VARIATION IN THE ESSENTIAL OIL COMPOSITION OF *CALENDULA OFFICINALIS* L.

BY

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DECLARATION

I declare that this dissertation is my own work except the acknowledged supervision and referred literature. It has not been submitted before for any degree or examination in any other University.

Omobola Okoh

January 2008, Alice

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Finally I would like to thank the National Research Foundation for funding my research work.

ABSTRACT

Variations in the yield and composition of the essential oils from *Calendula officinalis* L. cultivated in Alice (Eastern Cape) are reported. Essential oils were obtained by hydrodistillation using the Clevenger apparatus and analysis was performed by GC-MS. The yield in essential oil revealed a maximum at the full-flowering stage (0.97%) and a minimum during the pre-flowering stage (0.13%). The composition showed different patterns at different phases of the vegetative cycle. Sesquiterpenes (α-cadinene, α-cadinol, T-muurolol, and *epi*-bicyclosesquiphellandrene) and monoterpenes (limonene, 1,8-cineole, and *trans*-β-ocimene) showed highest correlations with the age of the plant. An interesting stage is the post-flowering period, the essential oil being rich in α-cadinene, α-cadinol, T-muurolol, limonene, 1,8-cineole, with *p*-cymene presenting lower levels.

The oils were extracted by hydrodistillation from fresh leaves, dry leaves, and fresh flowers yielding 0.06 %, 0.03 %, and 0.09 %, respectively. The analysis of oils by GC-MS revealed a total of 30, 21, and 24 compounds from the fresh leaves, dry leaves, and the flowers, respectively, representing 91.7, 89.8, and 87.5% of the total oil composition. Sesquiterpenoids dominated in the fresh leaves (59.5 %) and flowers (26 %), while the monoterpenes dominated in the dry leaves (70.3 %). T-Muurolol (40.9 %) predominated in the fresh leaf oil, α-thujene (19.2 %) and δ-cadinene (11.8 %) were present in high quantities. In contrast, 1,8-cineole (29.4%), γ-terpenene (11.6 %), δ-cadinene (9.0 %), β-pinene (6.9 %), and α-thujene (6.3 %) were the major components in the dry leaf oil, while in the fresh flower oil, α-thujene (15.9 %), δ-cadinene (13.1 %) and γ-cadinene (10.9 %) were the major components. The significance of the effect of drying and age on essential oil composition is discussed.

CHAPTER 1

INTRODUCTION

1.1 NATURAL PRODUCTS

All over the world, natural products have found great usefulness in industry as well as in herbal medicine. In Africa, for example, the majority of inhabitants depend on the available plants for their primary health care. Most of these remedies are natural products.

Plants through scientific researches have been found to contain valuable chemicals (Morrison and Boyd, 1987). These natural chemicals and their synthetic counterparts have continued to serve as feed stock in relevant industrial fields. While some are used in pharmaceutical, food, and chemical industry, others are applied as food flavors and fragrances, sweeteners, or even pesticides. Although western technologists have transformed many medicinal plants into more palatable forms like tablets, capsules, and syrups, many traditional healers still use plants in their crude form (herbal remedies). Extracts from some of the medicinal plants being used by traditional healers have been found to contain properties that inhibit the growth of bacteria, viruses, and other microbes (Ndubani & Hojer, 1999).

The global markets of natural products for industrial and medicinal uses have been growing rapidly in recent years. Today medicinal and aromatic plants have become an integral component of research and pharmaceutical industry. Such research focuses on the isolation and direct use of active medicinal constituents of plants, semi-synthetic drugs, and pharmacologically active compounds. As a result, industry is investing vast

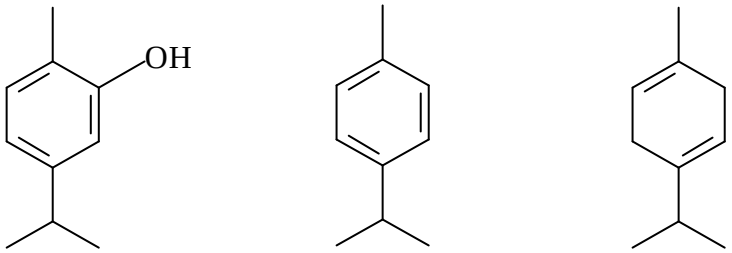
resources into screening of the active constituents of medicinal and aromatic plants from all over the world. For example, about half of the world's 25 best selling pharmaceuticals in 1991 originated from natural source materials and about 25% of the prescribed drugs were from the plant kingdom (Balick, 1990). In addition to the medicinal and industrial uses of natural products from plants, many phytovolatile compounds are used in cosmetics (Table 1).

Table 1: Some Plants Used as Cosmetics in Mozambique (Bodeker, 1994)

Scientific Name	Family	Use	Part Used
Diwrocaryum zanguebarium	*Pedaliacea*	Shampoo	Leaves, stem
Sesanum alatum	*Pedaliacea*	Shampoo	Leaves
Albizia versicolor	*Leguminosae*	Detergent	Bark, roots
Securidaca longepediculata	*Polygalaceae*	Detergent	Roots
Olax dissitiflora	*Olacaceae*	Beauty cream	Stem's powder
Euclea natalensis	*Ebenaceae*	Dentifrice	Roots
Diospyros vellosa	*Ebenaceae*	Dentifrice	Roots
Vepris laceolata	*Rutaceae*	Aromatic	Leaves
Zanthoxylon Capensis	*Rutaceae*	Aromatic	Leaves, bark

Among the plants whose essential oils are widely used in South Africa for food flavor, pharmaceuticals, cosmetics, and medicinal purposes is *Calendula officinalis L*. It has been well documented that there exist dramatic variations in the yields and composition of essential oils within and between natural plant populations. According to Viljoen *et al.* (2005), since the antimicrobial activity of these oils may be directly related to their specific composition, they may also fluctuate. It is a well known phenomenon in several plant species that the yield and composition of the volatile oils vary both quantitatively and qualitatively at different phases of the vegetative cycle (Moldao-Martins *et al,* 1999).

This has been demonstrated for *Dracocephalum moldavica*, *Thymus capitatus*, *Artemisia judaica and Thymus vulgaris (*Holm *et al*, 1988; Arras *et al*, 1993; and McGimpsey *et al*. 1994; Ravid *et al*, 2006). In these reports, higher yields were observed in the flowering or post-flowering period. In *Thymus capitatus*, carvacrol **1** (the main compound) was present at higher levels before flowering and until the post-flowering period. Some other compounds, such as *p*-cymene **2** and γ-terpinene **3** also showed seasonal variations. *p*-Cymene **2** showed a minimum level before flowering and a maximum after the flowering period whereas γ-terpinene **3** showed the opposite variation. It was also reported that the content of hydrocarbons in this plant decreased with increase in the size of leaves, while the content of oxygenated hydrocarbons showed the opposite variation.



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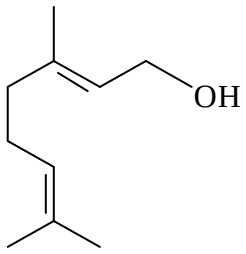
1.2 MOTIVATION / JUSTIFICATION OF THIS RESEARCH

Viljoen *et al*. (2005) in the report on the essential oil chemistry of *Lippia javanica* growing in South Africa observed dramatic variation within and between natural plant population and suggested that as the antimicrobial activity may be directly related to the specific composition of the oil, the activity may also fluctuate.

Although several investigations have been carried out on *Calendula officinalis* as shown above in other parts of the world, the studies are exhaustive, not exhausted, and there is a dearth of information in the literature on systematic studies of the chemical

composition of essential oils from the plant found growing in the Eastern Cape Province of South Africa. Yet, there have been reports of variability in the chemical composition of essential oils of same plants from different regions, with seasonal differences also significantly affecting such compositions (Miguel *et al*, 2004).

Before the commencement of this study, there was no information in the literature where variation in the chemical composition of the essential oil of *Calendula Officinalis* was reported. Yet, since the essential oil of this plant is used for flavoring, its acceptability is important. Panel test results have shown that essential oil rich in geraniol **4** and geranyl acetate **5** is well accepted, but not accepted or badly scored when *p*-cymene **2** and γ-terpinene **3** are present at high levels (Moldao-Martins *et al*, 1999). This study could therefore not have come at a more auspicious time to eminently fill in these gaps, and it is expected to give for the first time, a comprehensive picture of the chemical composition of the essential oils of the leaves and flowers of the plant cultivated in the Eastern Cape province of South Africa and at different stages of growth.



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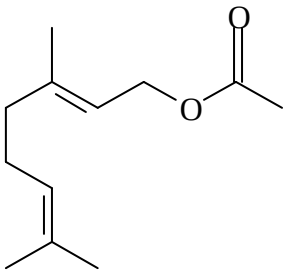

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1.3 AIMS AND OBJECTIVE OF THE STUDY

This study therefore aims at investigating the chemical profile of the essential oil of *Calendula officinalis* growing in the Eastern Cape Province of South Africa. Specifically, the objectives of this project are:

- To carry out a comparative investigation of the chemical composition of *Calendula officinalis* growing in the Eastern Cape Province of South Africa.
- Isolation of essential oils from the leaves and flowers of *Calendula officinalis* plant at different stages of growth and season.
- To investigate the influence of drying on the quantity and quality of its essential oil.
- To carry out structural elucidation of key components of the essential oil using traditional spectroscopic methods (IR- and NMR-spectroscopy).

CHAPTER 2

LITERATURE REVIEW

2.1 *CALENDULA OFFICINALIS L.*

Calendula officinalis also known as marigold or pot marigold is an annual or biennial aromatic herb with soft glandular leaves and attractive yellow or orange heads. It belongs to the Asteraceae family and grows wild in the Southern, Eastern, and Central Europe (van Wyk and Wink, 2004). The botanical classification of the plant is as shown below in Table 2 (USDA, 2005):

Table 2. Botanical Classification of *Calendula officinalis* L.

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Asteridae – Subclass
Order	Asterales – Composite family
Family	Asteraceae – Aster family
Genus	Calendula L. – marigold
Species	Calendula officinalis L. – pot marigold

The annual form is more widely grown and is usually multi-stemmed with a strong taproot. The plant grows one to two feet tall and requires full to partial sunlight. The vegetative parts of the plant are mid-green in color and the stems are angular and covered by fine hair. The lower leaves of the plant are paddle-shaped whilst the upper leaves are smaller and more pointed. The composite flowers are yellow and orange (Gilman and Howe, 1999) and are born on multi-stock stalks. The flower heads are heterogamous, *i. e.* the outer flowers are female whilst the inner flowers are disk flowers which are pseudo-

hermaphroditic and sterile female. The flowers blossom in the spring-summer seasons as shown below in Fig. 1.

Figure 1: *Calendula officinalis* L.

The seeds are grey or light-brown in color and vary in shape, decreasing in size towards the centre of the head. As a herb, the petals are much prized for their color and flavor, and have been used to color butter and cheese and to flavor soup (Gilman and Howe, 1999). *Calendula officinalis* is generally planted in the fall of winter and spring, and their seeds are recognized as an important source of fatty acids with conjugated double bonds with tremendous potential for use as industrial oil (Beerentrup and Robbelen, 1987).

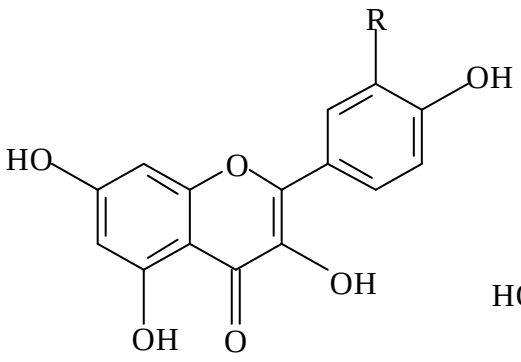
2.2 PROPAGATION OF *CALENDULA OFFICINALIS*

Calendula officinalis is actually a biennial, but it is cultivated as an annual plant. The seeds are best sown as soon as it is ripe in a green house. Stored seeds are usually sown in

early spring in a greenhouse or in the field, and no treatment is needed. Seeds planting could be commenced in-doors and then transplanted using 10 to 12 inches spacing. The seed germinates well under conditions of high temperature and full sun. The seeds germinate in one to two weeks and usually have about 80% germination. However, calendula pests exist and they include whitefly, aphids, and thrips. Cucumber beetles and blister beetles may also be a problem (Janke and DeArmond, 2004). Flowers are usually harvested by hand when they are completely open and have not gone to seed, as medicinal properties are usually not active in plants that have gone to seed. Also, harvesting can be done any time in the growing season but preferably in early summer in order for the new plant to become established before winter.

2.3 CHEMICAL CONSTITUENTS OF *CALENDULA OFFICINALIS*

Some of the chemical constituents of *Calendula officinalis* have been reported to include flavonoids (*O*-glycosides of quercertin **6** (R = OH), kaempferol **6** (R = H), and isohamnetin **6** (R = OMe) up to 0.8 %, bisdesmosidic and monodesmosidic saponins (glycosides of steroids, steroid alkaloids (steroids with a nitrogen function) or triterpenes found in plants (up to 10 %), hydroxylated and esterified triterpenes (taraxasterol **7** and faradiol **8**).



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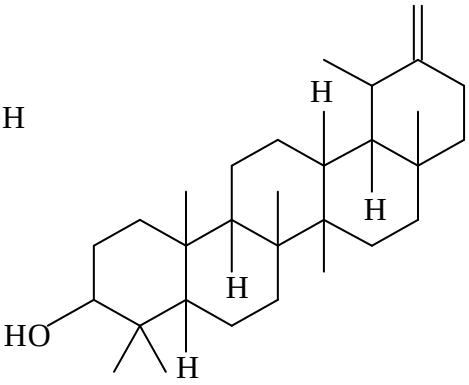

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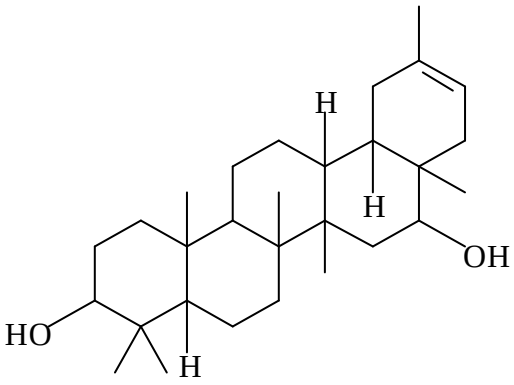

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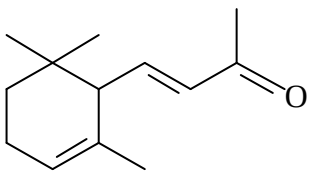
The essential oil contains mainly sesquiterpenoids (α-ionone **9**, β-ionone **10**, and many others). Essential oils are volatile odorous concentrated aromatic extracts, which are distilled from plants (Atherden, 1969). They are soluble in alcohol but to a very limited extent in water. They have very strong aromatic components. Chemically, essential oils are mixtures of esters, aldelydes, alcohols, ketones, and terpenes. The major difference between essential oils and fixed oils is their volatility. They are secreted in oil cells, in secretion ducts or cavities, or in glandular hair. They are colorless particularly when fresh, but on prolong standing, may oxidize and become darkened in color (Trease and Evans, 1978). In some volatile oils, *e. g.* that of thyme, a separation into a solid and a liquid portion occurs on standing in the cold. The solid portion frequently is known by the

name stearoptene, and the liquid portion is called eleoptene. Some of the stearoptene is of commercial importance (*e. g.* thymol, camphor, and menthol).



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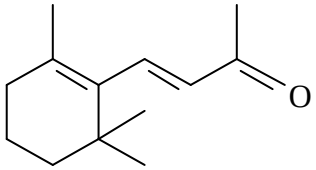

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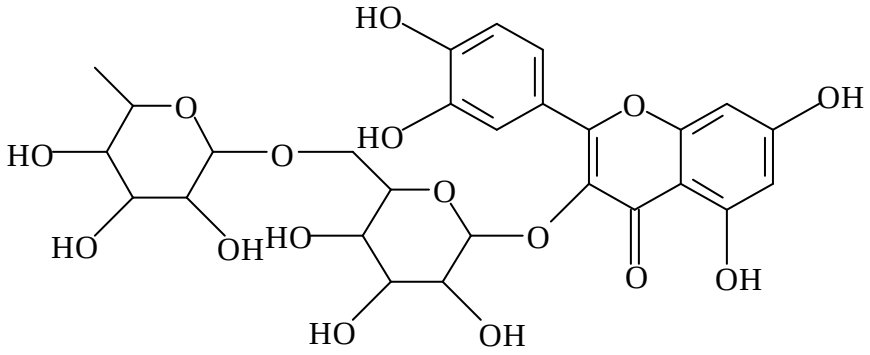
The pharmacological activity of marigold is related to the content of several classes of secondary metabolites such as essential oils, flavonoids, sterols, carotenoids, tannins, saponins, triterpene alcohols, polysaccharides, a bitter principle, mucilage, and resin. Vidal-Ollivier *et al*., 1989, Bilia *et al*., 2001 found that marigold flowers contain rutin **11**, isoquercitrin **12**, and others.



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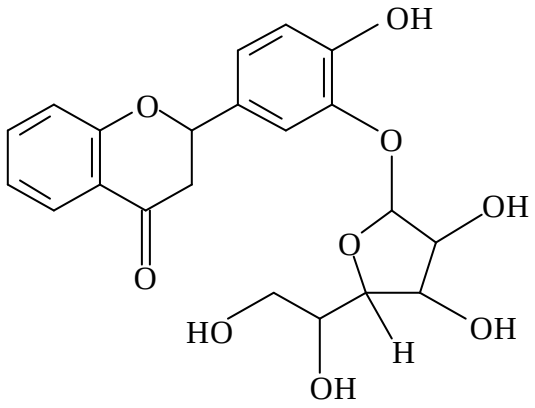

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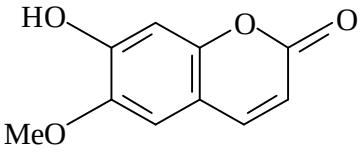
Also present are coumarines (scopoletin **13**), carotenoids, and polysaccharides (van Wyk and Wink, 1997). The saponins, triterpenes, and flavonoids appear to be responsible for wound-healing effects as they show anti-inflammatory and anti-microbial properties (Jimenez-Medina *et al*., 2006). Also, Crabas (2003) reported that the essential oil of *Calendula officinalis* obtained from Italy contained methyl hexadecanoate **14** (23.8%), methyl linoleate **15** (18.6%), methyl 9,12,15-octadecatrienoate **16** (17.2 %), methyl octadecanoate **17** (4.8 %), methyl tetradecanoate **18** (4.6 %), γ-cadinene **19** and cubenol **20** (4.0 %), δ-cadinene **21** (3.2 %), α-cadinol **22** (1.8 %) and oplopanone **23** (1.3 %).



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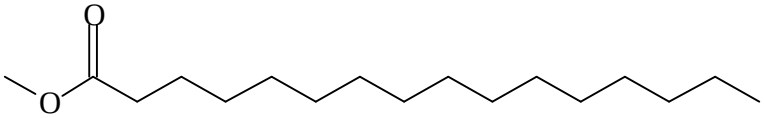

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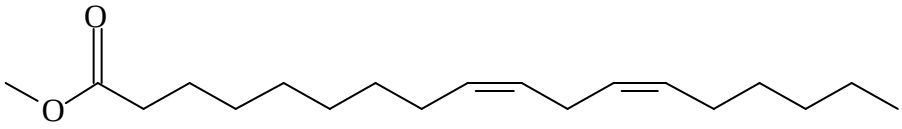

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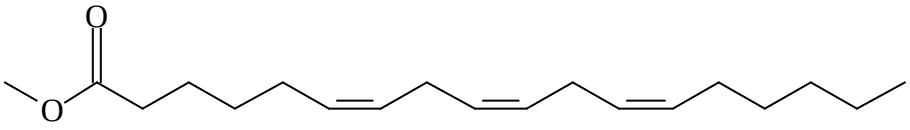

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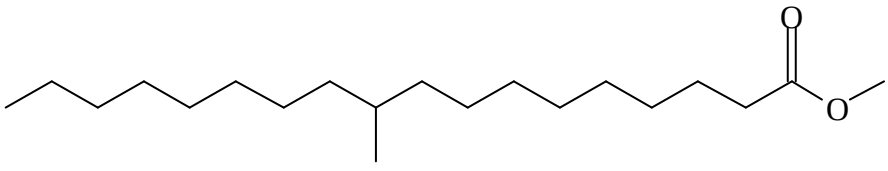

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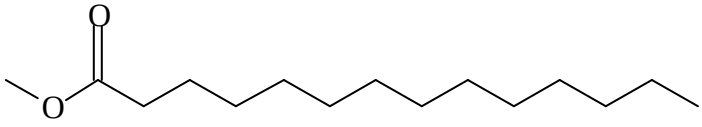

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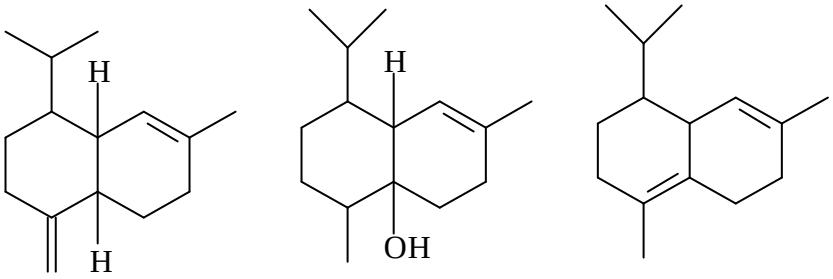

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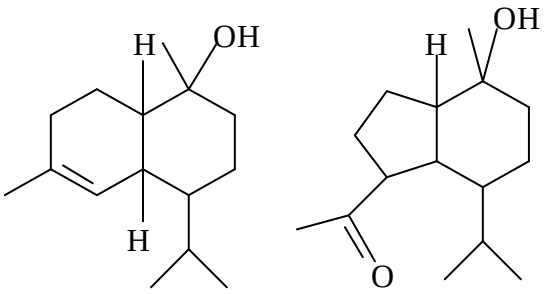

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2.4 USES OF *CALENDULA OFFICINALIS*

Calendula officinalis products are mainly used for external and local application to treat slow-healing wounds, burns, dry skin, eczema, oral thrush, and hemorrhoids. It is applied locally as a tincture, oil, or lotion and is considered an antiseptic. Taken internally it has anti-inflammatory (Dumenil *et al*., 1980) and spasmolytic effects and is effective against inflammation of the mouth and throat. It also improves digestion, stimulates bile production, heals gastric ulcers, and regulates menstrual disorders. The flowers are used in foods to color and add flavor to local dishes, and contain essential oil, fatty acids, organic acids, bitter substances, mucilage, resin, rubber, cholesterolic esters, saponins, triterpenic alcohols, ascorbic acid, and a mixture of natural dyes (Marczal *et al*., 1987). The dried flowers are included in herbal teas to improve their appearance (van Wyk and Wink, 2004). The crushed petals may be combined with olive oil to form an ointment for

external application to cuts, bruises, sores, and burns. The infusion is used to soothe watery irritated eyes, to relieve bronchial complaints, to treat liver disorders, and to induce perspiration in case of fever.

Several clinical studies have shown that calendula has antimicrobial and antiviral activity and wound healing capacity in skin tissue by inducing the formation of new blood vessels, and has been approved in Europe for use in inflammation of the mouth and pharynx, and for healing wounds and burns (Janke and DeArmond, 2004). Historically, calendula blossoms were used to color broth, rice, and other foods as a substitute for saffron, but are now primarily used as skin cream, oil, or lotion (Janke and DeArmond, 2004).

Some of the other non-food applications of *Calendula officinalis* include their use in paints, coatings and cosmetics (Muuse *et al.* 1992) and industrial nylon products. It is also considered an ornamental plant in Cuba (Svanidze *et al*., 1975) and across Europe (Cromack and Smith, 1998), and more than 35 properties have been attributed to the decoctions and tincture from the flowers such as anti-inflammatory, analgesic, antitumor, antiulcer, bactericide, diuretic, tonic, and the healing of wounds and skin eruptions (Duke, 1991). The seeds of *Calendula officinalis* are recognized as an important source of fatty acids with conjugated double bonds with tremendous potential for use as industrial oil (Beerentrup and Robbelen, 1987). The vast medicinal uses of this plant are probably due to the yield, quality and general properties of its essential oil.

Marigold is a herb of ancient medicinal repute. In traditional and homeopathic medicine it has been used for skin complaints, wounds, and burns, conjunctivitis and poor eyesight, menstrual irregularities, varicose veins, hemorrhoids, duodenal ulcers, etc.

(Wichtl, 1994). Marigold grows as a wild and common garden plant throughout Europe and North America. The yellow or golden-orange flowers of marigold are used as spice, tea, and medicine. They may be used either as fresh or dried, and can be made into tea, tinctures, ointments, and creams.

2.5 RISK INVOLVED IN CALENDULA USAGE

Chemicals in calendula may result in a miscarriage if taken by a pregnant woman. They may also interfere with conception if taken by either member of a couple trying to conceive a child. Women who are breast-feeding and small children are advised to avoid taking calendula orally and individuals who are allergic to any members of the daisy family of plants may also have allergic reactions to calendula (*E*Drug Digest, 2004).

2.6 ISOLATION OF ESSENTIAL OILS

Several methods are available for the extraction of essential oils from plants. The isolation of essential oils is facilitated by the properties of a compound such as vapor pressure, solubility, polarity, and molecular size. The following methods can be used to isolate or extract essential oils: effleurage, pneumatic method, maceration, expression, solvent extraction, distillation procedures, and liquid carbon dioxide method (Trease and Evans, 1978; Srivastava, 1991; Igwe and Osinowo, 1996).

2.6.1 Effleurage

The most important center for the extraction of flower oils is Grasse, in the South of France where the effleurage method is used and has its root. This method involves extrac-

tion of the volatile oil with cold fat. In the effleurage process, glass plates are covered with a thin layer of purified fixed oil or fat upon which the fresh flowers are spread. The essential oil gradually passes into the fat and the exhausted flowers are removed and replaced by a fresh supply until the fat is saturated with the volatile oil. In this process the volatile oil is obtained in a fatty base. Then successive extractions with alcohol are fulfilled. The alcoholic extracts may be put on the market as flower perfumes or the oil obtained in a pure form by recovery of the alcohol (Trease and Evans, 1978; EB, 1990).

2.6.2 Pneumatic Method

This method is similar in principle to the effleurage process. It involves the passage of a current of warm air through the flowers. The air, laden with suspended volatile oil, is then passed through a spray of melted fat in which the volatile oil is absorbed. The volatile oil, as in effleurage, is obtained from the fat by three successive extractions with alcohol.

2.6.3 Maceration

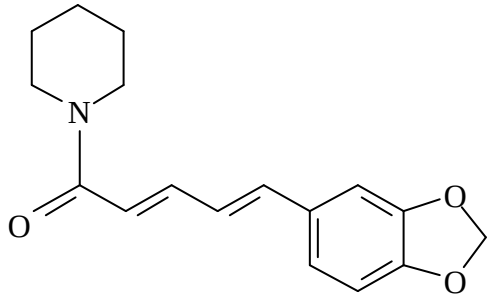
This involves the immersion of the flower into a melted fat at a temperature of about 40° to 80°C. This process, which is similar to effleurage, takes a shorter period of one to two hours. The volatile oil can be sold in fatty base or extracted with alcohol to obtain the pure oil (EB, 1990).

2.6.4 Expression

This method of isolation is often applied to citrus oils, *e. g.* oils from lemon, lime, grape, tangerine, sweet and bitter orange, *etc*. Citrus oils are isolated from the peel by expression or cold pressing. This process involves the abrasion of peel and the removal of the oil in an aqueous emulsion, which is subsequently separated in a centrifuge. Centrifuging of the aqueous emulsion separates the aqueous component and cell debris (EB, 1990). Expressed citrus oils have superior fragrance characteristics compared with distilled oils, because of the absence of heat during processing and the presence of components that would not be volatile in steam. They are also more stable to oxidation because of the presence of anti-oxidants, such as tocopherol, which are not volatile in steam. The lack of heat damage to the oil is also significant. The citrus oils are one of the most natural perfume materials in the sense that they can be used exactly as they occur in nature.

2.6.5 Solvent Extraction

An essential oil that is sensitive to heat, *e. g.* jasmine or tuberose, or that contains an essential monovolatile constituent, *e. g.* piperine **24** is extracted with a solvent.



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A proper solvent is low-boiling, free of odor and impurities, and does not react with the extract. Volatile solvents such as benzene, alcohol, or *n*-hexane, are primarily used.

The freshly picked flowers are placed in specially constructed vessels and extracted at room temperature. The dissolved oil carries waxes and coloring matter along with it. Distillation of semi-solid dark–colored mass can remove the solvent, which is called flora concrete. In order to remove plant waxes from the floral concrete, the latter is dissolved in alcohol. The mixture is cooled and filtered to remove solidified waxes. The filtrate is then distilled to recover the viscous oil known as absolute (Conn and Stumpf, 1976).

2.6.6 Distillation Procedures

Distillation is by far the most common and important method of isolating essential oil from a plant material. There are three types of distillation, namely steam distillation, water distillation, and dry distillation (Igwe and Osinowo, 1996).

2.6.6.1 *Steam Distillation*

Essential oils are produced by a variety of methods as described in the above sections of this chapter. Steam distillation is the most widely used. In this process the plant material is suspended on a grid above the water level. The steam, which is normally generated in a separate boiler, is passed through the plant material *via* a pipe under the grid. The steam and volatile oil are then condensed and the oil separated. The basic principle behind the distillation of two heterogeneous liquids, such as water and an essential oil, is that each exerts its own vapor pressure as if the other component were absent. When the combined vapor pressure equals the surrounding pressure, the mixture starts to boil. Essential oil components boil at a temperature close to the boiling point of water. The steam and essential oil are condensed and separated. Essential oils produced in this way are frequently different from the original in the plant material in a number of respects. Chemi-

cals, which are not volatile in steam, for example, phenyl ethanol ($C_6H_4CH_2CH_2OH$) in rose oil, are mainly left behind in the still. Many of these non-volatile components are responsible for the taste rather than fragrance effects. Some very volatile chemicals may be lost in the distillation, and the process itself may induce chemical changes such as oxidation or hydrolysis.

2.6.6.2 *Water Distillation*

In this process, a vessel containing water and the crushed plant material is heated by direct flame. The water vapor and volatile oil are condensed and recovered by a water-cooled condenser. This process is disadvantageous in that coming in contact with the sides of the vessel can burn the material and this imparts a bad odor (still odor) to the finished product. The burnt character of still odor gradually reduces on storage of the oil.

2.6.6.3 *Dry Distillation*

Dry distillation is only suitable for a small range of essential oils and is often used to distil the oil from exudates such as balsams. In the process, the vessel containing the plant material on a grid is heated to prevent condensation of steam under vacuum (EB, 1990).

2.6.6.4 *Hydrodiffusion*

Hydrodiffusion is a variation of the normal steam distillation process and involves the pulsing of the steam through the top of the vessel containing the plant material; the oil

and water mixture is then condensed from the bottom. This method reduces distillation time and is particularly suitable for distilling seeds (Srivastava, 1991).

During distillation, the boiling water penetrates the plant tissues and dissolves a part of the essential oil present in the oil-containing structures (cells, secretion ducts, cavities, or glandular hairs). The aqueous solution diffuses through the cell membrane by the process called hydrodiffusion. Immediately upon arrival at the surface, the essential oil is vaporized. The process cycle continues until all the enclosed essential oil is removed from the oil cells. The various components of the essential oils are liberated based on their solubility in the boiling water rather than the order of their boiling points (Srivastava, 1991). The oxygenated oil constituents, which are more soluble in boiling water than the hydrocarbon carbon analogues, remain associated with the plant material to a lesser extent (Beckett and Stenlake, 1986; Srivastava, 1991).

2.6.7 Liquid Carbon Dioxide Extraction Method

Extraction of the oils with supercritical or liquid carbon dioxide is a new process and it provides the advantages of a cold process and the incorporation of some of the non-volatile components. It is expensive in terms of plant and, in some cases, results in an unusual balance of extracted oil components.

The process is carried out using a specially designed high-pressure soxhlet apparatus for extraction with carbon dioxide. The plant materials are charged into the extraction columns, which are under high pressure (55–58 bar). The required amount of carbon dioxide is then slowly introduced into the column before commencing the extraction process. The liquid CO_2 flows through the extraction columns in turn and the last is satu-

rated with the essential oil. At the end of the extraction the column is taken and liquid carbon dioxide is drained from it. The essential oils obtained by this method have been found to be superior in quality and flavor as compared with the conventional steam distilled essential oils (Srivastava, 1981).

2.7 METHOD OF ANALYZING ESSENTIAL OILS

2.7.1 Gas Chromatography

Developed largely since 1951, this technique has become the preferred method for rapid and accurate analysis of many volatile substances (Conn and Stumpf, 1976; Beckett and Stenlake, 1986). The introduction of capillary gas chromatography to essential oil analysis has unraveled the complete essential oil profiles, giving an overview of the different column types used in recent times.

In gas chromatography, the sample, *e. g.* essential oil, is introduced into a stream of an inert gas, which is the mobile phase. The vaporized sample is swept through the liquid stationary phase, which is held on an inert support in the column and the separated analytes flow through a detector, whose response is displayed on a computer or recorder. The column must be hot enough to provide sufficient vapor pressure for analytes to be eluted in a reasonable time. The detector is maintained at a higher temperature than the column, so that the analytes are gaseous (Harris, 1999). In gas-liquid chromatography, separation occurs as the vapor constituent's partition between the gas and the liquid phases in the same manner as other liquid–liquid chromatographic processes. The carrier gas must be chemically inert and available in pure form, *e. g.* argon, helium, or nitrogen. A high-

density gas gives best efficiency but a low-density gas gives faster speed (Christian, 1977). The type of detector often dictates the choice of a gas.

The vast majority of analyses use long, narrow open tubular columns made of fused silica (SiO_2) and coated with polyimide (a plastic capable of withstanding 350°C) for support and protection from atmospheric moisture. Column inner diameters are typically 0.10 to 0.53 mm. The open tubular designs offer higher resolution, shorter analysis time, and greater sensitivity but have a lower capacity for sample when compared with packed columns. Narrow open tubular columns provide higher resolution than wider open tubular columns, but they require higher pressure to operate and have less sample capacity. These open tubular columns are also known as capillary columns.

The capillary columns can either be wall-coated or support-coated designs. The wall-coated column features a 0.1–5 mm-thick film of stationary liquid phase on the inner wall of the column. Decreasing the thickness of the stationary phase increases resolution, decreases retention time, and decreases sample capacity. The support-coated design has solid particles coated with stationary liquid phase attached to the inner wall of the column (Harris, 1999).

The packed columns contain a fine solid support coated with non-volatile liquid stationary phase; or the solid itself may be the stationary (in gas solid chromatography). Despite their inferior resolution, packed columns are useful for preparative operations, when a great deal of stationary phase is required, or to separate gases that are poorly retained. Columns are usually made of stainless steel, nickel, or glass and are typically 3 – 6 mm in diameter and 1–5 m in length (Harris, 1999).

Several common liquid stationary phases are listed in Table 3. The choice of liquid phase for a given problem is based on the rule "like dissolves like". Non-polar columns are best for non-polar solutes. Columns of intermediate polarity are best for intermediate polarity solutes, and strongly polar columns are best for strongly polar solutes. As a column ages, stationary phase bases off, surface silanol (Si-O-H) are exposed, and tailing increases. Exposure to oxygen at high temperatures also leads to degradation and tailing. To reduce the tendency of stationary phase to bleed from the column at elevated temperature, it may be bonded (covalently attached) to the silica surface or covalently cross-linked to itself (Harris, 1999). Below are some of the commonly used stationary phases.

Table 3: Some Common Stationary Phases (Skoog and West, 1980)

Name	Chemical Composition	Maximum Temperature °C	Polarity	Type of Separation
Squalene	$C_{30}H_{62}$	150	NP	Hydrocarbons
OV-1	Polymethylsiloxane	350	NP	General purpose non-polar
DC 710	Polymethylphenyl-siloxane	300	NP	Aromatics
Q7-1	Polytrifluoropro-pylmethylsiloxane	250	P	Amino acids, steroids, nitrogen compounds
XE-30	Polycyanomethylsi-loxae	275	P	Alkaloids, halogenated compounds
Carbowax 20M	Polyethylene glycol	250	P	Alcohol, esters, essential oils
DEG adipate	Diethyleneglycol adipate	200	SP	Fatty acids, esters
-	Dionyl phthalate	50	SP	Ketones, ethers, sulfur compounds

NP = non-polar; SP = semi-polar; P = polar

The material chosen as the inert support should be of uniform granular size and have good handling characteristics (*i. e.* be strong enough not break down in handling) and be

capable of being packed into a uniform bed in a column. The surface area of the material should be large so as to promote distribution of the liquid phase as a film and ensure the rapid attainment of equilibrium between the stationary and mobile phases. The material should be inert at elevated temperatures and be readily wetted by the liquid phase to give a uniform coating. The most common supports are made from diatomaceous earths, *e. g.* firebrick and kieselguhr. Firebrick which is solid under trade names such as chromosorb P, C 22, and sterchamol, has the better strength and larger specific area (4 m^2/g); its disadvantage lies in the fact that it is more active and, therefore, cannot be employed on polar compounds. Kieselguhr is more fragile and has a smaller specific surface area (1 m^2/g) but is less reactive; it is sold under such trade names as chromosorb W, celite, embacel, and celatom.

The function of the detector, which is situated at the exit of the separation column, is to sense and measure the small amounts of the separated components present in the carrier gas stream leaving the column. The output from the detector is fed to a recorder, which produces a pen-trace called a chromatogram. The choice of detector depends on various factors such as the concentration level to be measured and the nature of the separated components. The most widely used detectors are the thermal conductivity and flame ionization detectors.

In thermal conductivity detector, the detection system is based upon changes in the thermal conductivity of the gas stream; an instrument employed for this purpose is sometimes called a katharometer. As a gas is passed over a heated filament wire, the temperature and thus the resistance of the wire vary according to the thermal conductivity of the gas. The purer carrier gas is passed over one filament, and the effluent gas containing the

sample constituents is passed over another. These filaments are in opposite arms of a Wheatstone bridge circuit that measures the difference in their resistance. So long as there is no sample gas in the effluent, the resistance of the wires is the same. But whenever a sample component is eluted with the carrier gas, a small resistance change occurs in the effluent arm. The change, which is proportional to the concentration of the sample component in the carrier gas, is registered on the recorder. Helium is the carrier gas commonly used with a thermal conductivity detector. Helium has the second highest thermal conductivity of any gas (after H_2), so any analyte mixed with helium lowers the thermal conductivity of the gas stream.

In the flame ionization detector, eluate is burned in a mixture of hydrogen and air. Carbon atoms (except carbonyl and carboxyl carbons) produce CH radicals, which are thought to produce CHO^+ ions in the flame:

$$CH + O \rightarrow CHO^+ + e^-$$

Only about one in 10^5 carbon atoms produce an ion, but ion production is strictly proportional to the number of susceptible carbon atoms entering the flame. Cations produced in the flame carry electric current from the anode flame tip to the cathode collector. This electric current is the detector signal. Most detectors other than flame ionization and thermal conductivity, respond to much limited classes of analytes. Other detectors are electron capture, nitrogen–phosphorus, flame photometric, photoionization, sulfur chemiluminescence, nitrogen chemiluminescence, and atomic emission detectors (Harris, 1999).

Certain parameters are used in gas chromatography for qualitative analysis of separated components. One of such parameters is the retention time index (KI). The retention

time index (KI) was proposed by Kovats (Goedert, 2006) as a qualitative parameter for general use in reporting chromatographic data. The retention index is based upon a comparison between the position of an analyte peak and the peak for two or more normal paraffins. That is, retention index relates the retention time of a solute to the retention times of linear alkanes. By definition, the Kovats retention index for a linear alkane is equal to 100 times the number of carbon atoms in the compound, regardless of the columns used or the chromatographic conditions.

A compound eluted between two linear alkanes has a retention index that can be computed by the formula (Harris, 1999) given below:

$$\text{Retention index: KI} = 100 \times \frac{\tau_a - \tau_z}{\tau_{z+1} - \tau_z} + 100\text{Z}$$

where Z is the number of carbon atoms in the smaller alkane; τ_a is the retention time in seconds for the compound of interest; τ_z is the retention time of the alkane with one carbon atom less than that of the compound of interest; τ_{z+1} is the retention time of the alkane with one carbon atom higher than that of the compound of interest.

The relative retention times of polar and non-polar solutes change as the polarity of the stationary phase changes. The retention index of a compound on non-polar columns is usually identical within the series and the retention index of a compound on polar columns is likewise similar in the range of similar compounds. The retention index system has the advantage of having readily available reference materials that cover a wide boiling range. In addition, the temperature dependence of retention indices is relatively small. Furthermore, the change in retention index between a polar and a non-polar stationary phase provide a measure of the relative polarity of different stationary phases (Skoog and West, 1980).

2.7.2 Gas Chromatography – Mass-Spectroscopy (GC – MS)

Gas chromatographic columns have been directly interfaced to rapid-scan mass spectrometers, thus permitting the instantaneous display of the spectrum of each species as it leaves the column. The excellent separation qualities of gas chromatography, combined with the powerful technique for the identification properties of mass-spectrometry, provide the chemist with a most useful tool for analyzing complex mixtures. The combination of the two methods gives a powerful technique for the separation of complex constituents of essential oils and other volatile substances.

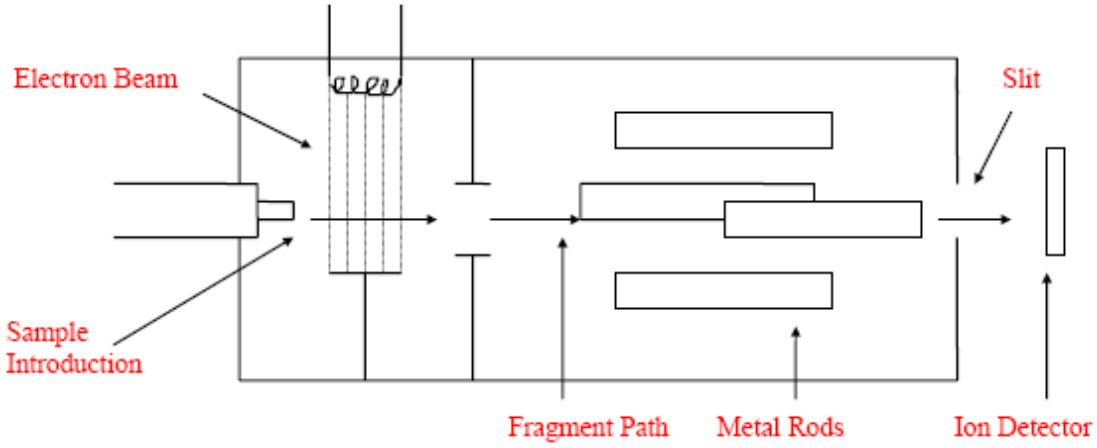
In mass spectrometry, gaseous molecules are ionized (usually to make cations), accelerated by an electric field, and then separated according to their mass (Harris, 1999). The ionization process usually imparts enough energy to the molecule to break it into a variety of fragments. Mass-spectrum is a graph showing the relative abundance of each fragment striking the detector of the mass spectrometer. The mass spectrometer consists of three major parts, which are the ion source, mass-analyzer and the detection system. A characteristic feature of mass-spectrometry, which is not encountered in most optical methods, is the need to maintain all of the components leading to the detector at low pressures (10^{-4} to 10^{-8} torr); thus, the elaborate vacuum systems are an important part of mass spectrometers. The operation of a typical analytical mass spectrometer is based on the following sequence of events:

(1) A micromole (or less) of a sample is volatized and allowed to leak slowly into the ionization chamber, which is maintained at a pressure of about 10^{-5} torr.

(2) The molecules of the sample are ionized directly or indirectly by a stream of electrons flowing from the heated filament toward an anode (both positive and negative ions

are formed by impact, but the former predominate; analytical methods are generally based upon positively charged particles).

(3) The positive ions are separated from the negative ions by the small negative potential at the slit and are then accelerated by a potential of a few hundred to a few thousand volts between the slit. A collimated beam of positive ions enters the separation area through the slit.



(4) In the analyzer tube, which is maintained at a pressure of about 10^{-7} torr, the fast–moving particles are subjected to a strong magnetic field which causes them to describe a curved path, the radius of which corresponds to their velocity and mass as well as to the field strength – particles of different mass can be focused on the exit slit by varying the accelerating potential or the field strength.

(5) The ions passing through the exit slit fall upon a collector electrode; the ion current that results is amplified and recorded as a function of field strength or accelerating potential.

The sample of the material to be analyzed is introduced into the ion source by either batch inlet or direct probe inlet systems. In batch inlet system, the sample is introduced as a gas into a reservoir which is at a pressure greater than that within the ionization chamber; while in direct probe inlet system, non-volatile or thermally unstable materials are often introduced directly into the ion source by means of a sample probe, which is inserted through a vacuum lock. The separated components in the effluent stream of the typical gas liquid chromatograph can be fed directly into the ion chamber.

When the gas stream reaches the ionization chamber, it is bombarded at right angles by a beam of electron emitted by a hot filament. This leads to the removal of an electron from the gaseous molecule to form the molecular ion (M^+) or parent ion.

$$M + \bar{e} \rightarrow M^{+\cdot} + 2\bar{e}$$

The molecular ion under electron bombardment of minimum energy reaches the detector and gives a mass-spectrum consisting almost entirely of a single peak corresponding to the mass of the original molecule. Increasing the energy of the electron beam yields a more highly excited ion that fragments if it is complex, or a second electron may be knocked out.

In the analysis of essential oil using mass-spectrometry, electron impact (EI) and chemical ionization (CI) methods are used for the ion production. Electron impact ionization usually creates molecular fragments. The molecular ion, M^+, might have a low abundance or even be absent which makes the identification of an unknown substance difficult. Extensive fragmentation of large molecules makes their mass-spectra difficult to interpret. Computer programs may be used to match the spectrum of an unknown to one or more similar spectra in a library (Harris, 1999).

The chemical ionization is a gentle technique that yields less fragmentation. In this case, the ionization source is filled with methane (CH_4) at a pressure of about 10^{-5} to 10^{-7} torr. Energetic electrons convert CH_4 to a variety of reactive products.

$$CH_4 + \bar{e} \rightarrow \quad CH_4^{+\cdot} + 2\bar{e}$$

$$CH_4^{+\cdot} + CH_4 \rightarrow CH_5^{+} + CH_3^{\cdot}$$

CH_5^+ is a proton donor that reacts with analyte to give MH^+, which is usually the most abundant ion in the methane chemical ionization mass spectrum.

$$CH_5^+ + M \rightarrow CH_4 + MH^+$$

The mass spectrum by CI methods is always a simpler profile than those produced by EI techniques. The CI spectrum displays a clearly visible protonated molecular ion $(M + 1)^+$. The presence of this quasi-molecular ion aids greatly in identifying the molar mass of the compound under investigation, particularly where EI techniques do not indicate any M^+ ions (Pecsoc. 1976).

GC–MS makes possible the identification of the hundreds of components that may be present in natural and biological systems. For example, the interfacing of chromatography with mass spectroscopy has permitted characterization of the odor and flavor components of foods, identification of pollutants, medical diagnosis based on breath components, and studies of drug metabolites (Skoog and West 1980).

A major problem in interfacing of a gas chromatograph with a mass-spectrometer arises from the presence of the carrier gas, which dilutes the eluted components enormously and tends to swamp the pumping system of the spectrometer. Several methods have

been developed for overcoming this problem. One of the solutions to the problem is that the exit gases flow through a fritted glass tube situated in an evacuated chamber. The smaller atoms or molecules of the carrier gas (He or H_2) diffuse readily through the walls of the tube and are pumped away, leaving the molecules of the eluted sample; these are then led directly into the ion source of the mass spectrometer (Skoog and West, 1980).

2.7.3 Microbial Assays

Microbial assay designates a type of biological assay, specifically, a biological assay performed with microorganisms, *e. g.* bacteria, yeast and moulds. Biological assay refers to the measurement of the relative potency of activity of compounds by determining the amount required producing a stipulated effect on a suitable test animal or organ under standard conditions (Katocs, 1995).

Antimicrobial agents are chemical or biological agents that can either destroy or inhibit the growth of microorganisms. Such agents can be antibacterial, antifungal, antiviral or antiprotozoan depending on the kind of microorganisms against which they are found effective.

The chemical agent at low concentrations should have a broad spectrum of antimicrobial activity, which implies that it should kill or inhibit the growth of many kinds of microbes. Antibiotics are initially referred to as substances, produced by one microorganism, which inhibit the growth of other microorganisms. The advent of synthetic method has, however, resulted in a modification of this definition and they are now referred to as substances produced by a microorganism, or to a similar substance (produced wholly or

partly by chemical synthesis), which in low concentration inhibits the growth of other microorganism.

Antimicrobial agents perform their work by killing or inhibiting the growth of microorganisms. The mechanism of their action is by damaging some structures of the cell like cell wall or the cytoplasmic membrane or substances within the cytoplasm, such as enzymes, ribosome or nuclear material. Microbial agents kill microorganisms while microbiostatic agents inhibit the growth of organisms.

Certain parameters may be considered to have effect on the antimicrobial assay of essential oils. These parameters are the method of assay, the medium, microorganisms and the composition of the essential oils.

2.7.3.1 *Antimicrobial Assay Of Essential Oils*

The antimicrobial activity measurement of essential oils poses some difficulties because of their volatility, complexity and water insolubility. The parameters, which may affect the antimicrobial assay of essential oils, are briefly discussed below.

2.7.3.1.1 The Assay Technique

The two assay techniques namely agar-plate and tube-dilution techniques are common laboratory techniques. For antimicrobial assay of essential oils, the agar-plate method is commonly used, as it does not require homogenous dispersion of the oil in water. The method requires reservoirs like the paper disc or cylinders placed on the surface of the medium and holes bored on the medium. A plate of nutrient agar medium is then inoculated with the test organism and the essential oil is instilled into the discs or cylinders.

On the other hand, small volume of the essential oils is placed in the holes bored on the medium. The plate is observed for zone of inhibition after incubation for 48 hours.

2.7.3.1.2 The Assay Medium

The growth medium is the artificially created habitat where an organism is expected to grow. The constituents of the medium must not react or alter the components of essential oil or *vice versa*. Any change in the constituents of the medium affects the growth of the organism.

2.7.3.1.3 Microorganisms

In the microbial assay of essential oils both Gram-positive and Gram-negative organisms are tested. The minimum inhibitory concentration (MIC) values may vary from one essential oil to another using the same microorganisms.

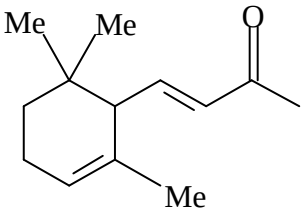
The test period for an organism may also affect the assay. The test period for fungi is generally long; this may facilitate the decomposition or evaporation of the oil and this may affect the zones of inhibition.

If any change occurs in the composition of essential oil, this will invariably affect the microbial activity of the essential oil. Essential oils are water insoluble and to enhance their solubility, solvents like ethanol, methanol, and dimethylsulfoxide are used to dissolve them so as to be able to measure their minimum inhibitory concentration. Essential oils have direct proportionality between their concentration and antimicrobial activity against test organisms.

2.8 PAST WORKS ON ESSENTIAL OIL

Problems of resistance and environmental degradation and pollution associated with irrational use of orthodox medicines have necessitated renewed interests in nature as source of effective and safe alternatives in the management of human infections. Thus, in recent years, there has been a phenomenal rise in the interest of scientific community to explore the pharmacological activities of medicinal plants and to confirm the claims made about them in folklore medicines (Chah *et al*., 2006). This has led to research works on the elucidation of the compositions of many essential oils.

A report on chemical constituents of the essential oils from ripe and unripe *Iriboaka capsicum* revealed the presence *n*-butanoic acid, 3–methyl–, and 4–methyl-*n*-pentylester as the most abundant constituents. Other acids and esters found in *Iriboaka capisum* include *n*-pentanoic acid, 1–methyl-*n*-pentylester; *n*-decanoic acid, and methylester. The ketones, α-lonone **25**, α - and γ–atlantone **26** and **27**, 4–fluoro-*n*-butylmethyl ketone; acetophenone; *n*-nonylmethyketone and 4-*n*-heptenylmethylketone as well as the aldehydes geranial **28,** *iso*-dodecyl aldehyde; 4-methylbenzaldehyde and tridecyl aldehyde were reported for the first time in the essential oils of *genus capisum*. The terpenes neryl acetate **29**, zingiberene **30**, (-)–α–, β, and γ– himachalene **31**-**33**, and the ethers 4–*n*-pentenyl methyl ether; 2–*n*-propyl-nona–5,7–dienylethyl ether, and 2–*n*-propyl-non–7-enylethyl ether were also reported for the first time in the essential oils of *genus capisum* (Agbakwuru, 1993).



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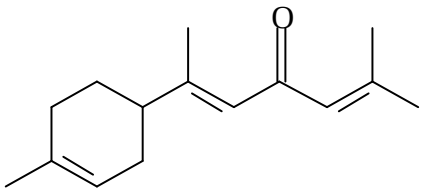

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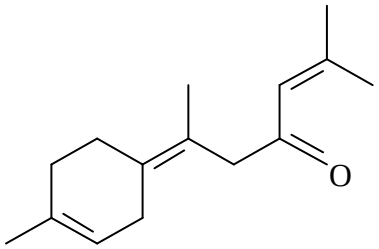

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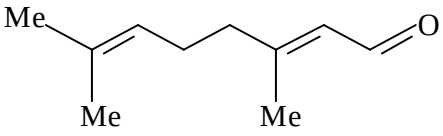

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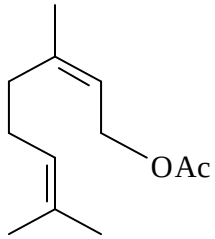

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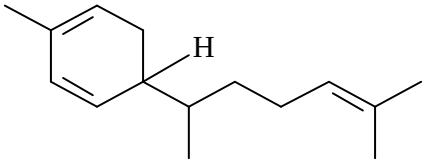

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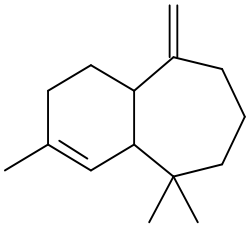

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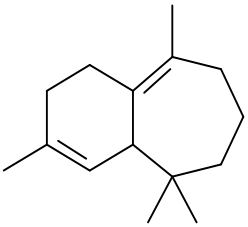

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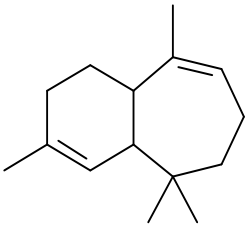

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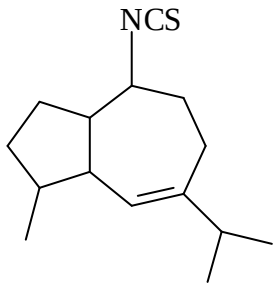
Marine sponge *Acanthella klethra* has been reported to contain sesquiterpene isothiocyanates, *e. g.* **34**. The lipophilic extract of the sponges of the genus *Acanthella* subjected to X-ray and spectroscopic analyses (NMR, IR, and MS) gave sesquiterpene isothiocyanates, (1R,5R,6R,8R)–dec(4.4.0)ane-1,5-dimethyl-8-(1-methyletheny)-5-isothiocyanate and (1R,5R,6R,8R)-dec(4.4.0)ane-1,5-dimethyl-8-(1′-methylethenyl)-5-isothiocyanate (Konig *et al*, 1992). Each of the compounds contained either an isonitrile or an isothiocyanate moiety. Before this work, earlier work done on sponges of the genus *Acuathella* revealed the presence of three sesquiterpenes.



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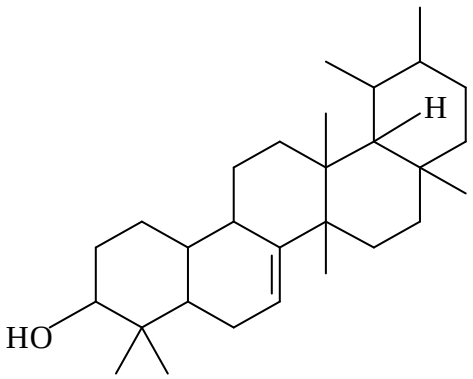
Research work has been done on the leaves of *Bosistoa brassii (Rutaceae)* for its essential oil components. The plant is confined to the rain forests of eastern Australia. It is a small tree found in the coastal strip of north-eastern Queensland. Four triterpenes and five flavonoids were isolated from the leaves of the plant and characterized using spectroscopic methods. The triterpenes were characterized as baurenol **35** and multiflorenol **36** (isolated as mixture). Four of the flavonoids were identified as kumatakenin (5,4′-dihydroxy-3,7-dimethoxyflavone) **37**, 5,7-dihydroxy-4′-(3-methyl-*n*-but-2-enyloxy) flavone, 5,7-dihydroxy-4′-methoxy-8-(3-but-2-enyl)flavone, and 5,7-dihydroxy-4′-methoxy-8-(2-hydroxy-3-methylbut-3-enyl)flavone. The fifth flavonoid is a diner (5,7-dihydroxy-4′-methoxy-8-(3-methylbut-2-enyl)flavonoid-6-yl – (5,7-dihydroxy-4′-methoxyflavanon-8-yl)methane, to which was assigned the trivial name bosistoabiflavanone (Parsons *et* al., 1993).



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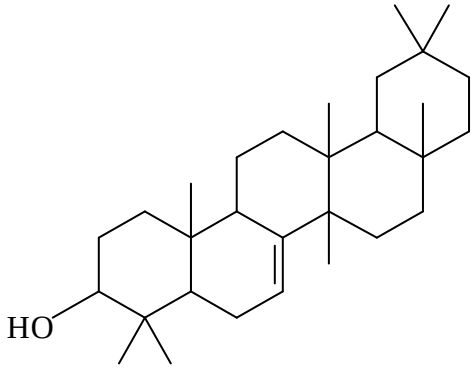

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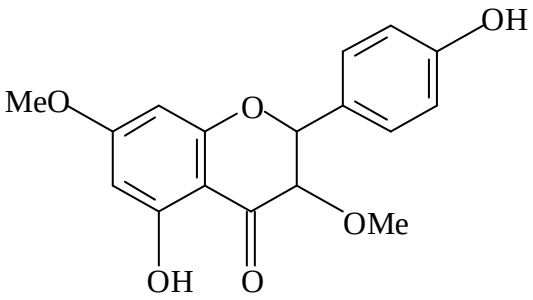
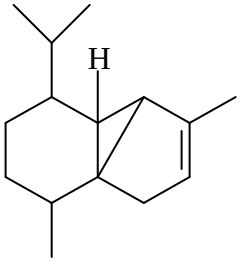


37

Similarly, Kasali and Eshinlokun (2002) reported on the chemical composition of the essential oil of *Dacryodes edulis*. The essential oil was obtained by hydrodistillation of the leaves and analyzed by gas chromatography (GC) and gas chromatography – mass spectrometry (GC–MS). Forty-two constituents accounting for 86.2 % of the total oil were identified by their Kovats retention indices on Cpsil-5 and by their mass spectra. α - Cubebene **38** (29.8 %) and δ-cadinene **21** (14.0 %) were the major constituents. Other compounds identified in appreciable amount include γ-terpinene **3** (6.8 %), germacrene B **39** (4.7 %) and (E)–nerolidol **40** (4.4 %). A diterpene alcohol, phytol **41** (0.44 %) was reported as the constituent of *D. edulis* volatile leaf oil for the first time.



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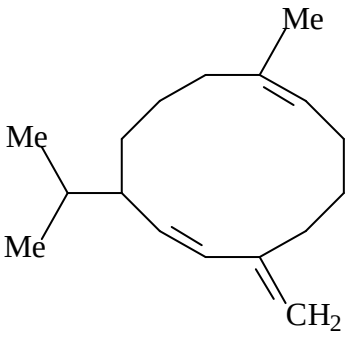

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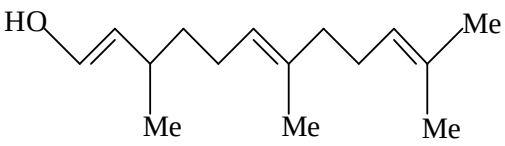

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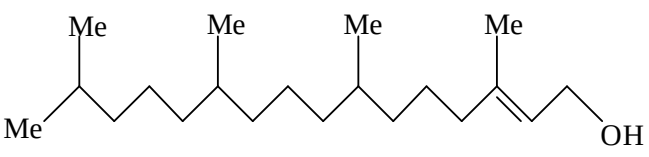
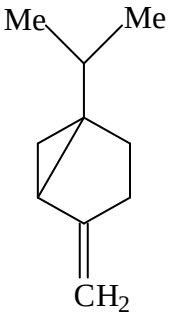


41

Asekun and Ekundayo (2000) reported on the chemical constituents of the essential oil of the leaves of *Hyptis suaveolens (L.) Poit.* from Nigeria. Of the 49 components, which were detected, 39 amounting to 89.5% were identified. The dominant components were sabinene **42** (16.5 %), *trans*-α-bergamotene **43** and β-caryophyllene **44** (19.8 %) terpinen-4-ol **45** (9.6 %), and β-pinene **46** (8.6 %).



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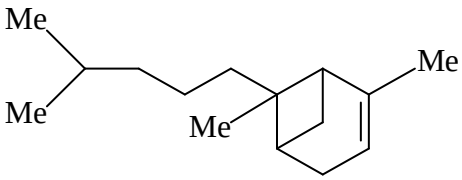

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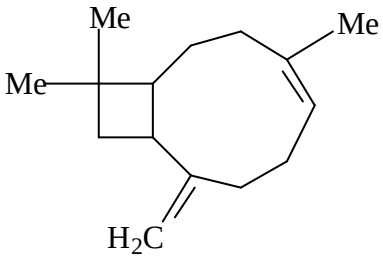

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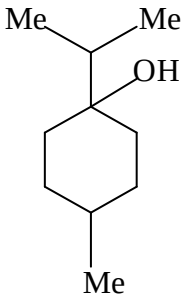

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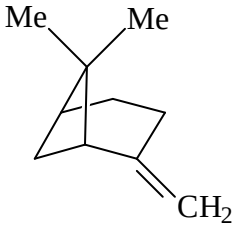
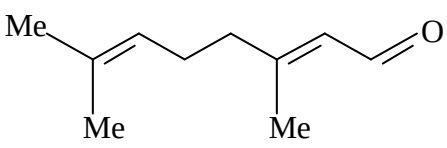


46

The work of Kasali *et al*. (2001) on the essential oil of *Cymbopogon citratus* needs to be mentioned. They identified twenty three (97.3 %) constituents in the leave oil, the main constituents being geranial **20** (33.7 %), neral **47** (26.5 %) and myrcene **48** (25.3 %). Small amounts of neomenthol **49** (3.3 %), linalyl acetate **50** (2.3 %), and β-ocimene **51** (1.0 %) were also detected.



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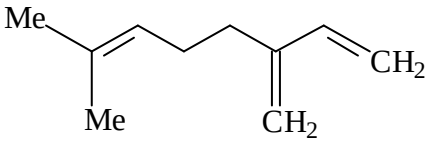

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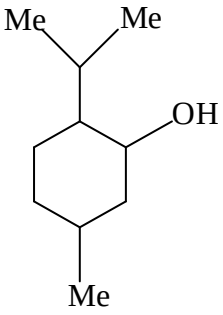

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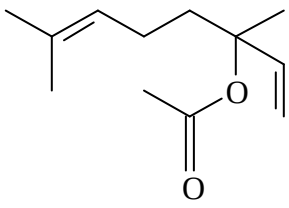

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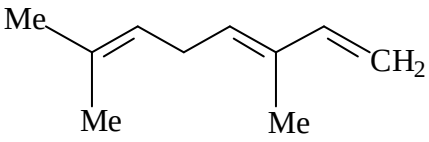
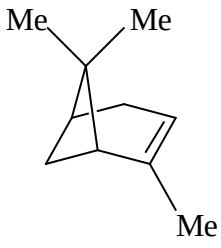


51

Kasali *et al*. (2002) reported on the composition of the essential oil of *Boswellia serrata*. The oil predominantly comprised monoterpenoids, of which α-pinene **52** (73.3 %) was the major constituent. Other monoterpenoids identified included β-pinene **46** (2.05 %), *cis*-verbenol **53** (1.99 %), *trans*-pinocarveol **54** (1.80 %), borneol **55** (1.78 %), myrcene **56** (1.71 %), verbenone **57** (1.71 %), limonene **58** (1.42 %), and *p*-cymene **2** (1.0 %), while α-copaene **59** (0.13 %) was the only sesquiterpene identified in the oil.



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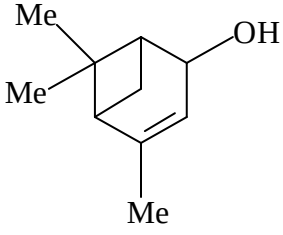

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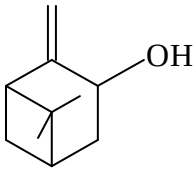

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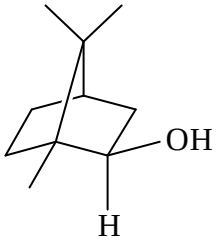

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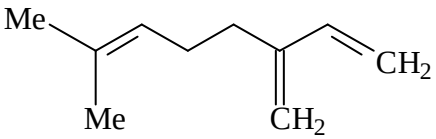

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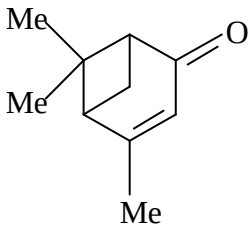

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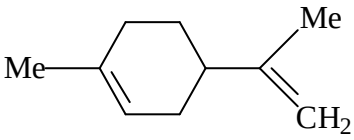

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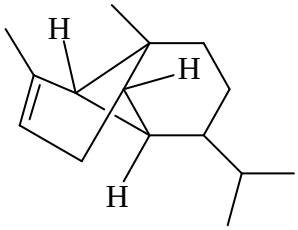
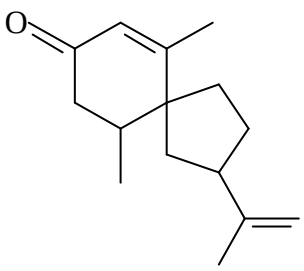


59

One of the works reported on *Solanum aethiopicum* is the one conducted by Nagaota *et al*., 2001. In this study, five known sesquiterpenoids, solavetivone **60**, lubimin **61**, lubiminoic acid **62**, aethione **63**, and lubiminol **64** were isolated from the root exudates recovered from *Solanum aethiopicum* by a newly proposed method using charcoal. Quantitative analysis of the sesquiterpenoids in the roof exudates of *S. aethiopicum* and *S. Melongena* suggested that relatively large amounts of the sesquiterpenoids were exuded from the roots. Antifungal activity of the sesquiterpenoids against *Fusarium oxysporum* and *Verticillium dahliae* was also examined. Nagase *et al*. (2000) made further report on this plant.



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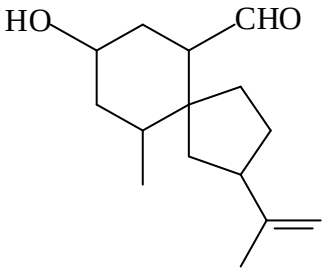

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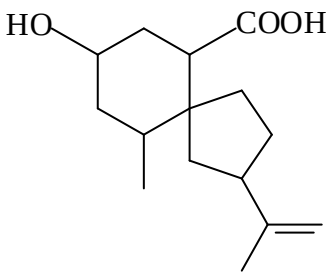

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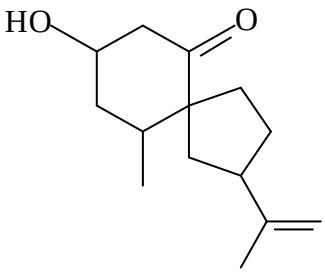

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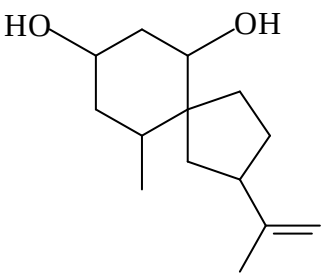


64

Long before mankind discovered the existence of microbes, the idea that certain plants had healing potential or, in modern words, antimicrobial principles, had been well accepted. Since antiquity, man has used plants to treat common infectious diseases and some of these traditional medicines are still included as part of the habitual treatment of various maladies (Rios and Recios, 2005). For example, the use of bearberry (*Arctostaphylos uvaursi*) and cranberry juice (*Vaccinium macrocarpon*) to treat urinary tract infections is reported in different manuals of phytotherapy, while species such as lemon balm (*Melissa officinalis*), garlic (*Allium sativum*), and tee tree (*Melaleuca alternifolia*) are described as broad-spectrum antimicrobial agents (Heinrich *et al*., 2004).

Essential oils of these plants rather than their extracts have had the greatest use in the treatment of infectious pathologies in the respiratory system, urinary tract, gastrointestinal and biliary systems, as well as on the skin. In the case of *Melaleuca alternifolia*, for example, the use of the essential oil (tee tree oil) is a common therapeutic tool to treat acne and other infectious troubles of the skin (Vanaclocha and Canigueral, 2003).

However, a common mistake in many papers is to claim positive activity for slight dilutions or excessively high concentrations. For example, experiments with quantities higher than 1 mg/mL for extracts or 0.1 mg/mL for isolated compounds should be avoided, whereas the presence of activity is very interesting in the case of concentrations

below 100 μg/mL for extracts and 10 μg/mL for isolated compounds (Rios and Recios, 2005).

CHAPTER 3

MATERIALS AND METHODS

3.1 SEED COLLECTION, SOIL PREPARATION, AND CULTIVATION OF THE PLANT

The seeds of *Calendula officinalis* were collected from a cultivated garden within the University of Fort Hare campus. They were planted in the nursery in the greenhouse of the Botany Department. Individual plants were grown in polythene bags. The soil was collected from the University Research Farm, dried for about 48 h, sieved through a 2 mm wire mesh (Ingram, 1993) and homogenized before filling the polythene bags. All plants were adequately watered as required. Harvesting was not done during the first two weeks following transplanting; this was to allow the seedlings overcome the shock of transplanting and establish themselves in the new soils. Thereafter, the leaves were harvested at weekly intervals until full flowering stage. After each harvesting the fresh leaves were weighed and hydrodistilled for 3 h in an all-glass Clevenger apparatus in accordance with the British Pharmacopoeia method (British Pharmacopoeia, 1980).

3.2 PLANT COLLECTION AND DISTILLATION OF THE ESSENTIAL OILS

Fresh materials of *Calendula officinalis* were collected from one population within the University of Fort Hare, Alice campus in the Eastern Cape Province of South Africa, latitudes 30°00′–34°15′S and longitudes 22°45′–30°15′E in September 2005. A voucher specimen (OKOH/01) was deposited at the University Herbarium.

The fresh plant materials were carefully separated into leaves and flowers. Some of the leaves were air dried at room temperature (18°C) for seven days. About 500 g, 200 g and 250 g of the fresh leaves, dry leaves, and fresh flowers, respectively, were hydrodistilled separately for 3 h in an all-glass Clevenger apparatus in accordance with the British pharmacopoeia method (British Pharmacopoeia, 1980).

3.3 SOIL ANALYSIS

The sieved soil samples were digested at 360°C for 2 h using the selenium powder, lithium sulfate, hydrogen peroxide, and sulfuric acid digestion mixture (Anderson and Ingram, 1993). Total phosphorus was determined from the digest using the colorimetric method without pH adjustment (Okalebo *et al*. 2002). Total K, Mg, Na, Ca, Fe, Cu, and Mn content were determined in the digest using the atomic absorption spectrometer. The soil particle size analysis was carried out using the hydrometer method while pH and electric conductivity were determined using the methods described by Okalebo *et al*. 2002.

3.4 GC-MS ANALYSES AND IDENTIFICATION OF COMPONENTS

The GC-MS analyses were carried out using Hewlett-Packard HP 5973 mass spectrometer interfaced with an HP-6890 gas chromatograph with an HP5 column. The following conditions were used: initial temperature 70°C, maximum temperature 325°C, equilibration time 3 min, ramp 4°C / min, final temperature 240°C; inlet: split less, initial temperature 220°C, pressure 8.27 psi, purge flow 30 mL / min, purge time 0.20 min, gas

type helium; column: capillary, 30 m × 0.25 mm, film thickness 0.25 µm, initial flow 0.7 mL / min, average velocity 32 cm / s; MS: EI method at 70 eV.

The components of the oils were identified by matching their mass spectra and retention indices with those of the Wiley 275 library (Wiley, New York) in the computer library and literature (Shibamoto, 1987). The yield of the oil was calculated per gram of the plant material, while the percentage composition was calculated from summation of the peak areas of the total oil composition.

3.5 ISOLATION OF COMPOUNDS FROM *Calendula officinalis*

3.5.1 Plant Materials

The leaves of *Calendula officinalis* were collected from a cultivated garden in Alice, South Africa. The plant was authenticated by Prof. Afolayan and a voucher specimen was deposited in the herbarium of the University of Fort Hare.

3.5.2 General Analysis

The ^{1}H, ^{13}C and DEPT 135 (Distortionless Enhancement of Polarization Transfer using a 135° decoupler pulse) NMR spectra (in methanol-d_4) were obtained on a Bruker Avance DPX 300 spectrometer (300 MHz); melting points were recorded on Stuart Scientific (SMPI) apparatus; vacuum liquid chromatography (VLC) and column chromatography (CC) experiments were achieved using silica gel 60 (particle size 0.063-0.200 mm, Merck); preparative TLC was carried out using silica gel 60 $PF_{254+366}$ precoated alumina sheets (Merck); visualization of compounds was done under UV lamp (254 and 365 nm) and using vanillin-sulfuric acid spray. The IR spectra were recorded using Per-

kin-Elmer 2000 FTIR spectrophotometer, spectrum version 5.3. The solutions of the isolates liquid were prepared (1×10^{-3} M concentration) and placed into a 1-cm spectral cuvette.

3.5.3 Extraction and Isolation

Fresh plant material were collected and air-dried at room temperature. The dried material (1 kg) was milled to a fine texture and extracted with ethanol for 48 hours at room temperature, with gentle and continuous shaking using a Labotec 201 orbital shaker. After filtering, the residue was again extracted four times. Filtrate were combined and concentrated to dryness under reduced pressure using a Buchi rotary evaporator at a maximum temperature of 40°C. The mass of the combined crude extracts was 67 g and this crude extract was subjected to vacuum liquid chromatography using an elution gradient as follows: petroleum ether (100%); petroleum ether / $CHCl_3$ (9 : 1); petroleum ether / $CHCl_3$ (7 : 3); petroleum ether / $CHCl_3$ (5:5); $CHCl_3$ (100%); $CHCl_3$ / EtOAc (8:2); $CHCl_3$ / EtOAc (5:5); EtOAc (100%); EtOAc / MeOH (8:2); EtOAc / MeOH (5:5), and finally MeOH (100%). A total of 113 fractions of 20 ml each were collected. The combined fractions 59 to 65 were loaded using 100% $CHCl_3$ then followed by chloroform / ethyl acetate (8:2) with increasing polarity to 50% ethyl acetate. It was later eluted with petroleum ether / toluene / ethyl acetate (3 : 5 : 10). Fourteen fractions each with 50 mL were collected. Fractions 1 to 6 were combined to give A, fractions 7 to 12 were combined to give B, fractions 13 to 21 were combined to give C, fractions 22 to 27 were combined to give D, fractions 28 to 35 were combined to give E, fractions 36 to 40 were combined to give F, fractions 41 to 52 were combined to give G, fractions 53 to 56 were combined to give

H, fractions 57 to 73 were combined to give I, fractions 74 to 83 were combined to give J, fractions 84 to 88 were combined to give K, fractions 89 to 97 were combined to give L, fractions 98 to 102 were combined to give M, fractions 103 to 113 were combined to give N. Fractions 13 to 21 were subjected to preparative TLC and developed three times using petroleum ether / toluene / ethyl acetate (3 : 5 : 10) to give a pure compound. Fractions 57 to 73 were also subjected to preparative TLC and developed three times using petroleum ether / toluene / ethyl acetate (3 : 5 : 10) to give a pure compound. The structures were obtained through NMR and IR spectral analysis and by comparing with the available data in the literature.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 EFFECTS OF AGE ON THE YIELD AND COMPOSITION OF THE ESSENTIAL OILS OF *Calendula officinalis*

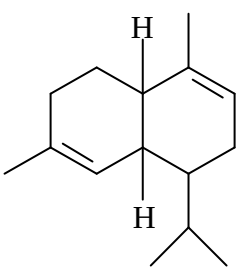
The pH of the soil was 6.20 and the electrical conductivity (EC) was 115.5 µS/cm. The total content of nitrogen, phosphorus, and potassium of the soil used for the cultivation of the plant was 0.20, 1.0, and 1.6 g / kg of soil, respectively; the content of the exchangeable cations, calcium, magnesium, and sodium was 2.1, 0.19 and 1.2 g / kg, respectively, while the content of iron, manganese, copper, and zinc was 1.2, 18, 41 and 1.5 g / kg, respectively (Table 4).

Table 4: Soil Parameters and the Values Obtained

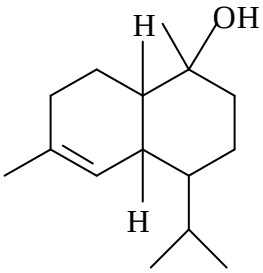
Soil Parameters	Values
pH	6.20
EC (us/cm)	115.5
Total N (g/kg)	0.20
Total P (g/kg)	1.00
Total K (g/kg)	1.60
Exc. Ca (g/kg)	2.10
Exc. Mg (g/kg	0.19
Exc. Na (g/kg)	1.20
Fe (g/kg)	1.20
Mn (g/kg)	18
Cu (g/kg)	41
Zn (g/kg)	1.50

The GC-MS analysis of the extracted essential oils during the growing phase of *C. officinalis* indicated the presence of 43 compounds (Table 5). These included 20 mono-terpenes and 23 sesquiterpenes. No diterpene was observed in all the samples. The total yields of the essential oils at different stages of the vegetative cycle increased with the age of the plant. Increase in essential oil yields has been observed to be a mechanism that favors the pollination of the plant. According to Harborne (1991), several terpenoids have been previously reported as pollination vectors in this plant.

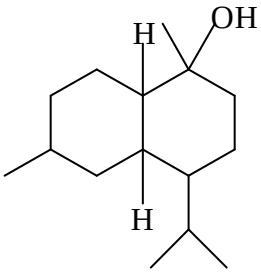
Of all the constituents observed in the oil, the sesquiterpenes (α-cadinene **65**, α-cadinol **66**, T-muurolol **67**, and *epi*-bicyclosesquiphellandrene **68**) and the monoterpenes (limonene **58**, 1,8-cineole **69**, and *trans*-β-ocimene **70**) showed the highest correlations with the age of the plant (Table 6). α-Cadinene is an important flavoring agent in baked food, candies and chewing gum and also a fragrance in cosmetics and detergents. T-Muurolol and α-cadinol are important antimicrobial agents (Chang *et al*,.2003). The concentration of both compounds increased with the age of the plant, this trend was similar to that reported elsewhere (EMEA, 2001).



65



66



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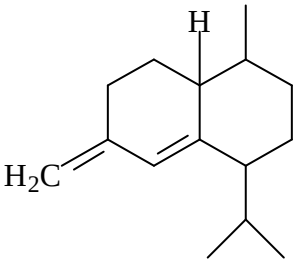

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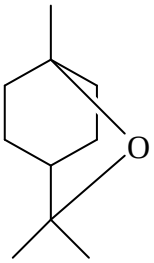
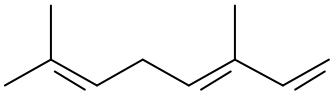
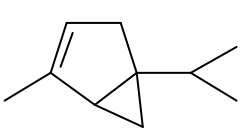


69

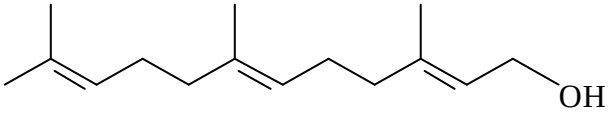


70

Thujenes are poisonous components usually present in essential oils. In *Calendula officinalis*, the concentrations of these compounds (*e. g.* α-thujene **71**) remained very low (0.1-0.2%) throughout the vegetative life of the plant, which is a measure of the good and safe quality of the oil. Geraniol **4** was the most prominent component of the oil from this herb. Its concentration increased slightly from the fourth week until the eighth week after which the compound increased sharply in concentration and eventually became the largest component of the oil. Geraniol is a natural antioxidant, which has been suggested to be useful in cancer prevention. According to Carnesecchi *et al* (2001), geraniol caused a 50% increase of ornithine decarboxylase activity, which is enhanced during cancer growth. In addition, geraniol has been observed to inhibit DNA synthesis. Together with farnesol **72**, geraniol suppresses pancreatic tumor growth (Burke *et al*. 1997).



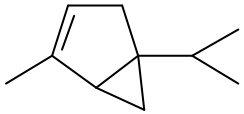
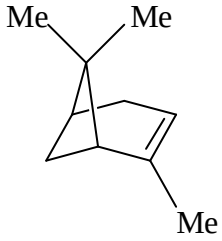
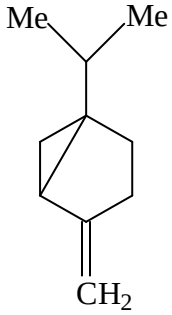
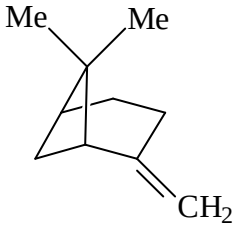
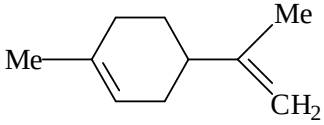
71

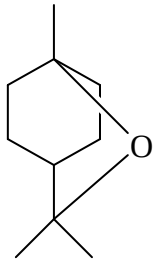
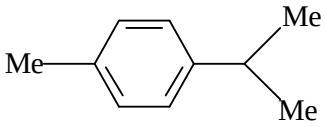
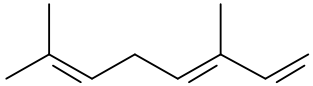
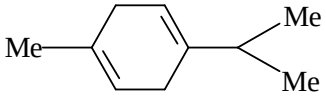
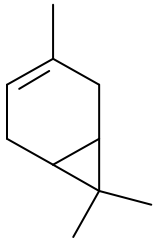
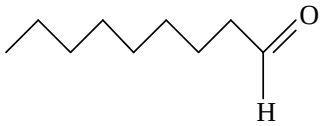
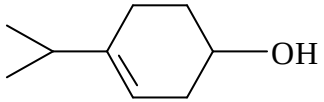
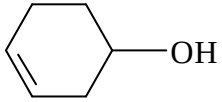


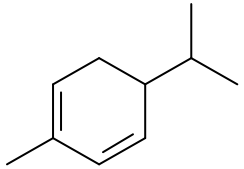
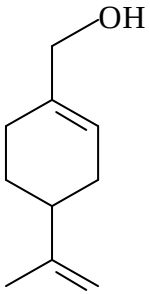
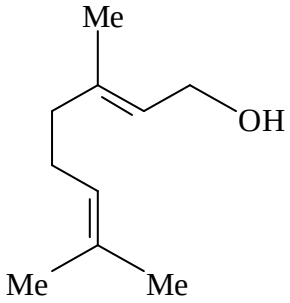
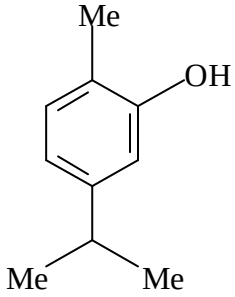
72

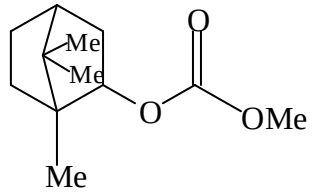
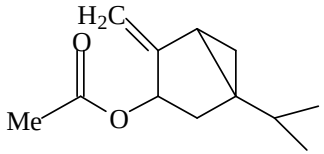
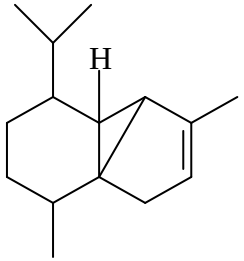
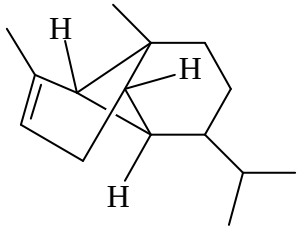
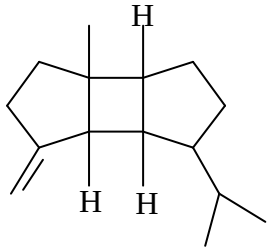
This study has shown that a correlation exists between the yield of *Calendula officinalis* essential oil and the age of the plant and that the yield is best during the flowering stage of the plant. Also, the relative abundance of the chemical constituents of its essential oil at this stage is a veritable indicator of the appropriate period for collection and harvesting of the plant for the mining of the desired mono- and sesquiterpenes.

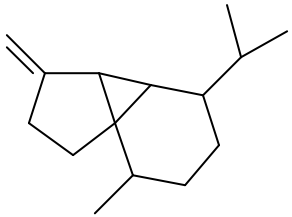
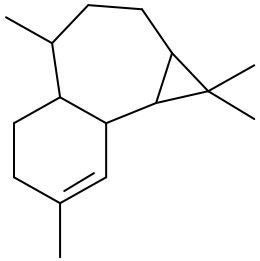
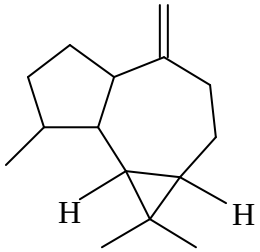
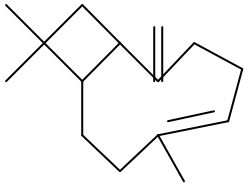
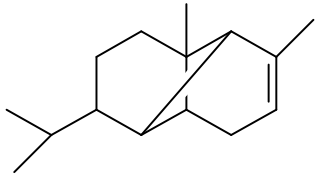
Table 5: Percentage Composition of the Major Essential Oil Constituents of *Calendula officinalis* at Different Stages of Growth [1]

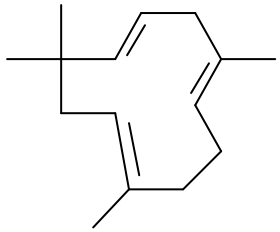
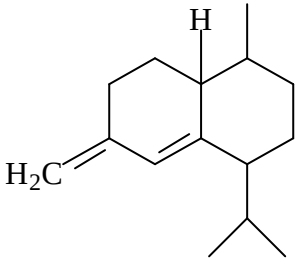
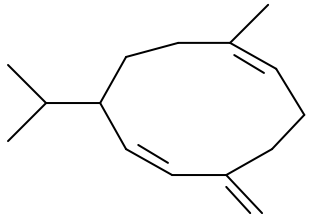
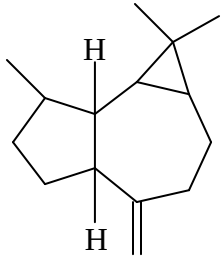
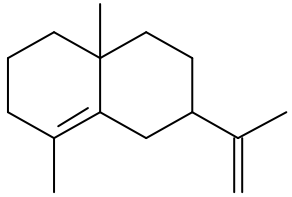
Compound	Structure	KI	Stages of Growth (Weeks)									
			3	4	5	6	7	8	9	10	11	12
α-Thujene		908	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.3
α-Pinene		928	0.1	1.2	1.4	1.6	2.2	2.3	2.5	2.7	2.7	2.9
Sabinene		960	0.1	0.1	0.2	0.3	0.4	0.2	0.5	0.6	0.8	0.9
β-Pinene		969	0.1	0.3	0.5	0.8	0 .9	1.3	1.1	1.2	1.3	1.4
Limonene		1020	10.2	11.0	12.0	14.5	13.5	11.7	21.6	22.0	22.4	22.6

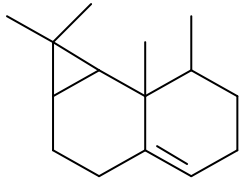
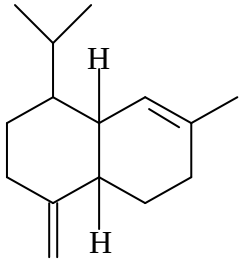
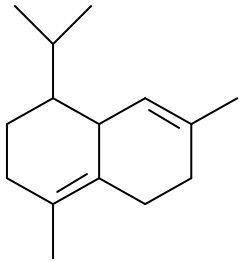
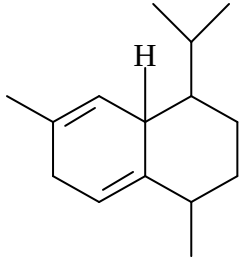
1,8-Cineole		1022	11.1	11.2	12.9	13.1	14.1	14.5	15.3	18.2	21.5	22.1
p-Cymene		1026	0.1	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.9	1.0
Trans-β-ocimene		1033	0.1	0.3	0.5	0.8	1.3	1.5	1.8	1.7	1.9	2.0
γ-Terpenene		1049	0.1	0.1	0.2	0.4	0.5	0.6	0.7	0.8	0.9	1.2
δ-3-Carene		1050	0.3	0.3	0.5	0.7	0.8	0.2	0.1	-	0.1	0.2
Nonanal		1099	-	-	0.1	0.2	0.2	-	0.3	-	-	0.3
Terpene-4-ol		1174	0.1	-	0.4	-	0.6	-	0.8	-	0.9	1.0
3-Cyclohexene-1-ol		1175	0.1	-	-	-	0.1	-	-	0.3	-	-

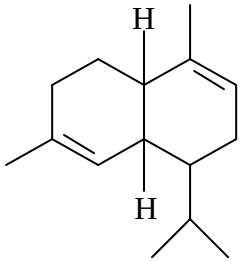
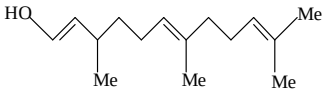
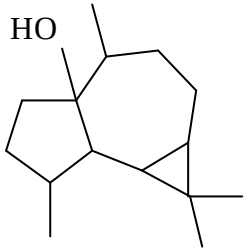
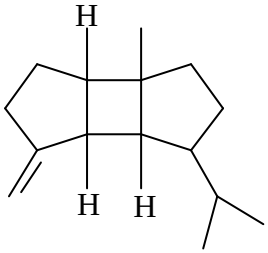
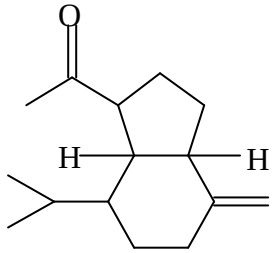
α –Phellandrene		1176	0.1	-	-	-	0.1	-	-	-	-	0.2
α-Terpeneol		1205	0.1	0.3	0.5	0.3	0.4	0.5	0.6	0.7	0.8	0.9
Geraniol		1257	0.5	0.6	2.3	3.3	4.5	5.2	10.2	10.2	10.3	10.5
Carvacrol		1272	-	0.2	-	0.1	-	0.1	-	-	-	0.1

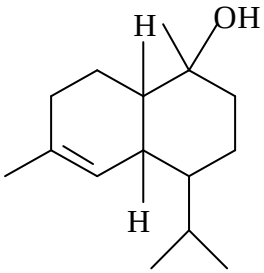
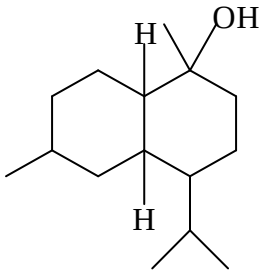
Bornyl acetate		1283	-	-	0.1	-	-	-	-	0.4	-	-
Sabinyl acetate		1288	0.1	-	-	0.1	-	-	0.1	0.1	-	-
α-Cubebene		1347	-	0.3	-	0.1	0.3	-	-	0.2	-	1.7
α-Copaene		1376	-	-	-	-	0.1	-	-	0.2	-	-
α-Bourbonene		1385	-	0.1	-	-	-	-	-	0.1	0.1	0.2

β-Cubebene		1389	-	-	-	-	2.5	-	-	1.6	-	-
α-Gurjunene		1409	-	-	1.0	-	-	1.2	-	1.5	-	0.1
Aromadendrene		1410	-	-	-	-	-	-	-	0.1	-	-
β-Caryophyllene		1420	-	-	-	-	-	-	-	0.4	-	0.9
α-Ylangene		1450	0.1	0.2	0.1	0.2	0.2	0.3	0.3	0.5	0.5	0.8

α-Humulene		1454	1.0	1.2	1.3	1.3	1.2	1.4	1.4	1.4	1.5	1.7
Epi-bicyclo-sesquiphellandrene		1463	-	0.1	0.2	0.1	0.2	0.3	0.4	0.4	0.5	0.5
Germacrene D		1481	--	0.1	0.1	0.2	1.0	1.2	1.3	1.9	1.9	11.5
Alloaromadendrene		1486	0.1	-	-	-	0.2	-	-	0.1	0.2	0.2
β-Selinene		1486	0.1	-	0.2	-	-	0.1	-	0.2	-	0.3

Calarene		1494	0.2	0.2	0.1	0.4	0.5	3.3	5.0	5.5	5.7	5.7
Muurolene		1498	-	0.1	-	0.3	0.4	0-.5	0.6	0.7	0.8	1.0
δ-Cadinene		1522	0.5	0.4	2.1	2.4	4.5	6.4	8.5	12.3	13.5	23.8
Cadina-1,4-diene		1531	0.7	-	0.8	-	0.1	-	0.2	-	-	12.2

α-Cadinene		1537	1.5	1.5	1.5	1.6	3.2	7.5	8.0	8.2	9.6	10.7
Nerolidol		1559	0.6	1.4	t	1.3	1.5	1.2	1.1	1.1	1.5	1.3
Palustrol		1569	0.2	0.3	-	0.2	0.4	-	-	-	-	0.7
β-Bourbonene		1575	0.1	-	-	-	0.2	-	-	0.1	-	1.0
Oplopenone		1609	-	0.1	-	-	0.2	-	-	t	-	t

α-Cadinol		1655	0.1	0.4	5.1	6.4	7.5	8.4	9.4	21.5	22.4	24.2
T-muurolol		1659	12.5	13.4	14.5	15.4	17.5	18.6	18.8	20.9	21.9	22.5
Yield (%w/w)			0.13	0.30	0.45	0.48	0.52	0.64	0.65	0.79	0.95	0.97

[1] t = traces, KI = Kovats indices

$$\text{Percentage Yield (\%)} = \frac{\text{Total Weight of Oil}}{\text{Total Weight of Plant}} \times 100$$

In addition the major components of the essential oils of this plant are 1,8-cineole, δ-cadinene, cadina-1,4-diene, germacrene D, T-muurolol, α-cadinene and α-cadinol as shown in Fig. 2.

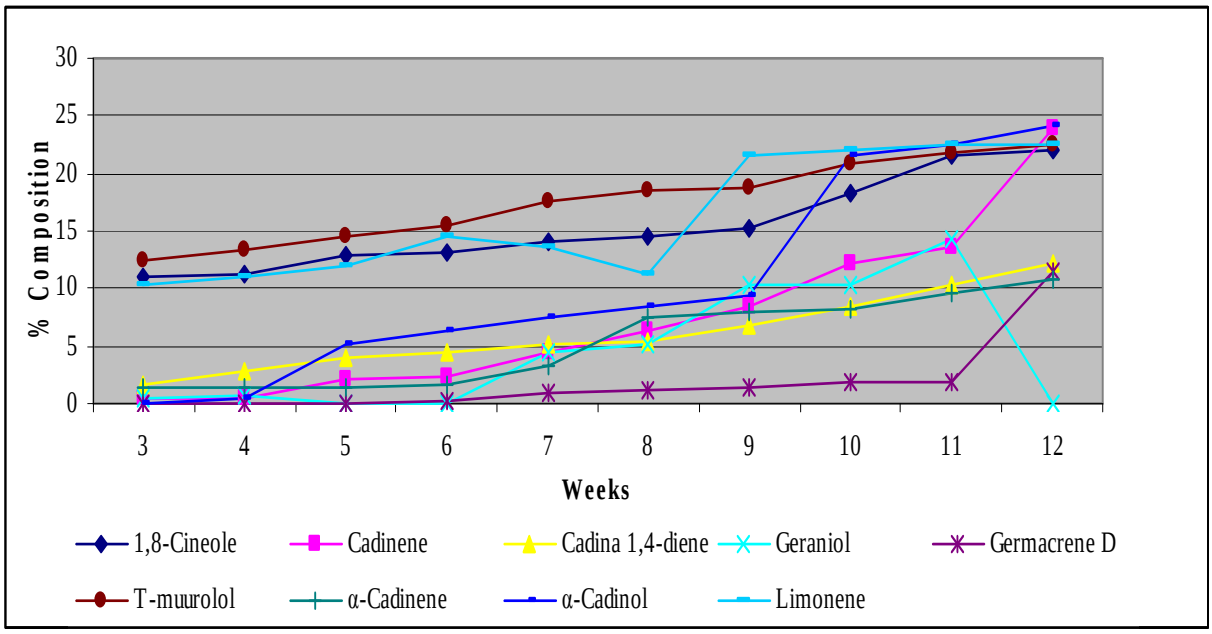
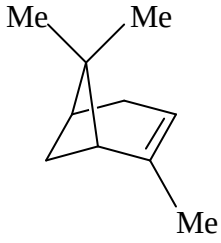
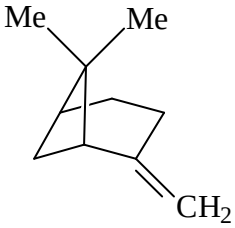
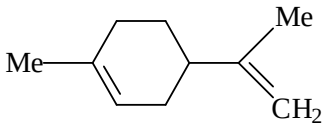
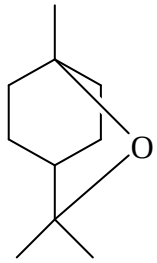
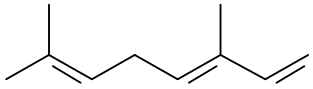
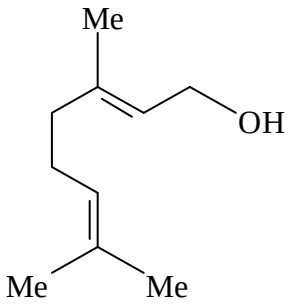
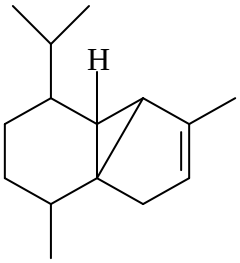
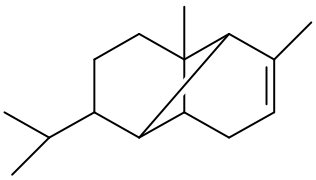
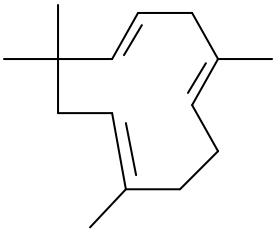
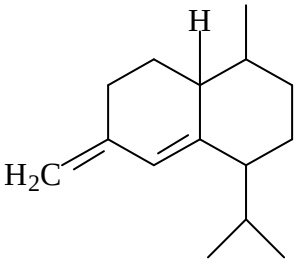
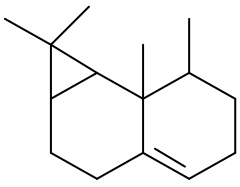
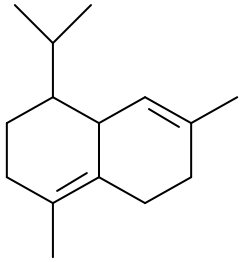
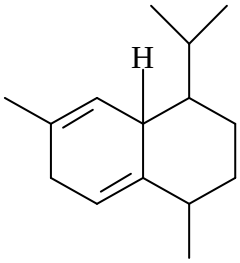
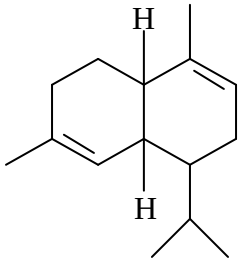
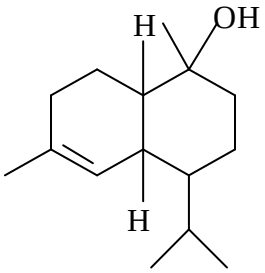
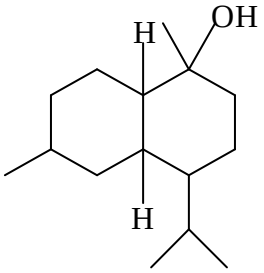


Figure 2: Major Components of Essential Oil of *Calendula officinalis*

Table 6: R^2 Values of the Main Components of the Essential Oil of *Calendula officinalis* During its Vegetative Life Cycle

Chemical Component	Structure	R-Squared Value (R^2) [1]
α-Pinene		0.8862
β-Pinene		0.8867
Limonene		0.9184
1,8-Cineole		0.9053
Trans-β-ocimene		0.944

Geraniol		0.8042
α-Cubebene		0.5024
α-Ylangene		0.8107
α-Humulene		0.8264
Epi-bicyclo-sesquiphellandrene		0.9075
Calarene		0.8623

δ-Cadinene		0.8577
Cadina-1,4-diene		0.5432
α-Cadinene		0.9028
α-Cadinol		0.9001
T-muurolol		0.9886
Yield (%w/w)		0.9686

[1] R^2 explains the relationship between concentration of chemical constituents and age of the plant

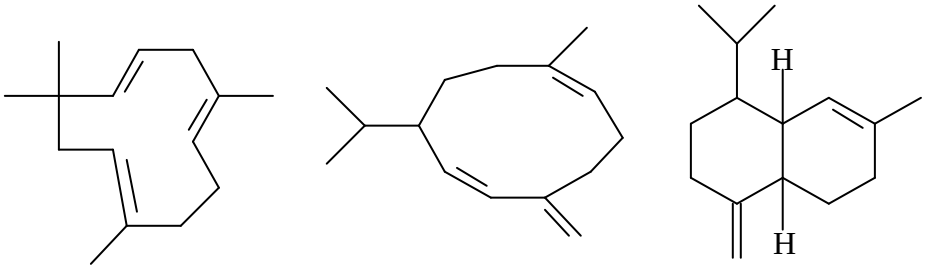
4.2 EFFECTS OF DRYING ON THE CHEMICAL COMPONENTS OF ESSENTIAL OIL OF *Calendula officinalis* L. GROWING WILD IN THE EASTERN CAPE PROVINCE OF SOUTH AFRICA

Pale yellow oils with yields of 0.06%, 0.03%, and 0.09% were obtained from the fresh leaves, dry leaves, and fresh flowers of the plant, respectively. The oils gave a total of 30, 21, and 24 identified compounds, representing 91.7%, 89.8%, and 87.5% of the total oil composition from the fresh leaves, dry leaves, and fresh flowers, respectively (Table 7).

Although the flowers had the greatest oil yield, the oil from the fresh leaves was richer in chemical constituents than that from the dry leaves and fresh flowers. A total of 30 chemical constituents were identified from the fresh leave oil, while 21 constituents were identified from oil from the dry leaves. This supports the observation by Loughrin and Kasperbauer (2003), who reported that there could be a 50-fold reduction in chemical composition when plant materials are dried.

The fresh leaf oil was dominated by T-muurolol (40.9 %), α-thujene (19.2 %), and δ-cadinene (11.4 %), while the dry leaf oil was found to be rich in 1,8-cineole (29.4 %), α-thujene (17.8%), β-pinene (6.9 %), and δ-cadinene (9.0 %). The fresh flower oil, on the other hand, has its major components as α-thujene (26.9 %), T-muurolol (24.9 %), and δ-cadinene (13.1 %). The complete absence of 1,8-cineole in the fresh leaves and its sudden appearance in the dry leaves (29.4%) is noteworthy. However, changes in oil composition are known to be dependent on a number of factors including the class of plant. The presence of 1,8-cineole in the dry leaf oil makes it superior to the fresh leaf oil due to the characteristic properties of the compound.

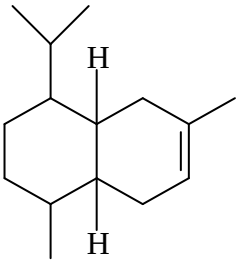
Generally, a lot of components were missing in the dried leaf oil as compared to the fresh leaf oil. The sesquiterpene hydrocarbons present in all the oil samples were α-humulene **73**, germacrene D **74**, α-cadinene **65**, and δ-cadinene **21**, while among the monoterpene hydrocarbons was α-thujene **71**. T-Muurolol **67**, the major component in the fresh leaf oil, was also present in the other oils. T-Muurolol is produced from the direct oxidation of α-muurolene **75**. β-Pinene present at 6.9% in the dry leaves occurred in minute amount in the fresh leaf oil, and α -terpenene present at values of 6.9% and 11.6%, in the dry leaf oil, occurred in minute amounts in the fresh leaf oil. The changes in the regimes of volatile compounds during drying have been reported to depend on several factors, such as drying method and class of plants (Asekun *et al*. 2007). According to Moyler (1994), the components of the essential oils that are lost in the dried leaves are those stored on or near the leaf surfaces. However, Ibanez *et al*. (1999) observed no difference in the essential oil composition of fresh and dry rosemary plant.



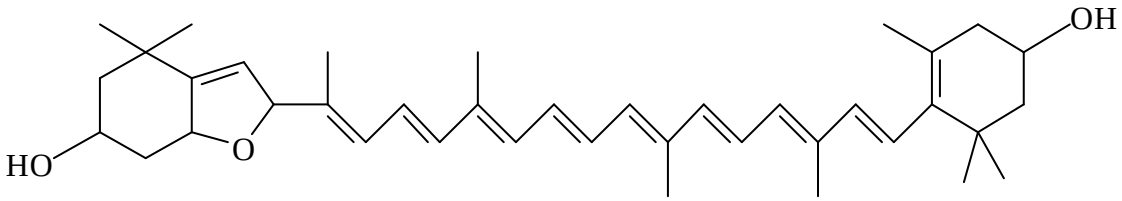
73 **74** **75**

The results of this study have reinforced the fact that there are quantitative and qualitative differences in the essential oil components of the same plant that may be growing in different parts of the world. For example, Crabas (2003) reported the presence of methyl hexadecanoate **14**, methyl linoleate **15**, methyl 9,12,15-octadecatrienoate **16**, 10-methyl octadecanoate **17**, methyl tetradecanoate **18**, γ-cadinene **19**, oplopanone **23**, cubenol **20**, ß-cadinene **76**, and α-cadinol **22** in the essential oil of *Calendula officinalis* growing wild in Italy. These compounds were not

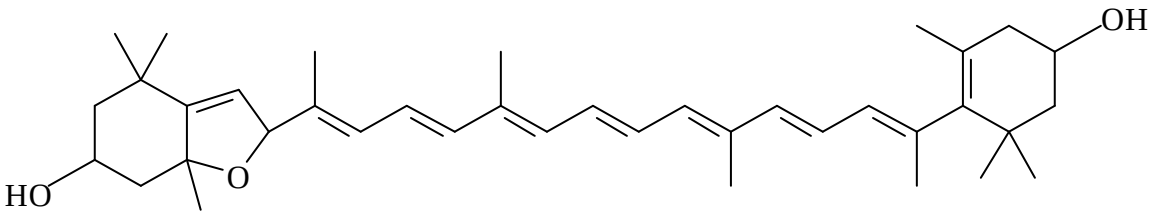
detected in the oil of this plant found in the Eastern Cape, except oplopanone **23** that was present only in the fresh leaf oil. In another study that assessed the carotenoid composition of different parts of *Calendula officinalis* (Bako *et al*. 2002), it was observed that in the petals and pollens, the main carotenoids were flavoxanthin **77** and auroxanthin **78** while the stem and leaves mostly contained lutein **79** and β-carotene **80**, thus supporting the hypothesis of variability in the oil composition of this herb depending on the part of the plant and geographical location.



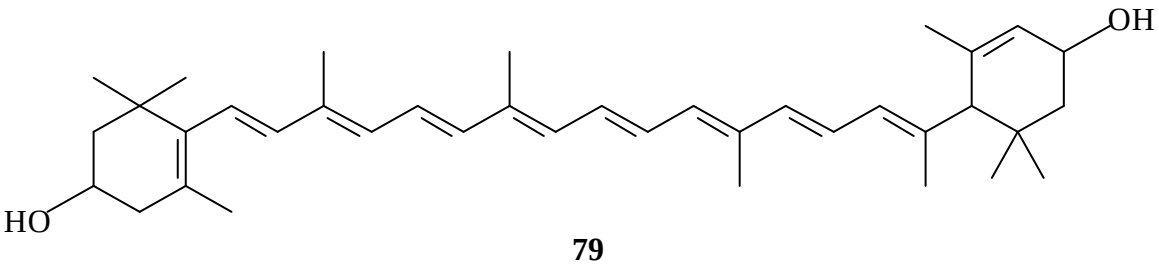
76



77



78



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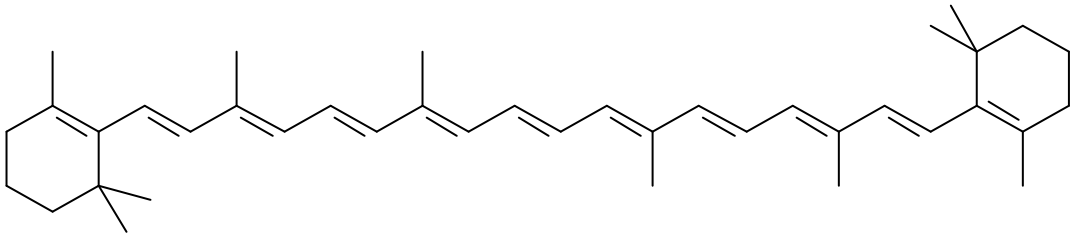
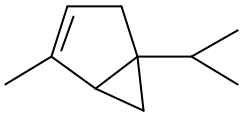
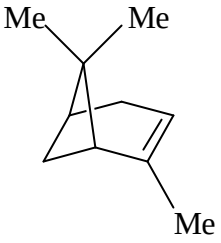
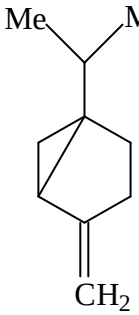
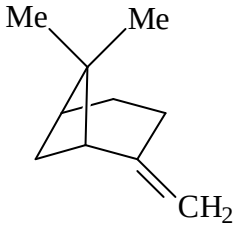
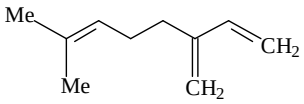
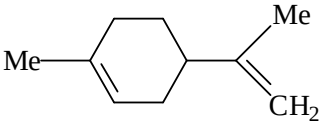
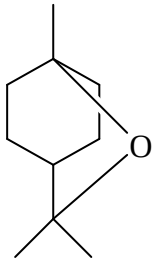
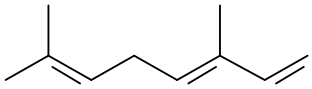
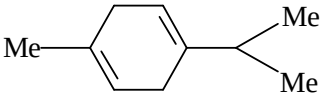
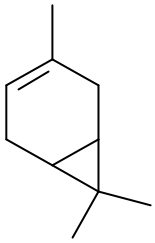
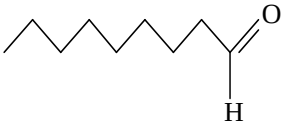
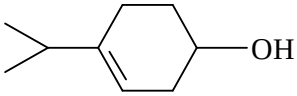
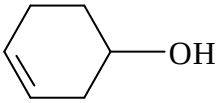
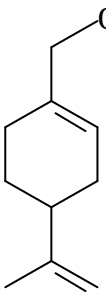
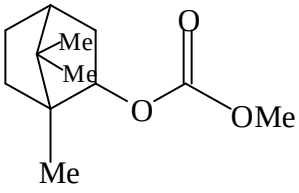
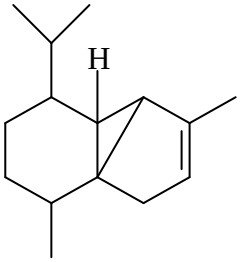
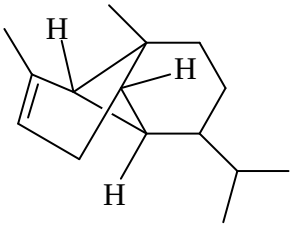
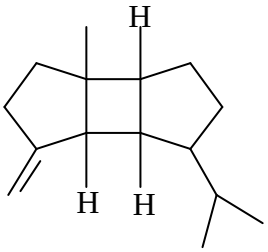
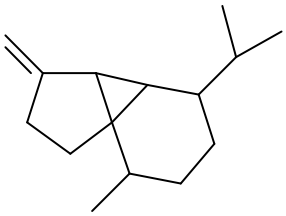


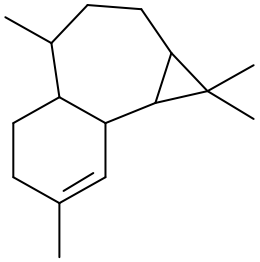
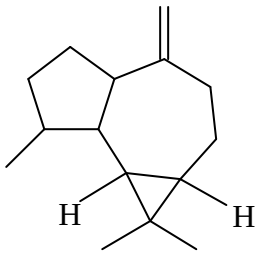
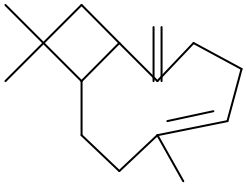
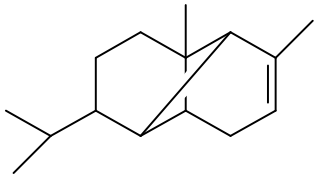
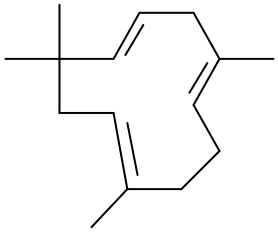
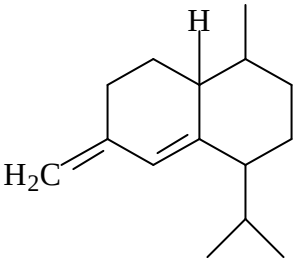
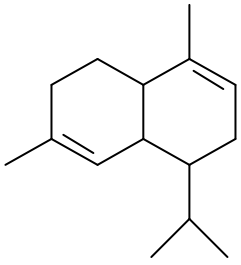
80

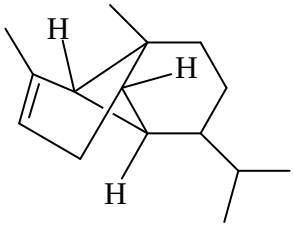
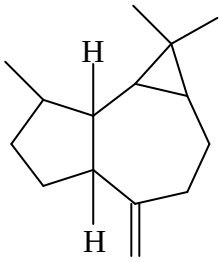
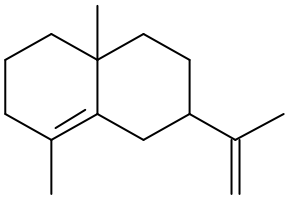
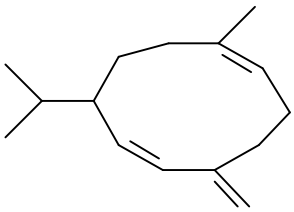
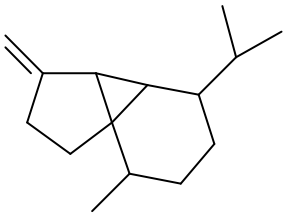
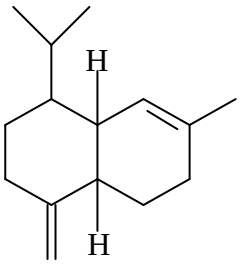
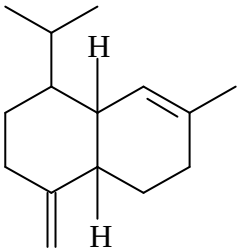
Table 7: Chemical Composition of the Essential Oil from *Calendula officinalis* L Growing in the Eastern Cape Province of South Africa.

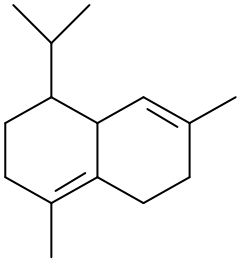
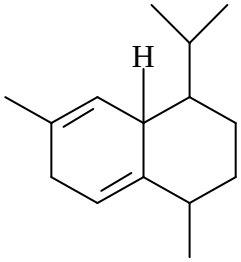
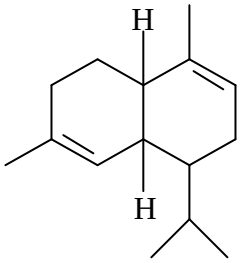
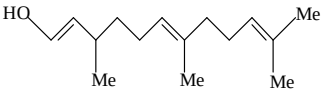
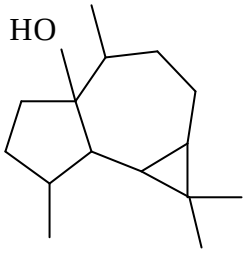
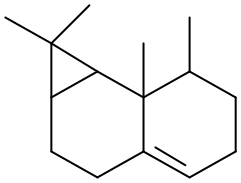
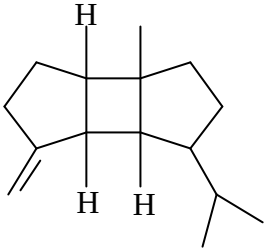
Compound [1]	Structure	KI [2]	% Composition (oil) Leaves		Flow-ers
			Fresh	Dry	
α- Thujene		908	19.2	17.8	26.9
α-Pinene		928	-	2.4	1.8
Sabinene		960	1.1	-	1.8

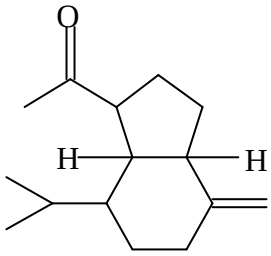
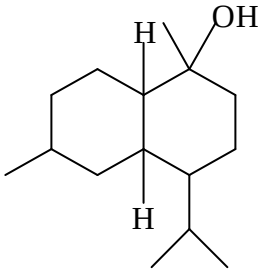
β- Pinene		969	0.6	6.9	-
Myrcene		971	-	-	1.1
Limonene		1020	0.8	-	-
1,8 Cineole		1022	-	29.4	1.7
Trans-β-ocimene		1033	0.2	-	-
γ-Terpenene		1049	0.4	-	0.7
δ-3-Carene		1050	-	0.3	-
Nonanal		1099	-	1.0	-
Terpene-4-ol		1174	0.4	-	0.6
3-cyclohexen-1-ol		1175	-	0.6	-

α-Terpeneol		1205	-	0.6	-
Bornyl acetate		1283	0.1	-	-
α-Cubebene		1347	0.2	-	-
α-Copaene		1376	0.3	0.2	0.2
α-Bourbonene		1385	0.3	0.2	-
β-Cubebene		1389	0.4	0.2	0.5

α-Gurjunene		1409	0.6	-	0.6
Aromadendrene		1410	-	0.2	-
β-Caryophyllene		1420	1.0	-	1.2
α-Ylangene		1450	0.2	-	-
α-Humulene		1454	1.7	1.2	1.5
Epi-bicyclosesquiph-landrene		1463	0.4	-	-
α-Amorphene		1513	0.6	-	0.5

α-Copaene		1376	-	-	2.7
Alloaromaden-drene		1486	-	-	0.3
β-Selinene		1486	0.5	-	-
Germacrene D		1481	1.1	0.6	2.8
β-Cubebene		1491	-	1.4	0.2
Muurolene		1498	2.1	1.6	-
γ-Cadinene		1513	2.7	2.2	2.2

δ- Cadinene		1522	11.4	9.0	13.1
Cadina-1,4-diene		1531	0.5	-	0.4
α-Cadinene		1537	0.6	0.4	0.4
Nerolidol		1559	-	-	0.9
Palustrol		1569	0.2	-	-
Calarene		1494	2.3	0.5	-
Endo-β-bourbonene		1575	0.6	-	0.5

Oplopenone		1609	0.3	-	-
T-Muurolol		1659	40.9	13.1	24.9
Yield (% w/w)			0.06	0.03	0.09

[1] In order of elution
[2] KI - Kovats retention indices on HP-5 (similar to DB-5)

4.3 ISOLATION OF MAJOR COMPOUNDS

Efforts were made to isolate some major compounds from this plant. The procedure for the extraction and partitioning, fractionation and purification are illustrated in the diagrams below (Fig. 3, 4).

Freshly collected plant material
↓
Air-dried and milled to fine texture (1 kg)
↓
Extracted with ethanol (4 times)
↓
Filtered (Whatman # 1)
↓
Filtrates combined and concentrated to dryness under reduced pressure
↓
Partitioned between hexane and water
↙ ↘
N-hexane fraction **Water fraction**
↘ ↙
Partitioned between EtOAc and water
↙ ↘
Ethyl acetate fraction **Water fraction**

Figure 3: Extraction and Partitioning Process

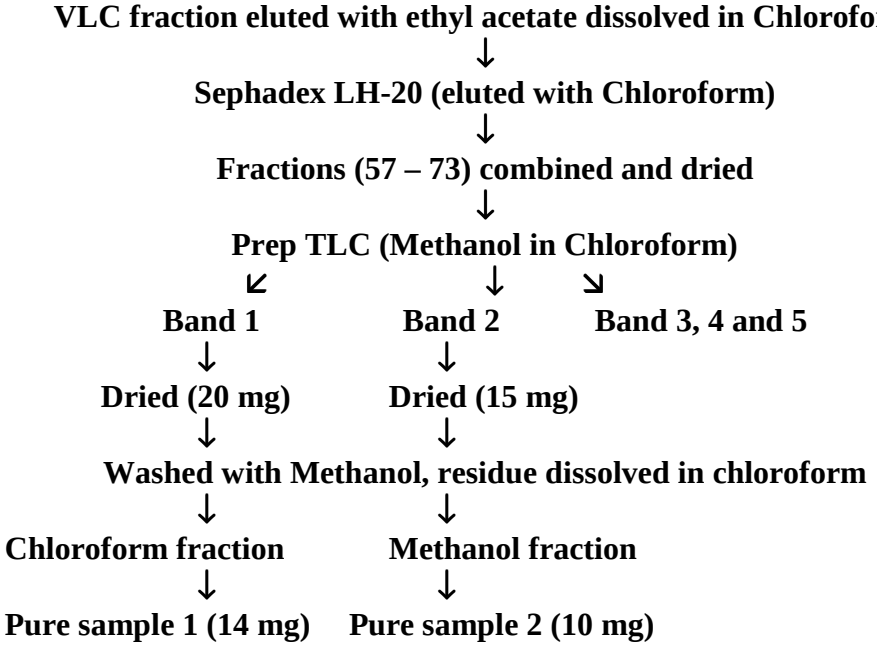


Figure 4: The Fractionation and Purification Process

4.4 FRAGMENTATION PATTERN

Using the mass spectra of the isolated liquid (Fig. 5), a fragmentation pattern was obtained (Fig. 6), which corresponds to that for 1,8-cineole. One way is related to the loss of methyl group from the parent ion with molecular mass of 154, the resultant epoxy structure being in tautomeric equilibrium with the structure containing the hydroxyl group. The latter is eliminated at the next stage, and further loss of the $CH_3CH^+CH_3$ group gives cyclohexene ion, which could be also the result of the loss of $CH_3C^+(OH)CH_3$ from the open tautomeric form. Additional process includes tautomerization of the parent ion itself followed by further elimination of $CH_3C(=O^-H)CH_3$ framework and then methyl group to yield the same cyclohexene ion, or alternative tautomerization with $CH_3CH^+CH_3$ loss to afford 4-methyl-4-hydroxycyclohexene.

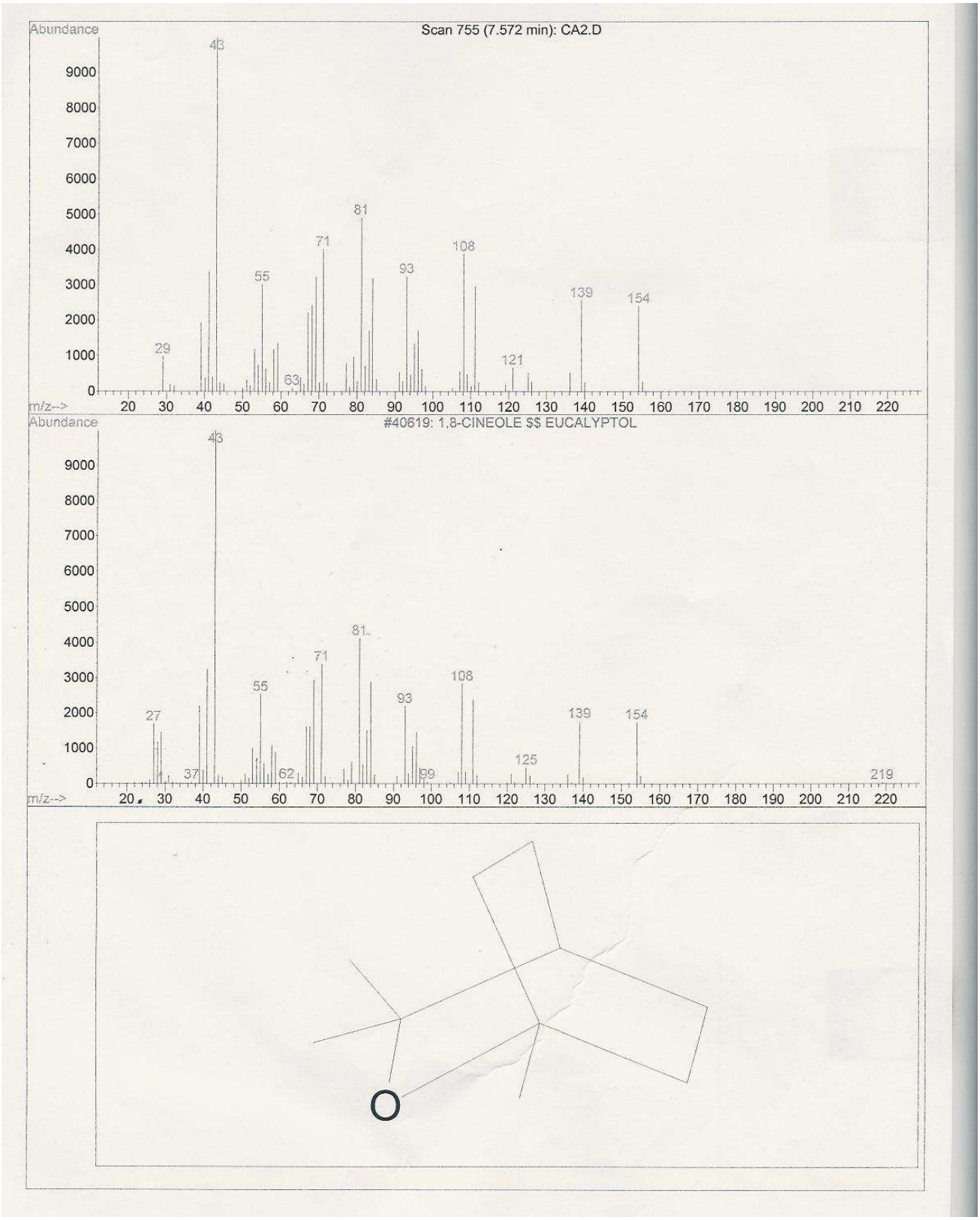

Abundance
Scan 755 (7.572 min): CA2.D
43
81
71
108
93
55
139
154
29
121
63
m/z-->
Abundance
#40619: 1,8-CINEOLE $$ EUCALYPTOL
43
81
71
108
55
93
27
139
154
125
37
62
99
219
m/z-->

Figure 5: GC-MS Peaks of 1,8- Cineole

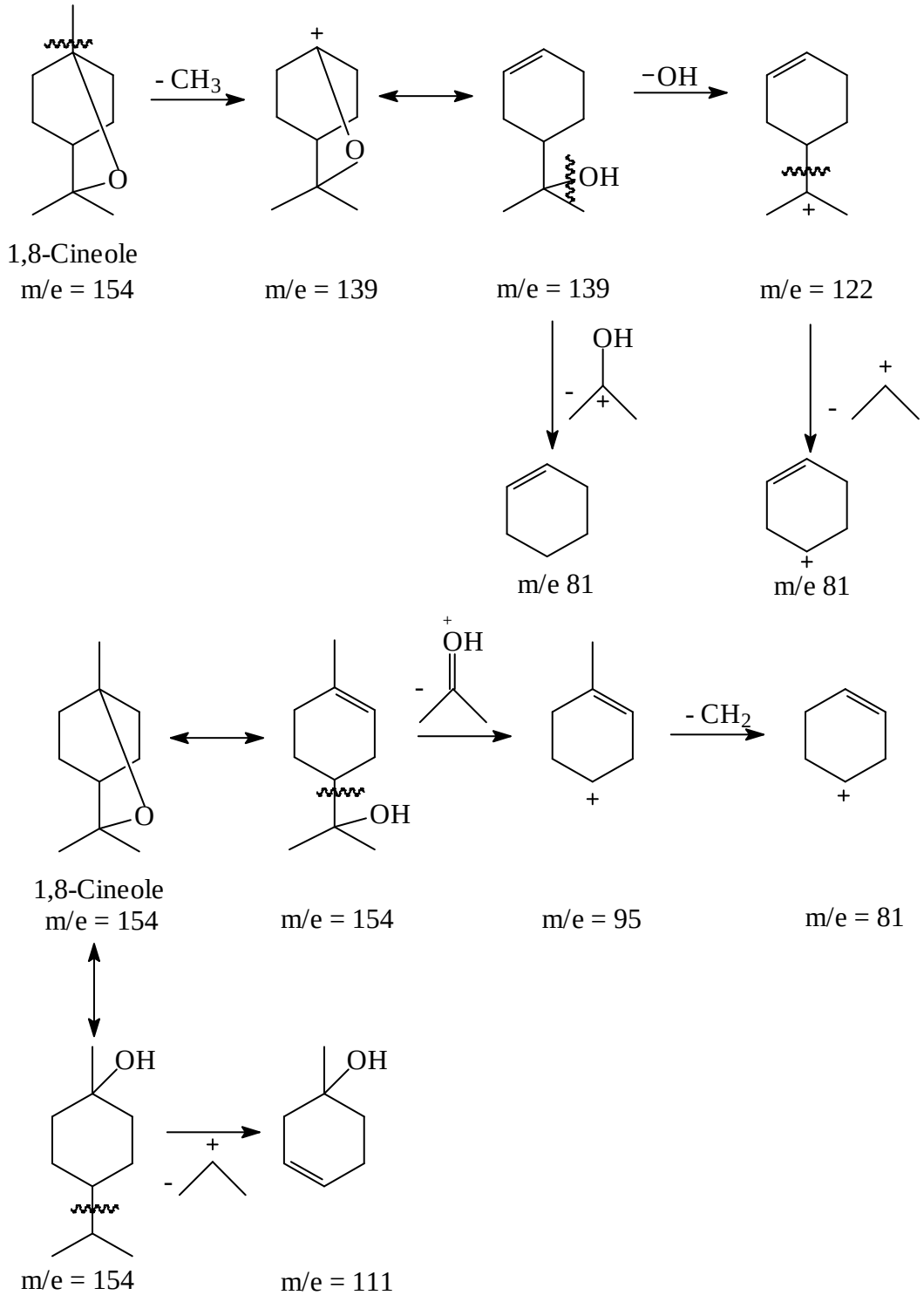


Figure 6: Fragmentation Pattern of 1,8-Cineole

4.5 SPECTRAL IDENTIFICATION OF 1,8-CINEOLE

The IR-spectral pattern is shown in Fig. 7. It consists of the asymmetric stretching vibrations of the alkane groups including those of $C(CH_3)_2$ framework in the region of 2972-2952 cm^{-1} (*s*) and 2936-2916 cm^{-1} (*s*), symmetric stretching vibrations 2900-2880 cm^{-1} (*w*), asymmetric stretching vibrations of the six-membered cyclic ether system C-O-C1110-1090 cm^{-1} (*s*), and symmetric stretching C-O-C vibrations in the range 820-805 cm^{-1} (*m*). This spectral picture is in full accord with the structure of 1,8-cineole and proves its identity of an individual isolated substance.

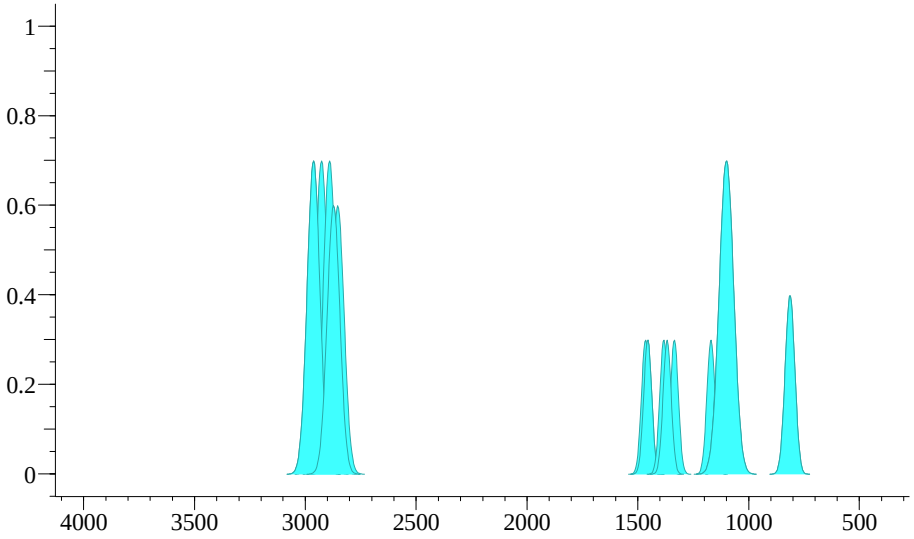


Figure 7: IR Spectrum of 1,8-Cineole

These analyses showed that 1,8-cineole was the major constituent obtained from the aqueous methanol extracts of *Calendula officinalis*. 1,8-Cineole (1,3,3-trimethyl-2-oxabicyclo[2.2.2] octane) is a monoterpene found in a variety of essential oils. In order to further characterize the isolated component, the extract was concentrated and pooled for NMR analysis. NMR data were collected on a Varian Inova 300 MHz instrument for ^1H spectra and illustrated in Fig. 8. The chemical shifts are reported in δ (ppm) and referred to residual $CHCl_3$ at 7.24 ppm in the NMR solvents. The ^1H NMR (300 MHz, $CDCl_3$) δ 0.70 (*s*, 3H, CH_3), 1.18 (*s*, 6H, 2 x CH_3), 1.21 (*m*,

1H, H_4), 1.26 (*m*, 4H), 1.3 – 2.22 (*m*, 3H). The chemical shift at 7.22 ppm is due to the solvent while the one at 0.001 ppm might be due to the presence of water or the possibility of another form of cineole that have the hydroxyl group.

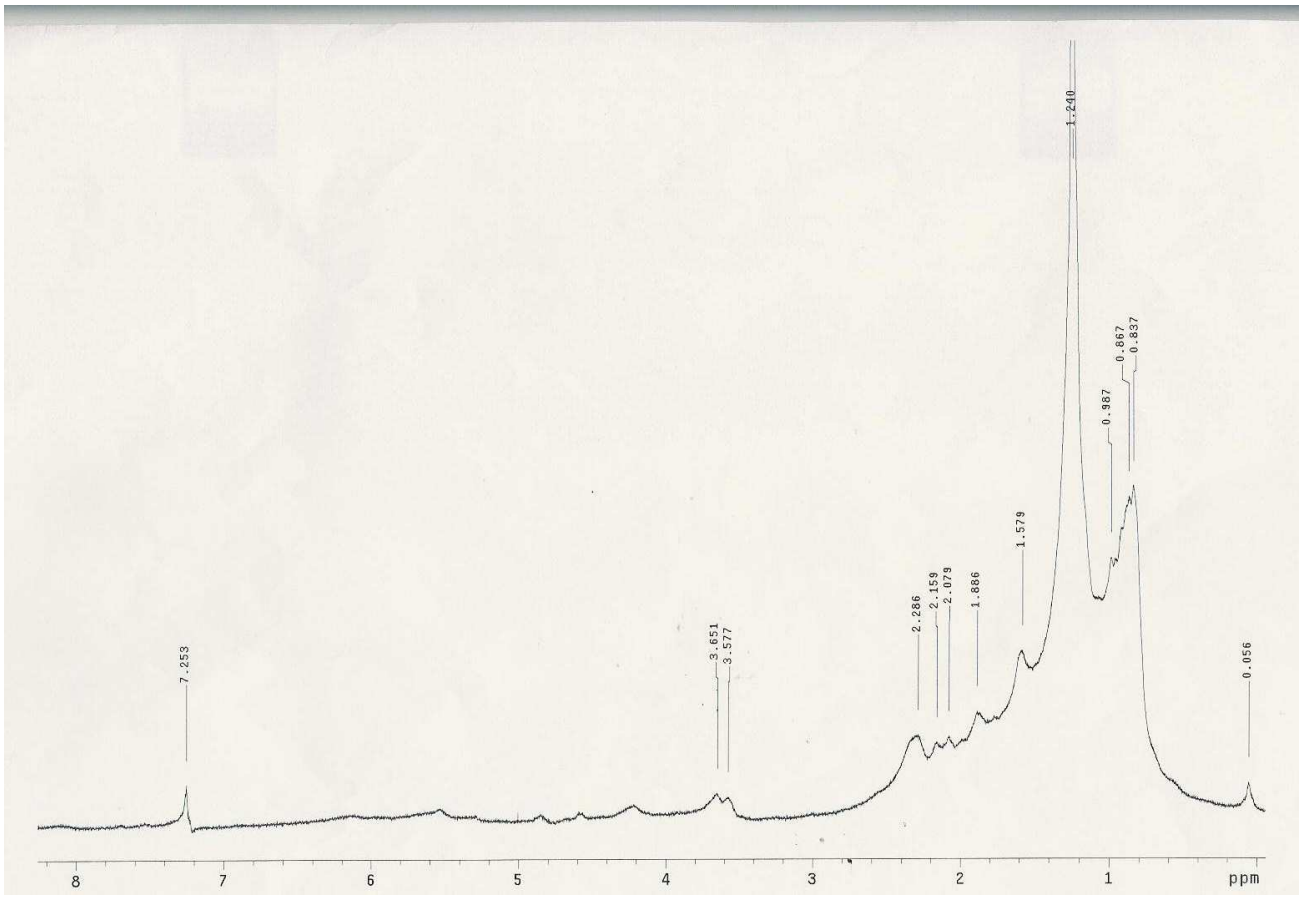


Figure 8: NMR Peaks for 1,8-Cineole

Nuclear magnetic resonance spectroscopy (NMR) is one of the most important and wide-spread analytical methods in the academic and industrial research. It enables a unique and, in principle, quantitative determination of the relative amount of molecular groups, thus offering a tool to quantify entire molecular structures even in mixtures. The recorded ^{1}H-NMR spectrum of the analyte correlates well with the known spectrum (Fig. 9), which was assigned to 1,8-cineole (Malz and Jancke, 2005), as well as the simulated ^{1}H NMR spectrum of 1,8-cineole done by us using Chemwindow software (Fig. 10). The published spectrum represents 1,8-cineole dissolved

in DMSO-d_6. The ^{1}H spectrum of the solvent showed the presence of impurities. The impurity signals appear in the 2D H, H-COSY spectrum which is located directly under the analyte signal 1.3-1.6 ppm (multiple of protons 2, 3a, 4, 5a and 6). These impurity signals were recognized by cross-peaks to other signals (two doublets at about 0.9 ppm) that have nothing in common with the 1,8-cineole spectrum (Malz and Jancke, 2005).

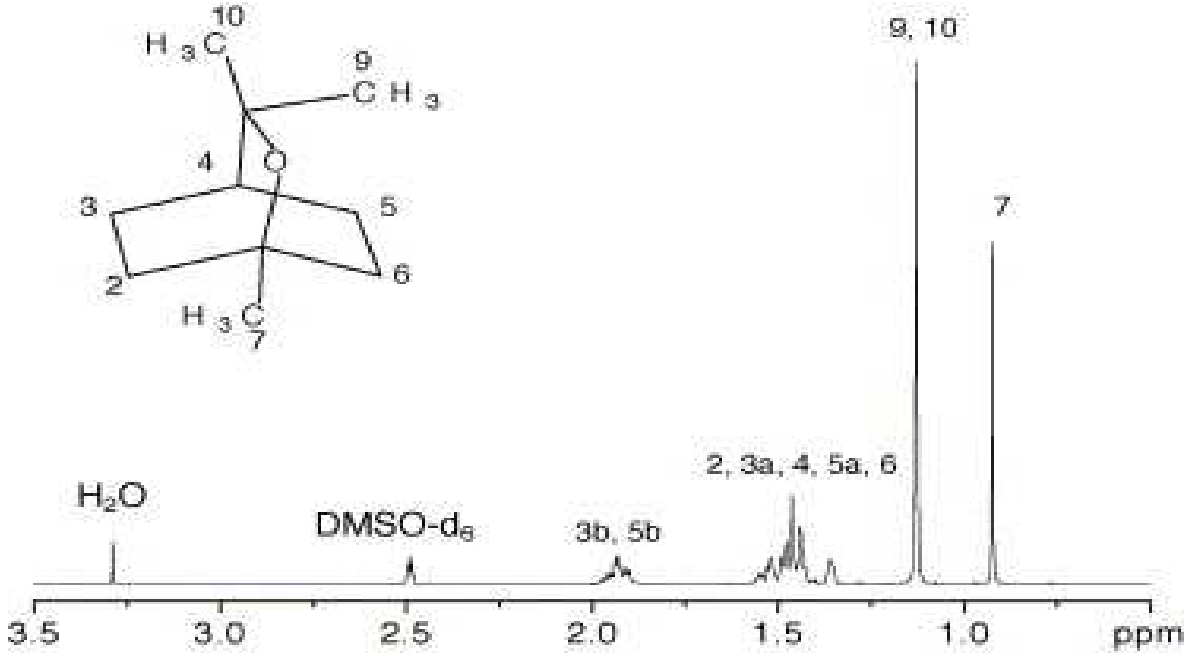


Figure 9: Published ^{1}H NMR Spectrum of 1,8-Cineole

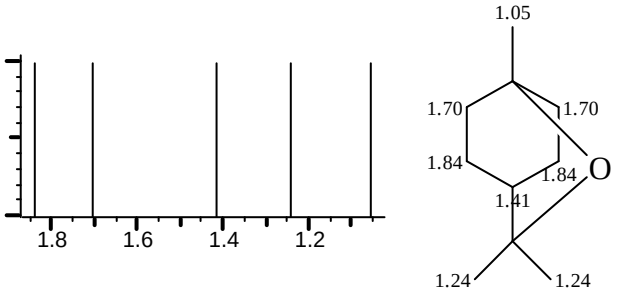
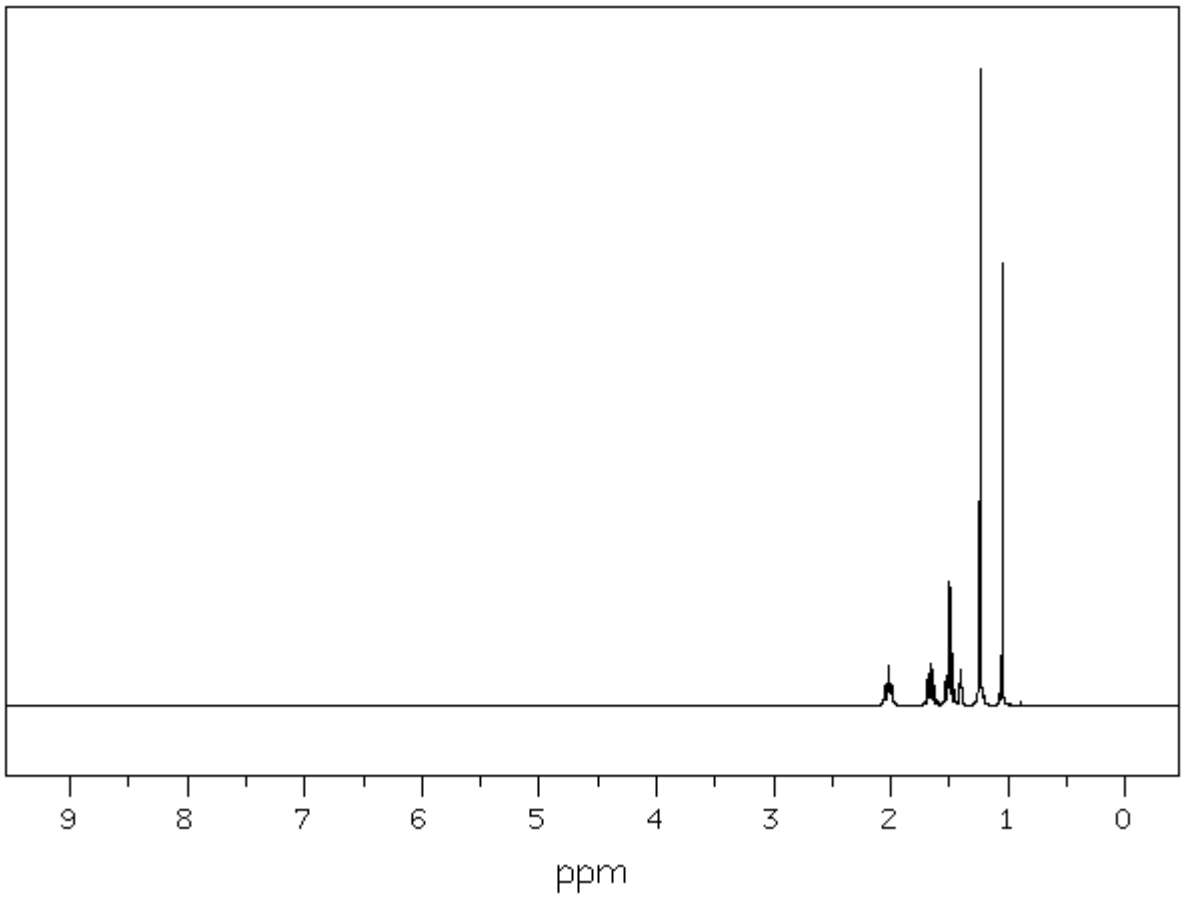
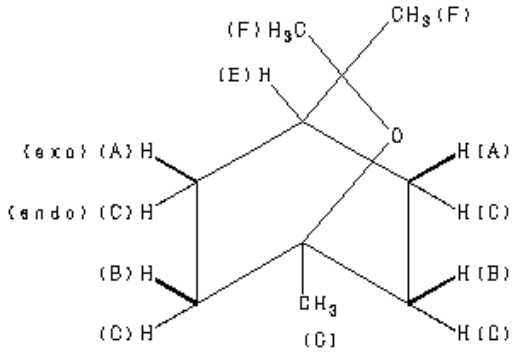


Figure 10: Simulated ^{1}H NMR Spectrum of 1,8-Cineole

Therefore the structure that is consistent with the above spectra is ,8 –cineole. 1,8-Cineole is an oxygenated monoterpenoid with a molecular mass of 154. The oxygen forms an ether linkage with carbon atoms at positions 1 and 8. It should be generally unreactive as any other ether. The fragmentation pattern of ether is favored by cleavages of the C-O bonds. Fragmentation is also favored by branching thus the cleavages at position 1 and 8 as shown in Fig. 6. ^{1}H and ^{13}C NMR spectra of some representative isolated components of the essential oil listed in Tables 5 and 8 are presented in Figures 7-15 together with their spectral peaks.

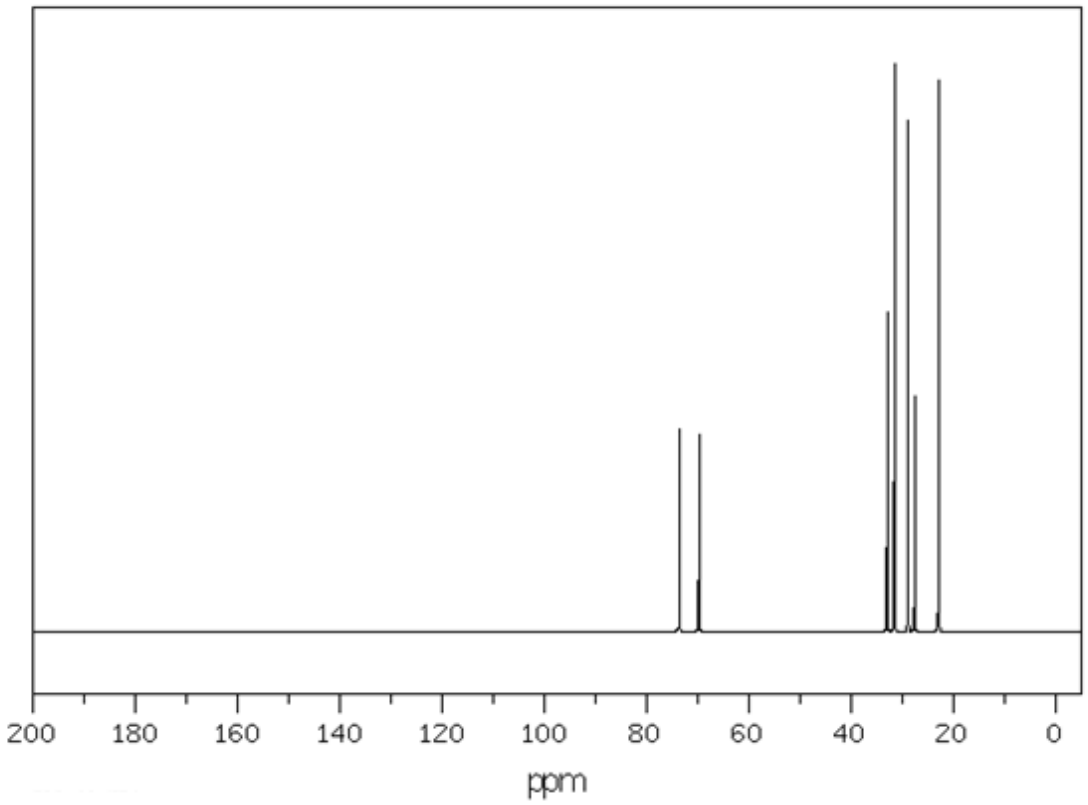
Figure 7: ^{1}H NMR **399.65 MHz C_{10} H_{18} O 0.05 ml: 0.5 ml $CDCl_3$** 1,8-cineole

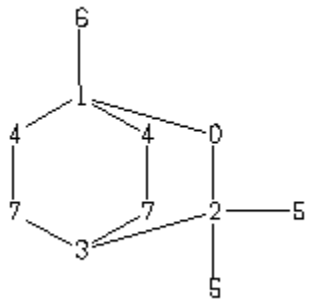




Assign.	Shift(ppm)
A	2.022
B	1.661
C	1.50
E	1.41
F	1.239
G	1.050

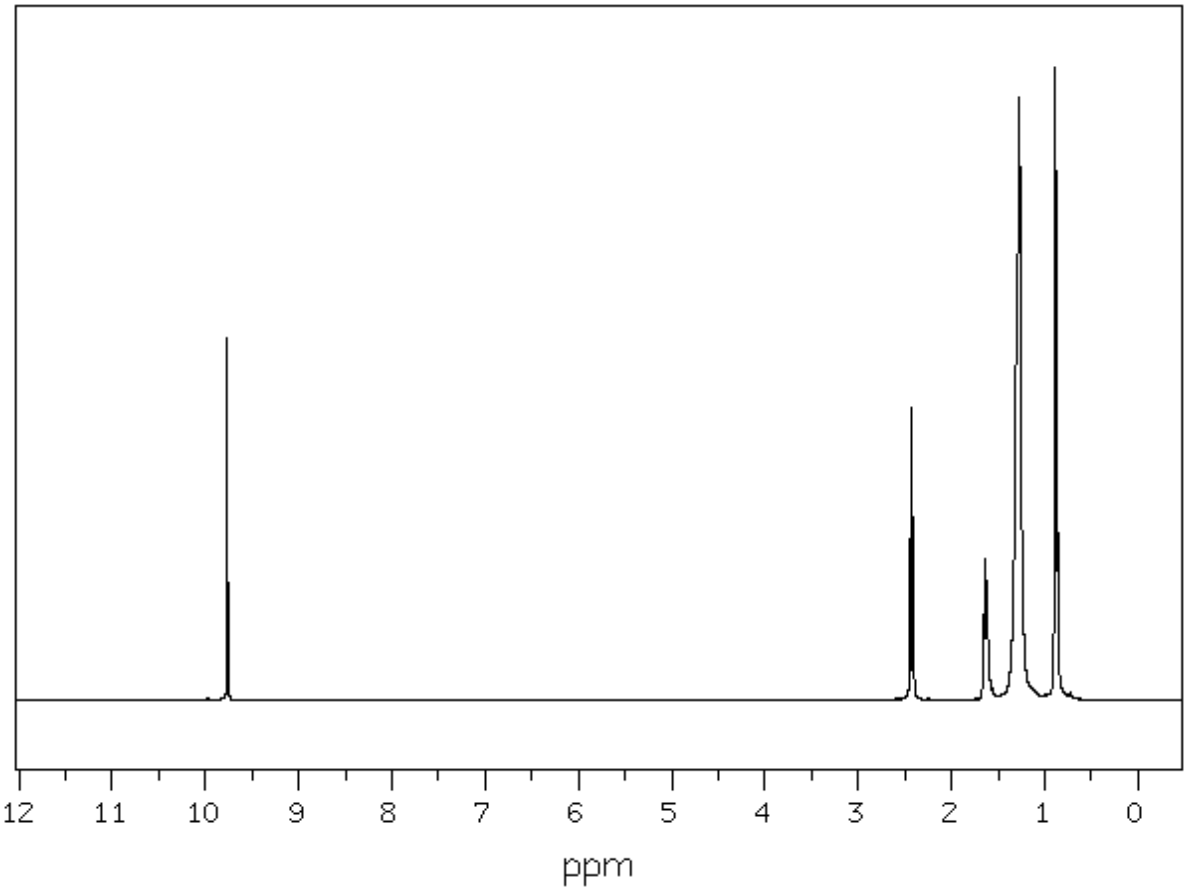
Figure 8: ^{13}C NMR 25.16 MHz C_{10} H_{18} O 0.5 ml : 1.5 ml $CDCl_3$ **1,8-cineole**

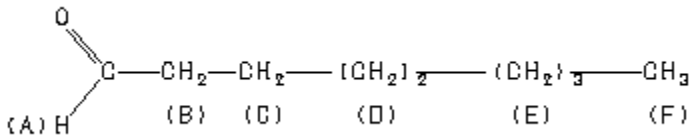




ppm	Int.	Assign.
73.62	355	1
69.77	345	2
33.00	560	3
31.57	1000	4
28.92	900	5
27.61	415	6
22.90	970	7

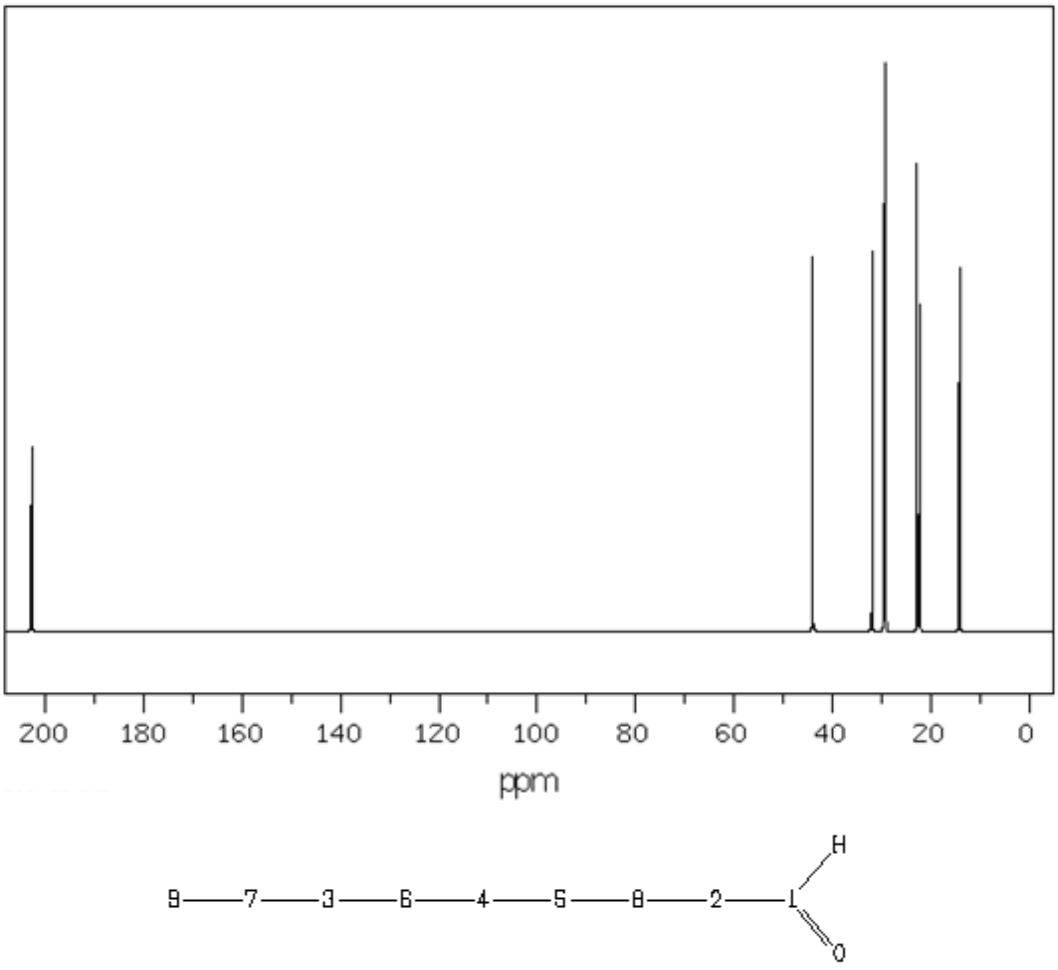
Figure 9: ^{1}H NMR 399.65 MHz $C_9 H_{18} O$ 0.05 ml : 0.5 ml $CDCl_3$ **Nonanal**





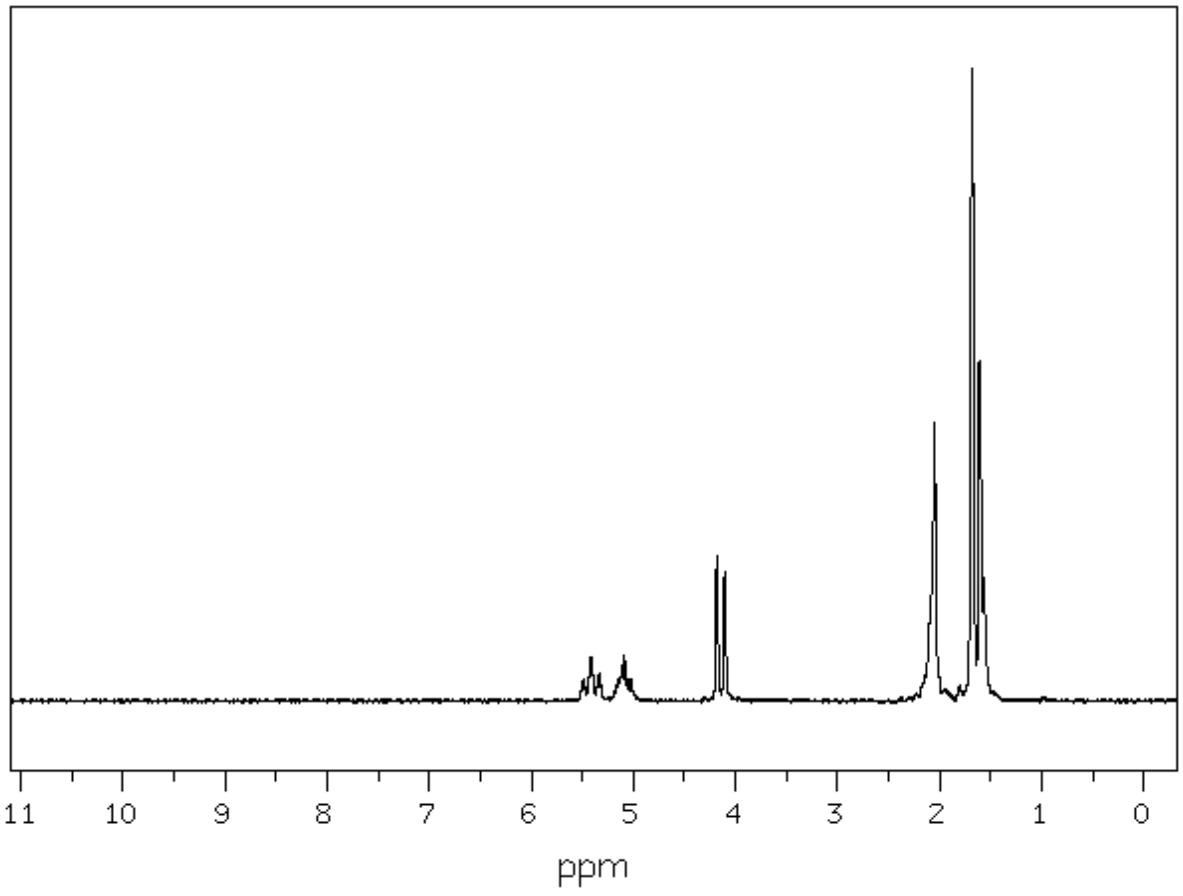
Assign.	Shift(ppm)
A	9.764
B	2.422
C	1.628
D	1.30
E	1.27
F	0.881

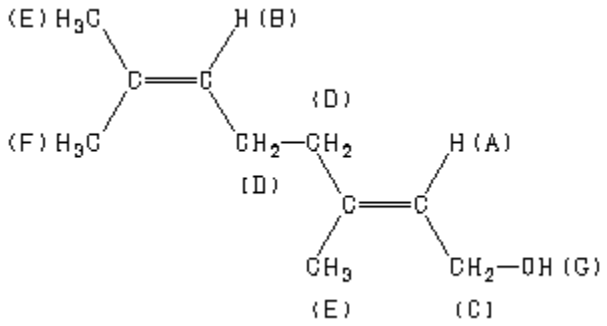
Figure 10: ^{13}C NMR 25.16 MHz $C_9H_{18}O$ 0.5 ml : 1.5 ml $CDCl_3$ **Nonanal**



ppm	Int.	Assign.
202.82	363	1
43.96	741	2
31.87	751	3
29.39	821	4
29.24	935	5
29.18	1000	6
22.70	925	7
22.15	647	8
14.11	721	9

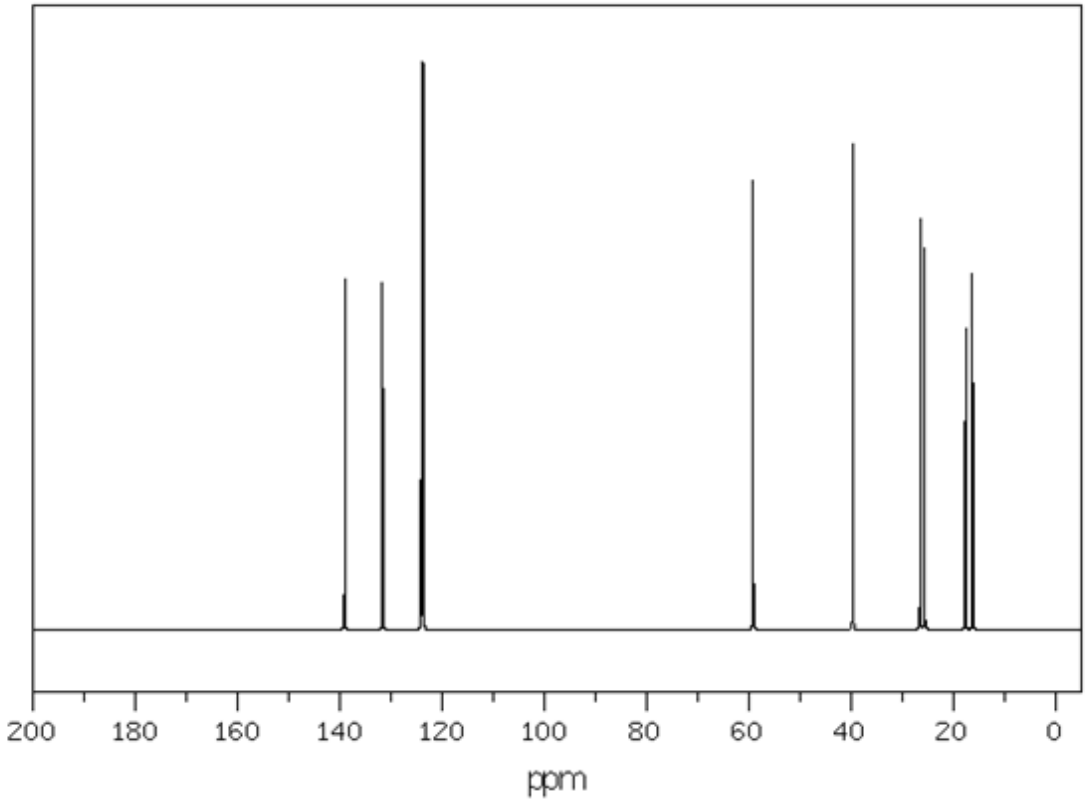
Figure 11: ^{1}H NMR 359 89.56 MHz C_{10} H_{18} O 0.04 ml : 0.5 ml $CDCl_3$ **Geraniol**

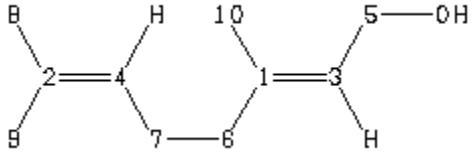




Assign.	Shift(ppm)
A	5.41
B	5.09
C	4.144
D	2.23 to 1.95
E	1.68
F	1.61
G	1.57

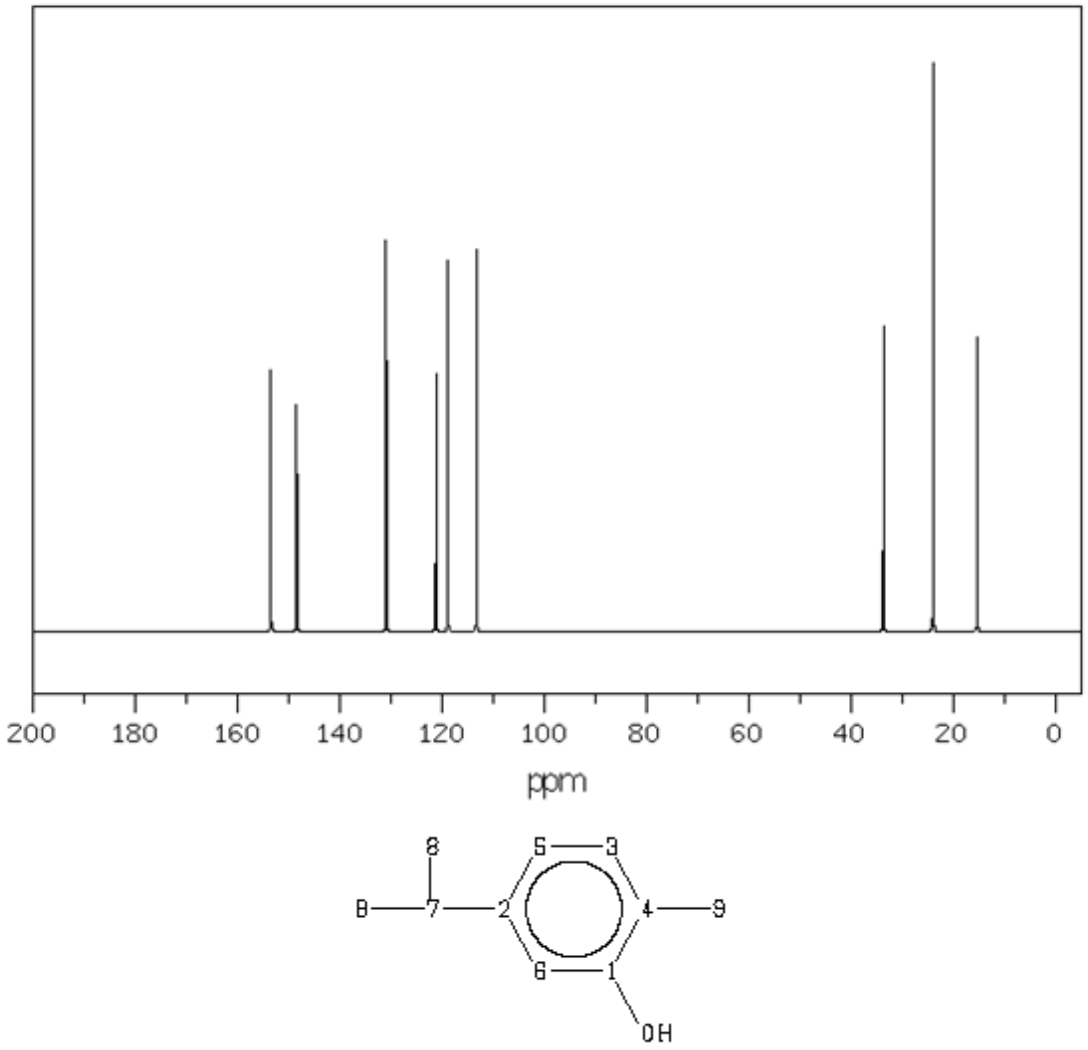
Figure 12: ^{13}**C NMR** 25.16 MHz C_{10} H_{18} O 0.5 ml : 1.5 ml $CDCl_3$ **Geraniol**





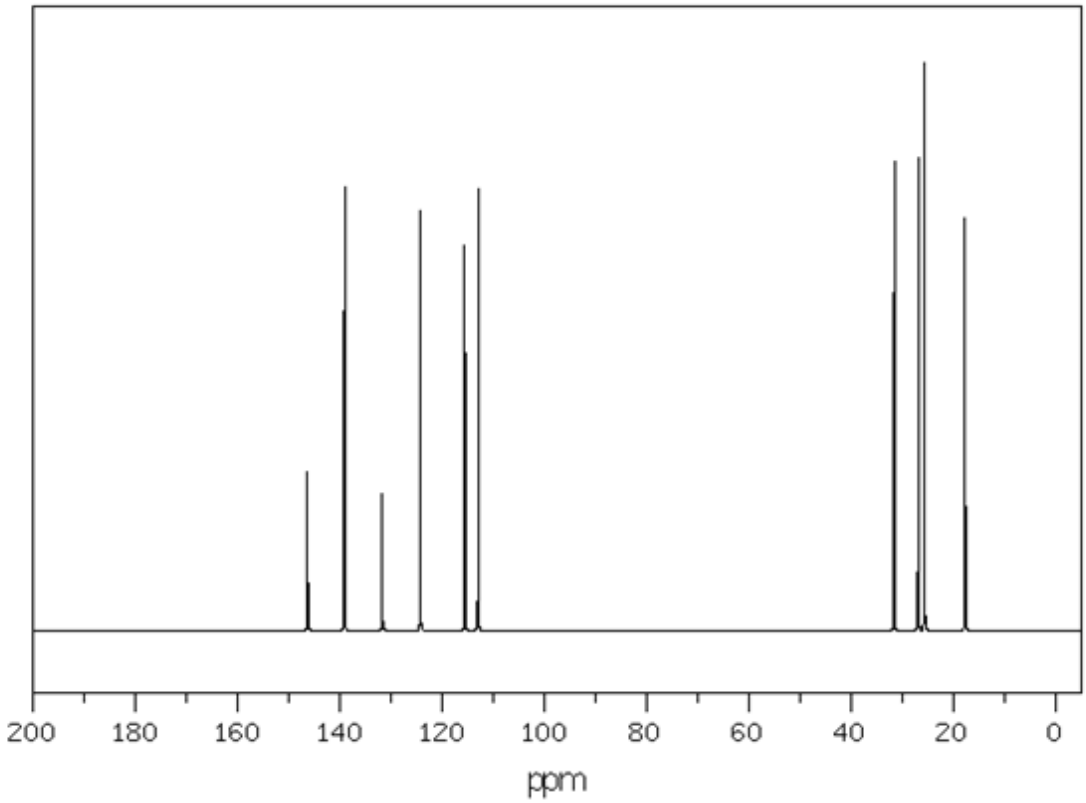
ppm	Int.	Assign.
139.07	617	1
131.62	612	2
124.07	1000	3
123.71	995	4
59.16	791	5
39.64	857	6
26.51	724	7
25.66	673	8
17.66	531	9
16.24	628	10

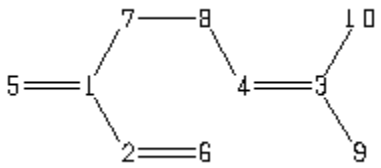
Figure 13: ^{13}C NMR 25.16 MHz C_{10} H_{14} O 0.5 ml : 1.5 ml $CDCl_3$ **Carvacrol**



ppm	Int.	Assign.
153.52	458	1
148.42	398	2
130.92	687	3
121.21	453	4
118.90	652	5
113.23	672	6
33.68	537	7
23.95	1000	8
15.35	517	9

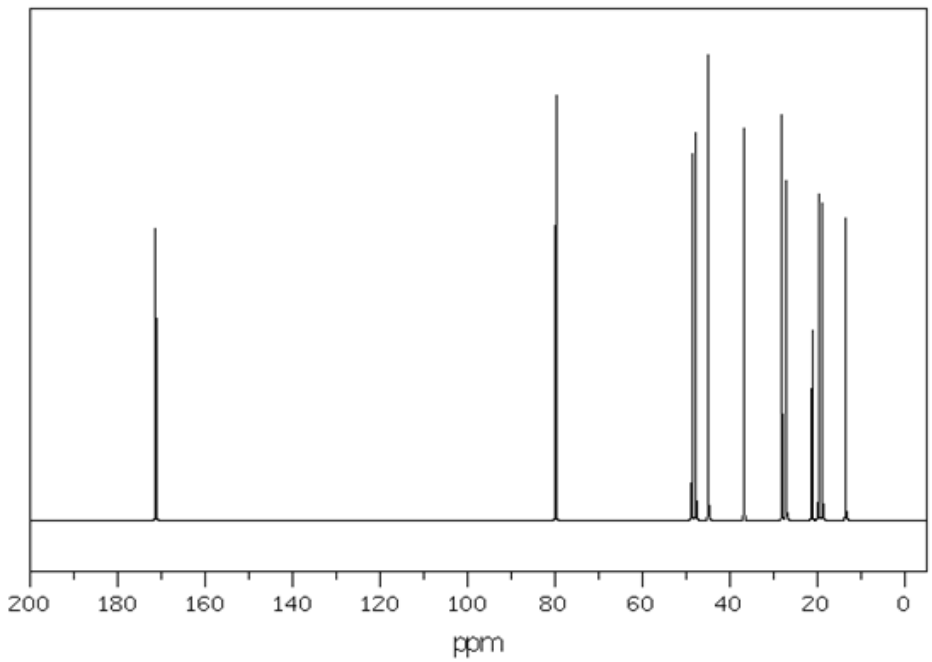
Figure 14: ^{13}C NMR 22.53 MHz C_{10} H_{16} 0.05 ml: 0.5 ml $CDCl_3$ **7-methyl-3-methylene-1, 6-octadiene (myrcene)**

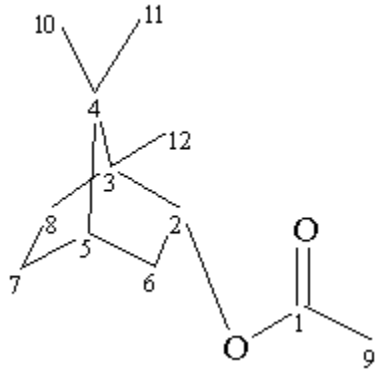




ppm	Int.	Assign.
146.28	277	1
139.10	779	2
131.69	241	3
124.27	739	4
115.55	676	5
112.96	776	6
31.59	825	7
26.89	832	8
25.67	1000	9
17.69	724	10

Figure 15: ^{13}C NMR 25.16 MHz C_{12} H_{20} O_2 0.5 ml : 1.5 ml $CDCl_3$ **Endo-bornyl acetate**





ppm	Int.	Assign.
171.26	625	1
79.80	913	2
48.69	788	3
47.77	832	4
44.92	1000	5
36.77	842	6
28.05	870	7
27.09	728	8
21.22	408	9
19.72	701	10
18.83	679	11
13.49	647	12

CONCLUSIONS

This study has shown that a correlation exists between the yield of *Calendula officinalis* essential oil and the age of the plant and that the yield is best during the flowering stage of the plant. The relative abundance of the chemical constituents of its essential oil at this stage is a veritable indicator of the appropriate period for collection and harvesting of the plant for the isolation of the desired mono- and sesquiterpenes.

The results of this study have reinforced the fact that there are quantitative and qualitative differences in the essential oil components of the same plant that may be growing in different parts of the world or of the fresh and dry plant materials. Finally, we were able to isolate and elucidate 1, 8-cineole and some other key components of essential oils from the plant. The dynamics of oxidation of the components like α-thujene to the components like 1,8-cineole can be envisaged and will be the topic of our further research in botanical and chemical laboratories.

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APPENDIX

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