International Congress of Biomedical Sciences, Albania, Maj 2013**

5- THE CORRELATIONS AMONG PHENOLIC MONOTERPENES AND THEIR PRECUSORS IN ESSENTIALS OLI OF THYMUS VULGARIS". L.Biotechnological **Developments-Book of abstracts pg 50 january 2012**

6- PHARMACEUTICAL ANALYSES OF PARACETAMOL SIRUPS IN ALBANIAN PHARMACEUTICAL" market, **AlbScience Institute Book of Abstracts, pg 528. Prishtine 2011**

7- COMPARATIVE ASSESSMENT OF QUALITY OF DIFFERENT BRANDS OF PARACETAMOL TABLETS COMERCIALLY " **vailable in Albania, Special issue of the "Macedonian Pharmaceutical Bulletin" poster dhe referim oral 2011**

8- CHEMICAL COMPOSITION OF ROSMARINE OILS FROM ALBANIA", **Special issue of the "Macedonian Pharmaceutical Bulletin". 2011**

9- DIFFERENT METHODS OF EXTRACTING CAPSAICIN FROM ALBANIAN'S CAPSICUM FRUITS**. 5thCongress of Pharmacy ofMacedonia . Ohrid, Macedonia. 2011**

10- QUALITATIVE IDENTIFICATIONS OF DIFFERENT DICLOFENAC SODIUM FORMULATIONS REGISTERED IN ALBANIA**".YoungChem 2010" Gdansk, Poland.2010**

11- PREPARATION, CHARACHETRIZATION AND INCLUSION IN B-CYCLODEXTRINE OF ESSENTIAL OIL**".YoungChem 2010" Gdansk, Poland.2010**

12- " CHEMICAL COMPOSITION OF ALBANIAN WILD OREGANOS OILS", V. PAPAJANI, U. ASLLANI, E. TROJA; World Congress of Pharmacy and Pharmaceutical

Sciences, 69th international congress of FIP, 3-8 september 2010, Istambul, Tukey, Book of Abstract.

13- ***CHEMICAL COMPOSITION OF ROSEMARY OILS FROM Albania", Vilma Papajani, Klodiola Dylgjeri, Erjon Troja, Entela Haloçi;*** *Fifth Congress of Pharmacy of Macedonia with International participations, 21-29. 09. 2011, Ohrid Macedonia, Macedonian Pharmaceutical Bulletin, 57 suppl., 2011, pg. 201.*

14- " ESSENTIAL OILS VARIABILITY IN SATUREA MONTANA L. FROM ALBANIA" V. Papajani, U. Asllani, E. Haloçi; *World Congress of Pharmacy and Pharmaceutical Sciences: 70th International Congress of FIP, 09/2010, Lisbonë, Portugali, Book of Abstract*

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1- PERGATITJA,AKTIVITETI ANTIBAKTERIAL DHE MIKROKAPSULIMI NE B-CD I VAJIT ESENCIAL TE SATUREJA MONTANA L. (TRUMZA, -poster- **Referim Ne Konferencen E 3-Te Kombetare Farmaceutike , Tetor Albania November 2013**

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2. ***D.ZELA, E.HALOCI,E.ALLUSHI , HANDBOOK of MARIN BARLETI UNIVERSITY, CO-AUTHOR/BOOK OF 450 PAGES***

PROJECTS

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Albanian Ministry Of Education And Science – Fondo Di Exellenza "Preparazione, Caratterizazione E Inclusione In Ciclodestrine Degli Olii Essenziali (Satureja Montana) Entela Haloci – on going

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Research Article

DEVELOPMENT AND VALIDATION OF A GC/FID METHOD FOR IDENTIFICATION AND QUANTIFICATION OF MAIN COMPONENTS OF SATUREJA MONTANA L. ESSENTIAL OIL

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ABSTRACT

Objective: *Satureja montana* L. is well-known for its essential oil contents and dermatological benefits. Main components of its essential oil are borneol, carvacrol, thymol, γ-terpinen, p-cymen. Our objective is to develop a Gas/Fid analytical method for quantification and identification of main components of *Satureja montana* L. essential oil.

Methods: Essential oils were obtained by hydrodistillation of *Satureja montana* L. using Clevenger apparatus. Analyses of essential oil and validation method were developed by GC/FID apparatus tip Varian 3800.

Results: The validation process; linearity, optimization of GC/FID parameters of method proposed, precision and accuracy results were statistically significant.

Conclusion: The method proposed were found appropriate for GC/FID analyses of *Satureja montana* L.

Keywords: *Satureja montana* L, essential oil, Gas/Fid,validation.

INTRODUCTION

Essential oils are lipid soluble well-known ingredient often applied to the skin for their important properties that ranges from antimicrobial to antinflammatory and skin whitening. Current applications of these volatile compounds turn out to be complicated because of chemical and physical properties.[6,7].

This is one of the major problems for their uses; therefore, microencapsulation could be the solution to problems of stability, evaporation and controlled release.[11] *Satureja montana* L. provides of interesting antimicrobial properties an on the other hand analytical method development and validation play important role in discovery, development and manufacture of herbal medicinal products. [8] The results from the validated test methods are used to ensure identity, purity, potency and performance of drug product. During encapsulation process is necessary the identification and quantification of main components of *Satureja montana* L. through a validated analytical method.

Identification and quantification of main components of *Satureja montana* L. essential oils by GC/FID method is our aim in this study. As far as we know there are no validated methods of performing such analyses of this essential oil. *Satureja montana* L. essential oil main components are [12,13,14,15] carvacrol (2.21 - 55.95%), thymol (0.38 - 40.51%), p-cymene (1.13 - 17.40%), borneol (1.35-9.64%), γ-terpinene (0.31 - 8.86%). As result we studied the GC/FID analytical method of identification and quantification of these components and developed the method validation.

MATERIALS AND METHODS

Plant Material

Herbal plants of *Satureja montana L.* were collected from different zones of Albania and were identified from our botanist Skerdilaid Xhulaj in Botanic Department, Faculty of Natural University of Tirana, Albania. Sample of drug is recorded in Herbarium Deposit in Natural University of Tirana.

Isolation of the essential oil

The hydrodistillation was carried out with a Clevenger-type apparatus according to the Hungarian Pharmacopiea VII. (1986). Drug quantity of 20 g was used; it was distilled with 500 ml of water for 3 hours. The resulting essential oil was dried over anhydrous sodium sulphate and stored at 4°C. [2,16,10]

Reagents

All reagents and solvents used were obtained from Sigma Aldrich Company. GC/FID Tip Varian CP3800, Stationary, Phase Capillary VF: 1ms, Film thickness 0.25 µm(L) 25 mx (ID) 0.25mmx(OD) 0.39mm. Mobile Phase is helium. Standards of carvacrol, p-cymen, y-terpinen, borneol, thymol were obtained from Sigma Aldrich company.

Gas/Fid method

GC/FID conditions GC analysis of the essential oil was performed using a Varian CP-3800 instrument equipped with a capillary column. Helium was used as the carrier gas at the constant flow of 1.2 ml/min and split ratio 1:30. The oven temperature was held at 50 °C for 1 min, and then programmed to 280°C at a rate of 5°C /min. Helium flux is 30ml/min and air flux is 300ml/min. The injector temperature is 280 and detector (FID) temperature is 300°C. Injection volume is 1µl. [3]

METHOD VALIDATION

Standard and sample Stock Solutions

Satureja montana L. essential oil stock solution was prepared dissolving 5 mg essential oil in 5 ml hexane and was stored in refrigerator (-4°C) for stability. Six samples were prepared and each one was injected three times. The standards stock solution were prepared in following concentration p-cimen 2mg/ml carvacrol 2mg/ml, γ-terpinen 2mg/ml, thymol 8mg/ml, borneol 0,5mg/ml.

Linearity – Calibration Curbes

We prepared serial dilutions of each standard. The calibration lines were constructed by plotting the areas of p-cymen, borneol, carvacrol, γ-terpinen and thymol against their corresponding concentration [5]. The concentration studies ranges between 0.5-5mg/ml for borneol, 1.0-8.0 mg/ml for γ-terpinen, 0.1-2.0mg/ml for carvacrol, 0.4-2mg/ml for p-cymen and 2.0-10 mg/ml for thymol. The statistical parameters slope, intercept, residual standard on deviation response correlation co-efficient and p- values were calculated by GraphPad 6.02 version. (Table 1). Their correspondative graph is shown below. (Fig.1)

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Screening the Antibacterial Activity of Satureja Montana Essential Oil before and after Inclusion in β-Cyclodextrine

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ABSTRACT

The aim of this study is to study the antimicrobial activity of Satureja Montana essential oil collected in different places of Albania, and how is it affected by microencapsulation with β-cyclodextrin (β-CD). Gas/Fid spectrometry analysis of the isolated oil resulted in the identification of twentyone compounds in the oil of S. montana. Carvacrol was the major constituent of the S. montana oil (around 60 %). Other important compounds were the monoterpenic hydrocarbons p-cymene, y-terpinene and the oxygenated compounds borneol and thymol. The screening of the antimicrobial activities of essential oil before and after microencapsulation were individually evaluated against three microorganisms Escherichia coli, Staphylococcus aureus and Proteus Vulgaris, using a disc diffusion method. Maximum activity of Satureja montana oil was observed against *Proteus Vulgaris* and *Staphylococcus aureus (inhibition zone > 13 mm)*. From the results obtained is evident that microencapsulation doesn't change the antibacterial activity of essential oil which gives us the possibility to have the optimal antibacterial activity and minimum side effects of essential oil usage and storage the same time due to benefits of microencapsulation by β -cyclodextrine.

Keywords- Satureja Montana, antibacterial, **β**-cyclodextrine

1. INTRODUCTION

Essential oils are lipid soluble well-known ingredient often applied to the skin for their important properties that ranges from antimicrobial, to antinflammatory and skin whitening. [7][Current applications of these volatile compounds turn out to be complicated because of chemical and physical properties. This is one of the major problems for their uses; therefore, microencapsulation could be the solution to problems of stability, evaporation and controlled release. Moreover, some of their components are also provided by side effects such are skin sensitizing activity, thus behaving as allergens. For these reasons, direct contact with skin should be avoided. Satureja Montana also provides of interesting antimicrobial properties and is used for topical treatment against incipient baldness and to treat arthritic joints.[2][14][18]. To explore their dermatological application we investigate the preparation of essential oil, their antimicrobial activity and their inclusion in cyclodextrine complexes, in order to achieve better stability in emulsions and better compatibility with skin application. The present study is done to compare the antibacterial properties before and after encapsulation of the essential oil. The ratio of oil: **β**-cyclodextrine used is 20:80 (w/w).

2. MATERIALS AND METHODS

Gas/Fid Tip Varian CP3800, Stationary, PhaseCapilar VF:1ms, Film thickness 0.25 μm(L)25mx(ID) 0.25mmx(OD)0.39mm. Mobile Phase is helium. Standards of carvacrol, p-cymen, y terpinen,borneol,thymol were obtained from Sigma Aldrich company. β-Cyclodextrin was purchased from Titolchimica (Italy). Bacterias Staphylococcus Aerus ATCC 29737 Lot 58312397, Proteus Vulgaris ATCC 1978 Lot 0876523C, Escherichia coli, ATTC 8456 LOT 6543109, Canida Albicans ATCC 2091 Lot 7051869, Mueller--Hinton agar (Lot 685C2S, Code 060098), Dimethylsulfoxide (DMSO), Cefuroxime 30ug lot 1A320, Tetracyclini 30ug lot OD3313, Cyprofloxacini 5 ug Lot OM3189.

Antibacterial and Antifungal Activity Assesment of Nigella Sativa Essential Oils

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***Abstract*—**Antifungal activities of ether and methanolic extracts of volatiles oils of Nigella Sativa seeds were tested against pathogenic bacterias and fungies strains.The volatile oil were found to have significant antifungal and antibacterial activities compare to tetracycline, cefuroxime and ciprofloxacin positive controls.The ether and methanolic esxtracts were compared to each other for antifungal and antibacterial activities and ether extracts showed stonger activity than methanolic one.

***Keywords*—**Antifungal, antibacterial, essential oils, extraction, Nigella Sativa.

I. Introduction

A LARGE number of medicinal plants have therapeutic potentials. Seeds of Nigella sativa L.(Ranunculaceae), known commonly as "black cumin" have been employed for thousands years as a spice and food preservative. The oil and seed constituents have shown potential medicinal properties in traditional medicine. Recently, many biological activities of Nigella sativa L. seeds have been reported, including: antioxidant, anti-inflammatory, anticancer and antimicrobial and antifungal ones. [2]

Several pharmacological effects have been attributed to active principles of Nigella sativa L. which includes thymoquinone, thymohydroquinone, dithymoquinone, thymol,carvacrol, nigellicine, nigellimine-x-oxide, nigellidine and alpha-hedrin (Aljabre et al. 2005). [3],[7],[11] Nigella sativa L. seed extract inhibits fungal strains. In our study we have tested the antifungal and antibacterial properties of Nigella Sativa.

II. Experiments

A. Extraction of the Essential Oils

25 g seeds were crushed and extracted with petroleum ether for 4 h in a Soxhlet apparatus.After extraction, the solvents were removed by rotary vacuum and dried in a vacuum oven at 30°C for.The same method was repeated by using methanol as extract agent.

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B. Materials and Methods

Staphylococcus Aerus ATCC 29737 Lot 58312397, Proteus Vulgaris ATCC 1978 Lot 0876523C, Escherichia coli, ATTC 8456 LOT 6543109, Canida Albicans ATCC 2091 Lot 7051869, Mueller--Hinton agar (Lot 685C2S, Code 060098), Dimethylsulfoxide (DMSO), Cefuroxime 30ug lot 1A320, Tetracyclini 30ug lot OD3313, Cyprofloxacini 5 ug Lot OM3189

C. Antimicrobial and Antifungal Activity af Essential Oil

The essential oil of samples M1- Ether extract and M2-methanol extract were tested for antibacterial activity by the disc diffusion method using 100μL of suspension of the tested microorganisms, containing 2.0 x 106 colony forming units (cfu mL–1) for bacteria and 2.0x105 spore mL–1 for fungalstrains. [11]

Fig.1 Essential Oils Inhibition Zone And Positive Controls vs /C.Albicans

Mueller--Hinton agar and dextrose agar were distributed to sterilized Petri dishes with a diameter of 9 cm.

The filter paper discs (6 mm in diameter) were individually impregnated with10μL and 30μL of the essential oils dissolved in dimethylsulfoxide (DMSO). (Fig 1, Fig 2)

The Petri dishes were kept at 4°C for 2 h. The plates inoculated with bacteria incubated at 37°C for 24 h and at 30 °C for 48 h for the yeasts. The diameters of the inhibition zones were measured in millimetres. Negative controls were set up with equivalent quantities of DMSO. Studies were performed in triplicate. In addition, positive controls antibiotic discs such as Cefuroxime, Ciprofloxacine, Tetracycline and Nystatin were used for comparison.

II. Results And Discussion

The results of disc diffusion assay are demonstrated on tab I.

COMPLEMENTARY AND ALTERNATIVE MEDICINE (CAM) FOR PAIN, HERBAL ANTI-INFLAMMATORY DRUGS

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Abstract

Introduction: Many specialists in pain management view the integration of traditional treatment approaches and a wide range of complementary and alternative medicine approaches as the ideal goal of many types of pain. Pain is a subjective, complex, multidimensional and unpleasant experience. It is one of the most prevalent conditions that require medical attention. Nowadays the number of patients that are using herbal remedies and complementary and alternative medicine for treatment of pain is growing rapidly.

Objectives: The aim of this investigation is to describe the concept of complementary and alternative medicine – CAM and the role of specific plants and their anti-inflammatory activity in curing inflammatory painful diseases.

Conclusions: Nonsteroidal anti-inflammatory drugs and opioid analgesics are normally used to treat inflammation and pain, but they can manifest a great number of adverse effects. Therefore herbal drugs can be a potential source to replace them. Many medicinal plants, some of them indigenous, are known for their analgesic and anti-inflammatory effect, but only a few are included in the health care system after clinical research. So, it is time to give an important place to the scientific uses of natural medicinal plants and investigation of the active phytoconstituents of them. Extraction of the active principle of herbal drugs and their pharmaceutical formulation must be the main direction of industrial research.

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CHEMICAL CHARACTERIZATION, ANTIBACTERIAL ACTIVITY ASSESMENT AND INCLUSION IN CYCLODEXTRINES OF SATUREJA MONTANA L. ESSENTIAL OILS

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INTRODUCTION

Essential oils are lipid soluble well-known ingredient often applied to the skin for their important properties that ranges from antimicrobial, to antinflammatory and skin whitening. Current applications of these volatile compounds turn out to be complicated because of chemical and physical properties. This is one of the major problems for their uses; therefore, microencapsulation could be the solution to problems of stability, evaporation and controlled release. Moreover, some of their components are also provided by side effects such are skin sensitizing activity, thus behaving as allergens. For these reasons, direct contact with skin should be avoided. *Satureja Montana* L. also provides of interesting antimicrobial properties. To explore their dermatological application we investigate the antimicrobial activity and their inclusion in cyclodextrine complexes, in order to achieve better stability in emulsions and better compatibility with skin application. This study is part of a major project divided in four steps. First step was the extraction of the essential oil by hydro-distillation by Clevenger type apparatus, second one was the characterization by GS/FID of essential oils obtained, and third one was screening the antimicrobial activity of the extracted essential oils (the presenting paper). The last one will be preparation of essential oil complexes with cyclodextrine and analysis of their chemical, physical, antibacterial activity and biodisponibility properties.

MATERIALS AND METHODS

MATERIALS and APPARATUS: Gas/Fid TipVarianCP3800, Clevenger Apparatus. Standards: Carvacrol, p-cymen, y-terpinen, borneol, thymol were obtained from Sigma Aldrich Company. Bacterias: Staphylococcus aureus, Escherichia coli, yeast Aspergillus flavia, Claudosporium herbarum, Samples of *S.montana L.*:

Origin	Traditional Pharmacy - M1	Elbasan - M2	Kerraba - M3	Kruja - M4	Scutatri Mauntains - M5
Altitude	150 m	300 m	950 m	740 m	1000 m

Extraction of the essential oils

A hundred grams of dried aerial plant material were subjected to hydro-distillation for 3 hours using a Clevenger-type apparatus to produce oils. The essential oils obtained were dried over anhydrous sodium sulphate and stored in sealed vials at low temperature (2°C).

GAS/FID Identification and quantification of Main components

GC/FID analysis of the essential oil was performed using a Varian CP-3800 instrument equipped with a capillary column.VF:1ms, Film thickness 0.25 mm (L) 25mx (ID) 0.25mmx (OD) 0.39mm.
Helium was used as the carrier gas at the constant flow of 1.2 ml/min and split ratio 1:30. The oven temperature was held at 50°C for 1 min, then programmed to 280°C at a rate of 5 °C /min. Injection volume was 1µl. The injector temperature was 250 and detector (FID) temperature 300°C. Identification was done by comparing the RI of samples with those of standards. Percentages of main components were obtained by serial dilution of standards in hexane. (Tab.2)

Antimicrobial activity of essential oils

The essential oil of samples was tested for antibacterial activity by the disc diffusion method using 100µL of suspension of the tested microorganisms, containing 2.0 x 106 colony forming units (cfu mL–1) for bacteria and 2.0x105 spore mL–1 for fungal strains. Mueller—Hinton agar and dextrose agar were distributed to sterilized Petri dishes with a diameter of 9 cm. The filter paper discs (6 mm in diameter) were individually impregnated with10µL and 30µL of the essential oils dissolved in dimethylsulfoxide (DMSO). The Petri dishes were kept at 4°C for 2 h. The plates inoculated with bacteria incubated at 37°C for 24 h and at 30 °C for 48 h for the yeasts. The diameters of the inhibition zones were measured in millimetres. Negative controls were set up with equivalent quantities of DMSO. Studies were performed in triplicate. In addition, positive controls antibiotic discs such as Cefuroxime, ciprofloxacin, tetracycline and nystatin were used for comparison.

Synergism of main components

Many studies deal with the fact that the components of *Satureja Montana* L. essential oil are in synergy with each other. To study this phenomenon we compared the inhibition zone of each standard and their mixture too.

RESULTS AND DISCUSSION

The sample from Scutari Mountains has the highest (0.65 ml) amount of essential oil and its yields have the highest % of carvacrol and thymol too.

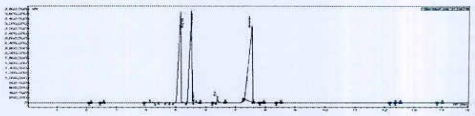
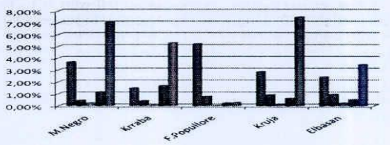


Fig. 1: Standarts chromatograms obtained by Gas/FID.

Table 2: Comparative % of important components of *Satureja Montana* L essential oil from different altitudes in Albania.



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ENCAPSULATION OF SATUREJA MONTANA ESSENTIAL OIL IN β-CYCLODEXTRINE

--Manuscript Draft--

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Abstract:	Inclusion complexes between the Satureja montana essential oil and β-cyclodextrin were prepared by co-precipitation method with the four oil to β-cyclodextrin ratios of 5:95, 10:90, 15:85 and 20:80 (w/w) in order to determine the effect of the ratio on the inclusion efficiency of β-cyclodextrin for encapsulating oil volatiles. The characterization of the complex involved the analysis of the initial essential oil, the surface and the total extracted oils. The retention of essential oil volatiles reached a maximum of 93.15% at the oil to β-cyclodextrin ratio of 15:85. Though, the maximum inclusion efficiency of β-cyclodextrin was achieved at the ratio of 20:80.The qualitative and quantitative composition of the volatiles in the total oil extracts was similar to the starting oil which means that essential may still have the antibacterial and antifungal properties after encapsulation to β-cyclodextrine This justifies the use of β-cyclodextrin as complexing agent for Satureja Montana essential oil in the food and pharmaceutical industrie

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MOLECULAR DYNAMICS OF THYMOSIN α-1: STRUCTURE STABILIZATION BY PEO-DIAMINE STAPLING.

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ABSTRACT

Thymosin α-1 belongs to the small molecule family of thymosines, which have been studied for a long time as molecules with very high potential use as drugs. In particular Thymosin α-1 is under intensive studies for its use in different disorders and diseases such as respiratory distress syndrome, hepatitis C, hepatitis B, AIDS, cytomegallovirus infection etc. Thymosin α-1 is implicated in the control of gene expression of MHC I, MHC II, cytokines and other immune system regulators, but its molecular mechanism remains still unclear. On the other hand several studies were conducted with the aim to identify the structure of the peptide. In fact it was found that thymosin α-1 presents an α-helix conformation in fluorinated alcool/water mixtures but it is highly unstructured in water solution. It is also presumed that very likely thymosin α-1 presents an α-helix conformation when bound to the receptor. Here we present a MD simulation of a Poly Ethylen Oxide Diamine stapled thymosin α-1 as a new strategy for the stabilization of its α-helix conformation in water solution. The PEO diamine stapled peptide presented a good overall stability also at high temperatures offering new insights into the design of more potent Thymosin α-1 derivative molecules.

INTRODUCTION

Thymosin α-1 (AcSDAAVDTSSEITTKDLKEKKEV VEEAEN) is a 28 residue long peptide isolated for the first time from the calf thymus. It has been reported that this peptide produces a strong activation of T-cell differentiation and function [1]. In fact it is implicated in the control of gene expression of MHC I, MHC II, cytokines and other immune system regulators [2], but the real mechanism of action remains still unclear since no specific receptors have been identified. Thymosin α-1 is currently used against infectious diseases such as respiratory distress syndrome, hepatitis C, hepatitis B, AIDS, cytomegalovirus infection and as antitumoral where usually is administered subcutaneously [3]. It is produced in vivo by the cleavage action of a lysosomial asparagynil endopeptidase on the N-terminal region of prothymosin α, a 109 residue nuclear protein [4] and presents an acetate cap on its N-terminus. High field NMR spectroscopy studies in DMPC/DMPA and in TFE/water mixtures demonstrated that the peptide adopts an α-helix conformation from residues 14 to 26 and two double β-turns in the N-terminal region involving the first 12 residues. Furthermore these NMR studies demonstrated that thymosin α-1 is unstructured in water solution and proposed a model in which the interaction with cellular membranes is

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