EXTRACTION OF ESSENTIAL OILS, AND ACTIVE COMPOUNDS FROM *MATRICARIA CHAMOMILLA L.* AND THEIR APPLICATION IN TOILET SOAP

BY

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DECLARATION

This thesis is my original work and has not been presented for a degree in any University.

P. K. CHEPLOGOI — Date 14/11/97

This thesis has been submitted for examination with our approval as University supervisors.

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DEDICATION

This thesis is dedicated to Kabargasau family for their efforts to educate me.

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SCHEMES

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NOTATION

$[a]^t_D$ - Specific rotation at temperature t in °C for the sodium line D

n^{20}_D - Refractive index at 20°C for the sodium line

ft - foot or feet

lb - pound

w/w - weight/weight

v/v - volume/volume

M Molecular weight

d_{20} - density at 20°C

Sp. gr - Specific gravity

atm - atmosphere

mg - milligram

in - inch

mm Hg - millimetre of mercury

nm - nanometres

*u*m - micrometers

U.V - ultra violet

lR - Infrared

b.p - boiling point at 760 mm Hg

tlc - thin layer chromatography

b_{12} - boiling point at a reduced pressure of 12 mm Hg

gri48 - griseofulvin introduced 48 hours after inoculation

cham48 - chamomile oil introduced 48 hours after inoculation

ABSTRACT

Essential oil was extracted from *Matricaria Chamomilla L.* Various compounds present in the essential oil were identified using thin layer and gas chromatographic techniques and then isolated into pure components using column chromatographic technique. Compounds present in the essential oil were confirmed by various spectrophotometric methods. IR and NMR spectra confirms the structures of four natural occurring compounds. The major and stable components isolated were farnesene, chamazulene, (-)-*alpha*-bisabolol and bisabololoxides.

The antifungal activity *in vitro* of the isolated compounds of the chamomile oil, crude chamomile oil, aqueous flower extracts and toilet soap impregnated with various concentrations of crude chamomile oil and bisabololoxides were tested against *Trichophyton mentagrophytes* and compared with the antifungal activity of griseofulvin, the most effective drug for the treatment of fungal diseases. The toilet soap used was prepared from 80% tallow, 20% coconut oil and small amounts of borax. The diameter of inhibition zone was used as a measure of the antifungal activity. Crude chamomile oil, farnesene, (-) -*alpha*-bisabolol and bisabololoxides were active and so were crude chamomile oil and bisabololoxides incorporated in soap. Chamazulene and aqueous

flower extracts were not active. After three days, farnesene, (-) - *alpha* - bisabolol, bisabololoxides and crude chamomile oil had 18.50 mm, 29.50 mm, 40.00 mm and 36.50 mm inhibition zone, respectively, while griseofulvin had 37.50 mm.

After two months of the observation, it was found that the inhibition zone for each of the tested components were 10.00 mm, 16.00 mm, 25.50 mm and 17.50 mm, respectively, while griseofulvin was completely inactive. Similar trend was observed for toilet soap prepared using various concentrations of crude chamomile oil and bisabololoxides. The active compounds of chamomile oil were found to have a better persistence than griseofulvin. Bisabololoxides had a better antifungal activity than griseofulvin. Chamazulene and its precursor from aqueous flower extracts showed inactivity against the fungus. The presence of chamomile oil and its components imparts some fragrance in the soap as well as good antiseptic properties.

CHAPTER 1

INTRODUCTION

1.0 General introduction .

A survey of the Literature on medicinal plants indicates that the relationship between these plants and man has been very close throughout the development of human culture. Medicinal plants are generally considered as those species used for medical care - be it in traditional or modern medicine (Mungaruline, 1990).

The Compositae or Asteraceae is one of the largest and most familiar of flowering plants and has been a subject of research for centuries. The family has attracted, fascinated and even repelled botanists for over two centuries. Compositae, in spite of its relatively uniform capitular and floral features, occupies a wide range of habitat types and is found in abundance on every continent except Antarctica. The family is most abundant, as to diversity in montane subtropical or tropical latitudes, this diversity being most evident in mountaineous areas that border semi-arid or desert region (Heywood et al., 1977).

The biological and therapeutical application of plants of the compositae with nearly 100 genera and about 15,000 species is more the result of systematically conducted chemical and pharmacological research than of tradition. In addition, the compositae has

attracted many chemists and biochemists and substantial research has been built up over the past four decades on the chemical constituents of individual species and genera. A study of the early herbals reveals that a surprisingly large number of plants of the compositae were used for their curative properties. Undoubtedly, the wide medicinal use of many compositae inspired the organic chemists at the turn of the century to explore the chemistry in order to identify the active constituents. Compositae in fact are exceptionally rich, both in the range of secondary compounds present and also in the number of complex structures known of any one class (Heywood et al., 1977).

Many of the substances elaborated by the family are toxic or show other significant physiological activity and this may be one reason why plants of the compositae are rarely used in human diets or for animal fodder. Several classes of plant compound are characteristic of this family notably the terpenoid - based sesquiterpene lactones, the fatty acid derived polyacetylenes and the polysaccharide fructans. Many structures discovered for first time in this family have served as models for the synthesis of biologically active compounds and have promoted research into the activity of analogous structures. New screening methods and isolation techniques have made it possible to elucidate the mode of action of old drugs and thereby reintroduce them into modern therapy (Heywood et al.,1977; Unesco, 1986).

There is a direct connection between the physico-chemical properties of the predominantly lipophilic compounds of the compositae and the biological and pharmacological activity. In addition to drugs known since antiquity from plants such as

chamomilla, cynara and silybum, there are over 25 species in the family which have found therapeutic application due to their antihepatotoxic, choleretic, spasmolytic, anthelmintic, antiphlogistic, antibiotic or antimicrobial activity. In food technology, some drugs containing bitter principles have achieved industrial significance. The insecticides pyrethrin from pyrethrum species exemplify how plants products serve as models for the developments of more active synthetic agents. Research on medicinal plants needs to be intensified in a multidisplinary approach which should embrace all stages leading finally to the production of formulated drugs. This would provide the stage for the growth of pharmaceutical and cosmetic industries based on pharmacopoae (Heywood et al., 1977; Unesco, 1986).

Matricaria chamomilla L. (fam. compositae), the so called German, Hungarian or small chamomile is a fragrant, low annual herb, with ligulate flower heads about 2 cm broad; up to about 0.6 m high; possesses a branching stem 1 or 2 ft. high, bearing very green smooth and alternate leaves; the inflorescence consists of ray florets. The flower heads appear singly at the ends of stems and branches. The flowers are smaller than those of *Anthemis nobillis L.*, the so called Roman (English) chamomile.

The plant thrives in moderately heavy soil, rich in humus and rather moist. It can withstand considerable cold. *Matricaria chamomilla L.* grows also on farmland and in grain fields around houses, along roads and elsewhere. Seed may be sown in drills and barely covered, or it may be broadcast, since the plants soon occupy the entire ground and exclude weeds (Heywood et al., 1977; Gasic et al., 1982; Leung, 1980).

Chamomile blooms from the end of April to the end of May about eight weeks after the seed is sown. A crop may thus be raised from seed sown early in the spring *Matricaria chamomilla L.* grown on farmland is hand picked which permits removal of the flowers without the stem. However, in large scale farms, it must be collected by means of flower scoops or strippers. When using these implements, the harvesters must gather the flowers as carefully and with as little stem material and extraneous matter as possible. A single worker with a "flower comb" can collect from 60 to 100 Kg of fresh flowers per day while by hand he can gather only 8 to 10 Kg. The harvested flowers are sifted in a suspended sieve (mesh diameter 7-11 mm) to separate the flower heads from the flowers with attached stems and from clinging bits of weed or grass. The flowers are spread out on the floor or on sheets in thin layers to dry. Artificial dryers may also be employed. Five Kilograms of fresh flowers give 1 Kg of air-dried flowers (Heywood et al., 1977; Gasic et al., 1982; Poucher, 1974a).

Objectives of the research work

(1) Extraction of essential oil from *Matricaria chamomilla L.*,

(2) isolation and characterization of chemical constituents of the oil

(3) study of the effect of chamomile oil compounds in the making of toilet soap and

(4) screening of the antifungal activities of the isolated compounds and the soap impregnated with the isolated compounds.

CHAPTER 2

LITERATURE REVIEW

2.0 Essential oil from *Matricaria chamomilla L.*

The highest content of essential oil and azulenic substances was found in normally ripe flower heads (Akacic and Kustrak, 1960). The essential oil content of chamomile flowers was 0.24 - 1.9%.(Heywood et al., 1977).

Active components of *M. chamomilla L.* is composed of lipophilic and hydrophilic nature. The lipophilic components are the essential oil of the *M. chamomilla L.* The oil derived from plant is a deep blue liquid of strong and characteristic odour and bitter aromatic flavor. Depending upon the temperature it is more or less viscous and under the influence of light and air the deep colour of the oil gradually changes to green and finally brown (Leung,1980).

Chamomile is found in different chemotypes. The content of biologically active components depended mainly on genetic factors while ecological factors had only slight influence (Suab,1972). The optimum harvest time for chamomile H-29 variety was established to be that time when 50% of the flowers were open. When 50% of the flowers were open the plants are normally expected to have reached their maximum content of essential oil. Maximum drying temperature was 50°C (Ottol,1974). The content of

essential oil decreases during storage of flowers. It is conceivable that in general the higher the light intensity the higher the essential oil. The increase due to high light intensity can be attributed to the increase in photosynthetic rate as light energy increases while respiration rate was not affected.

Owing to increase in the vegetative growth (the photosynthetic capacity of the plants) the dry weight of the flower heads was found to increase with age of the plant up to a maximum and then decreased (Saleh, 1974). Frozen flower heads could be stored without loss of volatile substance. In contrast dried chamomile flowers lost 70% of their matricin content within 2 years even when stored under controlled conditions. Furthermore, freezing the chamomile flowers resulted in higher yield of essential oil and its compounds than any of the various drying procedures tested (Carle et al.,).

2.1 Extraction, analysis and isolations of chamomile oil and its compounds

Chemical literature contains numerous references about investigations of the various methods of assaying the volatile oil contents of chamomile flowers and of the yield of oil obtained from different qualities of flower material. Numerous methods of assaying the contents of essential oil in plant material have been suggested. The literature offers many modifications of these methods all of which aim at a quantitative yield of the oil (Guenther, 1948).

Several methods have been studied and the use of Clevenger apparatus was most

effective (Kaiser and Lang, 1981). Clevenger apparatus offers the following advantages: compactness, cohobation of distillation and separation of the essential oil (so that certain chemical and physical properties may be determined and so that the odour and flavour of the oil may be studied.) and an accurate determination of the essential oil content using only small quantities of plant material. Clevenger apparatus permits assaying quantitatively the content of essential oil in a small amount (50 to 500 g) of plant material. Furthermore this apparatus may be used to advantage for steam rectification of small amounts of essential oils (Guenther, 1948).

The active ingredients were extracted undecomposed and in good yield from chamomile using super critical gases (especially CO_2 and N_2O) at 72-500 bar and 31-80°C. The percentage of the active ingredients in extracts thus obtained was higher than extracts obtained by conventional solvents. The composition of the extracts would be varied by stepwise extraction and separation (Stahl et al., 1978).

Steam distillation method was also used to obtain oil in chamomile flowers. Yields of the oil obtained by these method from different qualities of flower materials were varied from 0.39-10.5% (Guenther, 1952).

Chamomile oils, despite literature reports to the contrary, do not assume buttery consistency on cooling, and do not congeal at 0°C to a solid mass. The chamomile oil were only viscous even at -20°C; none solidified, none hardened to even a butter-like consistency at this temperature. However, a sample of pure German chamomile oil congealed to a solid mass when cooled to 0°C. The oils normally are too dark in colour to

permit the determination of optical rotation or refractive index (Heywood et al.,1977).

Chamomile oil flower material of German origin exhibited the following properties (Guenther, 1952).

specific gravity at 15°C .....0.9326 to 0.9459

Acid number .....18.7 to 31.7

Ester number ..... 1.9 to 12.1

Ester number after acetylation ..... 66.3 to 115.7

Solubility .....Soluble in 90% alcohol with separation of paraffin.

The boiling point of various fractions are (Guenther, 1952).

b.p. 105 - 180°C ..... 4.5 percent of a slightly bluish oil with a strong odour

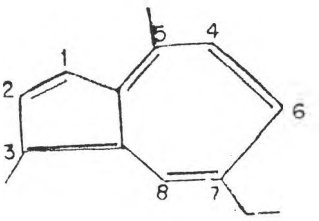
b.p. 180 - 255°C ..... 8.3 percent of oil;

b.p. 255 - 295°C ..... 42 percent of oil, with development of a blue vapour.

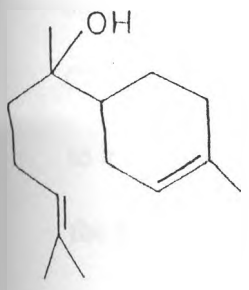
b.p. Above 295°C ..... 25 percent of a very viscous oil, with development of a violet colour;

Residue ..... 20 percent of a brown mass.

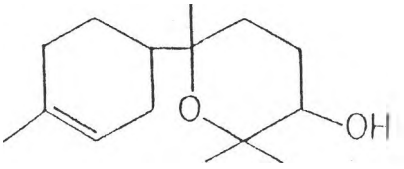
The volatile oil contains chamazulene (1), (-)- *alpha*-bisabolol (2), bisabolol oxide-A (3), bisabolol oxide-B (4), *cis*-spiroether (5) (en-in-dicycloether) and farnesene (6) as the major components with their relative concentration varying considerably depending on sources (Gasic et al., 1982).



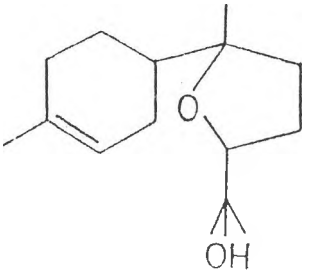
Chamazulene (1)



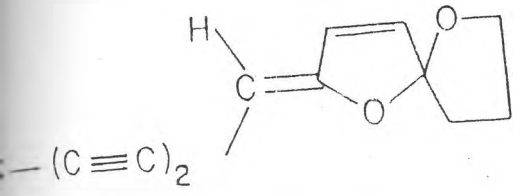
alpha-Bisabolol (2)



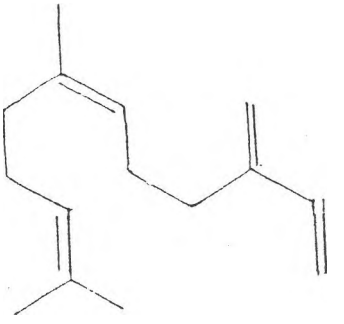
Bisabolol oxide-A (3)



Bisabolol oxide-B (4)



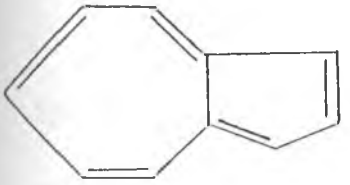
(*cis*-Spiroether 5)



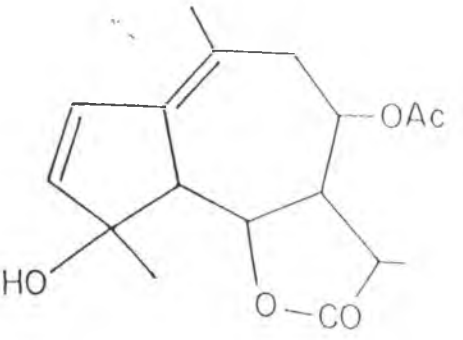
Farnesene (6)

For a long time the only known active principle in the chamomile oil was azulene (7). The name azulene was given to the parent compound of the azulene series $C_{10}H_8$ (Maxwell and Gordon, 1952). Azulene from *M. chamomilla L.* has been named chamazulene (camazulene) (4) in order to differentiate it from the azulenes contained in other essential oils (Guenther, 1952). One of the most striking properties of the azulenes is their intense blue or blue-violet colour, noticeable even at very high dilution. Azulenes are decomposed by permanganate, even at low temperature, to small fragments and therefore there is no six membered aromatic ring in azulenes (Maxwell and Gordon, 1952). Chamazulene (1,4-dimethyl-7-ethylazulene) is of intensive characteristic blue colour owing to its conjugation system of five double bonds. Chamazulene is a bicyclic hydrocarbon of the formula $C_{14}H_{16}$, $b_{11}159$, $d^{18}{}_4$ 0.9881 and M 184 (Heeger et al., 1946).

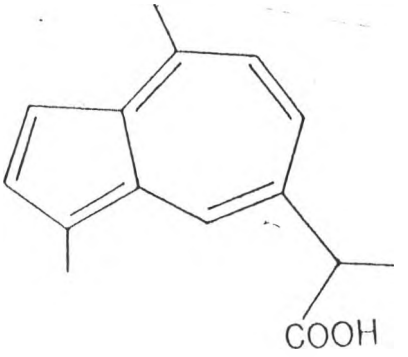
The plant itself does not seem to contain the azulene in free form. Normal preparation of chamomile, infusion and decoction made with water, contains practically no chamazulene but a precursor of it known as matricin (prochamazulene) (2). Chamazulene is produced from matricin during steam distillation via the equally unstable chamazulene carboxylic acid (3), $C_{15}H_{16}O_2$ (Ottol, 1974; Guenther, 1952). The formation of chamazulene from prochamazulene proceeds satisfactorily (in 70-75 % yield) by steam distillation from faintly acid solution (Cekan et al., 1954).



Azulene (7)



Matricin (8)



Chamazulene carboxylic acid (9)

The prochamazulene forms colourless well - developed platelets (from ethanol-isopropyl ether) which melts at 158-160°C. The content of matricin decreases during storage. Infusion with H_2O contains only 0.2 to 0.6 mg per 100 c c and even soon decomposes on storage. The same was found with most other solvents (Koch, 1942).

Chamazulene-high and chamazulene-low varieties were found but no chamazulene free chamomile was found (Kustrak et al., 1977). However, comparing the chemical composition of flowers of wild and domesticated *M. chamomilla L.*, the wild samples to differed from the domesticated samples only by the absence of prochamazulene (Tauker et al., 1977).

All azulenes have the same chromophore and absorp at 580 nm. Spectrophotometry is, therefore, suitable to be used in the determination of total azulenes content when applied to an extract previously purified by preparative thin layer chromatography. Moreover, the absorbance/concentration relationship was shown to be linear thus allowing the use of the absorptivity value. This one is easily calculated on the basis of a standard solution of chamazulene. The measure of azulene, as chamazulene is justified because it is by far the main azulene present in chamomile oil. (Padula et al., 1976).

(-)-*alpha*-Bisabolol is an unsaturated monocyclic sesquiterpene alcohol. NMR and gas chromatographic comparison of (-)-*alpha* -bisabolol isolated from chamomile oil with two synthetic diastereoisomers showed that the natural material possesses the 6S, 7S stereochemistry in contrast to a previously reported assingnment of 6S,7R stereochemistry. Laevorotatory form of (-)-*alpha*-bisabolol exists in chamomile oil (Swanson and Schwartz, 1979).

A sesquiterpene alcohol-oxide (bisabolol oxide-A) $C_{15}H_{26}O_2$ has been isolated from *Matricaria chamomilla L.* oil of Indian origin. A structure has been assigned to

bisabolol oxide-A on the bases of chemical studies and spectroscopic data. More recently three isomeric bisabolol oxides were separated using thin layer chromatography. Bisabolol oxide-A (liquid isomer) has been isolated from the solvent extracted out of *M. chamomilla L.* of Indian origin (Sampath et al,. 1969a)

Bisabolol oxide-B, isolated from *M. chamomilla L.* has shown its relation with (-)-*alpha*-bisabolol. The structure of bisabolol oxide B is reported by its mass spectral fragmentation pattern (Sampath et al,. 1969b) . *cis*-Spiroether (en-in-dicycloether) is a very unstable compound. Dicycloethers are present in the receptacle of chamomile flowers. The *cis* form of spiroether occurs in chamomile flowers though *trans*-spiroether is mostly present in surbodinate concentration (Vane and Ferreira, 1979; Murray, 1969)

Farnesene, consists of *alpha*-isomers and *beta*-isomers. *beta*-Farnesene has been isolated from the oil of *Matricaria chamomilla L.*

2.2 Biological activities

German chamomile has been reported to have numerous pharmacological properties some of which are the following: The oil has bactericidal and fungicidal activities, particularly against gram-positive bacteria (e.g., *Staphyloccus aureus*) and *Candida albicans*. It also reduced blood urea concentration in rabbits to a normal level. It has been reported that the chamomile oil causes contact dermatitis in humans Leung, 1980).

Aqueous and alcoholic extracts have been used since antiquity, internally and externally for anti-inflammatory activity (Heywood et al., 1977). Available data indicate that chamomile oil are generally non toxic when applied externally (Leung, 1980).

Chamazulene, one of the major component of the oil, has pain-relieving, wound-healing, antispasmodic, antiinflammatory antimicrobial properties (Leung, 1980). Antiphilogistic activity of prochamazulene has been determined and found to be at least equal to that of chamazulene (Cekan et al., 1954)). Chamazulene when tested on dextran-induced rat paw oedema showed the highest antiinflammatory activity (Verzar et al., 1979). Chamazulene show only slight activity and ineffective against tetanus toxin (Mose and Luka, 1957). Chamazulene is a special histamine-releasing substance. Chamazulene or the blue oil of chamomile does not have an antiinflammatory effect, but it enhances sluggish inflammatory reaction and make them more intensive (Vane and ferreira, 1979).

(-)-*alpha*-Bisabolol, another constituent of the oil, has antiinflammatory, antimicrobial and antipeptic activities (Leung, 1980). Laevorotatory form which is found in chamomile has more effective antiphlogistic and spasmolytic effect then the racemate (dextrarotatory form). The chemistry of bisabolol has been fruitfully extended by the partial synthesis of bisabolol ethers and esters. Most of these synthetic compounds are more active and usually have lower toxicity than natural (-)-*alpha*-bisabolol (Heywood, 1977). Bisabolol exhibited varying degrees of fungistatic activity. It has significant effects at only 100 mg/ml and is fungicidal to *Candida albicans* following a 30 minutes exposure of the yeast to a 1000 mg/ml concentration (Szalontai et al., 1977).

(-)-*alpha*-Bisabolol promotes epithelization and granulation. (-)-*alpha*-Bisabolol inhibited the ulcer formation induced by indomethacin alcohol or stress in rats and increased the rate of healing of ulcers caused by acetic acid or heat cauterisation of the stomach. A composition for the treatment of acne comprises (-)-*alpha*-bisabolol an only substance from volatile oil (Unesco, 1986; Saeki et al., 1988). Bisabolol is antiphlogistically stronger than guaiazulene on carrageenin-oedema of a rat's paw. The acute toxicity of (-)-*alpha*-bisabolol is very low in mice and rats after oral administration. Side effects occurs at high doses only (Unesco, 1986). (-)-*alpha*-Bisabolol when tested on dextran-induced rat paw oedema showed the highest antiinflammatory activity (Verzar et al., 1979).

The cyclic ether also have antimicrobial, antiinflammatory, antianaphylactic and antispasmodic properties (Leung, 1980). It has an effect on the generalized dextran oedema in rats. Dextran causes an anaphylactic reaction on rats. Oedema are formed on paws and snouts but their appearance may be suppressed by a retreatment with spiroethers. Spasmolytic effect is superior to papaverine which can be observed on the intestine of guinea pigs and rabbits (Unesco, 1986). Apparently only the cis -isomer exerts a spasmolytic action, which is at least ten times higher than papaverine (Murray, 1969). Spiroether has a better antiphlogistic activity than chamazulene (Heywood et al., 1977). Cyclic ethers had significant effects at only 100 mg/ml (Szalontai et al., 1977).

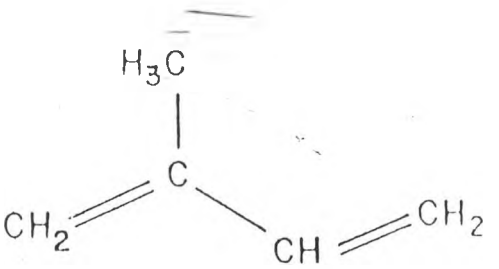
Bisabolol oxides A and B show a papaverine-like spasmolytic action. (-)-*alpha*-Bisabolol had an activity of the same intensity as papaverine and was twice as potent as

the A and B bisabolol oxides (Unesco, 1986). When tested on dextran-induced rat paw oedema bisabolol oxides showed the highest antiinflammatory activity (Verzar et al , 1979).

2.3 Biosynthesis of chamomile oil compounds

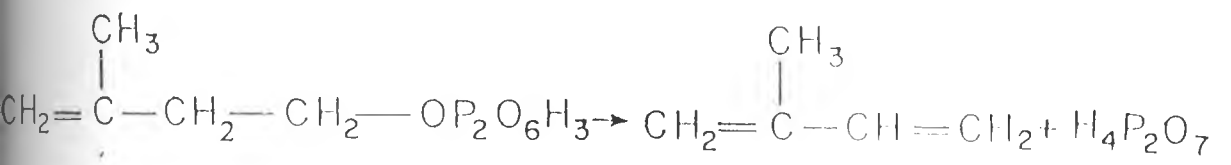
The volatile compounds from flowers of *Matricaria chamomilla L.* are essential oils which should not be confused with other well known oils including vegetable, fatty and petroleum based oils. They are composed mainly of terpenes both aliphatic and cyclic together with some oxygenated terpenoids and aromatic and heterocyclic compounds. The odour of essential oil is due to the presence of terpenes C_{10} and C_{15} compounds (Roberts, 1965).

Terpenes are derived from isoprene (10) and each molecule is built up in multiples of isoprene units(10). These isoprene units(10). are usually joined head to tail, the resulting compound having varying degrees of saturation (Roberts, 1965)



Isoprene (10)

The designation "terpene" is actually reserved for the C_{10} compounds, the C_{15} compounds being known as sesquiterpenes, the C_{20} as diterpenes, C_{30} as triterpenes. C_{10} and C_{15} compounds which are important components of essential oils, are in reality members of a much larger class of substances with carbon skeleton made up of isoprene units and occurring in both plants and animals. The so called "isoprene rule" which correlates the structures of these substances, speaks for their synthesis in living systems from some common precursor with five carbon atoms It is interesting to note however, that isoprene does not occur naturally. The actual five carbon intermediate of terpene biosynthesis appears to be isopentenyl pyrophosphate(11) Elimination of pyrophosphoric acid would lead to isoprene (10) (Roberts, 1965).

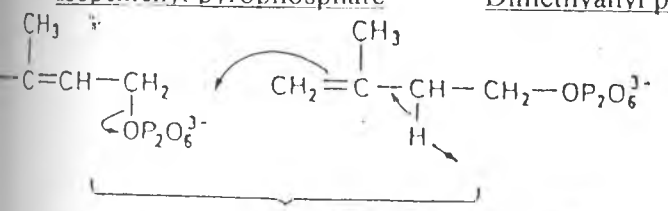


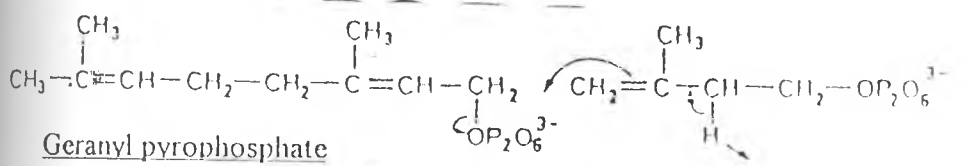
Isopentenyl pyrophosphate (11) Isoprene (10)

Scheme 2.3.1 Formation of isoprene

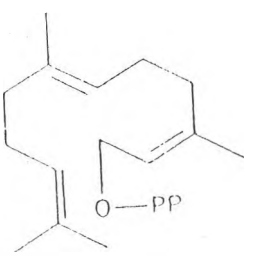
The synthesis of terpenes takes place by the condensation of several molecules of isopentenyl pyrophosphate. It is common to refer to all members of these group as isoprenoid compounds. Isoprenoid compounds are biogenetically related. The Scheme 2.3.2 shows the synthesis of isoprenoid involving isopentenyl pyrophosphate. The synthesis of the terpenoid carbon chains involves C-alkylation. This is made possible by the bifunctional nature of isopentenyl pyrophosphate, based on the nucleophilic reactivity of the double bond and the potential electrophilic character of the pyrophosphate ester. The process is initiated by the isomerization of isopentenyl pyrophosphate to *gama*-dimethylallyl pyrophosphate by a shift of the double bond. With the formation of dimethylallyl pyrophosphate the electrophilic reactivity of the precursor isopentenyl pyrophosphate is fully realized. Ionization of the carbon-oxygen bond of the dimethyallyl pyrophosphate creates a cationic centre which then attacks the electrons available in the exomethylene group of isopentenyl pyrophosphate. Subsequently, the elimination of a proton leads to the first condensation product, geranyl pyrophosphate. This homologous allyl pyrophosphate by acquisition of another isopentenyl pyrophosphate is converted into farnesyl pyrophosphate, the direct precursor of the sesquiterpenes (ISCNP, 1966).

Isopentenyl pyrophosphate Dimethyallyl pyrophosphate





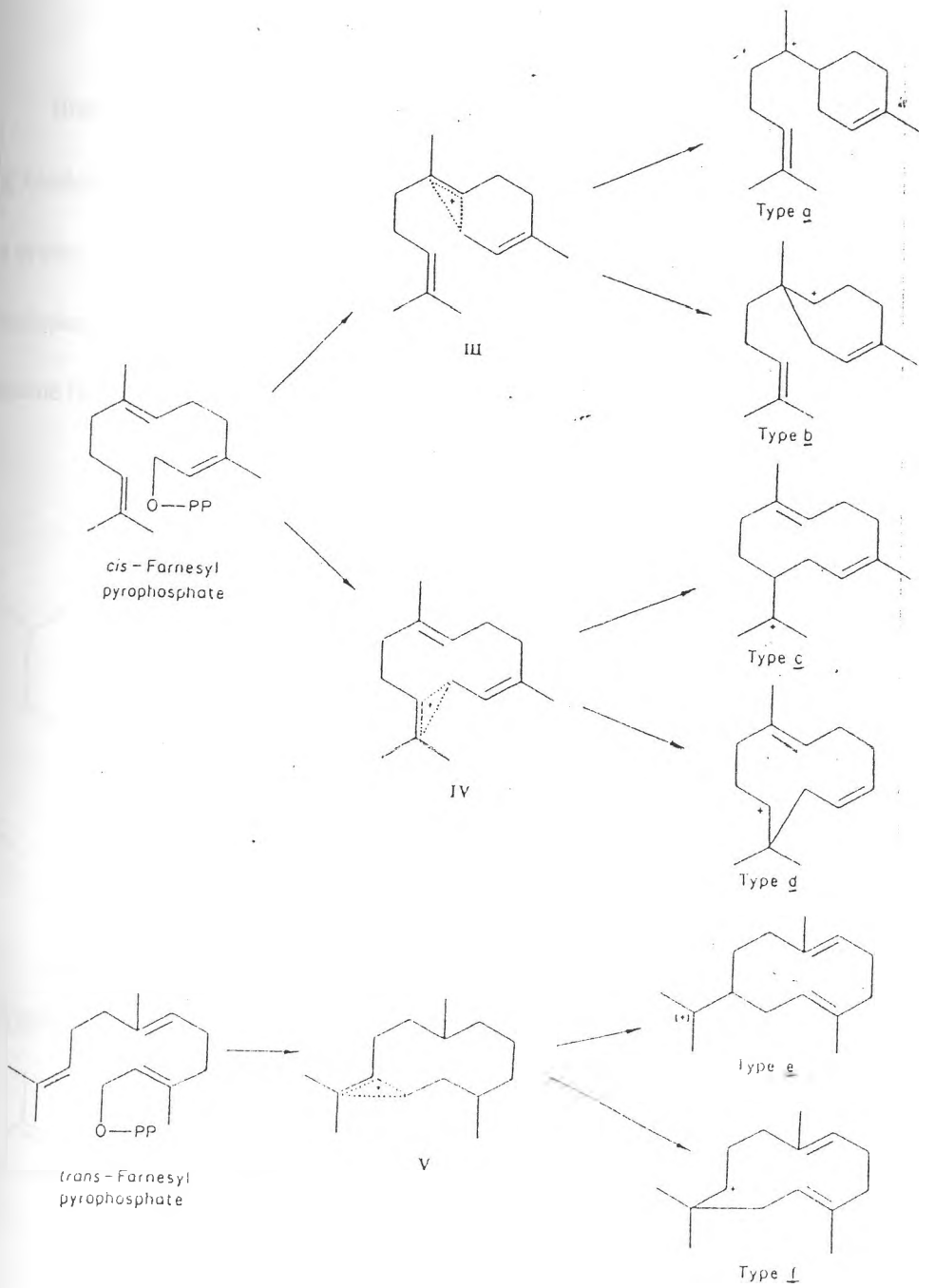
Geranyl pyrophosphate



Farnesyl pyrophosphate

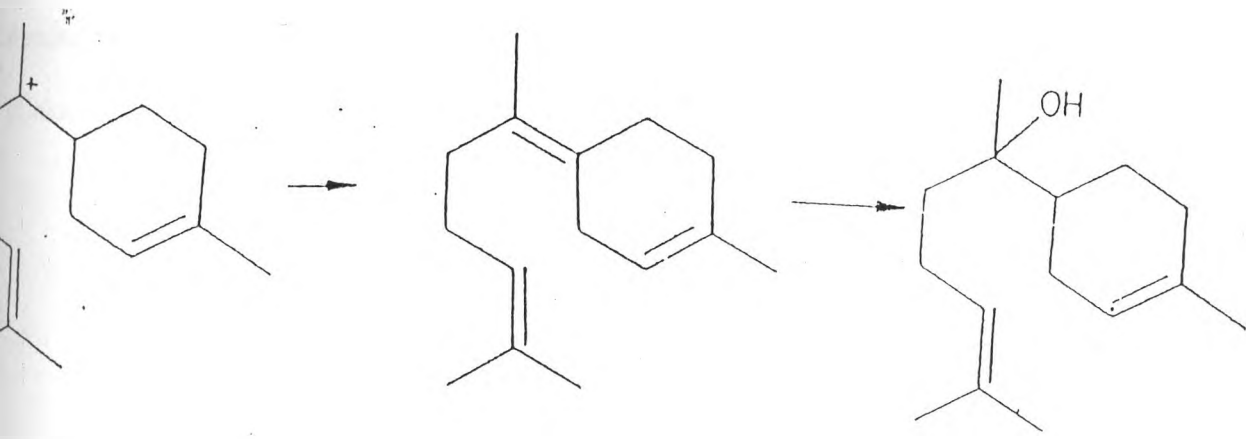
Scheme 2.3.2 The mechanism of the synthesis of isoprenoid carbon chains

The sesquiterpenes originate from farnesyl pyrophosphate. The ring systems most frequently found may be directly formed after elimination of the pyrophosphate group via the unstable cations III-V shown in the scheme 2.3.2. The positively charged intermediate products of the type a-f may stabilize by elimination of a proton or by reaction with another compound.

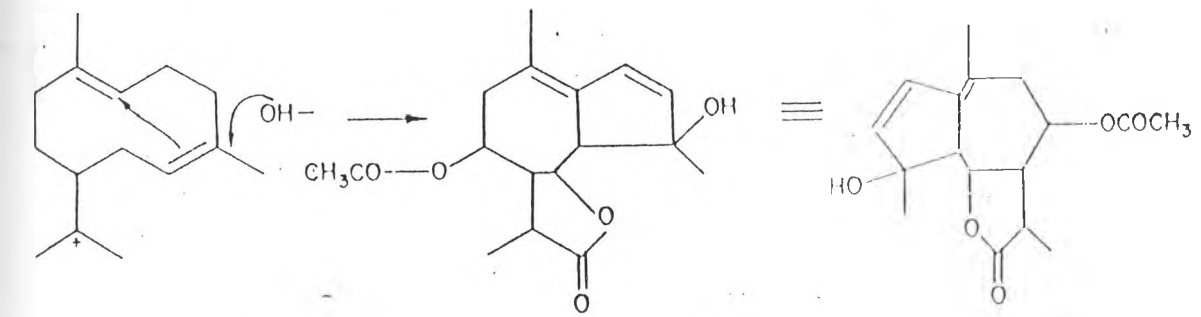


Schemes 2.3.3 Possible ways for the formation of different types of cyclic sesquiterpenes from farnesyl pyrophosphate.

Bisabolene, whose ring system is the basis of a great number of sesquiterpenes (e.g. bisabolol), may be formed from the condensation product of the type a by elimination of a proton. From type c, guajanolides (e.g matricin), which are important as precursors of azulenes, may be synthesized. Scheme 2.3.4 is a possible way for the formation of matricine (Luckner, 1972).



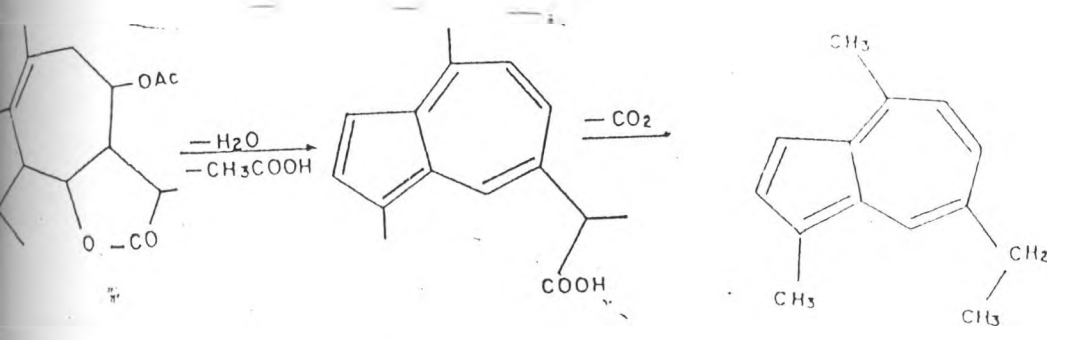
Condensation product of type a Bisabolene Bisabolol



Condensation product of type c Matricin

Scheme 2.3.4 possible ways for the formation of some sesquiterpenes from chamomile oil.

The blue compound (chamazulene) is closely related to sesquiterpenes but has some aromatic properties. The formation of chamazulene may be explained as involving the elimination of three hydroxyl groups and subsequent decarboxylation. One mole of carbon dioxide is formed. The formation of chamazulene is found to proceed most satisfactorily in faintly acid solution. In neutral media pro-chamazulene is relatively stable and does not decompose even on prolonged heating. In faintly acid solution decomposition begins at 50° to 60°C as indicated by the appearance of a blue emulsion (Cekan et al., 1954).

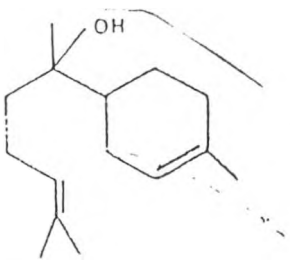


Matricin chamazulene carboxylic acid Chamazulene

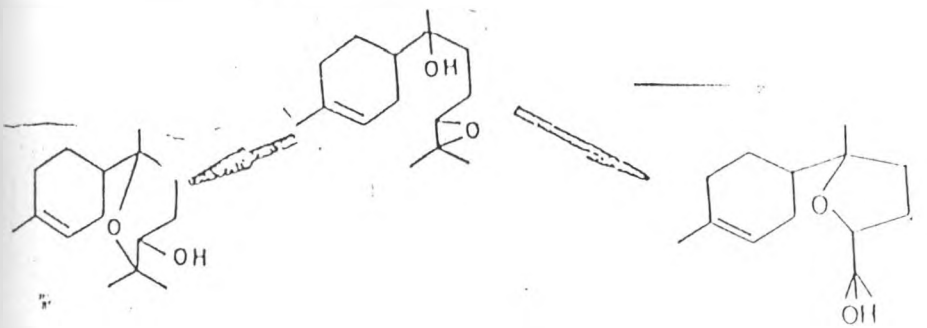
Scheme 2.3.5 Formation of chamazulene from matricine

(-)-*alpha*-Bisabolol is an unsaturated monocyclic sesquiterpene alcohol. The biogenetic formation of (-)-*alpha*-bisabolol the condensation product of type a via bisabolene.

The biogenetic formations of bisabolol oxides seems to be accomplished by first the formation of double linkage on the side chain of the bisabolol molecule epoxized, then ring closure. Such cyclisation are also known with other natural substances (Ottol, 1974; Bohlmann et al., 1961). It will be postulated that the step from (-)-*alpha*-bisabolol to the bisabolol oxide A, and B takes place either during the pathway of the biomembranes in the cytoplasma or in the excretory glandular trichomes. Probably both biosynthetic routes are possible *in vivo* (Schilcher, 1977). Bisabololoxide A, and B are sesquiterpene alcohol-oxides.



(-)- *alpha*-Bisabolol

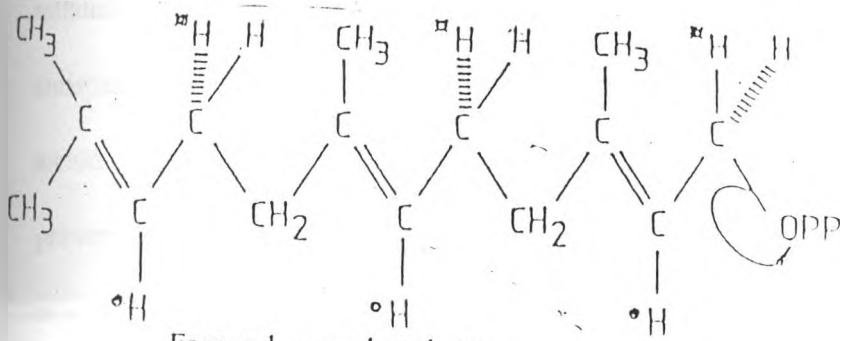


Bisabolol oxide-A

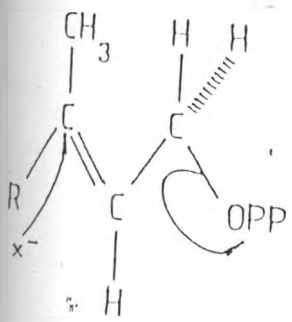
Bisabolol oxide-B

Scheme 2.3.6 Biogenetic formation of bisabololoxides from (-)-*alpha*-bisabolol

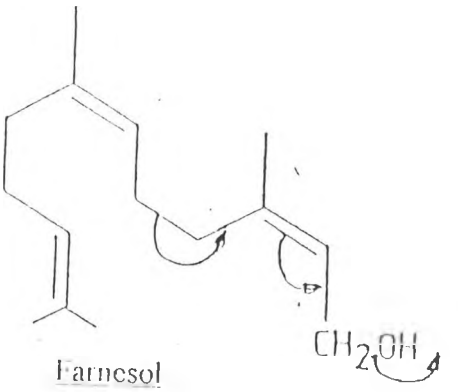
Farnesene is also a sesquiterpene containing 4 double bonds Farnesyl pyrophosphate is converted to the widely occurring alcohol farnesol by hydrolytic elimination of the pyrophosphate group. Farnesene is via an intermediate product which corresponds to cation shown in the scheme 2.3.7



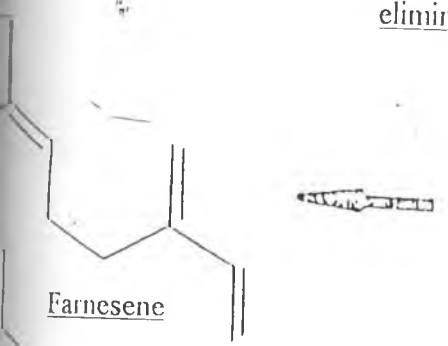
Farnesyl pyrophosphate



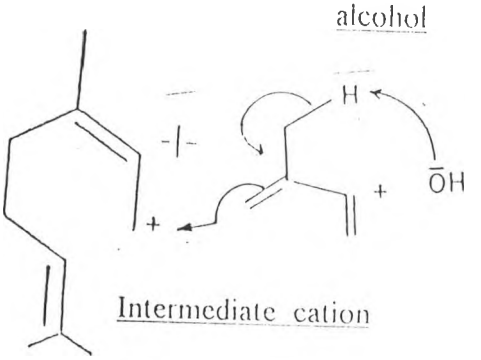
Hydrolytic elimination



Farnesol alcohol



Farnesene



Intermediate cation

Scheme 2.3.7 Formation of farnesene from farnesyl pyrophosphate

2.4 General uses

German chamomile extracts are used in pharmaceutical preparations. They are used in antiseptic ointments, creams, and gels to treat cracked nipples, sore gums and inflammations and for wound healing. The volatile oil are used in carminative, antispasmodic, and tonic preparations, among others. Extracts are also used in cosmetics including bath preparation, hair dye formulae (for blond hair) shampoos, preparations to prevent sun burns, mouth washes and others. The oils are used as fragrance components or active ingredient in soaps, detergents, creams, lotions and perfumes. User levels reported range from a low of 0.0005% in detergent to a maximum of 0.4% in perfumes (Leung, 1980).

Matricaria chamomilla L. has become well-established as a household medicine and is used with the following objectives; convulsions in children; diarrhoea, colic and acidity; hysteria and sleeplessness; the steam from the tea for sore throat; hot compresses for croup and diphtheria; rheumatism, sciatica, gout and lumbago; as a cold or warm compress for inflammation of the eye; with buchu for pains in the bladder region and for colic. In Europe it has been used mainly as a bitter aromatic tea and as a poultice and in the United States of America as a bitter tonic a carminative, a diaphoretic and a counter-irritant. The flower has been used in Europe as an antispasmodic and nerve tonic, for fever particularly typhus, for skin conditions and as an emollient in the bath (Watt and Breyer-Brandwijk, 1962).

The essential oils from *Matricaria chamomilla L.* are used as flavour components in most major food categories, including alcoholic (e.g bitters vermouths, Benedictine liqueurs, etc) and non alcoholic beverages, frozen dairy desserts, candy, baked goods, and gelatins and puddings. Average maximum use levels reported are usually less than 0.002% for the oils (Leung, 1980).

The flowers are used in teas. The tea is prepared by diluting chamomile flowers in hot water. This tea is mainly drunk to make use of its light spasmolytic effect, though it is hard to draw a line between the use of the tea for medicine and for pure enjoyment (Unesco, 1986; Leung, 1980).

2.5 Fungal diseases

Fungi constitute one of the two groups of plants in the Phylum *Thallophyta.* The other group, the algae, are distinguished from the fungi by the presence of chlorophyll in the body, whereas the fungi lack this material and are either saprophytic or parasitic. *Dermatophytes* are a closely related group of fungi which cause specific infections of man and animals by invading only the superficial Keratinized areas of the body such as the skin, hair and nails. Three genera now recognised are: *Microsporum*, *Trichophyton* and *Epidermophyton* (Dubos,1948).

Dermatophytes like bacteria exist only as saprophytes or parasites and being

essentially necrophilic. Anthropophilic species of dermatophytes tend to produce milder and more chronic cutaneous lesions whereas zoophilic fungi which usually infect people from an animal source evoke a more inflammatory skin lesion (Schwartz et al., 1978)..

Trichophyton mentagrophytes parasitizes Keratinized tissues such as horny layer of the epidermis, hair and nails. It exist in either a hyphal form or an arthrosporic form (Tadashi et al.,1993). *Trichophyton mentagrophytes* variety *granulosum* is a zoophilic organism isolated from domestic animals and particularly involves population of wild rodents and other mammals, and is widespread in farming areas throughout the world. Tinea capitis caused by *T. mentagrophytes* variety *granulosum* and variety *asteroides* show prominent inflammation and development of Kerion is common. Scalp lesions usually appear as ill-defined, scaling areas with alopecia. Painful, edematious, and boggy lesions characterise the development of Kerion (Baker, 1977).

Tinea pedis (athlete's foot) is usually caused by *Trichophyton mentagrophytes*. The process begins between or under the toes as a slight maceration and scaling that is invariably pruritic and is usually followed by fissuring and accummulation of hyperkeratotic debris. Acute episodes are marked by increased erythema, vesiculation and extension of the vesiculobullous lesions to the plantar surfaces. Secondary bacterial infection may occur in the fingers or deep-seated button-like vesicles. If the patient tries to treat himself, the infected acute tinea pedis is likely to be further irritated by various antifungal medications. Plantar hyperhidrosis, warm weather and poor bathing habits are contributing factors to acute process (advanced state) (Schwartz et al.,1978). Other

diseases caused by *T. mentagrophytes* are: *Tinea barbae* which affects the bear and coarse body hair, *Tinea corporis* which attack the smooth body skin, *Tinea manuum* responsible for some palm and finger diseases and *Tinea Unguium* (nail invasion) attacks the nails (Campbell and Stewart, 1980).

2.5.1 Treatment of dermatophytosis

One of the difficulties in developing antifungal (antimycotic) drugs is that drugs that are toxic to fungi are also toxic to animals. (Both fungi and animals have eukaryotic cells). There are presently only a few drugs that are established as effective in the treatment of fungal diseases. The skin, unlike most other organs, is available for the application of various medicines directly onto lesions requiring treatment (Mtulia, 1992).

The few drugs that are used for treatment are; griseofulvin, for infaneous fungal disease caused by dermatophytes (dermatomycoses), hystatin for cutaneous and mycoinfaneous yeast infection, and amphotericin B, a highly toxic drug which is nonetheless, the drug of choice for all systemic fungal diseases. Other antimycotic drugs are either of limited effectiveness or are still in the experimental stage. These include miconazole, clotrimazole, primaricin and candicidin (Baker, 1971; Campbell and Stewart, 1980).

Griseofulvin is an antifungal drug (fungistatic) derived from the species *Penicillium griseofulvum*. It is the only drug effective orally against the species of

Epidermophyton, *Microsporum* and *Trichophyton*. Griseofulvin is an effective and convenient but very expensive way of treating superficial fungus infections (Mtulia, 1992; AMA, 1977).

It is most valuable in the treatment of tinea captis where the curative dose for children is one gram four times daily for a total dose of 4 grams. Higher blood levels are obtained if griseofulvin is taken with a fatty meal. The disease caused by these fungi are contagious, and the patient should be advised not to share his hat, comb, brush and towel with others. Griseofulvin is not without side-effects but appears to be especially well tolerated by children. Headache, gastrointestinal distress, and diarrhea are the most complaints but they can be eliminated by lowering the dosage or stopping and then starting the medication again. The cream form should be applied three times daily to lesion of the glabrous skin whereas lotion is preferable in intertriginous infection in order to reduce maceration (Schwartz et al., 1978).

2.6 Manufacture of toilet soap

Soap is an essential cleaning agent helping people to keep themselves and their surrounding clean. Cleanliness helps diseases from spreading. When soap is mixed with water it forms a later which washes out dirt and grease far better than water alone (Vita,1980).

Consumers are faced with large selective choice of both brands and products of

soap available in the market. The initial preference is primarily based upon the visual attraction mainly relating to the package- its concepts, the wrapper or carton design and colour: then the fragrance and finally the soap itself. Apart from the last-named, all of the features play an unequal part in the initial selection, but it is the basic performance of the soap that will finally determine any future repeat purchases. If the soap does not fulfil the original claims, the customer will not purchase it again (Whalley, 1980).

2.6.1 Raw materials

2.6.1.1 Fats and Oils

The essential materials for making soap are fats and/or oils and an alkali which are found throughout the world. Optional additions are perfumes, dyes, abrasives, sudsing agents, etc. In the manufacture of the best quality soap it is necessary to employ the purest, whitest and least odorous fats and oils. The price and quality of the soap stock varies according to the proportion of raw material employed (Poucher, 1977b).

Fats and oils used in soap making come from animal or vegetable sources. Oil derived from another source, such as mineral oil cannot be used. Soap can be made by using only one kind of fat or oil or by mixing animal or vegetable oil. Animal fats are hard fats, and soap which uses only animal fat is hard, tends to be grainy and lathers poorly. Soaps made from only vegetable oils lather well but do not harden properly. A mixture of

the two types of fats brings out the best quality of both (Vita, 1980).

The following oils and fats are those chiefly employed in soap making: Coconut oil, Palm Kernel oil, Babassu oil, Castor oil, Olive oil, Groundnut oil, Tallow and Lard. Other oils and fats that can be used are: cottonseed, maize, soyabean, sunflower, sesame and linseed. The Coconut, Palm Kernel oil and Babassu oil are vegetable oil and contain mainly lauric acid glycerides. Normally up to 20% of vegetable oil are used in a toilet soap making. The purpose of vegetable oil in the toilet soap is to give the good lathering properties even in cold or hard water. Soaps made with these vegetable oil are rather hard, being slow to rub down or become slushy, but they bind large amounts of common salt. If the percentage of the vegetable oil is increased, the electrolyte content would exceed the maximum allowed, so 20% is the limit. Moreover. cost does not usually permit higher dosages of these oils. It may also be mentioned that a number of individuals react to an increased content of such fatty acids with erythema and other forms of irritation (SPC, 1970).

In order to enhance plasticity, castor oil and olive oil are added to the soap mass. However, addition of castor oil are limited to a maximum of 2%, since it considerably increases the electrolyte content. Tallow is the framework substance of a soap, but also slow to form lather in cold water. A toilet soap may consists of up to 85 percent of tallow, provided that the colour of the soap and the average titre of the batch are no hinderance. Tallow having a titre of approximately 41-43°C, 3% free fatty acids is the most economical (SPC, 1970).

Fats and oil raw materials for toilet soap should always be bleached prior to the manufacturing process. The melted fats are dried in a vacuum, and afterwards bleached - also in a vacuum with 3-8% bleaching earth and an addition of activated charcoal. An alternative method is to split oils and fats in a highly pressure splitting plant with subsequent distillation of the raw fatty acids (vacuum of 4-5 mm Hg.) This method makes possible better glycerin recovery, whereas a larger amount of glycerin remains in the soap if neutral fats have been employed (SPC 1970).

All vessels, pipes, valves and cocks coming in contact with oils, fats and fatty acids should no be made of iron but of aluminium or stainless steel, in order to prevent traces of iron from getting into the soap. It is advisable to filter the raw fats as well as the corresponding soda lyes and salt solutions and pass them over a magnet to extract all iron particles (SPC, 1970).

2.6.1.2 Alkali

Common alkali used in the saponification reaction are caustic soda (sodium hydroxide) and potash (potassium hydroxide). The alkali used in soap making can be from two sources; lye caustic soda or potash purchased at the market; or lye obtained by leaching or washing water through the ashes of plants (Vita, 1980).

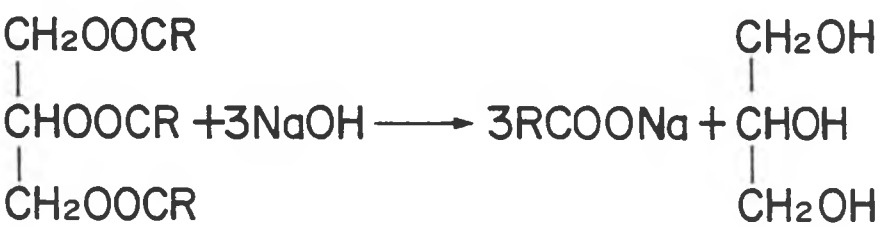
2.6.1.3 Other ingredients

Borax, soda, ammonia, Kerosene, naptha and rosin are sometimes added, but these

substances are not necessary to soap. They increase the quantity of suds and improve the appearance of the soap. The addition of sand and pumice increase the soap's cleaning power. Perfumes are sometimes added to make the soap more pleasant smelling. Either essential oil or colouring matter can also be added. Antiseptive and medicated soaps are made up with all kinds of medicaments. Many of the essential oils used in perfuming the soaps are highly antiseptic (Vita, 1980; Poucher, 1974b).

2.6.2 Production of base soap by saponification

Soap can be made very simply using readily available equipment and common ingredient (Vita,1980). The manufacture of toilet soap may be divided broadly into two stages (Poucher,1974b). (1) the production of the soap base by saponification of fats and oils and (2) the perfuming, milling, plodding and stamping of this base soap yielding the finished tablets of toilet soap. Soaps are salts of the fatty acids or a mixture of such salts. The conversion of fat into soap proceeds according to the equation below.



Fat Sodium hydroxide Soap Glycerol

Although today's industry has developed a number of methods whereby neutral fats and oils and their fatty acids can be saponified or neutralized within 15-60 minutes and almost all the glycerin recovered resulting in a finished basic soap with a fatty acid content of 62-64%, the majority of soap manufacturers still employ the traditional boiler method. Usually the tallow, the lard, groundnut oil or olive are saponified first followed by the coconut oil e.t.c. The entire fat charge may also be saponified at once. After saponification is completed, the soap should boil with easy mobility in the pan or kettle, with sufficient rising space allowed. After the saponification process has been completed, the batch must be allowed sufficient time to saponify the very last oil and fat particles. When saponification has been completely effected, less than 0.1% free alkali should be present (Poucher, 1974b).

Thereupon one begins the salting out of the soap, either to brighten it or to recover the glycerin if neutral fats were employed. It is advisable to add 0.5% of sodium hyposulphite for bleaching. A slight surplus of free alkali must be present in the soap. The quantity of bleaching agent is determined by the quantity of fat employed: (1 Kg bleaching agent per 1000 kg fat). Depending on which fats have been employed, the boil is salted out once or repeatedly. Either sodium chloride solution in hot water is used or solid salt diluted to the desired strength, dependent on the lye limit concentrations with hot water. Before ending each washing process one should ascertain the perfect homogenity (Poucher, 1974b).

The last washing lye should unfailingly be stored overnight before being evaluated by the bottom cock. The curd is now boiled until it reaches the rim of the pan and the operator makes sure that sufficient free alkali is present. Then as much hot electrolyte solution (NaOH + NaCl) is added in the required concentration as is necessary to separate. A further bleach with 0.5% sodium hyposulphite can be applied (Poucher, 1974b).

The cold process soaps are seldom an unqualified success since they are rarely neutral. Small makers sometimes manufacture them owing to their easier production. The cold process consists in the saponification of coconut oil at low temperatures with high strength alkali. Sometimes small quantities of other fats and oils are added to the coconut oil, and for some super fatted soaps lanolin is added at this stage. The exact quantity of alkali necessary for complete saponifcation is worked out previously, so that a neutral soap results. In making these soaps it is customary to melt part of the fats, remove the source of heat and then add the remainder so that when all is liquid the temperature is between 30° and 40°C. The lye at about 40°C is run in gradually while the whole is gently agitated. As saponification takes place the mass becomes uniform and finally translucent. The perfume is now added and after stirring the whole is run into frames. Complete saponification is effected when the temperature rises to about 85°C (Poucher, 1974b).

2.6.3 The fitting operation

The fitting operation is without doubt, one of the most difficult part of the soap - making process to describe in a simple manner; as well as being difficult to achieve in

practice, without some considerable degree of first-hand experience. It is very essential stage of the soap making technique, and one which has a significant bearing upon the quality of the resultant soap, and its final yield. (SPC, 1979).

In the simplest of terms the word 'fitting' is used to describe that part of the soap - making process, which takes place at the completion of the washing stages when both glycerol and the brine soluble impurities have essentially been removed. It consists of addition of a dilute electrolyte solution, or even water, to a well - settled curd, in such quantities that after the necessary homogenisation, a two phase system is produced. These two phases, which are quite immiscible liquids having different densities, can be readily separated under the influence of gravity or by centrifugal means. The two phases separate into a light phase of neat soap and heavy phase nigre. The latter contains a high concentration of impurities (SPC, 1979)

The purpose of the fitting operation is two fold. Firstly it serves as a cleansing operation, and secondly it is a means of obtaining a soap which can be successfully processed in all subsequent operation and which has a relatively constant total fatty matter content. The cleaning aspect is related to the fact that although the bulk of the impurities released after saponification are removed with the soap lye certain colour and odour materials are only soluble in dilute soap solution, and they are therefore collected in the nigre. True skill of the soap boiler is required to predict from physical appearance of the boiling soap as to what extent will be the final state of the fitted soap mass when it is allowed to separate by simple gravitational means, into neat soap and nigre layers (SPC, 1979).

The method of fitting depends on the quantity of soap or pan size, and this in turn determines the length of time the soap stays in the pan. A normal base soap suitable for subsequent finishing with toilet soap usually has the following values (SPC, 1970).

Total fatty acids - 62 - 63%

Sodium chloride content - 0.30 - 0.35%

Titre of the total fatty acids 38 - 39°C

Iodine number of the total fatty acids - 40 - 50

Saponification index of the total fatty acids 207-220

Unsaponified - 0.1%

Unsaponifiable - 0.15%

2.6.4 Drying method and milling

The free alkali, however, does fluctuate depending on the drying method for the base soap. Three different procedure are in use today .

(1) The soap is cooled in frames and afterwards cut into bars. Large quantities of air are used with this method. It requires the soap base to contain more free alkali 0.10-0.12%, in order to prevent acidity of the soap due to excess of air during drying.

(2) The liquid base soap is solidified by a water-cooled roller and then reaches a steam-heated roller. A scraping knife peels off the soap. The resultant solidified soap flakes are dried within 30 minutes. The freshly dried flakes are then conveyed to a silo.

(3) The most modern method is vacuum drying; the liquid base soap is filtered into a medium storage tank and passes by means of an apportioning pump, through one or several superheaters. The warm soap expands in the vacuum spray container releasing its surplus moisture within seconds and after passing through t the plodder worm and refining plates, the dried soap noodles travel by worm conveyor to soap storage silos.

A comparison of the three drying methods clearly shows the need for keeping the free alkali in the base soap at different levels. Freshly dried flakes for a fine toilet soap should contain not more than 0.4-0.5% free sodium hydroxide. The base soap having been completely dried, has to be stored for 12 hours in order to permit a moisture exchange before processing is continued (SPC, 1970).

The milling process is followed by mixing. Different methods can be employed. The traditional method calls for a mixer. Then the soap is milled twice or three times by a water-cooled roller mill. Thereupon it is pressed in a simple extruder with a worm 200-300 mm in diameter, passes a refining plate and the water cooled extrusion head, and

emerges finally as a continuous bar. The bar is either cut manually or with a cutter to the desired length and after brief surface cooling, it is moulded. The more modern method do without a mixer and repeated milling, employing a refining plodder. Here, the apportioning of the soap, dye, superfatting agents, perfume, the optical brightener and titanium white takes place fully automatically. In spiral, the soap is mixed so intimately with the rest of the ingredient as to assure a perfectly homogeneous bar of soap (SPC, 1970).

2.6.5 Perfuming

Virtually all toilet soaps contain a perfume or fragrance to mask the natural odour of the soap base and to make soap more acceptable to the consumers. It also imparts a degree of fragrance to the skin after use. The fragrance should not be the cause of discolouration spots nor should it produce an overall discolouration when exposed to light or air or during prolonged storage. When comparing fragrance in soaps, known standard should always be used. This standard should also be kept sealed in an airtight container or wrapped in metal foil and stored in a refrigerator. All subsequent production samples should then be compared with the accepted standard which has been allowed to reach room temperature, for fragrance acceptance (Whalley, 1980).

The cost of a toilet soap is partially influenced by perfume. The perfuming of toilet soap is an art acquired by long practice, keen observation of the relationship existing

between aromatic substances and the soap and attention to small details in the course of milling, plodding, e.t.c. While some cheap soaps are perfumed in the course of the manufacture of the base, all finer products exhibited on the shop counters are perfumed afterwards, and today constitutes, in many cases, a separate business (Poucher, 1974b). The inclusion of a perfume is a necessity, since unperfumed soap, however, has little appeal to the average consumer, who is accustomed to highly scented soaps containing 0.5 - 5% of perfume. Other fragrance types and often associated with deodorant, medicated or special scalp products. The final selection of a toilet soap perfume must satisfy the following requirement (SPC, 1975).

(1) Effectively mask, or complement, the original soap base odour

(2) Be retained during the average lifetime of the soap

(3) Not discolour the soap

(4) Not cause any skin irritation during and after use.

(5) Its addition to the soap must not seriously interfere with the plasticity/temperature relationship during the manufacture.

(6) Possesses a degree of substantivity to the skin of the user.

It can be postulated that there exist some type of bond formation between the soap base and certain perfumery ingredients at the molecular level. However, it must be borne in mind that the soap base is not a single material, but consists of a number of differing entities including the presence of about 12% of water; and that also the perfumes are a collection of widely differing chemical components of varying reactivities. Now if the

odour intensity of the perfumed soap A is less than that of soap B, it is suggested that bonding in soap A is much stronger than that in soap B. It is extremely difficult to prove this, however, since it is highly probable that other factors present in the system can influence the perfume retention capabilities of a particular soap composition (SPC, 1976).

Nevertheless, one type of molecular bonding can be envisaged at the outset, since the soap molecule has polar head and a non-polar tail. In a similar manner, many perfumery constituents are also polar in character, often possessing more than one highly polar group in their molecular structure. These facts suggest that a molecular attraction between certain perfume molecules and the molecules of the soap could well exist. This simple type of association could be due to the weak van der Waal's forces or perhaps even hydrogen bond formation. Whenever bond formation takes place between perfume compounds and soap, this could give rise to the soap A and soap B effects. On the basis of this hypothesis, molecules that have a tendency to bond formation would be expected to exhibit lower odour yields in a given soap, and constituents having a minimum polarity, showing only a small ability for bond formation would have high odour yield, since they can rapidly diffuse towards the surface of the soap (SPC, 1976).

Another factor which probably has some effect on the rate of diffusion, is the individual size of the constituent perfume molecules; the larger the size, the slower will be the rate of diffusion, causing less release of vapour at the surface of the soap. The odour yield of a given soap decreases with time although this undoubted decrease is much greater for unwrapped tablets than with wrapped tablets. It was also stated that for an

aged soap the odour intensity is much greater in the interior of the soap than at its surface. This was interpreted as an indication of migration of the odorous material does not always readily diffuse to the soap surface. It is possible for the free alkali to saponify a perfumery ester or to cause aldol formation with an aldehyde or perhaps assists in a condensation reaction. There are many instances whereby perfumery ingredients are incorporated into soaps, and after a relatively short period of time at room temperature, the odour has literally disappeared and the soap appears unperfumed (SPC, 1976).

The odour is measured quantitatively when the soap is wet, i.e. under conditions as normally obtained when the is being used. The odour given off by a perfumed soap does not always corresponds to the odour of the original perfume blend. This feature is not always accounted for by the contributory value of the basic soap odour (SPC, 1976).

CHAPTER 3

EXPERIMENTAL

3.0 Plant materials

Matricaria chamomilla L. currently being grown in the semi arid regions of Kibwezi, Kenya, were used for this study. The plant originated from Yugoslavia. These were identified at the Botany Herbarium, University of Nairobi. Ripe flower heads which usually have the highest yields were harvested and dried in their natural conditions. The dried flowers used were stored for a period of ten months before extraction. Loss of essential oil in the plant material due to the length of storage and storage conditions were, therefore, expected. The dried flower heads were ground mechanically.

3.1 Extraction

The Clevenger apparatus (Fig 3.1.) give high yields and little variation (Stahl et al., 1978).

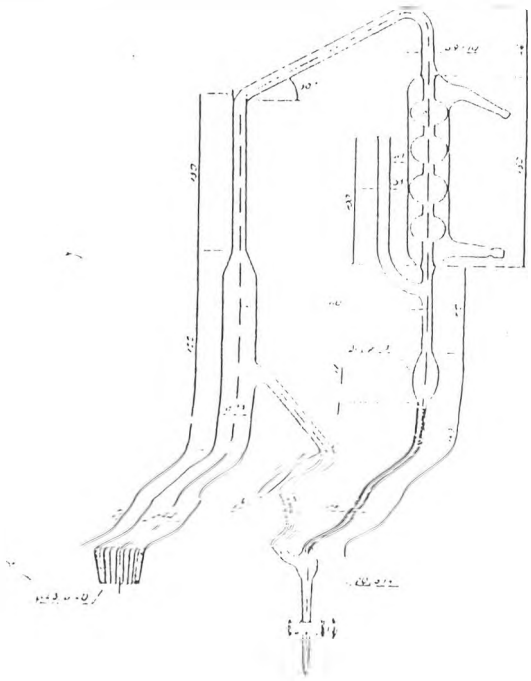


Fig 3.1 Clevenger apparatus

30 g of ground flowers of chamomile were placed in a one litre round bottom flask and 600 ml of water were added. The contents of the flask were heated to boiling point and subsequently distilled for two hours as recommended (Unesco, 1986). The ratio of water to flower were varied as follows: 13:1, 20:1 and 40:1 w/w. Distillation times were varied from 2, 4 to 6 hours. For each run, distillation was carried out until no further increase of oil was observed. The oil was permitted to stand undisturbed so that a good separation was obtained and also to cool to room temperature to avoid loss of volatile components of the oil. 10 ml petroleum ether was added to extract the chamomile oil from the water. The graduated tube and the condenser were washed three times with 3 cm^3 petroleum ether into the chamomile extract. The separation of the organic and aqueous layers were carried out using a separatory funnel. Petroleum ether was removed using the rotatory evaporator. The organic layer was dried with anhydrous sodium sulphate. The weight of the isolated oil was determined gravimetrically.

3.2 Analysis, isolation and purification

A variety of processes based on differential distribution of the sample components between stationary and mobile phases called chromatography were employed in the analysis, isolation and purification of the chamomile oil. The chamomile oil and the isolated components were subjected to GC- and UV-VIS spectrophotometer. The procedures most frequently applied to isolate the constituents of essential oils were

fractional distillation and chemical methods such as formation of decomposable derivatives. The major limitations of both techniques were that the pure compounds were relatively seldom obtained and that isomerisation and decomposition took place . Introduction of chromatographic methods for analysis were a substantial improvement. Due to its simplicity and speed thin layer chromatography along with gas chromatography are still most important methods in the analysis of essential oils. Thin layer chromatography were used as a pilot technique for column chromatography of essential oils.

3.2.1 Thin layer chromatography

The oil was separated into various components using thin layer chromatography (tlc). Commercial aluminium plates of 20 cm x 20 cm were covered with a thin layer of silica gel 60 were used. The plates were cut into various sizes. The width of the plate used depended upon the number of spots to be run at a time. The distances between each spot were 0.5 cm. A tlc spotter was made by drawing out a melting point tube to about 0.5 mm using a bunsen burner. The spotter was used many times but washed between the runs with clean methanol or any other appropriate solvent. The oil to be analysed was diluted with a solvent. Samples were spotted 0.5 cm at the starting end of the plate..

The mobile phases used were benzene: ethylacetate 95:5 dichloromethane: ethyl acetate 98:2 and chloroform: benzene 75:25. The chromatograms were developed and

the plates were then viewed under U.V. lamp to show any U.V. active spots. The plates were also sprayed with anisaldehyde reagent for detection of active components. Anisaldehyde acts as a locating reagent. The anisaldehyde reagent was prepared in the following procedure: 0.5 cm^3 anisaldehyde was dissolved in 10 cm^3 96% glacial acetic acid and then 4.5 cm^3 concentrated H_2SO_4 and 85 cm^3 methanol was added. After spraying with anisaldehyde the tlc plates were left for 5 minutes in an oven at a temperature of 120°C.

3.2.2 Column chromatography.

Column chromatography technique was used for isolation and purification of the main component from chamomile oil. A ratio of 20:1 (silica: oil mixture) was sufficient.

Column: 3.2 cm in diameter 96 cm long.

Adsorbent: Kieselgel 60 (Merck, 0.063-0.02 mm)

Sample weight: 3.0 g of chamomile oil.

Fractions collected: 15 ml

The solvent system used was hexane, diethyl ether and methanol. The solvent system was changed successively from pure hexane, 20% diethyl ether in hexane, 50% diethyl ether in hexane to 100% diethyl ether before flushing with methanol. The separated components was collected continuously. The size of fractions collected

depended mainly on the size of the columns and were about half the weight of silica or less (i.e. for a 60 g column 30 ml or less fraction were collected). Care was taken not to allow the column to run to dryness. Fractions of 15 ml were collected continuously in test tubes number 1-121. The column was monitored by spotting onto an analytical thin layer chromatographic plate, which was then developed in the following way: Benzene; ethyl acetate 9:1. The plates were sprayed with anisaldehyde reagent to locate the active components. When all the compounds of interest had been eluted and identified, fractions with similar R_f values were combined, concentrated and spotted again on tlc.

The crude chamomile oil was also spotted along with the eluents to help identify the spots. Similar fractions were combined, dried into sample vials for further analysis. Further purifications were carried out for chamomile components with R_f values close together. A small column with diameter of 1 cm and 40 cm long was filled with 30 g Kieselgel 60 (Merck 0.063-0.02 mm) and eluated with a solvent system (Hexane/dichloromethane) 8:2. The fractions were analysed using tlc and identical fractions were combined. The pure components were obtained when further purification was carried out in test tube number 48-74 and 75-95. pH of the compounds were estimated with pH papers.

3.2.3 Spectrophotometry

The chamomile oil and isolated chamazulene was dissolved in hexane and petroleum ether. Scanning range was in the region of 350 - 800 nm. SP8-150 UV-VIS spectrophotometer PYE UNICAM model was used under the following conditions.

Chart speed	20 sec/cm
Band width	1 mm
Wavelength speed	2 mm/sec

3.2.4 Gas chromatography

Gas chromatography of the essential oil as an analytical technique was used for both qualitative and quantitative determination. Analysis of the essential oil and its isolated components was done using a GC. The volume injected was 0.5 ml for gas chromatography. The following were the conditions used in gas chromatography:

Apparatus: Perkan-Elmer 8500

Column: Dimension: 30 m x 0.316 mm

Column type: Metal

Film thickness: 0.25 mm

Packing: Fused silica capillary

Temp: linear temp programming, 80-250°C 10°C/minute

Detector: flame ionization detector

Injector: temp 300°C

Detector: temp. 350°C

Carrier gas: nitrogen (flow 25 ml/min).

Total run time: 18.0 min.

3.2.5 NMR and IR Spectrometry

NMR for isolated compounds 1, 2, 3 and 6 was recorded at 300 MHz and 75 MHz for ^{1}H NMR and ^{13}C NMR using deuterated chloroform for all the analysis respectively. NMR spectra were determined at the University of Botswana. The chemical shifts are given in ppm relative to the internal standard tetramethyl silane (TMS).

Pure compounds were run as neat in IR spectrometer since they were liquids. A thin film was spread evenly on the IR. sodium chloride windows and then the windows were pressed while turning.

3.3 Mycology

Growth of a microorganism in pure culture mandates that all other microbial species be eliminated. Since microorganisms are distributed in nature, obtaining and maintaining pure culture required the elimination of other organisms from the growth medium. There were three distinct operations carried out.

(i) The preparation of suitable media

(ii) The removal of other organism from the medium and its containers e.g glass ware etc. by sterilization

(iii) The cultivation of the organism and its isolation from others present in the material to be examined.

3.3.1 Sterilization using autoclave

All the glassware, culture dishes, test tubes, pipettes, transfer loops and other equipment were cleaned before sterilizing. Physical sterilization most commonly used was heating. The autoclave used operates on the same principle as the domestic pressure cooker. Moist heat in the form of pressurized steam was highly effective in killing micro-organisms. The advantage of the autoclave method of sterilizing was that lower temperature (121°C) used and a pressure of 10 1b/in^2 ensured less danger of damage to the material. The saturated atmosphere enables liquids to be sterilized without evaporation.

Water was poured into the chamber so that the level was always above the electrically heated element and just below the resting plate. This was to ensure that enough steam was generated during sterilization. The controls on the autoclave were used to adjust the pressure and temperature and a safety valve to avoid explosions. Pressure of 10 lb/in^2 for 20 minutes was used. Articles to be sterilized were placed loosely in the

autoclave so that the steam could envelop them effectively. Glassware and other utensils to be sterilized were sealed with aluminium foil before sterilizing to prevent contamination when removed. When the lid had been tightly screwed down, the heating element was switched on. Steam was generated and escaped through an open valve at the base of the chamber. As the steam was formed, it rose to the top of the chamber and forced all the imprisoned air down and out through the valve which was only closed when pure steam started escaping. At the required pressure, the heating element cut out automatically and further heating was controlled thermostatistically. The noting of time was done when the autoclave reached the temperature and pressure required. The pressure gauge was left until a zero reading before the valve was opened. The autoclave was allowed to cool a little further before the lid was opened.

3.3.2 Media preparation

Three steps that followed in media preparation were

(i) preparing a solution of the media, (ii) dispensing the

the media and (iii) sterilizing.

The media (singular medium) suitable for fungus employed in this experiment were glucose (40 g), Peptone (10 g), Agar (20 g) Water (1 litre).

The media was adequate for the isolation and study of most fungi pathogenic for man.

The prepared media was constant and reproducible.

The ingredients, glucose, peptone and agar were weighed into a one litre stock bottle. One litre of distilled water was added to the ingredients. The contents were dispensed in the stock bottle and autoclaved at 121°C and pressure of 10 lb/in^2 and the sterilization was carried out for 20 minutes. After autoclaving the medium was allowed to cool to 50°C or allowed to solidify in bulk if it was to be used later. The high concentration of sugar in the media could only be melted once with minimum heating because repeated heating tends to char the sugar.

3.3.3 Pouring of plates

Pouring of plates was performed in "microbiological safety cabinets", inoculation hood or simply "hood". The hood was used to minimize aerial contamination. The cabinet was fitted with ventilation system that would not allow contamination of the laboratory. Bulk medium was prepared and sterilized in an autoclave. The media was allowed to cool to 50°C to reduce water condensation on the petri dish lids. The lids of the petri dish was lifted slightly and the agar was poured in quickly. Care was taken not to allow the agar to fall on the table. Immediately, the petri dish was rotated so that the agar could spread evenly before it cooled, but splashing over the edges was avoided.

3.3.4 Inoculation

Pure culture of *Trichophyton mentagrophytes* was used. Wire needle or a loop was sterilized in a flame and allowed to cool for about five seconds. The process of inoculation were accomplished quickly and efficiently to minimize the time of exposure during which contamination of the culture could occur. The stopper was removed from one of the tubes containing the fungi and the neck was flamed. The stopper was grasped between the small finger of the right hand. A loopful of culture was removed with the wire loop. Again the neck of the tube was flamed and the stopper was replaced. The lids of the petri dishes were raised at the nearest side just high enough to insert the wire loop. With a free arm movement from elbow, the loopful of culture was spread at the upper end of the plate; then streaks back and forth over the surface of the agar were made. The spreading was done evenly. The plates were then incubated until a thick mycelium on the surface was observed.

3.3.5 Antifungal test of chamomile oil and its components

The crude chamomile oil, bisabololoxides, chamazulene, (-)- *alpha*-bisabolol, farnesene, dimethylsulfoxide (DMSO) and griseofulvin were used. Griseofulvin was dissolved in DMSO. DMSO was used as a control while Griseofulvin antifungal activity was compared with the other compound because it was the recommended drug for the

treatment of diseases caused by the fungi of interest. In petri dishes (diameter = 0.88 mm) filled with the adequate medium and seeded with the *Trichophyton mentagrophytes* a penicillin bioassay disc were placed. The disc was impregnated with 0.5 ml of the compounds to be tested. After the incubation period the total diameter of the inhibition zone, if present, was measured. The petri dishes were labelled in accordance with the compound used.

The fungus was incubated at 25.6°C for a few days and the growth of *Trichophyton mentagrophytes* was noted. The diameter from the centre of the petri dish were measured when full growth was attained. The measurements were taken for several months.

Similar procedures were repeated for chamomile oil and its components incorporated in a toilet soap. The soap solution was made by warming the soap with water at 80°C, The concentration of each component was noted. The sample of various components at different concentration were compared with Griseofulvin. The plates were then incubated under favourable conditions to permit the growth of the fungi. The conditions for incubation were 25.6°C. The antimicrobial test of the components were compared with a soap sample which was impregnated with the components.

3.4 Making of toilet soap

3.4.1 Raw materials and pretreatment

The raw material were coconut oil, beef tallow, sodium hydroxide, sodium chloride, borax and chamomile oil and its compounds. The fatty raw materials used was subjected to various pretreatment. Colouring matter were removed from the oil by activated charcoal. The charcoal was added to the melted beef tallow and coconut oil in separate containers. The contents were heated to a temperature of 80oC for one hour. The amount of activated charcoal used for bleaching was 5% by weight. The activated charcoal was filtered off after bleaching.

3.4.2. Determination of the saponification value (S.V)

The beef tallow and coconut oil were each rendered homogeneous as possible by turning their containers upside down several times. Each of the samples was kept in an oven at 50°C and filtered. The coconut oil and beef tallow were heated to 10°C above the melting point of the fat. Into a round-bottomed or conical flask about 2 g of the sample was weighed. 25 ml of the 0.5 M ethanolic potassium hydroxide solution was pipetted into the contents in the flask. The flask was fitted with reflux condenser. The contents were boiled gently with occasional shaking. After 60 minutes the heating was stopped and 0.5 ml of phenolphthalein, 10 g/L solution in 95% v/v ethanol was added. Titration with hydrochloric acid solution (0.5 N) until the colour of the indicator changed was carried out. Blank test was carried out in the same way.

3.4.3. Soap preparation

400 g of beef tallow and 100 g of coconut oil were mixed in a 3 litre flask. The mixture was boiled and 100 g of sodium hydroxide dissolved in 500 ml distilled water was cautiously added in small amounts. As this was being done the whole mixture was stirred slowly and evenly in one direction to maintain a homogeneous emulsion. By gradual addition of sodium hydroxide solution, saponification took place rapidly and the addition of the alkali was completed in one and the half hours. After the saponification process was over, the mixture was allowed sufficient time to saponify the fat. Saturated solution of sodium chloride was added cautiously until the soap separated in form of a thick curd, 70 minutes was allowed for settling. The lower layer of aqueous liquid (nigre) was withdrawn. The soap was salted out four times. Washing was done with dilute sodium chloride solution to brighten the soap. Before ending each washing perfect homogenization of the batch was ascertained. The last washing was stored overnight before being evacuated. Evacuation was stopped when clear curd started to show. The curd was boiled until it reached the rim of the container. Hot electrolyte solution (sodium hydroxide and sodium chloride) was added to separate the layers.

3.4.4. The fitting operation

Partial salting out with diluted brine termed as fitting was performed after two days. Dilute electrolyte solution or even water was added to well settled curd, in such

quantities that after the necessary homogenization, a two phase system was produced. These two phases which are quite immiscible liquids having different densities, were readily separated under the influence of gravity. The two phases were separated into a light phase of neat soap and a heavy phase of nigre. The latter contained a high concentration of impurities.

3.4.5. The milling process and drying

The milling process was preceded by mixing. The soap chips, chamomile oil or its individual components were mixed in a mortar until a uniform mix was obtained. Water was added to the soap chips and boiled at 80°C to allow uniform mixing. The soap was cooled in frames and afterwards cut into bars. The bars, were cut manually to the desired length.

CHAPTER 4

RESULTS AND DISCUSSION

4.0 General discussion

Ground flower heads of *Matricaria chamomilla L.* were extracted with water using Clevenger apparatus. The flowers to water ratios of were varied as follows 1:13, 1:20 and 1:40 w/w yielded 0.293, 0.308 and 0.310 percentages of oil, respectively. Hydrodistillation of chamomile flowers require a certain minimum amount of water. The minimum amount of water was thirteen times the weight of the flowers. Optimum volume of water and weight of flowers for 1 litre flask was found to be 600 ml and 40 g, respectively. It was noted that on adding water, vigorous shaking was required to wet the flowers.

During the period of the extraction process, the aroma from the chamomile flowers diffused and filled the laboratory. Highly developed sense of smell could detect the fresh aroma of chamomile oil as far as ten metres from the source. The strong aroma is, therefore, lost during the extraction. The extracted oil was blue in colour with characteristic sweet odour. The oil was lighter than water and nongreasy. The distillation water flowing off the oil separator contained some volatile oil in solution or suspension. The fragrance of the chamomile oil was also detected in the distillation water.

The active ingredients can be extracted undecomposed and in good yield from chamomile flowers using super critical gases at 72-500 bar and 31-80^{0}c. The yield were higher than the extracts obtained by Clevenger apparatus. The yield obtained with

supercritical gases was 1.4% and the essential oil content was 0.5 ml/100g.

4.1 Extraction of chamomile oil using Clevenger apparatus with water

There was a small increase in the yield of chamomile oil when the water used for extraction was increased, however, the difference in increase was not big. This is shown in Table 4.1.1 for 1:13, 1:20 and 1:40 flower to water ratios.

Sample No.	Flowers to water ratio w/w	Grams of chamomile oil per 120 g of the flowers	% yield of chamomile oil	Average yield (%)	Standard deviation
1	1:13	0.35	0.29		
2	1:13	0.33	0.28		
3	1:13	0.35	0.29	0.293	0.009
4	1:13	0.36	0.30		
5	1:13	0.35	0.29		
6	1:13	0.37	0.31		
7	1:20	0.37	0.31		
8	1:20	0.36	0.30		
9	1:20	0.36	0.30		
10	1:20	0.35	0.29		
11	1.20	0.37	0.31	0.308	0.010
12	1:20	0.38	0.32		
13	1:20	0.38	0.32		
14	1:20	0.38	0.32		
15	1.20	0.36	0.30		
16	1:40	0.36	0.30		
17	1:40	0.35	0.29		
18	1:40	0.37	0.31		
19	1:40	0.38	0.32	0.310	0.011
20	1:40	0.38	0.32		
21	1:40	0.37	0.31		
22	1:40	0.38	0.32		

TABLE 4.1.1 Effects of flowers to water ratio on the yield of camomile oil extracted for two hours.

Sample No.	Time of extraction (hours)	Grams of camomile oil per 120g of the flowers	% yield of camomile oil	Average yield (%)	standard deviation
1	2	0.35	0.29		
2	2	0.33	0.28		
3	2	0.35	0.29	0.293	0.009
4	2	0.36	0.30		
5	2	0.35	0.29		
6	2	0.37	0.31		
23	4	0.37	0.31		
24	4	0.38	0.32		
25	4	0.37	0.31		
26	4	0.38	0.32		
27	4	0.38	0.32	0.315	0.007
28	4	0.39	0.33		
29	4	0.37	0.31		
30	4	0.37	0.31		
31	4	0.37	0.31		
32	6	0.40	0.33		
33	6	0.38	0.32		
34	6	0.37	0.31		
35	6	0.40	0.33	0.321	0.008
36	6	0.39	0.33		
37	6	0.37	0.31		
38	6	0.38	0.32		

TABLE 4.1.2 Effects of time of extraction on the yield of camomile oil with Clevenger apparatus Flower:water 1:13 (w/w).

The yield increased with distillation time as shown in table 4.1.2. The average yield of oil for 2,4 and 6 hours of flower to water ratio 1:13 was 0.293, 0.315 and 0.321, respectively. The presence of water in the extraction was distinctly beneficial in that it increases the rate of removal of essential oils by distillation. All essential oils are slightly degree soluble in hot water. Therefore, the amount of water present will determine the extent to which the oil yield will be decreased as a results of the retention of various constituents of the oil

In the case of water distillation using Clevenger apparatus the water is automatically returned into the distillation flask. Loss of the chamomile oil in the water is minimized by returning the distilled water back to the distillation flask. The high temperature of about 100°C used during extraction suggested that the oil is relatively stable.

4.2 Thin layer chromatography

The compounds present in chamomile oil were identified with thin layer chromatography using dichloromethane: ethyl acetate (98:2) benzene: ethyl acetate (95.5) and chloroform: benzene (75:25) as mobile phases. The chromatograms were developed and non coloured compounds were viewed under UV lamp and anisaldehyde reagent was used as a locating reagent.

The Tables 4.2.1, 4.2.2 and 4.2.3 shows the R_f values of the chamomile oil components on a tlc plate. Ten components were present in the oil mixture.

The ten components have average R_f values from the Table 4.2.1 of 0.98, 0.96, 0.85, 0.76, 0.62, 0.51, 0.43, 0.41, 0.32 and 0.05. Using dichloromethane : ethyl acetate ratio of 98:2 as the mobile phase. The average Rf values with other different mobile phases is given in Tables 4.2.2 and 4.2.3. The compounds give various colours on spraying with anisaldehyde reagent.

	Components (colour with spraying reagent)									
Sample No	Violet	Purple	Brown	Brown	Brown	Red-violet	-*	Yellow	Yellow	Red
1	0.99	0.97	0.87	0.80	0.59	0.47	0.38	0.33	0.27	0.07
2	0.99	0.96	0.85	0.83	0.71	0.57	0.49	0.48	0.37	0.05
3	0.97	0.95	0.80	0.74	0.68	0.59	0.55	0.54	0.46	0.04
4	0.97	0.95	0.80	0.71	0.64	0.56	0.53	0.47	0.36	0.05
5	0.98	0.94	0.79	0.76	0.60	0.48	0.41	0.39	0.21	0.06
6	0.98	0.96	0.90	0.78	0.65	0.55	0.40	0.38	0.32	0.03
7	0.97	0.96	0.87	0.68	0.56	0.46	0.34	0.32	0.25	0.04
8	0.98	0.96	0.82	0.70	0.56	0.48	0.36	0.34	0.27	0.05
9	0.99	0.98	0.92	0.83	0.62	0.48	0.42	0.40	0.33	0.08
Average	0.98	0.96	0.85	0.76	0.62	0.51	0.43	0.41	0.32	0.05
Standard deviation	0.008	0.010	0.044	0.052	0.048	0.048	0.071	0.071	0.071	0.015

MOBILE PHASE: Dichloromethane: Ethylacetate 98:2

SPRAYING REAGENT: Anisaldehyde reagent

* a brown colour was only observable with UV lamp

TABLE 4.2.1 Rf values of chamomile oil components

Components (Colour with spraying reagent)

Sample No	Violet	Purple	Brown	Brown	Brown	Red -violet	*	Yellow	Yellow	Red
1	0.97	0.95	0.87	0.83	0.77	0.68	0.58	0.56	0.43	0.00
2	0.98	0.95	0.88	0.75	0.72	0.69	0.68	0.58	0.40	0.00
3	0.96	0.94	0.91	0.87	0.85	0.76	0.69	0.67	0.60	0.00
4	0.94	0.87	0.79	0.76	0.73	0.58	0.48	0.45	0.38	0.00
5	0.98	0.95	0.77	0.74	0.70	0.60	0.48	0.46	0.38	0.00
6	0.97	0.95	0.77	0.73	0.69	0.57	0.48	0.43	0.37	0.00
7	0.97	0.95	0.72	0.69	0.67	0.53	0.38	0.33	0.26	0.00
8	0.97	0.92	0.68	0.65	0.62	0.49	0.38	0.32	0.29	0.00
9	0.99	0.94	0.72	0.69	0.67	0.46	0.39	0.31	0.21	0.00
Average	0.97	0.94	0.79	0.75	0.71	0.60	0.50	0.46	0.37	0.00
Standard deviation	0.013	0.025	0.076	0.065	0.063	0.093	0.114	0.119	0.106	0.000

MOBILE PHASE: Benzene: Ethylacetate 95.5
SPRAYING REAGENT: Anisaldehyde
* a brown Colour can only be observed with UV lamp.

TABLE 4.2.2 Rf values of chamomile oil components

Components (Colour with spraying reagent)

Sample No	Violet	Purple	Brown	Brown	Brown	Red-violet	-*	Yellow	Yellow	Red
1	0.97	0.94	0.74	0.67	0.47	0.39	0.31	0.26	0.21	0.00
2	0.97	0.94	0.76	0.69	0.67	0.44	0.40	0.38	0.31	0.05
3	0.99	0.96	0.71	0.66	0.57	0.35	0.31	0.29	0.25	0.02
4	0.97	0.96	0.80	0.74	0.63	0.47	0.42	0.37	0.32	0.00
5	0.97	0.95	0.69	0.65	0.55	0.38	0.33	0.28	0.24	0.03
6	0.97	0.93	0.73	0.69	0.64	0.48	0.32	0.38	0.30	0.00
7	0.98	0.94	0.80	0.78	0.59	0.50	0.47	0.44	0.39	0.00
8	0.96	0.92	0.60	0.48	0.37	0.26	0.22	0.27	0.14	0.04
9	0.98	0.92	0.69	0.59	0.52	0.41	0.38	0.37	0.32	0.02
10	0.96	0.89	0.71	0.53	0.48	0.43	0.39	0.36	0.29	0.02
11	0.95	0.87	0.60	0.45	0.37	0.27	0.24	0.21	0.14	0.00
12	0.96	0.92	0.62	0.46	0.39	0.30	0.25	0.22	0.18	0.02
Average	0.97	0.93	0.70	0.67	0.52	0.39	0.34	0.31	0.26	0.02
Standard deviation	0.010	0.026	0.066	0.107	0.101	0.078	0.074	0.070	0.075	0.016

MOBILE PHASE: Chloroform: Benzene 75:25
SPRAYING REAGENT: Anisaldehyde
* a brown colour can only be observed with UV lamp.
TABLE. 4.2.3 Rf values of chamomile oil components

4.3 Column chromatography

Column chromatographic technique was used for isolation and purification of the components of chamomile oil. The solvents used were hexane diethyl ether and methanol. The solvent systems used were pure hexane, hexane-diethyl, ether and diethyl ether and finally methanol. The isolated fractions were found to have various colours and odours as shown in Table 4.3.1. Some of the compounds were not pure. Further purification was carried out by repeating column chromatography.

The tlc analysis indicated that the chamomile oil contains ten compounds. Four of these compounds were separated in good yield by column chromatography. Compounds 1, 2, 3 and 6 were stable and in good yields. Each has its own characteristic colour and odour as shown in Table 4.3.1

test tubes	Odour	Colour	Colour of the compound on tlc on tlc plate with anisaldehyde	Weight (g)	% yield	Coding
1-15	non	non	non	0.03	1.00	*
17-31	odourless	colourless	violet	0.14	4.67	compound 6
35-45	strong	blue	purple	0.08	2.67	compound 1
50-55	strong	brown	brown	0.45	15.00	*
57-73	strong	colourless	red violet	0.12	4.00	compound 3
77-95	weak	yellow	yellow	0.48	16.00	compound 2
99-121	non	brown	brown	0.41	13.67	*

*- Compounds were not pure and the yield were poor

TABLE 4.3.1 Isolation of chamomile oil components.

The uncoded compounds were unstable and poor in yield. Therefore no further analysis were done on the three minor components.

4.4 Spectrophotometry

The blue coloured compound was dissolved in hexane and analysed with UV-VIS spectroscopy. The presence of strong absorption in the area 560-680 nm indicated a highly conjugated system. The parent azulene structure in chamazulene was responsible for the absorption. In the two Kekule type structure of chamazulene, there are ten mobile *pie*- electron, one from each carbon atom; thus the seven membered ring has seven and the five membered ring has five *pie*- electrons, respectively. Owing to the tendency of each ring to acquire a sextet of *pie*-electron, an electron will pass from the seven membered ring; thus the molecule will tend towards dipolar structure each having an aromatic sextet. The fine structure of azulene is probably a hybrid of the two Kekule forms.

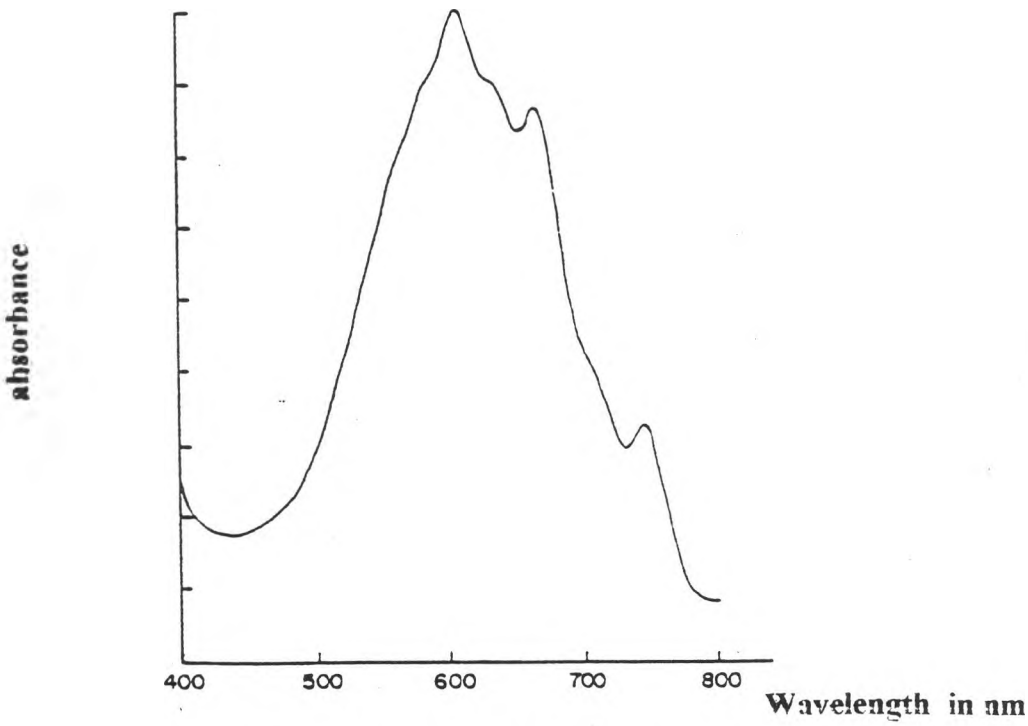
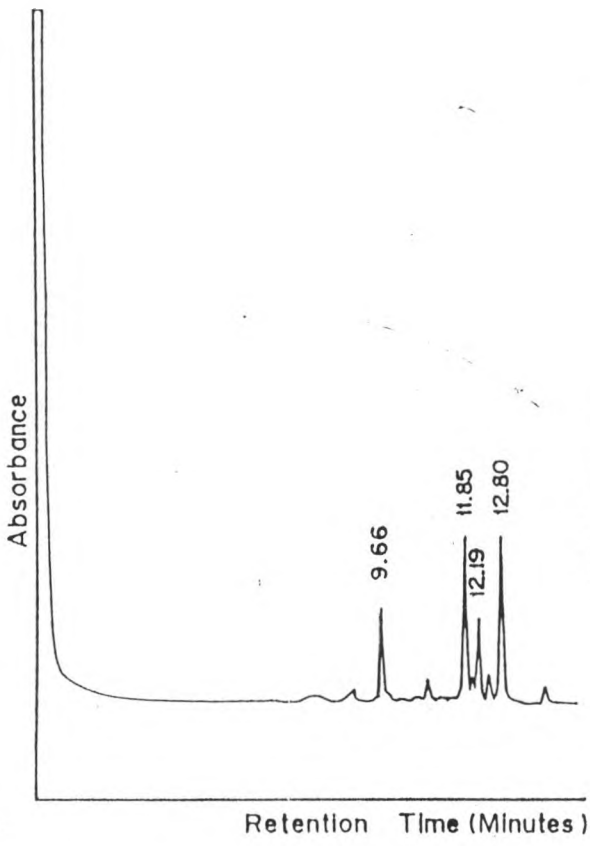


FIG. 4.4.1 UV/VIS spectrum of chamazulene

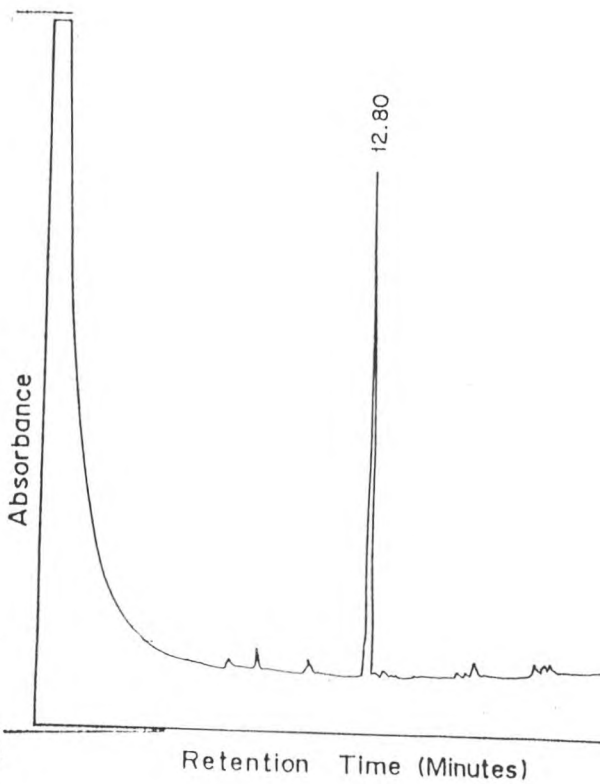
4.5 Gas chromatography

Chamomile oil and its compounds 1,2,3 and 6 were analysed with gas chromatography using hexane as the solvent. The gas chromatographic analysis of chamomile oil indicates the presence of ten components of which four were the major and stable components.

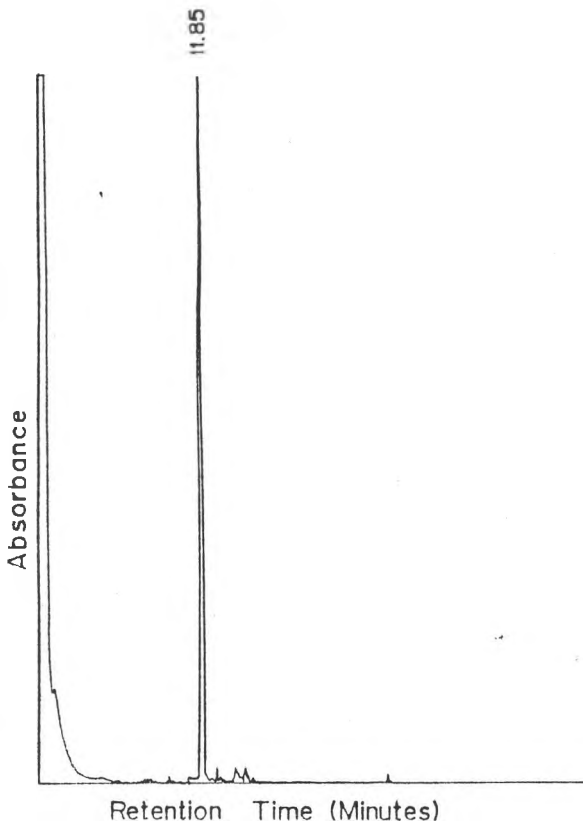
Observation of a single large peak for each of the isolated sample 1, 2, 3 and 6 at various temperatures was a strong indication of their purity



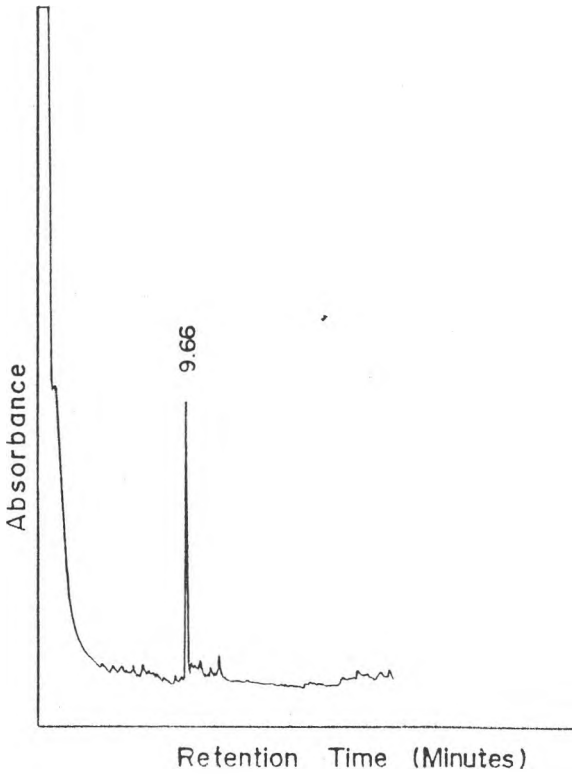
4.5.1 Gas chromatogram of chamomile oil



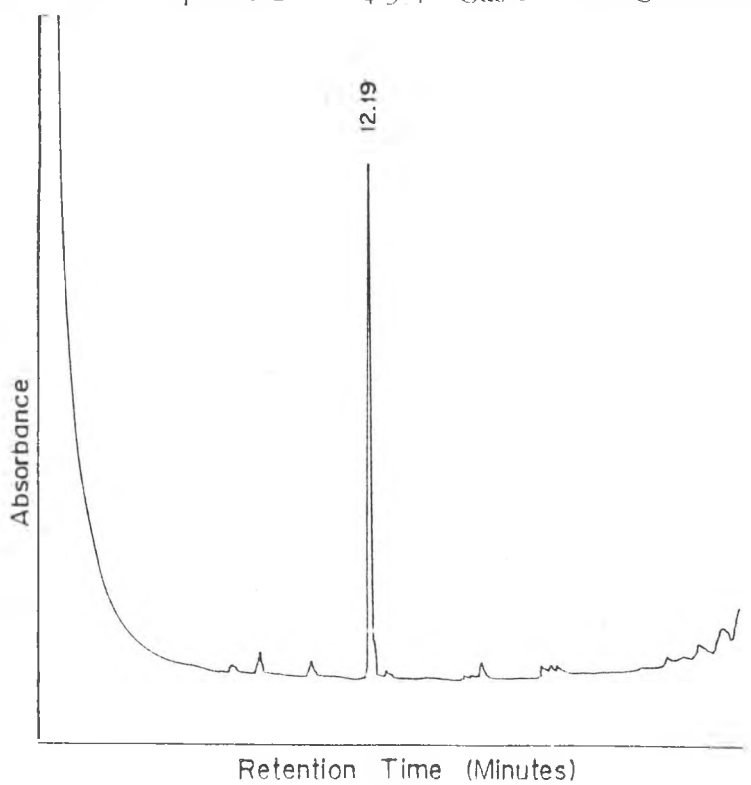
4.5.2 Gas chromatogram of isolated compound 1



3 Gas chromatogram of isolated compound 2



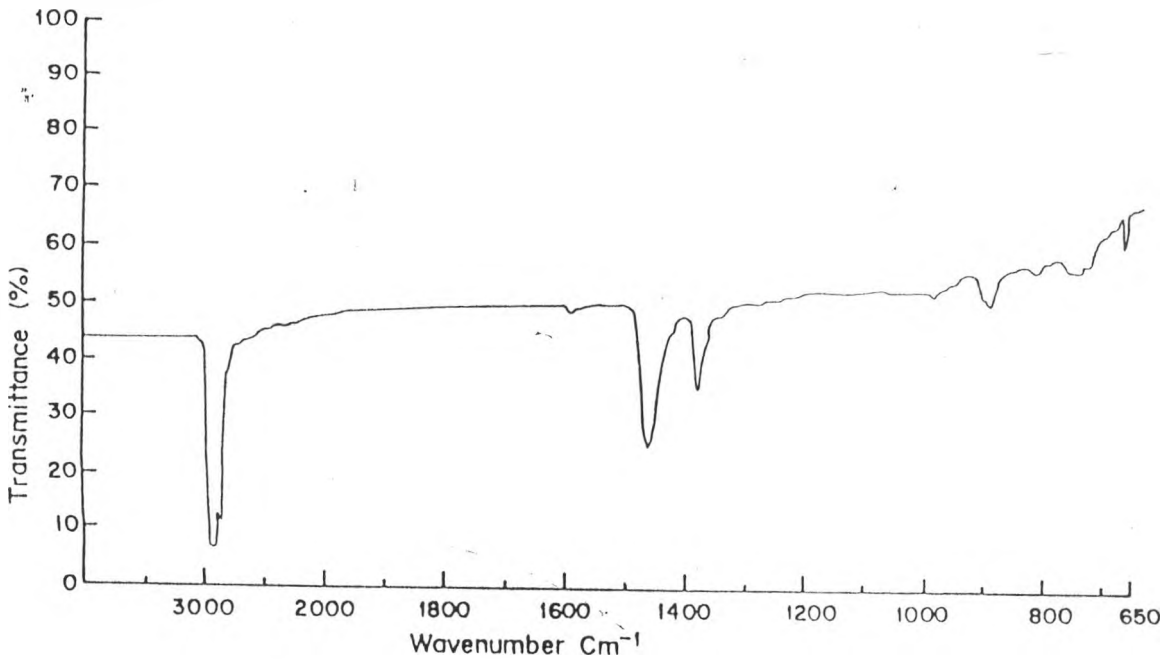
4 5 4 Gas chromatogram of isolated compound 3



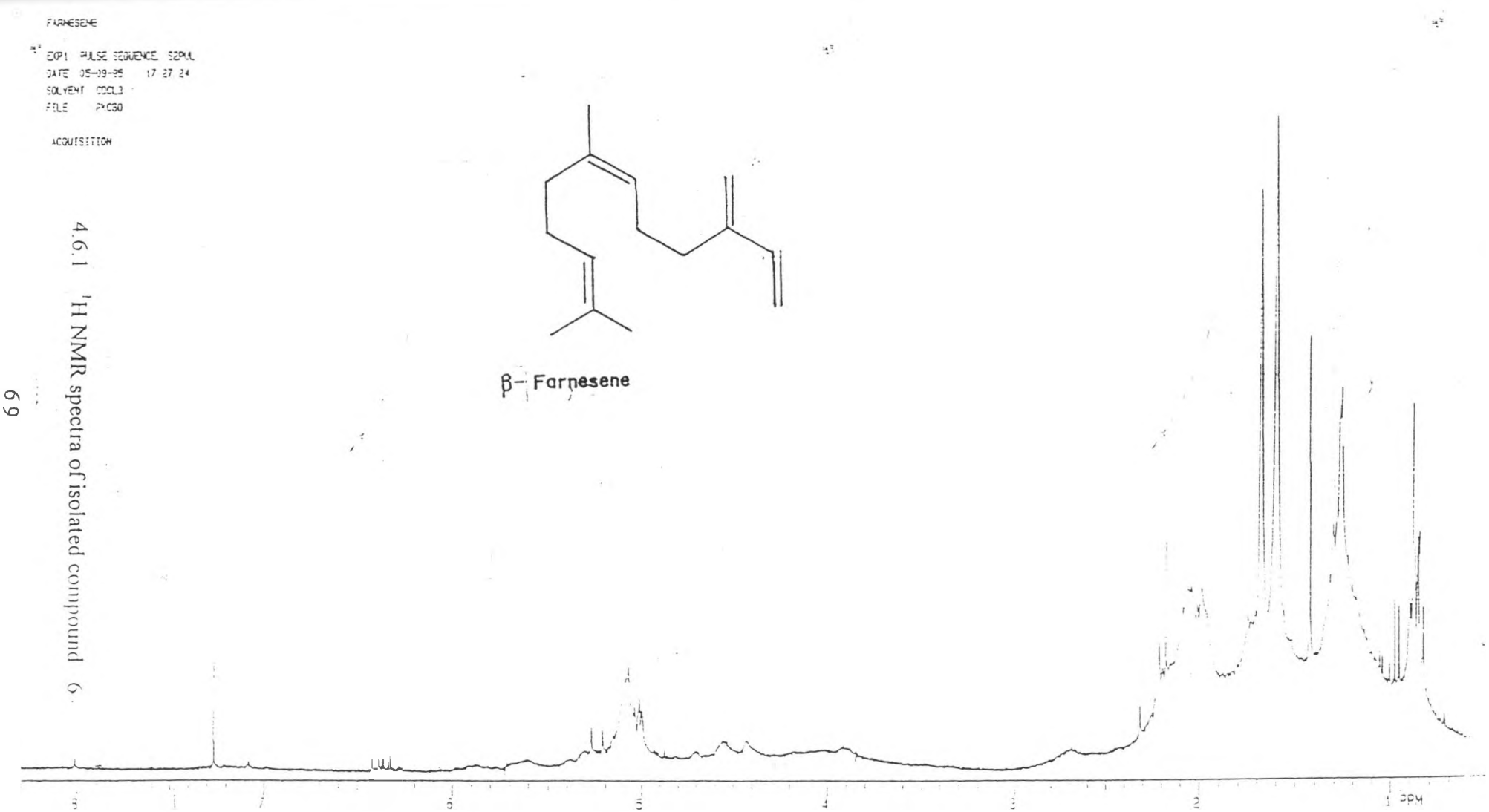
4.5.5 Gas chromatogram of isolated compound 6 -

4.6 NMR and IR spectrometry

^{1}H NMR spectrum of compound 6 shows a signal at 1.65 ppm (m, 9H) for the protons of three vinylic methyl groups in compound 6. Unresolved doublet (8H) at 2.05 ppm is attributed to the protons of four allylic methylene groups. A complex structure (6H) between 4.90 ppm and 5.40 ppm is attributed to vinylic protons. Finally a quartet (1H) at 6.40 ppm is a characteristic of the lone proton in the system R-CH = CH_2. Its IR spectrum shows a characteristic bands of an unsaturated aliphatic hydrocarbon. Absorption at 1598 cm^{-1} suggest further conjugated diene. One of the bands at 895 s or 906 s cm^{-1} arises from the methylene group and the other from vinyl group. IR and NMR spectra agrees with those found in the literature (Roberts, 1965). Compound 1 is assigned *beta*-farnesene.



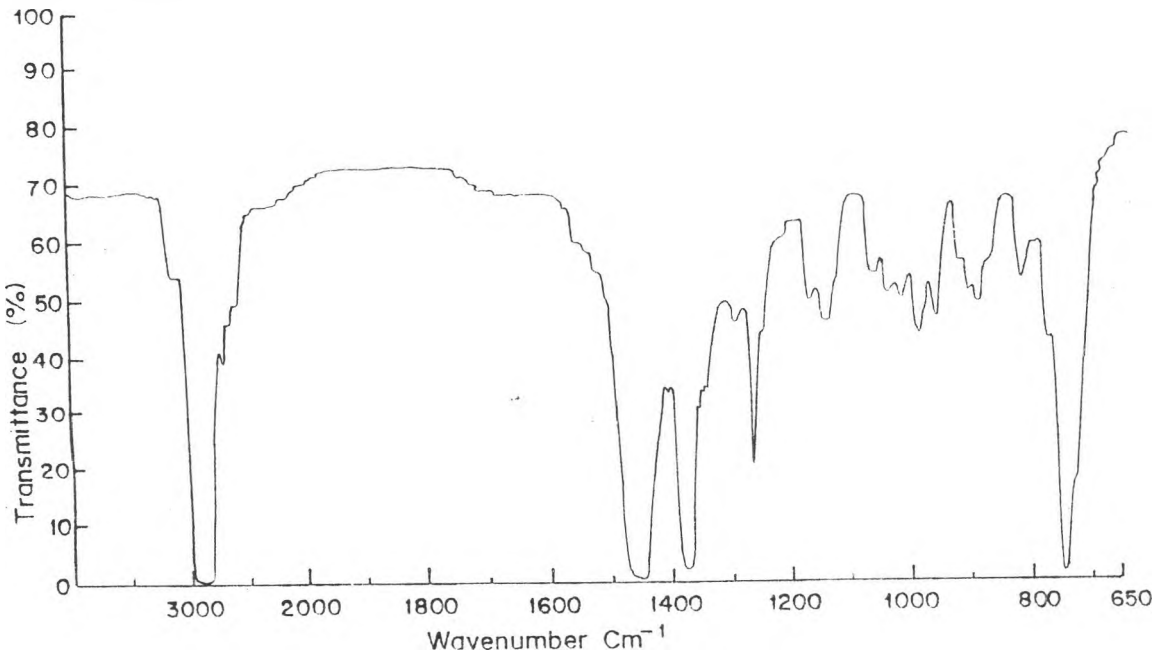
4.6.2 IR spectra of isolated compound 6



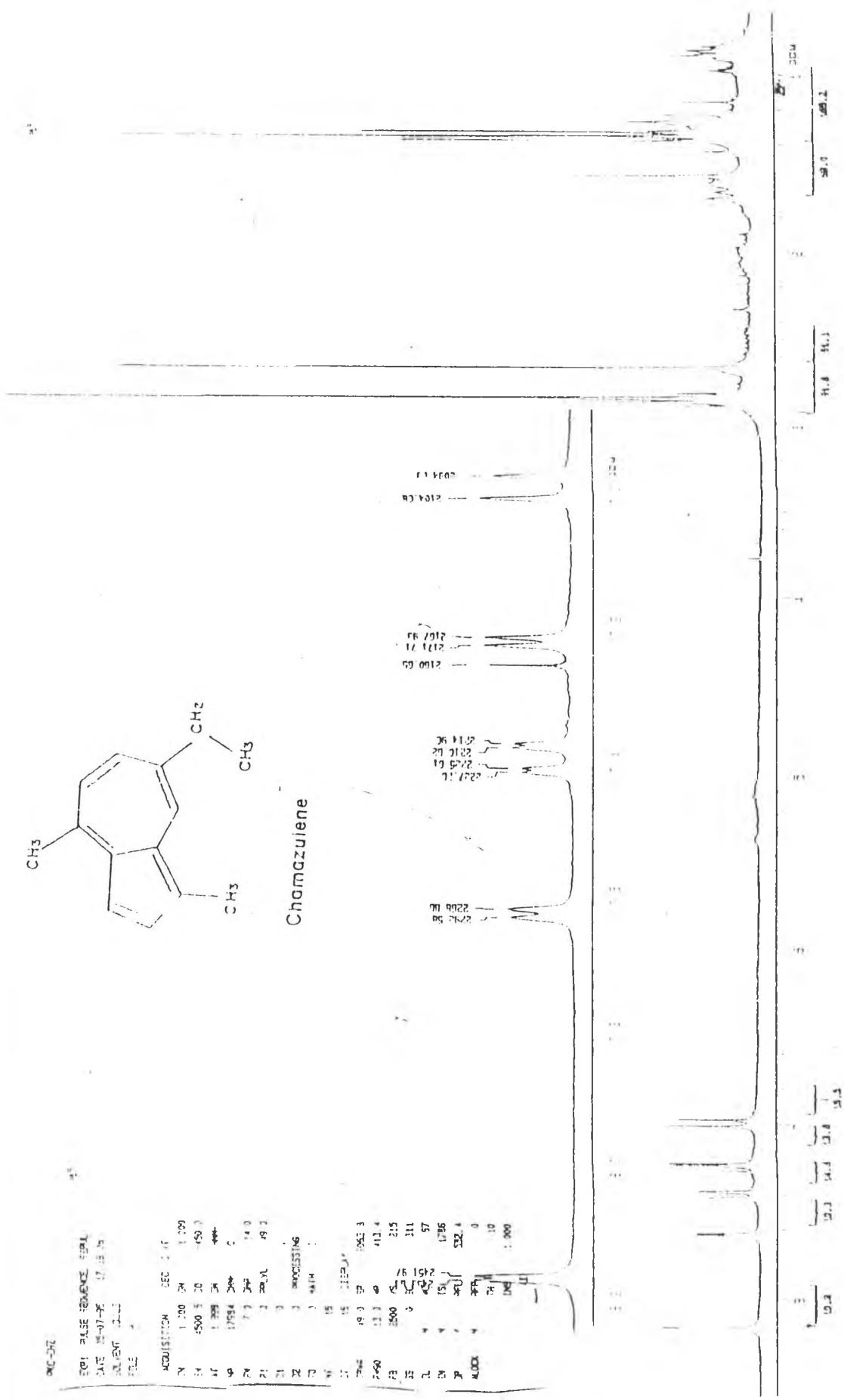
4.6.1 ^{1}H NMR spectra of isolated compound 6

The ^1H NMR of compound 1 shows a signal at 8.18 ppm (d, 1H) is assigned to the C-8 proton which is coupled to the C-6 proton by (1.86 Hz). This serves to identify the C-5 proton at 7.40 ppm ($J_{5,6}$ = 10.44 Hz, $J_{6,8}$ = 1.65 Hz) which are the AB part of the ABX system of the seven membered ring. The AB system at 7.22 ppm (J=3.78 Hz) and 7.63 ppm is attributed to the C-2 and C-3 protons respectively. Sharp peak at 7.27 is due to the solvent ($CDCl_3$) The singlet (2H) at 2.85 ppm is attributed to methylene ($-CH_2-$). The signal at 2.65 ppm (s, 6H) is due to the methyl groups of C-1 and C-4. The signal (t, 3H) is attributed to the methyl in the system (CH_2 CH_3).

The ^{13}C NMR spectra indicated the presence of fourteen peaks of non equivalent carbon atoms which is in conformity with the structure of chamazulene. IR spectra shows absorption at around 1450 cm^{-1} which is due to the -C=C- stretch vibrations of the conjugated system of five double bonds.



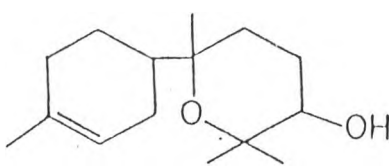
4.6.5 IR spectra of isolated compound 1



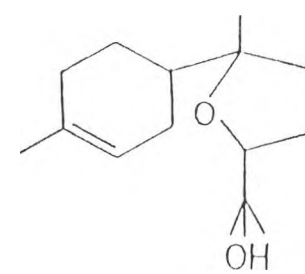
4.6.3 ^{1}H NMR spectra of isolated compound I

4.6.4 ^{13}C NMR spectra of isolated compound 1

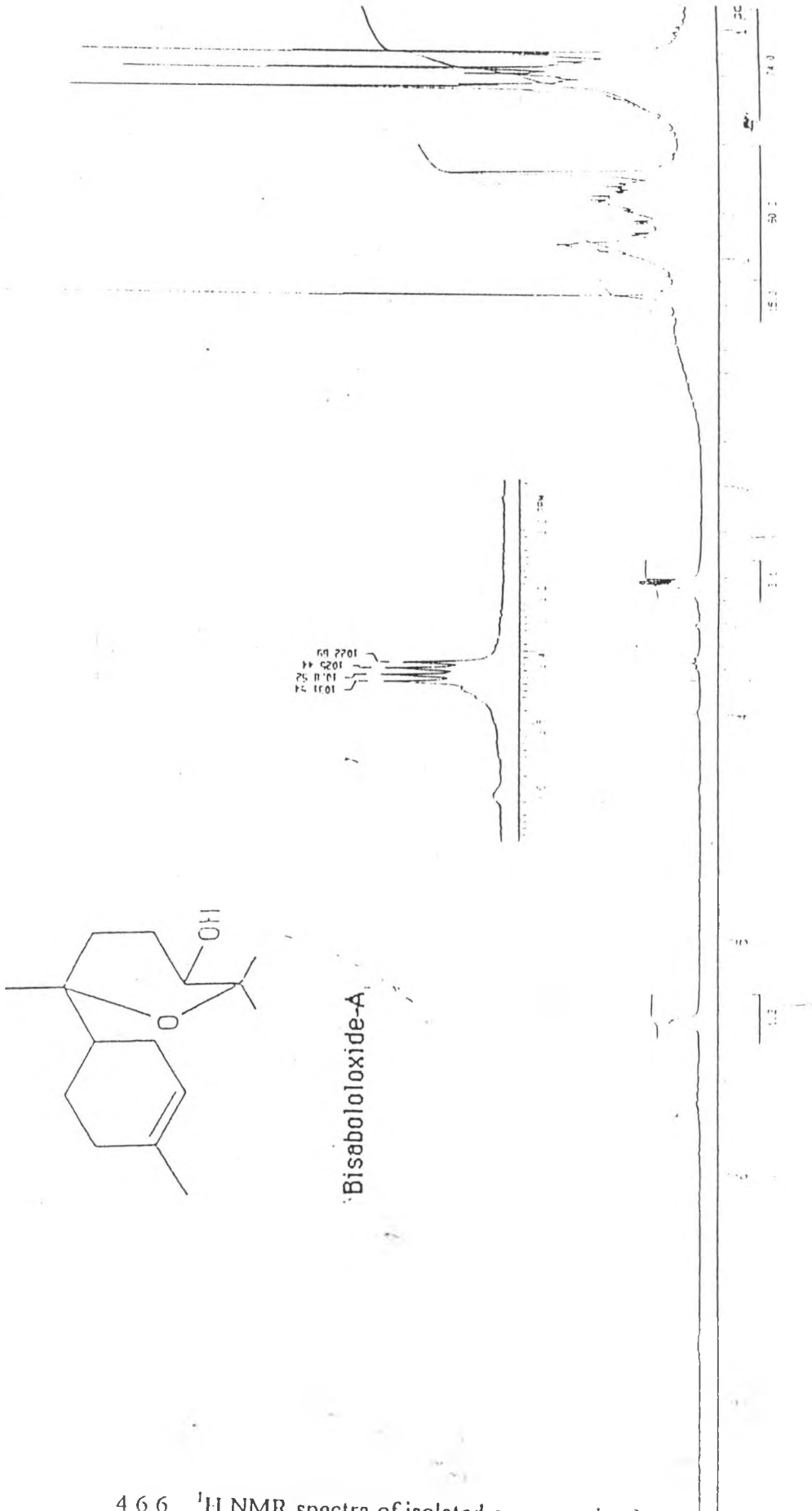
^{1}H NMR of compound 3 shows a broad signal at 5.31 ppm (s, 1H) and is attributed to the vinyl proton. The signal at 3.43 ppm (dd, 1H) is associated with the proton (-CH-OH). The broad signal at 1.65 ppm is assigned to the allylic methyl group. Peaks at 1.15, 1.20 and 1.25 ppm are attributed to the three methyl groups. The sharp singlet at 2.15 ppm (1H) is associated with the hydroxyl proton. The ^{13}C NMR spectra shows a singlet at 134.213 ppm and 120.773 ppm which is due to the olefinic carbon atoms. The three singlets at 75.0262, 74.2336 and 71.8241 ppm are attributed to the three groups (C-O-) in the compound. The spectra is in agreement with the literature data (Mose and Luka, 1957; Szalontai et al., 1977) Chamomile oil contains the two isomers (bisabololoxide A and B). Bisabololoxide B is in abundant (Unesco, 1986) Compound 3 is assigned bisabololoxide A. Compound 2 was not fully identified



Bisabolol oxide-A (3)



Bisabolol oxide-B (4)



4.6.6 ^{1}H NMR spectra of isolated compound 3

4.6.7 ^{13}C NMR spectra of isolated compound 3

4.7 Toilet soap

Determination of the saponification value (S.V)

The saponification value (S.V) is given by the formulae (IUPAC, 1979)..

$$S.V. = 56.1 \times T (V_o - V_1)/M$$

where V_o - number of ml of the HC1 used for blank test

V_1 - number of ml of HC1 used for test with the fat.

T - exact normality of HC1 used (T = 0.5 N)

M - mass in g, of the test portion (M = 2 g)

In the determination of saponification values for this analysis, 0.5 N HCl was put in the burette and titrated against 25 ml of alcoholic KOH which was accurately pipetted into a conical flask. Saponification values were calculated using the equation shown above.

The saponification values of the beef tallow and coconut oil were 220.61 and 251.05, respectively. From the saponification values, 220 mg of potassium hydroxide was required to saponify 1 gram of beef tallow while 251.1 mg of potassium hydroxide was required to saponify 1 gram of coconut oil. The result indicates that the saponification value of vegetable oil is higher than that of animal fat. 400 g of the beef tallow and 100 g of coconut oil were saponified with sodium hydroxide. Partial salting out with diluted brine termed as fitting was performed. The soap base obtained boiled was at 80°C to

allow uniform mixing with the chamomile oil and its compounds

When the soap was impregnated with chamomile and its component, the chamomile imparts a fragrance on the soap. The odour yield of the soap was increased when the soap was wet. The fragrance lasted long and the blue characteristic colour is not stable. The odour of chamomile oil in the soap did not correspond with the odour of chamomile oil on its own. The chamomile oil imparts a degree of fragrance to the skin after use. The concentration may be modified to suit individual tastes. The behaviour of chamomile oil indicates that it may be used as a perfume. The sweet smell of the chamomile oil could be detected at low concentration of 0.05%. The fragrance from the chamomile oil was fresh and appealing to individuals who used the soap. The chamomile oil had an ability to musk the soap very well. The soap when used topically did not show any signs of body irritation even at high concentration of 4.76%. The chamomile oil in the soap was retained in the soap for a long time. The chamomile oil when used as a perfume satisfies the requirement of a good perfume as stated (SPC, 1979).

On the basis of hypothesis, molecules that have less tendency to bond formation would be expected to exhibit high odour yields in a given soap since they can rapidly diffuse towards the surface of the soap. These facts suggests that a molecular attraction between chamomile oil molecules and soap are weak and could be due to the weak van der waal's forces. Majority of chamomile oil compounds are sesquiterpenes which are small in size. The rate of diffusion is affected by the individual size of the constituents perfume molecule; the smaller the size, the faster will be the rate of diffusion.

4.8 Antifungal test of chamomile oil and its compounds

In petri dishes filled with agar medium and seeded with pure culture of *Trichophyton mentagrophytes* a penicillin bioassay disc was placed. The disc was impregnated with 3 drops of the compounds to be tested. The plates were then incubated until a thick mycelium on the surface was observed. Chamomile oil, bisabololoxides, chamazulene, (-)-*alpha*-bisabolol, farnesene, griseofulvin, dimethylsulfoxide and soap impregnated with chamomile oil compounds were screened for antimicrobial activities.

The diameter of the inhibition zone was measured. A remarkable effects of the oil samples and some of their main components was observed in particular against fungi that are known to cause skin infections in humans.

The average inhibition zone in mm, for chamomile oil, isolated compounds and griseofulvin are given in table 4.8.1 (pg 81) . The crude chamomile oil and most of the compounds isolated from chamomile oil showed antifungal activity. Chamazulene showed no activity . After successful antimicrobial tests, it became necessary to investigate the most active compounds in toilet soap. Our investigation shows marked difference between crude chamomile oil and its isolated compounds. The average inhibition zone of griseofulvin decreases sharply with time while chamomile oil and its compound except for chamazulene were very stable with time. Dimethyl sulfoxide (solvent for griseofulvin) was used as a control and no antimicrobial activity was observed. Griseofulvin had no activity against *Trichophyton mentagrophytes* when introduced after 48 hours of inoculation while

crude chamomile oil showed a stable antimicrobial activity against *Trichophyton mentagrophytes* introduced 48 hours after inoculation. The chamomile oil and its components has a capability of persisting for a long time compared to griseofulvin. Toilet soap usually has excess sodium hydroxide (0.02-0.05%), therefore it is possible for a reaction to occur with the chamomile oil components. The positive activity of the chamomile oil component in the toilet soap suggests that most of the components are stable in the soap. The effectiveness of microcide is dependent on their continuing presence at effective dose levels. The inherent volatility of chamomile oil may require good formulation techniques to ensure that the products retain their activity for a longer period.

The inhibition zones exhibited by the pure isolated bisabololoxide shows maximum activity followed by (-)-*alpha*-bisabolol and farnesene. Aqueous extracts from chamomile flowers have no antifungal activity. The data presented in Table 3.8.2 (pg 82) indicates that the activity of the chamomile oil and bisabololoxide in toilet soap was effective at low concentrations applied. It is, therefore suggested that there is no major reaction between chamomile oil components and toilet soap components.

Griseofulvin failed to show antifungal activity when introduced 48 hours after inoculation. In contrast, the crude chamomile oil showed some antifungal activity when introduced 48 hours after inoculation. The chamomile oil and its components have a capability of persisting for a long time as compared to griseofulvin.

The small and short lived inhibition caused by soap base without chamomile oil

was due to the alkalinity of the soap. The fungus are inhibited by low and high pH values. The inhibition zone of soap with 0.11%, 0.002% and 0.00038% bisabololoxides immediately after incubation period had diameters 50.00 mm, 41.00 mm and 33.00 mm, respectively. After two weeks the diameters had reduced to 23.50 mm, 17.00 mm and 12.50 mm, respectively. Griseofulvin and base soap without chamomile oil had 47.50 mm and 20.00 mm, respectively after incubation period and about two weeks later the diameters were 0.00 mm for each. The crude chamomile oil was active up to a concentration of 0.05% in the soap. The inhibition zone are slightly less than that of bisabololoxides.

Because of the lipoid solubility of chamomile oil, it is easily resorbed via the skin. Terpenes are known to penetrate skin easily and majority of the chamomile oil compounds are sesquiterpenes. The lipoid solubility and their possibility to penetrate into the cells may give rise to influence on the metabolism of the microorganisms responsible for skin infections. The antiseptic property of the chamomile oil and its compounds is dependent on their lipoid solubility. The presence of high proportions of unsaturated compounds may be responsible for toxicity of chamomile oil compounds against the fungi responsible for skin infections. *Trichophyton mentagrophytes* invades the superficial Keratinized areas of the body such as the skin, hair and nails. The ability of chamomile oil to penetrate the skin suits it to be introduced in topical preparations and toilet soap.

Date of measurement

Sample/ Compound	30/9	2/10	6/10	11/10	14/10	18/10	21/10	2/11	11/11	16/11	28/11
Griseofulvin	37.50	14.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Chamomile oil	36.50	25.50	22.00	18.00	17.50	17.50	17.50	17.50	17.50	17.50	17.50
Dimethyl-sulfoxide	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Farnesene	18.50	13.50	13.50	10.50	10.00	10.00	10.00	10.00	10.00	10.0	10.00
Chamazulene	6.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bisabololoxide	40.00	34.50	30.00	29.50	27.00	25.50	25.50	25.50	25.50	25.5	25.50
Bisabolol	29.50	26.50	22.50	17.00	16.00	16.00	16.00	16.00	16.00	16.0	16.00
Gri(48)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aq extracts	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cham (48)	12.00	12.00	11.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00

Sample 1-4 represents the isolated compounds of chamomile oil

Gri(48) and cham(48) represents griseofulvin and crude chamomile oil introduced 48 hours after inoculation respectively.

Aq. extracts: aqueous extracts from chamomile flowers

TABLE. 4.8.1 The average inhibition zone for test compounds. Diameters given in the table were in mm. Trichophyton mentagrophytes were used as test organism

Date of measurement

Sample/ Compound	30/9	2/10	6/10	11/10	14/10	18/10	21/10	2/11	11/11	16/11	28/11
Griseofulvin	37.50	14.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Chamomile oil	36.50	25.50	22.00	18.00	17.50	17.50	17.50	17.50	17.50	17.50	17.50
Dimethyl-sulfoxide	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Farnesene	18.50	13.50	13.50	10.50	10.00	10.00	10.00	10.00	10.00	10.0	10.00
Chamazulene	6.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bisabololoxide	40.00	34.50	30.00	29.50	27.00	25.50	25.50	25.50	25.50	25.5	25.50
Bisabolol	29.50	26.50	22.50	17.00	16.00	16.00	16.00	16.00	16.00	16.0	16.00
Gri(48)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aq extracts	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cham (48)	12.00	12.00	11.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00

Sample 1-4 represents the isolated compounds of chamomile oil

Gri(48) and cham(48) represents griseofulvin and crude chamomile oil introduced 48 hours after inoculation respectively.

Aq. extracts: aqueous extracts from chamomile flowers

TABLE. 4.8.1 The average inhibition zone for test compounds. Diameters given in the table were in mm. Trichophyton mentagrophytes were used as test organism

Date of measurements 1995

compound	15/2	17/2	20/2	22/2	24/2	2/3
Griseofulvin	47.50	42.50	37.00	10.00	0.00	0.00
bisabololoxide (0.11%)	50.00	45.00	37.50	33.50	30.00	23.50
bisabololoxide (0.002%)	41.00	37.50	27.00	22.00	20.00	17.00
bisabololoxide (0.00038%)	33.00	26.50	15.50	13.50	13.00	12.50
Chamomile oil (4.76%)	39.50	35.00	17.50	16.50	15.50	15.00
Chamomile oil (0.05%)	26.70	22.50	11.00	10.00	0.00	0.00
Chamomile oil (0.0049%)	20.50	14.00	9.50	0.00	0.00	0.00
Soap base (no chamomile oil)	20.00	14.00	10.00	0.00	0.00	0.00

* First measurement was taken three days after inoculation

* All the measurements were taken in duplicate.

TABLE 4.8.2 The average inhibition zone for soap containing chamomile oil and its components with *Trichophyton mentagrophytes* as the test organism. Diameter was given in mm. The chamomile components were impregnated with soap.

The photographs of the plates below were taken 10 days after the start of the experiment. The plates show the strong antifungal activity of chamomile oil and its components. The chamazulene (blue component) considered formely to be the most active component in chamomile oil is not active in this case. Bisabololoxides exhibited the highest antifungal activity. The diameter of the inhibition zone was used as a measure of the antifungal activity. The longer the diameter the stronger the chemical against the fungus. The inhbition zone decreases with time. It is suggested that the fungus develops resistance with time.

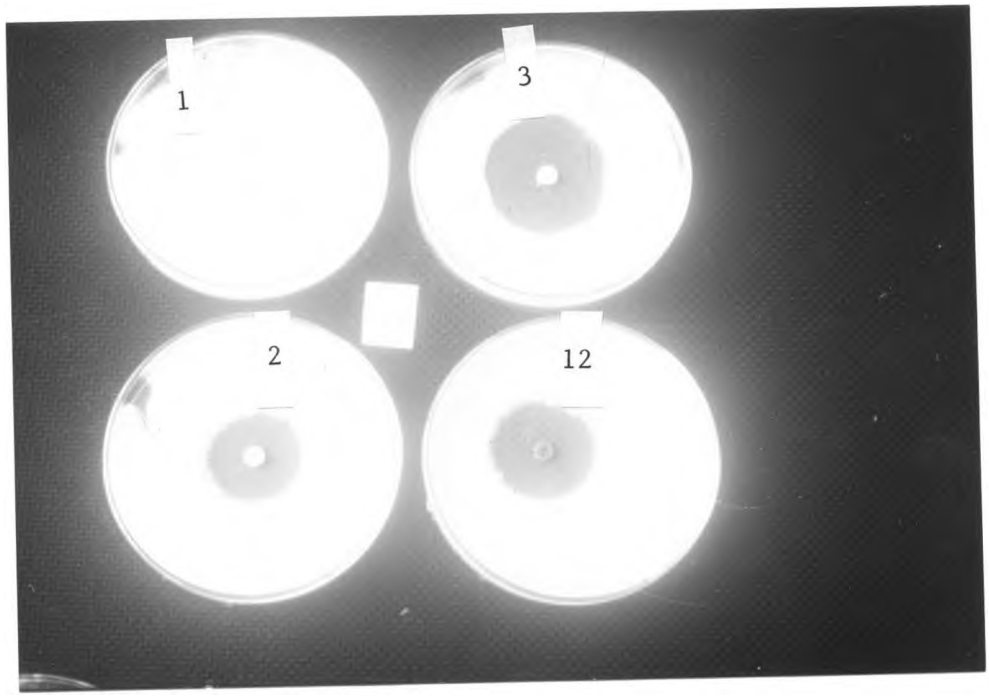


Plate 4.8.3 Antifungal effects of chamomile oil (12), chamazulene (1), bisabololoxide (3) and (-)-*alpha*- bisabolol (2)

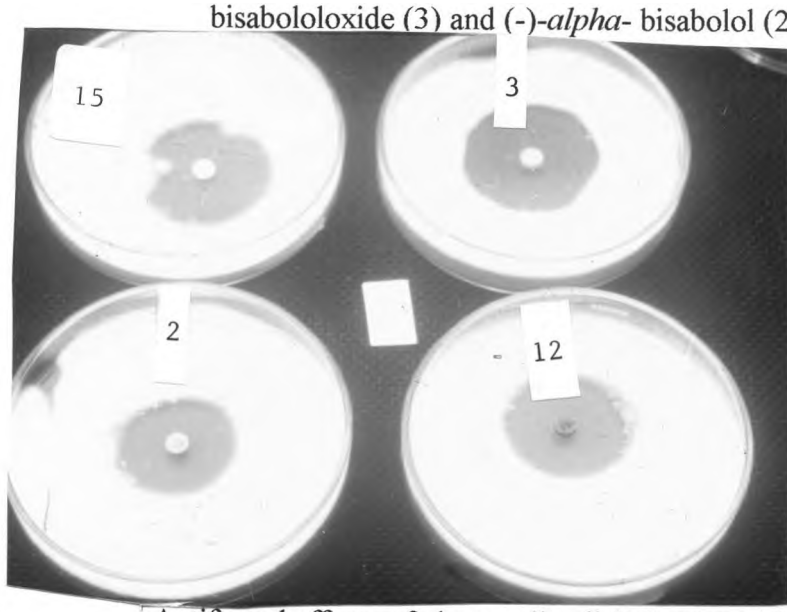


Plate 4.8.4 Antifungal effects of chamomile oil (12) griseofulvin (15), bisabololoxide (3) and (-)-*alpha*-bisabolol (2).

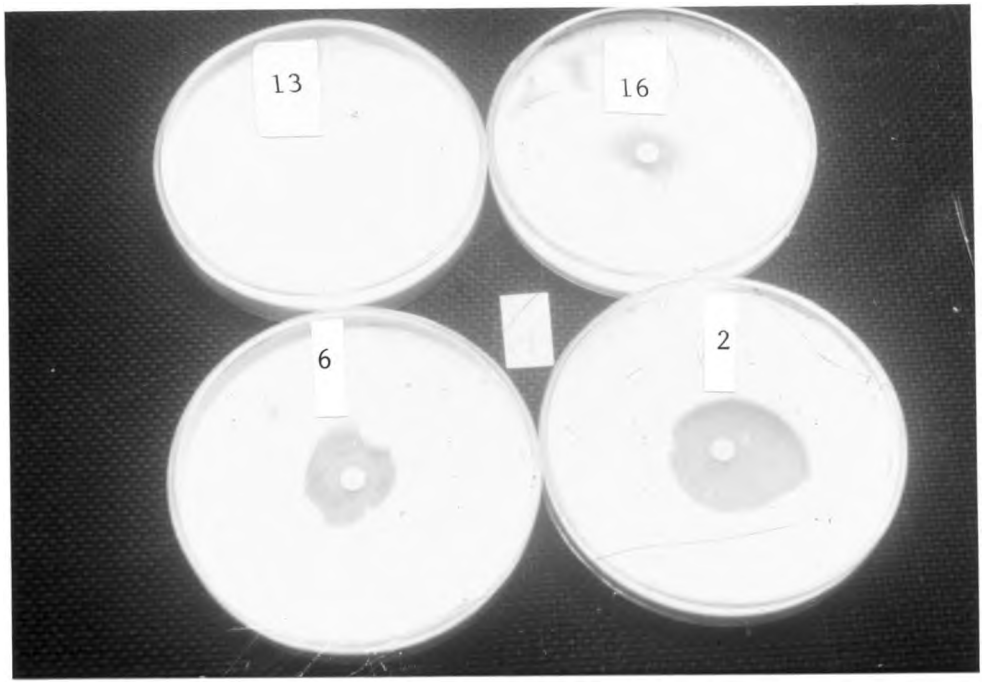


Plate 4.8.5 Antifungal effects of aqueous chamomile (13), gri48 (16), (-)-*alpha*-bisabolol (2) and farnesene (6)

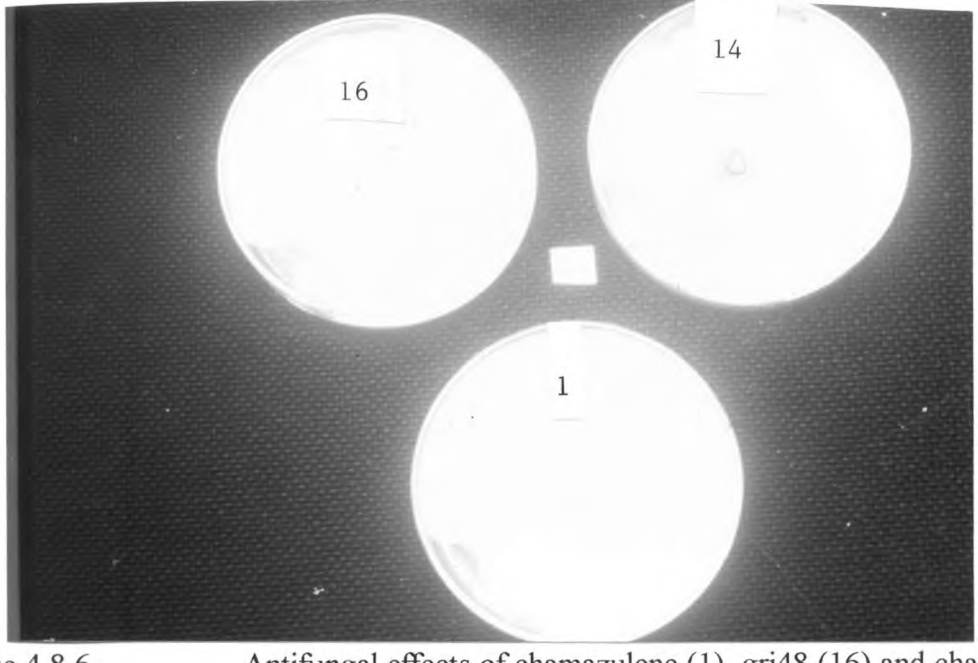


Plate 4.8.6 Antifungal effects of chamazulene (1), gri48 (16) and cham48 (14).

4.9 Conclusion and Recommendations

The NMR, UV-VIS and IR indicates that the compounds 1,3 and 6 were identified as chamazulene, bisabololoxide-A and farnesene respectively. Gas chromatography was used as a purity probe. Observation of a single sharp peak was used as an indication of the purity of each compound. Chamomile oil have been shown to contain many volatile compounds. Study could be carried out to find better methods of extraction of the oil to maximize the yield and to fully isolate and study all the most important compounds. Chamomile oil and its components exhibits good antimicrobial activity against *Trichophyton mentagrophytes*. These results support the use of the investigated plant in the local traditional medicine to treat microbial diseases. Majority of the compounds of chamomile oil from the results obtained and literature survey shows numerous pharmacological properties. The numerous rich properties of the family is expected to attract organic chemists.

To some degree there is a correlation between anti-microbial activity and spoilage is necessary to confirm the preservative nature of chamomile oil. Similar screening may be extended to bacteria and fungi known for skin infections. The minimum inhibitory concentration may be determined against *Trichophyton mentagrophytes*. Also microbial activity may be extended to other essential oils.

Since the percentage yield of chamomile oil is about 0.3%, the extraction is a very expensive process. We recommend that chamomile oil may be used without isolation of the major components. It is suggested here that chamomile oil would be best utilized in

creams, lotions, hair formulas and not in toilet soap. This is because soap is washed away after use while body lotions are maintained in the skin for a long time. Topical preparations with chamomile oil may do well because the consumer will enjoy the fragrance and medical care from the product. The concentration of chamomile oil may be varied depending on the purpose of the product. It is hard to draw a line between the use of the chamomile oil for perfume and medical needs. Better solvents should be used to re-introduce chamomile oil when it is used as a drug to ensure inherent volatility. Toilet soap can provide such property. A good microcide is dependent on the volatility of the compound in the solvent. Chamomile oil may do well in chronic infections of *Trichophyton mentagrophytes*. The result indicate that chamomile oil exerts excellent therapeutic efficacy due to potent antifungal activity and good penetration. Inspite of the in vitro anti-fungal activity of the chamomile oil, experiments on its ability to control fungal diseases *in vivo* are required.

The overall picture of the results obtained from the study confirms that from the present state of knowledge, the scientific and medical potential of the compositae family has yet to be fully exploited.

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