Faculty of Resource Science and Technology

STUDIES ON ESSENTIAL OILS FROM *CITRUS* SPP.

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DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

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ABSTRACT

Peels and leaves of five species of citrus, *C. grandis, C. medica, C. mitis, C. paradisi* and *C. reticulata* were hydrodistilled using the modified Clevenger type apparatus. The yields of essential oil were ranged from 14.26 % to 27.35 % for peels and 0.41 % to 4.14 % for leaves. The essential oils obtained were analysed by Gas Chromatography/Flame Ion Detector (GC/FID). A total of 47 and 100 components were identified from the oils of peels and leaves, respectively. The major component identified was (+)-limonene for peel while for leaves were limonene, α-terpinene, (Z)-nerolidol, (E)-α-bergamotene and 3,5-octadienone. The relationship among the species was analysed by Hierarchical Cluster Analysis (HCA) of the gas chromatographic data without using any auxilially morphmetric data. The dendogram indicated that the peels essential oils of *C. medica* and *C. grandis* were closely related, while the leaves oils of *C. reticulata, C. grandis* and *C. mitis* found to have similarity. The toxicity test found that essential oils from the leaves of *C. medica* are the higher toxic with LC_{50} at 199 ug/mL due to high percentage of limonene.

Key words: *Citrus* spp., essential oil, GC/FID, (+)-limonene, toxicity.

ABSTRAK

Kulit dan daun bagi lima spesies citrus iaitu C. grandis, C. medica, C. mitis, C. paradisi dan C. reticulata telah disuling hidro menggunakan alat radas Clevenger yang telah diubahsuai. Minyak pati yang diperolehi adalah dalam julat dari 14.26 % ke 27.35 % untuk kulit dan dari 0.41 % ke 4.14 % untuk daun. Minyak pati yang diperolehi telah dianalisis menggunakan Kromatografi Gas/Pengesan Nyalaan Ion (KG/PNI). Sebanyak 47 dan 100 komponen kimia telah dikenalpasti dalam minyak pati dari kulit dan daun, masing-masingnya. Komponen utama dikenalpasti dalam kulit adalah (+)-limonena, manakala limonena, α-terpinena, (Z)-nerolidol, (E)-α-bergamotena dan 3,5-oktadienon adalah komponen utama dikenalpasti dalam daun. Hubungan antara spesies telah dianalisis secara statistik menggunakan Analisis Hierarki Gugusan (AHG) terhadap data gas kromatografi tanpa menggunakan sebarang bantuan data morphometrik. Dendogram menunjukkan bahawa minyak pati dari kulit C. medica dan C. grandis adalah berkait rapat, manakala minyak pati dari daun C. reticulata, C. grandis dan C. mitis didapati mempunyai kesamaan. Ujian ketoksikan mendapati bahawa minyak pati dari daun C. medica adalah lebih toksik dengan LC_{50} *pada 199 ug/mL disebabkan oleh peratus limonena yang tinggi.*

Kata kunci: Citrus spp., minyak pati, KG/PNI , (+)-limonena, ketoksikan.

CHAPTER ONE

INTRODUCTION

Volatile oils or essential oils are defined as complex mixtures of odorous and steam-volatile compounds, which are excreted by glandular hairs or deposited in the plant body cell organelles (Mahran, 1991). They are natural oils with distinctive scents secreted by the glands of aromatic plants (Isaacs *et al.*, 1999). Essential oils differ from fixed oils, which are mainly triglycerides of fatty acids. They have been extracted and used for flavouring, incense, and medicinal purposes for many centuries (Parker, 1993).

Citrus belongs to the family Rutaceae, subfamily Aurantioideae, which comprises 33 well-known and well-described genera and 203 species. The genus *Citrus* provides nearly all of the commercial cultivars grown throughout the world (Forsyth and Cope, 1993). This genus includes several important citrus fruits such as oranges, mandarins, limes, lemons and grapefruits (Kale and Adsule, 1995). Different species of citrus fruits have different chemical compositions. The principal constituents of the edible portions in the sweet citrus fruits are sugars (glucose and sucrose) and acids (primarily citric acid and little of malic acid), while the sour citrus fruits contain primarily the acids in the fruit juice. The rind (peel) of citrus fruits contains certain glucosides, such as herperidin in oranges and lemons and naringins in grapefruit (Ghosh, 1990).

The essential oils of citrus fruits have been used both in the pharmaceutical and food industries as an aroma and/or flavouring agent (Prasad and Mustaffa, 1993). There is lack of previous studies on the essential oils of *Citrus* spp. was found in the literature. Therefore, this study is considered necessary to determine whether chemical compositions may have significance chemotaxonomical values and its biological activity against *Artemia salina*.

The main objectives of this study are:

i) To extract and identify the chemical composition of essential oils from citrus fruit (peel and leaves) by using GC data.

ii) To carry out chemometric analysis by using hierarchical cluster analysis (HCA) to find out whether the essential oils can be used as chemotaxonomical tool for *Citrus* spp., and

iii) To determine the toxicity of essential oils from *Citrus* spp.

CHAPTER TWO

LITERATURE REVIEW

2.1 Essential Oils

Essential oils are generally understood to be volatile compounds, which are freely soluble in alcohol, ether, and vegetable oils and are usually assumed to be the result of distillation or steam stripping process. The most common essential oil used as a food aroma and flavour is orange essence (Hernandez, 2000). Most of the chemical components of essential oil are sensitive to light, heat, air and water. Thus, they need to be stored under carefully controlled conditions (Sankarikutty and Narayanan, 1993). An essential oil is internationally defined as the product obtained by steam distillation, hydrodistillation or expression (for citrus fruits) of a plant or a part of it (Bicchi, 2000). Extraction of volatile oils by hydrodistillation has long been practised in many African countries (Mahran, 1991). Other methods used for the selective extraction of volatile oils (essential oils) are advanced phytonics method, supercritical fluid extraction and extraction into fixed oils. However, steam distillation is the most popular method for the extraction of volatile oils from plant material (Houghton and Raman, 1998).

2.2 Importance of *Citrus* spp.

Citrus is the most important genus of the family Rutaceae. It consists of some 20 species with edible fruit, all rich in vitamin C, flavonoids and essential oils (Pamplona, 2001). The oil sacs in the peel of citrus fruits contain the bulk of citrus essential oils with much less being found in the juice sacs (Hui, 1992). The rind of citrus fruits is rich in pectin and certain essential oils. The flowers, leaf and rind of citrus contain oils of good fragrance and have good commercial value. Lemon and orange oils are most important citrus oils used for flavouring purposes, followed by lime, grapefruit and tangerine oils (Ghosh, 1990). Flavonoids are abundant in citrus, having remarkable taste and nutritional properties. Flavonoids occur as flavanones, flavones, and anthocyanins, the latter of minor importance in citrus and occurring only in blood oranges (Hui, 1992). Citrus fruits such as lemons, grapefruits, and limes are important sources of several vitamins, minerals and trace elements, as well as some essential oils, which are extensively used in the pharmaceutical industry (Prasad and Mustaffa, 1993). Citrus fruits are remarkable for their balanced combination of anticarcinogenic substances such as vitamin C, flavonoids, limonoids, and pectin (Pamplona, 2001). Limonoids are among the many aromatic substances primarily found in the peel of citrus. Chemically these are terpenes, the most abundant of which is d-limonene. Studies on animals have demonstrated that it is capable of neutralizing carcinogens, which cause stomach cancer and breast cancer. Meanwhile, orange peel contains a very aromatic essential oil that is rich in flavonoids. Orange peel extracts is used to protect the vascular system, improve the appetite, and calm the nerves (Pamplona, 2001).

2.3 Hydrodistillation

Essential oils are classically obtained by steam or hydrodistillation via equipment based on the circulatory distillation apparatus introduced by Clevenger in 1928 (Bicchi, 2000). Suitably ground plant materials are placed in a boiler with completely covering them. As heat is slowly applied, steam alone will be initially formed and the distillate will be clear. With continued heating the essential oils start to distill over with the steam and the distillate becomes milky white. Distillation is continued until the distillate becomes clear, with no more oil distilling over from the material. Even though the essential oils have relatively high boiling points, codistillation like this brings about a satisfactory recovery of the oil because, in accordance with Dalton's law, a mixture boils when the sum of the vapour pressures of the individual component equal as the atmospheric pressure (Sankarikutty and Narayanan, 1993).

2.4 Gas Chromatography

Components of an essential oil are generally medium to highly volatile with medium to low polarity, and as a consequence gas chromatography (GC) is the technique of choice for their analysis (Bicchi, 2000). Gas chromatography is a highly appropriate technique for quantitative analysis of complex mixtures. For example, GC has been utilized in the analysis of amino acids or minor components such as vitamins, flavonoids and phenolic compounds. While the use of GC in combination with MS has been of fundamental importance in analyzing flavour and aroma compounds and has made possible the successful identification of many new compounds (Hernandez and Juarez, 1993). Consequently, GC-mass spectrometry (GC-MS) is most frequently and effectively used to identify the essential oil constituents by using data base libraries of both retention indices and mass spectral fragmentation patterns (Nakatsu *et al.*, 2000). Meanwhile, there are different systems for calculating the results of chromatographic data. Normalization is used to compensate for difference in components responses by correcting peak area measurements with response factors based on a major component. The response factors are calculated by injecting known quantities of pure components mixed in a proportion similar to that of the sample and then analysed under the same chromatographic conditions (Hernandez and Juarez, 1993).

2.5 Chemometric Analysis

Chemometrics is the use of statistical and mathematical techniques to analyze chemical data. There are many techniques in chemometrical tool such as Hierarchical Cluster Analysis (HCA) and Principal Components Analysis (PCA) (Beebe *et al.*, 1998). The uses of chemometric data in the processing of essential oils are to monitor the progress of mixtures of oils in foods, cosmetics and pharmaceuticals (Hibbert, 1997). PCA is successful in analyzing multivariate data, since it can investigate relationships between large numbers of variables, and it is useful for reducing the numbers of variables in a data set by finding linear combinations of those variables that explain most of the variability. These characteristics make PCA successful in comparing and discriminating groups of essential oils (Bicchi, 2000). Cluster analysis tries to group objects by their proximity in this variable space. A typical aggregation method would have the following step: find the two objects closest together and place them by a cluster with coordinates midway between them. This step is repeated until the entire set is clustered. The groupings that build up may be displayed as a graph, called a dendogram, showing the groups as a function of similarity. In the essential oil literature where hierarchical clustering is one of the most commonly used pattern recognition methods (Hibbert, 1997).

2.5 Biological Activity

The action mode of the toxicity for citrus peel oils to several insect species has recently been investigated. The results indicated that an efficient way to use citrus peel essential oils to control insects would be as a fumigant in relatively enclosed or air-tight systems (Nakatsu *et al.*, 2000). Limonoids such as azadirachtin and gedunin, present in species from the Meliaceae and Rutaceae are recognized for their toxic effects on insects and are used in several insecticide formulations in many parts of the world (Dua *et al.*, 1995; Nagpal *et al.*, 1996). In additions, limonene is known to have Glutathione S-Transferase (GST) and anti-carcinogenic activity. It is also found to be bioactive against fungi (Nakatsu *et al.*, 2000).

CHAPTER THREE

MATERIAL AND METHODS

3.1 Sampling

The samples of *Citrus* spp. were collected randomly from Agriculture Research Center (ARC) at Semongok, Kuching. Five species of citrus fruits were studied which are *C. paradisi, C. reticulata, C. grandis*, *C. mitis*, and *C. medica*. This study was focus on rind (peel) and the leaves of citrus fruits.

3.2 Extraction of Essential Oils

The extraction of essential oils was carried out using a modified Clevenger type apparatus based on Lee and Ogg method (Datta, 1987). 100 g of fresh sample were transferred to a 2 L flat bottom flask and mixed with 1.5 L of distilled water. The flask then was assembled to the clevenger trap and connected to the condenser. Hydrodistillation was carried out for 5 hours. The flask was heated on hot plate to maintain the distillation rate of two drops per second. After 5 hours, the oil, which trap in the clevenger were then cooled to room temperature. The oily layer was separated and dried over anhydrous sodium sulphate and stored at 4 – 5^{o}C. The hydrodistillation process was repeated for two times and the average yield (v/w) of the oils was determined.

3.3 Analysis of Essential Oil

3.3.1 Gas Chromatography- Flame Ion Detector (GC/FID)

The oils was analyzed using the Hewlett Packard gas chromatogram system 6890 series equipped with a FID detector using a fused silica DB-5 capillary column (25 m x 0.22 mm x 0.25 μm). Nitrogen was used as the carrier gas with velocity of 2 ml/min. The initial temperature was programmed at 50°C and hold for 2 minutes. The temperature was then increased to 250°C at a rate of 10.0°C/min. The final temperature was hold for ten minutes. The temperature for the injector and detector were set at 280°C and 320°C respectively. The components were identified by comparing their Kovat's retention indices published in literature (Acree and Arn, 2004) using similar DB-5 capillary column.

3.3 Qualitative and Quantitative Analysis

3.4.1 Percentages of Essential Oils

The yield of the essential oil was calculated based on the dry weight of plant material and the volume of oils. The equation used for calculation is as following:

$$\% \text{ of Essential Oil} = \frac{V}{W} \times 100$$

where,

V = volume of essential oil

W = weight of dry sample

3.4.2 Qualitative Analysis

Gas chromatographic data was analyzed to identify the chemical components in the essential oils using Kovat Index (Braun, 1987) calculated using the following equation:

$$K.Ix = 100\left[\frac{\log tRx - \log tRn}{\log tRn+1 - \log tRn}\right] + K.In$$

where,

tR_{n+1} and tR_n = retention time of alkanes with n+1 and n number of carbon, respectively.

tR_x = retention time for component x

3.4.3 Semi-Quantitative Analysis

The percentage of individual chemical components in the essential oils was calculated using the normalization method (Miller, 1988):

$$\%X = \frac{Ax}{\sum Ai} \times 100\%$$

where,

Ax = peak area of chromatogram for X component

$\sum Ai$ = sum of all the peak areas of chromatogram

3.5 Chemometric Analysis

3.5.1 Hierarchical Cluster Analysis (HCA)

Hierarchical Cluster Analysis was performed to decide whether the component was related to each other so that the chemical profile can be used in chemotaxonomy classification. This analysis was performed by using SPSS ver. 11.0 for windows.

3.6 Bioassay

3.6.1 Toxicity against *Artemia salina*

The biological activity testing against the larvae of *A. salina* was carried out using a protocol established by McLaughlin *et al.* (1991). The brine shrimp eggs were hatched by placing 20 g of *A. salina* eggs into 9 L of seawater in an aquarium. These aquariums were supplied with continuous airflow to make sure the hatching was successful. The test samples prepared by dissolving separately 20 mg of each extract in 2 mL of methanol. 500, 50 and 5 μL samples from these solutions were transferred to vials in duplicates. The solvent was then removed under high vacuum rotary evaporator. Subsequently, the samples were then diluted with 5 mL seawater resulting the final concentration of 1000, 100 and 10 μg/mL, respectively. 10-second instar larvae of *A. salina* was then placed in each solution. After 24 hours, the numbers of survivors were counted and the LC_{50} was calculated. Seawater and 10-second instar larvae of *A. salina* (without sample) was used as control.

CHAPTER FOUR

RESULT AND DISCUSSION

4.1 Yields of essential oils

The average percentage of essential oils is calculated over two replicates. Percentages of essential oils for peel and leaves for five *Citrus* spp. are given in Table 1. Both peels and leaves of *C. mitis* gave the highest percentage of essential oils at 27.35% and 4.14%, respectively. The lowest percentage of essential oils for peel is *C. paradisi* while the leaves of *C. grandis* show the lowest percentage of essential oils.

Table 1: Yields of essential oils from peel and leaves of *Citrus* spp.

Citrus spp.	Percentages of essential oils (v/w)	
	Peel	**Leaves**
C. grandis	15.68	0.41
C. medica	27.29	3.71
C. mitis	27.35	4.14
C. paradisi	14.26	2.10
C. reticulata	20.37	2.72

4.2 Chemical Composition of the Essential Oil

The component of the essential oil from *Citrus* spp. such as monoterpenes, sesquiterpenes, esters, aldehydes, ketones, alcohols and acids were identified by using Kovat Index calculated based on the retention time of GC/FID data. The volatile components identified are shown in Table 2 and Table 3.

The chromatograms of essential oils from peel of five *Citrus* spp. are shown in Figure 1 to Figure 5. The figures showed that monoterpene is the most abundant component with (+)-limonene as a major component with percentage ranged from 81.8 – 97.1%. This finding agreed with the previous published data which (+)-limonene was predominant compound in the mature fruit (Attaway *et al.*, 1968). It is also interesting to find that only δ-terpinene is found in all the species with percentage ranged from 0.30 – 0.91%.

The chemical composition analysis from peel also showed some components that were only present in certain species. β-pinene is one of the chemical components, which is found in *C. medica, C. paradisi* and *C. mitis* with 0.3%, 1.3% and 0.31% respectively. While *C. grandis* have high percentage of limonene oxide with 2.08 % followed by *C. paradisi* with 0.54%. However, no limonene oxide was found in the essential oils of other species.

Table 2: Chemical components in essential oils from peel of *Citrus* spp.

	Kovat Index		% of Individual Chemical Component				
Components	**DB-5[a]**	**DB-5[b]**	**C.A**	**G.A**	**K.A**	**L.A**	**P.A**
Nonane	900	900	0.81	-	0.31	0.3	0.31
2-hepten-1-ol	953	953	0.61	-	0.52	0.61	0.42
Heptanol	962	962	-	0.32	-	-	-
4-methylthio-2-butanone	969	969	1.82	-	1.77	1.73	1.15
β-pinene	981	979-982	0.3	1.3	0.31	-	-
Octanone	999	998	-	0.54	-	-	-
(+)-limonene	1030	1030-1032	87.7	81.8	95.9	97.1	89.2
Dihydrolinalool	1052	1054	6.69	-	-	-	-
2-octenal	1060	1060	-	0.22	-	-	-
Artemisia ketone	1062	1064	-	-	-	-	0.21
p,α-dimethylstyrol	1069	1069	-	0.43	-	-	-
Dihydromyrcenol	1080	1082	0.51	-	-	-	0.21
δ-terpinene	1090	1090-1094	0.91	0.32	0.52	0.3	0.31
Octenol	1104	1103	-	1.08	-	-	-
α-p-dimethylstyrene	1118	1116	-	-	-	-	0.52
Limonene oxide	1132	1129-1132	-	0.54	-	-	2.08
p-2-menthen-1-ol	1145	1145	-	1.19	-	-	-
Methyl dithiofurane	1172	1172	-	-	-	-	0.42
Dimethyloctadienal	1183	1182	-	0.32	-	-	-
Butyl hexanoate	1192	1192-1194	0.2	-	-	-	0.73
6-decenal	1203	1205-1208	0.41	0.65	0.31	-	0.52
(Z)-piperitol	1220	1220	-	1.19	-	-	-
Linalyl formate	1223	1225	-	-	-	-	1.04
Hexyl metylhylbutyrate	1239	1238	-	1.08	-	-	0.63
2-decenal	1252	1250	-	0.65	-	-	-
Citral	1254	1253	-	-	-	-	1.46
p-anisaldehyde	1263	1266	-	1.08	-	-	-
p-menthenethiol	1283	1279-1284	-	0.32	0.31	-	0.31
Methyl quinoxaline	1289	1289	-	0.22	-	-	-
Dihydroterpinyl acetate	1299	1297-1298	-	0.43	-	-	0.52
Cinnamyl alcohol	1312	1316	-	0.32	-	-	-
Methyl decanoate	1324	1324	-	0.32	-	-	-
Benzyl butanoate	1335	1335	-	0.22	-	-	-
(Z)-3-hexenyl hexanoate	1381	1380	-	0.32	-	-	-
Ethyl decanoate	1398	1399	-	0.65	-	-	-
(E)-β-damascone	1415	1414	-	0.22	-	-	-
(-)-γ-elemene	1425	1425	-	0.22	-	-	-
β-farnesene	1445	1446	-	0.22	-	-	-
Methyl laurate	1509	1510	-	0.32	-	-	-