Faculty of Resource Science and Technology

Studies on the Essential Oils and Biological Activity of *Citrus* Spp

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Studies on the Essential Oils and Biological Activity of *Citrus* Spp

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Studies on the Essential Oils and Biological Activity of *Citrus* spp

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ABSTRACT

Leaves and peels of four different *Citrus* spp (*Citrus hystrix*, *Citrus microcarpa*, *Citrus lemon*, *Citrus reticulata*) were subjected to hydrodistillation using Clevenger-type apparatus. The average yields of essential oils in the leaves and peels ranged from 0.08 % to 0.73 % and 0.30 % to 1.01 % respectively. The oils obtained from the fresh leaves and peels sample were analyzed by Gas-chromatography-mass spectrometry (GC-MS). Total of 23 and 20 chemical constituent were identified respectively. The major compounds found in the leaves oils were d-limonene, citronellal, linalool, caryophyllene and humulene. Major compounds found in the peels oils of *Citrus* spp were d-limonene, beta-bisabolene, alpha-bisabolol and alpha-cadinol. The cytotoxic effect of *Citrus* spp with brine shrimp given LC_{50} more than 100 µg/mL show that *Citrus* spp oils practically non-toxic. All the *Citrus* spp oils have good antioxidant activity except leaves of *Citrus microcarpa* and *Citrus reticulata* which have moderate antioxidant activity.

Key word : Citrus spp., Essential oils., Toxicity., Antioxidant., Chemical constituent

ABSTRAK

Daun dan kulit daripada 4 berlainan species Citrus (Citrus hystrix, Citrus microcarpa, Citrus lemon, Citrus reticulata) telah menjalani penyulingan hydro dengan menggunakan alat radas Clevemger. Peratus minyak pati yang diperoleh daripada daun and kulit Citrus dalam skala 0.08 % sehingga 0.73 % untuk daun dan 0.30 % sehingga 1.01 % untuk kulit. Pati minyak dianalisa menggunakan Gas Karomatografi-spektoskopi jisim (GC-MS). Jumlah komponen yang telah dikenalpasti sebanyak 23 dan 20 untuk daun dan kulit masing-masing. Komposisi utama yang dikenal pasti di daun pati minyak ialah d-limonene, citronellal, linalool, caryophyllene dan humulene. Komponen utama yang dikenal pasti dalam kulit Citrus ialah d-limonene, beta-bisabolene, alpha-bisabolol dan alpha-cadinol. Ujian kesan ketosikan Citrus terhadap anak udang menunjukan LC_{50} lebih daripada 100 µg/mL. Ini membuktikan bahawa Citrus tidak memiliki tosik. Semua pati minyak Citrus menunjukan ciri antioksidan yang baik kecuali untuk daun Citrus microcarpa dan Citrus reticulata yang menunjukan ciri antioksidan sederhana.

***Kata Kunci**: Citrus spp., Pati minyak., Ketosikan., Antioksidan., Composisi Kimia*

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

spp: species

L: Liter

g: gram

mL: mililiter

µg: microgram

nm: nanometer

GC-MS: gas chromatography-mass spectrometry

CHAPTER 1: INTRODUCTION

The *Citrus* plant are the family of Rutaceae. About 1300 species of *Citrus* plant can be found throughout the tropical, subtropical and temperate regions (Chanthaphon *et al.*, 2008). According to Food and Agriculture Organization (2010), the world production of *Citrus* in year 2012 was estimated about 131 million metric tons and grown over 140 countries around the world. The leading producer country include China, Brazil, USA, India, Mexico and Spain. The genus *Citrus* includes different important fruits such as orange, mandarins, limes, lemons and grapefruits (Chanthaphon *et al.*, 2008). In Malaysia, available *Citrus* fruit in market are include *Citrus aurantifolia, Citrus reticulata, Citrus microcarpa, Citrus lemon, Citrus hystrix* and *Citrus sinensis*. They are different color *Citrus* fruit, size, apperance and taste.

Citrus has enormous number of biological activities such as anti-cancer, anti-diarrhea, antibacterial, antifungal and antioxidant (Lawal *et al.*, 2013). According to Oussalah *et al.* (2006), numerous studies have shown the efficiency of plant extract even in low dose in the fight against bacterial pathogens (as cited in Hesham *et al.*, 2016). Antimicrobials derived from plant materials are often known as natural and safe compared to the industrial chemicals. According to Cerutti *et al.* (1985), antioxidant properties of essential oils from aromatic plants such as *Citrus* play an important role in preventing cancer which is free radical induced disease (as cited in Choi *et al.*, 2000).

Various techniques have been performed to obtain *Citrus* plant extract. For examples hydrodistillation method, solvent extraction and supercritical fluid extraction. Each technique has particular advantages and disadvantages. *Citrus* oil commonly extracted by hydrodistillation because it is safe to operate and environmentally friendly with better yield of 0.21 % compared to cold pressing which yield 0.05 % of essential oils (Ferhat *et al.*, 2007).

Besides, the hydrodistillation method will completely immersed plant materials in boiling water. The surrounding water acts as a barrier to protect the oil from overheating. Hydrodistillation method obtain the essential oil from plants by using the principle of the osmotic pressure to diffuse oil from plant material.

The pure essential oils can be obtained from both vascular and non-vascular part of a plant, for example from the plant roots, stem, fruit, leaves and flowers. These essential oils have a very high commercial value due to its properties. They are widely used in the various fields of industries such as food industries, perfumery industries and pharmaceuticals. Essential oils are plant-based volatile oils with strong aromatic components that are made up of different chemical compounds. Bozkurt *et al.* (2017) studied reported α-pinene, sabinene, β-pinene, β-myrcene, d-limonene, linalool, m-cymene and 4-terpineol as the main components of the *Citrus* essential oils.

The main objective of this research are:

1. to extract the essential oils from leaves and fruit peels of *Citrus* spp.
2. to characterize and identify the chemical composition of essential oils from several *Citrus* spp by using gas chromatography (GC).
3. to evaluate the biological activity of essential oils toward the brine shrimp larvae, *Artemia salina* for toxicity test and DPPH radical scavenging activity for antioxidant test

CHAPTER 2: LITERATURE REVIEW

2.1 Essential oils from *Citrus* spp

Citrus essential oils are an economic, eco-friendly and natural alternatives to chemical preservatives and other synthetic antioxidants, such as sodium nitrites, nitrates or benzoates, commonly utilized in food preservation. *Citrus* based essential oils are obtained mainly from the peels of *Citrus* fruits which are largely discarded as wastes and cause environmental problems. The essential oils have important application as a natural antibacterial agent for food industry, particularly pasta manufacturing industry. Pasta manufacture have serious spoilage challenge because of lactic acid bacteria activity (Kademi and Garba, 2017). Besides, *Citrus* essential oils have nutraceutical and economic importance, numerous research has been conducted to studied their chemical composition and the biological activities (Vasek *et al.*, 2015).

The essential oils of *Citrus* fruits are present in great quantity in the flavado portion, the layer consisting of the epidermis covering the exocarp of irregular parenchymous cells which are completely enclosing numerous glands or oil sacs (Chanthaphon *et al.*, 2008). Essential oils are also mainly present at different depths in the peel and cuticles of the fruit. Essential oils released when oil sacs are crushed during juice extraction. According to Arabhosseini *et al.* (2007), the storage on the essential oil have it effect on the content and color of essential oils. The results showed a reduction of the oil content and changed color parameters during the storage period. The largest changes of the essential oil content about 50 % after 30 days. (As cited in Rowshan *et al.*, 2013).

2.2 Chemical constituent of *Citrus* oils

Variation in chemical composition of essential oils in particular and extracts of medicinal plants may be observed due to the origin, the environmental conditions, and the developmental stage of collected plant materials (Miguel *et al.*, 2004).

Citrus essential oil are a complex mixture of approximately 400 compounds. The content and composition of the *Citrus* oils are depends on species,variety and cultivar, extraction and separation methods (Nannapaneni, *et al.*, 2009). They are rich sources of flavanoids, alkaloids, coumarins, limonoids, carotenoids, phenolic acid and many polymethoxylated flavones which are not mostly found in other plants (Sawamura *et al.*, 2004). *Citrus* essential oils contains 85 % to 99 % volatile and 1 % to 15 % non-volatile components. According to Muriel-Galet *et al.* (2015), the active compounds in *Citrus* oils are highly volatile and labile to oxygen, heat, or light (as cited in Mahota *et al.*, 2017). The volatile constituents are a mixture of monoterpene include limonene. Sesquiterpene hydrocarbons and their oxygenated derivatives, including aldehydes, ketones, alcohols and esters also the volatile compounds of *Citrus* oils. (Flamini *et al.*, 2007). The non-volatile fraction includes long chain hydrocarbons, fatty acids, sterols, carotenoids, and oxygenated heterocyclic compounds. The major component of the *Citrus* essential oils are d-Limonene, which is used as a green solvent for the determination of fats and oils and considered safer than petroleum solvents (Ueno *et al.*, 2008).

2.3 Extraction of essential oils by hydrodistillation

Distillation is the most widely used methods for the extraction of aroma producing compounds. Other methods used for the extractions are fractionated distillation, steam distillation at atmospheric pressure or vacuum distillation. Different extraction methods, as well as analysis result in different percentage composition of aroma producing compounds in the essential oils. Hydrodistillation is the easiest, oldest and usually used method to obtain and extract the essential oils from a plant part. The simplest way to obtain the essential oils from plants and the process use the principle of the osmotic pressure to diffuse oil from plant material. Hydrodistillation is a physical method where the important parameters evaluated are the extraction time and the characteristic of the plant material (Atti *et al.*, 2005). Hydrodistillation extraction method requires more time and also energy consumption. However, prolong extraction time will affect the chemical composition of the essential oil. The volatile molecules present in the essential oils may gradually decrease in concentration (Ferhat *et al.*, 2007).

During the distillation process, the plant material are exposed to the boiling water and released the essential oils from the plant part. The mixture at the condenser of Clevenger-type apparatus will flow into the separator part where the plant oils will separated directly from the distillate water. The advantages using the hydrodistillation method are inexpensive and easy to construct. However, the disadvantages of this hot method states by Reverchon and Marco (2006) are it can cause objectionable odor to extracts and affect the minor components present in the essential oil (Citing from Bagheri *et al.*, 2014). Essential oils obtained by distillation also deteriorate easily and develop offflavours because of the instability of the terpene hydrocarbons, particularly d-Limonene (Yamauchi & Sato, 1990).

2.4 DPPH Antioxidant Test

Antioxidants are usually used in the food industry to delay the oxidation process. Natural antioxidants in food industry used to replace the commonly used synthetic antioxidants such as BHA (Butylated hydroxyanisole) and BHT (Butylated hydroxytoluene). The synthetic antioxidant are tend to be cytotoxic and cause an increase of cancerous cells (Kang *et al.*, 2004). Numerous method can be used to test the antioxidant properties. For example, TEAC (Trolox equivalent antioxidant capacity), DPPH (2,2-diphenylpicrylhydrazyl) and PCL (Photochemiluminescence assay) are same method commonly used for assessments of antioxidant activity (Frassinetti *et al.*, 2011).

Mostly the antioxidant activity are determined by DPPH test because it is the simplest method. To evaluate the antioxidant activity of the extracts or specific compounds, it will allow to react DPPH in methanol solution (Brand-Williams *et al*, 1995). DPPH is a stable free radical and it is dark violet color. DPPH maximum absorption are at 517 nm (Chahardehi *et al.*, 2010). When the extracts and some pure compounds react with antioxidant that can donate hydrogen or electron donation, it will decolorized the purple-color methanol solution of DPPH. Also resulting decrease in absorbance at 517 nm when measured using UV-VIS spectrometer (Huang *et al.*, 2012).

2.5 Toxicity Test

High dose of bioactive compounds are mostly toxic. Thus Michael *et al.* (1956) proposed *in vivo* brine shrimp lethality bioassay (Citing from Morshafe *et al.*, 2009). This method is based on the ability of the plant extracts to kill the brine shrimp that's been cultured in a laboratory (Morshafi *et al.*, 2009). *Artemia salina* eggs are hatched in the artificial seawater. Active components that present in the plant extracts are exhibited cytotoxic activity against the brine shrimp (Olowa and Nuneza, 2013). Dead brine shrimp after 24 hours are counted and percentage of mortality calculated. Accoring to Gupta *et al.* (2006), for LC_{50} which is lethality concentration that less than 100 ppm shown that the extracts contain potent compounds (Citing from Olowa and Nuneza, 2013). According to Meyer *et al.* (1982) LC_{50} value for the toxic are less than 100 μg/mL and LC_{50} greater than 100 μg/mL value mean it are non-toxic.

Brine shrimp lethality assay are easy to perform and it is low cost. The brine shrimp eggs are commercially available and it is inexpensive. This assay being used to analyze the pesticide residue (Solis *et al.*, 1993) and also useful to monitor the toxicity of organic waste towards organism that live in the marine (Chahardehi *et al.*, 2010). Besides, brine shrimp lethality assay are successfully used as preliminary studies for the antitumor agent according to Ramachandran *et al.* 2010 (Citing from Olowa and Nuneza, 2013).

CHAPTER 3: MATERIALS AND METHODS

3.1 Sample collection

The sample of *Citrus* spp. were collected from Miri and Kuching. Four different species of *Citrus* were studied which include *Citrus hystrix*(Kaffir lime), *Citrus microcarpa* (kasturi lime), *Citrus lemon* (Lemon) and *Citrus reticulata* (Sweet orange). In this study was focus on peels and leaves of *Citrus* spp.

3.2 Extraction of Essential Oils

Hydrodistillation with Clevenger type apparatus was used to extract and isolate the essential oils from *Citrus* spp. Approximately 100 g of *Citrus* spp samples (leaves and peels) were weighed and transferred to 2 L flask and mixed with 1.5 L of distilled water. The flask was assembled to the Clevenger trap and connected to condenser. The hydrodistillation processes were carried out for 6 hours (Hamadan *et al.*, 2013). After 6 hours, oil that trapped in the Clevenger were cooled to room temperature. The oily layer was separated and dried with anhydrous sodium sulphate (Na_2SO_4) (Kordali *et al.*, 2005). The essential oils were stored in vials at 4°C. The extraction process was repeated three times and the average yield (w/w) of the oils was calculated.

3.3 Analysis of Essential Oils

3.3.1 Gas Chromatography- Mass Spectrometry (GC-MS)

Gas chromatography-mass spectrometry (GC-MS) analyses were performed using Shimadzu QP2010 Plus. A capillary column of BPX-5 (30.0 m× 0.25 mm i.d. × 0.25 μm film thickness) and an automatic injection system were used. The analysis was carried out at 280 °C, the oven temperature at initial 50 °C for 5 minutes increasing 50 °C per minute until 300 °C was reached. The injector temperature was set to 280 °C. Helium was used as a carrier gas at flow rate of 3.0 mL/min. Before the analysis, essential oils were diluted 1:200 ratio with dichloromethane (DCM). Individual GC peaks and Mass spectra were identified by computer. Identification of components was based on computer matching with NIST libraries.

3.4 Qualitative and Quantitative analysis

3.4.1 Percentage of Essential Oils

The yields of the essential oils were calculated based on the wet weighed of plant material used and weighed of oils obtained after 6 hours. The equation used for the calculation was as following:

$$\text{Percentage yield} = \frac{W1}{W2} \times 100\%$$

Where W1 = Weight of essential oils (g)

W2 = Weight of plant materials (g)

3.4.2 Semi-Quantitative analysis

The percentage of individual chemical components in the *Citrus* essential oils was determined using the following equation:

$$\text{Percentage (\%)} = \frac{Ax}{\sum A} \times 100\%$$

Ax = Peak area of chromatogram for compound X

$\sum A$ = Total peak area of all chromatogram

3.5 Bioassay Test

3.5.1 Antioxidant test

The free radical scavenging activity of compound 2,2-diphenyl- 1-pycryl-hydrazyl (DPPH) was used to evaluate the antioxidant properties of the *Citrus* essential oil. DPPH radical-scavenging activity of *Citrus* spp essential oils was adopted from Umaru *et al.* (2018). Sample was prepared by diluting 6 μg of essential oils with 6 mL methanol to produce concentration of 1000 μg/mL. Different concentration of the essential oils in methanol was prepared from the prepared stock solution (10, 50, 100, 500 μg/mL). About 3 mL of the prepared solution was then mixed with 1 mL of 0.1 mM DPPH reagent and mixed throughly. After 30 minutes of incubation at room temperature in the dark, absorbance of the mixture at 517 nm was measured. Ascorbic acid (10, 50, 100, 500 μg/mL) was used as positive control. The negative control contained 1 mL of 0.1 mM of DPPH and 3 mL of methanol without contained any essential oils. The concentration of the sample required to inhibit 50 % of the DPPH free radical was calculated as IC_{50} and the value was determined from the linear equation Y=mX + C based on the graph plotted DPPH scavenging activity (%) of the sample vs log concentration. DPPH scavenging activity (%) was calculated with following formula

$$\text{Radical scavenging activity (\%)} = \left[\frac{A_C - A_S}{A_C}\right] \times 100$$

where A_C: the absorbance of the control

A_S: the absorbance in presence of the sample.

3.5.2 Brine Shrimp toxicity test

Toxicity test against brine shrimp (*Artemia salina*) was adopted from Chieng *et al.* (2008). *Artemia salina* cysts was hatched in the glass container that contained filtered seawater. Air pump was fitted to the water to ensure complete aeration of the cysts after 48 hours. The freshly hatched nauplii were harvested and used for the bioassay.

The essential oils of *Citrus* spp were prepared by dissolving 2 mg of each sample with 2 mL of methanol. From the prepared stock solution, about 500, 50 and 5 μL were transferred to NUNC multidisc. The solvent was dried overnight in running fumehood to evaporate the solvent. Then 5 mL of seawater was added to the NUNC multidisc resulting in final concentration of 1, 10 and 100 μg mL^{-1} respectively.

Ten nauplii were transferred into each concentration of NUNC multidisc. After 24 hours, the number of dead nauplii in each NUNC multidisc was counted and the percentage of death nauplii was plotted against the concentration (on log scale). Thymol was used as the positive control while sea water used as negative control. The data was analyzed to determined LC_{50}. The LC_{50} is defined as the lethal concentration of the sample at which 50 % of the brine shrimp was killed at 24 hours. All experiments were run in triplicate.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Yields of Essential Oils

The average percentage yields of essential oils were calculated from the three replicates. Table 1 shows the percentage of essential oils from leaves and peels of four different *Citrus* spp by hydrodistillation. The amount of percentages of oils was calculated based on wet weight of plant materials. Wet weigh basis was the plant material which contained high water content without any drying process. Generally, all the *Citrus* spp in this study gave 0.08-0.73 % of essential oils from the leaves and 0.30-1.01 % from the peels. Highest percentage yields of essential oils obtained from both leaves of *Citrus hystrix* and *Citrus microcarpa* with 0.72 % and *Citrus reticulata* peels at 1.01 %. *Citrus reticulata* gave the lowest yields of essential oils from the leaves and *Citrus hystrix* from the peels.

Table 1: Yield of *Citrus* spp essential oils from leaves and peels

Citrus spp	Percentage of essential oils (W_1/W_2)	
	Leaves	Peels
Citrus hystrix	0.7355 ± 0.073	0.2967 ± 0.041
Citrus microcarpa	0.7233 ± 0.040	0.6130 ± 0.045
Citrus lemon	0.1313 ± 0.057	0.4797 ± 0.055
Citrus reticulata	0.0865 ± 0.024	1.0102 ± 0.238