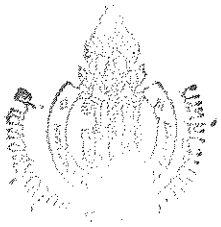
Characterization of Antioxidants from Some Herbs and Green Leaves



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Master of Science Thesis in Food Technology

Prince of Songkla University

2001

Thesis Title Characterization of Antioxidants from Some Herbs and Green Leaves

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ชื่อวิทยานิพนธ์ การจำแนกลักษณะของสารกันหืนจากสมุนไพรและพืชใบเขียวบางชนิด

ผู้เขียน นางสาว วีรณา ซิมอารีย์รัตน์

สาขาวิชา เทคโนโลยีอาหาร

ปีการศึกษา 2543

บทคัดย่อ

จากการศึกษากิจกรรมการเป็นสารกันหืนของสมุนไพรจำนวน 8 ชนิด และใบพืชจำนวน 9 ชนิด ในระบบเลซิตินไลโปโซมและลิโนเลอิค/เบตา-แคโรทีน พบว่า สารสกัดจากชะเอมและใบมะม่วงให้กิจกรรมการเป็นสารกันหืนสูงสุด ทั้งในระบบไลโปโซมและลิโนเลอิค/เบต้า-แคโรทีน การใช้สารละลายเมธานอล ร้อยละ 80 ในการสกัดสารกันหืนจากชะเอมและใบมะม่วงเป็นเวลา 5 ชั่วโมงให้กิจกรรมของสารกันหืนสูงสุด สารสกัดจากชะเอมและใบมะม่วงคงทนต่อความร้อนและให้กิจกรรมการเป็นสารกันหืนสูงที่พีเอชเป็นกลางและด่าง กิจกรรมการเป็นสารกันหืนสูงขึ้นเมื่อปริมาณสารสกัดเพิ่มขึ้น สารสกัดจากชะเอมและใบมะม่วงสามารถเสริมฤทธิ์กับแอลฟาโทโคฟีรอล อย่างไรก็ตาม ไม่พบการเสริมฤทธิ์ระหว่างสารสกัดกับกรดแอสคอร์บิคและกรดซิตริก สารสกัดจากชะเอมและใบมะม่วงมีความสามารถในการกำจัดอนุมูลของ 1,1-diphenyl-2-picrylhydrazyl(DPPH)และอนุมูลไฮดรอกซิล ส่งผลให้สามารถยับยั้งปฏิกิริยาลูกโซ่ของการเกิดออกซิเดชันของไขมันได้ โดยประสิทธิภาพขึ้นอยู่กับปริมาณของสารสกัดที่ใช้ สารสกัดจากชะเอมและใบมะม่วงสามารถกำจัดไอออนของโลหะ รวมทั้งสามารถยับยั้งการทำงานของเอ็นไซม์ไลปอกซิจิเนส โดยขึ้นอยู่กับปริมาณที่ใช้ สารสกัดจากชะเอมและใบมะม่วงสามารถยับยั้งการเจริญเติบโตของแบคทีเรียแกรมบวกได้ดีกว่าแบคทีเรียแกรมลบ และสามารถชะลอการเพิ่มขึ้นของไซโคโทรฟิคแบคทีเรีย ไตรเมธิลเอมีน ปริมาณด่างที่ระเหยได้ทั้งหมดและการเกิดออกซิเดชันของไขมันในเนื้อปลาบดระหว่างการเก็บรักษาที่อุณหภูมิ 4 องศาเซลเซียส เป็นระยะเวลา 10 วัน สารกันหืนจากชะเอมและใบมะม่วงเป็นสารจำพวกฟีนอลิคที่ไม่มีหมู่ไฮดรอกซิลอิสระที่ตำแหน่งออโธหรือพารา และมีคุณสมบัติเป็นสารรีดิวซิ่ง

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Author	Miss Weerana Sim-areerat
Major Program	Food Technology
Academic Year	2000

Abstract

Lecithin liposome oxidation system and β-carotene linoleic acid system were used to determine the antioxidant activities of methanolic extracts from 8 cultivars of herbs and 9 cultivars of green leaves. Liquorice and mango leaves showed the highest antioxidant activity ($p<0.05$). The extraction of liquorice and mango leaves could be conducted at room temperature using 80% methanol for 5 hrs. Extracts from liquorice and mango leaves had high thermal stability. Higher antioxidant activities of both extracts were observed at neutral and alkaline pHs. Antioxidant activity of both extracts increased with increased amount of the extracts. Synergistic action of liquorice and mango leaves extracts with α-tocopherol in lecithin liposome oxidation system was observed. However, no sysnergistic action between the extracts and ascorbic acid or citric acid in lecithin liposome oxidation system was observed. Both extracts showed marked activities as a radical scavenger toward 1,1-diphenyl-2-picrylhydrazyl radical and hydroxyl radical in a concentration-dependent manner. Therefore, these extracts possibly worked as primary antioxidant, which potentially react with free radical. Liquorice and mango leaves extracts also possessed metal ion chelating activity and were able to inhibit lipoxygenase activity, depending on amount of the extracts. Liquorice and mango leaves extracts showed higher inhibitory effect on growth of gram-positive bacteria than gram-negative bacteria. Liquorice and mango leaves extracts retarded the increase in psychotrophic count, trimethylamine (TMA), total volatile bases (TVB) and lipid oxidation in raw ground fish during 10 days

of storage at 4 °C. Antioxidant components of liquorice and mango leaves were tentatively identified as phenolic compounds without free ortho- and para- hydroxy groups, which were reducing compounds.

Acknowledgement

I would like to express my deep appreciation and sincere gratitude to my advisor, Asst. Prof. Dr. Soottawat Benjakul of the Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, for his kindness, guidance and assistance in reading, correcting and criticizing the manuscript.

I also would like to express my profound gratitude to my co-advisor, Dr. Suganya chanthachum of the Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, for her kindness and helpful suggestion.

I am also very grateful to my examination committees, Asst. Prof. Saowaluck Jitbunjerdkul and Asst. Prof. Dr. Niwat Keawpradub.

I am grateful to my dear friends and technicians who encouraged and helped in various ways.

Finally, I would like to express my deepest appreciation to my parents and my sisters for the great understanding, encouragement and support.

This study could not be succeeded without the financial support from the Graduate School, Prince of Songkla University.

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Chapter 1

Introduction

Lipid peroxidation is an important deteriorated reaction in food during storage and processing. It not only causes a loss of food quality but is also strongly associated with carcinogenesis, mutagenesis, aging and altherosclerosis (Yen and Wu, 1999; Ohara *et al.*, 1993). Addition of synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butylhydroquinone (TBHQ) can control lipid oxidation in foods and improve the stability of lipid (Khalil and Mansour, 1998). However, the use of these synthetic antioxidants has begun to be restricted because of their health risks and toxicity (Buxiang and Fukuhara, 1997). Some physical properties of BHA and BHT, such as their high volatility and instability at elevated temperatures, strict legislation and consumer preferences have shifted the attention of manufactures from synthetic to natural antioxidants (Pokorny, 1991; Dapkevicius *et al.*, 1998; Mansour and Khalil, 2000). Natural antioxidants from dietary plants have been reported to prevent oxidative damage by free radical and active oxygen, and prevent the occurrence of disease, cancer and aging (Yen and Wu, 1999; Fejes *et al.*, 2000; Stajner *et al.*, 1999). Many polyphenols such as flavonoids, phenolic acid and lignans express strong antioxidative activities (Schubert *et al.*, 1999; Seidel *et al.*, 2000). Moreover, some polyphenolic components in natural antioxidants showed antimicrobial activity against viruses, retroviruses, bacteria, yeasts (Vivas *et al.*, 1997; Aziz *et al.*, 1998; Chaibi *et al.*, 1997; Chanthachum and Beuchat, 1997; Koutsoumanis *et al.*, 1998).

Extraction, characterization and utilization of natural antioxidants have been conducted (Kim *et al.*, 1994; Haraguchi *et al.*, 1992; Osawa *et al.*, 1992; Ogata *et al.*, 1997; Shobana and Naidu, 2000). Recently, phytochemicals in food materials and their effects on health has been intensively studied

(Frankel *et al.*, 1997; Yen and Wu, 1999; Adegoke and Krishna, 1998; Mansour and Khalil, 2000; Castillo *et al.*, 2000).

Extracts from herbs and leaves such as clove, rosemary, sage, green tea leaves, barley leaves and olive leaves have been reported to possess antioxidant activity (Lean and Mohamed, 1999; Goupy *et al.*, 1999; Zandi and Gordon, 1999; Economou *et al.*, 1991; Ho *et al.*, 1994). Since Thailand has a variety of herbs and green leaves, the study on antioxidant from those sources should be initiated to gain new promising natural antioxidant which can be used as the potential antioxidants.

Therefore, the objectives of this investigation were to screen for herbs and green leaves with high antioxidant activity, to study the properties, mode of action and antimicrobial activity as well as application of the antioxidant extracts to prevent oxidation in ground fish.

Literature review

1. Lipid oxidation

The oxidation of lipid in food system can initiate the other changes in nutritional quality, wholesomes, safety, color, flavor and texture. Lipid oxidation mainly involves a free radical chain reaction that can be described in terms of initiation, propagation, branching and termination processes (Kanner *et al.,* 1987 ; Decker, 1998).

A. Initiation

Lipid autoxidation is thought to be initiated with the formation of free radicals. When in contact with oxygen, an unsaturated lipid gives rise to free radicals. Initiation reactions take place either by the abstraction of a hydrogen radical from an allylic methylene group of an unsaturated fatty acid or by the addition of a radical to a double bond.

$$RH + \text{initiator} \rightarrow R^{\bullet} + H^{\bullet} \qquad (1)$$

$$ROOH \rightarrow RO^{\bullet} + HO^{\bullet} \qquad (2)$$

$$2ROOH \rightarrow RO^{\bullet} + ROO^{\bullet} + H_2O \qquad (3)$$

The formation of lipid radical $R^{\bullet}$ is usually mediated by trace metals, irradiation, light or heat (Eq. 1, 2, 3). The rearrangement of the double bonds resulted in the formation of conjugated diene (-CH=CH-CH=CH-), showing a characteristic UV absorption at 232-234 nm (Figure 1) (Nakayama *et al.,* 1994).

B. Propagation

In propagation reaction, free radicals are converted into other radicals. In fact, propagation of free radical oxidation process occurs in the case of lipids by chain reactions that consume oxygen and yield new free radical species (peroxy radicals, $ROO^{\bullet}$) or by the formation of peroxides (ROOH) as in Eqs. (4) and (5).

$$R^{\bullet} + {}^{3}O_2 \rightarrow ROO^{\bullet} \quad (4)$$

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet} \quad (5)$$

Lipid peroxy radicals ($ROO^{\bullet}$) initiate a chain reaction with other molecules, resulting in the formation of lipid hydroperoxides and lipid free radicals. Lipid hydroperoxides, the primary products of autoxidation, are odorless and tasteless (Jadhav *et al.,* 1996; Giese, 1996).

C. Termination

$$R^{\bullet} + R^{\bullet} \rightarrow R\text{-}R \quad (6)$$

$$R^{\bullet} + ROO^{\bullet} \rightarrow ROOR \quad (7)$$

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2 \quad (8)$$

Although radical coupling is associated with very low enthalpy activation, the occurrence of termination reactions is controlled by radical concentration, which is responsible for the frequency of encounters between radicals and also by steriochemistry which causes radicals to collide with the correct orientation. Termination reactions may become important in edible oils heated at elevated temperatures, when large amounts of polymers are formed in frying oils. Hydroperoxides may also decompose to produce alcohols, aldehydes, allyl formates, ketones, hydrocarbons, etc.

1.1 Factors influencing lipid oxidation.

The autoxidation reaction is influenced by various factors such as fatty acid compositions, enzymes, oxygen tension, presence of metal catalysts and water.

1.1.1 Fatty acid compositions

Hydrogen abstraction occurs much easier in unsaturated fatty acids than in their saturated counterparts. The number, position and geometry of double bonds affect the rate of oxidation. Relative rates of oxidation for arachidonic, linolenic, linoleic and oleic acid are approximately 40 : 20 : 10 : 1, respectively. Cis acids are oxidized more readily than their trans isomers and conjugated double bonds are more reactive than non conjugated (Nawar, 1996).

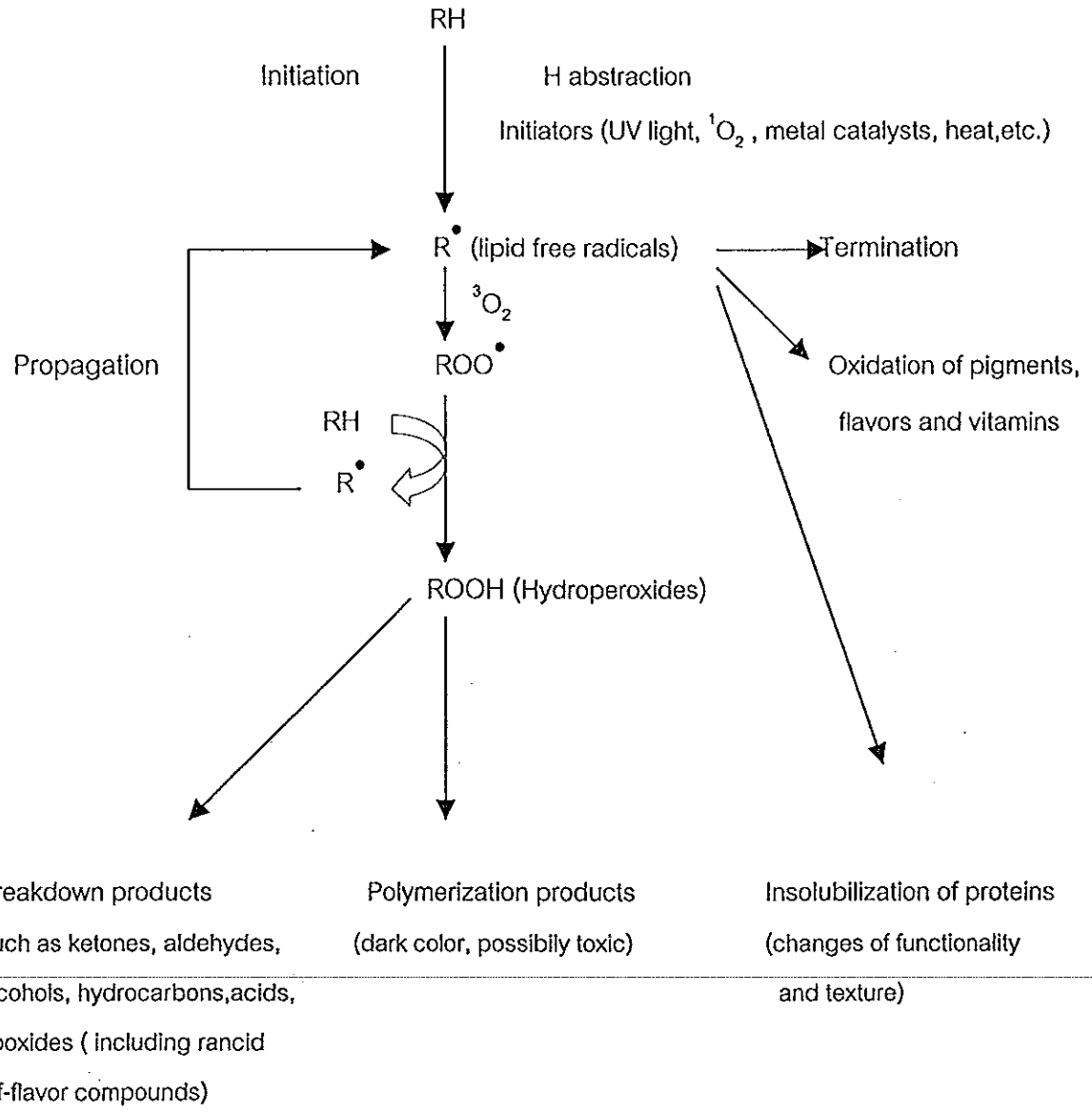


Figure 1 Autoxidation of polyunsaturated fatty acids and their consequences.
Source : Shahidi and Wanasundara (1992).

1.1.2 Pro-oxidants

Transition metals, particularly possessing two or more valence states and a suitable oxidation-reduction potentials between them e.g., cobalt, copper, iron and manganese are effective pro-oxidants. If present, even at concentration as low as 0.1 ppm, they can decrease the induction period and increase the rate of oxidation (Nawar, 1996). Several mechanisms for metal catalysis of oxidation have been postulated as follows:

a) Acceleration of hydroperoxide decomposition:

$$M^{n+} + ROOH \rightarrow M^{(n+1)} + OH^{-} + RO^{\bullet}$$
$$M^{n+} + ROOH \rightarrow M^{(n-1)} + H^{+} + ROO^{\bullet}$$

b) Direct reaction with the unoxidized substrate:

$$M^{n+} + RH \rightarrow M^{(n-1)} + H^{+} + R^{\bullet}$$

c) Activation of molecular oxygen to give singlet oxygen and peroxyl radical:

$$M^{n+} + O_2 \rightarrow M^{(n+1)} + O_2^{-} \xrightarrow{-e^{-}} {}^1O_2$$
$$M^{n+} + O_2 \rightarrow M^{(n+1)} + O_2^{-} \xrightarrow{+H^{+}} HO_2^{-}$$

Transition metal ions such as iron and copper can accelerate peroxidation by decomposing lipid hydroperoxide in both their lower (Eq. 9) and higher (Eq. 10) oxidation states (Jadhav *et al.,* 1996).

$$ROOH + Fe^{2+}\ (Cu^{+}) \rightarrow RO^{\bullet} + Fe^{3+}\ (Cu^{2+}) + OH^{-} \quad (9)$$

(Alkoxyl radical)

$$ROOH + Fe^{3+}\ (Cu^{2+}) \rightarrow ROO^{\bullet} + Fe^{2+}\ (Cu^{+}) + H \quad (10)$$

(Peroxyl radical)

Lipoxygenase (LOX ; linoleate : oxygen oxidoreductase, EC 1.13.11.12) is a non-heme iron-containing enzyme that catalyzes the dioxygenation of the cis,cis-1-4,pentadiene structure in fatty acid and fatty acid esters by molecular oxygen (Toyasaki, 1996). Lipoxygenase is an active lipid oxidation catalyst found in plants and some animal tissue (Decker, 1998).

1.1.2 Other factors

A. Temperature

In general, the rate of oxidation increases as the temperature increases. Tian and White (1994) found that peroxide values of soy and cottonseed oils kept at 60^{o}C were higher than those of soy and cottonseed oils stored at 30^{o}C.

B. Surface area

The rate of oxidation increases in direct proportion to the surface area of the lipid exposed to air. Furthermore, as surface-volume ratio is increased, a given reduction in oxygen partial pressure becomes less effective in decreasing the rate of oxidation (Nawar, 1996).

C. Moisture

Generally, water has a prooxidation effect at low (a_w<0.1) and high activities (a_w>0.55) and an antioxidant effect at medium water activities (a_w=0.3) on lipid autoxidation in foods (Nawar, 1985). Several hypotheises have been advanced to explain the protective effect of water in retarding lipid oxidation. Water reduces oxygen diffusion by forming a barrier for oxygen over the lipid surface. Additionally, water lowers the effectiveness of metal catalysts such as copper and iron. Water also forms hydrogen bonds with hydroperoxides and retards hydroperoxide decomposition (Krishna and Prabhakar, 1992 ; Lindely, 1998). Krishan and Prabhakor (1992) found that higher water activity values, particularly water activity of 0.67, tended to stabilize peroxides. Water activity had considerable influence on the formation of secondary products of autoxidation as evidenced by the variation

in the type and quantity of carbonyl compounds at different water activity values (Nawar, 1996).

D. Radiation

Radiation generates radicals, including hydroxyl radicals. Hydroxyl radicals are capable of abstracting hydrogen atoms from membrane lipid and bring about peroxidic reaction of lipid (Jadhav, 1996).

2. Antioxidants

2.1 Classification of food antioxidants.

Based on their functions, food antioxidants are classified as primary or chain-breaking antioxidants, synergist, secondary antioxidants and miscellaneous antioxidants (Rajalakshmi and Narasimnan, 1996).

2.1.1 Primary antioxidants

Primary antioxidants terminate the free radical chain reaction by donating hydrogen or electrons to free radicals and converting them to more stable products. Many of phenolic compounds, e.g. BHA, BHT, TBHQ, tocopherol, flavonoids, eugenol and rosemary also have chain-breaking properties (Rajalakshmi and Narasimhan, 1996 ; Ill *et al.,* 1998). Primary antioxidants are effective at very low concentrations. At higher levels, they may become prooxidants (Rajalakshmi and Narasimhan, 1996).

2.1.2 Synergistic antioxidants

Synergistic antioxidants can be broadly classified as oxygen scavengers and chelators. Hence phenolic antioxidants can be used at lower levels if a synergist is added simultaneously to the food product. Some synergists also provide an acidic medium that improves the stability of primary antioxidants. Oxygen scavengers include ascorbic acid, ascorbyl palmitate, sulfites and erythorbates. Chelators e.g. ethylenediaminetetraacetic acid, citric acid and phosphates are not antioxidants but they are highly effective as synergists with both primary antioxidants and oxygen scavengers.

Han *et al.* (1991) reported that ascorbic acid (0.02%) with α-tocopherol (0.4%) lengthened the induction period of fish oil by 22-fold, due to their synergism.

2.1.3 Secondary antioxidants

Secondary or preventive antioxidants such as thiodipropionic acid and dilauryl thiodipropionate function by decomposing the lipid peroxides into stable end products (Rajalakshmi and Narasimhan, 1996).

2.1.4 Miscellaneous antioxidants

Compounds listed under miscellaneous antioxidants e.g. flavonoids and related compounds and amino acids, function as both primary antioxidants and synergists. Nitrites and nitrates, which are used mainly in meat curing, probably function as antioxidants by converting heme proteins to inactive nitric oxide forms and by chelating the metal ions, especially nonheme iron, copper and cobalt that are present in meat. β-carotene and related carotenoids are effective quenchers of singlet oxygen and also prevent the formation of hydroperoxides.

2.2 Mode of action of antioxidants.

2.2.1 Free radical scavenger

Some antioxidants can act as free radical scavenger by donating a hydrogen to a free radical (Buettner, 1993 ; Namiki, 1990 ; Osawa, 1994). Antioxidants may either delay or inhibit the initiation step by reaction with the peroxy ($ROO^{\bullet}$) or alkoxy ($RO^{\bullet}$) radicals. The compounds which have the reduction potential lower than that of a free radical are capable of donating a hydrogen to the free radical unless the reaction is kinetically unfeasible (Buettner, 1993).

Hydrogen donor

$$ROO^{\bullet} + AH_2 \rightarrow ROOH + AH^{\bullet}$$

$$RO^{\bullet} + AH_2 \rightarrow ROH + AH^{\bullet}$$

$$AH^{\bullet} + AH^{\bullet} \rightarrow A^{\bullet} + AH_2$$

Electron donor

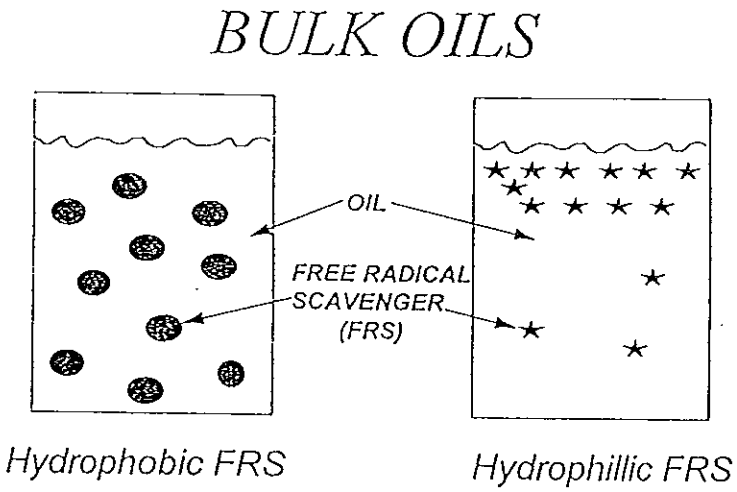
$$ROO^{\bullet} + AH_2 \rightarrow (AH_2 - ROO)^{\bullet}$$

$$(AH_2 - ROO)^{\bullet} + ROO^{\bullet} \rightarrow \text{stable products}$$

Hydroxyl groups associated with phenolic compounds are the most common and effective free radical scavengers in food. These phenolic compounds are effective antioxidants because of their ability to both donate hydrogen and form stable phenolic free radicals (Shahidi and Wanasundara, 1992). The solubility characteristics of phenolic antioxidants are also crucial towards activity. The antioxidant paradox as a phenomenon where hydrogen hydrophilic free radical scavengers were more effective antioxidants than hydrophobic free radical scavengers in bulk oil while hydrophobic free radical-scavengers were more effective in emulsified oils (Figure 2) (Decker, 1998).

2.2.2 Peroxide decomposer

Plant phenolic can delay the onset of lipid and decomposition of hydroperoxides in food products as well as in living tissue (Wettasinghe and Shahidi, 1999). Furthermore, amine, dithiopropionic acid and thiopropionic acid function by decomposing the lipid peroxide into stable end products such as aldehyde and alcohol (Namiki, 1990). Peroxides are important intermediates of oxidative reactions since they decompose via transition metals, irradiation and elevated temperatures to form free radicals (Decker, 1998). Hydroperoxide formation and decomposition in emulsion system are dependent on the effective concentrations of antioxidants in the oil and water phase and the interface (Wang *et al.,* 1996; Wu *et al.,* 1994).



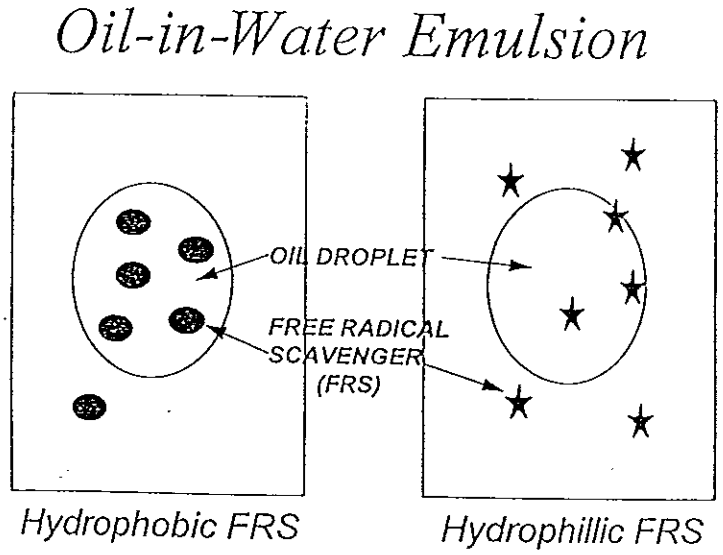


Figure 2 Physical location of hydrophilic and hydrophobic free radical scavengers in bulk oils and oil-in-water emulsion.

Source : Decker (1998).

2.2.3 Singlet oxygen quencher

Oxidation occurs in the presence of molecular oxygen in both the triplet and singlet states. Atmosphere oxygen that is in the triplet state contains two unpaired electrons, while oxygen in the singlet state has no unpaired electrons. The electron arrangement of triplet oxygen does not allow for a direct reaction with organic compounds, such as unsaturated fats. Singlet oxygen generated from triplet oxygen by excitation is suggested to be responsible for initiating lipid oxidation of food products, due to its ability to directly react with the electron-rich double bonds of unsaturated fats and other singlet-state compound (Bradley and Min, 1992). The importance of singlet oxygen as an initiator of oxidation has led to a growing interest in the prevention of singlet oxygen reaction, especially by quenching to ground state oxygen. β-carotene has been shown to be an inhibitor of singlet oxygen oxidation. Energy transfer from singlet oxygen to a quencher such as β-carotene leads to the formation of triplet state oxygen and an excited triplet state quencher is found as follows (Stahl and Sies, 1992 ; Woodall *et al.*, 1997) :

$$^{1}\text{Carotenoid} + {}^{1}O_2 \rightarrow {}^{3}\text{Carotenoid} + {}^{3}O_2 \quad (1)$$

$$^{1}\text{Carotenoid} + {}^{3}\text{Sen} \rightarrow {}^{3}\text{Carotenoid} + \text{Sen} \quad (2)$$

$$^{1}\text{Carotenoid} \rightarrow {}^{3}\text{Carotenoid} \quad (3)$$

Phenolic antioxidants including TBHQ, BHT and BHA do not effectively protect lipid oxidation induced by singlet oxygen (Yasaei *et al.*, 1996).

2.2.4 Enzyme inhibitor

Lipoxygenase specifically oxidizes polyethenoid acids containing methylene-interrupted double bond that are in the cis geometrical configuration such as those in linoleic, linolenic and arachidonic acids but not oleic acid (Oomah *et al.*, 1997). Several lipoxygenases have been described and the major distinction between isomers of these enzymes found in a

variety of cell types is the positional specificity of the hydrogen abstraction and oxygen addition site on the substrate fatty acid (Kanner *et al.,* 1987). Free radical intermediates occur during lipoxygenase catalysis and these can lead to cooxidation of easily oxidized compounds such as carotenoids and polyphenols (Rajalakshmi and Navasimhon, 1996). Lipoxygenase is present in spices, soybean, fish, wheat flour and vegetable. It is able to catalyze the oxidation of unsaturated fats, increasing the rate at which peroxides and volatile breakdown products are generated (Zougari *et al.,* 1995; Jadhav *et al.,* 1996). Phenolics are capable of indirectly inhibiting lipoxygenase activity by not only acting as free radical inactivators but also by reducing the iron in the active site of the enzyme to the catalytically inactive ferrous state (Decker, 1998; Laughton *et al.*, 1991).

2.2.5 Chelating agents

Many compounds are capable of chelating metals, however not all chelators inhibit oxidation (Decker, 1998). An unshared pair electrons in their molecular structure promotes the chelating action (Rajalakshmi and Narasimhon, 1996). Chelators which exhibit antioxidative properties inhibit metal-catalyzed reaction by one or more of the following properties : prevention of metal redox cycling ; occupation of all metal coordination sites ; formation of insoluble metal complexes ; steric hindrance of interactions between metal and lipids or oxidation intermediates (e.g. peroxides) (Graf and Eaton, 1990 ; Decker, 1998).

3. Natural antioxidants.

A multitude of natural antioxidants have already been isolated from different kinds of plant materials such as oil seeds, cereal crops, vegetables, fruits, leaves, roots, spices and herbs (Nicoli *et al.,* 1999 ; Pietto *et al.,* 1998).

A. Herbs and Spices

Many herbs and spices are known to exhibit antioxidant activity in food lipid (Jaswir and Man, 1999 ; Man and Tan, 1999 ; Zhang *et al.,* 1999). Economou *et al.* (1991) reported that the extracts from plants of the family

Labiatae including oregano, dittany, thyme, marjoram, spearmint and lavender showed antioxidant property. Farag *et al.* (1989) reported that essential oils from some spices including caraway, clove, cumin, rosemary, sage and thyme inhibited linoleic oxidation. Screening of 180 different types of oriental herbs revealed that 44 spices had quite strong antioxidant activities (Kim *et al.*, 1994). Some aromatic herbs that can be grown in Lithuania including thyme, marjoram, sage, rosemary, oregano, lavender basil, catrip, hyssop and anise hyssop have been found to possess antioxidant activity (Banias *et al.*, 1992 ; Svoboda and Deans, 1992 ; Vekiri *et al.*, 1993 ; Senatore, 1996 ; Dapkevicius *et al.*, 1998). Milovanovic *et al.* (1998) also found the antioxidant activity in the extract of *Anthriscus sylvestris.* The medicine plant extract from bilberry, ginkgo, sweet clover, witch hazel, artichoke and hawtorn exhibited antioxidant activity in vivo (Pietta *et al.*, 1998).

The medicinal herb *Thonningia sanguinea* and taushen (*Salvia miltiorrhiza* Bunge) showed strong antioxidant activity (Ohtani *et al.*, 2000 ; Weng and Gordon, 1992). Ogata *et al.* (1997) reported that herb extract of Magnolia cortex contained antioxidative compounds, magolol and honokiol. Three herbs extracts, including the flower of *Chrysanthemum morifolium* Ramat (FCMR), the calyx of *Hibiscus sabdariffa* L. (CHSL) and roasted seed of *Hordeum vulgare* L. (RSHVL) showed marked antioxidant activity in both linoleic acid and liposome model system indicating that those herb extracts may protect the cell from damage by lipid peroxidation (Figure 3) (Duh and Yen, 1997). Cao and Zhu (1997) reported that tetradrine isolated from herb *stephania tetrandra* S. Moore. (root) inhibited phospholipid liposome peroxidation and inhibited DNA damage.

Antioxidative compounds have been characterized in many sources (Table 1). Nishi *et al.* (1991) noted that musizin, an active component isolated from *Rumex japonicus* Houtt, had higher antioxidant activity than BHT and α-tocopherol. The antioxidant activity of rosemary extracts primarily related to two phenolic diterpenes, carnosic acid and carnosol (Aruoma *et al.*, 1992 ;

Chen *et al.*, 1992). Man and Tan (1999) found that rosemary strongly inhibited oxidation in bleached and deodorized palm olein, compared to BHT and BHA.

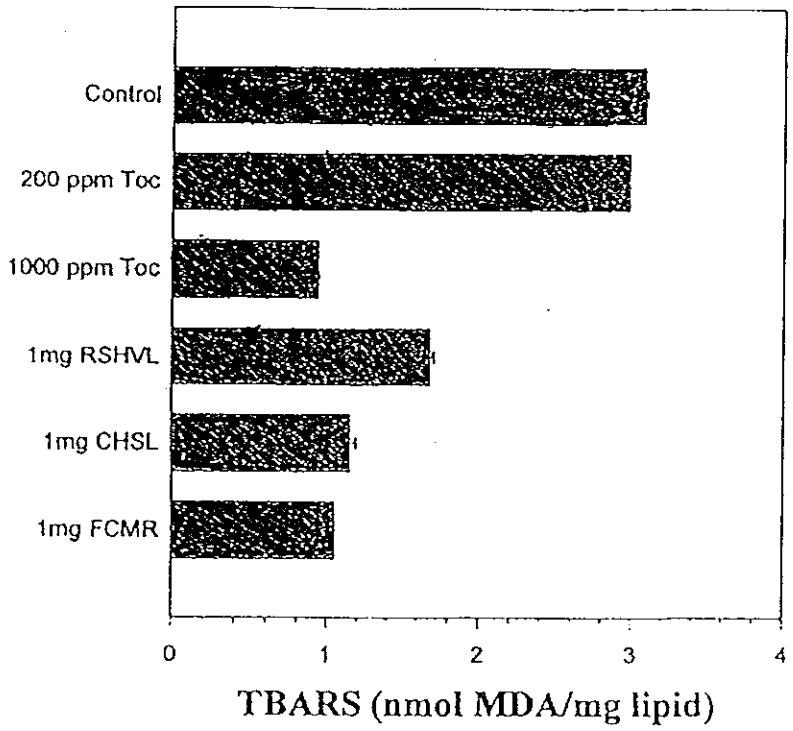


Figure 3 Antioxidative action of extracts of herbs in the liposome model system as determined by thiobarbituric acid method.

Source: Duh and Yen (1997).

Antioxidant extracts prepared from spices *Aframomum danielli* were more effective than BHT and α-tocopherol in stabilizing refined peanut oil. Antioxidant components of *Aframomum danielli* were tentatively identified as phenolic compounds of the trihydroxy type with reducing properties. Schwaz and Ernst (1996) found that 2,3-dihydroxy-4-isopropyl-1-methylbenzene isolated from thyme exhibited greater antioxidant activity than α-tocopherol and BHA. Mansour and Khailil (2000) found that the freeze-dried extracts from fenugreek seed and ginger rhizomes appeared to possess antioxidant activity against a carotene-linoleic acid emulsion. Ginger rhizome extract had an antioxidant activity comparable to commercial antioxidants. Fejęs *et al.* (2000) reported that herb extract of chervil (*Anthiscus cerefolium* L. Hoffm)

showed antioxidant and anti-lipoperoxidant activity, which indicates its effectiveness in prevention of disease caused by overproduction of radicals.

Table 1 Antioxidant compounds from natural sources.

Sources	Antioxidant compounds	Reference
Rosemary leaves	carnosol, carnosic acid	Aroma *et al.* (1992)
Sage	rosmanol, rosemarinic acid	Hopia *et al.* (1996)
	isorosmanol, epirosmanol	CuVelier *et al.* (1992 ;1996)
	rosmaridiphenol	Wang *et al.* (1999)
	rosmariquinone	
	rosmadial, and methyl carnosate	
Thyme	p-cymenene-2,3-diol, thymol	Schwarz and Ernst (1996)
	and carvacrol	
Buckwheat	catechin and rutin	Watanabe (1998)
Dittany	carvacrol and β-phellandrene	Moller *et al.* (1999)
Ginger	curcumin, demethyxycurcumin	Jitoe *et al.* (1992)
	and bisdemethoxycurcumin	Kikuzaki and Nakatani (1993)
Tanshen	rosmariquinone,miltrone,	Weng and Gordon (1992)
	rosmariquinone,miltrone,	
	tanshinone, cryptotanshinone,	
	dihydrotanshinone	
	dehydrorosmariquinone	
Clove	euganol, gallic acid	Kramer (1985)
Herb *Thonningia sanguinea*	ellagitannin and thonningianins	Otani *et al.* (2000)

B. Leaves.

Many phenolic antioxidants are found in a variety of plant leaves. From screening of 76 different kinds of plant leaf waxes, the extract from *Eucalyptus* and *Prunes* plant showed the highest antioxidant activity. 4-hydroxy-tritriacontane-14,18-diene and n-tritriacontane 16,18-dione were identified as the two major antioxidants in leaf waxes of *Eucalyptus* (Osawa and Namiki, 1981 ; 1985). The extracts of banana leaves, *Orephea enneandra, Daphiphyllum calycinum* and *Allium nutans* L. showed antioxidant property (Cavin *et al.,* 1998 ; Sekiya, 1985 ; Gamez *et al.,* 1998 ; Stainer *et al.,* 1998). Chevolleau *et al.* (1992) screened antioxidant activity in leaves of Mediterranean plants and found that myrtle showed highest antioxidant activity. 7,4-dimethylquercetin, 3′-methylquercetin, quercetin and isoquercitin were isolated from the leaves of *Polygonum hydropiper*, a culinary herb, (Haraguchi *et al.,* 1992). The active antioxidant component in young green barley leaves was identified as 2″(3″)-O-glycosylisovitexin and its antioxidative activity was almost equivalent to that of α-tocopherol in lipid peroxidation system (Osawa *et al.,* 1992). Takahashi *et al.* (1999) reported that gallic acid was an active component in leaves of *Casuarina equisetifolia*. Castillo *et al.* (2000) found that antioxidant activity of flavonoids from olive leaf was influenced by the presence of functional groups in their structure. Antioxidant activity of natural polyphenols depends upon the number and location of their aromatic hydroxyl groups (Chen *et al.*, 1996). Masuda *et al.* (1999) noted that an electron donor and/or bulky groups at the ortho- or para-position of the phenol were required for inhibition of lipid oxidation. Ogata *et al.* (1997) found that the antioxidant activities of catechol derivatives with phenolic hydroxyl group at the ortho position are much higher than those at meta position and phenolic compounds that contain allyl groups may be effective antioxidants. The extract of leaves of *Gulera senegalensis* inhibited peroxidation of phospholipid liposome (Bucar *et al.,* 1998).

C. Tea

Tea has been known to be rich in antioxidants. Regular intake of tea may improve antioxidant status and lower risk of chronic disease associated with increased oxidative stress, such as coronary heart disease. Twenty-seven types of dried tea leaves had widely different antioxidant activity in vivo (Szeto *et al.*, 1998). Yen and Chen (1995) showed that the antioxidant activity of various tea extracts is related to their antimutagenicity. Chen *et al.* (1996) studied the antioxidant property of the extracts from various Chinese teas including green, yellow, white, dark-green, black and oolong teas. Ethanolic extracts from yellow, white and green teas strongly inhibited oxidation of canola oil, compared to BHT. This was probably due to the presence of natural polyphenols. In contrast, an extract from oolong tea exhibited only moderate antioxidant activity because of the partial destruction of natural polyphenols by semifermentation. The extracts of dark-green, black and ginseng teas showed little or no protection to canola oil from lipid oxidation, probably due to the complete destruction of natural polyphenols by low activity fermentation during manufacturing processes. Benzie and Szeto (1999) reported that the extract of green tea leaves exhibited strong antioxidant activity than black tea and oolong teas. Frankel *et al.* (1997) found that the extract of green teas exhibited antioxidant activity in soybean lecithin liposome system with copper catalyst at 37°C. Matsuzaki and Hara (1985) reported that the major antioxidants from green tea were catechin and its derivatives. The hydrophilic catechol-type flavonoids of green tea were more effective in inhibiting oxidation of liposomes composed of egg phosphatidylcholine than was the lipophilic α-tocopherol (Terao *et al.*, 1994). Gardner *et al.* (1998) observed that green tea leaves had higher antioxidant activity than black tea in lipophilic systems. Chen *et al.* (1998) reported that antioxidant activity of green tea catechin extract was more effective than that of rosemary. The methanolic extracts of old tea leaves (0.1%) was as active in retarding the deterioration of oil as a rosemary extract (0.1%) during

repeated frying of potato crisps (Zandi and Gordon, 1999). Yen and Hsich (1998) found that extracts of Du-zhong tea from leaves exhibited stronger antioxidant activity than roasted cortex and raw cortex extracts. The antioxidant activity of extract of Du-zhong correlates to their polyphenol content. The extracts of Du-zhong leaves inhibited lipid peroxidation of linoleic acid, lecithin liposome and mitochondria. The extracts of Du-zhong leaves may be useful in inhibiting membrane lipid peroxidation and preventing free radical-linked disease (Sasaki *et al.,* 1996 ; Slage, 1995).

D. Fruits and Vegetables.

Many fruits and vegetables have been reported to contain various antioxidants. Wang *et al.* (1996) reported that the extract of fresh strawberries had highest total antioxidant capacity compared to those of plum, orange, red grape, kiwi fruit, pink grape fruit, white grape, banana, apple, tomato, pear and honeydew melon. The extracts of mango fibers, lemon fibers, sweet cherry, blueberry, tart cherry and 14 varieties of grapes showed antioxidant property (Velioglu *et al.*, 1998 ; Wang *et al.,* 1999 ; Bonilla *et al.,* 1991). The antioxidant activity for LDL was associated directly with anthocyanins and flavonols, while it correlated with the hydroxycinnamate content in berries and grapes when liposome was used (Abuja *et al.,* 1998 ; Frankel *et al.,* 1998 ; Meyer *et al.,* 1998 ; Ghiselli *et al.,* 1998). However, anthocyanins, flavan-3-ols and hydroxycinnamic acids were shown to inhibit liposome oxidation (Huang and Frankel, 1997 ; Satue-Gracia *et al.,*1997 ; Heinonen *et al.,* 1998).

Cao *et al.* (1996) screened antioxidant activity of 22 common vegetables. Kale was found to have highest antioxidant activity, followed by garlic, spinach, brussels sprouts, alfalfa sprouts, broccoli flowers, beets, red bell pepper, onion, egg plant, corn, cauliflower, potato, sweet potato, leaf lettuce, cabbage, string bean, carrot, yellow squash, iceberg lettuce, celery and cucumber, respectively. Furuta *et al.* (1997) reported that the ethanol extracts from yellow and red sweet pepper, red cabbage and red onion had higher antioxidative activity than the extracts from green and white cultivars.

Gazzani *et al.* (1998) found that carrot, cauliflower, celery, eggplant, garlic, mushroom, onion, white cabbages, white potato and zucchini showed antioxidant property. Antioxidant activity was also present in the extract from *Feijoa sellowiana* Berg, fruit which is widely used for human food (Vuotto *et al,*. 2000). Addition of burdock extracts resulted in lower malondialdehyde in both linoleic acid and liposome model system, compared to control (Duh, 1998). Yin and Cheng (1998) reported that several members of the *Allium* family, including garlic bulb, bakery garlic bulb, Chinese leek, Chinese chive, onion bulb, scallion and shallot bulb showed antioxidant activity in lecithin liposome system.

E. Cereals and oil seed.

Cereals are a potential source of both phenolic antioxidants and various antioxidatively acting compounds capable of encapsulating lipids (Lopez-Nicolas *et al.,* 1997 ; Lin *et al.,* 1995). Phenolic acids such as ferulic and ρ-coumaric acids, which are known to be naturally occurring antioxidants, have been proved to exist as carbohydrate esters in the Gramineae cell wall such as rich endosperm, zea shoot, barley straw and bamboo shoot (Cuvelier *et al.,* 1992 ; Ishii *et al.*, 1990).

Wu *et al.* (1994) and Asamarai *et al.* (1996) reported that the extract of wild rice possessed antioxidant activity. Isovitexin was major antioxidant in rice hull (Ramanathan *et al.,* 1989). Ohta *et al.* (1994) reported that sage extract had a greater antioxidant activity than that of polished rice extract. The extract of oat grout inhibited the oxidation of soybean and cotton seed oils (Tian and White, 1994 ; Xing and White, 1997). Oomah and Mazza (1996) found that the extract of buckwheat seed possessed antioxidant activity

Schubert *et al.* (1999) reported that the pomegranate seed showed greater antioxidant activity than red wine. The extract from canola and borage seed exhibited antioxidant activity in linoleic acid/β-carotene model system (Wanasundara *et al.*, 1994 ; Wettasinghe and Shahidi, 1999; 2000).

4. Extraction of antioxidants from plants.

Some authors demonstrated a variation in the activity of antioxidant extracts when different solvents were used for extraction. Many different organic solvents have been used for the extraction of antioxidant compounds from plants (Table 2). Chang *et al.* (1977) reported that sage and rosemary had a higher antioxidant activity when more polar solvents were used. Julkunen-Titto (1985) found that 80% methanol extract from leaves of *Salix cv. aquatica* showed higher total phenolic content than those prepared with 80% acetone, distilled water, 0.2 M NaCl and diethyl ether (Figure 4). Nishi *et al.* (1991) noted that hexane extract of *Rumex japonicus* Houtt showed higher antioxidant activity than the ethyl acetate and chloroform extracts, while the water and n-butanol extracts exhibited no antioxidant activity. Chen *et al.* (1992) found that the hexane extracts of rosemary leaves had a lower yield than methanol extracts. The hexane extract from 16 types of leaves of Mediterranean plants had a higher antioxidant activity than methanol extract. However, higher yields were observed for methanol extract, compared to hexane extract (Chevolleau *et al.*, 1992). Methanol is a widely used and effective solvent for extraction of antioxidant from herbs, leaves and other plants (Guillen and Manzanos, 1998 Takahashi *et al.*, 1999 ; Zandi and Gordon,1999). Methanol and ethanol extracts of wild rice exhibited better inhibition of lipid peroxidation in beef and lard than ethyl acetate extract (Wu *et al.*, 1994). Przybylski *et al.* (1998) observed that the methanol extract of buckwheat seed had stronger antioxidant activity than hexane, diethyl ether, ethyl acetate and acetone extracts. Wang *et al.* (1999) noted that methanol extracts of dried Balaton and Montmorency tart cherries had stronger antioxidant activity than haxane and ethyl acetate extracts. Azizah *et al.* (1999) reported that methanol was proved to be the best solvent in extracting antioxidants from cocoa by-products, followed by mixtures of chloroform, ether and dichloroethane and mixtures of chloroform, methanol and dichloroethane.

Table 2 Antioxidant activity of natural extracts.

Sources	Extraction method	Substrate	Analytical method	Relative activity
Sage, Rosemary, Clove, Cumin, Caraway,Thyme	Steam distillation	linoleic acid emulsion	Bleaching of β-carotene conjugated diene, TBARS	Clove>Thyme >Rosemary >Cumin>Sage >Caraway
Rosemary, Sage	Hexane, benzene ethyl ether, chloroform	Lard, chicken fat, vegatable oils	Peroxide value	Polar solvent >non-polar solvent
Rosemary, Sage	n-Hexane, Dichloromethane Ethanol	Linoleic acid	Bleaching of β-carotene	Hexane >Dichloromethane or Ethanol
Oregano, Thyme Marjoram, Basil, Spearmint, Lavender Dittany	Methanol	Lard	Oven test (75°C) Peroxide value	Oregano>Thyme> Marjoram>Dittany >Spearmint >Lavender >Basil
Nutmeg, Rosemary, sage	Commercial	Lecithin emulsion	Diene bond	Rosemary>Sage >Nutmeg
Leaves, Bulb and Root of Garlic	Methanol	Malonyldialdehyde, Fenton .HO	ESR	Leaves >Bulb or Root
Leaves, Flowers Stem of Thyme	Dichloroethane	Hexanal	GC	Leaves>Flowers >Stems

Sources : Madsen and Bertelsen (1995) ; Stajner *et al.* (1999)

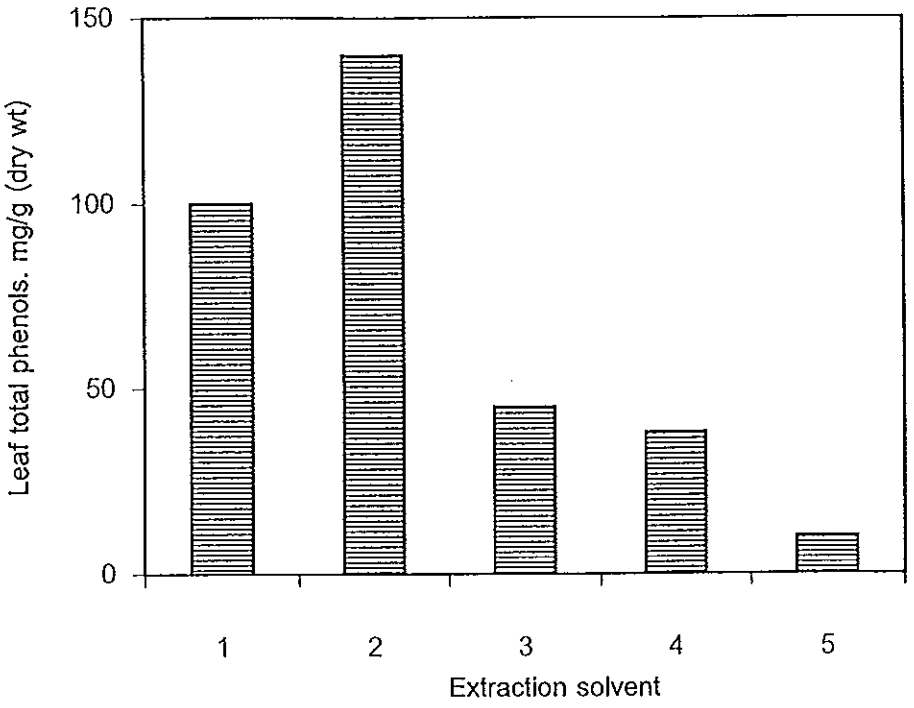


Figure 4 Effect of extraction solvent on total phenolics in the leaves of *Salix cv.* aquatica ; 1= 80% acetone, 2= 80% methanol, 3= distilled water, 4= 0.2 M NaCl, 5= diethyl ether.

Source : Julkunen-Titto (1985).

Many phenolic compounds are soluble in polar solvents (Moller *et al.*, 1999). Duh (1998) studied the effect of various solvents including water, methanol, ethanol, chloroform and n-hexane on the extraction of antioxidant compounds from burdock. Among the solvents used for extraction, water yielded the greatest amount of extract that exhibited the strongest antioxidant activity. Water extracts and hot water extracts of burdock showed antioxidant activity in both linoleic acid and lecithin liposome model systems. However, the hot water extract of *Porphyra yezoensis* showed low antioxidant activity (Figure 5) (Nakayama *et al.*, 1999). Pure water itself is not a very good solvent for extraction from leaves (Julkunen-Titto, 1985).

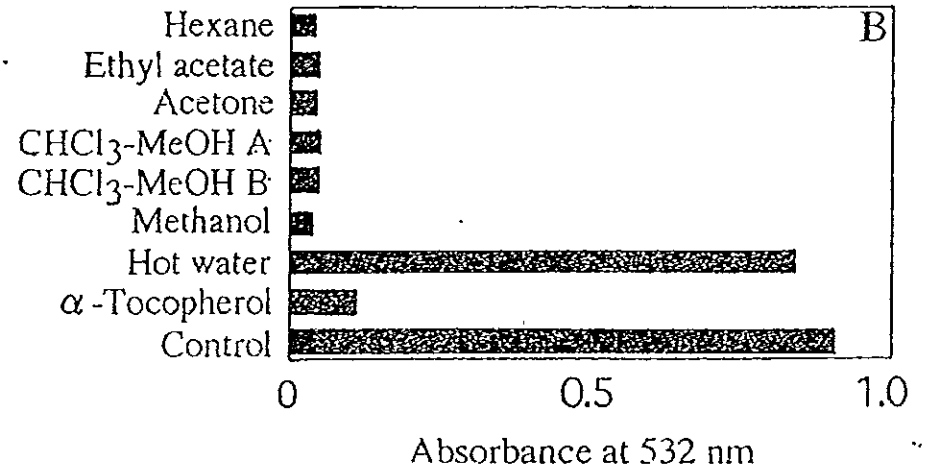


Figure 5 Antioxidant activities of the extracts from *Porphyra yezoensis.*
Source : Nakayama *et al.* (1999)

5. Some properties of natural antioxidants.

5.1 Heat stability

The action of the antioxidant depends on its participation in a series of reaction involving radicals. The rate of these reactions may have different activation energies (Denisov and Khudyakov, 1987). Hammama and Nawar (1991) reported the thermal decomposition of some phenolic antioxidant including BHA, BHT, TBHQ and propyl gallate (PG). After heat treatment at 185°C for 1 hr, the loss of antioxidant activities for BHT, PG, BHA and TBHQ was 20.4, 37.1, 42.8 and 47.7 % respectively. Marinova and Yanishlieva (1992) evaluated the efficiency of α-tocopherol and ferulic acid as antioxidant

in lard at 25, 50, 75 and 100°C. The results showed that an increase in temperature led to change in antioxidative effectiveness of ferulic acid, while the antioxidative effectiveness of α-tocopherol increased with temperatures. Prasad *et al.* (1996) studied the influence of heating on the hydroxyl radical-scavenging property of garlic and reported that its activity was reduced by approximately 10% when heated at 100°C for 20, 40 or 60 min. Several degradation products were reported to be formed from the phenolic diterpenes of rosemary extracts between 100°C and 170°C and under steaming conditions at 200°C some of these thermal degradation products were active as antioxidants (Schwarz, 1992). After heating of soybean oil treated with ajowan seed extract at 180°C for 14 days, the conjugated diene value of oil with ajowan seed extract was significantly lower than that of control (Mehta *et al.,* 1994). Man and Tan (1999) found that the uses of rosemary and sage extract at a level of 200 ppm in frying oil prolonged the storage life of potato chips fried at 180°C. Jawir and Man (1999) reported that sage and rosemary extract showed good thermal resistance and strong antioxidative characteristics. The ethanol extract from *Saturejae hortensis* L. improved the oxidative stability of sunflower oil even after 50 hrs at 180°C (Yanishlieva *et al.,* 1997). Yen and Lee (1997) observed that the ethyl acetate extract from *Aspergillus candidus* broth filtrate showed good thermal stability when heated at 185°C for 2 hrs. Mansour and Khalil (2000) indicated that antioxidants in potato peel and fenugreek seed extract were fairly heat-stable with 63.2 and 58.0% activity, respectively, remaining after 120 min heating at 100°C, while ginger rhizome extract was about 28% after heating at 100°C.

5.2 Effect of pH

Efficiency of antioxidant is dependent on a range of environmental factor such as pH of foods. Lee *et al.* (1975) reported that oxygen uptake rate increased two and four times at pH 7.5 and 8.0, respectively. Liu (1980) reported that hemoprotein-catalyzed oxidation is most active at alkaline pH.

Lee *et al.* (1986) found that the antioxidant effectiveness of extract from ginger rhizome was dependent on pH. The activity increased with increased pH ranging from 5.0 to 7.0. Mansour and Khalil (2000) observed that the antioxidant activity of freeze-dried extracts from ginger rhizomes and fenugreek seeds was maximum at pH 7.0, while for potato peel extract, it was maximum at pH ranging from 5.0 to 6.0 then decreased at neutral and alkaline pH values (Figure 6).

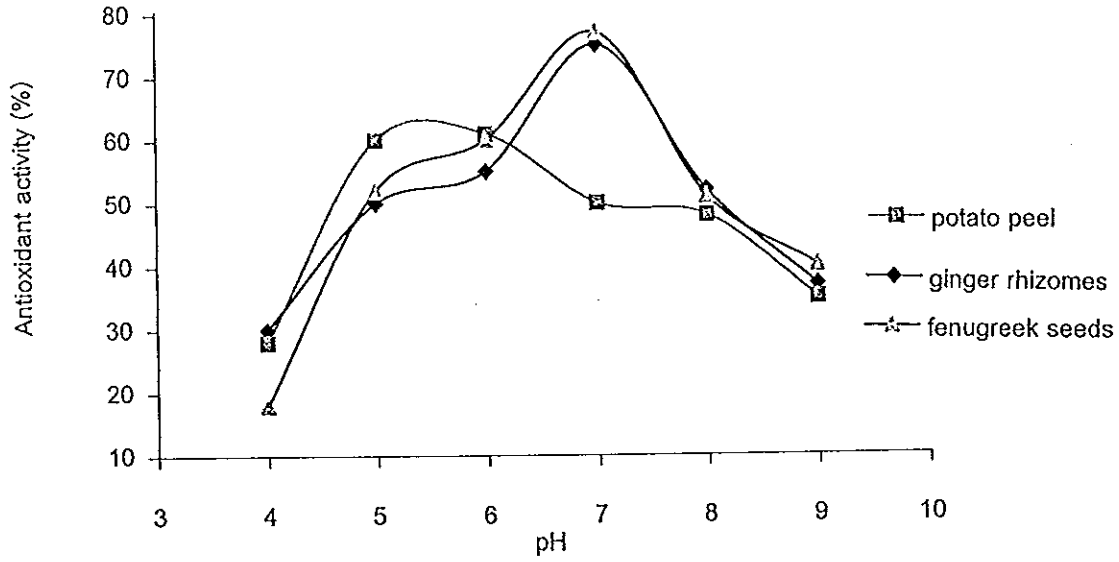


Figure 6 Effect of pH on antioxidant activity of freeze-dried extracts from potato peel, fenugreek seeds and ginger rhizomes.

Source : Mansour *et al.* (2000)

Barclay and Vinquist (1994) indicated that the antioxidant activity of Trolox is slightly better at pH 4.0 than at pH 7.0, but Trolox was completely dissociated at pH 11.0. Yen and Lee (1997) reported that the ethyl acetate

extract from *Aspergillus candidus* broth filtrate exhibited strong antioxidant activity at the neutral and acidic pH values but it was unstable at the alkali pH values. Carnosol and carnosic acid were much more active antioxidant in emulsion at pH 4.0 and 5.0 than at pH 7.0 (Frankel *et al.*, 1996). These antioxidants may have better reducing capacity at the lower pH values. Yen and Chen (1995) reported that pHs of 4.0, 6.0 and 8.0 did not affect the antioxidant of plant food in lecithin liposome system while pH of 2.0 reduced the antioxidant activity. Azizah *et al.* (1999) found that the methanol extract from cocoa by-products showed high antioxidative activities at neutral and alkaline pH.

5.3 Effect of concentration

Aruoma *et al.* (1993) reported that gallic acid has dual effects, as an antioxidant or prooxidant, depending on its concentration in liposome system. The rate of substrate oxidation decreases with increasing ferulic acid concentration (Marinova and Yanishlieva, 1992). The activity of 6,7-dihydroxyflavone was shown to be antioxidant at low concentrations and prooxidant at higher concentrations (Magnani *et al.*, 2000). Haroguchi *et al.* (1992) found that isoquercithin (Ia) and 7,4′-dimethylqucetin (Id) isolated components from the leaves of *Polygonum hydropiper* inhibited the oxidation of linoleic acid in a concentration dependent manner (Figure 7). Yanishlieva *et al.* (1999) reported that thymol and carvacrol inhibited the peroxidation in a concentration dependent manner. Wanasundara and Shahidi (1998) noted that dechlorophyllized green tea extract at 200 ppm exhibited excellent antioxidant activity in oil and its efficacy was higher than that of BHT, BHA at 200 ppm and α-tocopherol at 500 ppm but less than that of TBHQ at 200 ppm. However, green tea extract exhibited a prooxidant effect in oil. The antioxidant effect of methanolic extract of old tea leaves increased with concentration. At concentration of 0.25%, the antioxidant activity was not significantly different ($p>0.05$) from that of the rosemary extract (0.1%) (Zandi *et al.*, 1999). Azizah *et al.* (1999) found that increasing the concentration of

the methanolic extracts from cocoa by-products up to 2000 ppm resulted in an increase in antioxidative activity. The antioxidant activity, reducing power and radical scavenging effect of extract from burdock increased with an increase in concentration. Thabrew *et al.* (1998) observed that the extract of *Osbekia aspera* was shown to have scavenging effect on DPPH and hydroxyl radical in a concentration dependent manner.

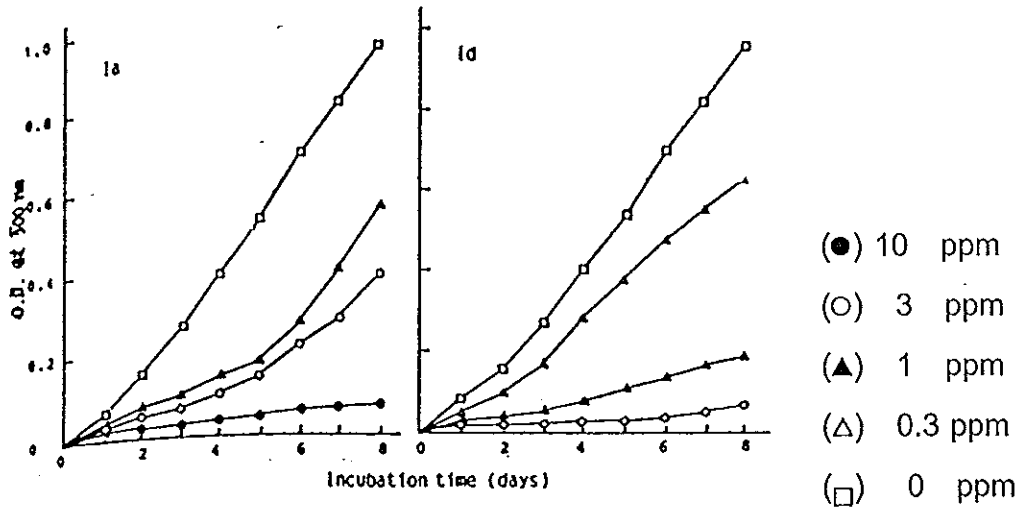


Figure 7 Concentration effects of antioxidative activity of compounds Ia and Id from the leaves of *Polygonum hydropiper.*

Source : Haraguchi *et al.* (1992)

5.4 Synergistic effect.

In general, the antioxidant effect of the mixture is greater than individual compound. Wada and Fang (1992) observed a synergistic effect between rosemary extract (0.02%) and α-tocopherol (0.05%). Gordon and Kourimska (1995) reported that the protective effect of carnosic acid on the oxidative stability of α-tocopherol, indicating the synergism between rosemary extract and tocopherols. Carnosic acid increased the oxidative stability of α-tocopherol in corn oil (Hopia *et al.*, 1996). Ishikawa *et al.* (1991) found that

atrovenetin-related antioxidants have been isolated from *Penicillium* species and exhibited excellent synergistic activities with tocopherol. The antioxidant activity of the extract from *Aspergillus candidus* broth filtrate in a combination with α-tocopherol, or citric acid was greater than that observed when the compounds were used alone (Yen and Lee, 1997). Duh (1998) observed that the mixtures of α-tocopherol, water extract of burdock and hot water extract of burdock exhibited a remarkable synergistic antioxidant effect in lecithin liposome system. However, no synergism was observed between rosemary extract and another organic acid, e.g. ascorbic acid (Han *et al.*, 1990). A very pronounced synergistic effect was found between citric acid and rosemary extract, while some effect was observed for combinations of citric acid and nutmeg or citric acid and sage but no synergism was observed between citric acid and oregano extract (Madsen and Bertelsen, 1995).

Banias *et al.* (1992) reported that the methanol extract of oregano, thyme, marjoram, dittany, rosemary and sage had a synergistic effect with ascorbyl palmitate but no synergism was observed for combination with α-tocopherol in the lard stored at 75°C. Citric acid showed high synergistic efficiency with marjoram extract but less effect with thyme extract.

6. Mode of action of natural antioxidants.

Natural antioxidants have different modes of action, however, their main mode of action has been shown to be as free radical scavengers. Two different mechanisms as radical scavengers have been proposed for natural antioxidants, either as hydrogen donor or as electron donor. Other mode of action for natural antioxidants has also been proposed in particular as peroxide decomposer, as singlet oxygen quencher and as enzyme inhibitor (Osawa *et al.*, 1994).

6.1 Radical scavenging activity

Among natural antioxidants, phenolic antioxidants have been reported to quench oxygen-derived free radical as well as the substrate-derived free radicals by donating a hydrogen atom or an electron to the free radical (Gamez *et al.,* 1988 ; Wang *et al.*, 1999 ; Teguo *et al.*, 1998 ; Wettasinghe and shahidi, 1999 ; Fogliano *et al.*, 1999). Cao *et al.* (1996) reported that green and black teas had much higher antioxidant activities against peroxyl radicals than 22 common vegetables. Unno *et al.* (2000) observed that tea catechins and their epimers served as powerful antioxidants for directly eliminating superoxide anion radicals. The presence of 3,4,5-trihydroxyl groups attached to the β-ring of the flavan skeleton of tea catechins elevated their radical scavenging efficiency in comparison to those with 3,4-dihydroxyl groups. Chen and Ho (1997) found that rosemarinic acid, which has four hydroxyl groups, showed the strongest 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging potency. Other antioxidants which have only one or two hydroxy groups, showed weaker DPPH scavenging activity.

Diphenyl such as caffeic acid, oleuropein and hydroxytyrosol had more hydroxyl radical scavenging capability than tyrosol, a monophenol. Moreover, methoxylation of hydroxyl group at the o-position, as in ferulic acid, resulted in a drastic decrease of the rate constant of phenolic antioxidant with hydroxyl radical (Chimi *et al.,* 1991). Masuda *et al.* (1999) reported that the radical trapping activity of vanillin and ferulic acid is lower than that of curcumin. The buckwheat extracts isolated with polar solvents, such as methanol, contained higher amounts of components which act as hydrogen donors than extracts isolated with nonpolar solvents, such as hexane (Przybylski *et al.,* 1998). Gao *et al.* (1999) showed that the direct free radical scavenging activities of the flour flavonoids decreased in the order of baicalein > baicalin > wogonin > wogonoside. The direct scavenging activity of baicalein on alkyl radical and DPPH radical suggested that flavonoid with o-di-hydroxyl structure in the A ring was an effective radical scavenger.

The rate constants of the reaction of phenolic with free radicals indicate the order of reactivity (Mareno *et al.*, 1998). The main reaction is shown as follows :

$$DPPH^{\bullet} + PheOH \rightarrow DPPHH + [\ PheO^{\bullet}\ (I) \leftrightarrow PheO^{\bullet}\ (II)\ ] \qquad (1)$$

Where (I), (II) are resonance structures.

$$DPPH^{\bullet} + PheO^{\bullet} \rightarrow DPPH - PheO \qquad (2)$$

Equation (2) may occur in some cases, but it also may be forbidden depending on molecular phenolic and aromatic ring substituent volumes. Equation (3) will complete with the $PheO^{\bullet}$ coupling termination reaction :

$$PheO^{\bullet} + PheO^{\bullet} \rightarrow PheO - PheO \qquad (3)$$

Moller *et al.* (1999) and Madsen *et al.* (1995) found that methanol extracts of dittany scavenged hydroxyl radical as generated in the Fenton reaction efficiently. The high efficiencies found in the methanol extracts of dittany were closely related to a high content of phenolic compounds, while the amounts of phenolics in ethanol and acetone extract were lower. Yen and Wu (1999) observed that methanolic extracts of *Ganoderma tsugae* showed a marked and concentration-dependent scavenging activity on the DPPH radical (Figure 8). The methanolic extracts of *Ganoderma tsugae* also exhibited a strong scavenging effect on the hydroxyl radical as measured by electron paramagnetic resonasnce spectrometry (EPR). The fremy's radical and galvinoxyl radical quenching ability of the green tea were more effective than that of the black tea in both aqueous and lipophilic systems (Gardner *et al.*, 1998).

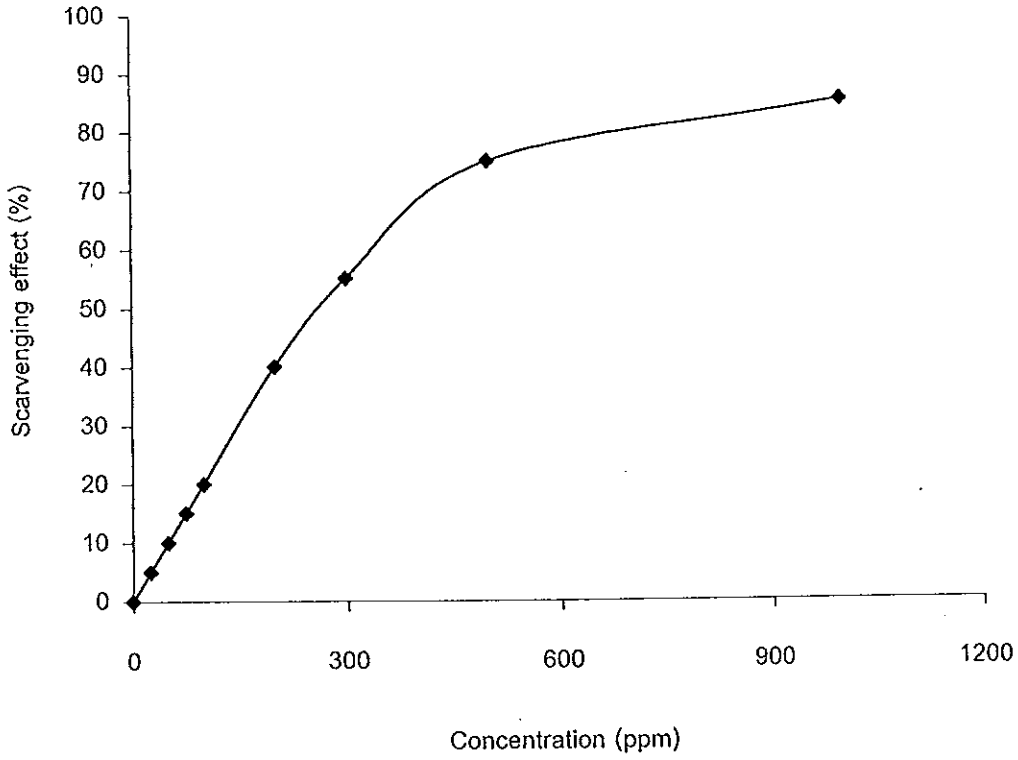


Figure 8 Scavenging effect of methanolic extracts from *G. tsugae* at different concentrations on DPPH radicals.

Source : Yen and Wu (1999).

Ogata *et al.* (1997) showed that an electron donor and/or bulky groups at the ortho-or para-position of the phenol compounds in crude kampo were required for inhibition of lipid oxidation. Phenolic compounds of crude kampo with an allyl substituent on their aromatic ring directly scavenged superoxide. Wettasingne and Shahidi (2000) found that crude of meals of borage and evening primrose and their fraction showed a concentration-dependent scavenging activities of reactive-oxygen species (ROS ; H_2O_2, $O^{\cdot}_2$, $OH^{\bullet}$) and DPPH.

6.2 Reducing power

The antioxidant effect has been reported by some investigators to be concomitant with development of reducing power (Yen and Duh, 1993 ; Duh *et al.*, 1997 ; Fejes *et al.*, 2000). The extracts of tree herbs, including the *Chrysanthemum morifolium* Ramat, *Hibiscus sabdariffa* L. and *Hordeum vulgare* L. had high content of phenolic compounds and exhibited reducing

Chrysanthemum morifolium Ramat, *Hibiscus sabdariffa* L. and *Hordeum vulgare* L. had high content of phenolic compounds and exhibited reducing power, revealing that these herbal extracts may contain reductones (Duh and Yen, 1997). The burdock extract had increased reducing power as concentration increased. Antioxidant activity correlated well with the reducing power of burdock extract (r^2 = 0.90) (Duh, 1998). Lugasi *et al.* (1998) and Lugasi *et al.* (1999) reported that the black radish and *Ginkgo biloba* L. extract exhibited strong reducing power. The green leaves of sweet potatoes and the outer leaves of onion showed higher reducing power and higher antioxidant activity when compared to cabbage, spinach and crown daisy (Chu *et al.*, 2000).

6.3 Chelating activity

Transition metal ions, especially iron and copper are powerful promoters of free-radical damage and lipid peroxidation in both the human body and foods (Figure 9) (Johnson *et al.*, 1992 ; Kitazawa and Wasak, 1999). Chelating agents bind metal ions and greater increase the energy of activation of the initiation reaction (Decker, 1998). Although chelating agents are not antioxidants, they play a valuable role in the stabilization of fatty food against rancidity (Yen and Duh, 1994). Shimada *et al.* (1992) reported that xanthan showed Fe^{2+}-binding activity. Li *et al.* (1997) found that the phenylpropanoid glycosides isolated from herb *Pedicularis striata* had Fe^{2+}-chelating activity. Chen and Ahn (1998) studied the antioxidant activities of six natural phenolics including quercetin, rutin, caffeic acid, ferulic acid, sesamol and catechin against lipid oxidation induced by Fe^{2+} and found that all the phenolics except sesamol and ferulic acid acted as Fe^{2+}-chelators. Furthermore, quercetin and myricetin showed chelating activity on Fe^{2+} and Cu^{2+} ion in sunflower oils and emulsion (Penman and Gordon, 1998). Yen and Wu (1999) observed that methanolic extracts of *Ganoderma tsugae* had a strong chelating effect on Fe^{2+} ion. The black radish root showed strong chelating activity on Cu^{2+} ion (Lugasi *et al.*, 1998). Fejes *et al.* (2000) found that the *Anthiscus cerefolium* L. (Hoffm) had a chelating activity on Cu^{2+}.

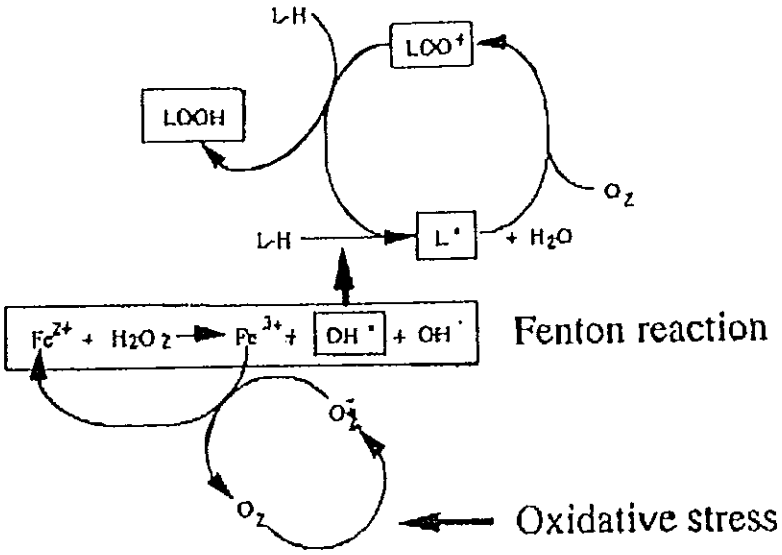


Figure 9 Generalized mechanism for iron-induced reactive oxygen species generation and lipid peroxidation.

Source : Kitazawa and Wasak (1999).

6.4 Lipoxygenase inhibitors

Lipoxygenase catalyzes the oxygenation of the *cis,cis*-1-4 pentadiene sequence of polyunsaturated fatty acids to produce their corresponding hydroperoxides (Siedow, 1991). Chen *et al.* (1992) reported that carnosic acid, ursolic acid and carnosol, isolated components from rosemary extracts, showed strong inhibitory effect on lipoxygenase activity. Carnosol inhibited lipoxygenase more effectively than cannosic acid and ursolic acid. Chlorogenic acid and flavoniods including kaempferol, quercetin, myricetin, quercitrin, isoquercitrin, isoquercitin, rutin, astragalin, (-)-epicatechin, (+)-catechin, epigallocatechin, dihydroquercetin and fisetin showed inhibitory effect on lipoxygenase oxidation of linoleic acid (Richard-Forget *et al*, 1995). Schubert *et al.* (1999) observed that the flavonoids extracted from cold pressed seed oil and pomegranate fermented juice showed strong inhibitory effect on lipoxygenase activity. The extracts of barley and malt also showed strong inhibitory effect on lipoxygenase oxidation of linoleic acid (Goupy *et al*,

1999). Bucar *et al.* (1998) found that the extract of *Guiera senegalensis* leaves showed a strong inhibitory activity on lipoxygenase in a concentration dependent manner. Hydroxytyrosol, oleuropein, caffeic acid and tyrosol, isolated components from the polar fraction of virgin olive oil, possessed a lipoxygenase-inhibitory activity (Puerta *et al.*, 1999).

7. Application of natural antioxidants.

Extracts of many plants have been reported to have varying degrees of antioxidant activities in oils or lipid containing foods (Nishina *et al.*, 1991 ; Ahn *et al.*, 1998). The plant extracts showed different antioxidant activities, depending on the substrate, the preparation procedure and the oxidation test.

7.1 Fish

Lipid oxidation is an important cause of quality deterioration in fish due to the high content of highly polyunsaturated fatty acid (Hultin, 1994).

Ramanathan and Das (1992) found that quercetin, morin, myicetin, kaempferol, tannic acid, ellagic acid, L-ascorbic acid and BHT effectively inhibited lipid oxidation in raw fish storage at 4°C for 14 days. Wada and Fang (1992) reported that α-tocopherol was a more effective antioxidant than rosemary extract in sardine dark muscle. A mixture of α-tocopherol and rosemary had a synergistic antioxidant effect in frozen crushed bonito meat during storage at 5°C. Canthaxanthin delayed TBARS formation in minced flesh from rainbow trout stored at 4°C for 6 days (Clark *et al.*, 1999).

7.2 Meat and meat products.

Guntensperger *et al.* (1998) found that rosemary extract inhibited lipid oxidation of heat-sterilized meat during storage at 20°C. Rosemary oleoresin in combination with sodium tripolyphosphate was proved to be the effective antioxidant during both refrigerated and frozen storage (Murphy *et al.*, 1998). Tipsrisukond *et al.* (1998) found that the ground pork treated with black pepper extract had lower TBARS values than the control. Britt *et al.* (1998) found that ground beef treated with tart cherries had less rancidity

development. TBA values of both raw or cooked ground beef containing fenugreek extracts were lower than the control when stored at 4°C ($p<0.05$). However, no differences in psychrotrophic bacterial counts between ground beef treated with fenugreek extracts and control were found (Hettiarachchy *et al.,* 1996). Extracts from ginger and fenugreek were more effective than potato peel extract in controlling lipid oxidation and color changes of beef patties during cold storage (Mansour and Khalil, 2000). The extracts of spices (sage, basil, thyme, marjoram and ginger) had a very strong antioxidative effect on lipid peroxidation in heat-treated chicken meat and pork patties during frozen storage at -18°C (El-Alim *et al.,* 1999). Karastogiannidou (1999) indicated that the dried onion reduced the TBARS in cooked chicken during refrigerated storage, when mixed before processing.

Lee and Hendricks (1997) reported that canosine effectively inhibited lipid peroxidation in lecithin liposome system and beef homogenates. Lee *et al.* (1998) observed that phytic acid was more effective than carnosine for inhibition of lipid peroxidation. However, carnosine inhibited lipid peroxidation in ground beef more effectively than ascorbic acid (Lee *et al.,* 1999). Chen *et al.* (1999) found that sesamol and quercetin were effective in oxidation retardation of both irradiated raw and cooked pork during storage of 7 days. Rosemary oleoresin and rutin were effective in prevention of oxidation in irradiated raw pork for 3 days.

7.3 Oil and lard.

Papadopoulos and Boskou (1991) reported that the hydroxyrosol and caffeic acid showed good inhibitory activity against lipid oxidation in refined olive oil when compared to BHT. The peroxide values of cottonseed and soybean oils treated with oat extract were lower than the control but it was slightly higher than the oils added with TBHQ. Wanasundara and Shahidi (1998) reported that the canola extract at 500 and 1000 ppm inhibited oxidation of canola oils more effectively than BHA, BHT and monoglyceride / BHT/ BHA but it was less effective than TBHQ. The extract of green, yellow

and white teas strongly inhibited oxidation of canola oil, compared to BHT (Chen and Chan, 1996). Richheimer *et al.* (1996) observed that soybean oil treated with carnosic acid were more stable than oils treated with carnosol, BHT and BHA but it was less effective than TBHQ. Frankel and Huang (1996) reported that the rosemary extracts, carnosol and carnosic acid effectively inhibited conjugated diene, hydroperoxide and hexanol formation in corn oil, soya bean oil, peanut oil and fish oil. In contrast, these compounds were either inactivated or promoted oxidation in vegetable oil-in-water emulsions. The green tea catechin extract was much more effective than BHT and rosemary extract against lipid oxidation in canola oil, pork lard and chicken fat (Chen *et al.,* 1998). Wanasundara and Shahidi (1998) found that green tea extracts inhibited the oxidation of seal blubber and menhaden oil. The peroxide value, TBARS value and weight gain of seal blubber oil and menhaden oil containing green tea extracts were lower than that of control. Green tea extracts at 200 ppm exhibited excellent antioxidant activity in both oils and its efficacy was higher than that of BHA, BHT and tocopherol but less than that of TBHQ. The peroxide values and conjugated dienes of sunflower oil added with myricetin were lower than the oils treated with quercetin, tocopherols and citric acid (Penman and Gordon, 1999). Yenishlieva *et al.* (1999) indicated that thymol and cavacrol inhibited oxidation of lard and sunflower oil kept at ambient temperature. Man and Tan (1999) found that rosemary oleoresin inhibited lipid oxidation in refined, bleached and deodorized palm olein more effectively than sage extract, BHA and BHT. The clove and lemon-grass inhibited lipid oxidation of butter cakes more effectively than BHA and BHT, while black pepper leaves appeared to be prooxidant (Lean and Mohamed, 1999).

Economou *et al.* (1991) reported that the oregano extract showed most effective in stabilizing lard, followed by thyme, dittany, marjoram and lavender extract in a decreasing order. Rosemary and sage inhibited formation of peroxides in lard more effectively than marjoram, thyme, dittany and oregano (Banias *et al.,* 1992). Kim *et al.* (1994) found that the extract from *Psoralea*

corylifolia and *Sorphora angustifolia* effectively inhibited peroxide formation in lard. Treatment with 0.2% methanol extract of *Psoralea corylifolia* exhibited significantly stronger antioxidant effect on the oxidation of lard than treatment with 0.02% BHA ($p<0.05$). Milovanovic *et al.* (1998) showed that the antioxidant activity of extract of *Anthriscus sylvestris* was superior to quercetin, apigenin or tocopherol mixture in lard.

7.4 Other

Oat meal cookies containing extruded potato peels had lower peroxide values (Arora and Camire, 1994). Cinnamic acid, vanillin and BHT were blended with degermed corn meal prior to extrusion in extruder to improve oxidative stability. All samples, except for 200 ppm BHT, had lower peroxide values and conjugated dienes after 12 weeks storage. BHT protected corn snacks against lipid oxidation less effectively than cinnamic acid and vanillin (Carmire and Dougherty, 1998).

8. Antimicrobial properties of plant extract

Several antimicrobial compounds occur naturally in plants (March *et al.,* 1991 ; Mahasneh and El-Oqlah, 1999 ; Ahmad and Holdsworth, 1995) are known to retard the growth or kill food borne pathogens (Aureli *et al.,* 1992 ; Pandit and Shelef, 1994 ; Sivropoulou, 1996).

Various phenolic compounds in plants extract used for preserving food, have an inhibitory effect on the growth and metabolism of microoganism (Table 3) (Walker, 1994 ; Vargas *et al.*, 1999 ; Rauha *et al.*, 2000). The mechanism responsible for antimicrobial activity of phenolic compound present in plants has not been fully defined, although activity has been attributed to inhibition of cellular enzymes, deprivation required for growth, inhibition of oxidative phosphorylation or iron deprivation (Scalbert, 1992).

Table 3 Antibacterial effects of phenolic compounds.

Phenolic Compounds	*Staphyloccus aureus*	*Bacillus subtilis*	*Micrococcus luteus*	*Escherichia coli*	*Pseudomonas aeruginosa*
Caffeic acid	-[a]	-	-	-	~[b]
Gallic acid	-	-	~	-	++
Protocatechuic Acid	-	-	+[c]	-	++
Flavone	++[d]	++	+++[e]	+	~
Quercetin	++	+	++	~	~
Rutin	-	-	-	-	-
Nafringenin	+++	+++	+++	+	~
Cathecin	~	-	-	-	~
Reference Activity	Penicillin +++	Tetracyclin +++	Bacitracin +++	Sulfonamide ++	

-[a] : No antimicrobial activity, inhibition zone (i.z.) of sample < i.z. of methanol + 1 mm.

~[b] : Slight antimicrobial activity, i.z. of sample 1-3 mm > i.z. of methanol

+[c] : Moderate antimicrobial activity, i.z. of sample 3-4 mm > i.z. of methanol

++[d] : Clear antimicrobial activity, i.z. of sample 4-10 mm

+++[e] : Strong antimicrobial activity, i.z. of sample > i.z. of methanol

Source : Rauha *et al.* (2000)

Jaiarj *et al.* (1999) found that water and methanol extract of dry guava leaves exerted a strong inhibitory effect on growth of *S. aureus*, by the disc diffusion method. The response was dose dependent. The higher the dose, the wider (10 mg/mL) the inhibition zone was observed. The methanolic leaf extract of *Vitex trifolia* L. (Verbenaceae) completely inhibited the growth of *S. aureus* and *E. coli* (Hernandez *et al.*, 1999).

Martinez–Vazquez *et al.* (1999) studied the antimicrobial activities of organic extracts of roots and stems of *Byrsonima crassifolia* (L.) H.B.K. The ethyl acetate extract of roots was the most active against *P. aeruginosa, S. typhi S. aureus* and *M. luteus*. The maximal zones of inhibition ranged from 17 to 26 mm. The methanolic extract of roots showed antimicrobial activity against *S. aureus, E. coli* and *S. typhi*, whereas the methanolic and hexanic extracts of stems were ineffective.

Perez *et al.* (1999) indicated that leaves of *Senecio graveolens* (Compositae) inhibited the growth of *M. luteus* and *S. aureus*. The minimal inhibitory concentration (MIC) values for *M. luteus* and *S. aureus* were 8.73 and 10.91 mg/mL, respectively.

Gnan and Demello (1999) found that the extract from Goiaba showed good antimicrobial activity. A complete inhibition of growth of *S. aureus* and *S. typhimurium* was caused by Goiaba leaf extract at a concentration of 8 mg/mL as tested by turbidity measurements as shown in Table 4. Kang *et al.* (1992) observed that the green leaves of *Perilla frutescen* showed antimicrobial activity. The major active constituent, perillaldehyde, inhibits both bacteria (Gram-positive and Gram-negative) and fungi.

Table 4 Antimicrobial activity of plant extracts (8 mg/mL) tested by turbidity measurements.

Microorganism	Control	Alhodo mato	Goiaba	Trapoeraba
S. aureus	+++++	+	-	+++++
S. typhimurium	+++++	+++++	-	+++
P. aeruginosa	+++++	+++++	++	+++++
S. pyogenes	+++++	+	+	+++++

+++++ : No inhibition ; ++++ : little inhibition ; +++ : moderate inhibition ; ++ : high inhibition ; + : very high inhibition ; - : complete inhibition

Source : Gnan and Demello (1999).

Mario *et al.* (1999) observed that thyme essential oils were demonstrated to have a good degree of bacteriostatic activity against the microorganisms. This activity was more marked against the gram-positive bacteria. Among gram-negative bacteria, the most sensitive bacteria were *E. coli* O157 : H7 and *Y. enterocolitica* (Chaibi, 1996). Nine plant essential oils (cedar, eucalyptus, vervain, savage carrots, camomile, artemisia, grapefruit, orange and rosemary) showed anti-bacterial effect on spores and vegetative cells of *C. botulinum* and *B. cereus.* Cedas oil was the most effective inhibitor at concentration of 100 and 300 ppm against *B. cereus* and *C. botulinum*, respectively. Spores of *C. botulinum* were more resistant than those of *B. cereus*. Oregano and thyme essential oils showed good antibacterial activity, which appeared to have more marked activity against the gram-positive bacteria (Paster *et al.,* 1990). Perrucci (1995) reported that the highest doses of essential oils of lavender and peppermint killed 100% of the mites of stored food. Eucalyptus oil was the least active. Among the essential oil constituent, menthol showed the highest activity, killing 100% of the mites at the lowest dose (0.25 μL). The essential oils of rosemary, sage, oregano, cinnamon, black pepper and clove inhibited the growth of *Aspergillus parasiticus* (Wendorff and Wee, 1997 ; Paster *et al.*, 1995). Cinnamon and clove oils can prevent growth of *Alternaria* and *Penicillium* species (Ryu, 1993). The major component of clove is eugenol. It was widely believed that the action would be similar to the phenolic compounds which affect cell membrane. The mechanism of microbial inhibition might also be related to the inactivation of enzymes or genetic materials (Thoroski *et al*, 1989 ; Henfnawy *et al.,* 1993).

The antimicrobial compounds in plant materials are often in the essential oil fraction. Among the compounds having wide spectra of antimicrobial activity, thymol [5-methyl-2 (1-methyl ethyl) phenol] from thyme and oregano, cinnamic aldehyde (3-phenyl-2-propenal) from cinnamon, and eugenol [2-methoxy-4-(2-propenyl) phenol] from cloves were found to show the high efficacy. Minimum inhibitory concentration of

these plant constituents was as low as 5 mg/mL (Beuchat and Golden, 1989 ; Karapinar, 1990). Structures of several antimicrobial compounds present in the essential oil of plants are illustrated in Figure 10.

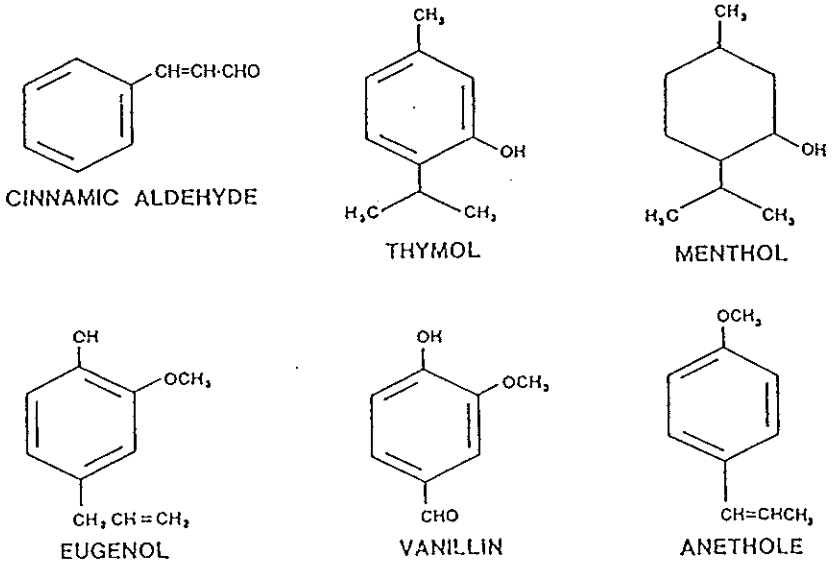


Figure 10 Structure of antimicrobial compounds occurring naturally in plants.
Source : Beuchat and Golden (1989).

Wendakoon and Sakaguchi (1992) observed that the clove and cinnamon inhibited the bacterial growth in fish muscle effectively. The inhibitory effects of clove (0.05%) on the growth and biogenic amine production of *Enterobacter aerogenes* were found in mackerel muscle (Wendakoon and Sakaguchi, 1992). Essential oil of clove, coriander and nutmeg at concentrations of 500, 1,250 and 10,000 µg/mL inhibited the growth of *Aeromonas hydrophila* on tryptic soy agar. Furthermore, the essential oils of clove and coriander could be used to control the hazard of *A. hydrophila* in noncured cooked meat at low and increased temperatures (Stecchini *et al,* 1993).

Objectives

1. To study the antioxidant activities of extracts from 8 cultivars of herbs and 9 cultivars of green leaves.
2. To study the extraction of antioxidants from selected herbs and green leaves.
3. To study some properties and mode of action of antioxidants from selected herbs and green leaves.
4. To study the application of herbs and green leaves extracts in ground fish.

Chapter 2

Materials and Methods

1. Materials

Herbs are purchased from herbal medicine store in Hat Yai. The herbs were kept at 4°C in polyethylene bag before used. Herbs used in this experiments are shown as follows :

- fennel (*Foeniculum vulgare* Mill.)
- cardamom (*Amomum krervanh* Pierre.)
- clove (*Eugenia caryophyllata* Thunb.)
- chinese aniseed (*Illicium verum* Hook .)
- cinnamon (*Cinnamomum zeylanicum* L.)
- long pepper (*Piper longum* L.)
- henna (*Lawsonia inermis* L.)
- liquorice (*Glycyrrhiza glabra* L.)

Leaves were collected from local orchard in Hat Yai area. Leaves used in this experiments are shown as follows :

- cashew (*Anacardium occidentale* L.)
- hog plum (*Spondias pinnata* L.)
- mango (*Mangifera indica* L.)
- asiatic pennywort (*Centella asiatica* L.)
- guava (*Psidium guajava* L.)
- cassod (*Cassia siamea* Brift.)
- neem (*Azadirachta indica* L.)
- tamarind (*Tamarindus indica* L.)
- luaranthanceae (*Loranthus pentadrus* L.)

2. Chemicals

2.1 Solvents used for extraction of the antioxidants from liquorice and green leaves.

- Methanol
- Ethanol
- Ethyl acetate
- Hexane

2.2 Chemicals for determination of antioxidant activity.

- Lecithin
- β-carotene
- Linoleic acid
- Tween 40

2.3 Chemicals for determination of total phenolic compound content.

- Folin and Ciocaltu' s Phenol Reagent
- Sodium carbonate

2.4 Chemicals for determination of mode of action.

- 1,1-diphenyl-2-picrylhydrazyl (DPPH)
- Potassium chloride (KCl)
- Copper sulphate pentahydrate ($CuSO_4.5H_2O$)
- Ferrous sulphate heptahydrate ($FeSO_4.7H_2O$)
- Potassium ferricyanide
- Tetrametylmurexide (TMM)
- Trichloroacetic acid (TCA)
- Potassium hydrogenphosphate
- Lipoxygenase
- Ferric chloride
- Tween 20

2.5 Chemicals for oxidation analysis.

- Thiobarbituric acid
- Hydrochloric acid

- Sodium thiocyanate
- Trichloroacetic acid
- Boric acid
- Potassium carbonate
- Formaldehyde

2.6 Chemicals for separation of compounds in liquorice and mango leaves.

- Thin-layer chromatography (precoated silica gel plate, 20 x 20 cm, Kieselgel 60 F_{254} 0.20 mm, E. Merck, Dramstadt, Germany).
- Dichloroethane : methanol : acetic acid (5.5 : 3.5 : 1)
- Dichloroethane : methanol (6 : 4)
- 2% Ferric chloride in ethanol
- 1% solution of potassium ferricyanide + 1% solution of ferric chloride
- Ammonical silver nitrate solution

Instruments

Instruments	Model	Company	Country
Spectronic 21	SP.21	Spectronic	U.S.A
Spectrophotometer	UV-1601	Shimadzu	Japan
Centrifuge	Hermle	Technical Science & Service	U.S.A
Freeze-dryer	Dura-Dry™ *µp*	FIS system	U.S.A
Shaker	GEL 1038	Gesellschaft fur	Germany
Rotary evaporator	Rotavapor-R	Brinkmann	Switzerland
pH meter	Denver 15	Denver Instrument	U.S.A
Shaker water bath	WB/OB 7-45	Memmert	Germany
Homogenizer	T25	Utra turrax	Malaysia
Stomacher	M.400	SEWARD	UK

3.Methods

3.1 Preparation of herbs and green leaves extracts.

Herbs and green leaves were cleaned with water and dried overnight in an air dryer at 50°C, then cooled to room temperature and ground to a particle size of 20 mesh and stored at 4°C in an airtight container until used. Herbs and green leaves powder (5.00 ± 0.01 g) was mixed with 100 mL of methanol and shaken at speed of 300 rpm at room temperature (28-30°C) for 24 hrs. The extracts were filtered using Whatman no. 1 filter paper. (Figure 11). Methanol extracts were prepared in duplicates for each herbs and green leaves and stored at 4°C until analysis.

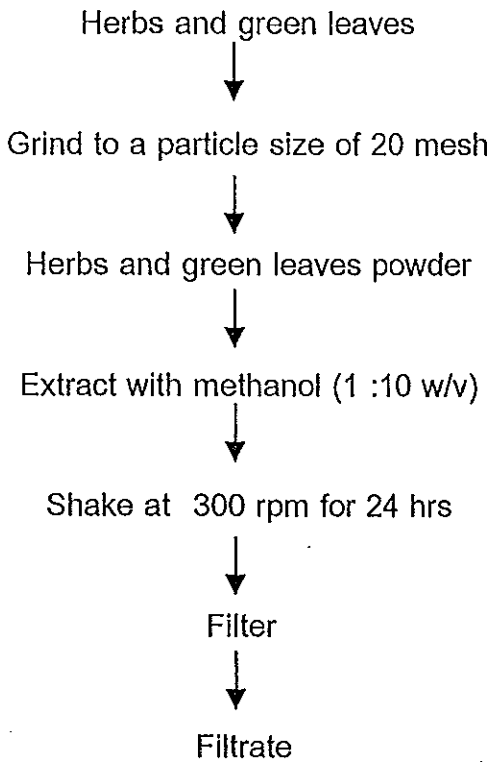


Figure 11 Extraction of antioxidants from herbs and green leaves.

Sources: Modified from Ganthavorn and Hughes (1997) ; Dapkevicius *et al.* (1998).

3.2 Primary screening of antioxidants in methanol extracts of herbs and green leaves.

3.2.1 Determination of antioxidant activity in lecithin liposome system (Frankel *et al.*, 1997 ; Yi *et al.*, 1997).

Lecithin (2.4 g) was suspended in deionized water at a concentration of 8 mg/mL by stirring with a glass rod and sonicating for 15 min. To test antioxidant activity, herbs and green leaves extracts (0.25 mL) were added to the lecithin liposome system. After addition of extracts, the liposome suspension was sonicated again for 2 min. To initiate the assay, 10 μL of cupric acetate (3.0 μM) was added. The mixtures were shaken (120 rpm) at 37°C in the dark. Liposome oxidation was monitored by determining thiobarbituric acid – reactive substances (TBARS) and conjugated diene.

3.2.1.1 Measurement of TBARS. Liposome sample (1 mL) was mixed with 20 μL of butylated hydroxytoluene (0.2 %) and 2 mL (15% TCA / 0.375% TBA / 0.025 N HCl). The mixtures were then heated for 10 min in a boiling water bath (95-100°C) to develop pink color, cooled with tap water, and centrifuged for 20 min at 5,500×g. The supernatant containing the pink chromogen was quantified at 532 nm (Lee and Hendricks, 1997; Duh, 1998).

3.2.1.2 Measurement of conjugated diene. Liposome samples (0.1 mL) were dissolved in methanol (5.0 mL), and conjugated dienes were measured at 234 nm. (Frankel *et al.*, 1997).

3.2.2 Determination of antioxidant activity in linoleic acid/β-carotene (Taga *et al.*, 1984)

β-carotene (1 mg) was dissolved in 10 mL of chloroform. A 3 mL aliquot of the solution was added to 20 mg linoleic acid and 200 mg Tween 40. Chloroform was removed by purging with nitrogen. Oxygenated distilled water (50 mL) was added to the β-carotene emulsion and mixed well. Antioxidant extract (0.2 mL) was mixed with oxygenated β-carotene emulsion (3 mL) and incubated at 50°C. Oxidation of the β-carotene emulsion was

monitored spectrophotometrically at 470 nm after 0, 10, 20, 30 and 40 min incubation at 50°C. Degradation rate of β-carotene was calculated according to following equation (Al-Saikhan *et al.*, 1995).

Sample degradation rate = ln (a/b) × 1/t

Where : ln = natural log

a = initial absorbance at time 0

b = absorbance at time 40 min

t = time (min)

Antioxidant activity (AA) was expressed as percentage inhibition relative to the control using the following equation :

$$AA = \frac{\text{Degradation rate of control - Degradation rate of sample}}{\text{Degradation rate of control}} \times 100$$

3.2.3 Determination of phenolic content and reducing power.

Total phenolic content in herbs and green leaves extracts were determined spectrophotometrically using Folin–Ciocalteu reagent (Julkunon–Titto, 1985; Weurman and Swain, 1955). Herb and green leaf, which provided highest antioxidant activity, were selected for further studies.

3.3 Extraction of antioxidants from selected herbs and green leaves.

3.3.1 Effect of extracting solvents.

The antioxidants from selected herbs and green leaves were extracted with different solvents including, hexane, ethyl acetate, ethanol and methanol. The extracts were evaluated for antioxidant activity by the lecithin liposome method (Frankel *et al.*, 1997) and β-carotene bleaching method (Taga *et al.*, 1984). Total phenolic content was determined according to the method of Julkunen–Titto (1985). The solvent rendering the highest antioxidant activity was chosen for further studies.

3.3.2 Effect of methanol / water ratio.

The different ratios of methanol to water (100 : 0 , 90 : 10 , 80 : 20 , 70 : 30 , 60 : 40 , 50 : 50 v/v) were used for extracting antioxidants from selected herbs and green leaves. The different extracts were evaluated for antioxidant activity by the lecithin liposome method (Frankel *et al.*, 1997) and β-carotene bleaching method (Taga *et al.*, 1984). Total phenolic content was determined according to the method of Julkunen–Titto (1985). The ratio of methanol to water, which gave an extract with the highest antioxidant activity, was chosen for further studies.

3.3.3 Effect of extraction time and repetition.

Different extraction times (2, 5, 10, 15 and 24 hrs) and repetition (1, 2 and 3) were used to prepare the extract with the selected solvent (3.3.2). The extracts obtained as above were evaluated for antioxidant activity by the lecithin liposome method (Frankel *et al.*, 1997) and β-carotene bleaching method (Taga *et al.*, 1984). Total phenolic content was determined according to the method of Julkunen–Titto (1985).

Optimum condition that rendered the highest antioxidant activity was chosen. Antioxidant extract from selected herbs and green leaves were prepared and freeze–dried to obtain the antioxidant powder. Resulting powder was stored at -20°C until used.

3.4 Some properties of the extracts from selected herbs and green leaves.

3.4.1 Effect of heat treatment

The powdered extracts were heated in hot air oven at 80°C and 100°C for 0, 10, 20, 30, 40, 50, 60, 80, 100 or 120 min. The samples were then cooled to room temperature, and dissolved in 0.2 mL of 80% methanol. The solutions were tested for antioxidant activity by the lecithin liposome system (Frankel *et al.*, 1997).

3.4.2 Effect of pH

The effect of pH on antioxidant activity of liquorices and mango leaves extracts were determined. The extracts were subjected to different pHs (3, 5, 7, 9, 11) using 0.2 M phosphate buffer. The antioxidant activity was determined using the lecithin liposome system (Frankel *et al.,* 1997).

3.4.3 Effect of concentration

Various concentrations of the extracts (0.05, 0.10, 0.30, 0.60, 0.90, 1.20 and 1.5 mg/mL) were used and antioxidant activity was determined according to the method of Frankel *et al.* (1997).

3.4.4 Synergistic effect

The synergists, including citric acid, ascorbic acid and α-tocopherol were used to study the synergism with the liquorice and mango leaves extracts. Some factors including extract concentration (0, 0.02, and 0.03 mg/mL) and type of synergists were evaluated. The effect of synergists on antioxidant of the extracts was determined in an aqueous system by using lecithin liposome system (Frankel *et al.,* 1997) and β-carotene bleaching method (Taga *et al.*, 1984).

3.5. Mode of action

3.5.1 Radical – scavenging activity

3.5.1.1 Scavenging effect on DPPH radicals.

The scavenging effect of liquorices and mango leaves extracts on 1,1–diphenyl-2-picryhydrazyl (DPPH) radicals was estimated according to the method of Chen and Ho (1997). Different concentrations of the extracts (0, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/mL) were tested and compared with BHT and α-tocopherol at a level of 200 ppm.

3.5.1.2 Scavenging effect on hydroxyl radicals.

The deoxyribose degradation caused by hydroxyl radicals was determined by the formation of TBARS (Aruoma, 1994; Halliwell *et al.*, 1987). Different concentrations of the extracts (0, 0.6, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/mL) were tested, and compared with BHT and α-tocopherol at a level of 0.1 mg/mL.

3.5.2 Reducing power

The reducing power of extracts was determined according to the method of Oyaiza (1986). Different concentrations of the extracts (0.05, 0.1, 0.15, 0.2, 0.25, 0.35, 0.4, 0.5 and 1.0 mg/mL) were tested, and compared with ascorbic acid at level of 0.05 mg/mL.

3.5.3 Chelating activity

The chelating activity of extracts on Fe^{2+} and Cu^{2+} was measured according to the method of Shimada *et al.* (1992). Different concentrations of extracts (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 mg/mL) was tested and compared with citric acid and EDTA at a level of 0.2 M

3.5.4 Inhibition of lipoxygenase activity

Inhibitory effect of extracts on lipoxygenase activity was studied spectrophotometrically, using linoleic acid as a substrate (Surrey, 1964). Different concentrations of extracts (0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.25 and 1.5 mg/mL) were added into reaction mixture and residual lipoxygenase activity was measured. The lipoxygenase inhibition of the extract was compared with that of BHT and α-tocopherol at level of 0.1 mg/mL.

3.6 Antimicrobial activity of the extracts from selected herb and green leaves.

The bacteria used in these studies were *Pseudomonas fluorescens, Bacillus cereus, Escherichia coli and Staphylococcus aureus.* Fresh cultures of bacteria were grown in nutrient broth at 37°C and stock cultures were maintained on nutrient agar slants at 4°C.

3.6.1 The disc diffusion method was used to detect the antimicrobial activity of extracts (250 and 500 ppm). The activity was evaluated by measuring the inhibition–zone diameters.

3.6.2 The minimum inhibition concentration (MIC) of extracts. The MIC is determined as the lowest concentrations with no growth. The MIC of extract was evaluated by turbidity measurements (Brock and Madigan, 1991; Davidson and Parish, 1989). Various concentrations of extracts (0.2, 0.4, 0.6, 0.8, 1.0 and 2 mg/mL) were added to the culture medium. The growth of microorganism after incubation at 37°C was determined as an increase in turbidity read at 660 nm.

3.7 Application of liquorice and mango leaves extract in ground fish.

The fish was obtained from local market in Hat Yai, Songkhla. After filleting, the fish (*Decapterus russelli*) was deskinned and deboned before grinding in a blender for 30 sec at maximum speed. Fat content was standardized to approximately 6.00% (AOAC, 1990). The extract powder was dispersed in ethanol for both liquorices and mango leaves. Aliquots (2 mL) of solution were then mixed well into the samples of ground fish (20 g) to obtain the final concentrations of 200, 500 or 1,000 ppm. All samples were placed in polyethylene bags and stored at 4°C. Ground fish were evaluated at 0, 2, 4, 6, 8 and 10 day for thiobarbituric acid reactive substances (TBARS) (Siu and Draper, 1978), total volatile base (TVB), trimethylamine (TMA) (Ng, 1987) and psychrophilic bacteria (Gilliand *et al.*, 1984). The effect of extract on ground fish quality was compared with that of BHT and α-tocopherol at a level of 200 ppm.

3.8 Separation of antioxidative compounds in liquorice and mango leaves extracts.

3.8.1 Fractionation of liquorice and mango leaves extracts by column chromatography.

The extract powder (5 g) was dissolved in methanol and mixed with Silica Gel, Kieselgel 100 (0.063 - 0.200 mm , E. Merck. Darmstadt. Germany). After drying, residue was loaded on a 75 cm glass column (4-cm diameter), packed with 100 g silica gel in dichloroethane slurries. The elution was carried out by using the following sequence of solvent mixture between methanol and dichloromethane as eluent with increasing methanol content 3, 6, 12, 15, 18, 25, 30, 45, 60 and 75%. The eluent was collected in 50 mL fractions and similar fractions (tested by TLC) were combined. Pooled fraction was evaporated to 1 mL. The antioxidant activity of the isolated fraction was evaluted using a β-carotene/linoleic acid bleaching system (Taga *et al.*, 1984).

Among seven fractions separated, fraction VI showed the highest antioxidative activity for liquorice extracts. For mango leaves extract, fraction VIII showed the highest antioxidative activity among nine fractions separated. Both fractions were used for further studies.

3.8.2 Identification of antioxidative compounds in selected fraction of liquorice and mango leaves extract.

Thin–Layer chromatography (TLC) silica gel G plates (20 × 20 cm 0.02 mm thick : Merck, Darmstadt. Germany) was used. Solvent mixtures used to develop TLC were the mixture of dichloroethane and methanol (6.5 : 3.5 vol/vol) for fraction VI of liquorices extract and the mixture of dichloroethane, methanol and acetic acid (5.5 : 3.5 : 1 vol / vol / vol) for fractions VIII of mango leaves extract. Fifty μL of the concentrated fractions were spotted on each precoated TLC silica gel plate, which had been activated for 30 min at 105°C.

To identify the chemical composition of the antioxidant extracts from liquorice and mango leaves, different sprays were used as follows :

Spray 1 : A solution of potassium ferricyanide (1%) in water and a solution of ferric chloride (1%) in water were sprayed to develop TLC plates. A blue color shown on the plate indicates that these compounds may be phenolic (Pratt and Miller, 1984).

Spray 2 : A solution of ferric chloride (2%) ethanol was sprayed to develop TLC plates. Blue coloration indicates the probable presence of trihydroxy phenolic compounds. Those with dihydroxy groups give a green colour with this reagent, while other phenolics give a red or brown colour (Reio, 1958).

Spray 3 : Ammonical silver nitrate solution was prepared by mixing 30 mL ammonium hydroxide and 70 mL water-silver nitrate (3.4 g in 100 mL water) and sprayed to develop chromatograms. After heating for 10 min at 105^{o}C, brown, black and gray spots developed indicate an evidence of reducing compounds.

3.9 Statistical analysis

The data were subjected to the analysis of variance (ANOVA). The differences among samples were determined by Duncan' s multiple range test. All statistical analysis were performed using the statistical Package for Social Sciences (*SPSS for windows* : SPSS Inc.)

Chapter 3

Results and Discussion

1. Primary screening of antioxidant activity in methanol extracts of herbs and green leaves.

The antioxidant activities of extracts from 8 different varieties of herbs are depicted in Figure 12. All methanol extracts of herbs showed antioxidant activity in inhibiting both lecithin liposome and β-carotene/linoleic acid systems. Herb extracts inhibited conjugated diene formation in lecithin liposome system ranging from 11.02 to 94.08% and TBARS formation ranging from 32.52 to 96.18%. In both oxidation systems, liquorice and clove extracts had highest antioxidant activity ($p<0.05$), while fennel, cardamom, long pepper and cumin extracts showed lower antioxidant activity ($p<0.05$). In general, the antioxidant activities measured using two systems were similar, particularly when using TBARS as oxidative indicator in liposome system. However, the results of conjugated diene were not concomitant with those of TBARS, especially for cardamom, fennel, henna and long pepper. In heterogeneous food system, the physical properties, such as lipophilicity, solubility and partition between the aqueous and lipid phase can become important in determining antioxidant activity (Frankel *et al.*, 1994). The type and polarity of the lipid system used as substrate significantly affect the activity of natural antioxidant (Frankel, 1993). In evaluation of natural antioxidants, varied results can be obtained with different lipid substrates, and with various methods measuring products at different stages of lipid oxidation (Huang *et al.*, 1994 ; Hopia *et al.*, 1996). Since liposome is a simple phospholipid biolayer, it is a useful model biomembrane for assay systems of antioxidants (New, 1990). Duh and Yen (1997) and Masuda *et al.* (1993) reported that three herbs extracts, including *Chrysanthemum morifolium* Ramat, *Hibiscus sabdariffa* L. and *Hordeum vulgare* L. and curcumin showed marked

antioxidative activity in both β-carotene/linoleic acid and lecithin liposome model systems. Kim *et al.* (1994) noted that the antioxidant activity of extracts prepared from herbs was dependent on the type of herb rather than the solvent used.

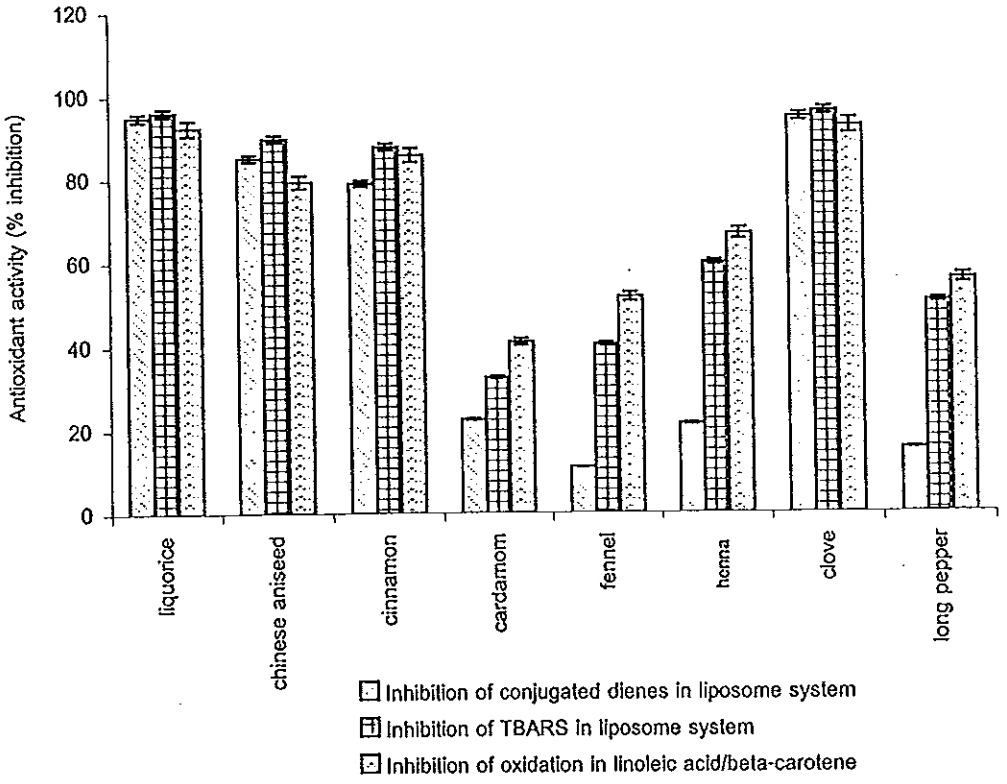


Figure 12 Antioxidant activity of methanol extracts from 8 cultivars of herbs.

Differences in phenolic content were observed among herb varieties (Figure 13). Total phenolic content of herbs in this study varied from 4.66 to 57.66 mg/g. Cinnamon contained the highest total phenolic content but it had a lower antioxidant activity than clove and liquorice ($p<0.05$). Among natural antioxidant, phenolics have been known as antioxidants widely distributed in the plant (Le and Guedon, 1991 ; Thomas *et al.*, 1992 ; Tsimidou *et al.*, 1992).

The amount and composition of the phenolic present in herbs vary greatly with the types of herbs (Duh and Yen, 1997 ; Mansour and Khalil, 2000). Plant phenolic can delay the onset of lipid oxidation and decomposition of hydroperoxides in food products as well as in living tissue (Frankel *et al.*, 1998 ; Chen *et al.*, 1996). Terao *et al.* (1994) showed that phenolic compounds in grapes have high and varying antioxidant activities in inhibiting lecithin liposome oxidation. Furthermore, phenolic antioxidants of plant materials are reported to quench oxygen derived free radicals as well as the substrate-derived free radicals by donating a hydrogen atom or an electron to the free radical in various model system. They also chelate metal initiators of oxidation (Wanasundara and Shahidi, 1998 ; Decker, 1998).

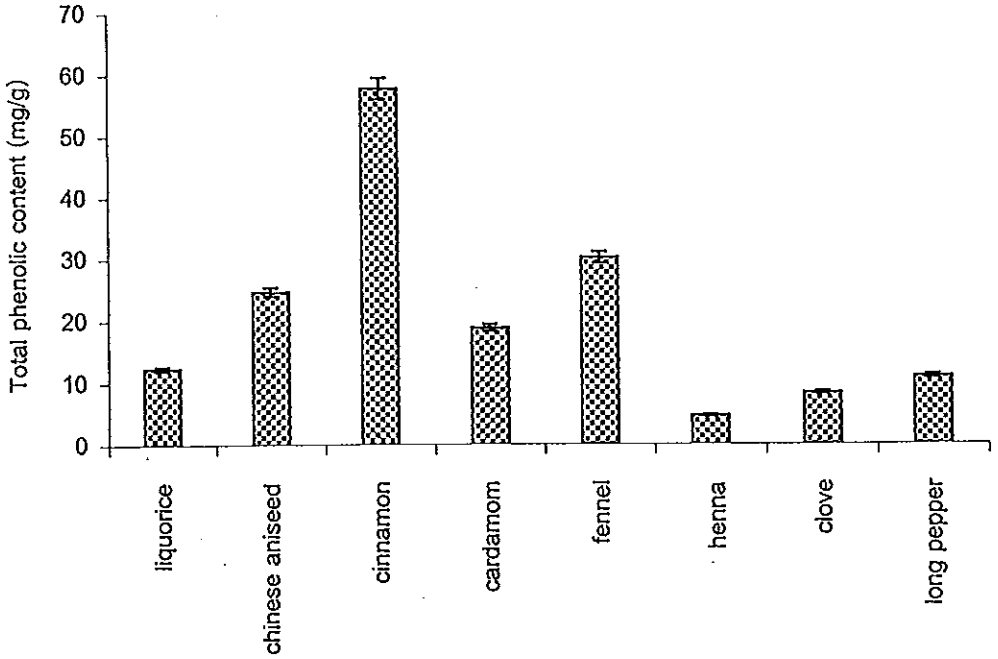


Figure 13 Total phenolic content of 8 cultivars of herbs.

Lee *et al.* (1995) reported that the phenolic compounds of fresh pepper correlated well with antioxidant activity. Velioglu *et al.* (1998) also found that the relationship between antioxidant activity and total phenolic content of 28 plant product extracts showed a positive correlation (r^2 = 0.4253).

Nevertheless, in this study, low positive correlation between the phenolic content of herbs extract and their antioxidant activities in both lecithin liposome and β-carotene/linoleic acid system was observed. This result indicated that herb extracts containing phenolic compounds may partially contribute to inhibition of lipid peroxidation. The result was in good agreement with Al-Soikhan (1995) who observed the low positive correlation between antioxidant activity and total phenolic content of the extracts from various varieties of potatoes.

The methanol extracts from different varieties of green leaves showed high antioxidant activities in both lecithin liposome and β-carotene/linoleic acid oxidation systems (Figure 14). The green leaves extract inhibited conjugated diene formation between 17.50 and 58.71% and TBARS formation between 59.88 and 91.72%. Mango leaves had highest antioxidant activity in both oxidation systems tested ($p<0.05$). Similar antioxidant activity of green leaves extracts was observed when determined by two oxidation model systems.

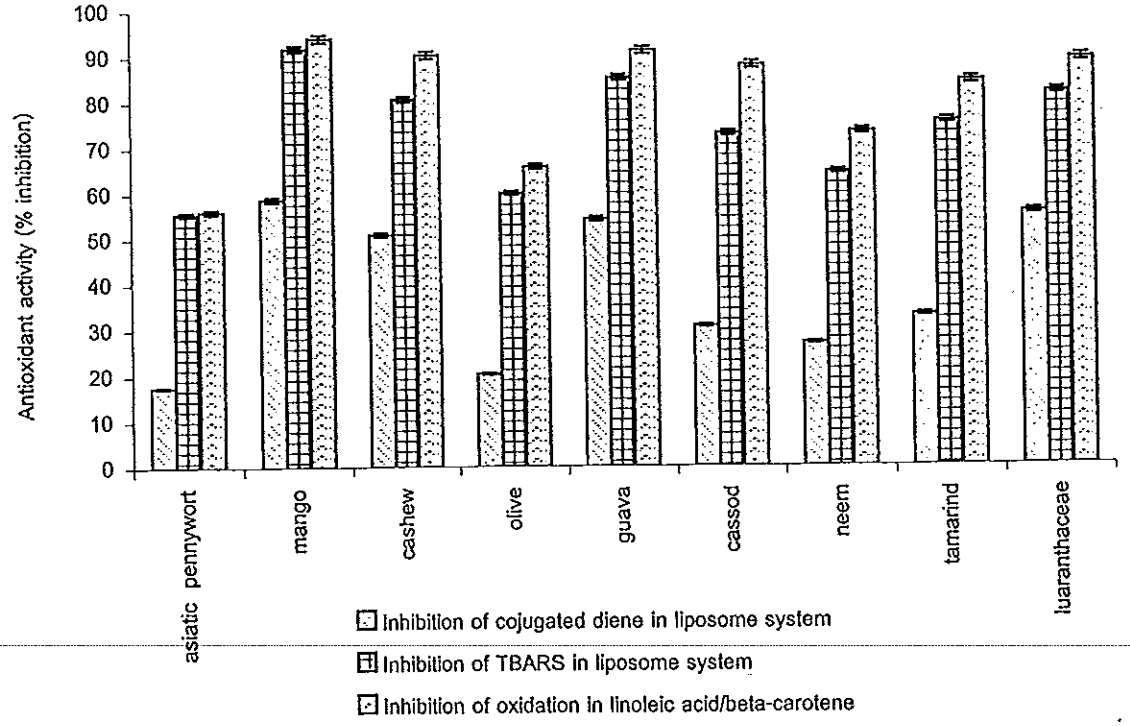


Figure 14 Antioxidant activity of methanol extracts from 9 cultivars of green leaves.

Differences in phenolic contents were observed among green leaves varieties tested (Figure 15). Total phenolic content of green leaves in this study varied from 4.36 to 57.66 mg/g. Low correlation between antioxidant activity and total phenolic content of green leaves extract was also found. Cashew leaves contained the highest total phenolic content but it exhibited lower antioxidant activity than mango leaves in lecithin liposome system. Yen and Chen (1995) noted that polyphenols are the most abundant group of compounds in tea leaf and seem to be responsible for antioxidative activity. Benzie *et al.* (1999) observed that different tea leaves were widely different in vitro antioxidant power and the antioxidant capacity was correlated with the total phenolic content. The antioxidant activity of of Du-Zhong leaves extracts correlated with their polyphenol content (Yen and Hsieh, 1998). The antioxidant activities of tea and banana extracts from old and young leaves were not significantly different (Zandi and Gordon, 1999; Mendoza *et al.*, 1990).

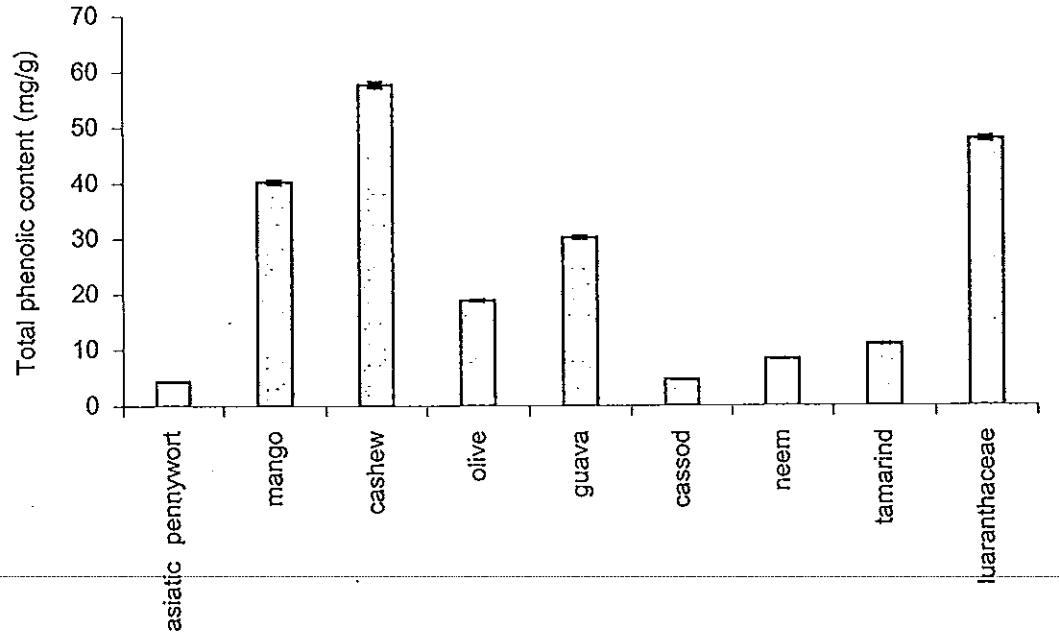


Figure 15 Total phenolic content of 9 cultivars of green leaves.

Our results clearly indicated that herbs and green leaves were rich in natural antioxidants. Different qualities or quantities of antioxidants in methanol extracts depended on the varieties of herbs and green leaves. Mango leaves showed highest antioxidant activity in lecithin liposome system. For herbs extract, liquorice and clove extract exhibited the highest antioxidant activity. Since a large number of researches have been done on clove (Farag *et al.*, 1989 ; Myint *et al.*, 1995 ; Lean and Mohanmed,1999) and clove renders the strong flavor, which can affect the general acceptance, we chose liquorice as a source of antioxidant instead of clove. Therefore, mango leaves and liquorice were chosen for further study.

2. Extraction of antioxidants from liquorice and mango leaves.

2.1 Effects of solvents on the antioxidant activities.

2.1.1 Liquorice

Antioxidant activity and the total phenolic content of liquorice extracts prepared by using different solvents are shown in Table 5. All liquorice extracts showed antioxidant activity in both liposome and β-carotene/linoleic acid sysytems. The extraction efficiency of solvents decreased in the following order methanol > ethanol > ethyl acetate > hexane. Among four solvent extracts, methanol extracts had highest antioxidant activity and highest total phenolic content. The inhibition of liposome oxidation and β-carotene/linoleic acid oxidation correlated with total phenolic contents in the extracts. Antioxidant activity generally increased with increasing polarity of solvents. Therefore, the polarity of extracting solvents was presumed to be a contributor to antioxidant activity. This result was in agreement with Moller *et al.* (1999) who reported that the antioxidant activity and total phenolic content were lowest in the dittany extracts made with the low polarity solvents, acetone. Antioxidant activities of *Aframomum danielli* were different when different solvents were used (Adegoke and Krishna,

1998). From the result, methanol was chosen as the solvent for extraction of liquorice antioxidant.

Table 5 Antioxidant activity and total phenolic content of liquorice extracts prepared with different solvents.

Solvents	Liposome system		β-carotene bleaching system	Total phenolic content (mg/g)
	%inhibition of conjugated dienes	TBARS	%inhibition of oxidation	
Methanol	96.11±0.40[a] c[b]	96.16±0.32 c	93.20±0.33 b	14.37±0.18 d
Ethanol	95.45±0.30 c	94.22±0.27 b	92.35±0.32 b	11.95±0.15 c
Ethyl Acetate	91.78±0.32 b	96.20±0.39 c	90.79±0.11 b	10.58±0.01 b
Hexane	17.49±0.21 a	13.60±0.15 a	76.10±0.18 a	1.04±0.01 a

[a] Mean±standard deviation from triplicate determinations.

[b] Different letters in the same column bear significant differences. ($p<0.05$)

2.1.2 Mango leaves

The antioxidant activity and total phenolic content of extracts from mango leaves prepared by using different organic solvents are shown in Table 6. From the result, the highest antioxidant activity and highest total phenolic content were obtained in methanol extract, followed by extracts prepared with ethanol, ethyl acetate and hexane, respectively. The inhibition of lecithin liposome oxidation and β-carotene/linoleic acid oxidation correlated with total phenolic contents. The polarity of solvents seemed to affect the antioxidant activity of the mango leaves extract. Antioxidant activity increased with increasing polarity of solvents. Duh (1998) reported that the water extract of burdock exhibited the greater yield and stronger antioxidant activity than methanol, ethanol, chloroform and hexane extracts. Moreover, water extracts of herbs showed marked antioxidant activity, not only in linoleic acid but also

in liposome model system (Duh and Yen, 1997). From our preliminary study, water extracts of liquorice and mango leaves had low antioxidant activity and offensive odor, possibly caused by deterioration of extract. Therefore, water was not used for extraction of liquorice and mango leaves. These results were in accordance with many researchers who found that methanolic extracts of ajorom, cocoa by-products, buckwheat, tea leaves, *Casyrina equisetifolia* leaves, *Polygonum hydropiper* leaves, oriental herbs and spices of family Labiatae showed highest antioxidant activity, compared to extracts prepared with other solvents (Mehta *et al.*, 1994; Zandi and Gordon, 1999 ; Azizah *et al.*, 1999 ; Przybylski *et al.*, 1998 ; Takahashi *et al.*, 1999 ; Haraguchi *et al.*, 1992 ; Kim *et al.*, 1994 ; Economou *et al.*, 1991). Methanol appeared to be best solvent for extracting compounds such as phenolics, flavonoids and other polar materials from plants materials (Kim *et al.*, 1994; Dapkevicius *et al.*, 1998). Toda *et al.* (1995) reported that methanol extracts prepared from rhizone of *Curcuma longa* L. showed the strongest antioxidant activity. Kallithraka *et al.* (1995) found that methanol was the best solvent for quantitative extraction of (+)-catechin, (-)-epicatechin and epigallocatechin from grape seeds. Chevollea *et al.* (1992) reported that hexane extracts from leaves of some Mediteranean plants had higher antioxidant activity than methanol extracts but higher yields were observed with methanol extracts than hexane extracts. Wettasinghe and Shahidi (1999) reported that an appropriate solvent with the right polarity can maximize the antioxidant activity of phenolic extracts of plant materials.

Our result suggested that the total phenolic content and antioxidant activity of liquorice and mango leaves were greatly dependent on the type of solvents used for the extraction. This was postulated to be due to the differences in solubility and polarity of antioxidative components of liquorice and mango leaves in different solvents. From the result, methanol extracts showed the highest antioxidant activity and highest total phenolic content. Therefore, methanol was chosen as extracting solvent for further study.

Table 6 Antioxidant activity and total phenolic content of mango leaves extracts prepared with different solvents.

Solvents	Liposome system		β-carotene bleaching system	Total phenolic content (mg/g)
	%inhibition of conjugated dienes	TBARS	%inhibition of oxidation	
Methanol	58.66±0.10 [a] d [b]	90.62±0.20 d	95.38±0.25 c	40.36±0.22 d
Ethanol	55.45±0.29 c	78.30±0.29 b	85.38±0.23 b	2.75±0.07 b
Ethyl Acetate	50.04±0.19 b	83.21±0.21 c	85.24±0.23 b	36.84±0.25 c
Hexane	4.41±0.21 a	27.62±0.31 a	20.00±0.14 a	0.49±0.22 a

[a] Mean±standard deviation from triplicate determinations.

[b] Different letters in the same column bear significant differences. ($p<0.05$)

2.2 Effect of water/methanol ratio on extraction of antioxidants.

2.2.1 Liquorice

The efficiency of antioxidant extraction by different mixtures of water and methanol was evaluated using total phenolic content and antioxidant activity in both testing systems. The 80-90% methanol extract had highest antioxidant activity in both systems ($p<0.05$). Maximum total phenolic content was observed when 80% methanol was used (Figure 16). Therefore, 80% methanol was found to be the most proper solvent for antioxidant extraction from liquorice. Alonso *et al.* (1991) found that the extraction of catechins and proanthocyanidins from grape seeds was more efficient when the ethanol content was increased. The differences in antioxidant activity of different extracts prepared using different ratio of methanol to water were due to different polarity and solubility of antioxidant compounds.

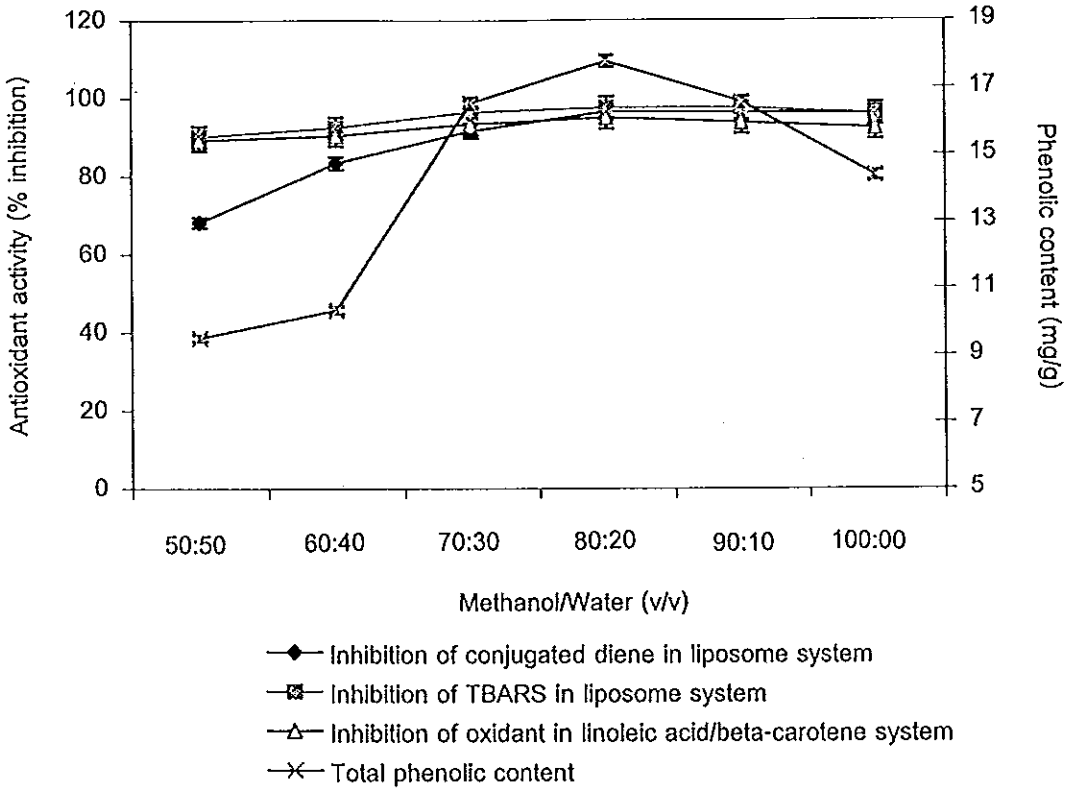


Figure 16 Effect of methanol/water ratio on antioxidant activity and total phenolic content of liquorice extract.

2.2.2 Mango leaves

Highest antioxidant activity in both systems tested was obtained when extracted with 70-80% methanol ($p<0.05$). Total phenolic content was highest when 80% methanol was used (Figure 17). From the result, 80% methanol extract of mango leaves had highest total phenolic content and highest antioxidant activity. Julkunen-Titto (1985) reported that the concentration of total phenols in leaves extract of *Salix cv.aquatica* reached a maximum when 50-80% methanol was used. Yi *et al.* (1997) reported that the concentration of total phenols in grape extract decreased with decreasing methanol content of different water-methanol mixtures and concentration reached a maximum when 50-70% methanol were used.

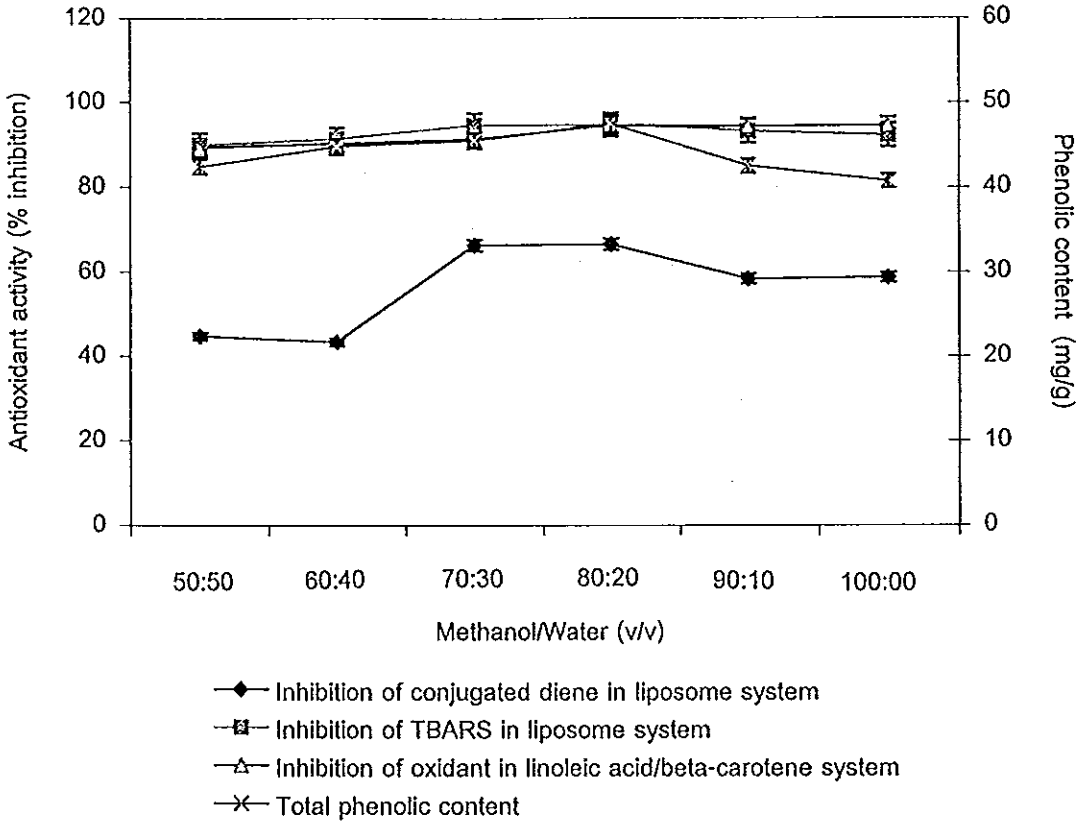


Figure 17 Effect of methanol/water ratio on antioxidant activity and total phenolic content of mango leaves extract.

From these results, the extracts of liquorice and mango leaves possibly contained a great variety of different phenolic compounds, which had different polarity and solubility. Thus, solvent with appropriate polarity can extract the antioxidant compounds selectively and effectively. From our result, 80% methanol was used as a proper solvent for extraction of antioxidants from both liquorice and mango leaves.

2.3 Effect of extraction time and repetition on the extraction of antioxidants.

2.3.1 liquorice

Antioxidant activity and total phenolic content in liquorice extracts prepared with different extraction times and repetitions are depicted in Figure 18 and Figure 19. No significant differences in antioxidant activity in both systems were found with different repetition ($p>0.05$) (Figure 18). Extraction time ranging from 5 to 24 hrs had no effect on antioxidant activity. An increase in total phenolic content was obtained with the increased repetition ($p<0.05$) while extraction time did not affect total phenolic content ($p>0.05$). This result was in agreement with Tian and White (1994) who reported that phenolic content in oat extract increased with increasing repetition. No differences in antioxidant activity were observed with increased extraction time and repetition. Thus, the optimum condition for extracting of antioxidant from liquorice involved extracting liquorice powder with 80% methanol for 5 hrs.

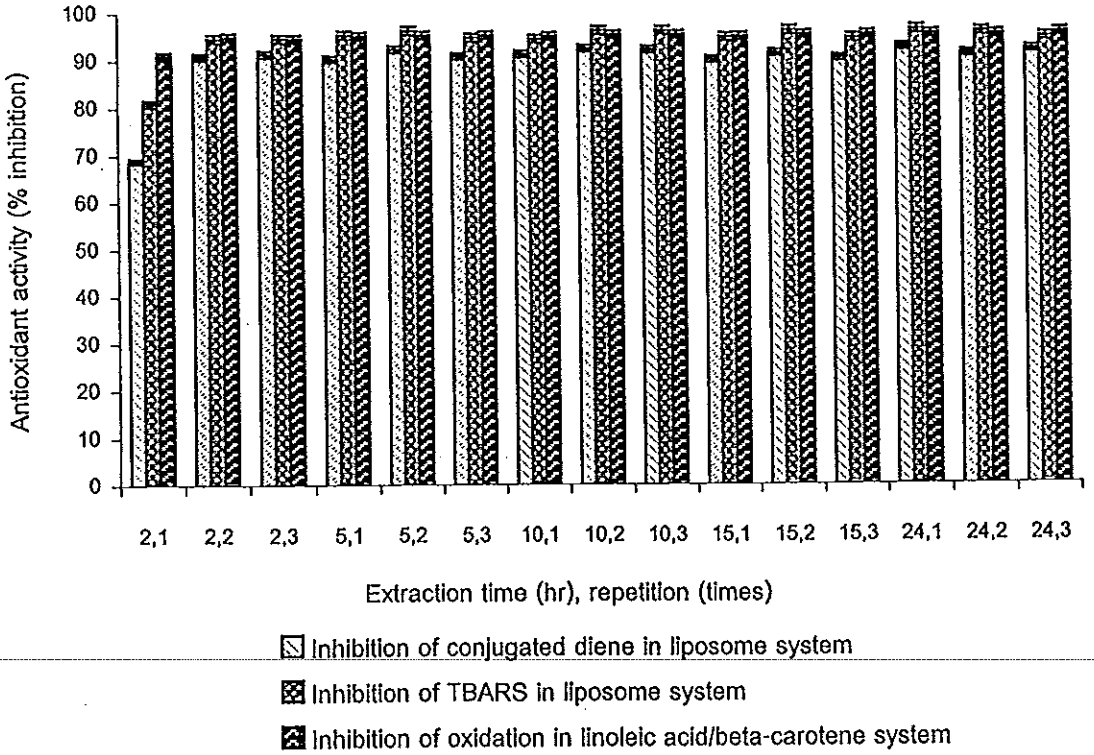


Figure 18 Effect of extraction time and repetition on antioxidant activity of liquorice extract.

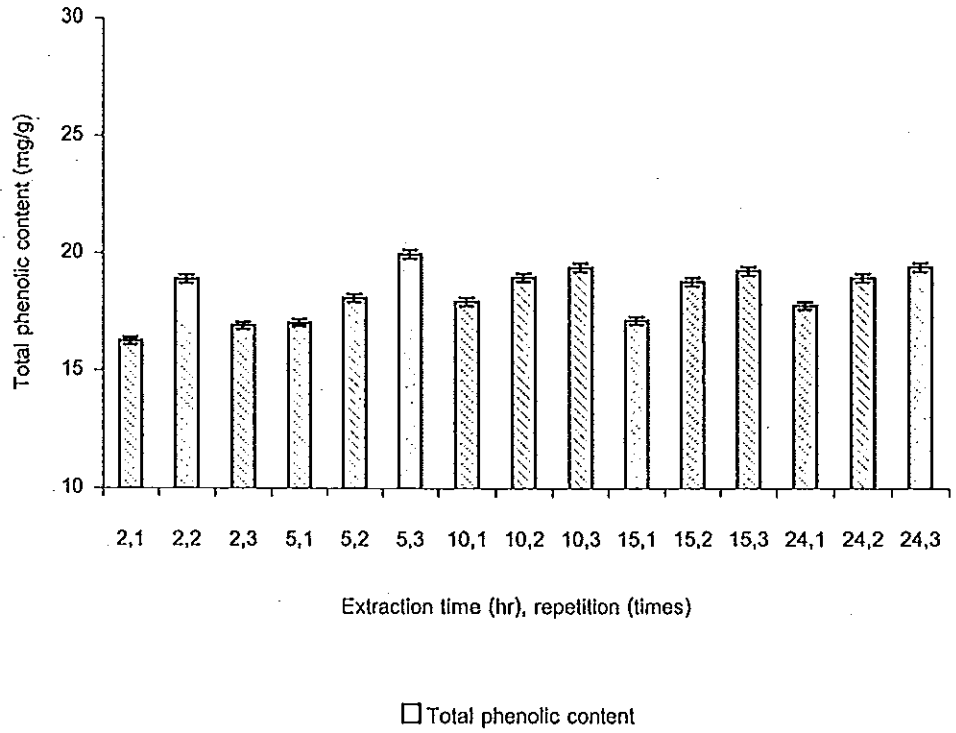


Figure 19 Effect of extraction time and repetition on total phenolic content of liquorice extract.

2.3.2 mango leaves

The effect of extraction time and repetition on the antioxidant activity and total phenolic content of mango leaves are shown in Figure 20 and 21. No significant differences in antioxidant activity in both systems were observed with different repetition and extraction time (5-24 hrs) ($p>0.05$). This result was similar to that observed in liquorice. An increase in total phenolic content was obtained with the increased repetition ($p<0.05$), whereas no changes in total phenolic content were obtained with increasing extraction time ($p>0.05$). Julkunen-Titto (1985) reported that 95% of the phenolic were extracted from the leaves of *Salix* sp. within 6 hrs and an additional 14 hrs gave only about 5% increase in total extractable phenolic. Longer extraction times increased the possibility of oxidation of phenolic unless reducing agents were added to the solvent system (Khanna *et al.*, 1968). However, Alonso *et*

al. (1991) found that the catechins and proanthocyanidins from grape seed increased as extraction time increased from 3 to 72 hrs.

No differences in antioxidant activity were observed with increased extraction time and repetition. Thus, the optimum condition for extracting of antioxidant from liquorice and mango leaves involved extracting both powders with 80% methanol for 5 hrs.

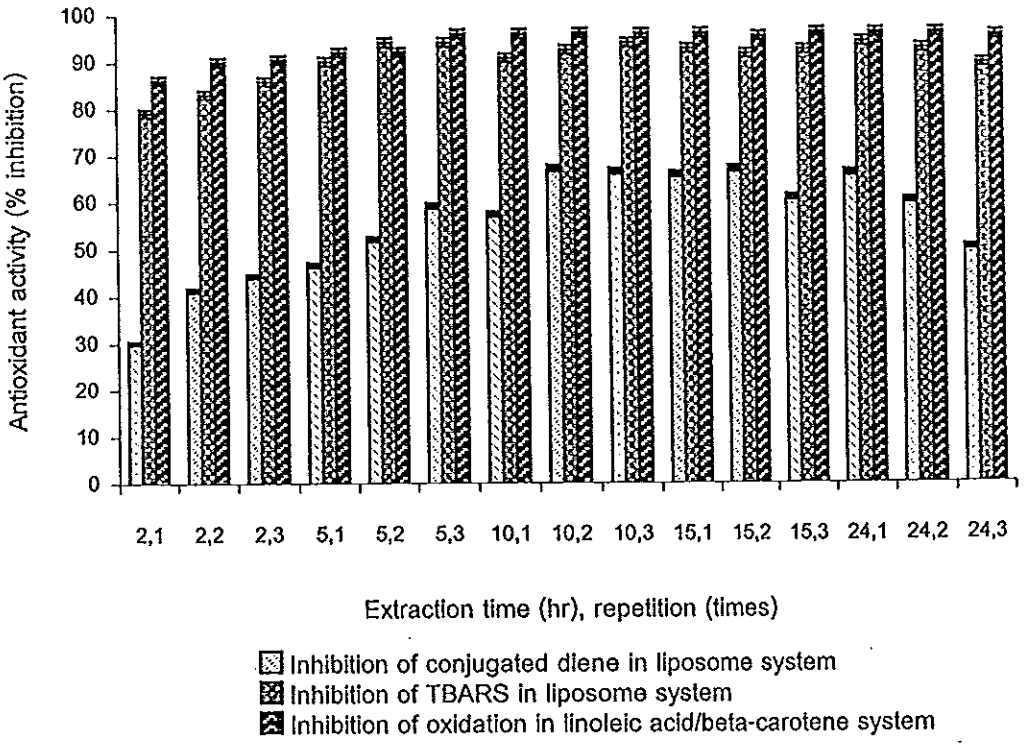


Figure 20 Effect of extraction time and repetition on antioxidant activity of mango leaves.

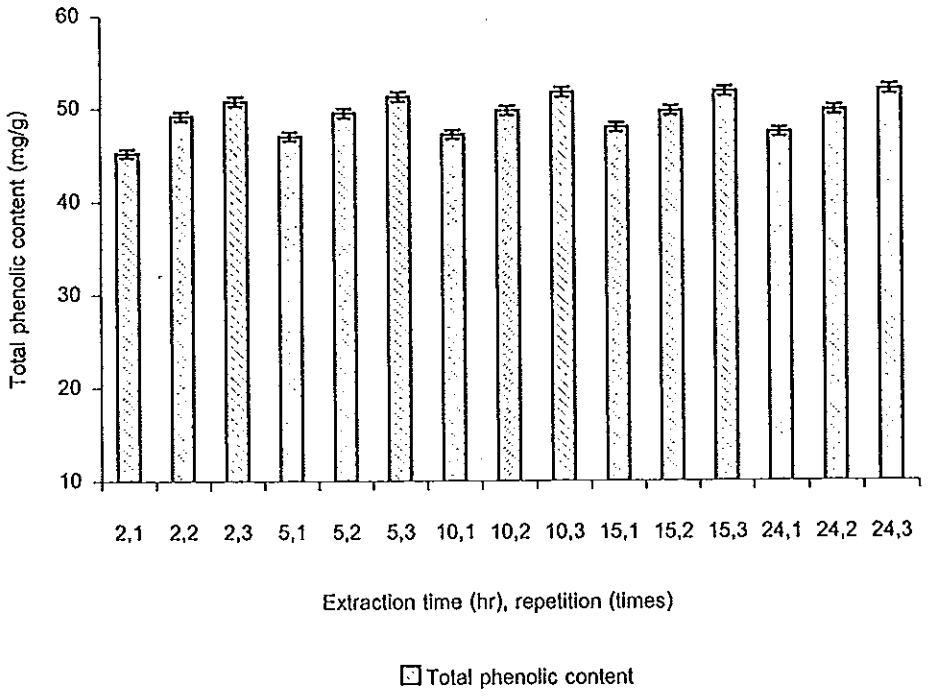


Figure 21 Effect of extraction time and repetition on total phenolic content of mango leaves.

After, the extraction of liquorice and mango leaves under the optimum condition, methanol in extracts was removed by a vacuum rotary evaporator at 40°C and then freeze-dried to obtain the antioxidants powder. Yields of liquorice and mango leaves extract powder were 9.30±1.05 % (w/w) and 10.75±1.20 % (w/w), respectively.

3. Some properties of liquorice and mango leaves extracts.

Although either lecithin liposome system or linoleic/β-carotene system can be used to measure antioxidant activity of the extracts, linoleic acid may not be a valid substrate for evaluating food antioxidants because it has unique physical properties in aqueous micelles that affect the concentration and location of antioxidants with different polarity (Huang *et al.*, 1996; Hopia *et al.*, 1996). In emulsions, the hydrophilic tea catechins in green teas partitioned into the water phase, oil-water interfaces, and Tween 20 micelles and its

concentration in the oil phase was reduced to protect lipid from oxidation (Frankel *et al.,* 1994; Yi *et al.,* 1997). Yi *et al.* (1997) reported that green tea had good antioxidant activity in lecithin liposome due to the interaction of polar tea catechins with the polar environment of lecithin liposome, which thus afforded better protection against oxidation. However, some of the less polar phenolic compounds appeared to be more effective antioxidants in lecithin liposome system than more polar compounds (Heinonen *et al.,* 1998). Though we had the similar result between two systems in this study, the liposome system was chosen for further study since liposome has similar characteristic to lipid membrane system (Hashimoto *et al.,* 1999 ; Asai *et al.,*1999).

3.1 Effect of heat treatment on antioxidant activity of liquorice and mango leaves extract.

Liquorice and mango leaves extract were incubated at 80°C and 100°C for 0, 10, 20, 30, 40, 60, 80, 100 and 120 min, and the residual antioxidant activity was determined in lecithin liposome system. With heat treatment at 80°C and 100°C, antioxidant activity of liquorice extract slightly decreased as the heating time increased (Figure 22). Heating at both 80°C and 100°C for 20 min did not significantly reduce antioxidant potency of extract ($p>0.05$). However, heating at 100°C for a longer time reduced the antioxidant activity of liquorice extracts. At the same heating time, liquorice extract exposed to 100°C showed lower antioxidant activity than that exposed to 80°C.

The antioxidant activity of mango leaves extract was slightly reduced when the heating time increased (Figure 23). The result was similar to those obtained in liquorice extract. The extracts heated at 80°C and 100°C for 10 min were not significantly different in antioxidant activity compared to the control ($p>0.05$). However, a decrease in antioxidant activity of mango leaves extract was observed when heated at 80°C and 100°C for 20 min or longer time.

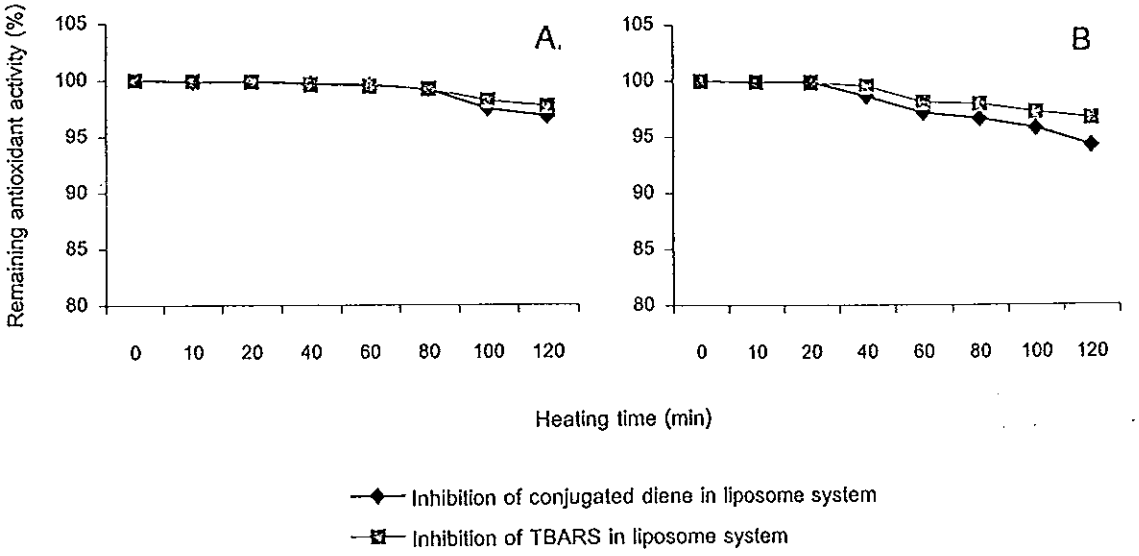


Figure 22 Remaining antioxidant activity of liquorice extract (2 mg/mL) as a function of heating time at 80°C (A) and 100°C (B).

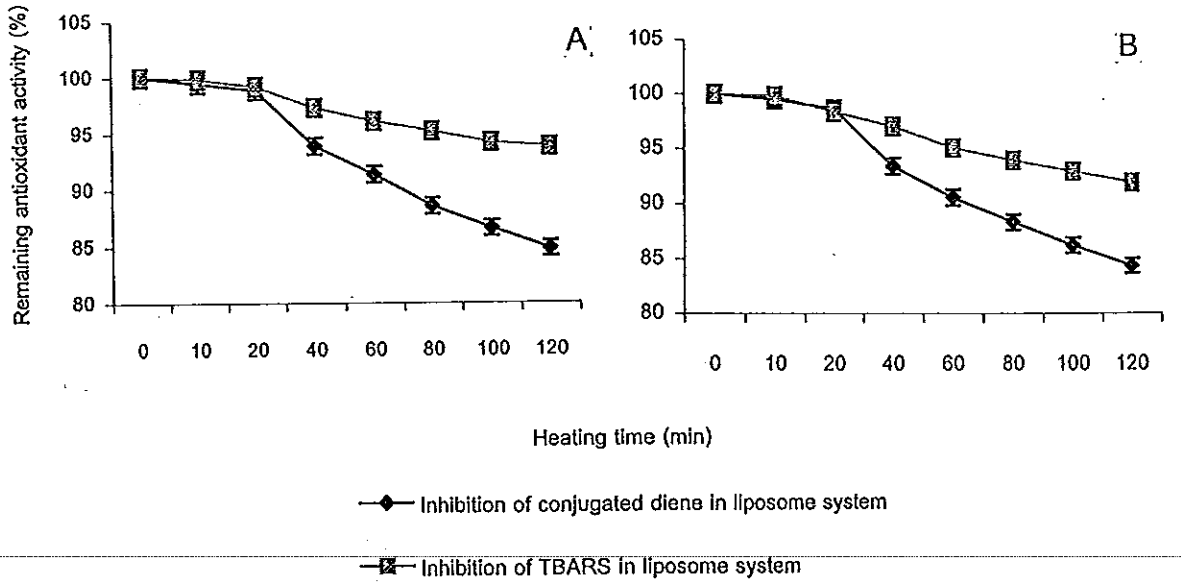


Figure 23 Remaining antioxidant activity of mango leaves extract (2 mg/mL) as a function of heating time at 80°C (A) and 100°C (B).

Generally, antioxidant activity of liquorice extract was much more heat stable than that of mango leaves extract. The antioxidant activity of liquorice and mango leaves extracts decreased at both temperatures, probably resulted from evaporation as well as chemical decomposition (Hamama and Nawar, 1991). Prasad *et al.* (1996) reported that the hydroxyl radical-scavenging property of garlic was reduced by approximately 10% when heated to 100°C for up to 60 min. Similarly, Yin and Chen (1998); Azizah *et al.* (1999) found that heating treatment reduced the antioxidant activity of garlic bulb and metanolic extract of cocoa by-product. Mansour and Khalil (2000) reported that antioxidant in potato peel and fenugreek seed extracts were heat-stable, still remaining after 120 min heating at 100°C. Thus, the results of this study showed that antioxidant activity of liquorice and mango leaves extracts were fairly heat stable.

3.2 Effect of pH on antioxidant activity of liquorice and mango leaves extracts.

The effect of pH on antioxidant activity of the liquorice extract in lecithin liposome system is shown in Figure 24. Maximum antioxidant activity of liquorice extract was observed in the wide pH ranges. Nontheless, the extract from liquorice exhibited lowest antioxidant activity at pH 3.0. Mansour and Khalil (2000) reported that the extract from ginger rhizomes and fenugreek seed showed maximum antioxidant activity at pH 7.0 and the activity was decreased at alkaline pH value. For DPPH radical scavenging, α-tocopherol had an effect immediately after its addition at both pH 5.5 and 7.5 (Kogure *et al.*, 1999). From the result, the antioxidant activity of extract was decreased at acidic pH, possibly due to either the loss of antioxidant property of extract or the enhancement of lipid oxidation in lecithin liposome system.

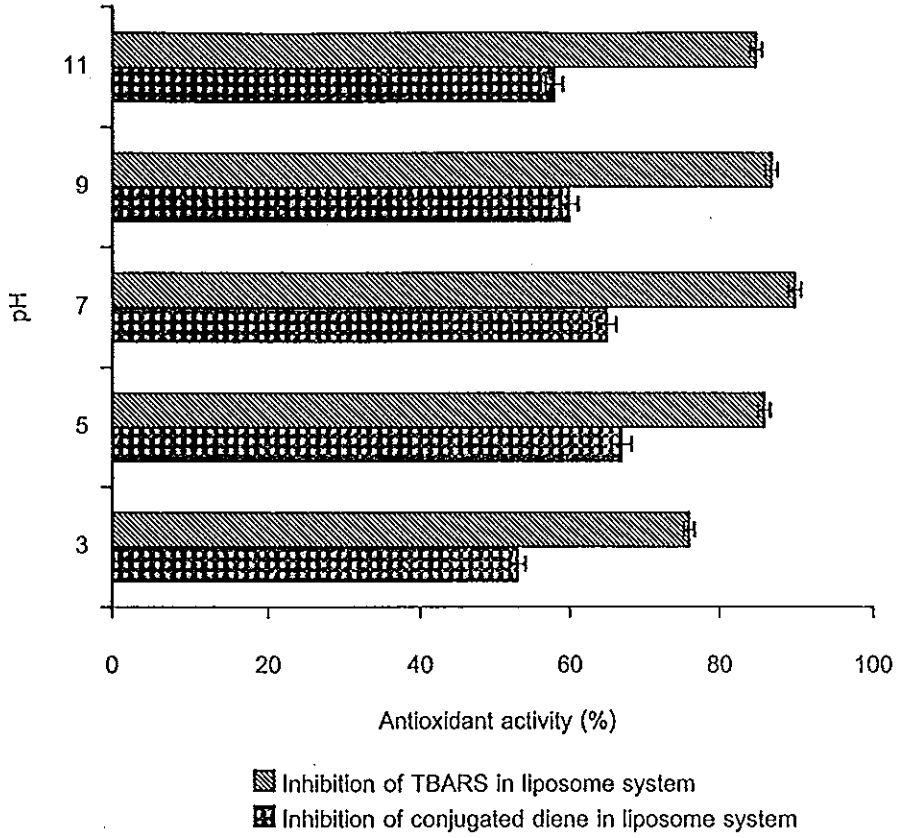


Figure 24 Effect of pH on antioxidant activity of liquorice extracts (0.5 mg/mL) in lecithin liposome system.

Figure 25 shows the effects of pH on antioxidant activity of the mango leaves extract. The mango leaves extract exhibited strong antioxidant activity at alkaline and neutral pHs and the activity decreased slightly at acidic pH. In this experiment, the extract from mango leaves was thermal stable and it was stable at all pHs tested. Flavonoids have been reported to function well in the pH range of 7.0 to 10.0 (Milovanovic *et al.,* 1994). Futhermore, Azizah *et al.* (1999) reported that antioxidant activities of cocoa by-products increased with increasing pH and were stable at all temperatures ranging from 50 ^{o}C to 90^{o}C.

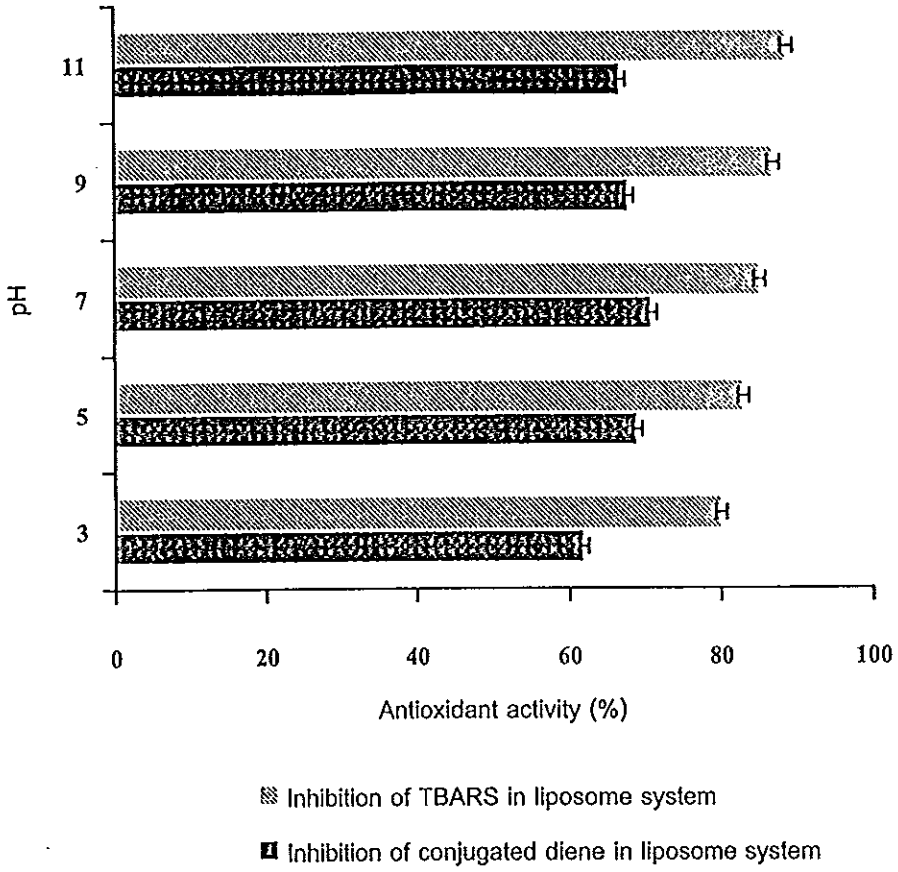


Figure 25 Effect of pH on antioxidant activity of mango leaves (0.5 mg/mL) extracts in lecithin liposome system.

3.3 Effect of concentration on antioxidant activity of liquorice and mango extracts.

The antioxidant activity of liquorice extract at different concentrations in lecithin liposome and β-carotene/linoleic acid system is shown in Table 7. The antioxidant activity of liquorice extract was compared with BHT, BHA and α-tocopherol. Antioxidant activity of liquorice extract increased as the concentration increased. No significant differences in antioxidant activity between liquorice extract with a concentration of 1.2 mg/mL, BHT and BHA (0.02 mg/mL) were observed. The activity of the extract with a concentration of 1.2 mg/mL was higher than that of α-tocopherol at 0.02 mg/mL ($p<0.05$).

Table 7 Effect of concentration of liquorice extract on antioxidant activity.

Concentration (mg/mL)		Inhibition (%)	
		TBARS	conjugated diene
0.05		70.03±0.13[a] a[b]	32.34±0.29 a
0.10		78.85±0.34 b	35.60±0.20 b
0.30		86.43±0.56 c	42.34±0.15 c
0.60		91.85±0.23 d	46.20±0.38 d
0.90		95.34±0.16 e	41.35±0.44 c
1.20		98.23±0.32 f	50.60±0.51 f
1.50		98.40±0.40 f	48.31±0.40 e
BHA	(0.02)	98.34±0.28 f	50.20±0.42 f
BHT	(0.02)	98.32±0.33 f	51.20±0.10 f
α-tocopherol	(0.02)	87.12±0.18 c	42.33±0.47 c

[a] Mean±standard deviation from triplicate determinations.

[b] Different letters in the same column bear significant differences ($p<0.05$)

Antioxidant activity of mango leaves extract increased with increasing concentration ($p<0.05$) (Table 8). The activity of the extract with concentration ranging from 0.6 to 1.5 mg/mL was higher than that of α-tocopherol at 0.02 mg/mL ($p<0.05$). However, mango leaves extract at all concentrations tested had lower antioxidant activity than BHT and BHA. Namiki (1990); Larrauri (1996) showed that natural antioxidants were less effective than synthetic ones. Yamaoka *et al.* (1991) reported that reactions of α-tocopherol with phospholipid peroxide within the liposomal membrane were inhibited to a different degree depending on the concentration of tocopherol.

Table 8 Effect of concentration of mango leaves extract on antioxidant activity.

Concentration (mg/mL)	Inhibition (%)	
	TBARS	Conjugated diene
0.05	67.21±0.34 a	30.24±0.21 a
0.10	75.56±0.23 b	33.85±0.30 b
0.30	84.32±0.12 c	40.30±0.17 d
0.60	89.25±0.18 e	37.24±0.29 c
0.90	93.62±0.29 f	43.55±0.34 e
1.20	96.30±0.57 g	49.61±0.38 g
1.50	96.24±0.43 g	46.20±0.56 f
BHA (0.02)	98.34±0.28 h	50.20±0.42 h
BHT (0.02)	98.32±0.33 h	51.20±0.10 h
α-tocopherol (0.02)	87.12±0.18 d	42.33±0.47 e

[a] Mean±standard deviation from triplicate determinations.

[b] Different letters in the same column bear significant differences ($p<0.05$).

The extract of liquorice exhibited slightly stronger antioxidant activity in both systems than mango leaves extract at same concentration. This was presumed to be due to different antioxidative compounds in both extracts. From this result, antioxidant activities of both extracts were dependent on the concentration used. The result was in agreement with Azizah *et al.* (1999) who found that antioxidant activity of methanolic extract from cocoa by-products increased with increasing concentration up to 2000 ppm and increasing concentration of extract thereafter had no effect on antioxidative activity. Yen and Lee (1997) reported that antioxidant activity of the extract

from *Aspergillus candidus* broth filtrate increased with an increasing concentration, but it reached maximum at 200 ppm.

3.4 Synergistic effect of liquorice and mango leaves extracts with some compounds.

3.4.1 Synergistic effect of liquorice and mango leaves extracts with α- tocopherol

Synergistic antioxidant action was found between the liquorice extract and α-tocopherol (Figure 26). The combination of liquorice extract (0.02 mg/mL and 0.03 mg/mL) and α-tocopherol (0.02 mg/mL and 0.03 mg/mL) rendered greater antioxidant activity than only α-tocopherol or liquorice extract ($p<0.05$). No synergistic effect of α-tocopherol with liquorice extract was observed with an increasing amount of the α-tocopherol. α-tocopherol, citric acid and ascorbic acid are reported to be synergists that enhance and improve the antioxidant activity of some natural plant extracts (Wada and Fang, 1992 ; Madsen and Bertelsen, 1995 ; Lee *et al.,* 1999).

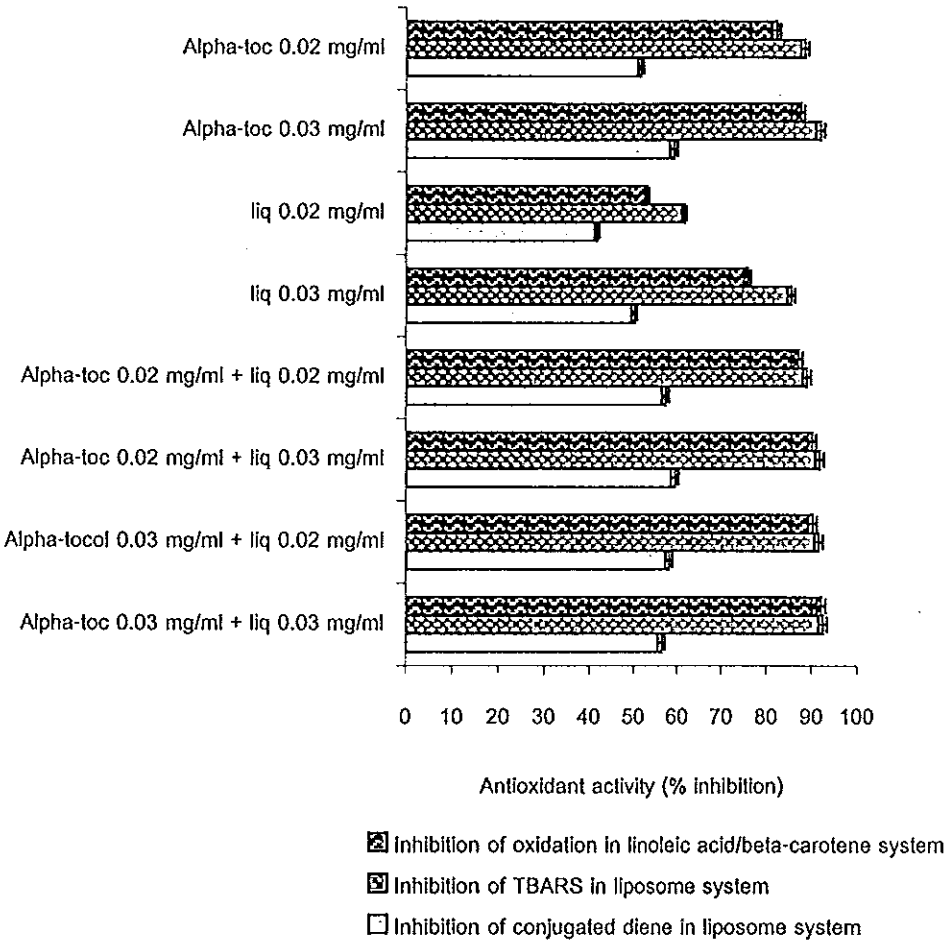


Figure 26 Synergistic antioxidant activity of liquorice extracts with α-tocopherol in lecithin liposome system and β-carotene/linoleic acid system.

Synergistic effect of mango leaves extracts with α-tocopherol is shown in Figure 27. The combination of mango leaves extracts (0.02 and 0.03 mg/mL) and α-tocopherol (0.02 and 0.03 mg/mL) exhibited higher antioxidant activity than only α-tocopherol or mango leaves extracts ($p<0.05$) in both lecithin liposome and β-carotene/linoleic acid systems.

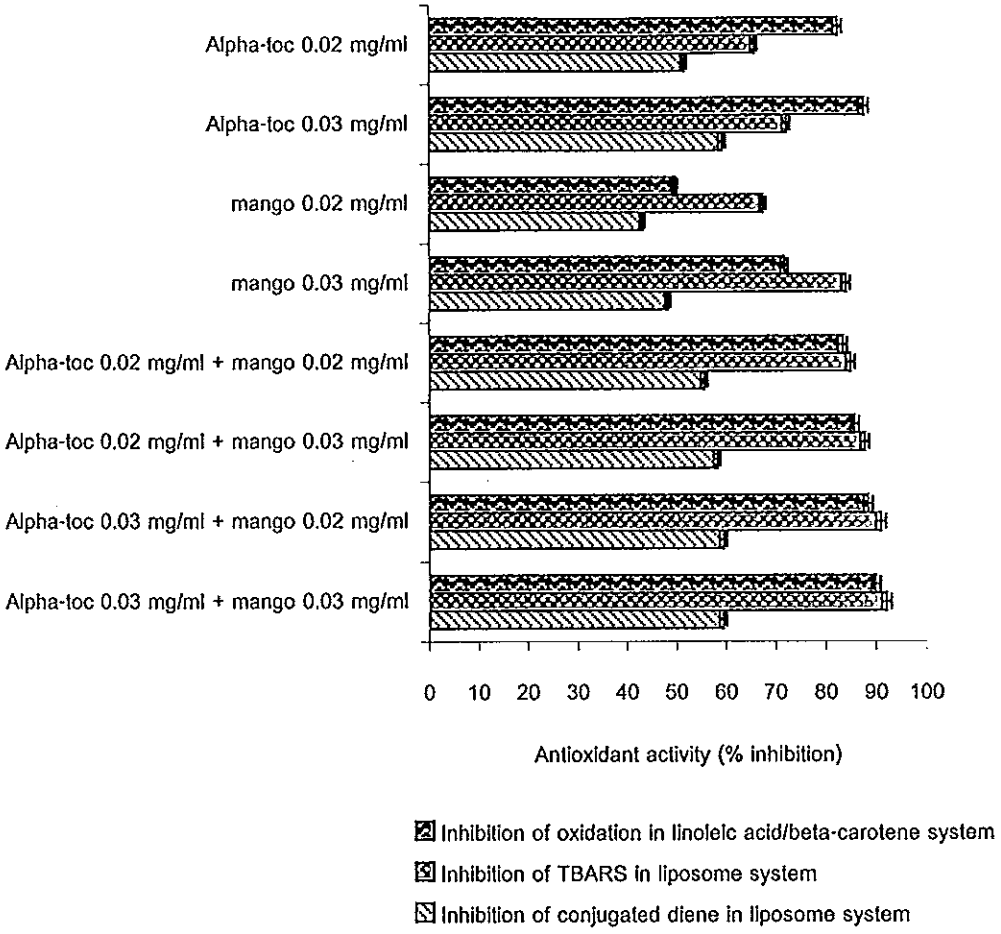


Figure 27 Synergistic antioxidant activity of mango leaves extracts with α-tocopherol in lecithin liposome system and β-carotene/linoleic acid system.

This result was in agreement with Duh (1998) who reported that the extract from burdock had a synergistic effect with α-tocopherol in lecithin liposome system. The burdock extract showed strong free radical scavenging activity on 2,2-diphenyl-1-picrylhydrazyl (DPPH), suggesting that the antioxidant substances in burdock extracts had hydrogen-donating ability.

Thus, the synergistic effect of burdock extracts with α-tocopherol might be due to a redox mechanism involving reduction of the α-tocopheroxyl radical intermediate by burdock extract to regenerate α-tocopherol.

From the result, it was postulated that synergistic effect of liquorice and mango leaves extract with α-tocopherol was propably due to a redox mechanism, leading to regeneration of α-tocopherol. Furthermore, α-tocopherol possibly functioned as a free radical quencher in biology cell and localized within the phospholipid bilayer of cell membranes to protect against biological lipid peroxidation (Hafeman *et al.*, 1997).

3.4.2 Synergistic effect of liquorice and mango leaves extracts with ascorbic acid.

Figure 28 presents the antioxidant activity of the extract from liquorice (0.02 and 0.03 mg/mL) ascorbic acid (0.02 and 0.03 mg/mL) and the combination of the extract and ascorbic acid. Ascorbic acid exhibited some antioxidant effect on lecithin liposome system and β-carotene/linoleic acid system. Antioxidant activity of ascorbic acid may depend on its concentration in a model system. Mitusumoto *et al.* (1991) reported that addition of 500 ppm ascorbic acid to ground beef decreased lipid peroxidation in ground beef. However, 50 ppm ascorbic acid caused increased the lipid peroxidation. Lee and Hendricks (1997) showed that the antioxidant or prooxidant activity of ascorbic acid depended on the concentration of ascorbic acid and Fe^{3+} in a system. Lee *et al.* (1999) and Yen *et al.* (1999) reported that in the presence of Fe^{3+}, ascorbic acid showed an antioxidant activity at high concentration while it showed a prooxidant activity at low concentration. The high concentration of ascorbic acid probably reduced all the added Fe^{3+} to Fe^{2+}, thereby restricting the redox activity of iron.

Ascorbic acid may act as a prooxidant in the presence of metal ions (Naiki, 1991). In the experiment, cupric acetate was added in lecithin liposome system and oxygenated water was added in β-carotene/linoleic acid system. Weak antioxidant activity of ascorbic acid was caused by cupric acetate in lecithin liposome system and excess oxygen in β-carotene/linoleic acid

system. The ascorbic acid seemed to act as primary antioxidant in an open system by stopping the free radical chain reaction as well as in closed system by absorbing free oxygen to prevent oxidation (Han *et al.*, 1990). Generally, ascorbic acid was not synergistic to the extract. Conversely, a lower antioxidant activity of liquorice extract was found in presence of ascorbic acid.

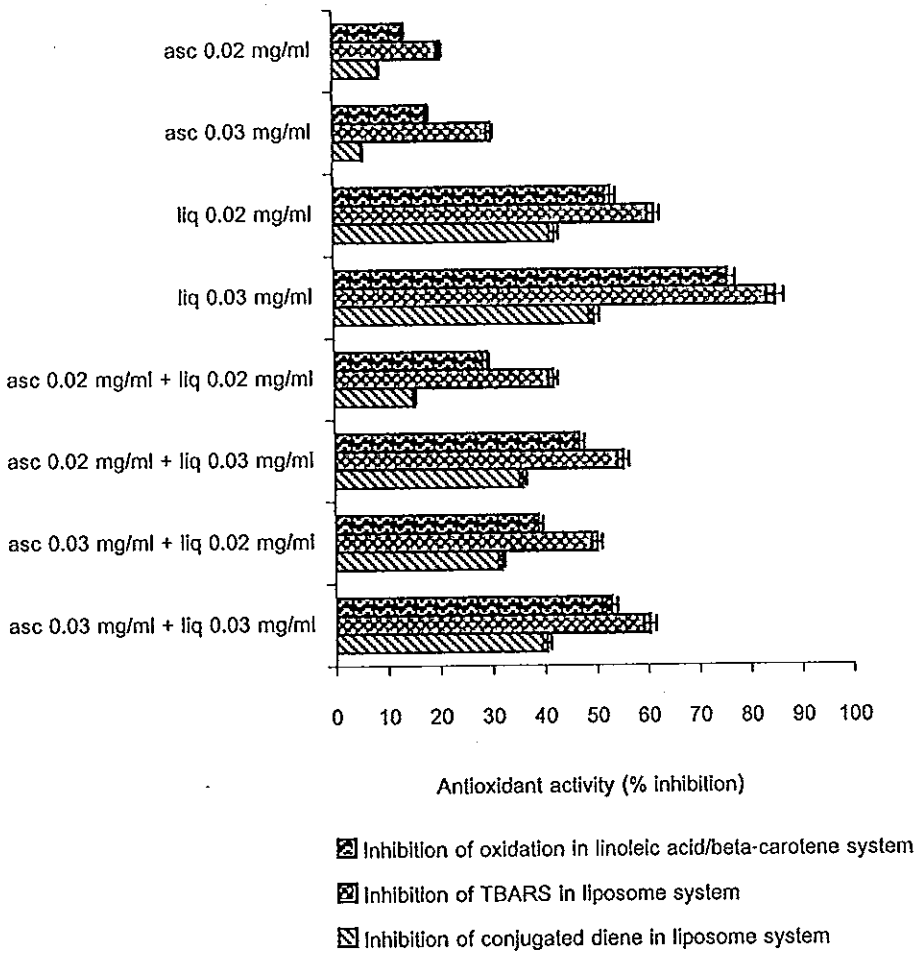


Figure 28 Synergistic antioxidant activity of liquorice extracts with ascorbic acid in lecithin liposome system and β-carotene/linoleic acid system.

Figure 29 shows the antioxidant activity of the extract from mango leaves (0.02 and 0.03 mg/mL), ascorbic acid (0.02 and 0.03 mg/mL) and the combination of the extract and ascorbic acid. No synergistic action was found between mango leaves extract and ascorbic acid. A lower antioxidant activity of mango leaves extract in presence of ascorbic acid was observed. Yamamura *et al.* (1997) found that ascorbic acid can directly scavenger free radicals that are generated in the aqueous phase and suppress oxidation of the liposome membranes, but ascorbic acid cannot efficiently scavenger peroxyl radicals located in the liposomal lipid region.

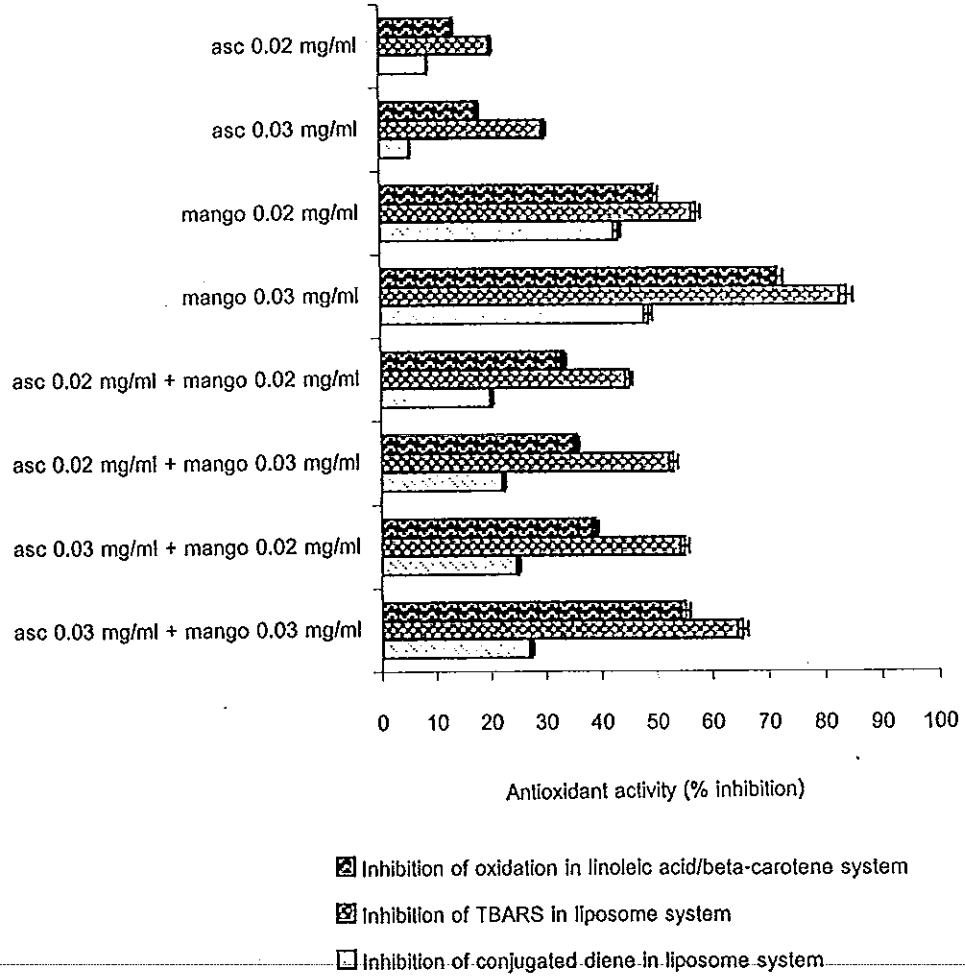


Figure 29 Synergistic antioxidant activity of mango leaves extracts with ascorbic acid in lecithin liposome system and β-carotene/linoleic acid system.

No synergism was observed when liquorice and mango extract was mixed with ascorbic acid in both lecithin liposome system and β-carotene/ linoleic acid system. On the other hand, antioxidant activity of ascorbic acid with liquorice or mango leaves extracts combination decreased. This was postulated that ascorbic acid possibly caused acidic pH, leading to weak antioxidant activity of both extracts.

3.4.3 Synergistic effect of liquorice and mango leaves extract with citric acid.

Citric acid is wildly used as a synergistic to antioxidants and chelator in food (Banias *et al.*, 1992). Citric acid is not hydrogen donor (Penman and Gordon, 1998). Synergistic effect of citric acid (0.02 and 0.03 mg/mL) on the antioxidant activity in lecithin liposome system and β-carotene/linoleic acid system of liquorice extract (0.02 and 0.03 mg/mL) is shown in Figure 30. Citric acid exhibited higher antioxidant in lecithin liposome system than that in β-carotene/linoleic acid system. This was presumed that citric acid inhibited iron-catalyzed oxidation of lecithin liposome system, probably by chelating of metal ions.

No synergism of citric acid on the antioxidant effect of liquorice extract was observed. Moreover, antioxidant activity of liquorice extract-citric acid combination was lower than that of only liquorice extract. Citric acid possibly caused an acidic pH. As the result, liquorice extract exhibited weak antioxidant activity in such as acidic pH.

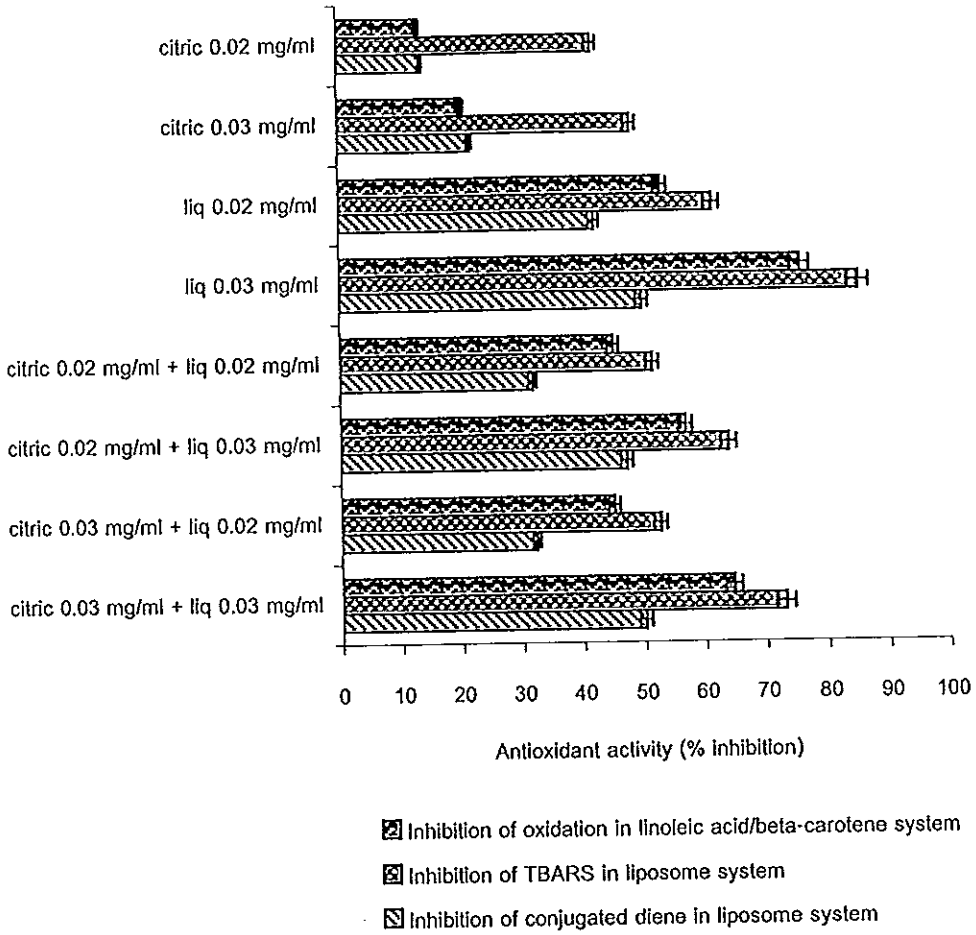


Figure 30 Synergistic antioxidant activity of liquorice extracts with citric acid in lecithin liposome system and β-carotene/linoleic acid system.

Synergistic effect of citric acid (0.02 and 0.03 mg/mL) on the antioxidant activity of mango leaves extracts (0.02 and 0.03 mg/mL) is shown in Figure 31. The result was similar to those obtained in liquorice extracts. Mango leaves extracts did not work synergistically with citric acid. Antioxidant activity of the mixture of citric acid and mango leaves extracts was lower than that of only mango leaves extracts.

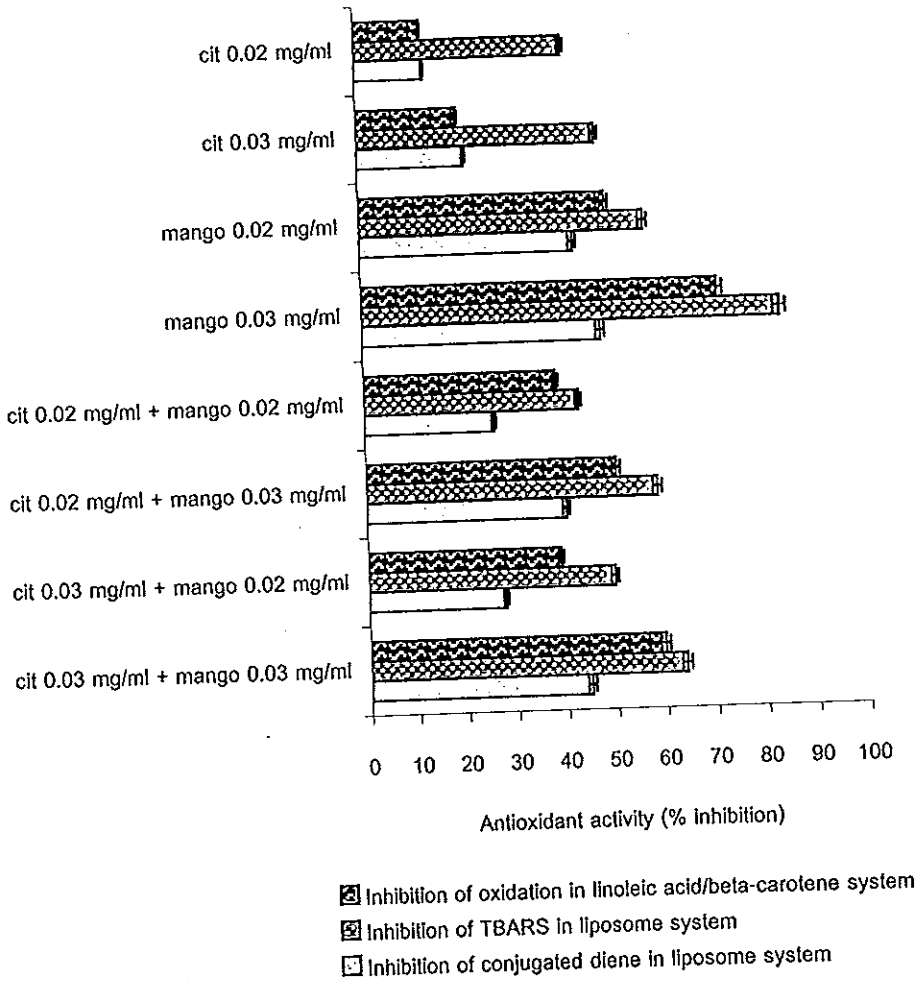


Figure 31 Synergistic antioxidant activity of mango leaves extract with citric acid in lecithin liposome system and β-carotene/linoleic acid system.

From the results, α-tocopherol showed higher synergistic effect with extracts in both lecithin liposome system and β-carotene system than citric acid and ascorbic acid.

4. Mode of action

4.1 Radical – scavenging activity of liquorice and mango leaves extracts.

4.1.1 Scavenging activity of liquorice and mango leaves extracts on 1,1 – diphenyl- 2 - picrylhydrazyl (DPPH) radical.

The scavenging effect of different concentrations of liquorice extract on the DPPH is presented in Table 9. The scavenging effect of liquorice extract on the DPPH radical increased with increasing amounts of extracts. The extracts exhibited 91.88 and 92.40% radical scavenging activity at concentrations of 4 and 5 mg/mL, respectively. The activity of extracts is therefore attributed to their hydrogen-donating ability (Shimada *et al.,* 1992). BHA BHT and α- tocopherol showed 94.47, 94.58 and 93.95 % radical scavenging activity at a concentration of 0.02 mg/mL. The scavenging activity of liquorice extract at all concentrations tested was significantly lower than that of BHA, BHT and α- tocopherol at 0.02 mg/mL.

The scavenging effect of mango leaves extract on the DPPH radical is shown in Table 10. The scavenging effect on DPPH radical of mango leaves extract at 5 mg/mL was significantly higher than that of α- tocopherol at 0.02 mg/mL but it was slightly lower than that of BHA and BHT at 0.02 mg/mL. Scavenging effects of mango leaves extract on DPPH radical was related to the amounts of the extracts added. Yen and Wu (1999) and Moon and Terao (1998) reported that methanolic extracts of *Ganoderma tsugae* and dittany showed a concentration – dependent scavenging activity on the DPPH radical. Moller *et al.* (1999) and Przybylski *et al* (1998) reported that the scavenging activity of dittany and buckweat extract increased when more polar solvent were used for extraction. The *Osbeckia aspera* was shown to scavenge DPPH free radical in a concentration – dependent manner (Thabrew *et al,* 1998). Furthermore, phenolic extracts of plant have been shown to neutralize free radicals in various model systems (Wettasinghe and Shahidi, 1999 ; Lissi *et*

al., 1999). The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability (Bran-Willams *et al.*, 1995).

Table 9 Scavenging effects of liquorice extract on the 1,1 diphenyl- 2 – picrylhydrazyl (DPPH) radical.

Concentration (mg/mL)	Asorbance at 517 nm	DPPH radical - scavenging activity(%)
0	0.382±0.008 j[a]	0.00 a[b]
0.20	0.195±0.002 i	48.95±0.19 b
0.40	0.103±0.003 h	73.18±0.30 c
0.60	0.046±0.002 g	88.11±0.44 d
0.80	0.043±0.002 f	88.98±0.20 e
1.00	0.040±0.003 e	84.57±0.12 f
2.00	0.036±0.001 d	90.67±0.15 g
3.00	0.033±0.001 c	91.46±0.16 h
4.00	0.031±0.002 c	91.88±0.24 hi
5.00	0.029±0.001 c	92.40±0.33 l
α-tocopherol (0.02)	0.023±0.002 b	93.95±0.21 j
BHA (0.02)	0.020±0.000 a	94.47±0.09 k
BHT (0.02)	0.020±0.001 a	94.58±0.17 k

[a] Mean±standard deviation from triplicate determinations.

[b] Different letters in the same column bear significant differences ($p < 0.05$).

The results demonstrated that the liquorice and mango leaves extract had effective activities as hydrogen donors and as primary antioxidants, which potentially react with free radical, particularly the peroxy radical, which is a major propagator of the autoxidation chain of fat, thus terminating the chain reaction (Yen and Wu, 1999). This may be responsible for the main cause of suppression of autoxidation in both β-carotene/linoleic acid and the lecithin liposome system.

Table 10 Scavenging effects of mango leaves extract on the 1,1 diphenyl- 2 - picrylhydrazyl (DPPH) radical.

Concentration (mg/mL)	Absorbance at 517 nm	DPPH radical-Scavenging activity(%)
0	0.382±0.008[a] j	0.00 a[b]
0.20	0.130±0.002 i	65.96±0.32 b
0.40	0.047±0.001 h	87.69±0.15 c
0.60	0.039±0.001 g	89.79±0.10 d
0.80	0.035±0.003 f	90.83±0.27 e
1.00	0.031±0.002 e	91.80±0.06 f
2.00	0.028±0.002 d	92.67±0.22 g
3.00	0.026±0.001 c	93.18±0.14 gh
4.00	0.023±0.003 c	93.82±0.16 h
5.00	0.021±0.001 c	94.50±0.30 i
α-tocopherol (0.02)	0.023±0.002 b	93.95±0.21 h
BHA (0.02)	0.020±0.000 a	94.47±0.09 i
BHT (0.02)	0.020±0.001 a	94.58±0.17 i

[a] Mean±standard deviation from triplicate determinations.

[b] Different letters in the same column bear significant differences ($p < 0.05$)

4.1.2 Hydroxyl radical ($OH^{\bullet}$) scavenging activity of liquorice and mango leaves extracts.

Liquorice extracts exhibited a concentration – dependent increase in hydroxyl radical scavenging activity up to a concentration of 5 mg/mL (Table 11). The hydroxyl radical scavenging activity of liquorice extracts at 5 mg/mL was significantly lower than that of α-tocopherol and BHT at 0.02 mg/mL. The hydroxyl radical is an extremely reactive free radical formed in biological systems. It can act on and damage almost every molecule found in living cells, such as sugars, amino acids, phospholipids,

DNA bases and organic acid (Namiki, 1990; Halliwell *et al.*, 1992). Lipid peroxidation is rapidly stimulated by hydroxyl radicals that are sufficiently reactive to abstract hydrogen atoms from unsaturated fatty acid (Halliwell *et al.*, 1995).

Table 11 Scavenging effects of liquorice extract on the hydroxyl radical in the deoxyribose assay.

Concentration (mg/mL)	Absorbance at 532 nm	Hydroxyl radical - scavenging activity(%)
0	0.419±0.003[a] j	0.00 a[b]
0.10	0.402±0.001 i	3.55±0.12 b
0.60	0.377±0.001 h	9.25±0.20 c
1.00	0.333±0.002 g	19.96±0.18 d
1.50	0.285±0.003 f	31.56±0.28 e
3.00	0.237±0.002 e	43.15±0.10 f
4.00	0.189±0.002 d	54.93±0.15 g
5.00	0.164±0.001 c	60.59±0.09 h
α-tocopherol (0.02)	0.125±0.001 b	70.25±0.11 i
BHT (0.02)	0.104±0.001 a	75.28±0.08 j

[a] Mean±standard deviation from triplicate determinations.

[b] Different letters in the same column bear significant different ($p < 0.05$).

Table 12 shows scavenging activity of mango leaves extracts (0.1-5 mg/mL) on hydroxyl radical. Mango leaves extracts showed scavenging activity on hydroxyl radical in a concentration – dependent manner. The hydroxyl radical scavenging activity of the extract at 5 mg/mL was significantly higher than α-tocopherol at 0.02 mg/mL. The results obtained revealed that

liquorice and mango leaves extracts inhibited deoxyribose degradation. Hydroxy radicals are known to be responsible for the breakdown of deoxyribose (Ueda *et al.*, 1996). Therefore, it indicated that liquorice and mango leaves extracts had hydroxyl radical scavenging activity.

Table 12 Scavenging effects of mango leaves extract on the hydroxyl radical in the deoxyribose assay.

Concentration (mg/mL)	Absorbance at 532 nm	Hydroxyl radical - scavenging activity(%)
0	0.419±0.003[a] i	0.00 a[b]
0.10	0.389±0.002 h	6.43±0.20 b
0.60	0.362±0.001 g	13.17±0.12 c
1.00	0.306±0.001 f	26.85±0.07 d
2.00	0.256±0.002 e	38.36±0.13 e
3.00	0.196±0.002 d	52.96±0.17 f
4.00	0.161±0.003 c	61.34±0.29 g
5.00	0.102±0.002 a	75.65±0.15 i
α-tocopherol (0.02)	0.125±0.001 b	70.25±0.11 h
BHT (0.02)	0.104±0.001 a	75.28±0.08 i

[a] Mean±standard deviation from triplicate determinations.

[b] Different letters in the same column bear significant different ($p < 0.05$).

The extract from *Osbeckia aspera* and burdock exhibited hydroxyl radical – scavenging activity in a concentration – dependent manner (Thabrew *et al.*, 1998 ; Duh, 1998). Cao and Zhu (1997) found that the herb *Radix Stephania tetrandra*, S Moore appeared to scavenge hydroxyl radical and suppressed the peroxidation of lecithin liposome system. Phenolic compounds in extract might play a role in hydroxyl radical scavenging. Polyphenol are hydroxyl radical scavenger because phenolic groups are excellent

nucleophiles and are also able to quench lipid peroxidation, acting as chain break antioxidants (Shi *et al.*, 1991 ; Bandyopadhyay *et al.*, 2000).

Therefore, the ability of liquorice and mango leaves extract to quench hydroxyl radicals seemed to relate directly to the prevention of propagation of the process peroxidation.

4.2 Reducing power of liquorice and mango leaves extracts.

The reducing power and antioxidant activity of liquorice extract increased with an increasing amount of extract (Table 13). Antioxidant activity correlated well with the reducing power of the extract. No significant differences in reducing power were found between the extract at a level of 1.00 mg and ascorbic acid at a level of 0.05 mg.

Table 13 Reducing power and antioxidant activity of different amounts of liquorice extract.

Amount (mg)	Reducing power Absorbance at 700 nm	Antioxidant activity(%)
0.05	0.045±0.011[a] a	38.71±0.36 a[b]
0.10	0.060±0.005 b	42.52±0.40 b
0.15	0.083±0.003 c	45.22±0.45 c
0.20	0.100±0.004 d	48.06±0.21 d
0.25	0.114±0.008 e	50.89±0.38 e
0.30	0.122±0.003 f	54.20±0.19 f
0.35	0.137±0.002 g	58.08±0.50 g
0.40	0.156±0.004 h	62.12±0.24 h
0.50	0.255±0.013 i	66.98±0.27 i
1.00	0.432±0.005 j	75.15±0.33 j
Ascorbic acid (0.05)	0.438±0.010 j	

[a] Mean±standard deviation from triplicate determinations.

[b] Different letters in the same column bear significant differences ($p<0.05$).

The reducing power and antioxidant activities of various amounts of mango leaves extract are shown in Table 14. The reducing power and antioxidant activity of mango leaves extract increased as the amount of extract increased. Correlation between reducing power and antioxidant activity of mango leaves extract was high. The mango leaves extract at 0.50 mg exhibited a greater reducing power than 0.05 mg of ascorbic acid, which is a reducing agent as well as a reductone (Shimada *et al.*, 1992). Mango leaves extract exhibited higher reducing power than liquorice extract at the same amount. This was postulated that mango leaves extract contained higher reductones than the extract of liquorice extract.

This result was in agreement with Duh (1998) who reported that the reducing power of burdock extract was dependent on concentration and correlated well with the extent of antioxidant activity. Fejes *et al.* (2000) found that antioxidant activity of *Anthriscus cerefolium* (L.) Hoffm. extracts were shown to be concomitant with the development of reducing power. Gordon (1990) reported that the antioxidative activity of reductones is believed to break radical chains by donation of a hydrogen atom. Lingnert and Eriksson (1981) noted that antioxidative properties are considered to be associated with the presence of reductones. Okada *et al.* (1983) reported that the reducing power of tannins prevents liver injury by inhibiting the formation of lipid peroxides. Reductones are believed not only to react directly with peroxides but also to prevent peroxide formation by reacting with certain precursors. Therefore the liquorice and mango leaves extracts were suggested to act as electron donors and can react with free radicals to convert them to more stable products and terminate radical chain reactions.

Our data indicated that the marked antioxidant action of liquorice and mango leaves extracts might be a result of their reducing power.

Table 14 Reducing power and antioxidant activity of different amounts of mango leaves extract.

Amount (mg)	Reducing power Absorbance at 700 nm	Antioxidant activity (%)
0.05	0.110±0.010[a] a	44.27±0.57 a[b]
0.10	0.140±0.009 b	47.53±0.32 b
0.15	0.186±0.005 c	51.34±0.19 c
0.20	0.214±0.010 d	53.88±0.30 d
0.25	0.245±0.003 e	56.61±0.57 e
0.30	0.287±0.004 f	59.03±0.52 f
0.35	0.322±0.008 g	63.49±0.45 g
0.40	0.356±0.008 h	66.24±0.11 h
0.50	0.440±0.005 i	70.03±0.27 i
1.00	0.563±0.013 j	78.85±0.19 j
Ascorbic acid (0.05)	0.438±0.010 l	

[a] Mean±standard deviation from triplicate determinations.

[b] Different letters in the same column bear significant differences ($p<0.05$)

4.3 Chelating activity of liquorice and mango leaves extracts.

4.3.1 Chelating activity of liquorice and mango leaves extracts on Fe^{2+} ion.

Fe^{2+} binding activity of liquorice extracts at various amounts is shown in Table 15. The absorbance of reaction mixture decreased with increasing amount of the extracts. This was caused by the complex between Fe^{2+} and the liquorice extract. Thus, Fe^{2+} binding activity of the extract was increased with increasing amount of the extract.

Table 15 Chelating effect of different amounts of liquorice extract on Fe^{2+} ion.

Amount (mg)	Absorbance at 480 nm	Fe^{2+} - chelating activity(%)
0	0.726±0.005[a] m	0.00 a[b]
0.10	0.711±0.009 m	2.06±0.76 b
0.20	0.699±0.012 l	3.72±0.97 c
0.40	0.657±0.012 k	9.50±0.99 d
0.60	0.602±0.003 j	17.08±0.45 e
0.80	0.558±0.005 i	23.14±0.55 f
1.00	0.503±0.003 h	30.71±0.42 g
1.20	0.452±0.002 g	37.74±0.27 h
1.40	0.393±0.010 f	45.31±0.89 i
1.60	0.330±0.010 d	53.72±0.82 j
1.80	0.273±0.012 c	62.04±1.00 k
2.00	0.208±0.004 b	71.35±0.50 l
citric acid 0.20 M	0.349±0.002 e	51.79±0.22 j
EDTA 0.20 M	0.077±0.004 a	89.39±0.46 m

[a] Mean±standard deviation from triplicate determinations.

[b] Different letters in the same column bear significant differences ($p<0.05$).

The chelating activity of the extract at 2.00 mg (71.35 ± 0.50%) was found to be significantly lower than that of EDTA at a level of 0.20 M (89.39 ± 0.46%) ($p<0.05$) but it was significantly higher than that of citric acid at the same concentration used (51.79 ± 0.22%) ($p<0.05$).

Fe^{2+}-binding activity of mango leaves extract at different amounts is shown in Table 16. Similar result was observed with liquorice extract. Fe^{2+}-binding activity of mango leaves extract increased with an increase in

amount of the extract. Fe^{2+}-binding activity of the extract at a level of 1.8 mg was equal to citric acid at a level of 0.02 M but it was significantly lower than that of EDTA at the same concentration ($p<0.05$).

Chen and Ahn (1998) found that natural phenolic including quercetin, rutin, caffeic acid and catechin acted as Fe^{2+} chelators. Yen and Wu (1999) reported that methanolic extracts of *Ganoderma tsugae* had a strong chelating activity on Fe^{2+} ion. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxide to reactive free radicals via Fenton type reactions (Miller *et al.*, 1990). Fe^{3+} ions also produce radical from peroxide, although the rate is ~10 – fold less than that of Fe^{2+} ions (Miller, 1996). Fe^{2+} ion is the most powerful prooxidant among various species of metal ions (Jadhav *et al.*, 1996). Hydroxyl radical and superoxide anion can also propagate the lipid peroxidation chain reaction via metal-catalyzed decomposition of lipid peroxides into peroxyl and alkoxyl radical (Minotti and Aust, 1989). Some phenolic compounds from plant have been reported to have a strong affinity to copper and iron ions (Morel *et al.*, 1993 ; Nardini *et al.*, 1995).

Table 16 Chelating effect of different amounts of mango leaves extract on Fe^{2+} ion.

Amount (mg)	Absorbance at 480 nm	Fe^{2+} - chelating activity(%)
0	0.726±0.010[a] m	0.00 a[b]
0.10	0.719±0.005 l	0.96±0.48 b
0.20	0.705±0.004 k	2.89±0.37 c
0.40	0.667±0.012 j	8.12±0.95 d
0.60	0.619±0.005 i	14.73±0.42 e
0.80	0.572±0.004 h	21.21±0.36 f
1.00	0.530±0.010 g	26.99±0.34 g
1.20	0.498±0.010 f	31.40±0.88 h
1.40	0.451±0.002 e	37.87±0.17 i
1.60	0.403±0.012 d	44.49±0.98 j
1.80	0.346±0.005 c	52.34±0.48 k
2.00	0.295±0.003 b	59.22±0.26 l
citric acid 0.20 M	0.349±0.002 e	51.79±0.22 j
EDTA 0.20 M	0.077±0.004 a	89.39±0.46 m

[a] Mean±standard deviation from triplicate determinations.

[b] Different letters in the same column bear significant differences ($p<0.05$).

The data obtained revealed that liquorice and mango leaves extracts acted as Fe^{2+}-chelators. The results indicated that the chelating activity of liquorice and mango leaves extracts on Fe^{2+} ion might play an important role in preventing lipid oxidation induced by Fe^{2+}.

4.3.2 Chelating activity of liquorice and mango leaves extracts on Cu^{2+} ion.

Chelating activity of liquorice extract (0.1-2 mg) on Cu^{2+} ion was evaluated (Table 17). Cu^{2+}-binding activity of liquorice extract increased with increasing amount of the extract. Cu^{2+}-binding activity of the extract at a level of 2.00 mg was equal to citric acid at a level of 0.20 M but it was significantly lower than that of EDTA at same concentration used ($p<0.05$). Table 18 shows Cu^{2+}-binding activity of mango leaves extract at different amounts. Similar result was observed, compared to that obtained in mango leaves extract. Mango leaves extract exhibited chelating activity on Cu^{2+} ion in a concentration – dependent manner. Chelating activity of extract at an amount of 2.00 mg was 59.22 $\pm$ 0.26%. It was significantly lower ($p<0.05$) than that of EDTA at a level of 0.20 M (89.39 $\pm$ 0.46%). However, it was higher than that of citric acid at the same concentration used (51.79 $\pm$ 0.22%) ($p<0.05$).

The results revealed that liquorice and mango leaves extracts acted as Cu^{2+}- chelator. Copper is reported as the most prooxidative effect in food and biological systems (Johnson *et al.*, 1992 ; Karahadian and Lindsay, 1989). Our data indicated that the chelating activity of liquorice and mango leaves extracts on Cu^{2+} ion probably played an important role in preventing lipid oxidation induced by Cu^{2+}.

Table 17 Chelating effect of different amounts of liquorice extract on Cu^{2+} ion.

Amount (mg)	Absorbance at 480 nm	Cu^{2+} - chelating activity(%)
0	1.130±0.005[a] l	0.00 a[b]
0.10	1.078±0.004 k	4.60±0.30 b
0.20	0.991±0.004 j	12.30±0.34 c
0.40	0.864±0.002 i	23.54±0.21 d
0.60	0.803±0.010 h	28.93±0.85 e
0.80	0.759±0.005 g	32.83±0.48 f
1.00	0.706±0.002 f	37.52±0.24 g
1.20	0.685±0.003 e	39.38±0.29 h
1.40	0.634±0.012 d	43.89±0.92 i
1.60	0.602±0.004 cd	46.64±0.37 j
1.80	0.565±0.003 bc	50.02±0.30 k
2.00	0.533±0.005 b	52.83±0.47 l
citric acid 0.20 M	0.530±0.010 b	53.09±0.85 l
EDTA 0.20 M	0.361±0.007 a	68.05±0.65 m

[a] Mean±standard deviation from triplicate determinations.

[b] Different letters in the same column bear significant differences ($p<0.05$)

Table 18 Chelating effect of different amounts of mango leaves extract on Cu^{2+} ion.

Amount (mg)	Absorbance at 480 nm	Cu^{2+} - chelating activity(%)
0	1.130±0.005[a] m	0.00 a[b]
0.10	1.071±0.010 l	5.22±0.80 b
0.20	0.908±0.007 k	19.64±0.67 c
0.40	0.846±0.004 j	25.13±0.35 d
0.60	0.784±0.004 i	30.62±0.32 e
0.80	0.735±0.002 h	34.96±0.17 f
1.00	0.671±0.002 g	40.62±0.15 g
1.20	0.621±0.012 f	45.04±0.94 h
1.40	0.577±0.007 e	48.93±0.69 i
1.60	0.534±0.003 b	52.74±0.26 j
1.80	0.485±0.003 d	57.08±0.24 k
2.00	0.426±0.005 c	62.03±0.44 l
citric acid 0.20 M	0.530±0.010 b	53.09±0.82 j
EDTA 0.20 M	0.361±0.005 a	68.05±0.46 m

[a] Mean±standard deviation from triplicate determinations.

[b] Different letters in the same column bear significant differences ($p<0.05$).

4.4 Lipoxygenase inhibitory effects of liquorice and mango leaves extracts.

The lipoxygenase-mediated reaction contributes to the formation of off-flavor and decreases in nutritional value of food (Whitehead *et al.*, 1995). Lipoxygenase inhibitory activities of different amounts of liquorice extract are presented in Table 19. Lipoxygenase inhibitory activities of liquorice extract increased with an increasing amount of the extracts. The inhibition of

lipoxygenase activity of extract at a level of 1.5 mg was not significantly different from that of α-tocopherol at a level of 0.10 mg. However, the lipoxygenase inhibitory activity of the extract was significantly lower than that of BHA and BHT at the same concentration ($p<0.05$).

Table 19 Inhibition effect of liquorice extracts at different amounts on lipoxygenase activity.

Amount (mg)	Absorbance at 234 nm	lipoxygenase inhibitory activity (%)
0	0.503±0.009[a] k	0.00 a[b]
0.05	0.491±0.004 k	2.40±0.33 b
0.10	0.470±0.002 j	6.56±0.21 c
0.20	0.454±0.004 i	9.74±0.35 d
0.40	0.436±0.003 h	13.32±0.27 e
0.60	0.423±0.005 g	15.90±0.46 f
0.80	0.405±0.001 f	19.48±0.17 g
1.00	0.382±0.005 e	24.05±0.49 h
1.25	0.360±0.002 d	28.43±0.12 i
1.50	0.332±0.002 c	33.99±0.15 j
BHA (0.01)	0.232±0.007 b	52.74±0.68 k
BHT (0.01)	0.217±0.005 a	55.80±0.51 l
α-tocopherol (0.01)	0.325±0.003 c	33.98±0.34 j

[a] Mean±standard deviation from triplicate determinations.

[b] Different letters in the same column bear significant differences ($p<0.05$)

Table 20 shows lipoxygenase inhibitory activities of mango leaves extract at different amounts. Mango leaves extract showed inhibitory effect on lipoxygenase in a concentration-dependent manner. The inhibition of lipoxygenase activity of extract at 1.25 mg was equal to that of α-tocopherol but lower than that of BHT and BHA at the same concentration used ($p < 0.05$).

This result was in accordance with that of Bucar *et al.* (1998) who reported that the extract of *Guiera senegalensis* leaves showed inhibitory effect on lipoxygenase in a concentration dependent-manner. The mango leaves extract showed higher lipoxygenase inhibitory activities than liquorice extract at same concentration. This was postulated due to the differences in structure of antioxidant compounds in each extract. Puerta *et al.* (1999) found that phenolic compounds in virgin olive oil including hydroxytyrosol, oleuropein, caffeic acid and tyrosol showed inhibitory effect on lipoxygenase activity. In each class of compounds, inhibition constants were strongly affected by structures of antioxidants. Lipoxygenase is non heme iron-containing oxidase that has been found in plants, animal tissue (including marine products) and more recently mushroom and other fungi (Oomah *et al.*, 1997 ; Nunez *et al.*, 1997). Lipoxygenase catalyzes the oxygenation of the *cis,cis*-1-4 pentadiene sequence of polyunsaturated fatty acids to produce their corresponding hydroperoxides (Siedow, 1991). The lipoxygenase pathways of arachidonic acid metabolism produce reactive oxygen species, and these reactive forms of oxygen and other arachidonic acid metabolites may play a role in inflammation and tumor promotion (Spector, 1995).

Table 20 Inhibition effect of mango leaves extracts at different amounts on lipoxygenase activity.

Amount (mg)	Absorbance at 234 nm	lipoxygenase inhibitory activity (%)
0	0.491±0.005[a] l	0.00 a[b]
0.05	0.478±0.001 k	2.65±0.15 b
0.10	0.455±0.002 j	7.12±0.22 c
0.20	0.442±0.001 i	9.97±0.17 d
0.40	0.424±0.003 h	13.64±0.38 e
0.60	0.401±0.004 g	18.33±0.42 f
0.80	0.378±0.002 f	23.42±0.25 g
1.00	0.349±0.001 e	28.94±0.18 h
1.25	0.322±0.002 d	34.62±0.23 i
1.50	0.292±0.003 c	40.52±0.24 j
BHA (0.01)	0.232±0.004 b	52.74±0.49 k
BHT (0.01)	0.217±0.002 a	55.80±0.20 l
α-tocopherol (0.01)	0.325±0.005 d	33.98±0.61 i

[a] Mean±standard deviation from triplicate determinations.

[b] Different letters in the same column bear significant differences ($p<0.05$)

Lipoxygenase, an enzyme that specially induces oxygen into free fatty acid, contains iron within its molecular structure (Shimoni *et al.,* 1994). Since lipoxygenase requires a non heme iron in the active site, the enzyme activity could be inhibited by iron chelator (Theerakulkait and Barrett, 1995 ; Wang *et al.,* 1991). Bucar *et al.* (1998) reported that lipoxygenase inhibition by antioxidants is due to scavenging of similar radicals that are generated within the active site of the enzyme. Phenolic compounds are capable of indirectly inhibiting lipoxygenase activity by acting as free radical inactivators but also by reducing the iron in the active site of the enzyme to the catalytically inactive ferrous state (Decker, 1998 ; Stector, 1995). Flavan-3-ols in barley (+)-catechin and (-)-epicatechin, were found to be good inhibitor of lipoxygenase activity (Goupy *et al.,* 1999).

From our results, liquorice and mango leaves extract functioned as metal-chelator. Therefore, the inhibition of lipoxygenase by the extracts was possibly via interaction with a ferric ion at active site of lipoxygenase, leading to inactivation of enzyme. Thus, liquorice and mango leaves extracts may inhibit oxidation caused by lipoxygenase.

5. Antimicrobial activity of liquorice and mango leaves extracts.

5.1 Effect of liquorice and mango leaves extracts on growth of microorganism.

The antimicrobial effect of liquorice and mango leaves extract on growth of gram-positive bacteria (*S. aureus and B. cereus*) and gram-negative (*E. coli and P. fluorescens*) was determined. The diameters of inhibition zones of *S. aureus* were 9.8 and 11.5 mm around wells containing 75 µL of 300 and 500 ppm of liquorice extract, respectively whereas those of *B. cereus* were 9.2 and 12.9 mm, respectively (Table 21). Growth of *E. coli* and *P. fluorescen* was not inhibited by any concentrations of extract tested. The liquorice extract showed no inhibitory effect on growth of gram-negative bacteria at all concentrations tested.

Table 21 Effect of liquorice extract on growth of microorganism as determined by the disc diffusion method.

Organisms	Inhibition zone diameter (mm)		
	0 ppm	250 ppm	500 ppm
S. aureus	0.00	9.80 ± 0.05[a]	11.50 ± 0.16
B. cereus	0.00	9.20 ± 0.13	12.90 ± 0.18
E. coli	0.00	0.00	0.00
P. fluorescens	0.00	0.00	0.00

In general, gram-negative bacteria are not sensitive to be inhibited by spices (Shelef *et al.,* 1980). The antimicrobial activity of mango leaves extract on growth of two gram-positive bacteria and two gram-negative was determined (Table 22). Zone of inhibition of *S. aureus* were 7.2 and 9.0 mm around wells containing 75 µL of 350 and 500 ppm of mango leaves extract, respectively, whereas those of *B. cereus* were 6.0 and 7.0 mm (Table 22). The extract of mango leaves showed no inhibitory effect on growth of *E. coli* at all concentrations used. Methanol extracts of dry guava leaves showed a stronger inhibitory activity on growth of *S. aureus* (Jaiarj *et al.,* 1999). The leaves of *Tagetes minuta* showed an effective antibacterial activity against *S. aureus* (Terschuk *et al.*, 1997).

At the same concentration, the liquorice showed higher gram-positive bacteria inhibitory activity than mango leaves extract. However, mango leaves extract at 500 ppm inhibited the growth of *P. fluorescens* more effectively than liquorice extract, presumably due to the differences in structure of antimicrobial components in each extracts, and differences in strains characteristics.

Table 22 Effect of mango leaves extract on growth of microorganism as determined by the disc diffusion method.

Organisms	Inhibition zone diameter (mm)		
	0 ppm	250 ppm	500 ppm
S. aureus	0.00	7.20 ± 0.08[a]	9.00 ± 0.05
B. cereus	0.00	6.00± 0.10	7.00 ± 0.11
E. coli	0.00	0.00	0.00
P. fluorescens	0.00	0.00	2.50 ± 0.10

Chanthachum and Beuchat (1997) reported that the gram-negative bacteria (*E. coli* 0157: H7 or *Salmonella*) did not appear to be sensitive to kiam extract, whereas the gram-positive microorganisms were sensitive, suggesting that differences in sensitivity may be associated with cell wall structure or phenolic compound in kiam wood that may be responsible for the antimicrobial activity. Phenol and phenolic compounds which is active against gram-positive act by coagulating proteins, especially in the cell membrane (Alcamo, 1997; Martinez–Vazquez *et al.* (1999). In this study, we found that liquorice and mango leaves extracts contained phenolic compounds, which showed stronger inhibitory effect on growth of gram-positive bacteria than gram-negative bacteria. Gram-negative cell wall is a multilayered structure and quite complex, while the gram-positive cell wall consists of primarily a single type of molecule and is often much thinner (Brock and Modigan, 1996). As a consequence, gram-positive bacteria such as *B. cereus* and *S.aureus* were particularly sensitive to antimicrobials (Hefnawy *et al.*, 1993).

Mario *et al.* (1999) found that thyme oils had high percentage of phenolic compounds that are responsible for the marked antimicrobial activity, particularly against the gram-positive bacteria. Inhibitory activity of the essential oils against the individual microbial species is thought to be the result of damages to proteins in the membrane, without interference with the

phospholipid components that would load to irreversible damage to the integrity of the membranes. Furthermore, Rauha *et al.* (2000) reported that phenolic compound including garlic acid, protocatechuic acid, flavone, quercetin, naringenin, naringin, +(-) cathecin, morin and kaempferol showed inhibitory effect on growth of bacteria. The inhibition constants were strongly affected by structure of antimicrobial. Lavanduly-, geranly- and prenyl-substituted flavanones have been shown to possess more effective antibacterial activity against *S. aureus* than simple flavonon and flavonols (Tsuchiya *et al.*, 1996).

5.2 Minimum inhibition concentration (MIC) of liquorice and mango leaves extracts on growth of microorganism.

MIC of liquorice and mango leaves extract on some microorganisms is reported in Table 23. MIC was defined as the lowest concentration tested that showed total inhibition after 48 hrs of incubation. This table shows that extracts affected the growth of two gram-positive and two gram-negative bacteria differently. MIC of extract on two gram-positive bacteria was significantly lower than those for two gram-negative bacteria. The liquorice extract did not completely inhibit the growth of *E. coli* and *P. fluorescens*, though high concentration was used.

Table 23 Minimum inhibition concentration of liquorice and mango leaves extracts on microorganism.

Organisms	Minimum inhibition concentration (MIC) (ppm)	
	Liquorice	Mango leaves
S. aureus	60	60
B.cereus	30	60
E. coli	> 1000	> 1000
P. fluorescens	> 1000	250

MIC of mango leaves extract for gram-positive was lower than that for gram-negative ($p<0.05$). MIC for *S. aureus* and *B. cereus* was as low as 60 µg/mL. The extract did not inhibit growth of *E. coli*, while completely inhibited growth of *P. fluorescens* at 250 µg/mL. Therefore, mango leaves showed more effective antibacterial activity against *P. fluorescens* than liquorice extract.

The crude leaves extract of *Acalypha wilkeniana* inhibited the growth of *S. aureus*, with MIC of 0.25 mg/mL (Alade and Irabi, 1993). Kang *et al.* (1992) found that the green leaves extract of *Perilla frutescens* exhibited broad spectrum activity in the range of 125-1000 µg/mL for both gram-positive and gram-negative. The MIC of leaves extract on *P. fluorescens* were 125 and 1000 µg/mL for *S. aureus* and *E. coli*, respectively.

Thus, the reduction of microbial population depended on the concentration and structure of antimicrobial components in each extract, and susceptibility of strain to active components in the extract.

6. Application of liquorice and mango leaves extract in ground fish

6.1 liquorice extracts.

Microbiological analyses

Total psychotrophic counts of raw ground fish treated with different concentrations of liquorice extract, BHT and α-tocopherol are shown in Figure 32. All treatments showed a logarithmic increase in total psychotrophic counts during storage at 4 $^{\circ}$C when the storage time increased. Sample treated with liquorice extract and other antioxidants had significantly lower total psychotrophic counts than the control ($p<0.05$).

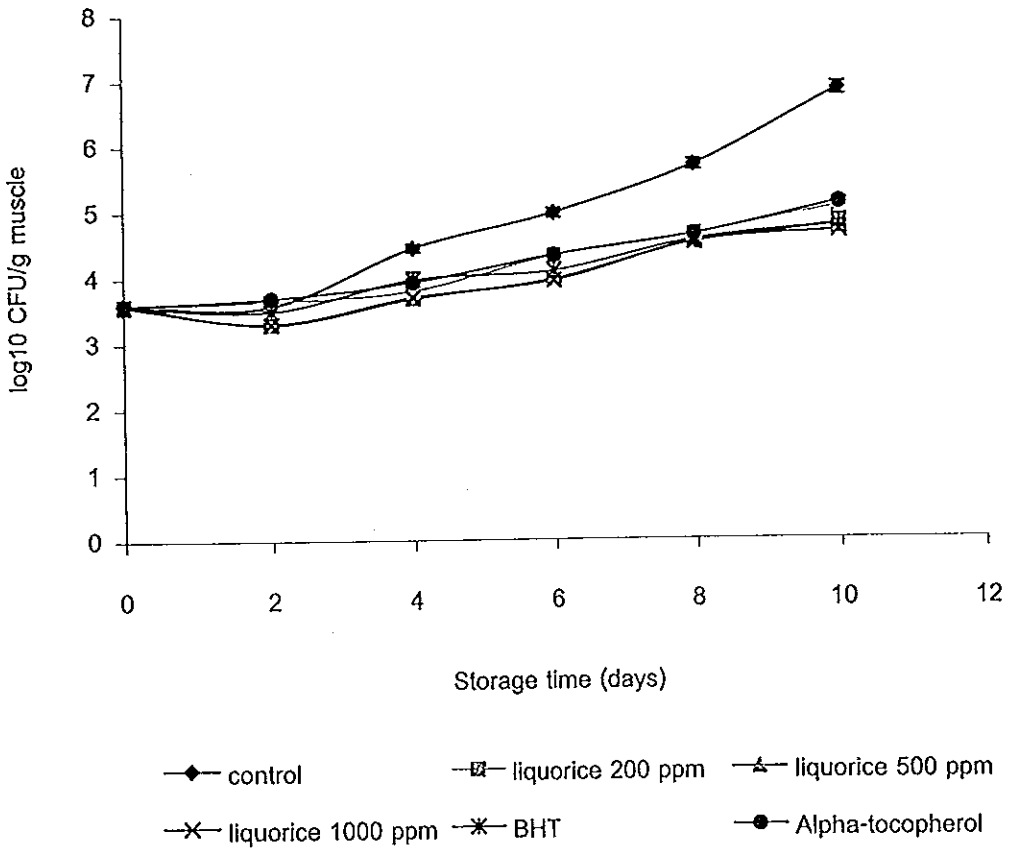


Figure 32 Changes in total psychotrophic counts of raw ground fish treated with liquorice extract at different concentrations during storage at 4° C for 10 days.

No significant differences in total psychotrophic counts were observed between sample treated with the extracts at 500 or 1000 ppm and BHT at 200 ppm. However, sample with α-tocopherol at 200 ppm had a slightly higher total psychotrophic counts.

The data obtained showed that liquorice extract at higher concentration (500 or 1000 ppm) retarded the growth rate of psychotrophic bacteria in raw ground fish during storage at 4°C for 10 days. Wou *et al.* (1998) reported that the extracts from ginger were effective in inhibiting growth of bacteria. However, Hettiarachchy *et al.* (1996) found that the fenugreek extract did not influence psychotrophic bacteria counts in raw patties stored at 4°C for 9 days.

Chemical analyses

TMA and TVB of raw ground fish treated with different concentratrions of liquorice extract (200 500 and 1000 ppm), BHT (200 ppm) and α-tocopherol (200 ppm) are depicted in Figure 33 and Figure 34. In this trial, TMA level of all treatments were constant during the first 4 days of storage. Generally, TMA of the control increased sharply after 4 days. The result coincided with TVB changes. The increase in TMA and TVB level may be due to bacteria and enzymatic action (Ben-gigirey *et al.,* 1999 ; Hernandez-Herrero, 1999). All samples treated with liquorice extract, BHT and α-tocopherol had significantly lower TMA and TVB than control ($p<0.05$). This was possibly due to the inhibition of bacterial growth caused by the active components in the extract, leading to lower TMA and TVB.

No significant differences in TMA and TVB between treatment with liquorice extract at 500 or 1000 ppm and BHT 200 ppm were observed during 10 days storage ($p>0.05$). Sample treated with the extract had lower TMA and TVB values, compared to that with α-tocopherol at 200 ppm. These results showed that liquorice extract retarded the formation of TMA and TVB in raw ground fish during storage. These reductions tended to correlate with the retardation of total psychotrophic counts. Both TMA and TVB are chemical indicator of microbial spoilage of raw ground fish. TMA is mainly generated post mortem by bacteria reduction of trimethylamine oxide and by endogenous enzymes (Perez-Villareal and Pozo, 1990). Levels of 10-15 mg TMA-N 100 g are usually regarded as the limit beyond which whole chilled fish can be considered too spoiled for most use (Connell, 1980).

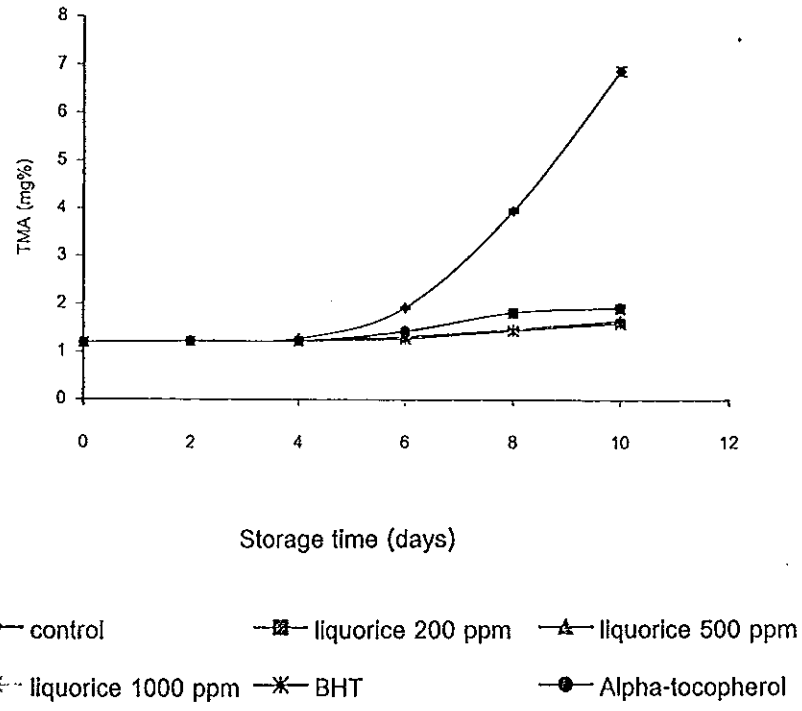


Figure 33 Changes in TMA of raw ground fish treated with liquorice extract at different concentrations during storage at 4°C for 10 days.

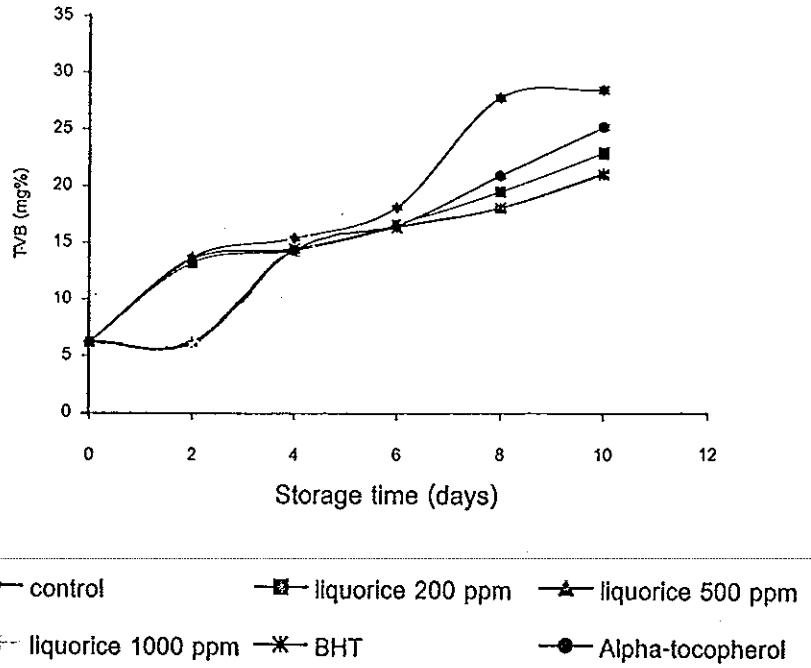


Figure 34 Changes in TVB of raw ground fish treated with liquorice extract at different concentrations during storage at 4°C for 10 days.

Lipid oxidation is another important aspect of fresh fish spoilage by causing off-flavors. TMA reacts with lipids in the fish muscle to produce the characteristic "fishy" ordor of low quality fish (Ashie *et al.*, 1996). Raw ground fish treated with different concentrations of liquorice extracts, BHT and α-tocopherol had significantly lower TBARS than the control ($p<0.05$)(Figure 35).

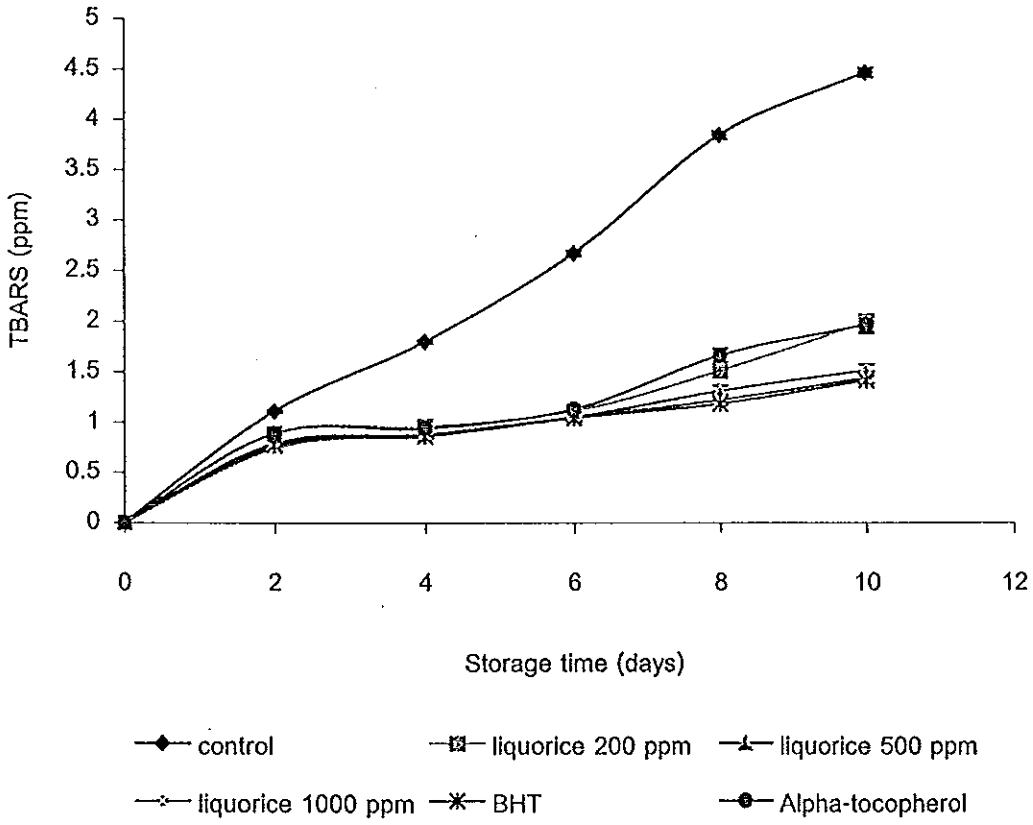


Figure 35 Change in TBARS of raw ground fish treated with liquorice extract at different concentrations during storage at 4°C for 10 day.

After 15 days of storage at 4°C, sample treated with extract at 1000 ppm showed the lower TBARS than the sample added with α-tocopherol but not significantly different ($p>0.05$) when compared to the sample treated with BHT at 200 ppm. The data obtained showed that the liquorice extract at high concentration (1000 ppm) inhibited oxidation in raw ground fish effectively during storage at 4°C for 10 days. Ramanathan and Das (1993) found that polyphenols had a concentration-dependent effect on inhibition of lipid oxidation. Das and Pereria (1990) ; Ramanathan and Das (1992 ; 1993) reported that several naturally occurring plant polyphenols including flavonoids, tannic acid and ellagic acid have been shown to have potent antioxidative properties in a variety of biological system. These results indicated that liquorice extract at high concentration (1000 ppm) retarded the increase in psychotrophic counts, TMA, TVB as well as lipid oxidation in raw ground fish during storage at 4°C. Chen and Gan (2000) reported that galangal extract at high concentration (0.05 or 0.10 %) inhibited lipid oxidation and microbial growth in raw minced meat during storage at 4 °C for 7 days.

6.2 Mango leaves extract.

Microbiological analyses.

Microbiological counts of raw ground fish treated with different concentrations of mango leaves extract, BHT and α-tocopherol are presented in Figure 36. All samples treated with extracts, BHT and α-tocopherol had significantly lower total psychotrophic counts than the control ($p<0.05$). Samples treated with the extract at levels of 200, 500 or 1000 ppm had significantly lower total psychotrophic counts than those treated with α-tocopherol ($p<0.05$). No significant differences in total psychotrophic counts were observed between the treatment with mango leaves extract at 200 ppm and BHT 200 ppm at day 10 of storage at 4°C.

The data obtained revealed that mango leaves extracts effectively inhibited the growth of psychotrophic bacteria in raw ground fish stored at 4°C

for 10 days. Generally, mango leaves extracts at 200, 500 and 1000 ppm were more active than α-tocopherol at 200 ppm.

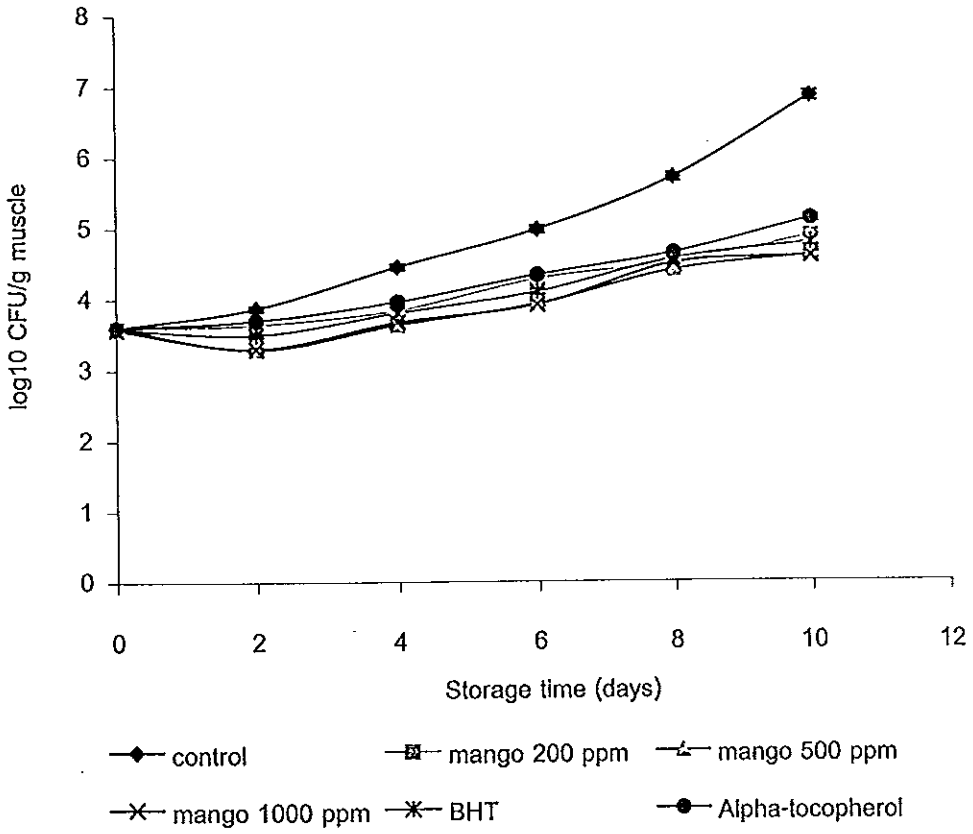


Figure 36 Changes in total psychotrophic counts of raw ground fish with mango leaves extract at different concentrations during storage at 4°C for 10 days.

Chemical analyses.

The results of TMA and TVB of raw ground fish treated with different concentrations of mango leaves extract, BHT and α-tocopherol are shown in Figure 37 and 38. All treatments added with the extract and other antioxidants

had significantly lower TMA and TVB value than the control ($p<0.05$). After 10 days of storage, treatment with mango leaves extracts at 500 or 1000 ppm showed the lower TMA and TVB value than that with α-tocopherol but not significantly different ($p>0.05$) when compared to the treatment with BHT 200 ppm.

The results indicated that mango leaves extracts effectively inhibited growth of spoilage microorganisms in raw ground fish during storage at 4°C for 10 days.

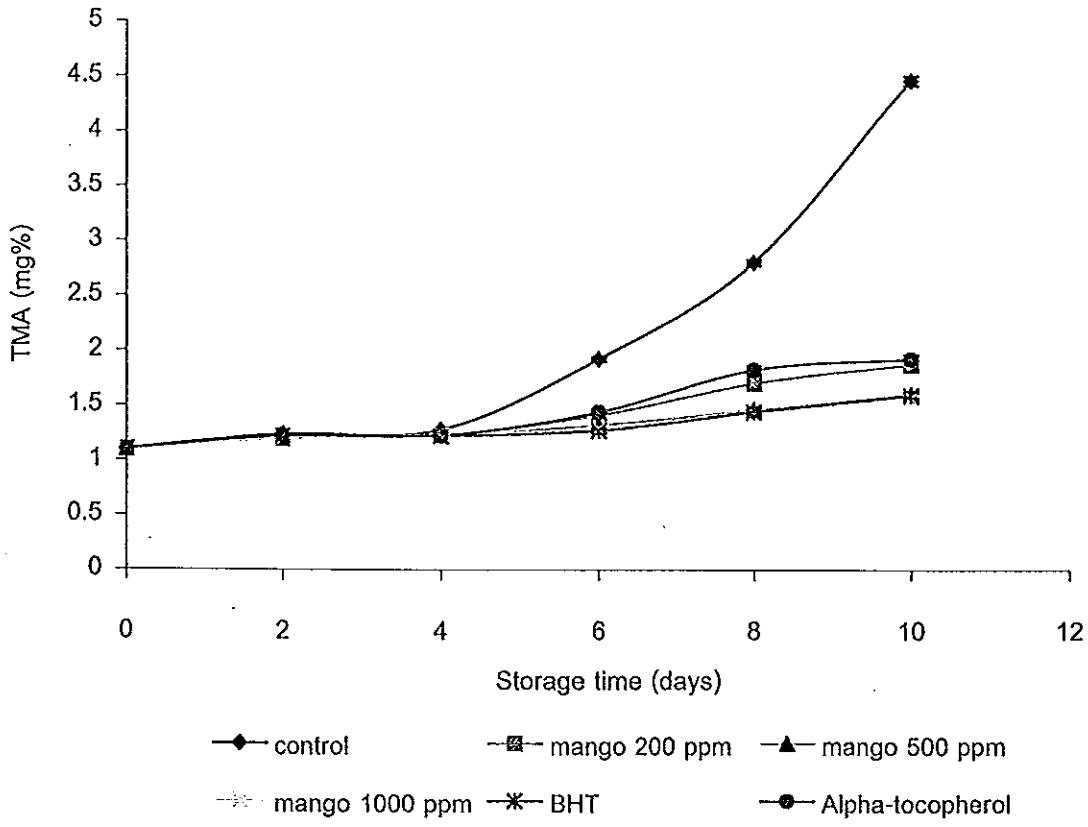


Figure 37 Changes in TMA of raw ground fish treated with mango leaves extract at different concentrations during storage at 4°C for 10 days.

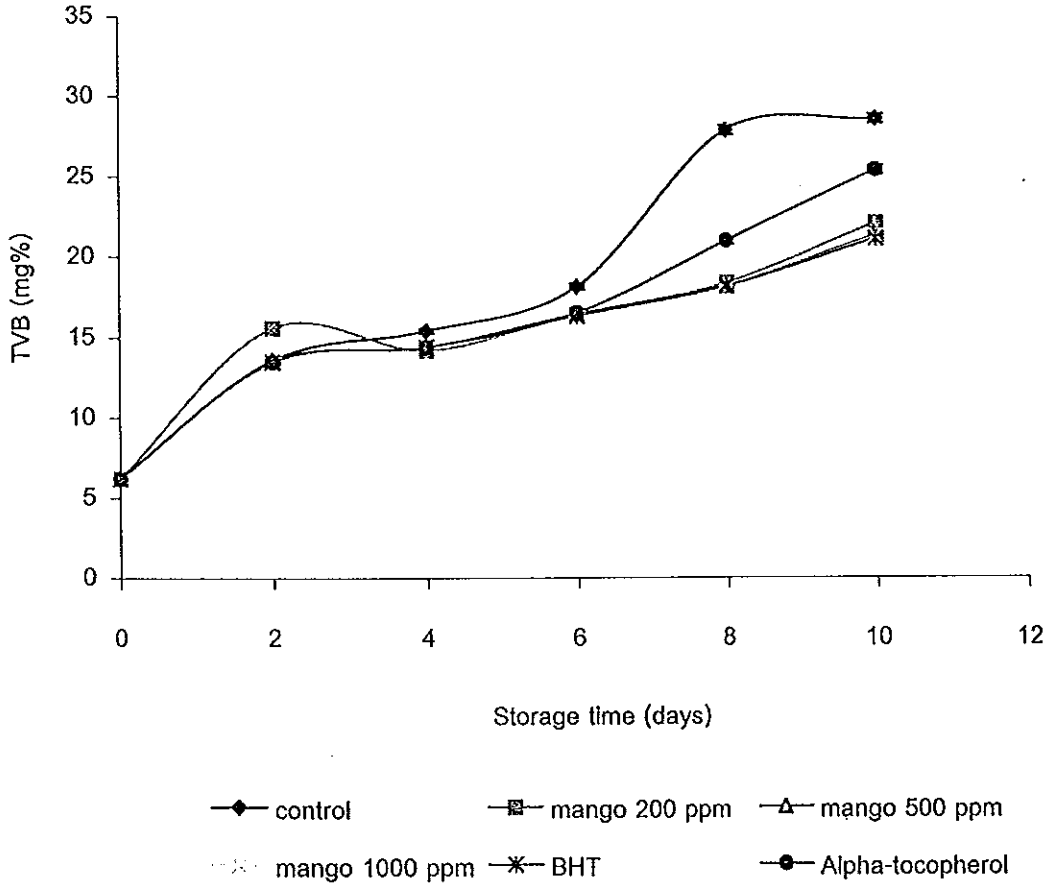


Figure 38 Changes in TVB of raw ground fish treated with mango leaves extract at different concentrations during storage at 4°C for 10 days.

TBARS of raw ground fish treated with different concentrations of mango leaves extract, BHT and α-tocopherol is shown in Figure 39. The TBARS of all samples treated with extracts, BHT and α-tocopherol were significantly lower than the control ($p<0.05$). No significant differences in TBARS between sample treated with the extracts 500 or 1000 ppm and BHT 200 ppm were observed. Moreover, treatment with mango leaves extract at 200 ppm had significantly lower TBARS value than the treatment with α-tocopherol at 200 ppm. The data indicated that mango leaves extract could inhibit lipid oxidation in fish muscle. The leaves of rosemary and sage were reported to inhibit lipid oxidation in beef hamburgers (Pizzacaro *et al.*, 1994).

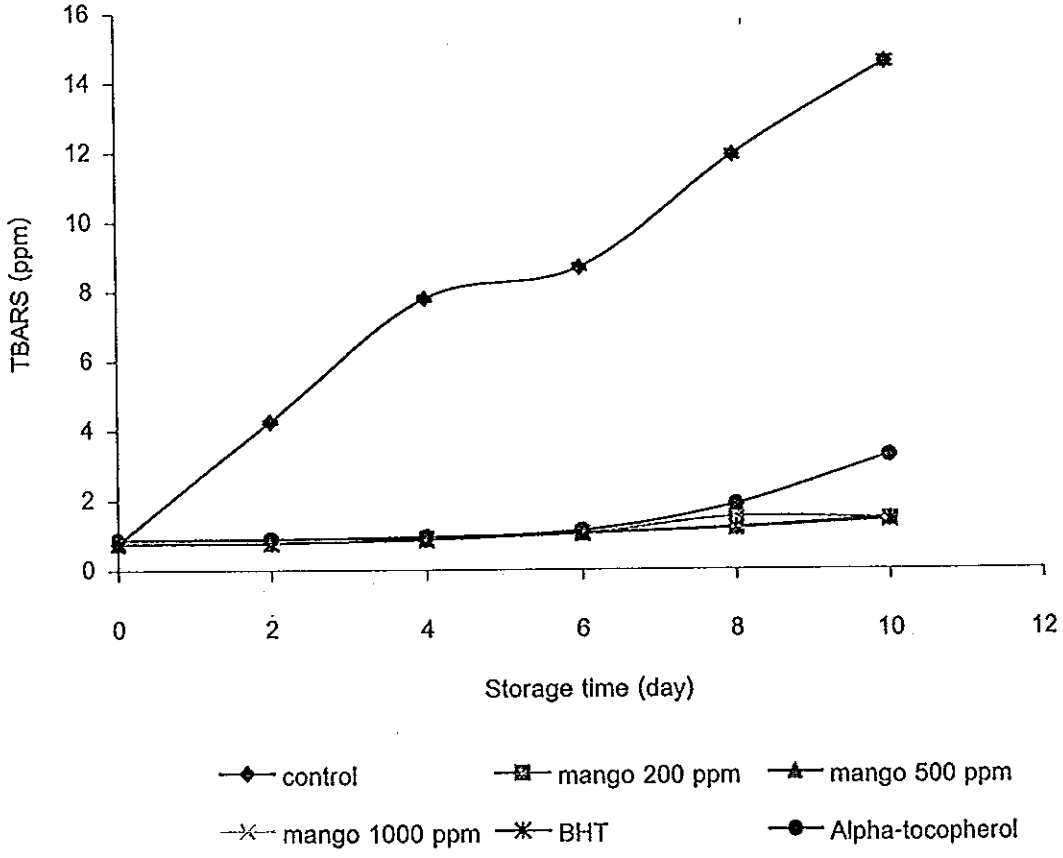


Figure 39 Changes in TBARS of raw ground fish treated with mango leaves extract at different concentrations during storage at 4°C for 10 days.

From our results, the mango leaves extract effectively inhibited lipid oxidation and showed inhibitory effects on growth of spoilage microorganisms in raw ground fish. Benjakul *et al.* (1997) reported that payorm wood extract showed effectively inhibited lipid oxidation in yellow-striped crevalle fillets during ice storage and showed slight effect on TVB, TMA and microbial inhibition. Polyphenolic compounds which occur naturally in plants are known to have antioxidative and antimicrobial properties (Nakatani, 1994 ; Vuotto *et al.* 2000).

Generally, mango leaves extract exhibited much higher antioxidant and antimicrobial activity in raw ground fish, compared to liquorice extract. The results indicated that mango leaves extract may be useful in inhibiting lipid oxidation and increasing microbial stability of raw ground fish. However, toxicological properties and safety of mango leaves extract must be studied prior to their approval for food use.

7. Separation of antioxidants from liquorice and mango leaves extracts.

Antioxidant compounds in liquorice extract were separated by silica gel column chromatography. Seven fractions (I, II, III, IV, V, VI, and VII) were obtained. The antioxidant activities of the seven isolated fraction and crude extract are presented in Figure 40. Fraction VI from liquorice extracts, which is a methanol / dichloromethane (6 : 4) eluate, showed the highest antioxidative activity. Amount of phenolic compounds is not the only factor when considering the antioxidative activity. Their molecular structure also plays an important role in their antioxidant activity (Zadernowski *et al.*, 1991). Fraction VI was further used for identification of antioxidant components.

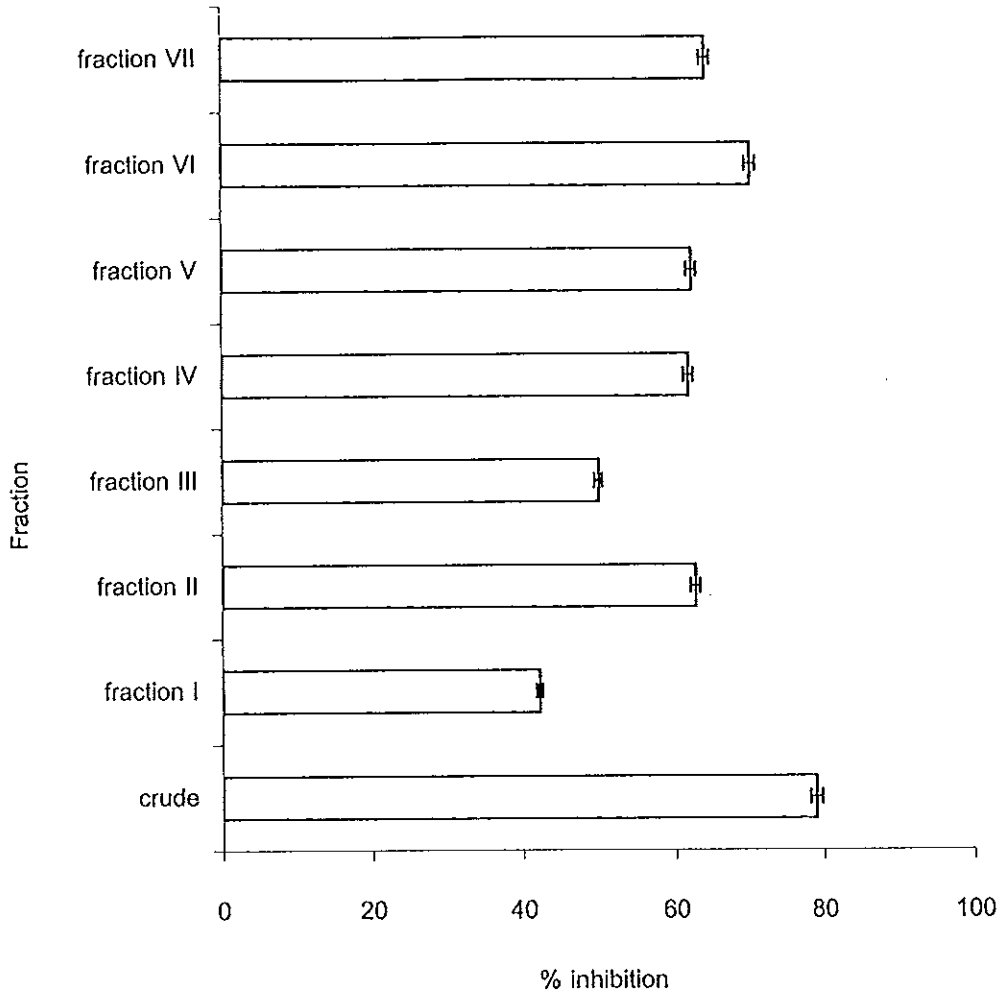


Figure 40 Antioxidant activity of individual fractions of liquorice extract separated using column chromatography.

Fraction VI was subjected to Thin-layer chromatography and different sprays were applied to tentatively identify the types of compounds in fraction VI of liquorice extract (Table 24). These sprays revealed three bands with Rf values of 0.80 (band A), 0.64 (band B) and 0.40 (band C) (Figure 41). Spray 1 revealed the presence of phenolic compound in all three bands found in fraction VI. Free ortho- and para- hydroxy groups were not present in the compounds separated as indicated by spray 2. The natural antioxidant extracts are primarily plant polyphenolic compounds that may occur in several parts of the plant (Shahidi and Wanasundara, 1992 ; Gamez *et al.*, 1988). Reducing compounds were identified by appearance of a brown color after spraying with an ammonical silver nitrate solution (spray 3).

Table 24 Tentative identification of antioxidant compounds in liquorice extract.

Sprays	Band[a]	Color	Compound
$FeCl_3$-$K_3Fe(CN)_6$	A, B, C	Blue	Phenolic
$FeCl_3$	A, B, C	Brown	Phenolic without di-, tri-hydroxy group
NH_4OH-$AgNO_3$	A, B, C	Brown	Reducing compounds

[a] The bands corresponded to positive identification of compounds at Rf values of 0.8 (band A), 0.64 (band B) and 0.4 (band C).

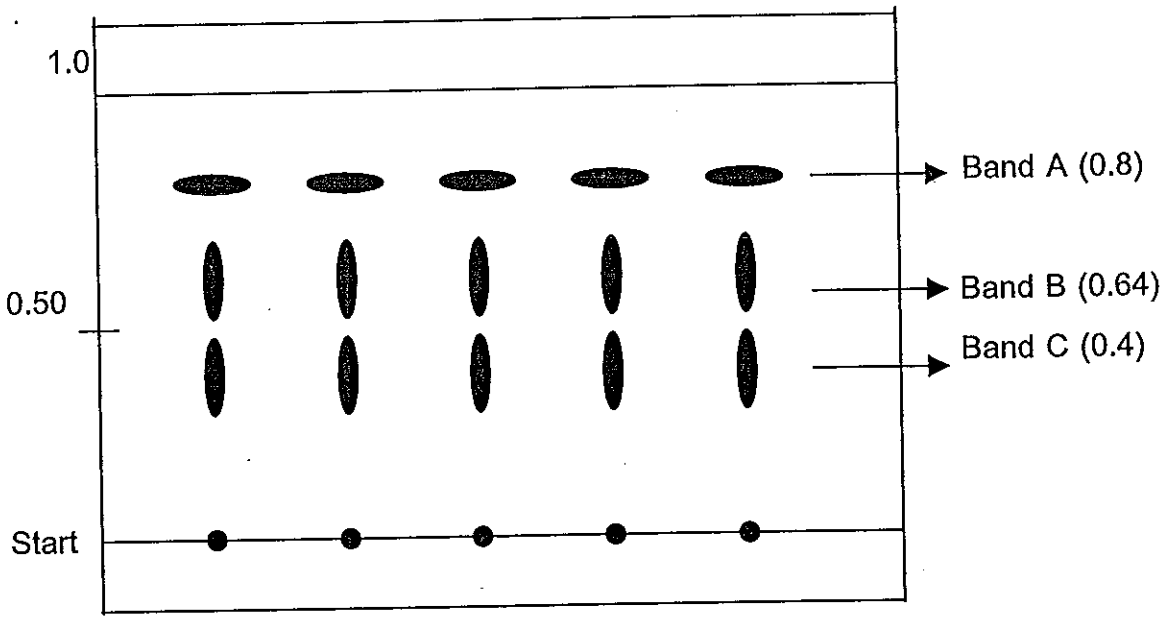


Figure 41 TLC chromatograms of fraction VI of liquorice extracts separated using column chromatography.

The mango leaves extract was separated by silica gel column chromatography. Nine fractions (I, II, III, IV, V, VI, VII, VIII and IX) were observed. The antioxidant activities of the nine isolated fraction and crude extract are presented in Figure 42. Fraction VIII from mango leaves extract, which is a methanol-dichloromethane (7.5 : 2.5) eluate, showed the highest antioxidative activity. Therefore, fraction VIII, which had the highest antioxidative activity, may contain several kinds of antioxidative compounds. Fraction VIII was further identified.

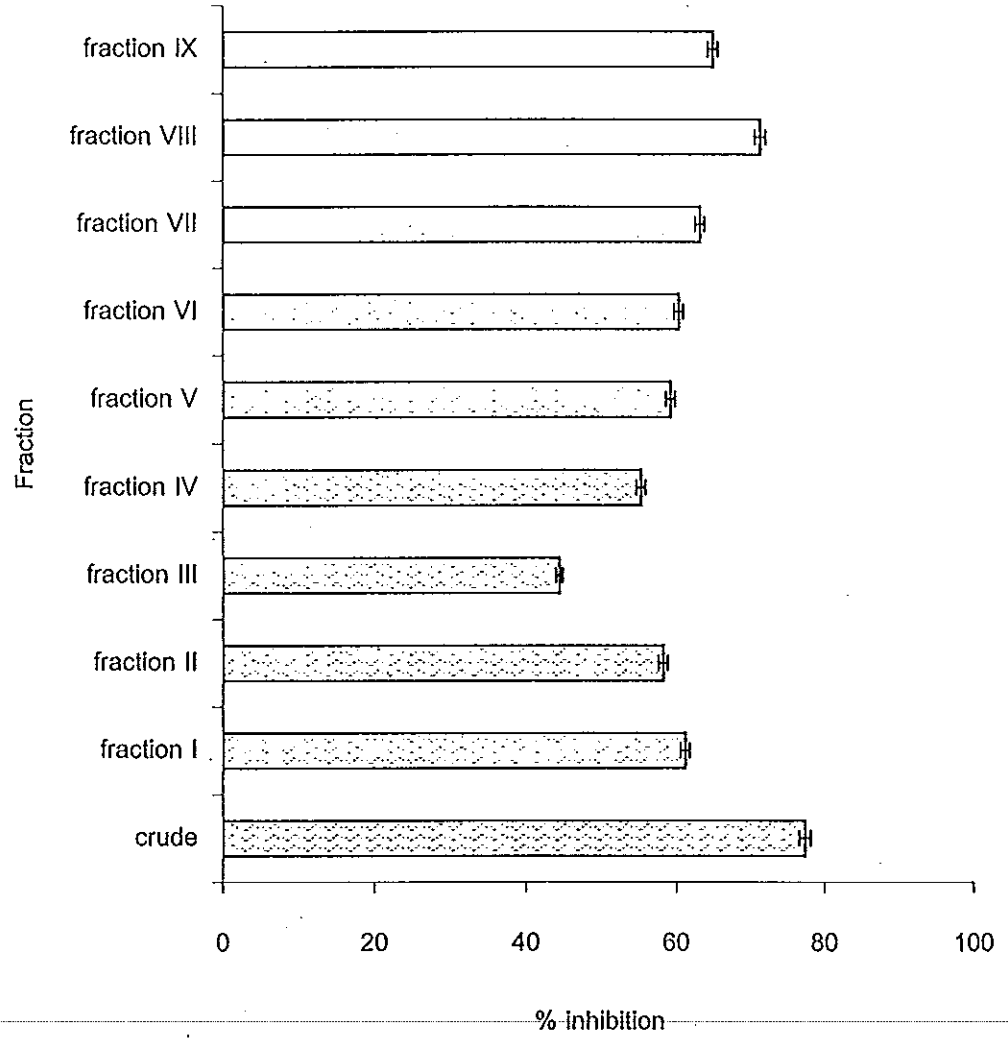


Figure 42 Antioxidant activity of individual fractions of mango leaves extract separated using column chromatography.

From the results, all isolated fractions showed a lower antioxidant activity than crude extract. Possible synergism of phenolics with one another or with other components present in the crude extract may be responsible for this observation. The crude canola extract exhibited a higher antioxidative activity than all isolated fractions in β-carotene-linoleate model system (Wanasundara *et al.,* 1996 ; Wanasundara *et al.,* 1994). Futhermore, pure compound isolated from young green barlay leaves showed less activity than the crude extract (Osawa *et al.,* 1992). However, pure compounds isolated from susabinori exhibited strong antioxidant activity as α-tocopherol at same concentration (Nakayama *et al.,* 1999). Wang *et al.* (1999) found that pure compounds isolated from tart cherries showed a higher antioxidant activity than BHT and TBHQ.

TLC was used to separate the antioxidant compounds in fraction VIII of mango leaves extracts and the types of compounds were identified using different sprays (Table 25). These sprays revealed three bands with Rf values of 0.65 (band A) 0.43 (band B) and 0.15 (band C) (Figure 43). All these bands were positive for phenolic compounds when sprayed which ferric chloride potassium ferricyanide reagent. Spray 2 indicated phenolic compounds without free ortho- and para- hydroxy groups. Reducing compounds were identified by the appearance of a black color with an ammonical silver nitrate solution (Spray 3).

Table 25 Tentative identification of antioxidant compounds in mango leaves extract.

Sprays	Band[a]	Color	Compound identified
$FeCl_3$-$K_3Fe(CN)_6$	A, B, C	Blue	Phenolic
$FeCl_3$	A, B, C	Brown	Phenolic without di-,tri-hydroxy group
NH_4OH-$AgNO_3$	A, B, C	Black	Reducing compounds

[a] The bands corresponded to positive identification of compounds at Rf values of 0.65 band A), 0.43 (band B) and 0.15 (band C).

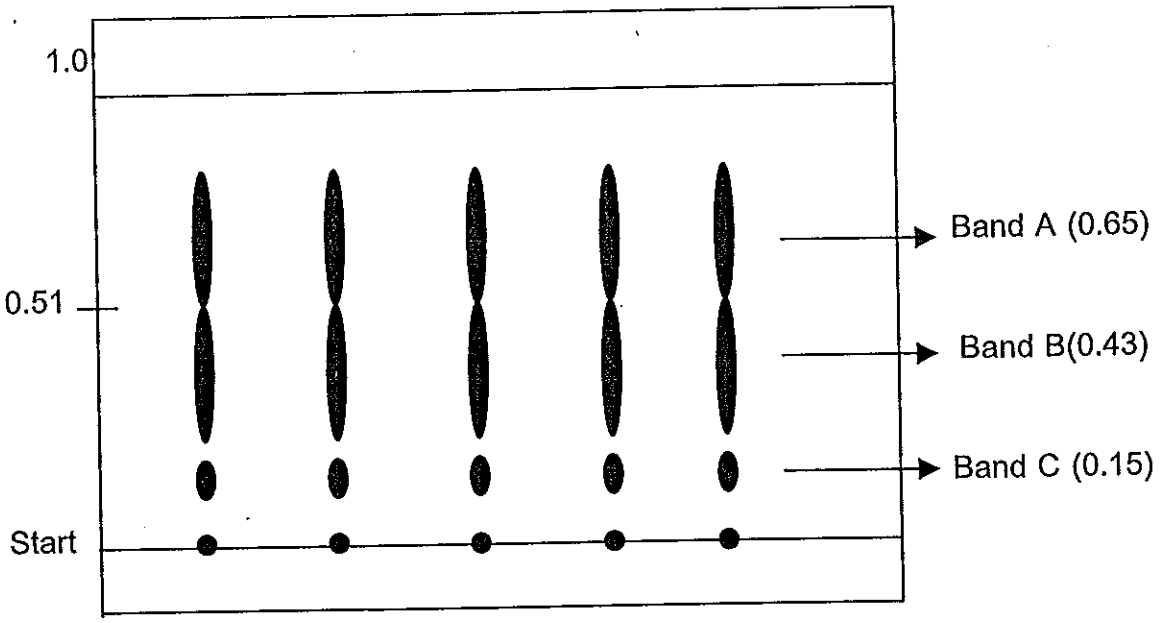


Figure 43 TLC chromatograms of fraction VIII of mango leave extract separated using column chromatography.

Chapter 4

Conclusions

1. Among 8 different cultivars of herbs and 9 cultivars of green leaves, liquorice and mango leaves showed the highest antioxidant activity in lecithin liposome system.
2. The methanol extract contained the highest total phenolic content and exhibited the strongest antioxidant activity in lecithin liposome system, followed by ethanol, ethyl acetate and hexane extracts.
3. The optimum condition for preparing both liquorice and mango leaves extract involved extracting liquorice and mango leaves powder with 80% methanol for 5 hrs.
4. Both extracts from liquorice and mango leaves had good thermal and pH stability.
5. Liquorice and mango leaves extracts exhibited higher antioxidant activity at neutral and alkaline pHs than acidic pHs.
6. Synergistic action of liquorice and mango leaves extracts with α-tocopherol in lecithin liposome system was obtained. However, no synergism was observed when the both extracts were used in combination with ascorbic acid and citric acid.
7. Antioxidant activities of liquorice and mango extracts increased with an increasing concentration of the extracts.
8. Liquorice and mango leaves extracts possessed scavenging activity toward DPPH radical and hydroxyl radical in a concentration-dependent manner.
9. Liquorice and mango leaves extracts acted as Fe^{2+} and Cu^{2+} chelator in a concentration-dependent manner.
10. The extracts of liquorice and mango leaves exhibited lipoxygenase inhibitor in a concentration-dependent manner.

11. The liquorice and mango leaves extracts showed higher inhibitory effect on growth of gram-positive bacteria than gram-negative bacteria.

12. Liquorice and mango leaves extracts retarded the increase in psychotrophic count, TMA, TVB and lipid oxidation in raw ground fish during storage at $4^{\circ}C$ for 10 days.

13. Antioxidant components of liquorice and mango leaves were tentatively identified as phenolic compounds without free ortho- and para- hydroxy groups, which were reducing compounds.

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Appendix 1

Analytical methods

1. Determination of total phenolic content (Julkunen-Titto, 1985)

Chemicals

- Folin and ciocalteu' s phenolic reagent
- Saturated sodium carbonate solution

Method

A certain amount (0.5 mL) of crude extract was diluted with distilled water to 5 mL. One milliliter of Folin-ciocalteu phenol reagent was added and shaken vigorously. After 5 min, 1 mL of saturated sodium bicarbonate solution was added and the color was allowed to develop for 1 hr. The absorbance was read at 640 nm. A standard curve was prepared at the same time by using various concentration of chlorogenic acid ranging from 0 to 100 µg/mL. Total phenolic content was calculated as chlorogenic acid equivalent by using standard curve.

2. Determination of reducing power (Oyaizu, 1986)

Chemicals

- 0.2 M Phosphate buffer, pH 6.6
- 1% Potassium ferricyanide
- 10% trichloroacetic acid (TCA)
- 0.1% ferric chloride

Method

The crude extracts (0.5 mL) was mixed with phosphate buffer (2.5 mL, 2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50 °C for 20 min. An aliquot of TCA (2.5 mL, 10%) was added to the mixture. The mixture was then centrifuged at 650xg f or 10 min. The

supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 0.1%) and the absorbance was read spectrophotometrically at 700 nm. Higher absorbance of the mixture indicated greater reducing power.

3. Determination of scavenging effect on 1,1-diphenyl-2-picrylhydracyl (DPPH) radical (Hatano *et al.,* 1988; Chen and Ho, 1997)

Chemical

- DPPH in methanol (0.2 mM)

Method

The extract (4.0 mL) was added to a DPPH solution (1 mL). The reaction mixture was shaken vigrously and incubated for 30 min at room temperature. The absorbance of the resulting solution was measured at 517 nm. All experiments were carried out in tripicate.

4. Determination of scavenging effect on hydroxyl radical (Aruoma, 1994)

Chemicals

- 2-deoxyribose
- 0.1 M sodium phosphate
- Stock solution [1% (w/v) TBA in 50 mM sodium hydroxy plus 2.8% (w/v) TCA]

Method

The reaction mixture (pH 7.4) containing 0.1 M phosphate buffered saline, 7 mM deoxyribose, 100 µM ascorbic acid, 10 µM ferric ions (1mL) was mixed with 0.1 mL of antioxidant extract. The mixture was incubated for 10 min at 37 ^{o}C. One mL stock solution was added to the reaction mixture. It was then heated for 10 min in the boiling water bath, cooled with tap water, and the absorbance of the pink chomogen was read at 532 nm.

5. Measurement of chelating activity on metal ions (Shimada *et al.*, 1992)

Chemicals

- 1 mM tetramethylmurexide
- 10 mM hexamine buffer containing 10 mM of potassium chloride and 3 mM either ferrous sulphate or copper sulphate.

Method

The extract (2.0 mL) was added to hexamine buffer (2.0 mL) and then tetramethylmurexide (0.2 mL) was added. The absorbance was measured at 234 nm. The reaction was carried out at 20 ^{o}C to prevent the oxidation of $Fe.^{2+}$

6. Determination of inhibition of lipoxygenase activity (Surrey, 1964)

Chemicals

- Enzyme solution: 1.2 mg of lipoxygenase was dissolved in 20 mL of 0.2 M borate buffrer (pH 9.0)
- Substrate solution; 0.05 mL of Tween 20 and 0.05 mL of linoleic acid in 10 mL of 0.2 M borate buffer (pH 9.0) were sonicated.

Method

The extract (0.2 mL) was added to substrate solution (8.0 mL), 0.5 mL of enzyme solution was added. The reaction mixture was shaken at room temperature for 15 min. Aliquot (1 mL) from these reaction mixture was transferred to test tube containing ethanol (2 mL), then 60% ethanol (7 mL) was added to make a total volume of 10 mL. The absorbance was measured at 234 nm.

7. Determination of antimicrobial activity by disc diffusion method (Ming *et al.*, 1997)

Microorganisms and culture media

- Soft agar : tryptic soy broth containing 0.75% agar
- Nutrient agar containing (1mg/L) : beef extract 3 g, peptone 5 g, peptone 5 g and agar 15 g.
- Fresh culture of each bacteria were prepared using tryptic soy broth and incubated at 37 °C for 24 hrs.

Method

The lawn was made by overlaying NA prepared plate with 7.5 mL of solft agar containing 0.5 mL of bacteria (about 10^5 CFU/mL). After 30 min at room temperature, sterile discs were placed on the agar surface. 75 µL of solution, prepared from liquorice and mango leaves extract was applied on the disc. Control discs contained only ethanol. These plates were incubated at 37°C overnight. After 24 hrs of incubation, the diameters of the inhibition zones were measured. A reading of more than control indicated growth inhibition.

8. Determination of control minimum inhibition concentration (MIC) (Brock and Madigan, 1991)

Microorganisms and culture media

- Sterilized tryptic soy broth
- Fresh culture of each bacteria were prepared using tryptic soy broth and incubation at 37 °C for 24 hrs.

Method

The extracts were dissolved in ethanol to obtain different concentrations. 30 µL of each extract was added to 3 mL of bacteria broth (about 10^5 CFU/mL). The tubes were incubated for 48 hrs at 37 °C. Growth after 48 hrs was decided as an increase in turbidity read at 660 nm. Initial concentrations of test extracts were varied between 100 and 1,000 ppm with

an increment of 100 ppm. When a test microorganism did not grow at 100 ppm, concentrations were varied between 10 and 100 ppm with 10 ppm increments. If no growth occurred at 10 ppm, concentrations were varied between 1 and 10 ppm with 1 ppm increments. The MIC was determined as the lowest concentration with no growth observed.

9. Determination of thiobarbituric acid reactive substances (TBARS) (Siu and Draper, 1978)

Chemicals

- 10% trichloroacetic acid (TCA)
- Thiobarbituric acid (TBA, 0.06 M)

Method

Ground fish (5 g) was homogenized in 12.5 mL of distilled water for 30 sec. At speed 2. TCA (12.5 mL) was then added to the homogenate, and the mixture was filtered. One milliliter of TBA was added to 4 mL aliquots of the filtrate which were then heated in a boiling water bath (10 min) for color development. The tubes were then cooled and the absorbance was read at 532 nm. The quantity of TBARS in each sample was calculated as mg MDA/kg sample using standard curve.

10. Determination of TMA-N and TVB-N by Conway's method (Ng, 1987)

Chemicals

- Inner ring solution -1% boric solution indicator: Take 10 g of boric in 1 litre flask, add 200 ml of ethanol. After dissolving boric acid, 10 mL of mixed indicator solution was added and then made up to 1 litre with distilled water.
- Mixed indicator solution: Bromocresol green (BCG) (0.01g) and methyl red (MR) (0.02 g) were dissolved in 10 mL of ethanol.
- 0.02 N HCl : Diluted 20 ml of 1 N HCl standard solution with distilled water and make up to 1,000 mL.

- Saturated K_2CO_3 solution : Take 60 g of potassium carbonate (K_2CO_3), and add 50 mL of distilled water. Boil gently for 10 min. After cooling down, obtain filtrate through filter paper.
- 50% K_2CO_3 solution twice with distilled water.
- 4% trichloroacetic acid (CCl_3COOH)(TCA) solution : Dissolved 40 g of TCA in 960 mL of distilled water.
- Neutralized 10% formaldehyde solution: Add 10 g $MgCO_3$ to 100 mL of formalin (35% formaldehyde solution) and shake in order to neutralized the acidity of formalin. Filter and diluted filtrate 3 times with distilled water.
- 1% $TiCl_3$ aqueous solution : Take 6.7 mL of 15% $TiCl_3$ solution into 100 ml with distilled water.
- Saturated KNO_3 aqueous solution: Dissolved about 55 g of KNO_3 in 50 mL of distilled water.

Sample extraction

Ground fish 2 g was homogenized in 8 mL of 4% TCA for 1 min at speed 2. The Homogenate was stood for 30 min and centrifuged at 3,000 rpm for 10 min. The supernatant was separated and stored at -20 °C until analyzed.

10.1 Determination of TVB-N

Apply sealing agent to Conway's unit, 1mL inner ring solution was added into inner ring and 1 mL sample extract was added into outer ring. The Conway's unit with cover was slanted. The saturated K_2CO_3 was added into outer ring at 1 mL. The unit was closed and mixed gently. The sample was incubated at 37 °C for 60 min and then titrated inner ring solution with 0.02 N HCl using micropipette until green color turned to pink. Blank was prepared by using 1 mL 4% TCA instead of sample extract.

10.2 Determination of TMA-N

Apply sealing agent to conway's unit, 1 mL inner ring solution was added into inner ring and 1 mL sample extract was added into outer ring. A 1

mL, neutralized 10% formaldehyde was added into outer ring. Conway's unit with cover was slanted, then 1 mL saturated K_2CO_3 was added to outer ring. The unit was closed and mixed gently. The sample was incubated at 37 °C for 60 min, titrated the inner ring solution with 0.02 N HCl until green color turned to pink. Blank was run using 1 mL of 4% TCA instead of sample extract.

11.Psychrotropic micrrorganisms analysis (Gilliland *et al.,* 1984)

Chemicals and media

- 0.85% normal saline
- plate count agar

Method

Fish sample (10 g) were removed aseptically and placed into plastic bags. Sterile saline solution (99 mL) was added, and samples were placed for 1 min in a stomacher blender. Decimal dilutions (10^{-1}, 10^{-2} and 10^{-3}) of sample suspensions was done using 9 mL of sterile saline. Plate count agar was poured into petri dishes containing the decimal dilutions. The medium in petri dishes was allowed to solidify and incubated at 10 °C for 7 day. Numbers of colony forming units (CFU) were counted and multiplied by dilution factor to determined CFU/g of sample.

Appendix 2

Analysis of variance

Table 1-A. Analysis of variance for antioxidant activity of 8 different varieties of herb in inhibition of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	7	45088.21	6441.173	7110.771**
Error	16	14.493	0.906	
Total	23	45102.71		

** = Significant at 1 % level

Table 2-A. Analysis of variance for antioxidant activity of 8 different varieties of herb in inhibition of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	7	40039.60	5719.943	9837.871**
Error	16	9.303	0.581	
Total	23	40048.90		

** = Significant at 1 % level

Table 3-A. Analysis of variance for antioxidant activity of 8 different varieties of herb in inhibition of oxidantion of linoleic acid/β-carotene system.

SV	DF	SS	MS	F
Treat	7	10276.73	1468.104	39.020**
Error	16	601.989	37.624	
Total	23	10878.72		

** = Significant at 1 % level

Table 4-A. Analysis of variance for total phenolic content 8 varieties of herb.

SV	DF	SS	MS	F
Treat	7	6145.254	877.893	7610.693**
Error	16	1.846	0.115	
Total	23	6147.100		

** = Significant at 1 % level

Table 5-A. Analysis of variance for antioxidant activity of 9 different varieties of green leaves in inhibition of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	8	31007.47	3875.934	9481.162**
Error	18	7.358	0.409	
Total	26	31014.83		

** = Significant at 1 % level

Table 6-A. Analysis of variance for antioxidant activity of 9 different varieties of green leaves in inhibition of conjugated dienes in liposome system.

SV	DF	SS	MS	F
Treat	8	13071.93	1633.991	4096.203**
Error	18	7.180	0.399	
Total	26	13079.11		

** = Significant at 1 % level

Table 7-A. Analysis of variance for antioxidant activity of 9 different varieties Of green leaves in inhibition of oxidation of linoleic acid/β-carotene system.

SV	DF	SS	MS	F
Treat	8	4054.153	506.769	1052.318**
Error	18	8.668	0.482	
Total	26	4062.821		

** = Significant at 1 % level

Table 8-A. Analysis of variance for total phenolic content 9 varieties of green leaves.

SV	DF	SS	MS	F
Treat	8	9593.356	1199.170	26934.18**
Error	18	0.801	0.0445	
Total	26	9594.158		

** = Significant at 1 % level

Table 9-A. Analysis of variance for antioxidant activity of mango leave prepared with different solvents in inhibition of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	3	7351.513	2450.504	16760.36**
Error	8	1.170	0.146	
Total	11	7352.682		

** = Significant at 1 % level

Table 10-A. Analysis of variance for antioxidant activity of mango leave prepared with different solvents in inhibition of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	3	5808.857	1936.286	23779.99**
Error	8	0.651	0.0814	
Total	11	5809.509		

** = Significant at 1 % level

Table 11-A. Analysis of variance for antioxidant activity of mango leave prepared with different solvents in inhibition of oxidation of linoleic acid/β-carotene system.

SV	DF	SS	MS	F
Treat	3	10799.12	3599.707	53361.94**
Error	8	0.540	0.0675	
Total	11	10799.66		

** = Significant at 1 % level

Table 12-A. Analysis of variance for total phenolic content of mango leave with different solvents.

SV	DF	SS	MS	F
Treat	3	4162.520	1387.507	23234.83**
Error	8	0.478	0.0598	
Total	11	4162.997		

** = Significant at 1 % level

Table 13-A. Analysis of variance for antioxidant activity of liquorice extract prepared with different solvents in inhibition of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	3	15197.11	5065.702	56036.53**
Error	8	0.723	0.0904	
Total	11	15197.83		

** = Significant at 1 % leve

Table 14-A. Analysis of variance for antioxidant activity of liquorice extract prepared with different solvents in inhibition of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	3	13355.99	4451.996	26957.29**
Error	8	1.321	0.165	
Total	11	13357.31		

** = Significant at 1 % level

Table 15-A. Analysis of variance for antioxidant activity of liquorice extract prepared with different solvents in inhibition of oxidation of linoleic acid/β-carotene system.

SV	DF	SS	MS	F
Treat	3	585.88	195.296	5.638**
Error	8	277.093	34.637	
Total	11	862.981		

** = Significant at 1 % level

Table 16-A. Analysis of variance for total phenolic content of liquorice extract with different solvents.

SV	DF	SS	MS	F
Treat	3	307.294	102.431	10994.41**
Error	8	7.453	0.9316	
Total	11	307.368		

** = Significant at 1 % level

Table 17-A. Analysis of variance for antioxidant activity of mango leave prepared with different ratio of methanol to water in inhibition of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	5	94.265	18.853	561.377**
Error	12	0.403	0.0336	
Total	17	94.668		

** = Significant at 1 % level

Table 18-A. Analysis of variance for antioxidant activity of mango leave prepared with different ratio of methanol to water in inhibition of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	5	1546.856	309.371	18599.47**
Error	12	0.200	0.0166	
Total	17	1547.055		

** = Significant at 1 % level

Table 19-A. Analysis of variance for antioxidant activity of mango leave prepared with different ratio of methanol to water in inhibition of oxidation of linoleic acid/β-carotene system.

SV	DF	SS	MS	F
Treat	4	88.880	22.220	378.748**
Error	10	0.587	0.0587	
Total	14	89.466		

** = Significant at 1 % level

Table 20-A. Analysis of variance for total phenolic of mango leave prepared with different ratio of methanol to water.

SV	DF	SS	MS	F
Treat	5	89.988	17.998	418.276**
Error	12	0.516	0.043	
Total	17	90.504		

** = Significant at 1 % level

Table 21-A. Analysis of variance for antioxidant activity of liquorice extract prepared with different ratio of methanol to water in inhibition of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	5	991.567	198.313	8436.877**
Error	12	0.282	0.0235	
Total	17	991.850		

** = Significant at 1 % level

Table 22-A. Analysis of variance for antioxidant activity of liquorice extract prepared with different ratio of methanol to water in inhibition of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	4	1704.653	426.163	9099.572**
Error	10	0.468	0.0468	
Total	14	1705.121		

** = Significant at 1 % level

Table 23-A. Analysis of variance for antioxidant activity of liquorice extract prepared with different ratio of methanol to water in inhibition of oxidation of linoleic acid/β-carotene system.

SV	DF	SS	MS	F
Treat	5	71.284	14.257	276.504**
Error	12	0.619	0.0516	
Total	17	71.903		

** = Significant at 1 % level

Table 24-A. Analysis of variance for total phenolic of liquorice extract prepared with different ratio of methanol to water.

SV	DF	SS	MS	F
Treat	5	182.552	36.510	5347.330**
Error	12	8.193	0.6828	
Total	17	182.634		

** = Significant at 1 % level

Table 25-A. Analysis of variance for synergistic antioxidant activity of liquorice extract with ascorbic acid in inhibition of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	7	12808.836	1829.833	3085.353**
Error	16	9.489	0.593	
Total	23	12818.320		

** = Significant at 1 % level

Table 26-A. Analysis of variance for synergistic antioxidant activity of liquorice extract with ascorbic acid in inhibition of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	7	6030.187	861.455	23.907**
Error	16	576.529	36.033	
Total	23	6606.716		

** = Significant at 1 % level

Table 27-A. Analysis of variance for synergistic antioxidant activity of liquorice extract with ascorbic acid in inhibition of oxidation of linoleic acid/β-carotene system.

SV	DF	SS	MS	F
Treat	7	13827.586	1975.369	2678.225**
Error	16	11.801	0.738	
Total	23	13839.387		

** = Significant at 1 % level

Table 28-A. Analysis of variance for synergistic antioxidant activity of mango leave extract with ascorbic acid in inhibition of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	7	12023.673	1717.668	3372.384**
Error	16	8.149	0.509	
Total	23	12031.822		

** = Significant at 1 % level

Table 29-A. Analysis of variance for synergistic antioxidant activity of mango leave extract with ascorbic acid in inhibition of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	7	6030.187	861.455	23.907**
Error	16	576.529	36.033	
Total	23	6606.716		

** = Significant at 1 % level

Table 30-A. Analysis of variance for synergistic antioxidant activity of mango leave extract with ascorbic acid in inhibition of oxidation of linoleic acid/β-carotene system.

SV	DF	SS	MS	F
Treat	7	12258.815	1751.259	4447.125**
Error	16	6.301	0.394	
Total	23	12265.115		

** = Significant at 1 % level

Table 31-A. Analysis of variance for synergistic antioxidant activity of liquorice extract with citric acid in inhibition of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	7	12979.197	1854.171	3521.358**
Error	16	8.425	0.527	
Total	23	12987.622		

** = Significant at 1 % level

Table 32-A. Analysis of variance for synergistic antioxidant activity of liquorice extract with citric acid in inhibition of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	7	7265.804	1037.972	1505.626**
Error	16	11.030	0.689	
Total	23	7276.835		

** = Significant at 1 % level

Table 33-A. Analysis of variance for synergistic antioxidant activity of liquorice extract with citric acid in inhibition of oxidation of linoleic acid/β-carotene system.

SV	DF	SS	MS	F
Treat	7	14535.847	2076.550	3931401**
Error	16	8.451	0.528	
Total	23	14544.299		

** = Significant at 1 % level

Table 34-A. Analysis of variance for synergistic antioxidant activity of mango leaves extract with citric acid in inhibition of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	7	11323.454	1617.636	4053.549**
Error	16	6.385	0.394	
Total	23	11329.839		

** = Significant at 1 % level

Table 35-A. Analysis of variance for synergistic antioxidant activity of mango leaves extract with citric acid in inhibition of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	7	6326.641	903.806	2483.886**
Error	16	5.822	0.364	
Total	23	6332.463		

** = Significant at 1 % level

Table 36-A. Analysis of variance for synergistic antioxidant activity of mango leaves extract with citric acid in inhibition of oxidation of linoleic acid/β-carotene system.

SV	DF	SS	MS	F
Treat	7	12546.112	1792.302	4911.313**
Error	16	5.839	0.365	
Total	23	12551.951		

** = Significant at 1 % level

Table 37-A. Analysis of variance for synergistic antioxidant activity of liquorice extract with α-tocopherol in inhibition of oxidation of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	7	2385.583	340.798	1027.698**
Error	16	5.306	0.332	
Total	23	2390.889		

** = Significant at 1 % level

Table 38-A. Analysis of variance for synergistic antioxidant activity of liquorice extract with α-tocopherol in inhibition of oxidation of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	7	754.154	112.022	110.871**
Error	16	16.166	1.010	
Total	23	800.320		

** = Significant at 1 % level

Table 39-A. Analysis of variance for synergistic antioxidant activity of liquorice extract with α-tocopherol in inhibition of oxidation of linoleic acid/β-carotene system.

SV	DF	SS	MS	F
Treat	7	3506.938	500.991	494.560**
Error	16	16.208	1.013	
Total	23	3523.146		

** = Significant at 1 % level

Table 40-A. Analysis of variance for synergistic antioxidant activity of mango leaves extract with α-tocopherol in inhibition of oxidation of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	7	2727.868	389.695	1141.073**
Error	16	5.464	0.342	
Total	23	2733.332		

** = Significant at 1 % level

Table 41-A. Analysis of variance for synergistic antioxidant activity of mango leaves extract with α-tocopherol in inhibition of oxidation of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	7	776.005	110.858	104.141**
Error	16	16.252	1.016	
Total	23	792.256		

** = Significant at 1 % level

Table 42-A. Analysis of variance for synergistic antioxidant activity of mango leaves extract with α-tocopherol in inhibition of oxidation of linoleic acid/β-carotene system.

SV	DF	SS	MS	F
Treat	7	3506.938	500.991	494.560**
Error	16	16.208	1.013	
Total	23	3523.146		

** = Significant at 1 % level

Table 43-A. Analysis of variance for antioxidant activities of mango leave prepared with different extraction time and repetition in inhibition of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	15	1.4263	0.0950	1.75**
Extraction time (T)	4	1.1046	0.2762	5.11**
Repetition (R)	2	0.1345	0.0672	1.24**
T x R	8	0.1218	0.0152	0.28**
Error	30	1.6206	0.0540	
Total	45	3.0469		

** = Significant at 1 % level

Table 44-A. Analysis of variance for antioxidant activities of mango leave prepared with different extraction time and repetition in inhibition of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	15	1.5287	0.1092	1.90^{ns}
Extraction time (T)	4	1.2093	0.3023	5.27**
Repetition (R)	2	0.1776	0.0888	1.55**
T x R	8	0.1417	0.0177	<1
Error	30	1.7204	0.0573	
Total	45	3.2491		

** = Significant at 1 % level

^{ns} = not significant

Table 45-A. Analysis of variance for antioxidant activities of mango leave prepared with different extraction time and repetition in inhibition of oxidation of linoleic acid/β-carotene system.

SV	DF	SS	MS	F
Treat	15	1.2243	0.0816	1.73**
Extraction time (T)	4	1.0215	0.2554	5.43**
Repetition (R)	2	0.1221	0.0610	1.29**
T x R	8	0.1123	0.0140	0.29**
Error	30	1.4105	0.0470	
Total	45	2.6348		

** = Significant at 1 % level

Table 46-A. Analysis of variance for total phenolic content of mango leaves with different extraction time and repetition.

SV	DF	SS	MS	F
Treat	15	1.1420	0.0761	1.88**
Extraction time (T)	4	1.0013	0.2503	6.21**
Repetition (R)	2	0.1116	0.0558	1.38**
T x R	8	0.1121	0.0140	0.34**
Error	30	1.2102	0.0403	
Total	45	2.3522		

** = Significant at 1 % level

Table 47-A. Analysis of variance for antioxidant activities of liquorice extract prepared with different extraction time and repetition in inhibition of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	15	1.4152	0.0943	1.75**
Extraction time (T)	4	1.1042	0.2761	5.11**
Repetition (R)	2	0.1346	0.0673	1.25**
T x R	8	0.1216	0.0152	0.28**
Error	30	1.6202	0.0540	
Total	45	3.0354		

** = Significant at 1 % level

Table 48-A. Analysis of variance for antioxidant activities of liquorice extract prepared with different extraction time and repetition in inhibition of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	15	1.5145	0.1009	1.76**
Extraction time (T)	4	1.2072	0.3018	5.27**
Repetition (R)	2	0.1756	0.0878	1.53**
T x R	8	0.1402	0.0175	0.31**
Error	30	1.7202	0.0573	
Total	45	3.2347		

** = Significant at 1 % level

^{ns} = not significant

Table 49-A. Analysis of variance for antioxidant activities of liquorice extract prepared with different extraction time and repetition in inhibition of oxidation of linoleic acid/β-carotene system.

SV	DF	SS	MS	F
Treat	15	1.2224	0.0814	1.75**
Extraction time (T)	4	1.0212	0.2553	5.48**
Repetition (R)	2	0.1215	0.0607	1.30**
T x R	8	0.1120	0.0140	0.30**
Error	30	1.4006	0.0466	
Total	45	2.623		

** = Significant at 1 % level

Table 50-A. Analysis of variance for total phenolic content of liquorice extract with different extraction time and repetition.

SV	DF	SS	MS	F
Treat	15	1.1210	0.0747	1.83**
Extraction time (T)	4	1.0011	0.2503	6.16**
Repetition (R)	2	0.1112	0.0556	1.36**
T x R	8	0.1135	0.0142	0.35**
Error	30	1.2205	0.0406	
Total	45	2.3415		

** = Significant at 1 % level

Table 51-A. Analysis of variance for relative antioxidant activity of mango leaves extracts as a function of heating time at 80°C in inhibition of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	7	220.043	31.435	3621.865**
Error	16	0.139	0.00868	
Total	23	220.182		

** = Significant at 1 % level

Table 52-A. Analysis of variance for relative antioxidant activity of mango leaves extracts as a function of heating time at 80°C in inhibition of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	7	113.106	16.158	774.193**
Error	16	0.334	0.002088	
Total	23	113.440		

** = Significant at 1 % level

Table 53-A. Analysis of variance for relative antioxidant activity of liquorice extracts as a function of heating time at 80°C in inhibition of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	7	17.221	2.460	480.813**
Error	16	0.082	0.00513	
Total	23	17.303		

** = Significant at 1 % level

Table 54-A. Analysis of variance for relative antioxidant activity of liquorice extracts as a function of heating time at 80°C in inhibition of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	7	15.018	2.145	472.392**
Error	16	0.073	0.00456	
Total	23	15.091		

** = Significant at 1 % level

Table 55-A. Analysis of variance for relative antioxidant activity of mango leaves extracts as a function of heating time at 100°C in inhibition of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	7	230.703	32.958	2715.346**
Error	16	0.194	0.012125	
Total	23	230.897		

** = Significant at 1 % level

Table 56-A. Analysis of variance for relative antioxidant activity of mango leaves extracts as a function of heating time at 100°C in inhibition of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	7	178.786	25.541	4043.415**
Error	16	0.101	0.00631	
Total	23	178.887		

** = Significant at 1 % level

Table 57-A. Analysis of variance for relative antioxidant activity of liquorice extracts as a function of heating time at 100°C in inhibition of conjugated diene in liposome system.

SV	DF	SS	MS	F
Treat	7	53.253	7.608	1450.221**
Error	16	0.084	0.00525	
Total	23	53.337		

** = Significant at 1 % level

Table 58-A. Analysis of variance for relative antioxidant activity of liquorice extracts as a function of heating time at 100°C in inhibition of TBARS in liposome system.

SV	DF	SS	MS	F
Treat	7	39.433	5.633	155.580**
Error	16	0.579	0.0362	
Total	23	40.012		

** = Significant at 1 % level

Table 59-A. Analysis of variance for relative antioxidant activity of liquorice extracts as a function of heating time at 100°C in inhibition of linoleic acid/β-carotene system.

SV	DF	SS	MS	F
Treat	7	30.553	4.365	555.433**
Error	16	0.126	0.00788	
Total	23	30.679		

** = Significant at 1 % level

Table 60-A. Analysis of variance for absorbance at 517 nm of mango leaves extract at different amounts.

SV	DF	SS	MS	F
Treat	13	3.139	0.241	6694.44**
Error	28	0.001	0.000036	
Total	41	3.140		

** = Significant at 1 % level

Table 61-A. Analysis of variance for Fe^{2+} - binding activity of mango leaves extract at different amounts.

SV	DF	SS	MS	F
Treat	13	35858.45	2758.342	1173.222**
Error	28	65.830	2.351	
Total	41	35924.28		

** = Significant at 1 % level

Table 62-A. Analysis of variance for absorbance at 517 nm of liquorice extract at different amounts.

SV	DF	SS	MS	F
Treat	13	3.521	0.271	7527.77**
Error	28	0.001	0.000036	
Total	41	3.522		

** = Significant at 1 % level

Table 63-A. Analysis of variance for Fe^{2+}- binding activity of liquorice extract at different amounts.

SV	DF	SS	MS	F
Treat	13	41003.65	3154.127	1343543**
Error	28	0.06	0.00214	
Total	41	41003.71		

** = Significant at 1 % level

Table 64-A. Analysis of variance for reducing power of mango leaves extract at different amounts.

SV	DF	SS	MS	F
Treat	11	3805.113	345.919	221191.8**
Error	24	0.038	0.00158	
Total	35	3805.151		

** = Significant at 1 % level

Table 65-A. Analysis of variance for absorbance of mango leaves extract at different amounts.

SV	DF	SS	MS	F
Treat	12	0.222	0.0185	313.559**
Error	26	0.001	0.000051	
Total	38	0.223		

** = Significant at 1 % level

Table 66-A. Analysis of variance for reducing power of liquorice extract at different amounts.

SV	DF	SS	MS	F
Treat	11	3902.355	354.760	227653.2**
Error	24	0.037	0.00158	
Total	35	3902.392		

** = Significant at 1 % level

Table 67-A. Analysis of variance for absorbance of liquorice extract at different amounts.

SV	DF	SS	MS	F
Treat	12	0.367	3.062	5768.092**
Error	26	0.001	0.0000384	
Total	38	0.368		

** = Significant at 1 % level

Table 68-A. Analysis of variance for absorbance at 480 nm of liquorice extract at different amounts.

SV	DF	SS	MS	F
Treat	13	2.089	0.161	258.242**
Error	28	0.017	0.000607	
Total	41	2.106		

** = Significant at 1 % level

Table 69-A. Analysis of variance for Cu^{2+} - binding activity of liquorice extract at different amounts.

SV	DF	SS	MS	F
Treat	13	13844.59	1064.968	272735.8**
Error	28	0.109	0.00389	
Total	41	13844.70		

** = Significant at 1 % level

Table 70-A. Analysis of variance for absorbance at 480 nm of mango leaves extract at different amounts.

SV	DF	SS	MS	F
Treat	13	2.280	0.175	10077.80**
Error	28	0.001	0.0000357	
Total	41	2.281		

** = Significant at 1 % level

Table 71-A. Analysis of variance for Cu^{2+} - binding activity of mango leaves extract at different amounts.

SV	DF	SS	MS	F
Treat	13	15946.70	1226.669	349288.9**
Error	28	0.1	0.00357	
Total	41	15946.80		

** = Significant at 1 % level

Table 72-A. Analysis of variance for absorbance at 234 nm of liquorice extract at different amounts.

SV	DF	SS	MS	F
Treat	10	0.288	2.878	259.591**
Error	22	0.002	0.000090	
Total	32	0.290		

** = Significant at 1 % level

Table 73-A. Analysis of variance for lipoxygenase inhibition of liquorice extract at different amounts.

SV	DF	SS	MS	F
Treat	10	10086.37	1008.637	36.769**
Error	22	603.503	27.432	
Total	32	10689.88		

** = Significant at 1 % level

Table 74-A. Analysis of variance for absorbance at 234 nm of mango leaves extract at different amounts.

SV	DF	SS	MS	F
Treat	10	0.319	0.0319	708.888**
Error	22	0.001	0.000045	
Total	32	0.320		

** = Significant at 1 % level

Table 75-A. Analysis of variance for lipoxygenase inhibition of mango leaves extract at different amounts.

SV	DF	SS	MS	F
Treat	10	10553.47	1055.347	38.913**
Error	22	596.653	27.121	
Total	32	11150.13		

** = Significant at 1 % level

Table 76-A. Analysis of variance for absorbance at 532 nm of liquorice extract at different amounts.

SV	DF	SS	MS	F
Treat	8	0.303	0.03785	688.636**
Error	18	0.001	0.000055	
Total	26	0.304		

** = Significant at 1 % level

Table 77-A. Analysis of variance for hydroxyl radical of liquorice extract at different amounts

SV	DF	SS	MS	F
Treat	8	17540.19	2192.523	688.636**
Error	18	0.04900	0.002722	
Total	26	17540.23		

** = Significant at 1 % level

Table 78-A. Analysis of variance for absorbance at 532 nm of mango leaves extract at different amounts.

SV	DF	SS	MS	F
Treat	8	0.356	0.04455	688.636**
Error	18	0.00023	0.0000127	
Total	26	0.357		

** = Significant at 1 % level

Table 79-A. Analysis of variance for hydroxyl radical of mango leaves extract at different amounts

SV	DF	SS	MS	F
Treat	8	17540.19	2192.523	688.636**
Error	18	0.06520	0.003622	
Total	26	17540.23		

** = Significant at 1 % level

Table 80-A. Analysis of variance for absorbance at 517 nm of mango leaves at different amounts.

SV	DF	SS	MS	F
Treat	11	0.347	0.03159	688.636**
Error	24	0.00021	0.00000875	
Total	35	0.348		

** = Significant at 1 % level

Table 81-A. Analysis of variance for DPPH of mango leaves extract at different amounts

SV	DF	SS	MS	F
Treat	11	23889.94	2171.813	365181.1**
Error	24	0.143	0.005947	
Total	35	23890.09		

** = Significant at 1 % level

Table 82-A. Analysis of variance for absorbance at 517 nm of liquorice extract at different amounts.

SV	DF	SS	MS	F
Treat	11	0.439	0.03995	688.636**
Error	24	0.000087	0.00000364	
Total	35	0.440		

** = Significant at 1 % level

Table 83-A. Analysis of variance for DPPH of mango leaves extract at different amounts

SV	DF	SS	MS	F
Treat	11	30146.51	2740.592	518996.9**
Error	24	0.127	0.005281	
Total	35	30146.64		

** = Significant at 1 % level

Table 84-A. Analysis of variance for antimicrobial with liquorice extract by diffusion method.

SV	DF	SS	MS	F
Treat	11	952.754	86.614	623.122**
Orga (O)	3	464.630	154.877	1114.22**
Concen (C)	2	242.489	121.245	872.26**
O x C	6	245.635	40.939	294.53**
Error	24	3.351	0.139	
Total	35	949.403		

** = Significant at 1 % level

Table 85-A. Analysis of variance for antimicrobial with mango leaves extract by diffusion method.

SV	DF	SS	MS	F
Treat	11	446.508	40.591	276.12**
Orga (O)	3	221.042	73.681	501.23**
Concen (C)	2	111.380	55.690	378.84**
O x C	6	114.086	19.014	129.35**
Error	24	3.542	0.147	
Total	35	442.966		

** = Significant at 1 % level

Table 86-A. Analysis of variance for TBARS of raw ground fish treated with liquorice extract and other commercial antioxidants.

SV	DF	SS	MS	F
Treat	29	64.463	2.223	344.65**
Concen (C)	5	33.176	6.635	1028.68**
Day (D)	4	19.429	4.857	753.02**
C x D	20	11.640	0.582	90.23**
Error	60	0.387	0.00645	
Total	89	64.850		

** = Significant at 1 % level

Table 87-A. Analysis of variance for TBARS of raw ground fish treated with mango leaves extract and other commercial antioxidants.

SV	DF	SS	MS	F
Treat	29	85.691	2.955	458.14**
Concen (C)	5	35.965	7.193	1115.19**
Day (D)	4	18.130	4.532	702.64**
C x D	20	31.596	1.579	244.80**
Error	60	0.387	0.00645	
Total	89	86.078		

** = Significant at 1 % level

Table 88-A. Analysis of variance for TMA of raw ground fish treated with liquorice extract and other commercial antioxidants.

SV	DF	SS	MS	F
Treat	29	37.464	1.292	200.31**
Concen (C)	5	11.588	2.3176	359.32**
Day (D)	4	12.529	3.132	485.58**
C x D	20	13.347	0.667	103.41**
Error	60	0.387	0.00645	
Total	89	37.077		

** = Significant at 1 % level

Table 89-A. Analysis of variance for TMA of raw ground fish treated with mango leaves extract and other commercial antioxidants.

SV	DF	SS	MS	F
Treat	29	37.213	1.283	202.05**
Concen (C)	5	11.673	2.335	367.72**
Day (D)	4	12.064	3.016	474.96**
C x D	20	13.476	0.674	106.14**
Error	60	0.381	0.00635	
Total	89	36.832		

** = Significant at 1 % level

Table 90-A. Analysis of variance for TVB of raw ground fish treated with liquorice extract and other commercial antioxidants.

SV	DF	SS	MS	F
Treat	29	2487.127	85.763	13484.74**
Concen (C)	5	404.482	80.896	12719.49**
Day (D)	4	1581.078	395.269	62149.21**
C x D	20	501.567	25.078	3943.08**
Error	60	0.382	0.00636	
Total	89	2486.745		

** = Significant at 1 % level

Table 91-A. Analysis of variance for TVB of raw ground fish treated with mango leaves extract and other commercial antioxidants.

SV	DF	SS	MS	F
Treat	29	2490.076	85.864	13629.21**
Concen (C)	5	424.961	84.992	13490.79**
Day (D)	4	1541.506	385.376	61170.79**
C x D	20	523.609	26.180	4155.55**
Error	60	0.380	0.0063	
Total	89	2489.696		

** = Significant at 1 % level

Table 92-A. Analysis of variance for total plate count bacteria of raw ground fish treated with liquorice extract and other commercial antioxidants.

SV	DF	SS	MS	F
Treat	29	48.037	1.656	260.79**
Concen (C)	5	13.555	2.711	426.93**
Day (D)	4	30.381	7.595	1196.06**
C x D	20	4.101	0.205	32.28**
Error	60	0.381	0.00635	
Total	89	47.656		

** = Significant at 1 % level

Table 93-A. Analysis of variance for total plate count bacteria of raw ground fish treated with mango leaves extract and other commercial antioxidants.

SV	DF	SS	MS	F
Treat	29	997.683	34.403	5409.28**
Concen (C)	5	184.173	36.835	5791.67**
Day (D)	4	85.027	21.257	3342.29**
C x D	20	728.483	36.424	5727.04**
Error	60	0.382	0.00636	
Total	89	997.301		

** = Significant at 1 % level

Table 94-A. Analysis of variance for antioxidant activity of individual fractions of liquorice extracts.

SV	DF	SS	MS	F
Treat	7	2043.287	291.898	562.365**
Error	16	8.305	0.519	
Total	23	2051.592		

** = Significant at 1 % level

Table 95-A. Analysis of variance for antioxidant activity of individual fractions of mango leaves extracts.

SV	DF	SS	MS	F
Treat	7	1282.961	183.280	223.466**
Error	16	13.123	0.820	
Total	23	1296.084		

** = Significant at 1 % level

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