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DETERMINATION OF ANTIOXIDANT ACTIVITIES IN POMELO PEEL (*CITRUS GRANDIS*) ESSENTIAL OIL

WONG YUET YENG

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SCHOOL OF FOOD SCIENCE AND NUTRITION
UNIVERSITI MALAYSIA SABAH
2009
DECLARATION

The materials in this thesis are original except for quotations, excerpts, summaries and references, which have been duly acknowledged.

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Essential oil was extracted out from pomelo (*Citrus grandis*) peels by hydro distillation and solvent extraction method. The percentage of oil yields obtained from the pomelo peel varied from 2.49 ± 1.23% to 4.2 ± 0.98%. The volatile components of the essential oils were characterized by means of GC-MS. GC-MS identified 43 compounds present in the essential oils, with main components being d-limonene (62.17-75.24%), α-myrcene (1.08-7.55%), α-linalool (0.93-3.26%), citral (0.55-4.14%), and terpinolene (0.98-2.71%). Differences in compositions and contents were observed in the essential oils extracted by these two different methods. Antioxidant and radical scavenging properties were tested by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, ferric reducing ability of plasma (FRAP), and β-carotene bleaching test. To examine the oils' relative activities compared with that of a standard antioxidant, BHT was employed. In the DPPH assay, the essential oils showed a free radical inhibition ranging from 86.174 ± 1.067%-40.260 ± 0.481%. In the FRAP assay, it was noticed that essential oils from the *Citrus grandis* have an average reducing capacity of 0.047 ± 0.001-0.270 ± 0.003 mM/mg oil. For β-carotene bleaching test, both hydro distilled and solvent extracted essential oils showed effective antioxidant activities, which were 83.817 ± 0.368% and 70.794 ± 0.596% respectively.
ABSTRAK

 PENENTUAN AKTIVITI ANTIOKSIDAN DALAM MINYAK PATI KULIT POMELO

Minyak pati telah diekstrak daripada kulit pomelo (Citrus grandis) dengan kaedah penyulingan hidro dan pengekstrakan dengan pelarut organik. Penghasilan minyak pati daripada kulit pomelo adalah anatra 2.49 ± 1.23% ke 4.2 ± 0.98%. Kandungan komponen-komponen merup telah dikaji dengan menggunakan alat instrumen GC-MS. GC-MS telah pun mengidentifikasikan 43 sebatian yang terkandung dalam minyak pati, di mana komponen utama adalah d-limonene (62.17%-75.24%), α-mycrene (1.08-7.55%), α-linalool (0.93-3.26%), citral (0.55-4.14%), dan terpinolene (0.98-2.71%). Perbezaan dalam komposisi dan kandungan dapat diperhatikan dalam minyak pati yang diekstrak dengan dua kaedah yang berlainan. Sifat-sifat antioksidan dan pemerangkapan radikal bebas minyak pati telah dikaji dengan menggunakan kaedah 1,1-diphenyl-2-picrylhydrazyl (DPPH), keupayaan penurunan ferum (FRAP), and ujian pelunturan β-carotene. Bagi membandingkan activiti minyak pati dengan satu antioksidan "standard", BHT telah digunakan. Dalam assay DPPH, minyak pati menunjukkan free radical inhibition dalam lingkungan 86.174 ± 1.067%-40.260 ± 0.481%. Dalam assay FRAP pula, ia menunjukkan minyak pati daripada Citrus grandis mempunyai kapasiti penurunan dari 0.047 ± 0.001 ke 0.270 ± 0.003 mM/mg minyak. Bagi ujian pelunturan β-carotene, kedua-dua sample minyak daripada penyulingan hidro dan pengekstakan pelarut menunjukkan aktiviti antioksidan yang efektif, di mana activiti tersebut adalah di antara 83.817 ± 0.368% dan 70.794 ± 0.596% masing-masing.
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LIST OF SYMBOLS

%  Percent
°C  Degree Celsius
kg  Kilogram
g  Gram
mg  Milligram
μg  Microgram
mL  Milliliter
μL  Microliter
mM  Millimolar
$A_{control}$  Absorbance of Control
$A_{sample}$  Absorbance of Sample
ΔA  Change of Absorbance
±  More or Less
<  Less Than
>  More Than
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CHAPTER 1

INTRODUCTION

1.1 Research Background

Health awareness of the public is growing nowadays. Healthy and natural way of living is leading the trend now. Diets rich in selected natural antioxidant such as polyphenols, flavonoids, vitamin C and vitamin E are related to reduced rate of incidence of cardiovascular, other chronic diseases and certain types of cancer are of current special concerns (Mata et al., 2007). This indeed has lead to the revival of interest in plant-based food. There are a large number of plant species that already been tested for their potential biological, therapeutic and pharmaceutical activities (Hussain et al., 2007).

The use of essential oils as functional ingredients in foods, drinks, toiletries and cosmetics is gaining momentum, both for the growing interest of consumers in ingredients from natural sources and also because of increasing concern about potentially harmful synthetic additives (Reische et al., 1998). The essential oil of a plant is the concentrated, volatile, aromatic mixture of chemicals that is formed in various organs or tissues, including leaves, bark, flowers, fruits, and roots. It is a mixture of known and partially unknown compounds such as hydrocarbons and contains terpene alcohols, aldehydes, ketones, phenols and esters (Lado et al., 2004).
Essential oils have an extensive range of uses, including uses in perfumery and food application. The value of flavouring herbs, condiments and spices is mainly due to the essential oils contained in them. Essential oils are the major raw material used by the soft drinks and confectionery industries. The main use for most essential oils is to give their own characteristic flavour to an end product (Ashurst, 1991). Hence, essential oils are extensively used for the flavouring of liqueurs, aerated beverages and other drinks. The commercial value of tea, coffee, wine and other beverages may be said to depend largely on the delicate aroma which they owe to the presence of minute quantities of ethereal oils. Apart from that, many of the essential oils are also find extensive use in medicine.

In recent years, the essential oils and herbal extracts have attracted a great deal of scientific interest due to their potential use as a source of natural antioxidants and biologically active compound (Bozin et al., 2006). Humans have benefited from plant volatiles throughout history, not only as sources of pleasant fragrances and flavors, but also as therapeutic agents against disease and protection against pests (Setzer, 2007). Many protocols for controlling the growth of foodborne pathogen, skin-resident pathogens, and food spoilage bacteria have also been developed using essential oil as natural food preservatives (Hao et al., 1998). The antimicrobial and antioxidant activities of essential oils have formed the basis of many applications, including fresh and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Bozin et al., 2006). Efforts have also been made to explore the potential of some essential oils for the treatment of infectious diseases in order to substitute standard pharmaceutical remedies (Celiktas et al., 2007). Essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use (Ormancy et al., 2001).

There are much attention has been given to Citrus components, since they present various pharmaceutical activities of anticarcinogenicity, antmutagenicity, antioxidative activity and antiaging (Nogata et al., 1996). Citrus plants are known to be rich in their flavonoid glycoside content in fruit, fruit juices and leaves (Manthey, 2002).
It is characterized by accumulation of large quantities of glycosylated flavanones, which are the first intermediaries in the flavonoid biosynthetic pathway (Maier & Hasegawa, 1970). From the studies of Koca et al. (2003), extract of young and old leaf tissue displayed the highest antioxidant potential among the other Citrus tissues. This might be due to the antioxidant flavonoids that evolved in plants to protect the photosynthetic machinery from oxidative damage (Demmig-Adams & Adams, 2002).

Limonoid concentration profiles to differentiate Citrus species limonin, nomilin, obacunone and deacetylnomilin, limonexic acid and deoxylimonin were found in varying amount of Citrus species (Hashinaga & Itoo, 1981). In citrus, the major part of the total antioxidant activity is due to hydrophilic compound (Cano et al., 2002), and some other authors have stressed the main role of hespiridin (HESP) in the total antioxidant capacity of orange juices (Dhuique-Mayer et al., 2005). Bocco et al. (1998) observed that Citrus seed possessed greater antioxidant activity than peel. Besides, β-cryptoxanthin was also extracted from the peel and flesh of Citrus fruits (Kyoung-Cheol et al., 2000). Polyphenolic compounds are known to be rich in Citrus peel (Kim et al., 1999) and methanolic extract of citrus peels also exhibited very strong antioxidant activity (Rehman, 2006). According to Mokbel (2005), glycosides and monosaccharides are important naturally occurring compounds specifically distributed in Citrus buntan fruit flavedo. Due to their polyphenolic structure, these compounds possess health-related properties, which are based on their antioxidant activity. In the case of Citrus essential oil, volatile components such as geraniol, terpinolene, γ-terpinene showed marked scavenging activities (Choi et al., 2000). The biological activities of these oils have suggested the potential uses of these natural materials in pharmaceutical preparations, perfumery, cosmetics, and agronomic fields (Caccioni et al., 1998).

According to previous studies, citrus peel has known to possess good antioxidant activity. The citrus peel is mainly the by-product from the production of citrus-based product. The industrial value of the citrus by product is expected to be increased if the industry can make use of it, such as extracting essential oil from the peel. This has enhanced and varied the usage of the citrus by-product. The extracted essential oil is potentially the alternative natural antioxidant source for food and nutraceutical industry.
Through this research, it is hoped to increase the popularity of pomelo essential oil in food flavouring and as a useful substance in food preservation, especially in the case of fighting against lipid oxidation. Although, there are a few researches have been done on the essential oil originated from different citrus species. These researches also concluded with an encouraging result in terms of their antioxidant activity. But somehow the components of the essential oils might not be totally the same even for the same species. This does apply for their antioxidant activity. They may vary depending on different environmental origins (e.g. temperature, humidity, soil condition), variety (e.g. species, hybrid), and essential oil extraction methods (Njogore et al, 2005). As far by the knowledge, in Sabah, the Citrus grandis fruit and its essential oil has not yet been investigated and understood in terms of antioxidant value. Hence, this research also aimed to study whether the essential oil extracted from the local pomelo (C. grandis) possess the similar extent of antioxidant activity of the citrus essential oil from previous studies. In terms of importance, this research offers a better understanding of Citrus grandis essential oil properties especially in terms of its antioxidant capacity. It also helps in determining possible alternative from Citrus cultivar towards synthetic antioxidants in food to prevent the oxidation. Besides, this study helps in promoting the nutritional aspect and functional properties of Citrus cultivar from sabah. For future research purpose, in which compound responsible for antioxidant activity can be further identified.

1.2 Research Objectives

These are the objectives of the study
1. To study the volatile components which are present in the essential oil from Citrus grandis.
2. To determine the antioxidant activity of the Citrus grandis essential oil.
3. To compare the antioxidant activity of the essential oil which is extracted from both hydro distillation and solvent extraction.
CHAPTER 2

LITERATURE REVIEW

2.1 Oxidation Process

Thermodynamic equilibrium strongly favors the net oxidation of reduced, carbon-based biomolecules. The kinetic stability of all biological molecules in an oxygen-rich atmosphere result from the unique spin state of the unpaired electrons in ground state molecular (triplet) oxygen in the atmosphere (German, 1999). This property renders atmospheric oxygen relatively inert to reduced, carbon-based biomolecules. In effect, reactions between oxygen and protein, lipids, polynucleotides, carbohydrates, etc. proceed at slow rates unless they are catalyzed. Catalysis generally takes the form of activating the oxygen to either an unpaired species, triplet oxygen or the organic molecule to a radical. Both reaction courses take place naturally, but the relative importance of these two oxidation catalysis differ depending on the food material and the conditions (German, 1999).

2.1.1 Oxidation Process on Harvested Commodities

Living organisms are the basis of the food supply. When an organism is harvested for food, the tendency of the tissues to maintain normal biochemical processes will have substantial effects on the subsequent oxidative event as a food commodity. The oxidative deterioration of lipids in harvested biomaterials occurs as a result of one of
three basic changes brought by harvesting: (i) oxidative enzymes are released that catalyze lipid oxidation, (ii) non-enzymatic but catalytically-active reduced transition metals are released that promote lipid oxidation and (iii) depletion of antioxidant enzymes, protectants and scavengers (German, 1999).

Progression of oxidation and meat flavour deterioration is dependent primarily on the species of the meat and its lipid content. Furthermore, interaction of oxidation and meat products with muscle food components may in turn bring about changes in their colour, texture and nutritional value (Spanier et al., 1992). While the warmed-over flavor associated with the liberation of hematin iron in muscle tissue is an example of the release of activated transition metals that accelerate oxidation in red meat. Oxidation is also very much affected the fish such as herring and mackerel in which these fish are extremely susceptible to oxidation due to the high content of long chain n-3 PUFA. These fatty acids are highly susceptible chemically is due to the large number of double bonds on a single fatty acid. The products from the oxidation of these fatty acids lead to a very distinctive volatile off-flavors (German, 1999). In gadoid fish, formation of formaldehyde from degradation of trimethylamine oxide leads to toughening of the muscle texture during frozen storage because of cross-linking of protein molecules (Shahidi, 1998). This is an example from the autoxidation process. The off-flavor of soybean oil due to the lipoxygenase enzyme of soybeans is an example of the liberation of an endogenous enzyme that accelerates lipid oxidation. Besides, green leafy tissues of plant that perform photosynthesis contain photosynthetic membranes that are highly enriched in α-linoleic acid. Contamination of the processed commodity by leaf tissue, therefore, can increase the susceptibility to oxidation as well (German, 1999).

Oxidation does play a role in enzymatic browning. Enzymatic browning is one of the most important colour reactions that affects fruits, vegetables and seafoods. It is catalysed by the enzyme polyphenol oxidase (1,2 benzenediol; oxygen oxidoreductase, EC1.10.3.1) (FAO, 2000). Undoubtedly, some enzymatic browning reactions are very beneficial to the overall acceptability of foods such as tea, coffee, and cocoa. Yet, enzymatic browning is one of the most devastating reactions for many exotic fruits and vegetables, in particular tropical and subtropical varieties. It is estimated that over 50
percent losses in fruit occur as a result of enzymatic browning (Whitaker & Lee, 1995). Green leafy vegetables, potatoes, mushrooms, apples, avocados, bananas, grapes, peaches, and a variety of other tropical and subtropical fruits and vegetables, are susceptible to browning and therefore cause economic losses for the agriculturist. Browning can also adversely affect flavour and nutritional value.

2.1.2 Processing Strategies to Prevent Oxidation of Commodities

Oxidation is one of the major causes of food deterioration. It is responsible for the degradation of odor, taste, texture, consistency, and appearance as well as the loss of nutritional value of most of our foods today (Goldberg & William, 1991). Many food-processing practices tend to solve these unwanted changes. Therefore, a food technologist has had to intervene in order to minimize the losses. A variety of strategies have developed over the history of food harvesting and manufacturing to improve the oxidative stability of commodities.

a. Minimize Cellular Destruction

In general, harvesting and processing methods should be designed to maintain as much as possible the native integrity of the tissues being harvested. The natural surfactant lipids in cells, including phospholipids, partial glycerides and sterols, are readily disassembled from their native structures in cells since these are held together by non-covalent forces. This not only destroys the native structure of the membranes but provides new surfaces and access of metals, oxidants, etc. The membrane lipids will reform into a variety of colloidal structures as a function of their concentration and the concentration of other components in the matrix (German, 1999).

b. Inactivate Cellular Enzyme Catalysts

For many raw food commodities, the liberation of endogenous enzymes is inevitable during harvest and affects the final product quality. Examples include the soybean lipoxygenase and lipase in rice bran. These enzymes are sufficiently active in the tissues
and can overwhelm other considerations for future stability. Therefore, inactivating these enzymes becomes a priority in the initial processing. Usually there is an incorporation of heating step early in the processing in order to inactivate these enzymes (German, 1999).

c. **Remove endogenous catalysts**

Examples of catalysts that are present in the commodity at harvest include chlorophylls in green plant tissue that are photosensitizers to photoxidation, metals liberated by hemolysis of blood in red meat, and in soybeans the extremely high levels of tocopherol in the raw material are net pro-oxidant (German, 1999). In such commodities, it is vital to know exactly the basis of the instability and process accordingly. Chlorophyll contamination can be dealt by selective extraction, bleaching or packaging with light barriers. Metal contamination must be complexed with effective chelating agents in order to get rid of those metals. The excessive tocopherol content in soybean oils is largely reduced by the degumming process. These excessive tocopherols of soybean and the need to remove them had led to the largest industrial source of tocopherols as antioxidants and supplements (German, 1999).

d. **Exclude Exogenous Substrates and Catalysts**

Oxidation requires unsaturated lipids, oxygen and initiation and decomposition catalysts. Oxygen is a necessary substrate to the oxidation reactions of lipids. Oxygen barriers, such as air-tight containers, have been employed to improve the stability of many lipid-containing foods. Unusual packaging materials, oxygen-scavenging systems and the availability of inert gases as blanketing atmospheres have all been applied to food materials, especially those containing unstable lipids (German, 1999).

Metals are the key initiation and decomposition catalysts present in raw food materials. They are also potential additives from various exogenous sources including processing equipment, flavorings, colorants and packaging materials. Two strategies have been applied to this problem. Great strides have been made in recognizing sources
of metals and carefully excluding them during processing. Alternatively, metals that cannot be excluded are inactivated by chelates. An antioxidant-acting chelate is a chemical that incapable of participating in redox reactions with lipids or oxygen. Citric acid is the most widely-used inactivating chelator, although there are many other alternatives that have been used in specific products (German, 1999). On the other hand, protecting light-sensitive food materials from light has a dramatic effect in reducing the rate of singlet oxygen production in the presence of photosensitizer.

e. Antioxidants Added to Foods

Soluble chain-breaking antioxidants used in foods include ascorbate as either the naturally-occurring free acid or as synthetic ascorbate, and various soluble and insoluble ester forms. The acid form is an excellent electron donor in foods. This property leads to its excellent antioxidant activity at low levels, but at high levels, its ability to reduce metal initiations can actually lead to a pro-oxidant effect (Frankel, 1989). In response to desire of consumers for less chemically-processed food ingredients, several naturally-occurring antioxidants are being introduced to accomplish the same effects as substituted phenols such as butylated hydroxyanisole and butylated hydroxytoluene (Aruoma et al., 1992). The natural antioxidants are primarily extracts of herbs or plant materials with high levels of particular polyphenolics that have good electron-donation and chain-breaking properties (Mukhopadhyay, 2000).

Their further addition in small quantities facilitates the delay, retardation, or prevention of the development of rancidity caused by atmospheric oxidation. Thus they preserve fats, oils, and fat-soluble components like vitamins, carotenoids, and other nutritive ingredients of foods. However, at high concentrations, antioxidants may act as pro-oxidants, since they themselves are susceptible to oxidation (Mukhopadhyay, 2000). Therefore, in order to ensure the quality, safety and shelf-life of food products, it is essential to strictly follow the standards with respect to the synergy between different classes of food product and antioxidants.
When food commodities are subjected to processing, the antioxidants naturally present in them often get depleted due to physical or by chemical degradation. Consequently, such foods are fortified with antioxidants, especially from natural sources. Antioxidants should be added to the product as early as possible (Mukhopadhyay, 2000).

2.2 Free Radical and Mechanism

Atoms contain a nucleus, and electrons move around the nucleus, usually in pairs. A free radical is an atom or molecule that contains one or more unpaired electrons (Halliwell, 1989). The unpaired electrons alter the chemical reactivity of an atom or molecule making it more reactive than the corresponding non-radical. Radicals are generally less stable than non-radicals, although their reactivity varies. The simplest free radical is an atom of the element hydrogen. The hydrogen atom contains one proton and a single unpaired electron, and hence it known as a free radical. Free-radical chain reactions are often initiated by removal of H* from other molecules (e.g. during lipid peroxidation).

Once radicals form, they can react either with another radical or with another molecule by various interactions. If two radicals meet they can combine their unpaired electron, thus forming a covalent bond. However, most molecules found in vivo are non-radicals. In this case a radical might donate its unpaired electron to the other molecule, or might take one electron from it, thus transforming its radical character. At the same time a new radical is formed. The feature that is becoming clear is that a radical generates another radical, leading to the chain reaction (Nonhebel et al., 1979). The rate and selectivity of these types of reactions depend on high concentrations of the radicals, on delocalization of the single electron of the radical (thus increasing its lifetime), and on the absence of weak bonds in any other molecules present with which the radical could interact (Aruoma, 1998). Free radicals of importance in living organisms include hydroxyl (OH·), superoxide (O2·−), nitric oxide (NO·), and peroxyl (RO2·). Peroxynitrite (ONOO·), hypochlorous acid (HOCl), hydrogen peroxide (H2O2), singlet oxygen (often written as 1O2), and ozone (O3) are not free radicals but can easily lead to free-radical reactions in living organisms. The term “reactive oxygen species” (ROS) is often used to include not only the radicals but also the nonradicals (Aruoma, 1998).
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