SEPARATION OF ESSENTIAL OIL FROM MIXTURES OF *MELISSA OFFICINALIS* AND *CYMBOPOGAN CITRATUS* LEAVES FOR MOSQUITO REPELLENT

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ASHWINDER CHELLIAH

Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
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JANUARY 2015

SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Pure).

Signature	:
Name of main supervisor	: DR. IR SAID NURDIN
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Date	: 19th JANUARY 2015

STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

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DEDICATION

To my dearest parents and siblings for their everlasting love and support.

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ABSTRACT

Synthetic based chemicals such as DEET is one of the most commercialised synthetic based mosquito repellents. This compound is effective against a wide range of insects and arthropods but its usage has raised several concerns to human health and surrounding environment.To combat its negative effects, there has been an overwhelming interest of producing essential oil products from different species of natural plant materials in order to access their repellent properties.Essential oils are odourless volatile mixture of hydrocarbons and their repellent properties have been linked to the presence of monoterpenes and sesquiterpenes.Therefore, it is important to identify a best technique to extract the essential oil without causing significant effect on their chemical constituents, since the conventional methods implied years back are time consuming and also record a low extraction yield.In order to enhance the extraction yield, an improvised approach namely Ultrasound Assisted Extraction-Hydrodistillation method will be exhibited to separate essential oil from mixtures of *Mellissa officinalis* and *Cymbopogan citratus* leaves. At the same time to evaluate the effectiveness of repelling properties in the essential oil, mixtures of leaves have been chosen in this extraction process.In this study, the effects of three main factors which are ultrasonic pre-treatment time, raw material to water ratio, and ultrasonic frequency were investigated.As a result, the best condition gives the highest yields of oil (0.3820%) were found at the ultrasonic frequency of 5kHz, pre-treatment time of 60 min and solid to water ratio of 1:6. The mixture of*Melissa officinalis* and *Cymbopogan citratus* leaves reflects higher essential oil yield compared with the yield of *Mellissa officinalis*leaves. Finally the oil samples were analysed by using Gas Chromatography Mass Spectrometry (GC-MS). From the analysis, almost similar chemical compounds with potentiating repellent activity such as linalool, citronellal, geraniol, citral, α-pinene, and limonene were found in their respective essential oils. Anyhow, the amount it contains differs from one another. Moreover, the repellency test carried out using the extracted oil against mosquito have also showed positive outcome.

ABSTRAK

DEET merupakan salah satu penghalau nyamuk yang dihasilkan berasaskan bahan sintetik dan paling dikomersilkan dalam pasaran sehingga masa kini.Kompaun ini amat berkesan digunakan terhadap pelbagai jenis serangga.Walaubagaimanapun, penggunaannya memberi kesan negatif kepada manusia serta alam sekitar di sekeliling kita. Dalam usaha untuk memerangi kelemahan tersebut, kebelakangan ini carian yang meluas menggunakan bahan berasaskan tumbuhan dijalankan.Justeru, pengeluaran produk minyak pati daripada spesies yang berbeza daripada bahan tumbuhan semulajadi untuk tujuan mengakses ciri-ciri penghalau nyamuk telah mendapat tumpuan orang ramai.Minyak pati adalah campuran menentu hidrokarbon, tidak berbau dan ciri-ciri penghalau mereka telah dikaitkan dengan kehadiran monoterpena dan sesquiterpenes. Oleh itu, adalah penting untuk mengenal pasti teknik terbaik untuk mengekstrak minyak pati tanpa menyebabkan kesan sampingan ke atas kandungan bahan kimia.Hal ini sedemikian kerana kaedah konvensional yang diaplikasikan bertahun –tahun lepas memakan masa dan juga mencatatkan kadar pengeluaran minyak pati yang rendah. Dalam usaha untuk meningkatkan hasil pengeluaran minyak pati , pendekatan yang terbaru iaitu kaedah rawatan pengekstrakan ultrasonik hidro-penyulingan akan digunapakai untuk mengekstrak minyak pati daripada campuran daun *Mellissa officinalis* dan *Cymbopogan citratus*.Dalam kajian ini,campuran daun *Mellissaofficinalis* dan *Cymbopogan citratus* telah dipilih untuk menilai keberkesanan ciri-ciri penghalau dalam minyak pati. Dalam masa yang sama kesan daripada tiga faktor utama iaitu masa pra - rawatan ultrasonik , bahan mentah kepada nisbah air , dan frekuensi ultrasonik telah dikenal pasti. Hasil kajian menunjukkan bahawa keadaan terbaik yang memberikan hasil minyak pati yang paling tinggi adalah pada kekerapan 5kHz , dengan masa pra - rawatan 60 minit dan pepejal kepada nisbah air 1:6.Campuran *Melissa officinalis* dan *Cymbopogan citratus* menggambarkan hasil minyak yang lebih tinggi berbanding dengan hasil *Mellissa officinalis* sahaja.Keputusan ini adalah berdasarkan kepada hasil tertinggi (0.3820 %) yang diperolehi dengan menggunakan campuran daun.Hasil analisis dengan menggunakan kaedah Gas Chromatography Mass Spektrometri (GC-MS)menunjukkan bahawa sebatian kimia yang hampir sama dengan aktiviti penghalau seperti linalool, citronellol, geraniol, citral, α - Pinene, dan limonene telah dikenal pasti. Walau bagaimanapun, jumlah kandungannya berbeza daripada satu

sama lain. Tambahan pula, ujian menghalau nyamuk yang dilakukan dengan menggunakan minyak yang diekstrak terhadap nyamuk juga telah menunjukkan hasil yang positif .

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^{o}C	degree celcius
kHz	kilohertz
min	minutes
g	grams

LIST OF ABBREVIATIONS

UAE-HD	Ultrasonic assisted extraction- Hydrodistillation
EO	Essential oil
HD	Hydro-distillation
UAE	Ultrasonic assisted hydrodistillation
PSE	Pressurized Solvent Extraction
MAE	Microwave Assisted Extraction
SFE	Supercritical Fluid Extraction

1 INTRODUCTION

1.1 Motivation and statement of problem

Insect transmitted disease remains a major source of illness and death worldwide. Mosquitoes alone transmit disease to more than 700 million of person annually (Taubis, 2000).Institute from Medical Research (2013), Ministry Of Health Malaysia has recorded that currently in Malaysia there are 5 human diseases transmitted by mosquitoes and they are namely Malaria, Dengue, Filariasis,Japanese Encophalitis and Chikungunya.According to the latest estimates released in December 2013, there were about 207 million cases of malaria in 2012 (with an uncertainty range of 135 million to 287 million) and an estimated 627 000 deaths (with an uncertainty range of 473 000 to 789 000).Most deaths occur among children living in Africa where a child dies every minute from malaria(WHO,2014).Although personal protective measures such as the use of repellents are widely used to prevent the transmission of arthropod-borne diseases, yet its usage has raised several concern related to environment and human health.

The most common mosquito repellent available in the market are based on DEET(N,N-diethyl-3.methylbenzamide) as it is not only a broad spectrum repellent but also the most effective and persistent on skin(Isman,2006).However, according to Qui et al.(1998) human toxicity reactions after the application of DEET varies from mild to severe. To avoid these adverse effects, there has been an increase in search efforts over the years for a natural and eco-friendly repellent. From the screening done, it has shown that quite a number of essential oil derived from plant species acts as a potential source of repellent and insecticides(Ayanoglu et al., 2005) to replace DEET.

A large number of EO extracted from different families has been shown to have high repellency against arthropod species.Among EO producing plants, some genus such as *Cymbopogon* spp., *Eucalyptus* spp. and *Ocimum* spp. have been widely studied(Luz ,2010).According to Jeanson et al. (2006),repellent properties of several essential oil appear to be linked with the presence of monoterpenes and sesquiterpenes.

Monoterpenes such as α-pinene, limonene, terpinolene, citronellal, citronellol, camphor and thymol are common constitutes which presents in mosquito repellent activities(Parket al.,2008).

There are several conventional methods which have been implied to extract essential oil from plant material. However those approaches are not reliable as the extraction times are long, potential loss of volatile constituents, high energy useand yield of oil produced is low. Hence, to improvise the separation process new extraction techniques such as ultrasonic assisted extraction have been applied to shorten extraction time, reduce organic solvent consumption, improve extraction yield and enhance extract quality(Hong, 2010).Mild ultrasonic stressing is introduced so that the desired components which are localised on plant material will ease the separation process (Toma et al., 2001). Therefore, in this research ultrasonic assisted extraction-hydrodistillation (UAE-HD) technique will be implied to evaluate its extraction process using a mixture of *Melissa Officialis* and *Cymbopogan Citratus*leaves. These mixtures of leaves are chosen as an alternative for synthetic based repellent (DEET) since their extracts containpotentiating insecticidal and therapeutic properties as reported by Adeniran et al.(2012).

1.2 Objectives

Thus, the objectives of this research are:

- To separate essential oil from mixtures of *Melissa O.* and *Cymbopogan C.* leaves using Ultrasonic Assisted Extraction – Hydrodistillation (UAE-HD) method.
- To investigate the effects of pre-treatment time, raw material to water ratio and ultrasonic frequency for a maximum oil yield.

1.3 Scope of this research

The following are the scopes which have been identified:

I. Separation of essential oil from *Melissa officinalis* and *Cymbopogan citratus* leaves by using ultrasonic assisted extraction-hydrodistillation (UAE-HD) method. In this process, essential oil will be separated first from *Melissa officinalis* leaves and only then from the mixtures of both leaves. This is done to compare the yield of oil between both leaves and determine the optimum parameters for maximum oil yield.

II. Determination of physical properties of essential oil and characterization of essential oil by using Gas Chromatography Mass Spectrometry (GC-MS) method.

III. Investigation of the effects of pre-treatment time, solid to solvent ratio and ultrasonic frequency on the maximum yield of oil. Graphs of yield against the parameters will be plotted.

IV. Repellence test against mosquito will be carried out.

1.4 Main contribution of this work

The main contribution of this work is to provide a new approach which combines conventional method together with a new technique in order to attain a maximum extraction yield. Besides that, a substitute to overcome the adverse effects of synthetic based repellents on users and to our surrounding environment is also presented.

1.5 Organisation of this thesis

The structure of the remainder thesis is outlined as follows:

Chapter 2 provides mainly the reviews from previous researchers regarding the use of chemical based mosquito repellents and its drawbacks to human and our environment. To overcome its adverse effects upon its usage on skin, a substitute repellent agent

which is more user friendly and environmental friendly is developed from essential oils derived from plant materials. Studies related to its chemical constituents in essential oil (EO) from plant materials are also discussed in this chapter. The techniques involved to separate EO using conventional methods as well as from new technologies are also discussed in detail.

Chapter 3 gives a review mainly on the separation technique using ultrasonic assisted hydro-distillation process in separating EO from plant materials. Essential oil yield collected from *Melissa officinallis* and its mixture with *Cymbopogan citratus* leaves will be compared using three different parameters. Further a brief discussion will be provided on the characterization and repellence test to be done after the separation of EO.

Chapter 4 provides the outcome of the work done using the experimental procedure stated in chapter 3.The analysis which has been conducted will be presented based on three unlike parameters in comparison to maximum yield obtained from the mixture of *Melissa officinallis* and *Cymbopogan citratus.*

Chapter 5 draws together a summary of the thesis and the significant of this research work.

2 LITERATURE REVIEW

2.1 Overview

DEET (N,N-diethyl-3-methylbenzamide) is an effective chemical based repellent against a broad spectrum of insects, and also the most effective and persistence on skin (Isman,2006).Although it is being used world widely, they still do have negative impact which causes environmental and human health risks. Due to the risks associated with DEET, a variety of plant based products have been developed as an alternative to meet the same purpose of DEET. Materials derived from plants could be a source for mosquito repellents agents because they constitute a rich source of bioactive chemicals (Kim et al., 2002).Besides that, they do also possess good efficacy and are environmental friendly. These alternatives to conventional extraction procedures may increase production efficiency and contribute to the protection of the environment by reducing the use of solvents and fossil energy, or generation of hazardous substances. Therefore, an improvise method namely ultrasonic assisted hydro-distillation is applied in this study to increase the yield of oil. Ultrasonic extraction is combined to the conventional method due to its ability to stimulate the release of components localized in the surface glands of the plant material by relatively mild ultrasonic stressing (Toma et al., 2001).

2.2 Introduction

This paper basically presents reviews on the types of mosquito repellents in use currently by users, its drawbacks, alternative repellents derived from plant materials, essential oil, reviews on its bio-actives studies, the extraction methods used to separate oil and its pros and cons.

2.3 Mosquito repellents

Repellents are substances that act locally or at a distance, deterring an arthropod from flying to, landing on or biting human or animal skin (or a surface in general) (Blackwell et al., 2003 ; Choochote et al., 2007).It plays an important role in preventing the transmission of vector-borne diseases by minimizing the contact between human and vectors (Pitasawat et al.,2003; Das et al., 2003).There are two types of repellent that are

commercially available and they are namely synthetic chemicals and plant-derived essential oils. The best known chemical based insect repellent is N,N-diethly-3-methyl benzamide (DEET) also known as N,N-diethyle-m-toulamide. DEET is the active ingredients of most commercially available mosquito repellent formulations throughout the world. It is used to repel a variety of animals such as mosquitoes, ticks, flies, gnats and midges. Being DEET not only a broad spectrum repellent, but also the most effective and persistent on skin (Isman, 2006).

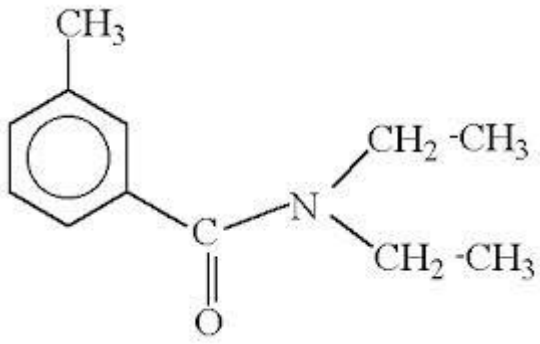

Figure 2.1:DEET

However, widely used synthetic chemical repellents are not safe for humans, especially children, and domestic animals because they may cause skin irritation, hot sensation, rashes or allergy (Das et al.,2003).The side effect after the application of DEET varies from mild to severe. Due to its drawbacks to human health and our ecosystem, more research has been done on repellents that are derived from plant essential oil to replace DEET. Plants may be an alternative source of mosquito repellent agents because they constitute a rich source of bioactive chemicals (Kim et al., 2002). According to Senthil Nathan et al.,(2004), botanical repellents are promising in that they are effective, safe to users, and also inexpensive.

2.4 Essential oil

Essential oil referred to as ‘essence’ are botanical extracts from various plant materials such as leaves, flowers, roots, buds, rhizomes, twigs, heartwood ,resin , seeds and woods. They are natural, volatile, complex compounds characterized by a strong odour and are formed by aromatic plants as secondary metabolite. Commercially, essential oils

are used in four primary ways such as pharmaceuticals, as flavour enhancers in many food products, as odorants in fragrances, and as insecticides. Particular emphasis has been placed on their antibacterial, antifungal and insecticidal activities (Chang et al., 2001; Chang & Cheng, 2002).Besides that, bioassays on a number of EO have also shown repellence against mosquitoes (Park et al., 2005, Trongtokit et al., 2005 and Yang et al., 2004) usually attributed to its main compounds and are recorded safe US Food and Drug Administration (Trongtokit et al., 2005).

However, synergistic phenomena between the diverse components of the EO may result in a higher bioactivity (an increased repellent response) of the oil as a whole compared to its isolated components (Hori, 2003). Thus, the fact that an EO contains specific main components may be an indication of its potential use, but does not warrant its use without confirmation of activity. According to Zygadlo and Juliani (2003), their composition may vary considerably between aromatic plant species and varieties, and within the same variety from different geographic areas.

Furthermore, repellent properties of several essential oils are also appeared to be associated with the presence of lower isoprenoids (monoterpenoids, sesquiterpenes and alcohols) (Jaenson et al., 2006). Some monoterpenes such as α-pinene, limonene, terpinolene, citronellol, citronellal, camphor and thymol are common constituents of a number of essential oils that show mosquito repellent activity (Yang et al., 2004). Among sesquiterpenes, β-caryophyllene is most cited as a strong repellent against *A. aegypti* (Gillij et al., 2008). Although there are several repellent properties of EO regularly appear to be associated with the presence of monoterpenoids and sesquiterpenes (Kiran and Devi, 2007; Jaenson et al., 2006 and Sukumar et al., 1991), a study by Odalo et al(2005) have found that phytol, a linear diterpene alcohol, has high repellent activity against *Anopheles gambiae*. Moreover, the oxygenated compounds phenylethyl alcohol, β-citronellol, cinnamyl alcohol, geraniol, and α-pinene, isolated from the essential oil of *Dianthus caryophyllum*, showed strong repellent activities against ticks (*I. ricinus*) (Tunón et al., 2006).

2.5 Methods of extraction

Extraction is a separation process consisting in the separation of a substance from a matrix. There are several techniques in which separation of EO from plant material can take place. The conventional ways employed to separate EO are hydro-distillation (HD), steam distillation, solvent extraction and also by using Soxhlet. Among these methods HD and steam distillation has been the most common way to extract EO from medicinal herb plants. From studies carried out by Aakanksha et al. (2013), the average percent of yield using hydro-distillation procedure was 0.8% as compared to steam distilled oil (0.7%). Although these techniques have been used since many years for EO's extraction, their application has shown several disadvantages like losses of some volatile constituents, low extraction efficiency, degradation of unsaturated, or ester compounds through thermal or hydrolytic effects, and possible toxic solvent residues in extracts or EO's (Temelli et al.,2007). However, in order to overcome the disadvantages and improve the production yield new extraction technologies such as microwave-assisted extraction(MAE) ,pressurized solvent extraction, supercritical fluid extraction , and ultrasound-assisted extraction has been introduced (Flamini et al.,2007).

2.5.1 Hydro-distillation

Hydro-distillation is one of the most commonly used techniques for volatiles isolation. This method utilises only water to obtain its isolates. In the HD process, the plant material is completely immersed in boiling water. The characteristic feature of this process is that there is direct contact between boiling water and the raw material(Fatima et al., 2014).The isolates namely its essential oil are distillation products that are immiscible with water and can be easily separated. However, some compounds of the EO are water soluble at an elevated temperatures and this may cause some loss in terms of its yield (Halim et al., 2010).

2.5.2 Steam distillation

Most EO's are also obtained from the plant material by a process known as steam distillation. The fundamental nature of steam distillation is that it enables a compound or mixture of compounds to be distilled at a temperature substantially below that of the boiling point of the individual constituents. The process starts when the sample to be extracted is placed in a chamber and hot vapour is allowed to pass through it. Both water and steam are utilized, but the plant material is not indirect contact with water

(Fatima et al., 2014).Heat which is supplied from the hot vapour aids to open the secretary structure which usually contains aromatic compounds and produces essential oil. When the secretary vessels opens up it releases the aromatic compound in the vapour form and rises along with the steam (hot vapour).As the vapour rises up to the condenser it is condensed by the flow of cold water into the condenser. Finally transforming from gaseous to liquid state and will be collected in a collecting funnel. Previous studies have also mentioned that steam distillation is known as one of the most utilized method for obtaining EO's at large scale; however, this technique which used to be considered reasonable for field operation in the past represents as an expensive method, waste of energy and time due to the increase in the cost of energy and the demand at industry for fast processes.

2.5.3 Solvent Extraction

Solvent extraction refers to the distribution of a solute between two immiscible liquid phases in contact with each other (Cox,2004). It is known as one of the simplest and effective techniques to extract EO. Its main disadvantage is contamination of the sample with the solvent (or impurities in the solvent) which needs to be completely removed either to characterize the olfactory qualities of the oil or to study its biological activity (Fatima et al., 2014). Plant material usually cannot withstand high temperature. This is because high sustained heat which is provided can cause the organic components in the plant to decompose and hence the essential oil cannot be extracted. In this process the sample of material is first washed using solvents such as ether, methanol and hexane. This step allows the organic compounds in the plant material to dissolve into the solvent. The solvent mixture is then filtered and distilled under low pressure to attain its EO.

2.5.4 Soxhlet Extraction

Soxhlet extraction is the removal and recovery of organic analytes from a permeable solid matrix by means of a solvent which is continually evaporated from a still-pot and condensed in such a manner that it falls into and permeates through the matrix which itself is held in a permeable container in a siphonable chamber. Soxhlet extraction has been used for over 120 years (since 1879) and is commonly used as a benchmark for total extractable organic residues. The operation of the Soxhlet extractor is intuitively easy to grasp. Extraction process by using this method is viewed to complete or close to complete.

2.5.5 Ultrasonic-assisted extraction

Ultrasonic extraction is the removal and recovery of organic analytes from a permeable solid matrix by means of a solvent which is energized by sound energy at frequencies in excess of those audible to the human ear. The application of ultrasound as a laboratory based technique for assisting extraction from plant material is widely published. There are also reviews which have been published in the past to extract plant origin metabolites (Knorr,2003) and bio actives from herbs (Vinatoru (2001). The enhancement of extraction efficiency of organic compounds by ultrasound is attributed to the phenomenon of cavitation produced in the solvent by the passage of an ultrasonic wave.

During the application of ultrasound, cavitation bubbles are produced and compressed. The increase in the pressure and temperature caused by the compression leads to the collapse of the bubble. With the collapse of bubble, a resultant "shock wave" passes through the solvent which eventually enhances the mixing (Paniwnyk et al.,2001). Schock waves and powerful liquid micro jets generated by collapsing cavitation bubbles near or at the surface of the sample also accelerates the extraction (Kellner et al., 2004). Moreover, ultrasound also exerts a mechanical effect, which allows greater penetration of solvent into the sample matrix, thus increasing the contact surface area between solid and liquid phase. This coupled with the enhanced mass transfer and significant disruption of cells, via cavitation bubble collapse, increases the release of intracellular product into the bulk medium.

Besides that, according to Paniwnyk et al (2001)and Palma and Barroso (2002)the use of higher temperatures in UAE can also increase the efficiency of the extraction process due to the increase in the number of cavitation bubbles formed. Ultrasonic assisted extraction has many advantages since it can be used for both liquid and solid samples, and for the extraction of either inorganic or organic compounds. The efficiency of the extraction depends on the instrument frequency, and length and temperature of sonication. The benefit of using ultrasonic pre-treatment before extracting oil from seeds of Jatropha curcas L.,and almond and apricot seeds by aqueous enzymatic oil was evaluated by Sharma et al., (2006) and its has shown significantly higher yield with reduction in extraction time.Ultrasonification is rarely applied to large-scale extraction; it is mostly used for the initial extraction of a small amount of material. It is commonly

applied to facilitate the extraction of intracellular metabolites from plant cell cultures (Kaufmann, 2002; Sarker, 2006).

2.5.6 Pressurized Solvent Extraction

Pressurized solvent extraction or ‘‘accelerated solvent extraction,’’ employs temperatures that are higher than those used in other methods of extraction, and requires high pressures to maintain the solvent in a liquid state at high temperatures. It is best suited for the rapid and reproducible initial extraction of a number of samples.In this extraction process the solid sample will be loaded into an extraction cell, which is placed in an oven. The solvent is then pumped from a reservoir to fill the cell, which is heated and pressurized at programmed levels for a set period of time. The cell is flushed with nitrogen gas, and the extract, which is automatically filtered, is collected in a flask. Fresh solvent is used to rinse the cell and to solubilize the remaining components. A final purge with nitrogen gas is performed to dry the material.High temperatures and pressures increase the penetration of solvent into the material and improve metabolite solubilization, enhancing extraction speed and yield. Moreover, with low solvent requirements, PSE offers a more economical and environment-friendly alternative to conventional approaches. As the material is dried thoroughly after extraction, it is possible to perform repeated extractions with the same solvent or successive extractions with solvents of increasing polarity. An additional advantage is that the technique can be programmable, which will offer increased reproducibility(Kaufmann, 2002; Tsubaki, 2010; Sarker, 2006).

2.5.7 Microwave assisted extraction

Microwave-assisted extraction (MAE) or simply microwave extraction is a relatively new extraction technique that combines microwave and traditional solvent extraction.MAE extractive processes are different from those of conventional methods because the extraction occurs as theresult of changes in the cell structure caused by electromagnetic waves. The microwave energy has been investigated and widely applied in analytical chemistry to accelerate sample digestion, to extract analytes from matrices and in chemical reactions.

Application of microwaves for heating the solvents and plant tissues in extraction process, which increases the kinetic of extraction, is called microwave-assisted extraction.According to Perino et al., (2010),the application of microwaves dramaticallyreduces both the extraction time and the volume of requiredsolvent, which automatically aids in reducing environmental burden by diminishing CO2 to the atmosphere. Microwave energy is a non-ionizing radiation that causes molecular motion by migration of ions and rotation of dipoles, without changing the molecular structures if the temperature is not too high. Nonpolar solvents, such as hexane and toluene, are not affected by microwave energy and, therefore, it is necessary to add polar additives.

Microwave-assisted extraction (MAE) is an efficient extraction technique for solid samples which is applicable to thermally stable compounds accepted as a potential and powerful alternative to conventional extraction techniques in the extraction of organic compounds from materials. The microwave-assisted extraction technique offers some advantages over conventional extraction methods. Compared to conventional solvent extraction methods, the microwave-assisted extraction (MAE) technique offers advantages such as improved stability of products and marker compounds, increased purity of crude extracts, the possibility to use less toxic solvents, reduced processing costs, reduced energy and solvent consumption, increased recovery and purity of marker compounds, and very rapid extraction rates.

2.5.8 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is one of the relatively new efficient separation method for the extraction of essential oils from different plant materials.SFE is a promising technique for industrial application (Reverchon,1997). This emergent extraction technique is usually faster, more selective toward the compounds to be extracted, as well as more environmentally friendly when compared to traditional methods. The new products, extracts, can be used for the production of pharmaceutical drugs and additives in the perfume, cosmetic, and food industries. Use of SFE under different conditions can allow selecting the extraction of different constituents.

The main reason for the interest in SFE was the possibility of carrying out extractions at temperature near to ambient, thus preventing the substance of interest from incurring in

thermal denaturation. SFE utilizes the ability of certain gases to behave as nonpolar solvents once acertain temperature and pressure combination has been reached. The most popular gas to be used as a solvent in SFE is carbon dioxide (CO2), because it is nonflammable, noncorrosive, inexpensive,and has generally recognized as safe (GRAS) status. However, extraction by using this technique requires higher investment but can be highly selective and more suitable for food products.

2.6 Melissa Officinallis

Melissa officinallis member of the family Lamiaceae (formerly Labiatae) is a perennial bushy plant and is upright, reaching a height of about 1 m. The soft, hairy leaves are 2 to 8 cm long and either heart-shaped . The leaf surface is coarse and deeply veined, and the leaf edge is scalloped or toothed (Turhan, 2006). It is commonly referred to as 'lemon balm' because of its lemon-like flavour and fragrance (Anonymous, 2003). Lemon balm is widely used in herbal medicine and is native to the eastern Mediterranean region and western Asia (Meftahizade and Sargsyan,2010).

Figure 2.2: Lemon balm

According to Meftahizade et al. (2010), the main constituent of the essential oil are citral (geranial and neral), citronellal, geraniol, beta-pinene, alpha-pinene, beta–caryophyllene, comprising 96% of the oil ingredients. Also Carnat et al. (1998), reported the chemical composition of essential oil of lemon balm, and found that major components are citral representing 48% of the essential oil, followed by citronellal with 39.47% and caryophyllene with 2.37% and in another investigation, the percentage of

the main constitute found by Sarer and Kokdil, are alpha-pinene (2.86%), beta-pinene (11.37%), linalool (2.74%), citronella (5.86%) borneol (0.62%), neral (12.22%), and geraniol (38.13%).

Melissa officinalis essential oils have shown notable biological activities including antiviral, antibacterial, antioxidant, antimicrobial activities (Allahverdiyev et al., 2004; Mimica-Dukic et al., 2004). In addition, it has traditionally been employedas a tonic, antispasmodic, carminative, diaphoretic, and sedative-hypnotic for strengthening the memory (Blumenthal et al., 2000). Currently it is used for the relief of stress-induced headaches and as an antiviral to improve the healing of herpes simplex cold sores. According to Farook et al. (2013), lemon balm also helps to improve cognitive function and decrease agitation in patients with Alzheimer's disease. It has also been reported to possess insect repellent properties (Sharafzadeh et al., 2011) due to the presence of citronella in its chemical constituents.

2.7 Cymbopogan Citratus

Cymbopogan citratus is a herb plant which belongs to the family Poaceae is a genus of about 55 species of grasses, native to warm temperate and tropical regions of the Old World and Oceania. *Cymbopogon citrates*, commonly known as lemongrass and other Cymbopogon species is a tall, clumped aromatic perennial coarse grass that can grow up to 1 m height. The leaf-blade is linear, tapered at both ends and can grow to a length of 50 cm and width of 1.5 cm (Sugumaran et al., 2005). Besides that, lemongrass is also known as barbed wire grass, silky heads, citronella grass or fever grass amongst many others.

Figure 2.3: Lemongrass

Essential oils are natural products obtained from plants. They were formed by varied and complex volatile mixtures of chemical compounds, with predominance of terpene associated to aldehyde, alcohols and ketone which were deposited in various structure of the plant (Linares et al., 2005).Fresh lemongrass contains approximately 0.4% volatile oil and rest of it are non- volatile components and nutritious such as calcium, iron, magnesium, manganese, phosphorus, potassium, selenium and zinc. Its oil has a lemony, sweet smell and is dark yellow to amber and reddish in colour with a watery viscosity.It constitutes mainly citral. Citral of lemongrass is a natural combination of two isomeric aldehydes, namely isomers geranial (α- citral) and neral (β- citral) (Pengelly,2004). According to Schaneberg and Khan,(2002) other unusual active components of the EO are limonene, citronellal, β-myrcene and geraniol.

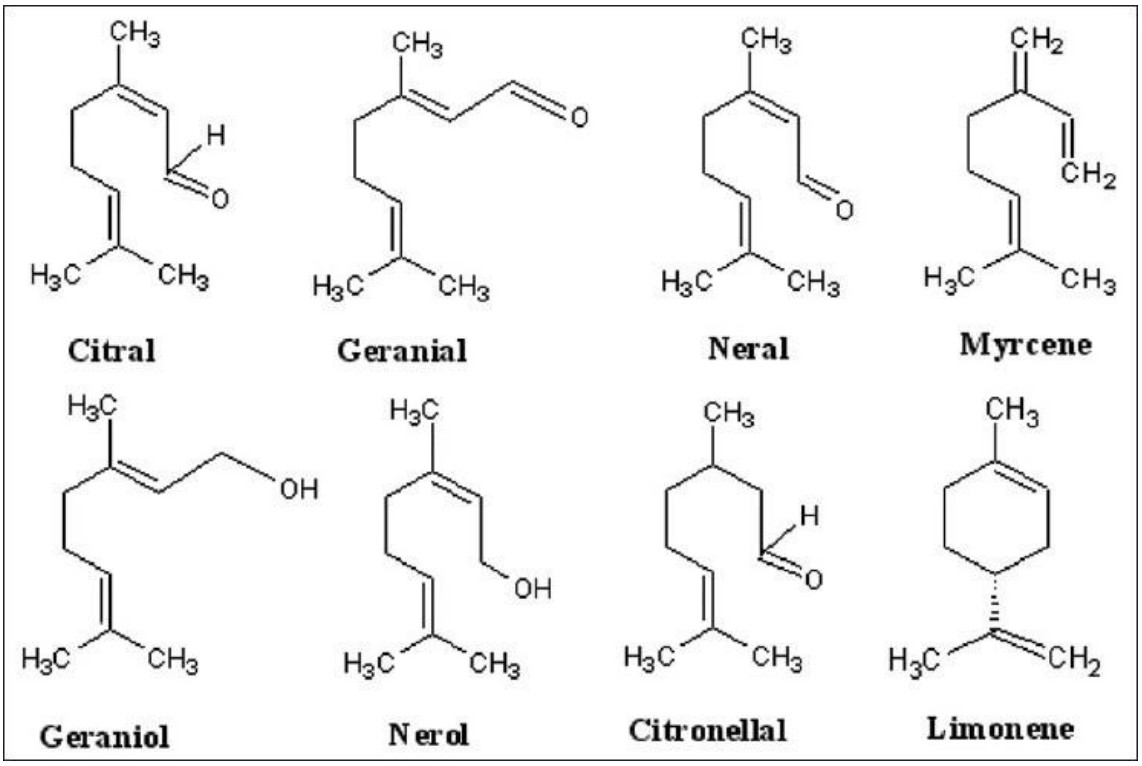

Figure 2.4: Chemical structures of major constituents in lemongrass oil (Shah et.al, 2011)

C. citrates has many uses as it is widely used in cooking, as perfumes due to its sharp lemony smell and it is commonly used in folk medicine for treatment of nervous and gastrointestinal disturbances. However, further bioactive studies has also revealed that various monoterpene constituent of the essential oil contains antifungal, antimicrobial, antibacterial and mosquito repellent properties (Shaneberg and Khan, 2002). Studies by Moore et al., (2007) on *Cymbopogon* plants have stated that the plant has been traditionally used to repel mosquitoes in jungle regions such as the Bolivian Amazon and this genus is also claimed to produce the most used natural repellents in the world (Trongtokit et al., 2005). Extract from *Cymbopogan c.* leaves also demonstrated antispasmodic, analgesic, anti-inflammatory, anti-pyretic, diuretic and sedative properties (Santin et al., 2009).

3 MATERIALS AND METHODS

3.1 Overview

This paper presents on the separation of essential oil from mixtures of *Melissa Officinallis* and *Cymbopogan citratus* leaves using UAE-HD method. Proper step by step experimental procedures will be described clearly in this chapter. Besides that, types of analysis to be carried out on the separated oil are stated too.

3.2 Introduction

This paper presents on the materials used and the precise experimental procedure to achieve the objective of this research.

3.3 Plant Materials

Lemon balm (*Melissa officinallis*) and lemongrass (*Cymbopogan citratus*) leaves were identified and collected from home garden in east of Malaysia. The fresh samples were minced and placed in a sample bag prior to the pre-treatment process. The sample bags are then kept in a cold refrigerator until it is being used for the study purpose. Before running the experiment, 150g of plant materials will be placed into a beaker containing de-ionized water according to its water to raw material ratio for ultrasonic pre-treatment process to be carried out.

3.4 Chemical Used

3.4.1 Dichloromethane

Dichloromethane solvent used for Gas Chromatography Mass Spectrometry (GC-MS) was of HPLC with an analytical grade (purity > 99%).

3.4.2 Diethyl Ether

In this research diethyl ether (purity >99%) is used to separate the extracted oil sample into oil and water phase for easy separation of oil.

3.5 Ultrasound-Assisted Hydrodistillation

Extraction of essential oil from mixtures of lemon balm (*Melissa oficinallis*) and lemongrass *(Cymbopogan citra*tus) leaves were conducted by using Ultrasound-Assisted Hydro-distillation method (UAE). For this distillation process, an ultrasonic bath (model) and Clevenger apparatus is set-up. A total of 150 grams of lemon balm leaves are minced and placed into a beaker containing 600ml of deionised water. The ratio of solid to raw material ratio is 1:6. This solution mixture is then placed into an ultrasonic bath cavity for sonication process to take place at a suggested pre-treatment time which is 30 min at a temperature of 50^{o} C. After the pre-treatment step, the sonicated mixture is removed from the ultrasonic bath and left to cool. Once the mixture cools down, the raw extracts were subjected to hydrodistillation process. The hydrodistillation was carried out by using the Clevenger equipment at 100°C for 5 h. After the extraction process, the extracted EO together with water solution is removed from the collecting vessel and transferred into a separating funnel together with 20ml of diethyl ether and left for a whole day for the oil, water and alcohol mixture to separate and form two distinguished phases namely water phase and mixture of solvent and oil phase. The addition of solvent into the mixture of oil and water is to separate both the phases. The same procedure will be repeated by manipulating the extraction operating conditions which is at different ultrasonic pre-treatment time ,raw material to water ratio and ultrasonic frequency .Then, the same method and operating condition is repeated using a mixture of lemon balm and lemongrass leaves.

3.6 Optimization of oil yield

In order to optimize the extraction operating conditions for achieving maximum oil yield, the study was conducted at three unlike conditions parameters namely at different raw material to water ratio(w/v)(1:2,1:4,1:6,1:8,1:10), different ultrasonic pre-treatment time (30,45, 60,75 and 90 min), and at different frequency of ultrasonic bath(1 kHz,3kHz,5kHz,7kHz and 9kHz). Graphs will then be plotted to analyse the operating conditions for achieving a maximum essential oil yield.

3.7 Analysis for the optimized operating conditions of essential oil

The extracted essential oils are then mixed with diethyl ether, and left to separate into two phases (water and oil miscible with diethyl ether).After separation, water is

removed and the solution mixture is kept in a fume chamber to allow diethyl ether solution to vaporise and left with only oil. The oil left is then weighed and stored in sample vials at 4^0C for the use of analysis. The amount of yield obtained from the extraction was analysed to evaluate the performance of UAE-HD. The yield of oil obtained for every run was then calculated by using Equation (1) (Shaneberg and Khan,2002).

$$\text{Yield of essential oil (\%)} = \frac{\text{amount of essential oil (g) obtained}}{\text{amount of raw materials (g) used}} \times 100\% \qquad (1)$$

3.8 Gas Chromatography Mass Spectrometry (GC-MS) Technique

After obtaining the distilled oil sample from lemon balm leaves and its mixture with lemongrass, the oil samples were characterized using GC-MS Agilent 7890A gas chromatography instrument coupled to an Agilent 5975C mass spectrometer and an Agilent Chem in order to identify the chemical constituents in the essential oil. This is an essential technique to evaluate the quality of the oil samples. The following operating parameters were used: system operating in EI mode (70eV) , equipped with a split/splitness injector (160 ºC, split ratio 120:1) , using DB -5 column (30 x 0.2 5mm i.d x 0 . 2 5 mm) . The temperature is programmed from 60ºC to 130 ºC at a rate of 10 ºC/min to 280 ºCat a rate of 30 ºC /min for 3 min .Injector and detector temperature was 160ºC at 0.2 µL .Helium was used as carrier gas at a flow rate of 0.3 mL/m.

3.9 Bioasssays Repellence Test

The repellence test was carried out in the laboratory using perforated plastic containers. Two plastic containers were prepared and same numbers of mosquitoes were placed in the respective containers. Each container was equipped with four mosquitoes caught around the university compound. One of the container was made as control while in the other container, the extracted oil from mixtures of leaves were introduced by placing a few drops of oil onto a filter paper. The filter paper is then introduced into the container. Both the containers were observed for a time frame of an hour. The repellent activity is then measured by observing the attractancy of the mosquito towards the oil.

3.10 Schematic process flow diagram

Melissa O. and *Cymbopogon C.* leaves are minced and are weighed 150 grams respectively.

Figure 3.1: Mincing sample process.

Mixture of leaves is put into a beaker containing distilled water according to water to raw material ratio. It is and then placed into the ultrasonic cleaner for sonication process.

Figure 3.2: Sonication process using Ultrasonic bath.

The sonicated material is then transferred into a Clevenger apparatus.

Figure 3.3: Clevenger apparatus set-up.

⇩

Analysis of essential oil using GC-MS technique.

Figure 3.4: Analysis using GC-MS.

⇩

Determination of physical properties and active components of essential oil and optimum operating parameters for a maximum oil yield.

⇩

Repellence test.

Figure 3.5: Repellence test on mosquitoes

4 RESULTS

4.1 Overview

This paper presents the results obtained after carrying out research on the performance of UAE-HD method on the extraction process of essential oil from lemon balm and a mixture of lemon balm and lemongrass leaves at three unlike parameters. The data's are plotted to depict its relationship with one and another parameter. Based on the results obtained the best operating condition which gives a maximum yield is chosen. Further, analyses performed on the essential oil yield are included and its results are discussed and compared with literature studies. Finally, a repellence test is carried out.

4.2 Introduction

This paper discusses the physical properties of essential oil, chromatogram of analysed essential oil using GC-MS and the effects of three different parameters namely ultrasonic pre-treatment time, raw material to water ratio and ultrasonic frequency on the essential oil yield.

4.3 Characterisation of extraction yield

Table below depicts the physical properties for essential oil extracted from mixtures of leaves.

Table 4.1 : Physical properties of essential oil from mixtures of *Melissa officinalis* and *Cymbopogan citratus* leaves

Physical properties	Standard		This work (mixture)
	Melissa o.	*Cymbopogan c.*	
Colour	Light yellow	pale yellow	pale yellow
Smell	lemony smell	lemony scent	lemony smell
Specific gravity	0.88-0.92	0.885-0.905	0.89

From the tabulated results, it shows that the physical characteristics of the essential oil from mixtures of essential oil extracted from *Melissa officinalis* and *Cymbopogan citratus* leaves do fulfil the properties possess by the standard essential oil of both the leaves.

Figure 4 presents chromatogram of essential oil separated from a mixture of *Melissa officinalis* and *Cymbopogan citratus* leaves.

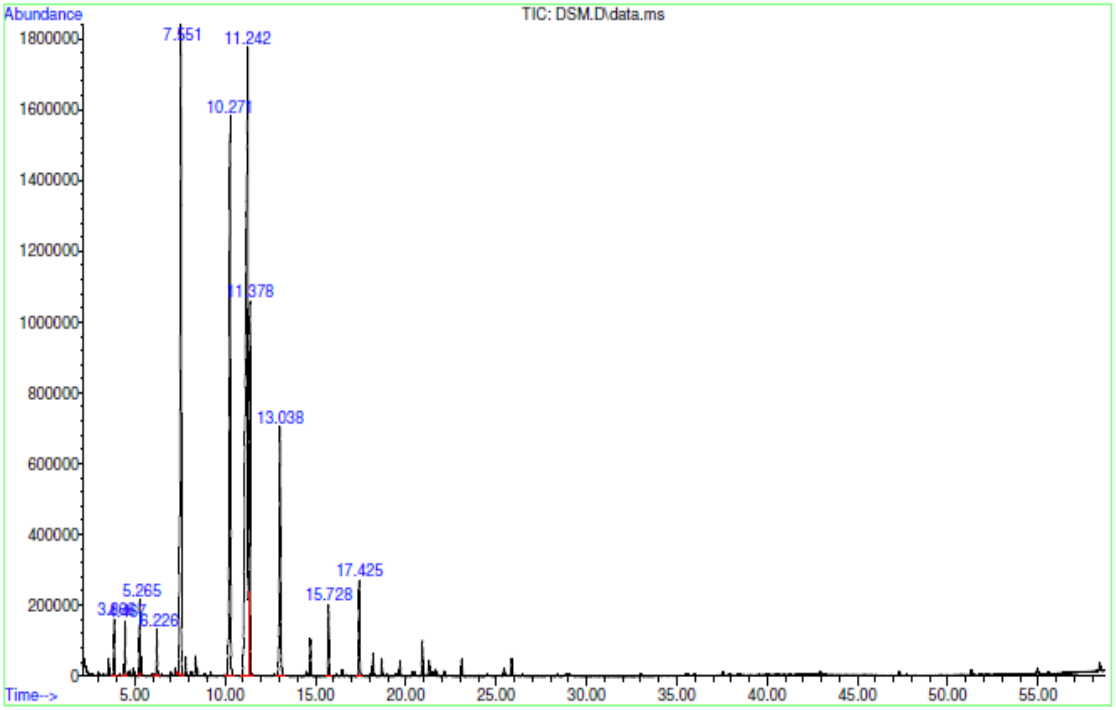

Figure 4.1 : GC-MS chromatogram for mixture of *Melissa officinalis* and *Cymbopogan citratus*leaves.

From the chromatogram shown above, it reveals that the oil was characterized with a higher percentage of monoterpenes, especially oxygenated monoterpenes in which geraniol (peak7-37.21%), citronellyl isobutyrate (peak 6-17.82%), citronellol (peak 5-16.98%) and geranial (peak 8-11.20%) which comprising of citral and neral were accounted as the major components. In contrast, the sesquiterpene fraction was lower, the hydrocarbons represented by α-terpinene (peak 3-1.28%) and caryophyllene (peak 112.69%) were detected in a higher concentration than the oxygenated sesquiterpenes, such as caryophyllene oxide.

Figure 5 presents chromatogram of essential oil separated from *Melissa officinalis leaves.*

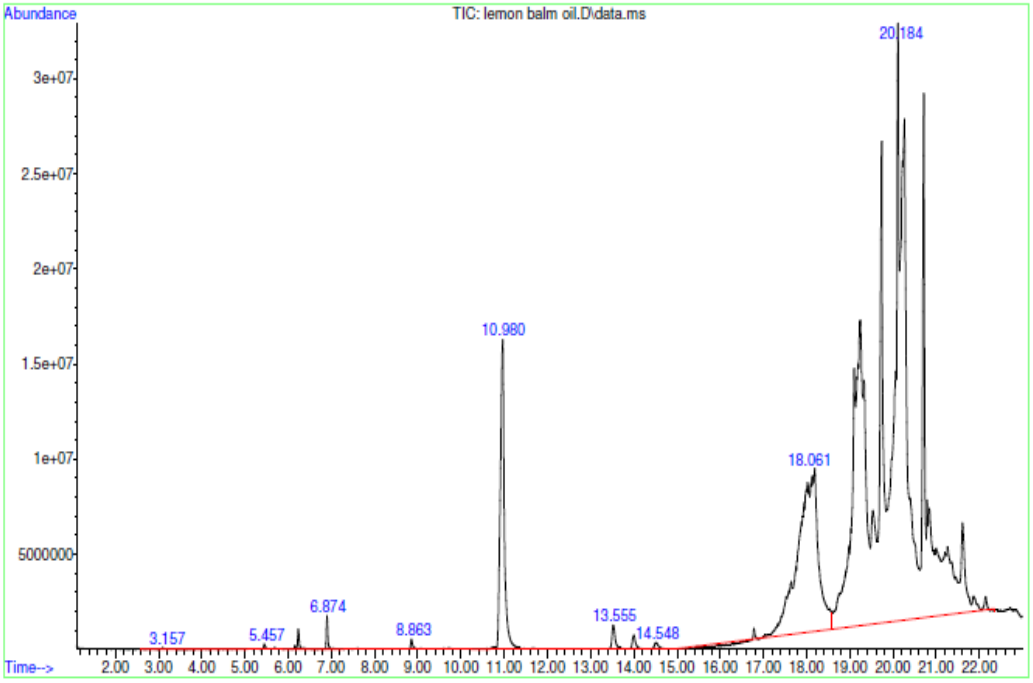

Figure 4.2: GC-MS chromatogram for *Melissa officinalis*

It shows that carvacrol (peak 5) followed by β-caryophyllene (peak 6) and gamma-terpinene(peak 3) dominated the extracted oil. However after carrying out the analysis, it revealed that there is no any traces of citronellol , neral , geranial and other monoterpenene and sesquiterpenene found in the separated oil as mentioned in the literature. Likewise, studies by Basta et al. (2005) reported that caryophyllene oxide (12.6 %) and β-pinene (18.2 %) were also the most abundant constituents in the oil of *M. Officinalis* from Greece but neral and geranial were not detected in the oil. Oils from Cuba (Pino et al., 1999) and Brazil (Da Silva et al.,2005) were dominated by neral (29.9 % and 39.3 %) and geranial (41.0 % and 47.3 %) respectively. A low content (0.2 %) of citronellal was found in leaves of Cuba (Pino et al., 1999) and it is not detected in oil from Brazil (Da Silva et al., 2005). Hence, it can be concluded that volatiles contained in leaves of *M. officinalis* which grows in the East of Malaysia shows similitude and differences with composition of oils from different countries in the world and is not necessary to possess similar constituents as the climate difference may influence the chemical composition of the essential oil.

4.4 Factors affecting extraction yield of oil

4.4.1 Ultrasonic pre-treatment time

Figure 4.3shows the yield of oil attained from lemon balm *(Melissa officinallis*) and mixtures of lemongrass (*Cymbopogan citratus*) and lemon balm *(Melissa officinallis*) leaves at different pre-treatment time(30 min, 45min, 60min, 75min, and 90 min) in a fix raw material to water ratio of 1:6 and ultrasonic frequency of 5kHz.

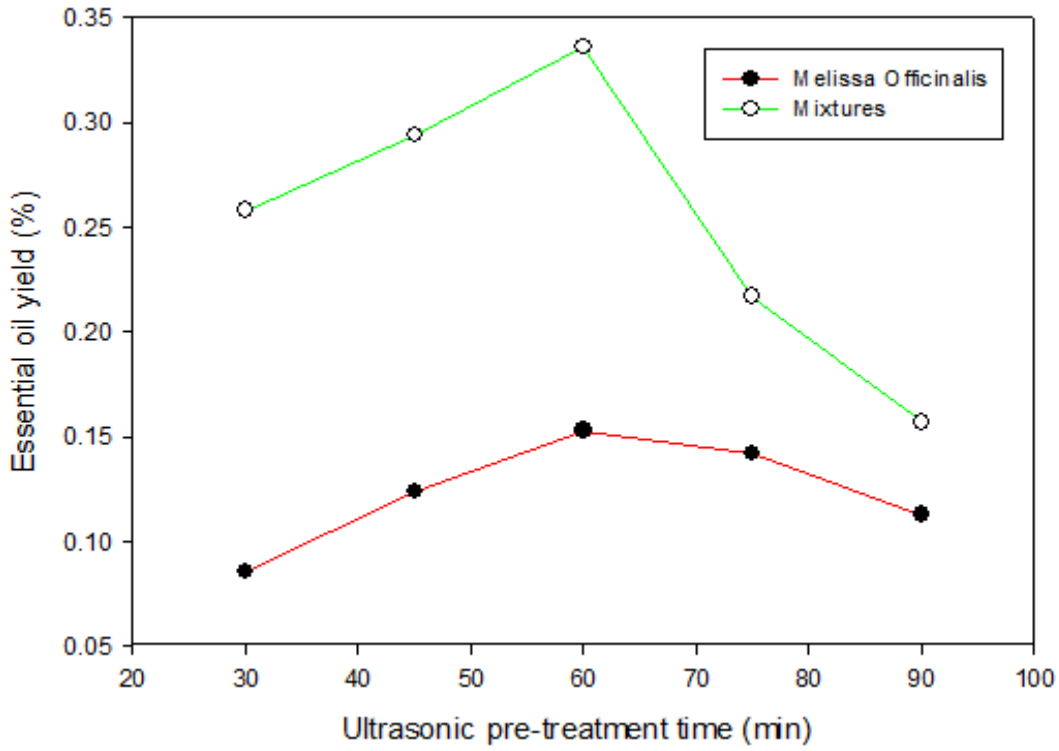

Figure 4.3: Essential oil yieldat different ultrasonic pre-treatment time using 5kHz and 1:6 ratio.

From the result, it clearly indicates that the extraction yield increased with increasing ultrasonic pre-treatment time. However, increasing pre-treatment time of more than 6o minutes tended to decrease the yield. The efficacy of the ultrasonic pre-treatment on the plant material was exhibited at the 60th minute .Similar results were obtained for lemon balm and the mixture of leaves by recording its highest extraction yield of 0.153% and 0.336%, respectively. It is observed from the trend line that, most of the oil was extracted after pre-treating the leaves samples for more than 45 minutes in the ultrasonic bath. The yield obtained was 0.086%, 0.124%, 0.153%, 0.147% and 0.113% at different pre-treatment time for lemon balm leaves.

Where else, for mixtures of leaves the extraction yield increased from 0.258% (30min) to 0.294% (45min) and eventually rising up to a maximum yield of 0.336% at 60 minutes. However, after the pre-treatment time was prolonged to 75 minutes and 90 minutes it showed that the extraction yield started decreasing significantly with a yield reading of 0.2170% and 0.1570%, respectively. Similar results were obtained in orchid seeds with ultrasonic pre-sowing treatment (Shin et al,2011). This could be due to the decomposition of the extracts by prolonged sonication or due to the initial rinsing effect of sonication, which facilitated the release of most of the active constituents inside the cells to the water (Annegowda et.al,2010) during the first 60 minutes. Therefore it can be concluded that ultrasonic pre-treatment (sonication) can increase extraction yield. Sharma and Gupta (2004) also found that ultra-sonication was a critical pre-treatment to obtain high yields of oil from almond, apricot and rice bran.

4.4.2 Raw material to water ratio

Figure 4.4 presents yield of oil obtained by manipulating raw material to water ratio (1:2, 1:4, 1:6, 1:8 and 1:10) by keeping the ultrasonic frequency constant at 5 kHz and the ultrasonic pre-treatment time at 60 minutes. The amount of raw material used is constant throughout the study and only the volume of water is varied. For every 150g of raw material used, 300ml of water will be used in the extraction process. Where else for the mixtures of leaves, a total of 300 g of raw materials will be used in which lemon balm and lemongrass leaves weighs 150g, respectively.

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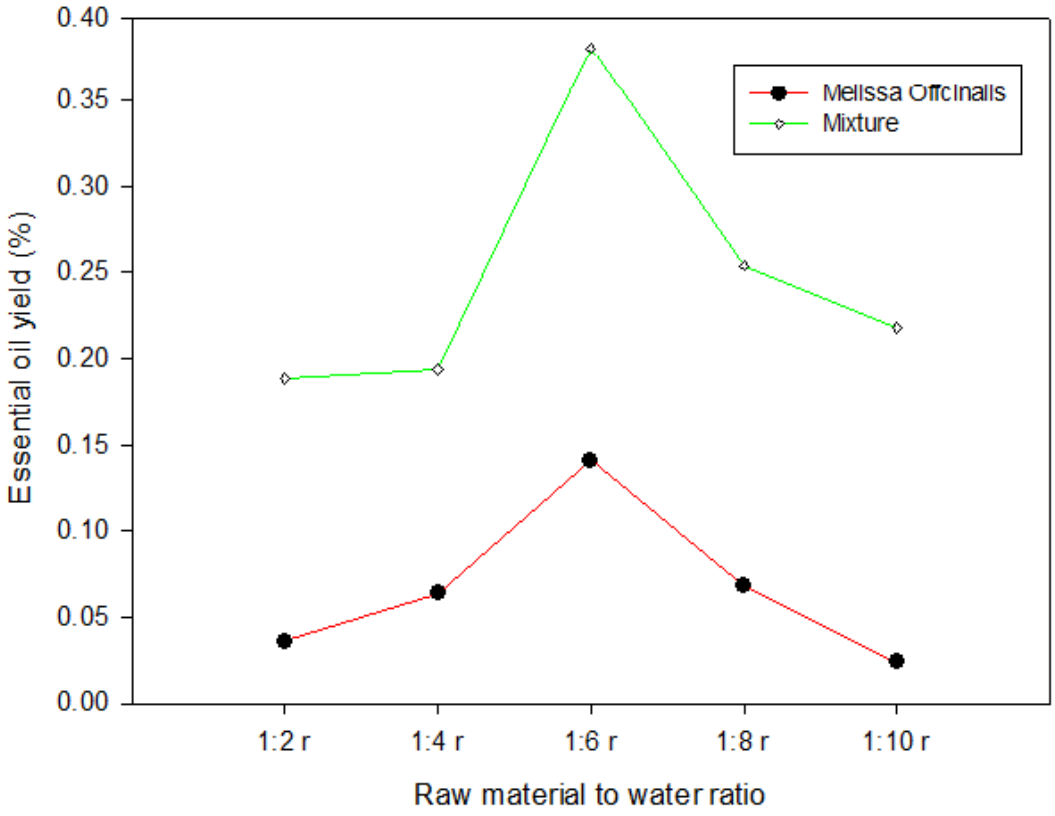

Figure 4.4: Essential oil yield at different water to raw material ratio using 60 min pre-treatment time at5kHz.

From the figure illustrated above, it can be said that lemon balm and mixtures of leaves shows similar trend upon manipulating the raw material to water ratio. Raw material to water ratio of 1:6 was resulted as the highest extraction yield for both lemon balm leaves and its mixture. As the raw material to water ratio was increased from 1:2 to1:4 and 1:6, the essential oil yield was found to also increase from 0.0360 to 0.0640 and 0.1410%, respectively. The extraction yield for lemon balm increases as the raw material to water ratio increases yet at a ratio of 1:8, the yield declined drastically to 0.0680% and followed by 0.0240% at 1:10 ratio. Therefore, the solvent-to-solid ratio of 1:6 (v/w) was found to be an optimum ratio for this study. For the mixture of leaves as depicted above, the extraction yield increased from 0.1887% and recorded a maximum yield at 0.3820%. However, the yield started dropping to 0.21800% at a ratio of 1:10. Thus, it can be concluded that the yield from plant materials increases when the amount of water which acts as a solvent is at its optimum level and beyond its ideal state yield of oil will decrease because the reduction would minimize the degradation, transesterification or oxidation process in plant material (Ranitha,2013).

4.4.3 Ultrasonic frequency

Effect of ultrasonic frequency (1 kHz, 3 kHz, 5 kHz, 7 kHz and 9 kHz) on the extraction yield of lemon balm and its mixture with lemongrass was studied by maintaining at ultrasonic pre-treatment time of 60 minutes and raw material to water ratio of 1:6 as shown in the Figure 4.5.

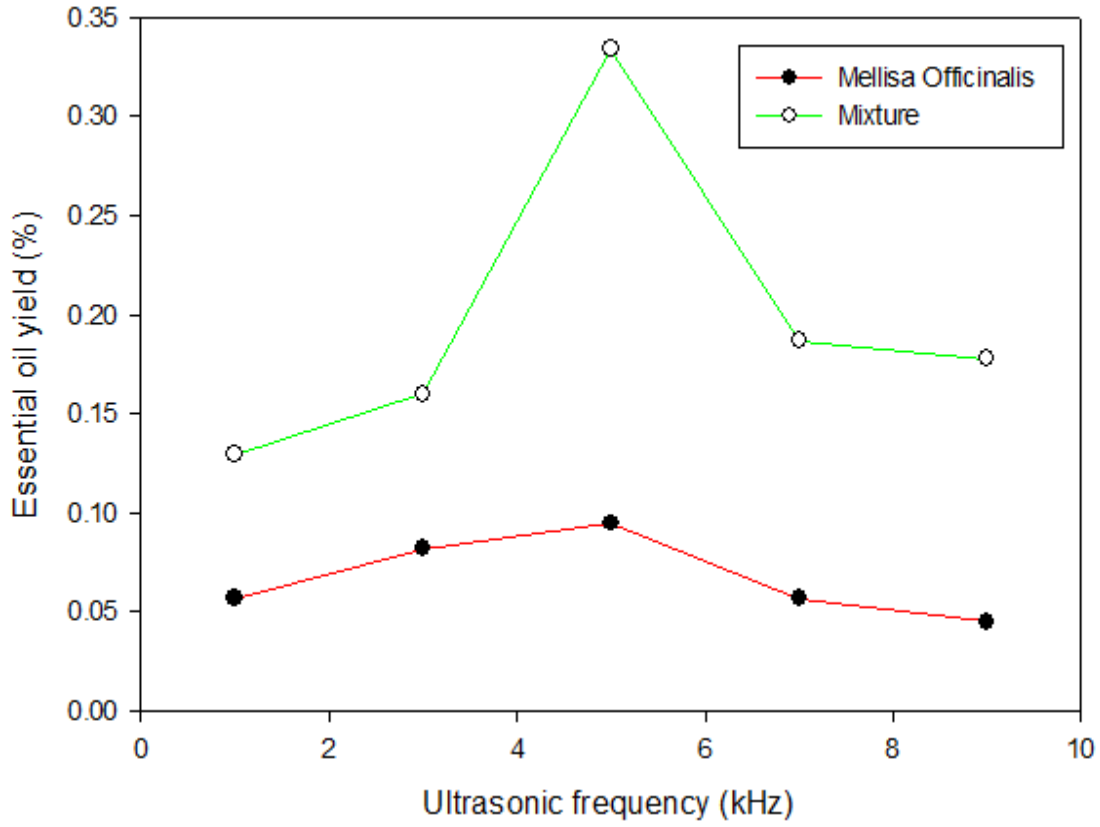

Figure 4.5: Essential oil yield at different ultrasonic frequency with 60min of pre-treatment time and 1:6 ratio.

From the graph it can be seen that both the graphs shows similar trend in which the yield increases up to an ideal frequency and beyond the point, extraction yield begin to show a decrement. The yield for lemon balm *(Melissa Officinalis*) are 0.0570% (1kHz), 0.0820% (3kHz), 0.0950% (5kHz), 0.0570% (7kHz) and 0.0450% (9kHz).While the extraction yield for mixture of leaves up to a frequency of 5kHz are 0.1295%, 0.1599% and 0.3340% ,respectively. Further increase in the ultrasonic frequency above 5kHz resulted in a reduced extraction yield of 0.1870% (7kHz) and 0.1780% (9kHz).This reveals that a frequency of 5kHz found to be the an optimum value for this study.

4.5 Mosquito Repellence Test

After chemical composition analysis, the oil sample was then tested for its repellence against mosquitoes. After introducing the oil into the container, it was observed that the mosquitoes started to move in all direction and when it flies towards the filter paper, the mosquitoes starts to change its direction and move away. In contrast, mosquitoes which were kept as control did not show much movement. After half an hour, it was noticed that out of four mosquitoes one of it started to fall and eventually collapsed. Another mosquito which accidently fell on the filter paper showed no movement and died. After an hour all the mosquitoes was found to be lying on the container and found dead. While the mosquitoes kept as control remain the same and was found dead after eight hours. Hence this repellence results shows that the separated oil managed to repel mosquitoes.

4.6 Summary

From the study, it has been proven that ultrasonic pre-treatment time, raw material to water ratio and ultrasonic frequency do influence the extraction yield of the separation process .Therefore, the optimum condition to attain a maximum yield for the separation of essential oil from *Mellisa officinalis* and the mixture *of Mellisa officinalis with Cymbopogan citratus leaves* are 60 minutes of pre-treatment time, raw material to water ratio of 1:6 and a ultrasonic frequency of 5kHz, respectively. From the study it has also shown that the maximum yield obtained from mixtures of leaves (0.3820%) is higher compared with only *Mellisa officinalis* leaves (0.1530%).Besides that, analysis carried out using GC-MS method has also shown that the oil extracted from *Mellisa Officianalis* and the mixture *of Mellisa officinalis with Cymbopogan citratus* leaves contains active compounds. After performing repellence test on the mosquitoes it has shown that those identified compounds are biologically effective as mosquito repellents.

5 CONCLUSION

5.1 Conclusion

This research was carried out to evaluate the performance of Ultrasonic Assisted Extraction – Hydrodistillation (UAE-HD) method in the extraction of essential oil from mixtures of *Melissa officinalis* and *Cymbopogan citratus* leaves by examining their yield. Three vital factors which may influence UAE-HD method on the extraction process were studied and optimized based on highest extraction yield from plant materials. Hence, the optimum operating condition which were attained to achieve a maximum yield of 0.1530% for lemon balm (*Melissa officinalis*) and 0.3820% for mixtures of lemon balm and lemongrass (*Melissa officinalis* and *Cymbopogan Citratus)*leaves were at 60 minutes of ultrasonic pre-treatment time, 1:6 ratio of raw material to water and 5kHz of ultrasonic frequency, respectively. The major chemical compounds which were found in the essential oil of lemon balm (*Melissa officinalis*) were gamma- Terpinene, carvacrol and caryophyllene. Wherelse, in the mixtures of leaves (*Melissa officinalis* and *Cymbopogan Citratus*), the main components were geraniol, geranial, citronellyl isobutyrate and citronellal. Even though, the characterization results obtained from both plant materials did not show similar results as provided in the literature yet the repellence test which was carried out against mosquito showed positive outcome. This proves the presence of potentiating repelling chemical compounds in the extracted oil from mixtures of leaves. Based on the results obtained, it can be concluded that improvised UAE-HD method using Clevenger apparatus can be implied for easier extraction process of essential oil and the mixture of plant material chosen effectively repels mosquitoes.

5.2 Future work

While the research work was in progress, some issues were raised regarding other factors which might influence the extraction yield. Those factors were the temperature of solvent used to extract EO, types of solvent used and also the time taken to obtain the EO. Due to shortage of time those factors could not be studied .By investigating those effects thoroughly; a clearer review on the optimal operating conditions for the extraction of EO using UAE-HD method can be gained. Therefore, it is recommended that further study to be done on the factors stated above.

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APPENDICES

A1: Essential Oil Yield for *Melissa officinalis*

Table A1. 1: Essential oil yield at different ultrasonic pre-treatment time.

Time (min)	Solid to water ratio	Ultrasonic frequency	Yield (%)
30	1:6 r	5	0.086
45	1:6 r	5	0.124
60	1:6 r	5	0.153
75	1:6 r	5	0.142
90	1:6 r	5	0.113

Table A1. 2: Essential oil yield at different raw material to water ratio.

Solid to water ratio	Time (min)	Ultrasonic frequency	Yield (%)
1:2 r	60	5	0.036
1:4 r	60	5	0.064
1:6 r	60	5	0.141
1:8 r	60	5	0.068
1:10 r	60	5	0.024

Table A1. 3: Essential oil yield at different ultrasonic frequency.

Ultrasonic frequency	Time (min)	Solid to water ratio	Yield (%)
1	60	1:6 r	0.057
3	60	1:6 r	0.082
5	60	1:6 r	0.095
7	60	1:6 r	0.057
9	60	1:6 r	0.045

A2 : Essential Oil Yield for mixtures of *Melissa officinalis and Cymbopogan citratus* leaves

Table A2.1: Essential Oil Yield at different ultrasonic pre-treatment time.

Time (min)	Solid to water ratio	Ultrasonic frequency	Yield (%)
30	1:6 r	5	0.258
45	1:6 r	5	0.294
60	1:6 r	5	0.336
75	1:6 r	5	0.217
90	1:6 r	5	0.157

Table A2.2: Essential Oil Yield at different raw material to water ratio.

Solid to water ratio	Time (min)	Ultrasonic frequency	Yield (%)
1:2 r	60	5	0.1887
1:4 r	60	5	0.1938
1:6 r	60	5	0.382
1:8 r	60	5	0.254
1:10 r	60	5	0.218

Table A2.3: Essential Oil Yield at different ultrasonic frequency.

Ultrasonic frequency	Time (min)	Solid to water ratio	Yield (%)
1	60	1:6 r	0.1295
3	60	1:6 r	0.1599
5	60	1:6 r	0.334
7	60	1:6 r	0.187
9	60	1:6 r	0.178

A3 : Spectrum of mixture of *Melissa officinalis* and *Cymbopogan citratus* leaves

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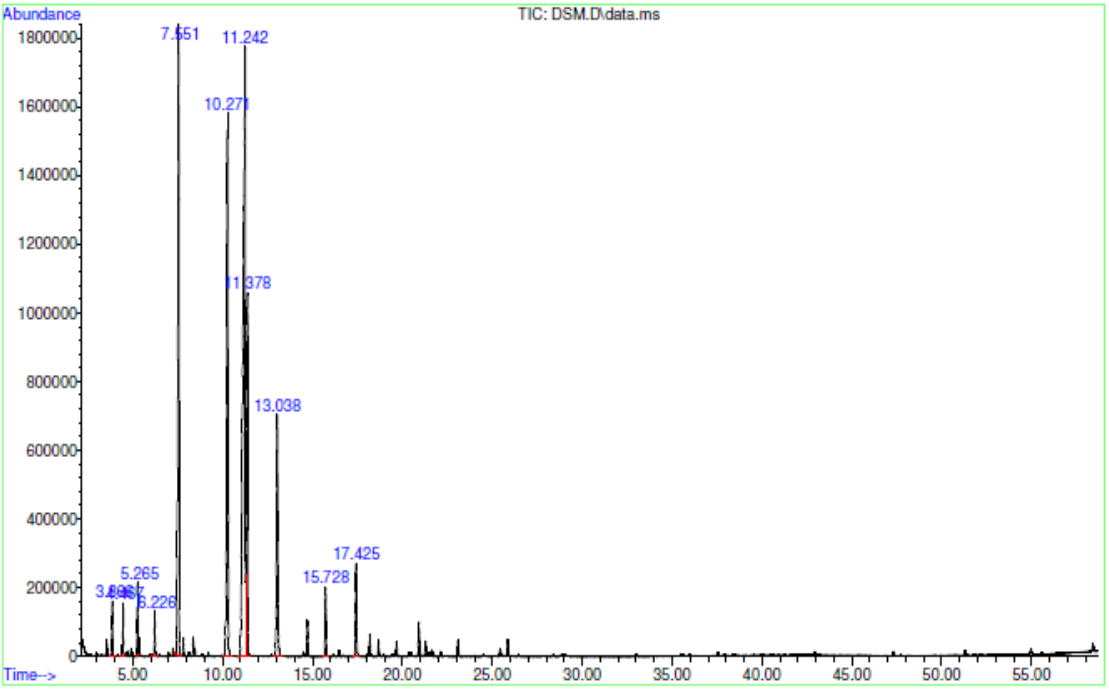
Instrument :GCMSD
Sample Name:
Misc Info :
Vial Number: 1



Library Search Report

Data Path : D:\Data\ashwinder\24nov14lmixture\
Data File : DSM.D
Acq On : 24 Nov 2014 10:36
Operator :
Sample :
Misc :
ALS Vial : 1 Sample Multiplier: 1

Search Libraries: C:\Database\NIST05a.L Minimum Quality: 0

Unknown Spectrum: Apex
Integration Events: ChemStation Integrator - autoint1.e

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
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			.beta.-Pinene	15175	000127-91-3	86
			.beta.-Myrcene	15179	000123-35-3	70
			.beta.-Myrcene	15177	000123-35-3	70
2	4.467	1.06	C:\Database\NIST05a.L			
			Benzene, 1-methyl-2-(1-methylethyl	14428	000527-84-4	95

```
          )-
          Benzene, 1-methyl-3-(1-methylethyl        14424 000535-77-3 95
          )-
          Benzene, 1-methyl-2-(1-methylethyl        14429 000527-84-4 94
          )-

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          methylethyl)-
          1,4-Cyclohexadiene, 1-methyl-4-(1-        15354 000099-85-4 91
          methylethyl)-
          1,4-Cyclohexadiene, 1-methyl-4-(1-        15355 000099-85-4 91
          methylethyl)-

  4   6.2270.94 C:\Database\NIST05a.L
          1,6-Octadien-3-ol, 3,7-dimethyl-         25643 000078-70-6 87
          1,6-Octadien-3-ol, 3,7-dimethyl-         25636 000078-70-6 86
          1,6-Octadien-3-ol, 3,7-dimethyl-,        107591 007149-26-0 53
          2-aminobenzoate

5     7.553 16.98 C:\Database\NIST05a.L
    6-Octenal, 3,7-dimethyl-, (R)-                 25617 002385-77-5 96
    6-Octenal, 3,7-dimethyl-                       25584 000106-23-0 94
    7-Octenal, 3,7-dimethyl-                       25575 000141-26-4 90

  6 10.270 17.82 C:\Database\NIST05a.L
    2,7-Octadiene, 4-methyl-                       10320 1000061-78-0 74
    2-Octen-1-ol, 3,7-dimethyl-                    27105 040607-48-5 45
    Citronellyl isobutyrate                        75860 000097-89-2  43

  7 11.244 37.21 C:\Database\NIST05a.L
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    (E)-
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    (E)-

811.377 11.20 C:\Database\NIST05a.L

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          2,6-Octadienal,     3,7-dimethyl-       24102 005392-40-5 94

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          Phenol, 2-methyl-5-(1-methylethyl)      22815 000499-75-2 95
          Phenol, 2-methyl-5-(1-methylethyl)      22822 000499-75-2
          3-Methyl-4-isopropylphenol              22746 003228-02-2

 10   15.7261.77 C:\Database\NIST05a.L
          2,6-Octadien-1-ol, 3,7-dimethyl-,        54284 000105-87-3
          acetate, (E)-
          4-Hexen-1-ol, 5-methyl-2-(1-methyl       54303 025905-14-0
          ethenyl)-, acetate
```

Library Search Report

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Operator :
Sample :
Misc :
ALS Vial : 1 Sample Multiplier: 1

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Unknown Spectrum: Apex
Integration Events: ChemStation Integrator - autoint1.e

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1117.427	2.69	C:\Database\NIST05a.L				
			Caryophyllene	59797	000087-44-5	
			Caryophyllene	59802	000087-44-5	99
			Caryophyllene	59800	000087-44-5	94

A4 : Spectrum of *Melissa officinalis*leaves.

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Operator: syuhada
Acquired: 16 Oct 2014 15:30using AcqMethod LEMONBALMOIL.M
Instrument :GCMSD
Sample Name: lemon balm oil
Misc Info :
Vial Number: 1
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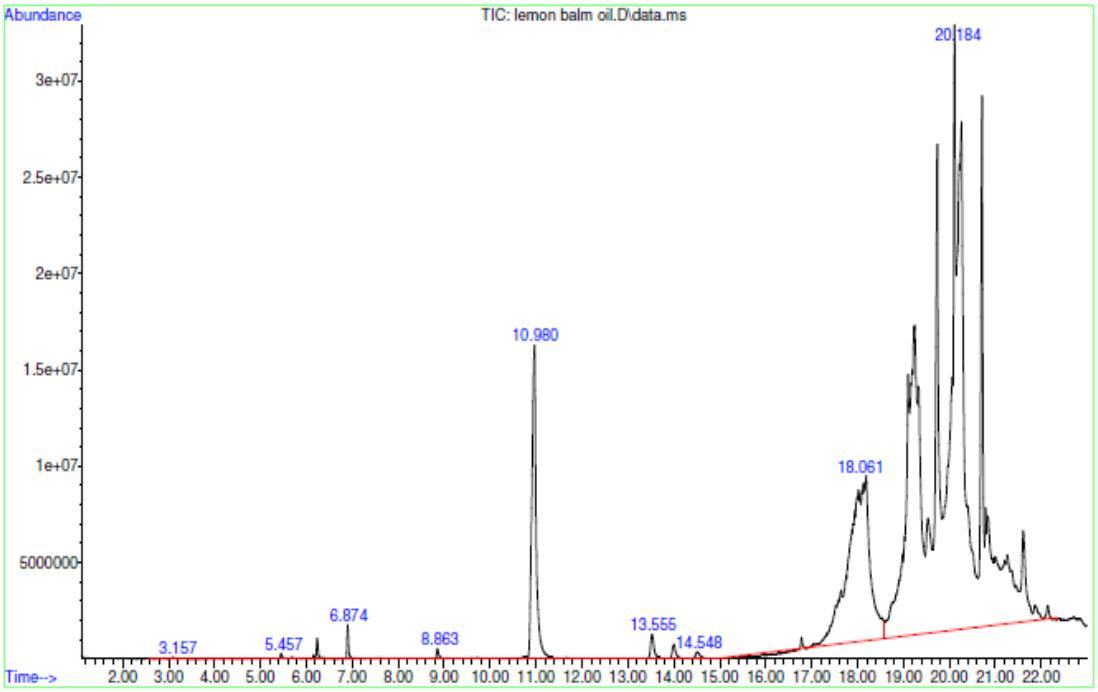


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                                    Library Search Report

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Operator    : syuhada
Sample      : lemon balm oil
Misc        :
ALS Vial    : 1    Sample Multiplier: 1

Search Libraries:      C:\Database\NIST05a.L                Minimum Quality:      0

Unknown Spectrum:Apex
Integration Events: ChemStation Integrator - autoint1.e

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                 1,3-Hexadiene,c&t1178 000592-48-3 18

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                   1-Octen-3-ol                          12054 003391-86-4 64
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			1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	15347	000099-85-4	96
			1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	15355	000099-85-4	96
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			3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	25781	020126-76-5	95
			3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	25784	020126-76-5	94
			3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	25745	000562-74-3	91
5	10.978	6.26	C:\Database\NIST05a.L			
			Phenol, 2-methyl-5-(1-methylethyl)	22815	000499-75-2	94
			3-Methyl-4-isopropylphenol	22746	003228-02-2	94
			Phenol, 2-methyl-5-(1-methylethyl)	22822	000499-75-2	91
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			Caryophyllene	59802	000087-44-5	99
			Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-	59917	242794-76-9	95
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