Antioxidants from Some Legume Seeds: Extraction, Some Properties and Application

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Thesis Title	Antioxidants from Some Legume Seeds : Extraction, Some Properties and Application
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บทคัดย่อ

จากการศึกษากิจกรรมการเป็นสารกันหืนจากเมล็ดถั่ว 19 สายพันธุ์ โดยใช้ระบบที่ประกอบด้วยเบต้า-แคโรทีนและกรดลิโนเลอิค พบว่า เมล็ดกระถินและเมล็ดถั่วดำมีกิจกรรมการเป็นสารกันหืนสูงสุด สภาวะที่เหมาะสมในการสกัดสารกันหืนจากเมล็ดกระถินและเมล็ดถั่วดำ คือ สกัดเมล็ดถั่วบดด้วยสารละลายเมธานอล ร้อยละ 50 เป็นเวลา 2 ชั่วโมง และสารละลายเมธานอล ร้อยละ 70 เป็นเวลา 5 ชั่วโมง ตามลำดับ สารสกัดจากเมล็ดกระถินและเมล็ดถั่วดำ มีความคงทนต่อความร้อนและพีเอชได้เป็นอย่างดี ที่พีเอชเป็นกลางและด่าง สารสกัดทั้งสองชนิดมีกิจกรรมการเป็นสารกันหืนสูงกว่าที่พีเอชเป็นกรด กิจกรรมการเป็นสารกันหืนของสารสกัดจากเมล็ดกระถินและเมล็ดถั่วดำเพิ่มขึ้นเมื่อปริมาณของสารสกัดเพิ่มขึ้น สารสกัดจากเมล็ดกระถินและเมล็ดถั่วดำสามารถเสริมฤทธิ์กับแอลฟ่า-โทโคฟีรอล ส่งผลให้สามารถยับยั้งการเกิดออกซิเดชั่นในระบบที่ประกอบด้วย เบต้า-แคโรทีนและกรดลิโนเลอิค ได้ดีกว่าการใช้แอลฟ่า-โทโคฟีรอล หรือสารสกัดเพียงอย่างเดียว อย่างไรก็ตาม ไม่พบการเสริมฤทธิ์ระหว่างกรดซิตริกและกรดแอสคอร์บิคกับสารสกัดทั้งสอง สารสกัดจากเมล็ดกระถินและเมล็ดถั่วดำ มีความสามารถในการกำจัดอนุมูลอิสระ โดยเป็นตัวให้ไฮโดรเจนและอิเล็กตรอนแก่อนุมูลอิสระ ส่งผลให้สามารถยับยั้งปฏิกิริยาลูกโซ่ของการเกิดออกซิเดชั่นของไขมัน โดยความสามารถในการให้ไฮโดรเจนและอิเล็กตรอนขึ้นอยู่กับปริมาณที่ใช้ สารสกัดจากเมล็ดกระถินและเมล็ดถั่วดำสามารถจับกับไอออนของเหล็กและทองแดง รวมทั้งสามารถยับยั้งการทำงานของเอนไซม์ไลพอกซิจีเนส โดยขึ้นอยู่กับปริมาณของสารสกัด สารกันหืนในสารสกัดของเมล็ดกระถินและเมล็ดถั่วดำเป็นสารจำพวกฟีนอลิคที่ไม่มีหมู่ไฮ

ดรอกซิลอิสระอยู่ที่ตำแหน่งออโธหรือพารา และมีคุณสมบัติเป็นสารรีดิวซ์ สารสกัดจากเมล็ดกระถินและเมล็ดถั่วดำสามารถชะลอการเกิดออกซิเดชั่นในเนื้อหมูบดสุกได้สูงขึ้นเมื่อความเข้มข้นของสารสกัดเพิ่มขึ้น และสามารถเพิ่มประสิทธิภาพในการยับยั้งการเกิดออกซิเดชั่นในเนื้อหมูบดสุกได้โดยใช้ร่วมกับกรดซิตริกหรือกรดแอสคอร์บิค

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Abstract

Extracts of 19 different varieties of legume seeds were tested for their antioxidant activities in β-carotene/linoleic acid system. Among legume seeds, wild tamarind seed and cow pea seed extracts exhibited highest antioxidant activity. The optimum condition for extracting antioxidants from wild tamarind seed and cow pea seed involved extracting seed powder with 50 % methanol for 2 hours and 70 % methanol for 5 hours, respectively. Wild tamarind seed and cow pea seed extracts had good thermal and pH stability. Both extracts showed higher antioxidant activity at neutral and alkaline pHs than acidic pHs. Antioxidant activities of both extracts increased with increased amount of the extracts. Synergistic action of wild tamarind seed and cow pea seed extracts with α-tocopherol in β-carotene/linoleic acid system was observed. However, no synergistic action between citric acid or ascorbic acid with both extracts were obtained. The extracts of wild tamarind seed and cow pea seed exhibited radical scavenging property and reducing power, depending on amount of the extracts. Therefore, these extracts possibly worked as primary antioxidants that reacted with free radicals. Furthermore, wild tamarind seed and cow pea seed extracts functioned as Fe^{2+} and Cu^{2+} chelators and also inhibited lipoxygenase activity. These properties of both extracts were dependent on amount of the extracts. Phenolic compounds without free *ortho*- and *para*- hydroxy groups with reducing activity were present and were postulated to exhibit antioxidant activity in both wild tamarind seed and cow

pea seed extracts. Wild tamarind seed and cow pea seed extracts retarded the oxidation of cooked ground pork in concentration dependent manner. Synergistic effect of both extracts with citric acid and ascorbic acid on inhibition of cooked ground pork oxidation was observed.

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List of Abbreviations

^{o}C	=	degree Celsius
g	=	Gram
hr.	=	Hour
min	=	Minute
mg/ml	=	Milligram per milliliter
nm	=	Nanometer
OD	=	Optical density
ln	=	Natural log
TBARS	=	Thiobarbituric acid substances
TBA no.	=	Thiobarbituric acid number
BHA	=	Butylated hydroxyanisole
BHT	=	Butylated hydroxytoluene
TBHQ	=	*tert*-butyl hydroquinone

Chapter 1

Introduction

One of the major changes that occur during processing, distribution, and final preparation of food is oxidation. Oxidation of fat initiates other changes in the food systems that affect its nutritional quality, wholesomeness, safety, color, flavor, and texture. In addition, it is thought that lipid oxidation is strongly associated with carcinogenesis, mutagenesis, aging and atherosclerosis (Yagi, 1987; Cutlar, 1984; 1992). The addition of antioxidants has become popular as a means of increasing the shelf life of food products and improving the stability of lipid. In living system, dietary antioxidants such as β-carotene, α-tocopherol, and ascorbic acid may be effective in protection from oxidative damage as well as in enzymatic protection by endogenous enzymes such as superoxide dismutase, glutathione peroxidase, and catalase. Synthetic antioxidants, e.g. butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butyl hydroquinone (TBHQ), are widely used in the food industry because they are effective and cheaper than natural antioxidants. Their safety, however, has been questioned. Ito *et al.* (1986) reported that BHA was shown to be carcinogenic in animal experiments. At high doses, BHT may cause internal and external hemorrhaging, which contributes to death in some strain of mice and guinea pigs. This effect is due to the ability of BHT to reduce vitamin K-depending blood-clotting factor (Ito *et al.*, 1986). TBHQ is allowed as a food antioxidant in the U.S. However, it is not permitted in the European Economic Community countries and Canada due to the lack of adequate toxicological information acceptable to those countries. Moreover, it showed mutagenic activity *in vivo* (VanEsch, 1986). Recently, several natural antioxidants from dietary plants,

e.g. polyphenol or β-diketone type have been reported to play an important role in prevention of carcinogenesis and to extend life span in animals (Osawa *et al.*, 1990). Dietary antioxidants were reported to offer effective protection against peroxidative damages in living systems (Cutlar, 1984; Osawa *et al.*, 1990; Hirose *et al.*, 1994). Therefore, much attention has been focused on natural antioxidants, and some polyphenol compounds, e.g. flavonoids, phenolic acid, and lignans. A number of antioxidants isolated from natural sources with high antioxidant activity have been reported (Duh *et al.*, 1992; 1997; Tsuda *et al.*, 1993a; 1994b; Yi *et al.*, 1997; Frankel *et al.*, 1997; Przybylski *et al.* 1998; Duh, 1998).

Legumes are cultivated throughout the world for their pods and seed and consumed as various dishes. Extracts from legume seeds, including soybean, peanut, mung bean, pea bean, navy bean, and tamarind seed, have been reported to possess antioxidant activities (Pratt, 1972; Hammerschmidt and Pratt, 1978; Duh *et al.*, 1992; Tsuda *et al.,* 1993a; 1994a; Duh *et al.,* 1997). Since Thailand has a variety of legumes, a study on antioxidant from legume seeds should be initiated. The objectives of this investigation were to screen for legume seeds with high antioxidant activities, to study the properties and mode of action as well as to apply the antioxidant extracts to prevent oxidation in food system.

Literature review

1. Lipid oxidation

Oxidative reactions naturally occurring within foods involve the removal of electrons from atoms or molecules and lead to the reduction of the recipient components. Oxidation mainly causes the development of rancid off-flavors and odors in fats, oils, and lipid containing foods (Dziezak, 1986). Autoxidation of polyunsaturated lipids involves a free radical chain reaction and could be described in term of initiation, propagation and termination processes (Angelo, 1996).

A. Initiation

At initiation step, fat free radicals are formed when loosely held hydrogen atom is abstracted from "active methylene" located between two double bonds. Initiators of this step include trace metal, light or heat (Eq. 1, 2, 3) (Angelo, 1996). The rearrangement of the double bonds results 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). The fat free radicals react with oxygen to form peroxy free radicals. These peroxy free radicals act as strong initiators or catalysts of further oxidation by extracting a hydrogen atom from another molecule triggering propagation (Frankel, 1984).

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

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

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

B. Propagation

In propagation, the peroxy radicals remove a hydrogen atom from a lipid to form a relatively stable hydroperoxides (ROOH) and a new unstable fatty radical (Eq. 7, 8). Lipid hydroperoxides, the primary products of autoxidation, are odorless and tasteless. The unstable fatty radical will then react with oxygen to form another new reactive peroxy radical. This reaction, when repeated many times, produces an accumulation of hydroperoxides. The propagation reaction becomes a continuous process as long as unsaturated lipid or fatty acid molecules are available. (Frankel, 1984; Giese, 1996; Jadhav *et al.*, 1996).

$$R^{\bullet} + {}^{3}O_2 \rightarrow ROO^{\bullet} \qquad (7)$$

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet} \qquad (8)$$

C. Termination

For termination, which is a final step in autoxidation, the hydroperoxides split into smaller short chain organic compounds e.g. aldehydes, ketones, alcohols, and acids which cause the off-odors and off-flavors characteristic of rancid fats and oils. The auto-oxidative process is ended when two unstable radicals react with each other (Eq. 9, 10 , 11) (Jadhav, 1996; Giese, 1996).

$$R^{\bullet} + R^{\bullet} \rightarrow R\text{-}R \qquad (9)$$

$$R^{\bullet} + ROO^{\bullet} \rightarrow ROOR \qquad (10)$$

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2 \qquad (11)$$

A generalized scheme for autoxidation of lipid is given in Figure 1.

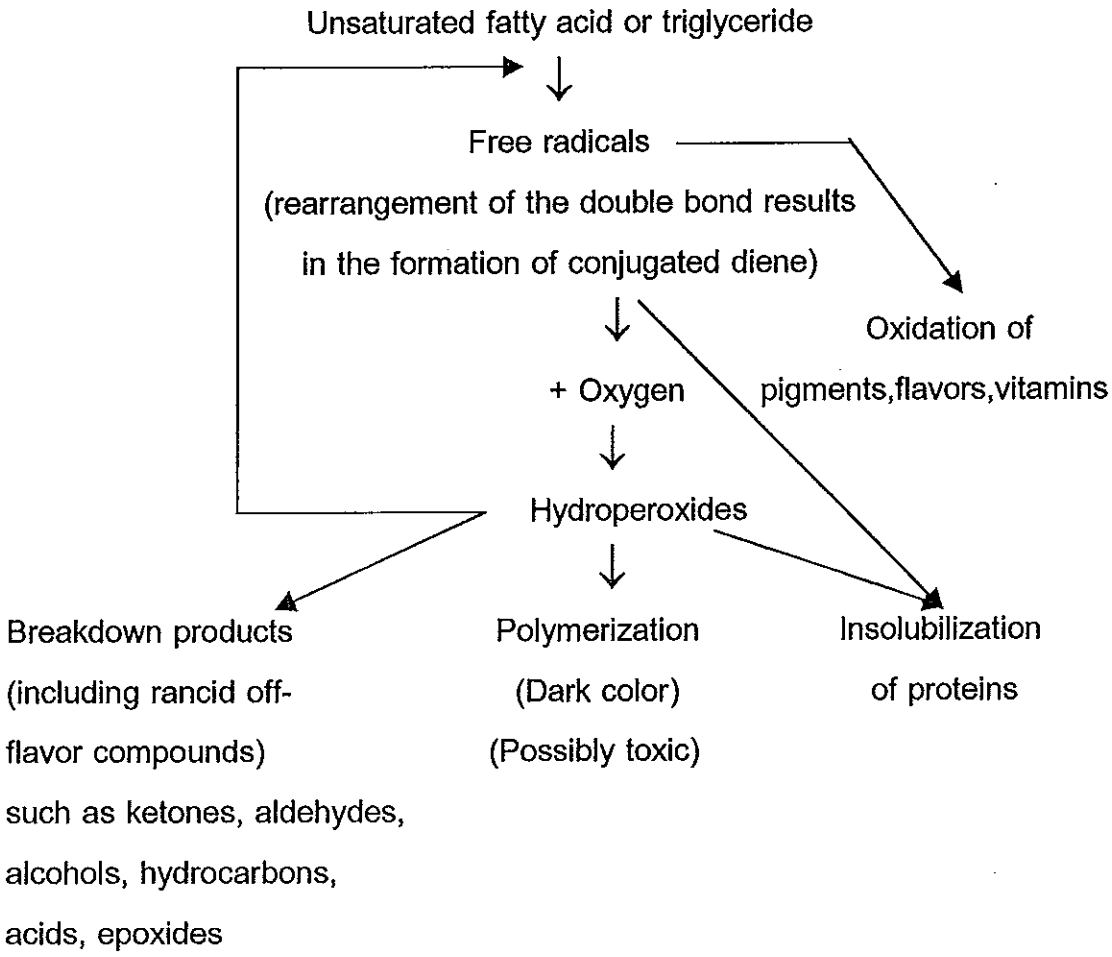

Figure 1 Overall mechanism of lipid oxidation.

Sources : Jadhav *et al.* (1996); Nakayama *et al.* (1994).

1.1 Factors influencing rate of lipid oxidation in foods.

The rate of lipid oxidation in foods are dependent on many factors as follows:

1.1.1 Fatty acid compositions

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 nonconjugated (Nawar, 1996).

1.1.2 Pro – oxidants

Transition metals, particularly those possessing two or more valency states and a suitable oxidation-reduction potential between them (e.g., cobalt, copper, iron, manganese, and nickle) are effective pro-oxidants. If present, even at concentrations as low as 0.1 ppm, they can decrease the induction period and increase the rate of oxidation (Nawar, 1996). Several machanisms 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:

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

Lipoxygenase is an enzyme found in vegetables such as beans and peas, specifically introduces oxygen into free fatty acids. Hemoproteins planer porphyrin structures that contain a centrally oriented iron atom, are also implicated as pro-oxidants (Dziezak, 1986).

1.1.3 Other factors

A. Temperature

In general, the rate of oxidation increases as the temperature is increased. Onyeneho and Hettiarachchy (1991) studied the oxidative stability of soy and sunflower oil at 26 and 37°C and found that the greater increases in peroxide formation were observed in oils stored at 37°C. Similarly, Tian and White (1994) reported that peroxide values of soy and cottonseed oil kept at 60 °C were higher than those of soy and cotton seed oil stored at 30 °C.

B. Surface area

The rate of oxidation increases in direct proportion to the surface area of the lipid exposed to air. The rate of oxidation increased with increasing surface area (Nawar, 1996).

C. Moisture

The rate of oxidation depends strongly on water activity. Foods with very low moisture contents (a_w value of less than about 0.1) undergo oxidation very rapidly. Increasing the a_w to about 0.3 retards lipid oxidation and often produces a minimum rate. This protective effect of small amount of water is believed to occur by reducing the catalytic activity of metal catalysts, by quenching free radicals, and/or by impeding access of oxygen to the lipid. At somewhat higher water activities (a_w = 0.55-0.85), the rate of oxidation increases again, presumably as a result of increased mobilization of catalysts and oxygen (Nawar, 1985).

D. Radiation

Radiation generates radicals, including hydroxyl radicals (Jadhav, 1996). Hydroxyl radicals are known to be capable of abstracting hydrogen atoms from membrane lipids and bring about peroxidic reaction of lipid (Fong *et al.*, 1973).

2. Antioxidants

Antioxidants are defined by the United State (U.S.) Food and Drug Administration (FAD) as substances used to preserve food by retarding deterioration, rancidity, or discoloration due to oxidation (Dziezak, 1986).

2.1 Classification of food antioxidants

Based on their functions, food antioxidants are classified as primary or chain-breaking antioxidants, synergist, and secondary antioxidants (Figure 2.) (Rajalakshmi and Narasimhon, 1996).

2.1.1 Primary antioxidants

Primary antioxidants terminate the free-radical chain reaction by donating hydrogen or electron to free radicals and converting them to more stable products. They may also function by addition in reaction with lipid radicals, forming lipid-antioxidant complexes (Giese, 1996). Primary antioxidants include BHA, BHT, TBHQ, propyl gallate and α-tocopherol. Moreover, recent reports have described the compounds with chain-breaking properties present in peanut hulls, mung bean hulls, buckwheat seed, rosemary, and burdock (Yen and Duh, 1994; Duh *et al.*, 1997; Przybylski *et al.*, 1998; Ill *et al.*, 1998; Duh, 1998). Primary antioxidants are effective at very low concentration and they may become pro-oxidants at higher levels.

2.1.2 Synergistic antioxidants

Synergists are substances that enhance the activity of antioxidants without having their own antioxidant activity (Shahidi and Wanasundara, 1992). Synergistic antioxidants can be broadly classified as oxygen scavengers or reducing agents and chelators (Rajalakshmi and Narasimhon, 1996; Dziezak, 1986). Oxygen scavengers or reducing agents include ascorbic acid, ascorbyl palmitate, sulfites, glucose oxidase and erythorbic acid. Chelators, e.g. citric acid, polyphosphate and ethylenediaminetetraacetic acid (EDTA) can act as synergists. Phenolic antioxidants can be used at lower levels if a synergist is added simultaneously to the food products (Rajalakshmi and Narasimhon, 1996; Dziezak, 1986).

2.1.3 Secondary antioxidants

Secondary or preventive antioxidants such as thiopropionic acid and dilauryl thiodipropionate function by decomposing the lipid peroxides into stable end products (Rajalakshmi and Narasimhon, 1996; Dziezak, 1986).

2.1.4 Miscellaneous antioxidants

Compounds listed under miscellaneous antioxidants such as flavonoids and related compounds and amino acids function as both primary antioxidants and synergists (Rajalakshmi and Narasimhon, 1996). 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 nonheam 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 (Rajalakshmi and Narasimhon, 1996). Zinc strongly inhibits lipid peroxidation at the membrane level, possibly by altering or preventing iron binding. Selenium is necessary for the synthesis and activity of glutathione peroxidase, a primary cellular antioxidant enzyme. Glucose oxidase and catalase function

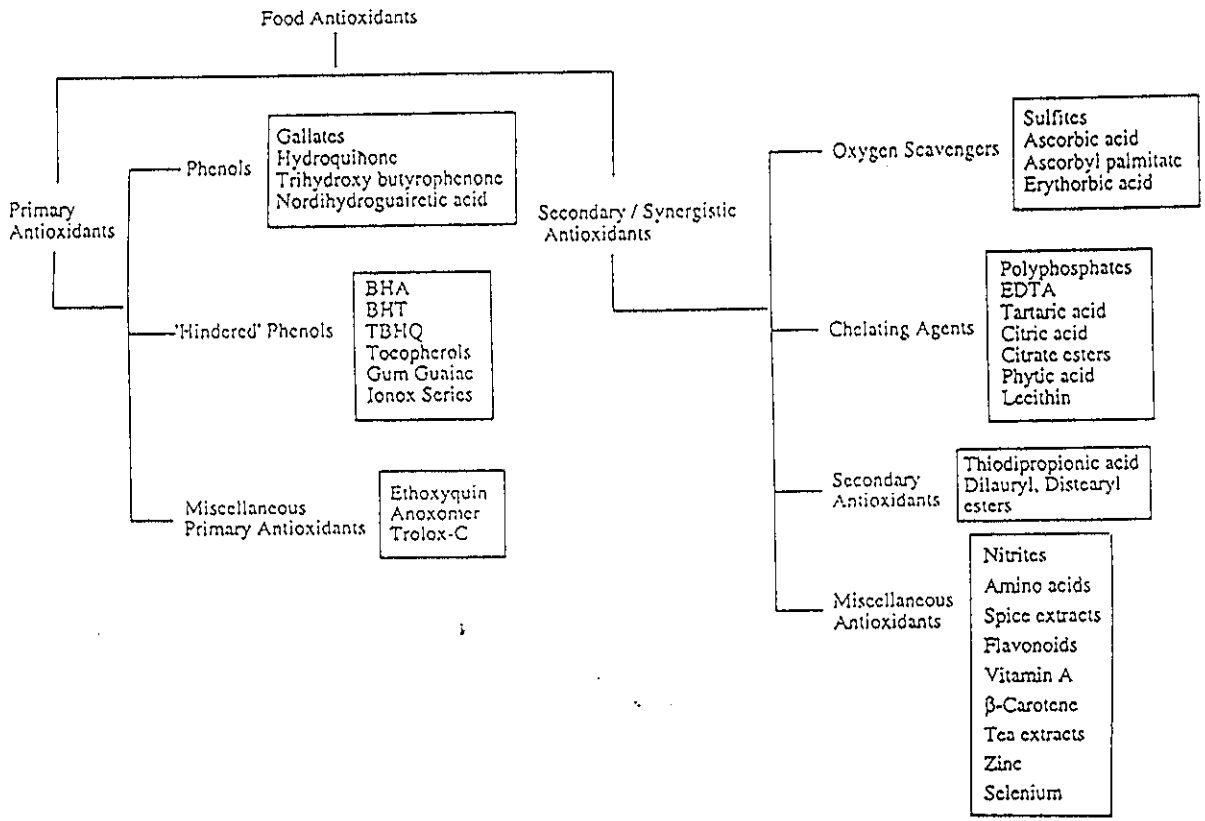

Figure 2 Classification of food antioxidants.

Source: Rajalakshmi and Narasimhon (1996).

by removing dissolved or headspace oxygen and preventing the accumulation of hydrogen peroxide, respectively (Rajalakshmi and Narasimhon, 1996).

2.2 Mode of action of antioxidants in food

Antioxidants have different modes of action. Several antioxidants function as discussed below.

2.2.1 Radical scavenger

Two different mechanisms as radical scavengers have been proposed for antioxidant: either as hydrogen donors or as electron donor that form charge-transfer complexes (Namiki, 1990; Osawa, 1994).

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

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

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

Antioxidants may either delay or inhibit the initiation step by reaction with the peroxy ($ROO^{\bullet}$) or alkoxy ($RO^{\bullet}$) radicals. Reaction of an antioxidant with free radical results in the formation of a free phenoxy ($A^{\bullet}$) radical (Rajalakshmi and Narasimhon, 1996). The phenoxy radicals are stabilized by delocalization of the unpaired electron in the aromatic ring and are further stabilized by bulky group at the ortho position (Figure 3) (Shahidi and Wanasundara, 1992).

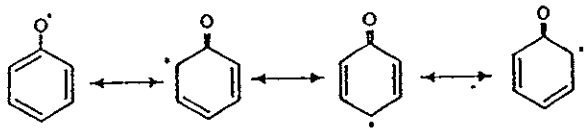

Figure 3 Delocalization of the unpaired electron in the aromatic ring of phenoxy radicals.

Source : Shahidi and Wanasundara (1992).

2.2.2 Peroxide decomposer

Some phenols, amine, dithiopropionic acid, and thiopropionic acid function by decomposing the lipid peroxide into stable end products such as alcohol, ketone and aldehyde (Dziezak, 1986; Namiki, 1990).

2.2.3 Singlet oxygen quenchers

Singlet oxygen is generated from the triplet state oxygen. The mechanism of conversion the triplet oxygen to singlet oxygen is initiated by the transfer of the photosensitizer to its electronically excited state due to the absorption of light in the visible or near-UV region. Subsequently, the photosensitizer is able to transfer its excess energy to an oxygen molecule, giving rise to singlet oxygen (Shahidi and Wanasundara, 1992). Thus, the singlet oxygen can react with a lipid molecule to yield a hydroperoxide. The sequence of events is indicated by the following reactions

$$S \xrightarrow{h\nu} {}^1S \rightarrow {}^3S^*$$

$${}^3S^* + {}^3O_2 \rightarrow {}^1O_2 + {}^1S^*$$

$${}^1O_2^* + RH \rightarrow ROOH$$

where ${}^1S$ is the singlet - state sensitizer, ${}^1S^*$ is the excited singlet - state sensitizer, ${}^3S^*$ is the excited triplet-state sensitizer, ${}^3O_2$ is normal triplet oxygen, ${}^1O_2^*$ is excited singlet - state oxygen, and *hv* is ultraviolet light energy in photons. It is observed that singlet oxygen reacts about 1,000-10,000 times as fast as normal oxygen with methyl linoleate (Jadhav *et al.*, 1996). β-carotene and related carotenoid and α-tocopherol are effective quenchers of singlet oxygen (Jadhav *et al.*, 1996). Lipid oxidation initated by xanthine oxidase can be inhibited by β-carotene because of its ability to quench singlet oxygen (Rajalakshmi and Narasimhon, 1996; Namiki, 1990). Yasaei *et al.* (1996) reported that phenolic antioxidants including BHA, BHT and TBHQ do not effectively protect fats from oxidation by singlet oxygen.

2.2.4 Enzyme inhibitor

Lipoxygenase is present in spices, wheat flour, and vegetables and catalyzes the oxidation of polyunsaturated lipid to hydroperoxide (Jadhav *et al.*, 1996; Ramarathnam rt al., 1986). Lipoxygenase specifically oxidizes polyethenoid acids containing methylene-interrupted double bonds in the cis geometrical configuration, e.g. those in linoleic, linolenic, and arachidonic acids but not in oleic acid. Free-radical intermediates occur during lipoxygenase catalysis, and these can lead to cooxidation of easily oxidized compounds, e.g. carotenoids and polyphenols (Rajalakshmi and Narasimhon, 1996). The lipoxygenase pathway of arachidonic acid metabolites may play a role in inflammation and tumor promotion. BHA, BHT, TBHQ, tocopherol,

propyl gallate and flavonoids showed an inhibitory effect on lipoxygenase activity (Chen *et al.*, 1992).

2.2.5 Synergists

A. Chelating agents.

Chelating agents are not antioxidants, however, they play a valuable role in stabilizing foods. Chelating agents form stable complexes with pro - oxidant metals such as iron and copper. An unshared pair electrons in their molecular structure promotes the chelating action (Dziezak, 1986; Rajalakshmi and Narasimhon, 1996).

B. Reducing agents or oxygen scavengers

Reducing agents or oxygen scavengers function by various mechanisms. They may act as hydrogen donors to the phenoxy radical, thereby regenerating the primary antioxidant or react with free oxygen and remove it in a closed system (Giese, 1996; Rajalakshmi and Narasimhon, 1996).

2.3 Ideal food grade antioxidant

Food grade antioxidants should possess a high antioxidant activity and provide other advantages without retarding or destroying the food quality. Coppen (1983) suggested the characteristics of the ideal food grade antioxidants as follows:

A. Safe

B. Not impart color, odor, or flavor to the fat even on long storage.

C. Effective at low concentration.

D. Easy to incorporate.

E. Effective for at least 1 year at a temperature of 25-30 ^{o}C.

F. Stable to heat processing and protect the finished product (carry-through effect).

G. Low cost.

3. Sources of food antioxidants

3.1 Synthetic antioxidants

Most of synthetic antioxidants are phenolic type. The commercially available and currently used synthetic antioxidants are BHA, BHT, TBHQ and propyl gallate (PG). The differences in their antioxidant activity are related to their physical properties such as volatility, solubility and thermal stability (Jadhav *et al.*, 1996; Shahidi and Wanasundara, 1992). Generally, BHT, BHA and TBHQ are used at level of 100-200 ppm, and gallates are used at levels up to 200-250 ppm for the stabilization of fat and oils (Jadhav *et al.*, 1996). A number of ready-to-use formulation essentially containing a food grade solvent (propylene glycol or glycerol monoleate) are commercially available (Rajalakshmi and Narasimhon, 1996).

3.2 Natural antioxidants

In recent years, consumers and food manufacturers have been interested in products with " all natural" labels. The volume of such products increased 175 % from 1989 to 1990, and the number of products claiming to be without additives or preservatives rose by 99 % during the same period (Rajalakshmi and Narasimhan, 1996). Consequently, a lot of emphasis has been given to the identification and incorporation of novel natural antioxidants in food products. The area of natural antioxidants has been developed enormously in the past decade, mainly because of the increasing limitation on the use of synthetic antioxidants and enhanced public awareness of health issues. Natural antioxidants are generally preferred by consumers since they are considered safe. Rajalakshmi and Narasimhan (1996) listed some advantages and disadvantages of natural antioxidants compared to synthetic antioxidants (Table 1). A wide range of natural antioxidants have been shown to occur in many sources (Table 2). Plants have been reported to contain a high amount of natural antioxidants (Namiki, 1990; Osawa, 1994)

Table 1 Advantages and disadvantages of natural antioxidants in comparison with synthetic antioxidants.

Advantages	Disadvantages
Readily accepted by the consumer, as considered to be safe and not a "chemical."	Usually more expensive if purified and less efficient if not purified
No safety tests required by legislation if a component of a food that is " generally recognized as safe" (GRAS).	Properties of different preparations vary if not purified.
	Safety often not known.
	Many impart color, aftertaste, or off-flavor to the product.

Source: Rajalakshmi and Narasimhan (1996).

Table 2 Source of natural antioxidants.

Sources	Example
1. Plant	
Oil seeds	Sesame, Sunflower
Grains	Rice, Wheat
Bean and nut	Soybean, Peanut
Germs	Rice, Wheat
Tea	
Vegetables and fruits	Onion, Potato, Tart cherries
Leaves and leaf waxes	*Eucalyptus, Prunus*
Bark and roots	*Eucalyptus*
Spices and herbs	Rosemary, Sage
Medicinal plants	Wakanyaku (*Osbeckia chinensis*)
Algae and marine products	Susabinori (*Porphyra yezoensis*)
2. Microbial products	
Fermented soybean products	Tempeh, Natto, Miso, Shoyu
3. Protein hydrolysates	
4. Amino-carbonyl reaction products	Melanoidine
5. Animal products	
6. Others	

Sources : Namiki (1990); Osawa (1994).

3.2.1 Plant antioxidants

A. Dry beans

Dry beans (*Phaseolus vulgaris*) contain considerable amounts of phenolic compounds that possess varying degrees of antioxidant activity. Onyenoho and Hettiarachchy (1991) reported an antioxidant property in the extract from navy bean hull. The extract was more protective against the oxidation of soy and sunflower oils than BHA-BHT mixture and rosemary AR but was less effective than TBHQ. Tsuda *et al.* (1994a) studied the antioxidant activity of three varieties of pea bean (*Phaseolus vulgaris*) with white, red and black seed coats. The extracts prepared from red and black seed coat exhibited strong antioxidant activities. The activities were not significantly different from α-tocopherol at 200 μg ($p < 0.05$). The main antioxidants in the extract prepared from red bean seed coat were determined to be cyanidin 3-*O*-*β*-D-glucoside and pelargonidine 3-*O*-*β*-D-glucoside. However, delphinidin 3-*O*-*β*-D-glucoside was the main antioxidant in the extract from black bean seed coat. On the other hand, seed coat extract from white bean had no antioxidant activity. These results were in contrast with the result of Tsuda *et al.* (1993a) who reported a high antioxidant activity in methanol extract from pea bean with a white seed coat. The activities of 0.2 or 1.0 mg extract were higher than 0.2 mg of α-tocopherol. Ganthavorn and Hughes (1997) also reported a considerable antioxidant activity in methanol extract from Great Northern bean with white seed coat. The extracts from dry beans (*Phaseolus vulgaris*) including pinto, kidney, pink, and black bean showed higher antioxidant activities than BHA, PG and ascorbic acid (Ganthavorn and Hughes, 1997).

B. Soybean

Soybean (*Glycine max* L.) has been used advantageously in many food products for nutritional and/or functional reasons. Many researchers have studied an antioxidant property of soybean and soy products. Pratt (1972) reported that the water extracts of fresh soybean, dried soybean, soy protein concentrate and defatted soy flour possessed antioxidant activities. Methanol extract of the dried whole soybean was found to exhibit potent antioxidant activity (Hammerschmidt and Pratt, 1978). Soy products including defatted flour, soy protein concentrate and soy protein isolate showed antioxidant property in lipid-aqueous system (Rhee *et al.*, 1981; Wu and Brewer, 1994). Furthermore, Murakani *et al.* (1984) reported that tempeh, a fermented soybean product, possessed antioxidant activity. Polyphenolic antioxidants from soybean and soy products were found to be isoflavone, chlorogenic acid, caffeic acid, furulic acid and *p*-coumaric acid. These compounds occurred primarily as glycosides. The isoflavone glycosides were identified as genistein, daidzein and glycitein. The antioxidant activity of these compounds in β-carotene/linoleic acid systems are illustrated in Figure 4 (Pratt and Birac, 1979).

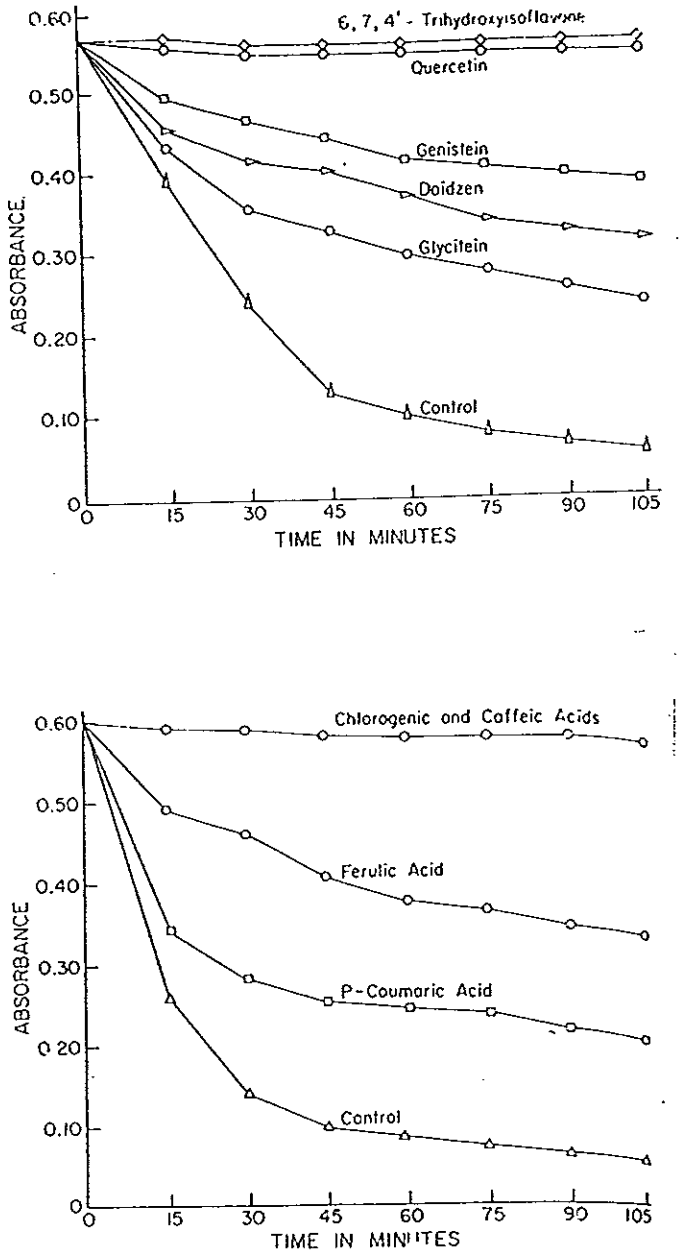

Figure 4 Antioxidant activity of isoflavones and cinnamic acid and derivatives of soybean and quercetin determined spectrophotometrically by the coupled oxidation of β-carotene and linoleic acid.

Source: Pratt and Birac (1979).

C. Peanut

Peanut (*Arachis hypogea,* Var. spanish) is one of principle agricultural plants in the world. Some researchers have studied the antioxidant property of peanut. Rhee *et al.* (1981) reported that peanut products including defatted flour, peanut protein concentrate and peanut protein isolate possessed antioxidant activities in lipid peroxidation model system (catalyzed by metmyoglobin and Fe^{2+}-EDTA) and autoxidation of safflower oil. Pratt and Miller (1984) reported dihydroquercetin as an antioxidant in methanol extract from peanut. Moreover, methanol extract from peanut hulls was found to exhibit marked antioxidant activity and an antioxidant component of methanol extract was identified as luteolin (Duh *et al.,* 1992).

D. Mung bean.

Mung bean (*Phaseolus aureus*) is a leguminous seed. The methanol extract prepared from mung bean hulls exhibited an antioxidant activity. The activity of extract at 100 ppm was stronger than α-tocopherol at 100 ppm or BHA at the same concentration (Duh *et al.,* 1997).

E. Tamarind

Tamarind (*Tamaridus indica* L.) belongs to Leguminosae, and the place of its origin is said to be Africa. The pulp is used as spices and seasoning, and accepted as a herb medicine in many parts of the world (Tsuda *et al.*, 1994b). Tsuda *et al.* (1993b) reported that the extract of tamarind seed showed antioxidant property. Antioxidant components of the extract were identified as 2-hydroxy-3′,4′-dihydroxyacetophenone, methyl 3,4-dihydroxybenzoate, and 3,4-dihydroxyphenyl acetate (Tsuda *et al.,* 1994).

F. Herbs and spices

Herbs and spices have been used for many centuries to improve flavor and to extend the shelf life of various foods. Several studies have demonstrated the antioxidant activity of herb and spice extracts. Nishina *et al.* (1991) found that musizin, an active component isolated from *Rumex joponicus* Houtt, had higher antioxidant activity than BHT and α-tocopherol. 7,4′-dimethylquercetin, 3′-methylquercetin, quercetin and isoquercetin isolated from the leaves of *Polygonum hydropiper*, a culinary herb, showed stronger antioxidant activity than α-tocopherol (Haraguchi *et al.*, 1992). Screening of 180 different types of oriental herbs revealed that 44 species had quite strong antioxidant activities (Kim *et al.*, 1994). Milovanovic *et al.* (1996) found that the extract of *Anthriscus sylvestris*, the Serbian plant, which has been widely used in folk medicine as tonics, diuretics and salad dressing possessed antioxidant activity.

The presence of antioxidants in spices, especially rosemary and sage, is well known (Chang *et al.*, 1977). Carnosol was an active antioxidant component in rosemary (Wu *et al.*, 1982). Cuvelier *et al.* (1994) reported that six active antioxidant components in sage were identified as carnosol, carnosic acid, rosmadial, rosemanol, epirosmanol and methyl carnosate. Gallic acid and eugenol were identified as the 2 major antioxidants in clove (Kramer, 1985). Farag *et al.* (1989) revealed that essential oils from some spices including caraway, clove, cumin, rosemary, sage and thyme inhibited linoleic oxidation. Curcumin, demethoxycurcumin and bisdemethoxycurcumin were active antioxidant components in ginger (Lee *et al.*, 1986; Jitoe *et al.*, 1992; Kikuzaki and Nakatani, 1993). Economou *et al.* (1991) revealed that the extracts from plants of the family Labiatae including, oregano, dittany, thyme, marjoram, spearmint and lavender showed antioxidant property.

G. Tea

Tea is one of the most popular beverages in the world. Tea is an excellent dietary resource for antioxidants known as polyphenolic compounds (Zhu *et al.*,1992). Chen *et al.* (1996) studied the antioxidant property of the extracts from various Chinese teas including green, yellow, white, black, dark-green, and oolong teas. Ethanolic extracts from green, yellow and white 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 black, dark-green and ginseng teas showed little or no protection to canola oil from lipid oxidation, probably due to the complete destruction of natural polyphenols by fermentation during manufacturing processes. Frankel *et al.* (1997) reported that the extract of green teas exhibited antioxidant activity in soybean lecithin liposome system without copper catalyst. The major antioxidants from green tea were catechin and its derivatives (Matsazaki and Hara, 1985). Antioxidant activity of green tea catechin extract was more effective than the rosemary extract against lipid oxidation in canola oil, pork lard and chicken fat (Chen *et al.*, 1998).

H. Cereals

Cereals are among the most common food components and can be added to many food products. Some researcher has studied the antioxidant properties of some cereal crops. Ramarathnam *et al.* (1988) reported that the methanol extracts of rice hull from Katakutara and Kusabue seeds exhibited stronger antioxidant activity than α-tocopherol. Isovitexin was a major antioxidant in rice hull (Ramarathnam *et al.*, 1989). Wu *et al.* (1994) found that the extract of wild rice possessed antioxidant activity. Furthermore, the extract from wild rice hull showed antioxidant property (Asamarai *et al.*, 1996). The extract of oat inhibited the oxidation of soybean and cottonseed

oils (Tian and White, 1994). Antioxidant activity was also present in buckwheat seed (Oomah and Mazza, 1996). Velioglu *et al.* (1998) found that buckwheat hull contained greater antioxidant activity than buckwheat seed.

I. Leaves

Osawa and Namiki (1981; 1985) carried out screening of 76 different kinds of plant leaf waxes. Strong antioxidant activity was found in those extracts from *Eucalyptus* and *Prunus* plant species. Two active components in leaf waxes of *Eucalyptus* were identified as n-tritriacontane-16,18-dione and 4-hydroxy-tritriacontane-16,18-dione (Osawa and Namiki, 1981; 1985). The extract from young green barley leaves possessed antioxidant activity and the active component was identified as 2″(3″)-*O*-glycosylisovitexin (Osawa *et al.,* 1992). The extract of banana leaf (*Musa zebrina*) showed antioxidant property (Sekiya, 1985). Chevolleau *et al.* (1992) screened antioxidant activity in leaves of sixteen Mediterranean plants and found that myrtle showed the highest antioxidant activity.

J. Vegetables and fruits

Phenolic antioxidants are found in many fruits and vegetables. Yi *et al.* (1997) screened 14 varieties of grapes and found strong antioxidant activity in those extract of red table grape varieties Red Globe and Emperor and white wine grape varieties Chardonnay and Sauvignon Blane. The extract of tart cherry, sweet cherry and blueberry exhibited antioxidant property (Wang *et al.*, 1999; Velioglu, 1998). Cao *et al.* (1996) screened antioxidant activity of 22 common vegetables and kale was found to have highest antioxidant activity, followed by garlic, spinach, brussels sprouts, alfalfa sprouts, broccoli flowers, beets, red bell pepper, onion, eggplant, corn, cauliflower, potato, sweet potato, leaf lettuce, cabbage, string bean, carrot, yellow squash, iceberg lettuce, celery and cucumber, respectively. Burdock, a vegetable which is consumed and has been used in beverages in China for centuries, has been reported to have antioxidant property (Duh, 1998).

4. Extraction of antioxidants from natural plants

Many different organic solvents have been used for the extraction of antioxidant compounds from plants. Pratt (1972) reported that the extracts of fresh soybean and dried soybean prepared from hot water possessed higher antioxidant index than the extracts prepared from cold water. However, the extracts of soy protein concentrates (SPC) and defatted soy flour prepared from hot and cold water had the same activity (Table 3).

Table 3 Antioxidant activity of extract from soy bean and soy products using different extract solutions.

Extract	TBA no. of roast beef slices at 30 °C		Antioxidant index
	3 days	6 days	
Fresh soybean –hot	2.1	4.0	38
Fresh soybean –cold	10.8	12.7	0.5
Dried soybean –hot	2.3	4.0	63
Dried soybean –cold	9.7	12.9	1.0
SPC – hot	1.3	1.7	80
SPC – cold	1.5	1.6	80
Defatted soy flour – hot	1.5	1.8	80
Defatted soy flour – hot	1.5	2.0	80
Control	9.0	14.7	-

Source : Pratt (1972).

Chang *et al.* (1977) found that rosemary and sage had a higher antioxidant activity when the more polar solvents were used for extraction. Nishima *et al.* (1991) observed that the 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. 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). Peanut hull was extracted using different solvents including methanol, ethanol, acetone, chloroform and n-hexane. Methanol extract exhibited the highest yield and strongest antioxidant activity (Duh *et al.*, 1992). Among four solvent extracts prepared from tamarind seed coat using ethyl acetate, ethanol, methanol and 1:1 ethyl acetate:ethanol, ethyl acetate extract showed the strongest antioxidant activity and its activity was stronger than α-tocopherol ($p < 0.05$) (Tsuda *et al.,* 1994b) (Figure 5).

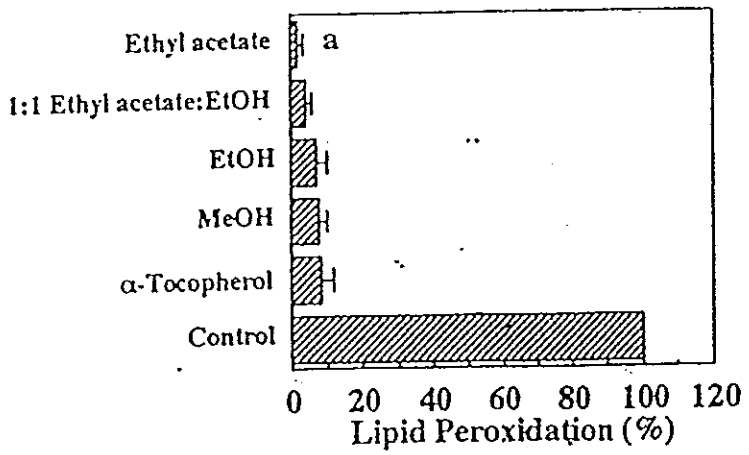

Figure 5 Antioxidant activity of tamarind seed extract with four solvents.
Source : Tsuda *et al.* (1994b)

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). Kallithraka *et al.* (1995) reported that methanol was the best solvent for the quantitative extraction of (+) – catechin, (-)- epicatechin, and epigallocatechin from grape seeds. Duh (1998) studied the effect of various solvents including water, methanol, ethanol, chloroform and n-hexane on the extraction of antioxidant components from burdock. Among the solvents used for extraction, water yielded the greatest amount of extract, which exhibited the strongest antioxidant activity (Table 4).

Table 4 Yield and antioxidant activity of burdock extracted with different solvents.

Solvents	Yield (g)[a]	Antioxidant activity (%)
Water	1.63 ± 0.012 A	96.3 ± 0.047 A[b]
Methanol	1.52 ± 0.014 B	94.4 ± 0.205 B
Ethanol	0.69 ± 0.047 C	92.8 ± 0.368 B
Chloroform	0.05 ± 0.001 D	15.9 ± 1.249 C
n- Hexane	0.03 ± 0.001 D	0.00 ± 0.000 D

[a] Based on 2.5 g of burdock for each solvent. Values are mean ± standard deviation of three replicate analyses.

[b] Means within a column with the same letter are not significantly different ($p > 0.05$).

Source : Duh (1998).

Przybylski *et al.* (1998) reported that the methanol extract of buckwheat seed had stronger antioxidant activity than hexane, diethyl ether, ethyl acetate and acetone extracts. Moreover, methanol, acetone, ethyl acetate, and hexane extracts, and the chloroform–soluble and water-soluble fraction from the chloroform-methanol extract of susabinori exhibited higher antioxidant activities than α-tocopherol, while the hot water extract showed low activity (Nakayama *et al.*, 1999).

5. Some properties of antioxidants from plants

5.1 Heat stability

Ideal food-grade antioxidants should be survived after processing and be stable in the finished product (carry-through) (Shahidi and Wanasundard, 1992). Thermal decomposition of some phenolic antioxidants including BHA, BHT, TBHQ and propyl gallate was reported by Lee *et al.* (1986). After heat treatment at 185 °C for 1 hr, the loss of antioxidant activities for BHA, BHT, TBHQ and propyl gallate was 42.8, 20.4, 47.7 and 37.1 %, respectively. Lee *et al.* (1986) found that the crude extract of ginger rhizome was fairly heat stable with 2/3 of the original activity remained after heating at 100 °C for 2 hrs. Methanol extract from peanut hull had good thermal stability and showed an 85.2 % inhibition of peroxidation of linoleic acid after heated at 185 °C for 2 hrs (Yen and Duh, 1993) (Figure 6). Tsuda *et al.* (1993a) reported that antioxidant activity in fraction 1 isolated from pea bean extract was completely stable after heating at 100 °C for 1 hr. 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). Similarly, Duh *et al.* (1997) reported that mung bean hull extract exhibited antioxidant activity in heated oil. Stability of soybean oils added with oat extracts was higher than that of oils treated with BHT and TBHQ (Duve and White, 1991). Asamarai (1996) evaluated the heat stability of wild rice hulls extract. Extracts exposed to 60 and 100 °C were not significantly different in antioxidant activity from the control ($p > 0.05$). Thus, the wild rice hull extract was heat stable. Yen and Lee (1997) noted that the ethyl acetate extract from *Aspergillus candidus* CCRC 31543 broth filtrate displayed good thermal stability when heated at 185 °C for 2 hrs.

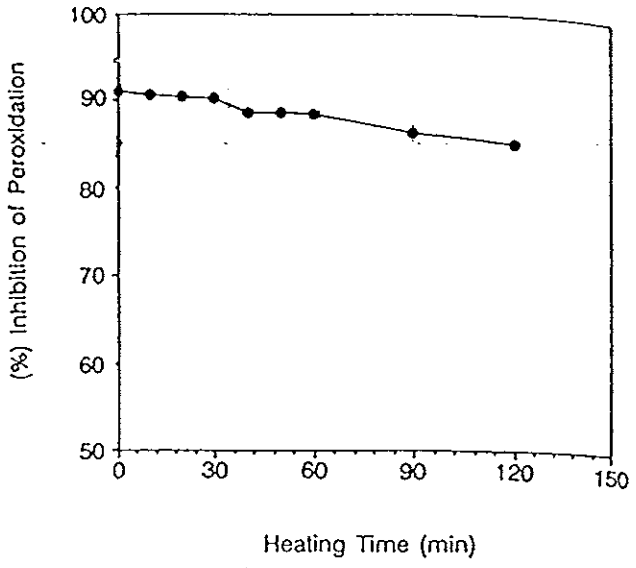

Figure 6 Antioxidant activity of methanol extract from peanut hull as a function of heating time at 185 °C.

Source: Yen and Duh (1993).

5.2 Effect of pH

When the antioxidants are used in food system, their effectiveness will depend on various factors, e.g. pH of food. Lipid peroxidation appears to be accelerated at the alkaline condition (Buck, 1985). Lee *et al.* (1975) reported that oxygen uptake rate did not change between pH 5.0 - 7.0 but increased two and four times at pH 7.5 and 8.0, respectively. Rate of linoleate oxidation catalyzed by beef homogenate at pH 7.0 was slower than that observed at pH 5.6 (Rhee *et al.*, 1979). Lee *et al.* (1986) observed 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. At alkaline pH, the antioxidant activity was decreased. The pigments isolated from red and black beans, pelargonidin 3-*o*-β-D-glucoside and delphanidin 3-*o*-β-D-glucoside, did not show antioxidant activity in linoleic acid system at pH 7.0,

but cyanidin 3-*o*-β-D-glucoside exhibited strong activity. Pelargonidin 3-*o*-β-D-glucoside and delphanidin 3-*o*-β-D-glucoside exhibited strong activity at both pH 3.0 and 5.0 (Tsuda *et al.,* 1994). Similarly, malvidin 3,5-diglucoside showed antioxidant activity in acidic condition (Igarashi *et al.*, 1989). The antioxidant activity of methanol extract from peanut hulls was dependent on pH. The activity decreased with an increase in pH from 3.0 to 9.0. The extract exhibited high activity at neutral and acidic pHs but no activity was observed at alkaline pH (Yen and Duh 1993). Yen and Lee (1997) reported that the ethyl acetate extract from *Aspergillus candidus* CCRC 31543 broth filtrate exhibited strong antioxidant activity at the neutral (pH 7.0) and the acidic pHs (pH 3.0 and 5.0). However, it was unstable at alkaline pH (pH 9.0). In addition, Barclay and Vinqvist (1994) indicated that the antioxidant activity of Trolox is slightly higher at pH 4.0 than pH 7.0, but Trolox was completely dissociated at pH 11. Carnosol and carnosic acid had higher antioxidant activity at low pHs (Frankel *et al.,* 1996). These antioxidants may have better reducing capacity at the lower pHs.

5.3 Effect of concentration

Antioxidant concentration is closely related to antioxidant activity, radical scavenging activity and reducing power. Dziezak (1986) reported that antioxidant activity of α-tocopherol was concentration-dependent, and the most effective concentrations were in the range of 0.01-0.02 %. However, Cillard *et al.* (1980) noted that α-tocopherol showed a pro-oxidant effect as the ratio of α-tocopherol/linoleic acid was $\geq 5 \times 10^{-3}$. Induction period of methyl linoleate is proportional to the concentration of musizin (Nishina, 1991). Similarly, the activity of extract from ginger rhizome, leaves of *Polygonum hydropiper*, pea bean, ajowan seed, canola seed, wild rice and *Anthriscus sylvestris* were concentration dependent (Lee *et al.*, 1986; Haraguchi *et al.*, 1992; Tsuda *et al.*, 1993; Mehla *et al.*, 1994; Wanasundara and Shahidi,

1994; Wu *et al.*, 1994; Milovanovic, 1996). Duh *et al.* (1992) observed that the antioxidant activity of the extract from peanut hull increased with increasing amount (50-100 µl), and then no significant differences in activity ($p > 0.05$) were obtained with concentration ranging from 100 to 500 µL (Figure 7). The activity of extracts from mung bean hull and *Aspergillus candidus* did not increase when the activity reached maximum at 100 ppm and 200 ppm, respectively (Duh *et al.*,1997; Yen and Lee, 1997). Furthermore, the reducing power of the extract from peanut hull increased with an increase in concentration. The antioxidant activity, reducing power and scavenging effect of mung bean hull and burdock extracts were concentration dependent (Duh *et al.*, 1997; Duh, 1998). Duh (1998) noted that the extract of burdock showed a higher antioxidant activity, reducing power and radical scavenging effect when concentration increased.

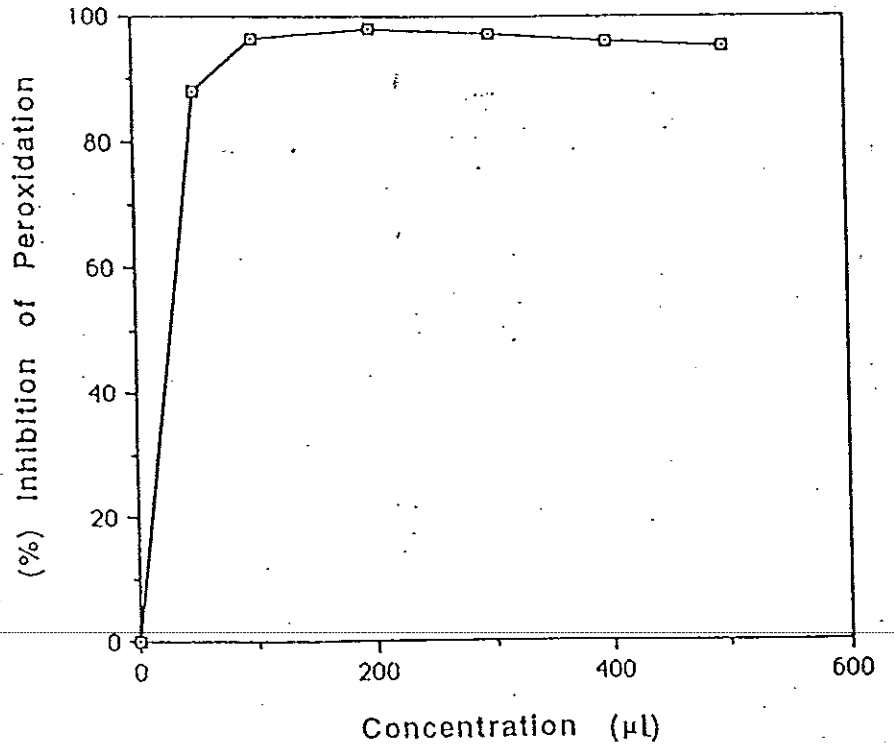

Figure 7 Antioxidant activity at different concentrations of peanut hull extract. Source : Duh *et al.* (1992).

5.4 Synergistic effect

In general, the actions of antioxidants may be influenced by the synergistic components in food system. Combination of the extracts with antioxidant activity and some known antioxidants may potentially retard lipid-peroxidation. Nishima *et al.* (1991) reported that the extract from roots of *R. japonicus* Houtt has a synergistic effect with tocopherol but no synergism was observed for the combination with L-ascorbic acid. Yen and Lee (1997) noted that 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. The extract from pea bean (*Phaseolus vulgaris* L.) had a synergistic effect with α-tocopherol in linoleic acid and liposome system (Tsuda *et al.*, 1993). The mixtures of α-tocopherol, water extract of burdock and hot water extract of burdock exhibited a remarkable synergistic antioxidant effect in a liposome system (Duh, 1998). Similarly, a synergistic effect was observed when 100 ppm of the extract from mung bean hulls was mixed with 100 ppm of α-tocopherol (Duh *et al.*, 1997). Moreover, the combination of carnosine and ascorbic acid was very effective in inhibition of metmyoglobin formation and brown color development in a ground beef pattie model system (Lee *et al.*, 1999). Nevertheless, no synergistic effect of ascorbic acid, citric acid, cysteine or α-tocopherol was observed on the inhibitory effect of the extract from peanut hull (Yen and Duh, 1993).

6. Mode of action of natural antioxidants

The addition of antioxidants to food is effective in retarding the oxidation of fats. Antioxidants have different modes of action, e.g. radical scavenger, peroxide decomposer, etc. However, their main mode of action has been known to be as free radical scavenger (Nakayama *et al.*, 1994). Much literature on the mode of action of antioxidants is available.

6.1 Radical scavenging activity

Yen and Duh (1994) reported the scavenging property of methanol extracts from peanut hulls (MEPH) on free radical. MEPH showed marked activity as a radical scavenger when 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), a stable free radical, was used. The radical scavenging activity was also observed in the extracts from *Vitis vinifera* cell cultures, corn bran hemicellulose fragments, mung bean and burdock (Teguo *et al.*, 1998; Ohta *et al.*, 1994; Duh *et al.*, 1997; Duh, 1998). The extract of buckwheat isolated with polar solvent (methanol) contained higher amount of components, which act as hydrogen donors, than the extract isolated with nonpolar solvent (hexane) (Przybylski *et al.,* 1998). Rosmariquinone, an *ortho*-quinone diterpenoid found in rosemary, acted as a hydrogen-donating antioxidant. Shimada *et al.* (1992) reported that ascorbic acid showed hydrogen-donating activity.

Therefore, the antioxidant activity of extracts may be attributed to its hydrogen-donating ability. Those extracts can work as primary antioxidant to react with lipid radicals.

6.2 Reducing power

The antioxidant effect has been reported to be concomitant with development of reducing power. The extract of peanut hull had reducing power and the correlation between antioxidant activity and reducing power was highly positive ($r = 0.9793$, $p < 0.05$) (Yen and Duh, 1993). Duh *et al.* (1997) observed that the reducing power in the extract of mung bean hull was contributing to its antioxidant activity. The extract of burdock had increased reducing power as concentration increased (Duh, 1998).

6.3 Chelating activity

Although chelating agents are not antioxidants, they play a valuable role in the stabilization of fatty foods against rancidity. Shimada *et al.* (1992) found that xanthan had Fe^{2+}-binding ability. Fe^{2+} ion is the most powerful pro - oxidant among various species of metal ions (Halliwell and Gutteridge, 1984). Ganthavorn and Hughes (1997) observed that the extracts of five cultivars of beans including pinto, kidney, Great Northern, pink, and black bean effectively inhibited iron-catalyzed oxidation of soybean oil, probably by chelating metal ions. Chen and Ahn (1998) studied the antioxidant activities of six natural phenolics including quercetin, rutin, catechin, sesamol, ferulic acid and caffeic acid against lipid oxidation induced by Fe^{2+} and found that all phenolics except sesamol and ferulic acid acted as Fe^{2+}-chelators. However, the extract from peanut hull showed no chelating effect on Fe^{2+} and Cu^{2+} (Yen and Duh, 1994).

6.4 Lipoxygenase inhibitor

Lipoxygenase catalyzes the oxygenation of polyunsaturated fatty acids containing a *cis,cis*-1-4 pentadiene system to hydroperoxide. The inhibition of lipoxygenase by the extract from rice hulls was different, depending on cultivars. Long – life types (Katakutara, Century Patna, Koshihikari) showed higher lipoxygenase inhibitory activity than short–life cultivars (Kusabue, Himenomochi, Sachiwatari, Koshihikari) (Ramarathnam *et al.*, 1986). Chen *et*

al. (1992) observed that carnosol, carnosic acid and ursolic acid, isolated components from rosemary extracts, showed strong inhibitory effect on lipoxygenase activity. Carnosol was more effective in lipoxygenase inhibition, compared to carnosic acid and ursolic acid. Chlorogenic acid and flavonoids including kaempferol, quercetin, myricetin, quercitrin, isoquercitrin, rutin, astragalin, fisetin, dihydroquercetin, (-)-epicatechin, (+)-catechin and epigallocatechin showed inhibitory effect on lipoxygenase oxidation of linoleic acid (Richard-Forget *et al.*, 1995).

7. Application of natural antioxidants.

Extracts from various natural sources have been shown to suppress lipid oxidation in different food systems as follow:

7.1 Oils

Sheabar and Neeman (1988) found that the extract from rape of olives at a level of 100 ppm inhibited the oxidative deterioration of refined olive or soybean oil stored in the dark at 100 ^{o}C. Farag *et al.* (1989) reported that the essential oil of thyme and clove inhibited cotton oil oxidation in concentration dependent manner, and the antioxidant activity of clove oil was higher than thyme oil. The antioxidant effect of musizin on the oxidation of six type of fats and oils was higher than δ-tocopherol and BHA (Table 5) (Nishima *et al.*, 1991).

Table 5 Comparison of antioxidant effect of musizin with that of BHA and δ-tocopherol on the oxidation of various oils.

	Induction period (days)			
Oil	Control	δ-tocopherol	BHA	Musizin
Corn	9.0	9.6	9.9	15.9
Rapeseed	9.3	11.4	10.2	15.9
Palm	35.4	39.6	34.5	48.6
Soybean	6.3	6.3	6.3	8.1
Beef tallow	12.0	52.5	24.0	54.0
Lard	5.4	15.6	19.2	30.6

Source: Nishina *et al.* (1991).

The extract of navy bean hull inhibited formation of peroxides in soy and sunflower oils more effectively than BHA-BHT conbination and rosemary AR but it was less effective than TBHQ (Onyeneho and Hettiarachchy, 1991). Wanasundara and Shahidi (1994) observed that the canola oils treated with the canola extract at 500 and 1000 ppm were more stable than oils treated with BHA, BHT, and BHA/BHT/monoglyceride but it was less effective than TBHQ. The peroxide values of soybean and cottonseed oils added with oat extract were lower than the control but it was slightly higher than the oils treated with TBHQ. However, the peroxide values of the emulsions of the same oils containing the oat extract were lower than the emulsions added with TBHQ (Tian and White, 1994). Frankel and Huang (1996) reported that the carnosol and carnosic acid effectively inhibited conjugated diene and hydroperoxide formation in corn oil, soybean oil, fish oil and peanut oil. In contrast, those test compounds were either inactive or promoted oxidation in the emulsions of the same oils. Furthermore, the soybean and peanut oils

with 0.48 and 1.2 % of the extract from peanut hull were superior to those added with 0.02 % BHA in reducing lipid oxidation (Duh and Yen, 1997).

7.2 Lard

Yen and Duh (1993) reported that the methanol extract of peanut hull showed good inhibitory activity in lard oxidation when compared to BHA. Addition of the extract from *Psoralea corylifolia* and *Sorphora angustifolia* Siab. & Zucc. greatly decreased the peroxide formation of lard during storage. Treatment with 0.02 % methanol extract of *Psoralea corylifolia* exhibited sinificantly stronger antioxidant effect on the oxidation of lard than treatment with 0.02 % BHA ($p < 0.05$) (Kim *et al.*, 1994). Milovanovic *et al.* (1996) showed that the extract of *Anthriscus sylvestris* was superior to quercetin, apigenin, or a tocopherol mixture in reducing oxidation of lard.

7.3 Meat and meat products.

Rosemary oleoresin inhibited oxidative change in the turkey sausage (Barbut *et al.*, 1985). No differences in TBA values between the turkey sausage treated with rosemary oleoresin and BHA/BHT/citric acid were found. Ginger rhizome prevented oxidation in fresh, frozen and precooked pork patties (Lee *et al.*, 1986). Ground beef containing 900 ppm of soy protein isolate antioxidant had lower TBA numbers, less rancid odor, hexanal and total volatile after 16 and 24 hr than samples containing 300 ppm of soy protein isolate antioxidant and control (Wu and Brewer, 1994). Wu *et al.* (1994) observed that wild rice extract substantially reduced rancidity in ground beef. Similarly, Asamarai *et al.* (1996) found that the ground beef treated with wild rice hull extract had lower thiobarbituric acid reactive substances values (TBARS) than the control. TBA values of raw or cooked ground beef containing fenugreek extracts were lower than that of control ($p < 0.05$) (Hettiarachchy *et al.*, 1996). Guntensperger *et al.* (1998) indicated that rosemary extract inhibited lipid oxidation of heat-sterilized meat during storage at 20 $^{\circ}$C. Furthermore, the rosemary oleoresin-sodium tripolyphosphate

combination in precooked roast beef slices was proved to be effective during both refrigerated and frozen storage (Murphy *et al.*, 1998). Lee *et al.* (1999) reported that ascorbic acid inhibited metmyoglobin formation on the surface of ground beef while carnosine inhibited metmyoglobin formation and brown color development throughout the product. However, carnosine was more effective on inhibition of lipid peroxidation than ascorbic acid. Moreover, Lee *et al.* (1998) observed that phytic acid and carnosine inhibited metmyoglobin formation in raw ground beef during storage at 4°C. Phytic acid was more effective than carnosine for inhibition of lipid oxidation.

7.4 Fish

Several antioxidants including quercetin, morin, myricetin, kaempferol, tannic acid, ellagic acid, L-ascorbic acid, and BHT were effective in inhibiting lipid oxidation in raw fish stored at 4 °C for 14 days, while L-ascorbic acid acted as a pro-oxidant in steam and microwave cooked fish (Ramanathan and Das, 1992). Li *et al.* (1998) found that the extract from shrimp shell waste showed antioxidant activity in β-carotene–linoleic acid assay. The antioxidant activity of crude extract was lower compared to the mixture of BHA, BHT and citric acid. Higher a* value and lower TBA values were found in rockfish sample treated with antioxidant from shrimp shell waste compared to the control.

7.5 Others

Ethoxyquin, δ-tocopherol and ascorbic acid were used for prevention of color loss in stored paprika. After 4 months storage at ambient temperature, the control lost 63 % of the initial extractable color. The δ-tocopherol treated samples lost 32 % and the ethoxyquin treated samples lost 6 % color (Osuna-Garcia *et al.*, 1997). BHT, cinnamic acid or vanillin was blended with degermed cornmeal prior to extrusion in an extruder to improve oxidative stability. After 12 weeks, all samples, except for 200 ppm BHT added sample,

had lower peroxide values and conjugated dienes. Cinnamic acid and vanillin protected corn snacks against lipid oxidation more effectively than BHT (Camire and Dougherty, 1998).

Objectives

1. To study the antioxidant activities of extracts from 19 varieties of legume seeds.
2. To study the extraction of antioxidants from some selected legume seeds
3. To study some properties and mode of action of legume seeds extracts.
4. To study the application of legume seed extracts in cooked ground pork.

Chapter 2

Materials and Methods

Materials

1. Legume seeds (19 varieties)

- Wild tamarind (*Leucaena leucocephala*)
- Cow pea (*Vigna unguiculata*)
- Pigeon pea (*Cajanus cajan*)
- Peanuts (*Arachis hypogea*) 2 varieties, including khongan 4 and SB. 38
- Mung bean (*Phaseolus aureus*)
- Black gram (*Phaseolus mungo*)
- Rice bean (*Phaseolus calcaratus*) 2 varieties, including Namung and Tropical.
- Green gram *(Phaseolus radiatus)*
- Horse bean (*Vicia faba*)
- Red bean (*Phaseolus vulgaris*)
- Red kidney bean (*Phaseolus vulgaris*)
- Garden pea (*Pisum sativum*)
- Soybean (*Glycine max*)
- Sword bean (*Canavalia ensiformis*)
- White bean (*Vigna unguiculata*)
- Bambara groundnut (*Voandzeia subterranea* or *Vigna subterranea*) 2 varieties, including Hat Yai 1 and Tropical.

2. Chemicals

2.1 Solvents used for extraction of the antioxidants from legume seeds.

- Methanol
- Ethanol
- Ethyl acetate
- Hexane

2.2 Chemicals for determination of antioxidant activity.

- β-carotene
- Linoleic acid
- Tween 40

2.3 Chemicals for determination of total phenolic compound content.

- Folin and Ciocalteu'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 ($FeSO_4.7H_2O$)
- Hexamethylenetetramine
- 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
- Starch
- Acetic acid : Chloroform 3:2 (vol/vol)
- Iso - octane

2.6 Chemicals for separation of compounds in seed.

- Thin-layer chromatography (precoated silica gel plate, 20 x 20 cm, Kieselgel 60 F_{254}, 0.20 mm, E.Merck, Dramstadt, Germany).
- Butanol : Acetic acid : Water (4:1:5 vol/vol/vol)
- Dichloromethane: Methanol : Water (5:3:2 vol/vol/vol)
- 1 % solution of potassium ferricyanide + 1 % solution of ferric chloride.
- Ammonical silver nitrate solution.

3. Instruments

Instruments	Model	Company	Country
Spectronic 21	SP.21	Spectronic	U.S.A
Spectrophotometer	UV-1601	Shimadzu	Australia
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

Methods

1. Preparation of legume seeds extracts.

All legume seeds (except wild tamarind) were cleaned with water and dried overnight in an air dryer at 50 °C. Dried legume seeds were ground to a particle size of 25 mesh and stored at 4 °C in an airtight container.

To extract antioxidant, seed powder (5.00 ± 0.01 g) was placed in a 250-ml erlenmeyer flask and mixed with 50 ml methanol. The flask was capped with aluminum foil and shaken at 300 rpm overnight at room temperature. The extract was filtered with filter paper (Whatman # 1) and the filtrate was filled to a volume of 50 ml with methanol (Figure 8). Methanol extracts were prepared in duplicates for each legume seed variety and stored at 4°C until analysis.

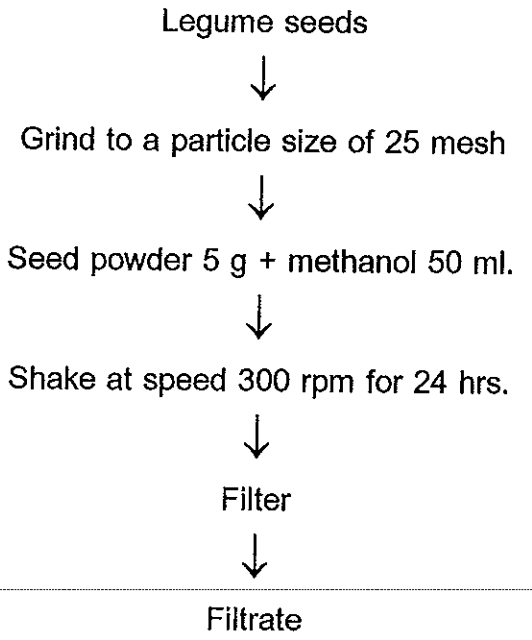

Figure 8 Extraction of antioxidants from legume seeds.

Sources : Modified from Yen and Duh (1992) ; Ganthavorn and Hughes (1997).

2. Primary screening of antioxidant activities in methanol extracts of legume seeds.

Evaluation of antioxidant activity based on coupled oxidation of β-carotene and linoleic acid was conducted as described by Taga *et al.* (1984) with some modification. β-carotene (1 mg) was dissolved in 10 ml of chloroform. A 1.5 ml aliquot of the solution was added to a 50 ml-beaker with 20 mg linoleic acid and 200 mg Tween 40. Chloroform was removed by purging with nitrogen. Oxygenated distilled water (50 ml) was added into the β-carotene emulsion and mixed well. Aliquots (3 ml) of the oxygenated β-carotene emulsion and 0.2 ml of the methanol extracts were placed in spectrophotometer tubes and mixed thoroughly. The tubes were immediately placed in a water bath and incubated at 50 °C. Oxidation of β-carotene emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm. Absorbance was measured at 0, 10, 20, 30 and 40 min. A control was prepared by using 0.2 ml methanol instead of seed extracts. Degradation rate of legume seed extracts was calculated according to first order kinetics with following equation: (Al-Saikhan *et al.*, 1995)

$$\ln (a/b) \times 1/t = \text{sample degradation rate}$$

where: ln = natural log

a = initial absorbance (470 nm) at time zero

b = absorbance (470 nm) at time 40 min

t = time (min)

Antioxidant activity (AA) was expressed as % inhibition relative to the control using:

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

The antioxidant activity of the extracts was compared with BHA, BHT, and α-tocopherol at the level of 0.02 mg/ml. Total phenolic content in seed extracts was determined spectrophotometrically by measuring absorbance at 640 nm (Weurman and Swain, 1955). Scavenging activity and reducing power of the extracts were measured according to the method of Blois (1958) and Oyaizu (1986), respectively. Correlation between antioxidant activity and total phenolic content was determined. Two legume seeds, which provided the highest antioxidant activity, were selected for further studies.

3. Extraction of antioxidants from selected legume seeds.

3.1 Effect of extracting solvents

The various solvents including methanol, ethanol, ethyl acetate and hexane were used for extracting antioxidants from selected legume seeds. The extracts were evaluated for antioxidant activity, total phenolic content and reducing power according to the method of Taga *et al.* (1984), Weurman and Swain, (1955) and Oyaizu (1986), respectively. The solvent rendering the highest antioxidant activity was chosen for further studies.

3.2 Effect of methanol to 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 legume seeds. The different extracts were evaluated for antioxidant activity, total phenolic content and reducing power according to the method of Taga *et al.* (1984), Weurman and Swain, (1955) and Oyaizu (1986), respectively. The ratio of methanol to water, which gave an extract with the highest antioxidant activity was chosen for further studies.

3.3 Effect of extraction time and repetition

Various extraction times (2, 5, 10, 15, and 24 hrs.) and repetitions (1, 2, and 3) were used to extract the antioxidants. Experimental design of this research is factorial (5 x 3) in CRD. The extracts prepared as above were evaluated for antioxidant activity and total phenolic content according to the method of Taga *et al.* (1984) and Weurman and Swain (1958), respectively. Optimum condition that rendered the highest antioxidant activity was chosen.

Antioxidants from selected legume seeds were prepared under optimum extracting condition. The extracts were freeze-dried to obtain the antioxidant powder and weighed to measure the yield.

4. Some properties of the extracts from selected legume seeds.

4.1 Effect of concentration

Different concentrations of the extracts (0, 0.02, 0.05, 0.1, 0.3, 0.6, 0.9, 1.2 and 1.5 mg/ml) were used and antioxidant activity was determined according to the method of Taga *et al.* (1984).

4.2 Effect of heat treatment

The extract powder (1.0 mg) was individually placed in 50 ml-beaker and heated in hot air oven at 80 ^{o}C and 100 ^{o}C for 0, 10, 20, 30, 40, 50, 60, 90 or 120 min. The samples were then cooled to room temperature, and dissolved in 0.2 ml 50 % methanol for wild tamarind and 70 % methanol for cowpea seeds, respectively. The solutions were tested for antioxidant activity by the β-carotene bleaching method (Taga *et al.*, 1984).

4.3 Effect of pH

The influence of pH on antioxidant activity of wild tamarind and cow pea seed extracts was studied using 0.2 M phosphate buffer, pH 3, 4, 5, 6, 7, 8 and 9. To reduce interference on β-carotene caused by pH, the control was performed using the same buffer without addition of extract samples. The net absorbance was used to calculate antioxidant activity.

4.4 pH stability

Extract powder (0.05 g) was dissolved in 0.2 M phosphate buffer (100 ml) at various pHs (3, 4, 5, 6, 7, 8 and 9), and kept at room temperature for 1 hr. The samples were then adjusted to a pH of 6.50 ± 0.5 before analysis. The antioxidant activity was determined by β-carotene bleaching method (Taga *et al.*, 1984).

4.5 Synergistic effect

Chemical compounds, including ascorbic acid, citric acid, and α-tocopherol were used to study the synergism with the seed extracts. Synergism of these compounds on antioxidant of the extracts was determined in an aqueous system by using β-carotene bleaching method (Taga *et al.*, 1984). Some factors including extract concentration (0, 0.02 and 0.03 mg/ml) and types of chemical compounds (citric acid, ascorbic acid or α-tocopherol) were evaluated.

5. Mode of action

5.1 Radical-scavenging activity

The radical-scavenging activity was measured from the reaction mixture containing 1,1-diphenyl-2-picryhydrazyl (DPPH), used as a radical source according to the method of Blois (1958). Different amounts of the seed (0.1, 0.2, 0.3, 0.4, 0.8, 1.2, 2, 3 and 4 mg) were tested, and compared with BHA, BHT, and α-tocopherol at a level of 1 mg.

5.2 Reducing power

The reducing power of different amounts of seed extract (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 mg) was measured according to the method of Oyaizu (1986). The reducing power of the extracts was compared with that of ascorbic acid (0.05 mg).

5.3 Chelating activity

The chelating activity of seed extracts was measured according to the method of Shimada *et al.* (1992). The reaction mixture contained 3 mM ferrous sulfate ($FeSO_4$. $7H_2O$) or 3 mM copper sulphate ($CuSO_4$. $5H_2O$) with different amounts of the extract (0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.4, 1.6, 1.8 and 2 mg). The chelating activity of the extracts was compared with that of citric acid, and EDTA at a level of 0.2 M.

5.4 Inhibition of lipoxygenase activity

The effect of seed extracts on the activity of soybean lipoxygenase (LOX) was studied spectrophotometrically, using linoleic acid as a substrate (Surrey, 1964). Different amounts of seed extract (0.05, 0.1, 0.3, 0.5, 0.8 and 1.0 mg) were added into reaction mixture and residual lipoxygenase activity was measured. The inhibitory effect on lipoxygenase activity of the extracts was compared with that of BHA, BHT, and α-tocopherol at a level of 0.1 mg.

6. Separation of antioxidative compounds of wild tamarind and cow pea seed extracts.

Thin-layer chromatography (TLC) with silica gel G plate (20 x 20 cm, 0.20 mm thick, E.Merck) was used. Solvent mixtures used as mobile phase were dichloromethane : methanol: water (5:4:1 vol/vol/vol) for wild tamarind seed extract and n-butanol : acetic acid : water (4:1:5 vol/vol/vol) for cow pea seed extract. Extract powder from wild tamarind seed and cow pea seed was dissolved in 50 % and 70 % methanol, respectively. A volume of 20 µl of 1 % extract was spotted on precoated TLC silica gel plate, which had been activated for 30 min at 105 ^{o}C. TLC plates with sample was developed ascendingly for 10 cm.

After development, the chromatograms were dried and different sprays were used to identify chemical compounds as follows.

Spray 1: 1 % solution of potassium ferricyanide in water and 1 % solution of ferric chloride in water gave a blue color upon reacting with phenolic compounds (Barton *et al.*,1952).

Spray 2: 2 % solution of ferric chloride in ethanol indicated the presence of trihydroxy-phenolics by turning blue, dihydroxy-phenolics by turning green or other phenolics by turning red or brown (Reio, 1958).

Spray 3: Ammonical silver nitrate solution was prepared by mixing 30 ml of ammonium hydroxide and 70 ml water-silver nitrate (3.4 g in 100 ml water). After heating for 10 min at 105 ^{o}C, brown, black and gray streaks were produced as evidence of reducing compounds (Duve and White, 1991).

Bands of interest were scraped from the plate. The silica gel residues which contained the separated compounds were soaked in excess 50 % methanol for wild tamarind seed extract and 70 % methanol for cow pea seed extract for 30 min, filtered using Whatman #42 filter paper. The filtrate

was evaporated to remove methanol by a rotary evaporator at 40 °C, then freeze-dried to obtain each antioxidant fraction. The powder fraction was redissolved in 1 ml of 50 % methanol for wild tamarind seed extract and 70 % methanol for cow pea seed extract. Antioxidant was determined by using the coupled oxidation of β-carotene and linoleic acid assay (Taga *et al.*, 1984).

7. Application of seeds extracts in cooked ground pork.

7.1 Antioxidant efficacy of extracts from seeds in cooked ground pork.

Raw ground pork was purchased from the Makro supermarket in Hat Yai, Songkhla. Fat content was standardized to approximately 20 % (AOAC., 1990). The extract powder was added to raw ground pork at concentrations of 0.02, 0.05 or 0.1 % (w/w). The extract powder was dispersed in 10 ml of 50 % ethanol for wild tamarind seed or 70 % ethanol for cow pea seed before adding to raw ground pork (100 g). The mixtures were mixed very well. A portion of each mixture (50 g) was placed in 200 ml-beaker and cooked to 80 ± 2 °C (core temperature) in a water bath, then cooled in iced bath. The samples were stored in a polyethylene bag for 15 days at 4 °C. Cooked ground pork samples were analyzed for lipid oxidation immediately after cooking (day 0) and after storage at 4 °C for 3, 6, 9, 12 and 15 days. Lipid oxidation was assessed by TBARS (Burge and Aust, 1978), peroxide value (IUPAC, 1979) and conjugated diene (Frankel and Huang, 1996). Lipid extraction was carried out at room temperature to avoid thermal degradation of products. Cooked ground pork (≈ 45 g) was mixed with 200 ml petroleum ether for 10 min. The petroleum ether extract was passed through a Whatman #1 filter paper into 250 round-bottomed flask. Petrolum ether was removed by rotary evaporator at 40-50 °C and lipid fraction was analyzed for peroxide value and conjugated diene. The effect of antioxidants from legume seeds on

lipid oxidation in cooked ground pork was compared with BHA, BHT, and α-tocopherol at a level of 0.02 % (w/w).

7.2 Synergist effect

Two synergists including ascorbic acid and citric acid were used to prevent the oxidation of cooked ground pork with and without seed extracts. Treatments used in this study were shown as follow:

- No additive
- 0.005 % ascorbic acid (w/w)
- 0.01 % ascorbic acid (w/w)
- 0.005 % citric acid (w/w)
- 0.01 % citric acid (w/w)
- Extract powder plus 0.005 % ascorbic acid (w/w)
- Extract powder plus 0.01 % ascorbic acid (w/w)
- Extract powder plus 0.005 % citric acid (w/w)
- Extract powder plus 0.01 % citric acid (w/w)

The extracts at the optimum concentration and synergists were mixed with ground pork, cooked and packaged in polyethylene bags. All samples were stored at 4 ^{o}C for 3, 6, 9, 12, and 15 days. Lipid oxidation in cooked ground porks was assessed by TBARS (Buege and Aust, 1978), peroxide value (IUPAC, 1979) and conjugated diene (Frankel and Huang, 1996).

8. Statistical analysis

The data were subjected to the analysis of variance (ANOVA). The differences among samples were determined by Duncan's multiple range test (Steel and Torrie, 1980).

Chapter 3

Results and Discussion

1. Primary screening of antioxidant activities in methanol extracts of legume seeds.

The decrease in absorbance of β-carotene in the presence of different legume seed extracts in methanol is shown in Figure 9. A sharp decrease in OD_{470} was obtained in the control, indicating a rapid oxidation of β-carotene/linoleic acid. However, a decrease in OD_{470} was retarded when methanol extracts of legume seeds were added. This result suggested that some antioxidants were available in the extracts and played an essential role in prevention of oxidation. The antioxidant activities of extracts from 19 different varieties are presented in Table 6. All methanol extracts from legume seeds showed high antioxidant activities ranging from 87.32 to 97.62 %. Wild tamarind seed extract had highest antioxidant activity, followed by cow pea seed extract. All legume seed extracts, except for the extract of wild tamarind seed, exhibited lower antioxidant activities than BHA and BHT but higher than α-tocopherol (Table 6). Antioxidant activities among different varieties of same seed, e.g. peanut (94.71 %, var. Khongan; 94.43% var. S.B.38), rice bean (93.71%, var. Namung; 94.09% var. Typical), or bambara groundnut (92.17%, var. Typical; 92.53%, var. Hat Yai 1) were not significantly difference ($p > 0.05$). This result is in agreement with Yen and Duh (1995) who reported that the antioxidant activity of peanut hull extract from various varieties were not significantly different ($p > 0.05$).

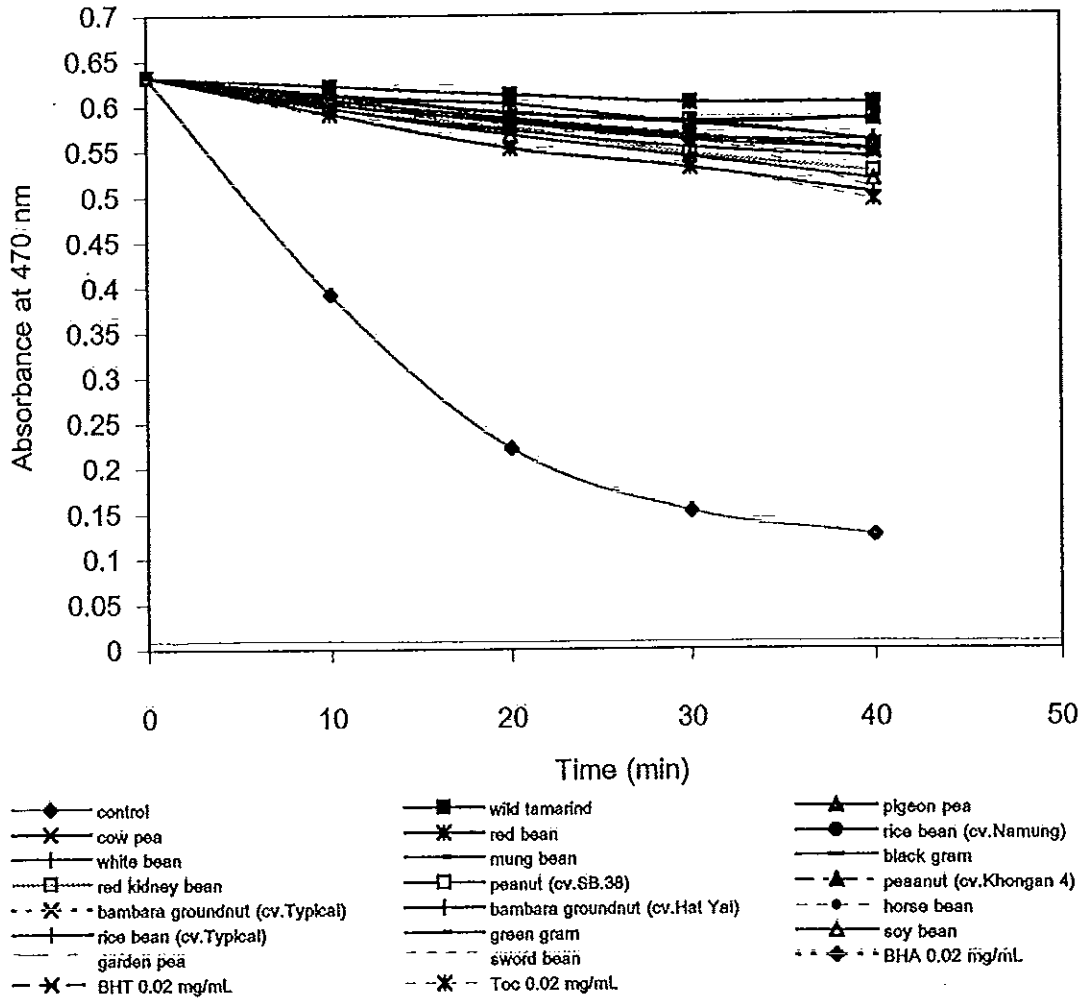

Figure 9 Antioxidant activity of methanol extracts of 19 different varieties of legume seeds assayed by β-carotene bleaching method. BHA, BHT and α-tocopherol at a level of 0.02 mg/L were used as references.

Table 6 Antioxidant activities and total phenolic content of 19 different varieties of legume seeds.

Legume seeds	Antioxidant activity (%)	Total phenolic content (mg/100g)
Wild tamarind	97.62 ± 0.53 k[a]	379.19 ± 0.85 o[b]
Cow pea	95.65 ± 0.57 j	137.68 ± 1.56 l
Pigeon pea	95.21 ± 0.95 ij	47.64 ± 1.66 g
Peanut (var. Khongan 4)	94.71 ± 0.55 hij	218.08 ± 1.73 n
Peanut (var. S.B. 38)	94.43 ± 0.30 hij	150.05 ± 1.01 m
Mung bean	93.57 ± 0.50 gh	42.10 ± 0.72 e
Black gram	93.62 ± 0.36 fgh	81.21 ± 0.98 j
Green gram	94.13 ± 0.73 ghi	117.27 ± 1.33 k
Rice bean (var. Namung)	93.71 ± 1.14 fgh	43.21 ± 0.61 e
Rice bean (var. Typical)	94.09 ± 0.54 ghi	45.45 ± 1.62 f
Horse bean	93.65 ± 0.65 fgh	43.93 ± 1.55 ef
Bambara groundnut (var.Hat Yai 1)	92.17 ± 0.64 e	75.59 ± 1.00 i
Bambara groundnut (var. Typical)	92.53 ± 0.64 ef	36.58 ± 0.90 c
Red bean	92.85 ± 0.06 efg	68.72 ± 0.93 h
Red kidney bean	90.79 ± 0.51 cd	40.07 ± 1.59 d
Garden pea	91.73 ± 1.03 de	25.25 ± 0.64 b
Soybean	89.84 ± 1.02 c	68.08 ± 0.30 h
Sword bean	88.64 ± 1.20 b	24.44 ± 0.37 b
White bean	87.32 ± 0.27 a	20.85 ± 0.78 a

[a] Mean ± standard deviation from triplicate determinations.

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

Table 6 (Continued) Antioxidant activities and total phenolic content of 19 different varieties of legume seeds.

Legume seeds	Antioxidant activity (%)	Total phenolic content (mg/100g)
BHA (0.02 mg/ml)	97.54 ± 0.47	-
BHT (0.02 mg/ml)	97.54 ± 0.47	-
α-tocopherol (0.02 mg/ml)	86.93 ± 0.27	-

[a] Mean ± standard deviation from triplicate determinations.

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

Major differences in phenolic content were observed among legume varieties (Table 6). Total phenolic content of legume seeds in this study varied from 20 to 397 mg/100g. Wild tamarind seed contained the highest total phenolic content. Legume seeds with white seed coat, e.g. sword bean and white bean had lower total phenolic content and antioxidant activity than legume seeds with colored seed coat. Drumm *et al.* (1990) and Ganthavorn and Hughes (1997) found that bean with white seed coat typically had lower polyphenolic compound content than beans with colored seed coats and the white-seed-coated bean had lower antioxidant activity than the colored seed. Additionally, Tsuda *et al.* (1993a) reported that no antioxidant activity in ethanol extracts from pea bean (*Paseolus vulgaris*) with a white seed coat was obtained. However, Onyencho and Hettiarachchy (1991) found that the extract of navy beans with white seed coat possessed antioxidant activity. Some phenolic compounds such as flavonoid have been reported to act as antioxidants (Duh *et al.*, 1993; Lee *et al.*,1995). When plotted, the total phenolic content and antioxidant activities showed a low positive correlation (r^2

= 0.4301) (Figure 10). This result indicated that phenolic compounds were partially contributed to antioxidant activity in legume seeds. Velioglu *et al.* (1998) noted that the relationship between antioxidant activity and total phenolic content of 28 plant product extracts showed a positive correlation (r^2 = 0.4253). Al-Saikhan (1995) reported that low positive correlation between antioxidant activity and total phenolic content of the extracts from various varieties of potatoes. Large variability in antioxidant activity of potato extracts indicated that the trait is related to genetics, implying a potential to breed potatoes with higher antioxidant. However, some reports revealed that a high total phenolic content was associated with high antioxidant activity (Ramarathnam *et al.*, 1986; Yen *et al.*, 1993). Significant differences in total phenolic content were observed between varieties of peanut (var. S.B. 38 and var. Khongan 4), rice bean (var. Namung and var. Typical) and bambara groundnut (var. Hat Yai 1, and var. Typical). For peanut, total phenolic content of var. Khongan4 was significantly higher than that of var. S.B.38. For rice bean, the amount of total phenolic in var. Namung was significantly lower than that found in var. Typical. In bambara groundnut, the total phenolic content of var. Hat Yai 1 was significantly higher than that observed in var. Typical. Differences in total phenolic content between the varieties of same legume seed may be due to differences in genetic, maturity and other factors. Ramarathnam *et al.* (1986) reported that content of total phenolic compound in rice hulls varied with various varieties. Yen and Duh (1995) found that no difference in antioxidant activity was observed among four varieties of peanut hull extracts but total phenolic content from various varieties differed significantly ($p < 0.05$). Yen *et al.* (1993) reported that difference in the total phenolic content of peanut hull extract might be mostly a result of maturity.

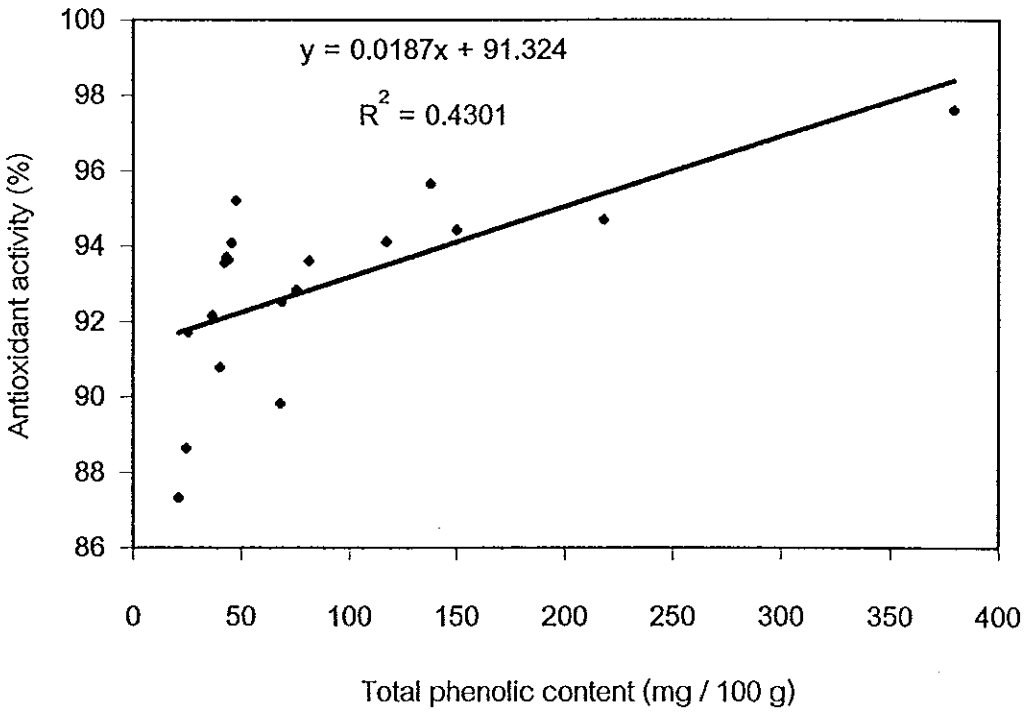

Figure 10 Relationship between total phenolic content and antioxidant activities of 19 different varieties of legume seeds.

Different seed extracts had different reducing power (Table 7). This was probably because the extracts may contain reductones (Duh, 1998). Moreover, all extracts showed marked activity as a radical scavenger in the assay using 1,1-diphenyl-2-picrylhydrazyl radical (Table 7), indicating that all seed extracts had effective activities as a hydrogen donor and acted as a primary antioxidant to react with lipid radicals. Wild tamarind seed extract showed highest radical-scavenging activity and reducing power, possibly leading to the highest antioxidant activity. Sword bean and white bean extracts showed weak radical scavenging and reducing power and the antioxidant activities of both extracts were lowest among all samples tested.

Table 7 Radical-scavenging activity and reducing power of 19 different varieties of legume seeds.

Legume seeds	Radical-scavenging activity (%)	Reducing power (ABS. at 700 nm
Wild tamarind	96.75 ± 0.55 g[a]	0.84 ± 0.00 n[b]
Cow pea	92.11 ± 0.53 de	0.67 ± 0.00 m
Pigeon pea	92.02 ± 0.40 de	0.21 ± 0.00 d
Peanut (var. Khongan 4)	91.75 ± 0.92 cd	0.65 ± 0.01m
Peanut (var. S.B. 38)	91.40 ± 0.30 cd	0.54 ± 0.00 k
Mung bean	90.91 ± 0.50 c	0.12 ± 0.00 b
Black gram	93.51 ± 0.15 ef	0.37 ± 0.02 i
Green gram	93.60 ± 1.29 efg	0.64 ± 0.04 l
Rice bean (var. Namung)	93.27 ± 0.46 ef	0.25 ± 0.02 ef
Rice bean (var. Typical)	92.46 ± 0.15 de	0.27 ± 0.01 fg
Horse bean	93.42 ± 0.65 ef	0.21 ± 0.02 d
Bambara groundnut (var.Hat Yai 1)	92.28 ± 1.06 de	0.15 ± 0.03 c
Bambara groundnut (var. Typical)	93.07 ± 0.92 ef	0.53 ± 0.02 k
Red bean	90.93 ± 0.06 c	0.45 ± 0.02 j
Red kidney bean	91.58 ± 0.26 cd	0.28 ± 0.01 g
Garden pea	91.23 ± 0.03cd	0.11 ± 0.01 b
Soy bean	93.84 ± 0.52 efg	0.34 ± 0.02 h
Sword bean	86.64 ± 0.20 a	0.06 ± 0.00 a
White bean	87.32 ± 0.56 ab	0.11 ± 0.00 b

[a] Mean ± standard deviation from triplicate determinations.

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

These results clearly indicated that legume seeds were rich in natural antioxidants. Qualities and/or quantities of the antioxidants in methanol extracts of the legume seeds seemed to be very different, depending on the varieties of legume seeds. From the results, wild tamarind and cow pea seed showed highest antioxidant activity. Therefore, both seeds were chosen for further study.

2. Extraction of antioxidants from wild tamarind and cow pea seeds.

2.1 Effects of extracting solvents on the antioxidant activities.

2.1.1 Wild tamarind seed.

The decrease in absorbence of β-carotene in the presence of extracts from wild tamarind seed prepared by using different organic solvents is shown in Figure 11. A marked decrease in OD_{470} was observed with the control and hexane extract, indicating a low efficacy in oxidation prevention. The rate of decrease was lowest in methanol extract, followed by ethanol and ethyl acetate extract, respectively. Antioxidant activity, reducing power and total phenolic content of wild tamarind seed extracts are shown in Table 8. Among four extracts, methanol extract had highest total phenolic content, strongest reducing power and highest antioxidant activity. The efficiency of solvents on the antioxidant extraction from wild tamarind seed was in the order of methanol > ethanol > ethyl acetate > hexane. Low positive correlation (r^2 = 0.36) between antioxidant activity and total phenolic content of the wild tamarind extracts prepared from various solvents was found, indicating that phenolic compounds were not entirely contributed to antioxidant activity in wild tamarind seed. Antioxidant activity increased with increasing polarity of solvent. Thus, the polarity of extracting solvents was postulated to be a contributor to antioxidant activity. The reducing power of wild tamarind seed extracts increased in following order: methanol > ethanol > ethyl acetate > hexane.

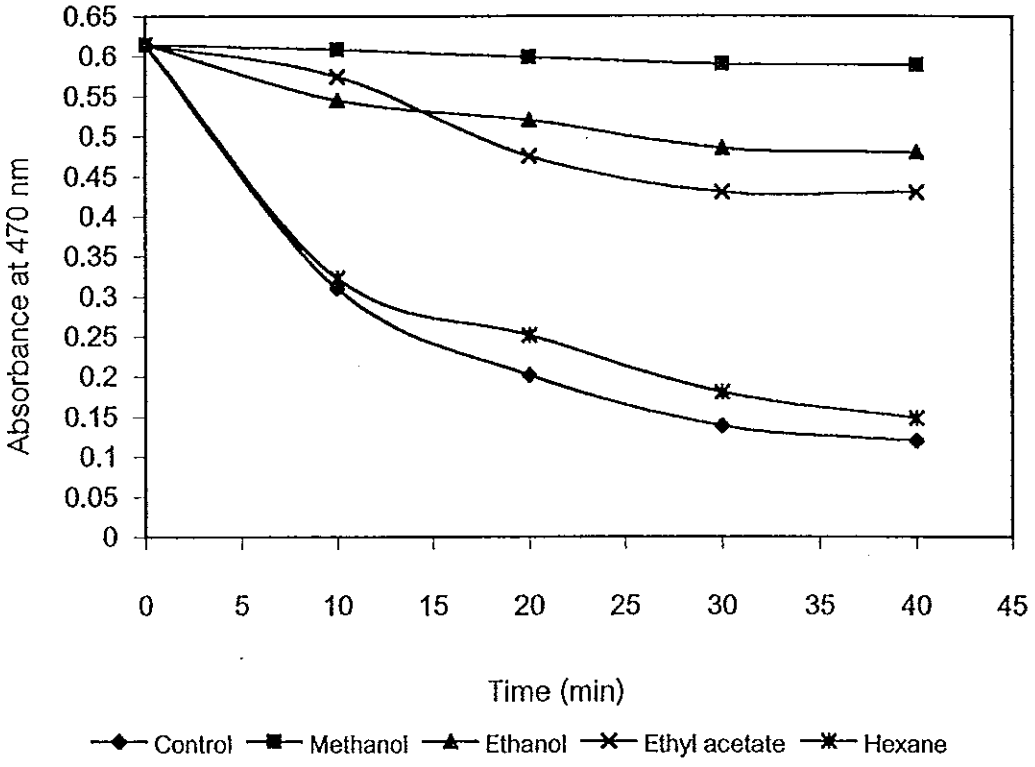

Figure 11 The decrease in absorbance of β-carotene in the presence of wild tamarind seed extracts prepared with different organic solvents assayed by the β-carotene bleaching method.

Table 8 Antioxidant activity, reducing power and total phenolic content of wild tamarind seed extract prepared with different solvents.

Solvent	Antioxidant activity (%)	Reducing power (ABS 700 nm)	Total phenolic content (mg/100g)
Methanol	97.62 ± 0.53 d[a]	0.84 ± 0.00 d[b]	379.19 ± 0.85 c
Ethanol	83.58 ± 0.92 c	0.34 ± 0.00 c	83.56 ± 0.63 b
Ethyl acetate	78.94 ± 1.11b	0.10 ± 0.00 b	9.01 ± 0.11 a
Hexane	11.91± 1.25 a	0.01 ± 0.00 a	8.63 ± 0.33 a

[a] Mean ± standard deviation from triplicate determinations.

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

This difference was possibly because the extracts isolated with polar solvent, such as methanol, contained higher amount of components which act as electron donor than extracts isolated with non polar solvent such as hexane. Moreover, the highest antioxidant activity of methanol extract from wild tamarind seed presumably resulted from their strongest reducing power. Przybylski *et al.* (1998) demonstrated that the radical scavenging activity of buckwheat seed extracted with polar solvents were higher than those prepared from non polar solvents.

2.1.2 Cow pea seed.

The decrease in absorbance of β-carotene in the presence of cow pea seed extracts prepared with different organic solvents is shown in Figure 12. Methanol extract showed the lowest rate of decrease, compared to other solvent extracts. From the result, the highest antioxidant activity was obtained in methanol extract, followed by extracts prepared with ethyl acetate, ethanol and hexane, respectively (Table 9). Methanol extract also rendered

the highest total phenolic content and strongest reducing power, followed by ethanol, ethyl acetate and hexane extracts, respectively. The relationship between antioxidant activity and total phenolic content showed a low positive correlation (r^2 = 0.23). The polarity of solvents seemed to affect the antioxidant activity of the cow pea seed extract. Antioxidant activity increased with increasing polarity the solvents. Moreover, reducing power of the extract also increased with increasing polarity of the solvent.

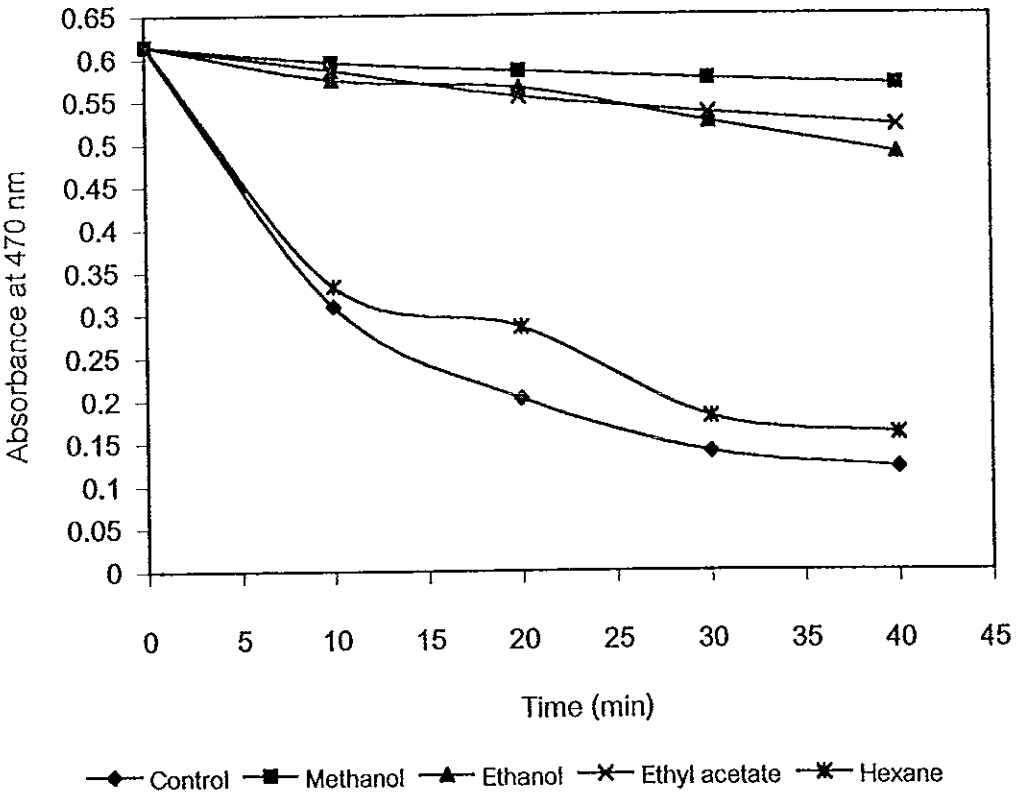

Figure 12 The decrease in absorbance of β-carotene in the presence of cow pea seed extract prepared with different organic solvents assayed by the β-carotene bleaching method.

Table 9 Antioxidant activity and total phenolic content of cow pea seed extract prepared with different solvents.

Solvents	Antioxidant activity (%)	Reducing power (ABS 700 nm.)	Total phenolic content (mg/100g)
Methanol	95.65 ± 0.57 d[a]	0.67 ± 0.00 c[a]	137.68 ± 1.35 d
Ethanol	85.24 ± 0.55 b	0.13 ± 0.02 b	19.02 ± 0.25 c
Ethyl acetate	89.07 ± 0.69 c	0.08 ± 0.06 a	9.00 ± 0.51 b
Hexane	16.57 ± 0.65 a	0.07 ± 0.00 a	7.08 ± 0.13 a

[a] Mean ± standard deviation from triplicate determinations.

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

These results are in agreement with many researchers who found that methanol extract of peanut, pea bean, wild rice, buckwheat and oriental herbs showed the highest antioxidant activity (Duh *et al.*,1992; Tsuda *et al.*, 1993; Wu *et al.*, 1994; Przybylski *et al.*, 1998; Kim *et al.*, 1994). Methanol is widely used as effective solvent for extracting antioxidant components such as phenolics, flavonoids, and other polar materials from plant materials (Economou *et al.*, 1991; Kim *et al.*, 1994). Killithraka *et al.* (1995) reported that methanol was the best solvent for the quantitative extraction of (+) – catechin, (-) – epicatechin and epigallocatechin from grape seeds. However, Chevolleau *et al.* (1992) noted that hexane extracts from leave of some Mediteranean plants had higher antioxidant activity than methanol extracts but higher yields were observed with methanol extracts than hexane extracts. Moreover ethyl acetate extracts of tamarind seed showed the stronger antioxidant activity than methanol, ethanol and 1:1 ethyl acetate:ethanol extracts (Tsuda *et al.*, 1994). Duh (1998) reported that the

water extract of burdock exhibited the greater yield and stronger antioxidant activity than the methanol, ethanol, chloroform and n-hexane extracts, and the extract yield increased with increasing polarity of solvents. The differences observed in different plant origins were presumed to cause by the differences in polarity of antioxidant compounds.

Hence, our data suggested that the antioxidant activity, reducing power and total phenolic content of wild tamarind and cow pea seed extracts were greatly dependent on the polarity of solvents used for the extraction. This was presumed to be due to the differences in solubility of antioxidative components of legume seeds in different solvents. Since water extracts of both seeds had low antioxidant activity and offensive odor, possibly caused by deterioration of extract. Thus, water was not used for extraction of antioxidants. From the result, methanol extracts provided the highest antioxidant activity and highest total phenolic content. Therefore, methanol was chosen as extracting solvent for further study.

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

2.2.1 Wild tamarind seed.

The efficiency of the antioxidant extraction was optimized by using the different ratios of methanol to water. Antioxidant activity, reducing power and total phenolic content were measured. No significant difference in antioxidant activity of 50-90 % methanol extracts were observed ($p < 0.05$) (Figure 13). The antioxidant activity of 100 % methanol extract was lowest ($p < 0.05$). However, total phenolic content was increased when methanol content decreased. This result was in agreement with Yi *et al.* (1997) who reported that the concentration of the total phenols in grape extract was increased with decreasing methanol content and the concentration reached a maximum between 50-70 % methanol. Reducing power was increased ($p < 0.05$) when methanol content increased (Figure 14). The 100 % methanol extract had highest reducing power, but lowest antioxidant activity. The

differences in antioxidant activity, total phenolic content and reducing power of resulted from a great variety of different phenolic compounds having different polarity and solubility.

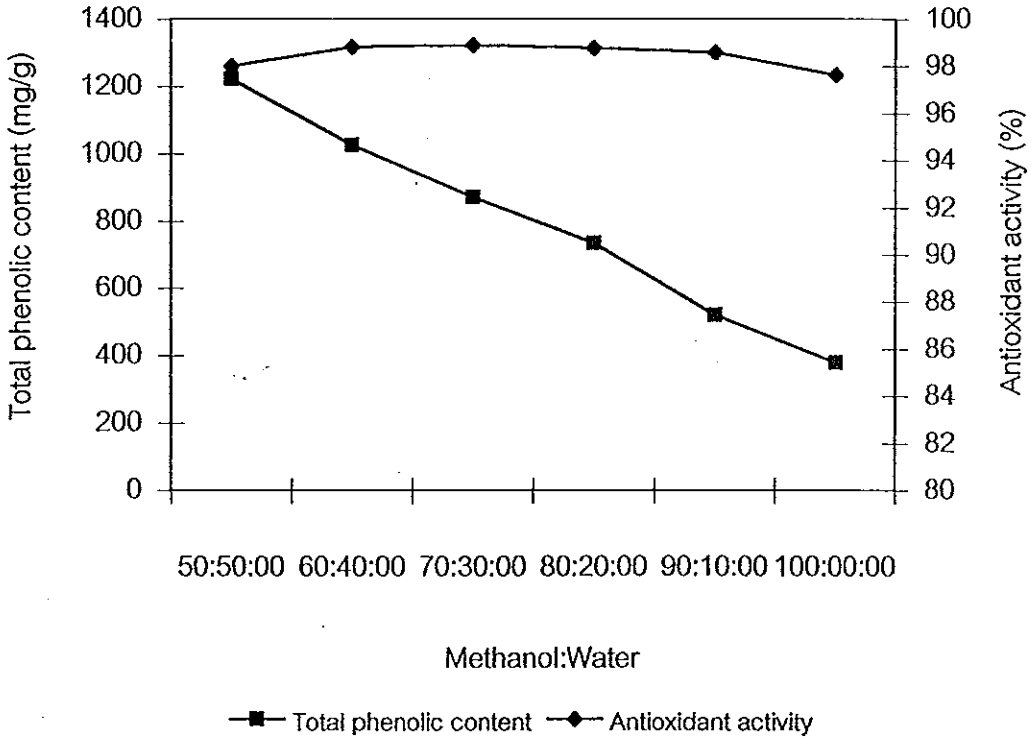

Figure 13 Effect of methanol/water ratio on antioxidant activity and total phenolic content of wild tamarind seed extract.

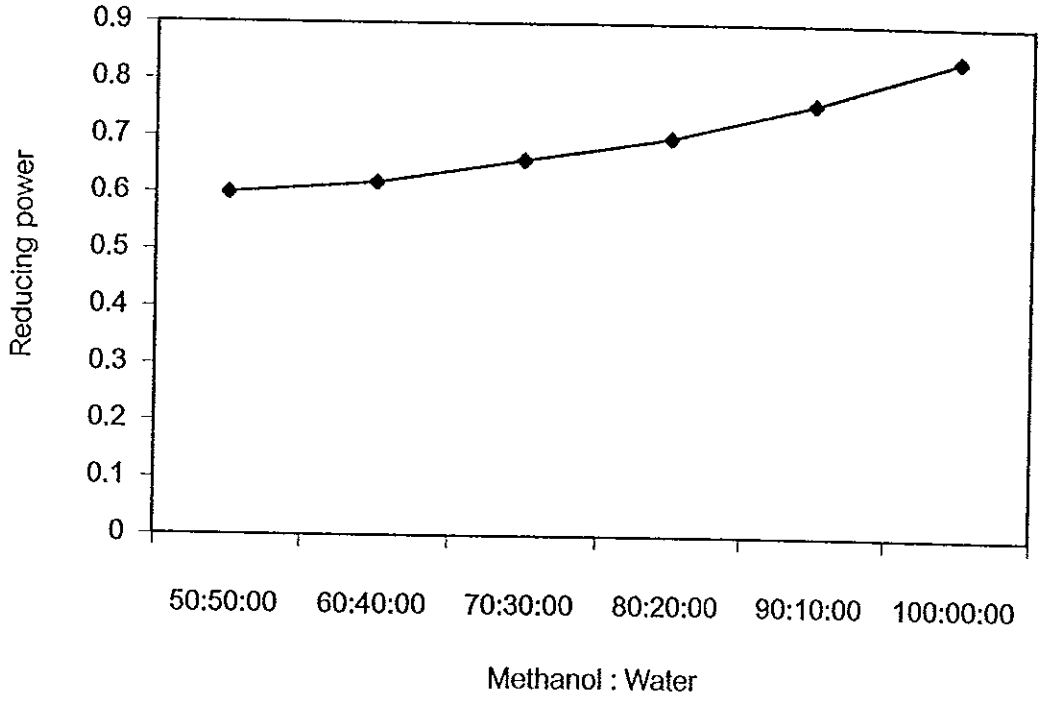

Figure 14 Effect of methanol/water ratio on reducing power of wild tamarind seed extract.

2.2.2 Cow pea seed.

For cow pea seed extracts, high antioxidant activity was obtained when extracted with 70 -100 % methanol and total phenolic content was increased with decreasing methanol content (Figure 15). The result was in accordance with that found in wild tamarind seed extract. Alonso *et al.* (1991) reported that the extraction of catechins and proanthocyanidins from grape seeds was more efficient when ethanol content of the extractant was increased.

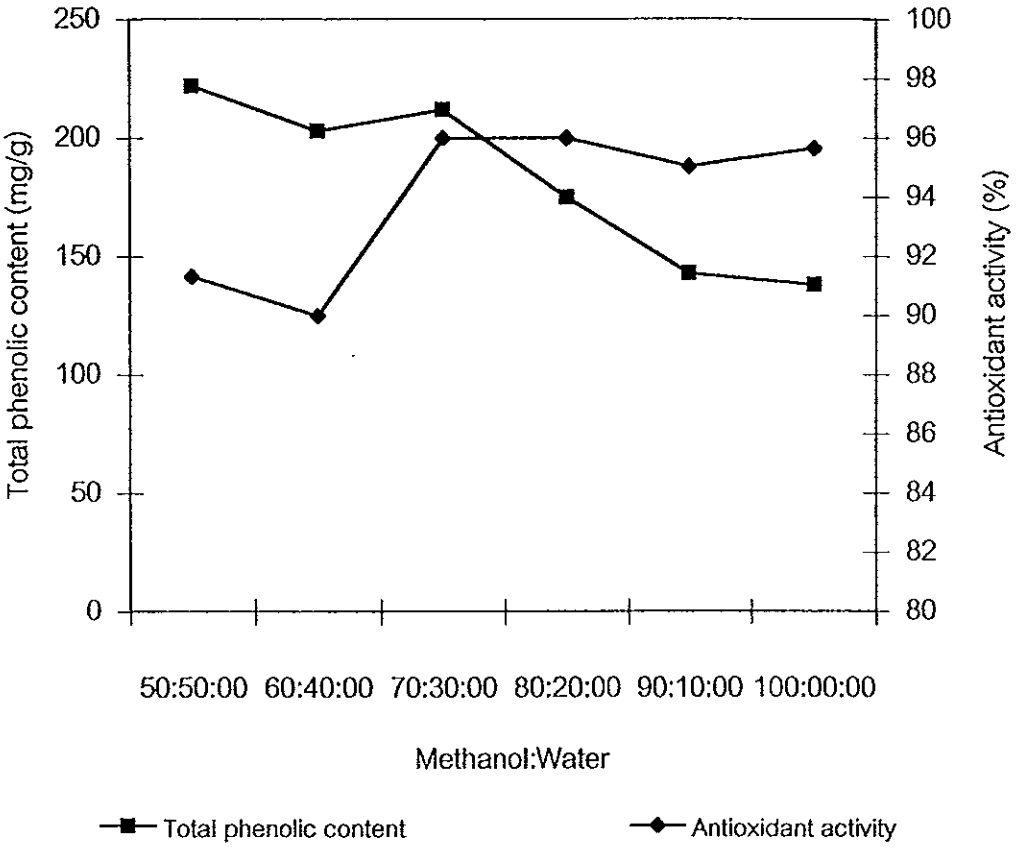

Figure 15 Effect of methanol/water ratio on antioxidant activity and total phenolic content of cow pea seed extract.

The reducing power of the extract prepared with 50 % methanol was significantly lower than the extracts prepared with 60-100 % methanol ($p < 0.05$) (Figure 16). No significant differences in reducing power among the cow pea seed extracts prepared with 60 -100 % methanol were obtained ($p < 0.05$). The 70 - 100 % methanol extracts exhibited higher antioxidant activity than the 50 - 60 % methanol extracts, possibly due to their stronger reducing power.

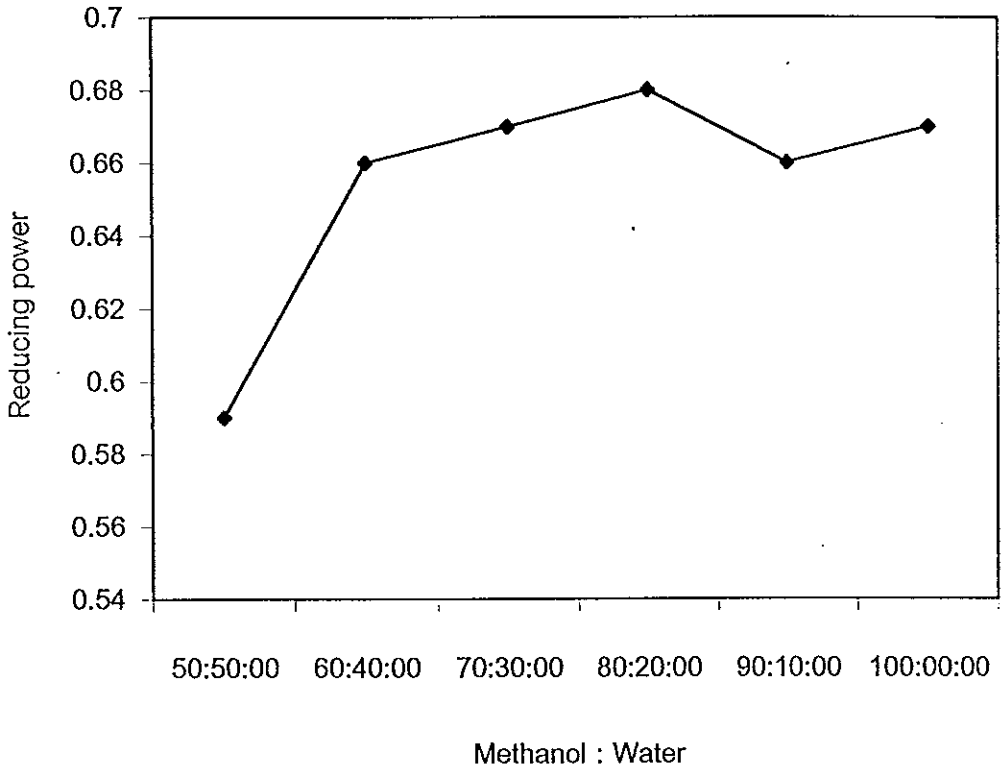

Figure 16 Effect of methanol/water on reducing power of cow pea seed extracts.

From these results, the extracts of wild tamarind and cow pea seed possibly contained a large variety of different phenolic compounds, which had different polarity. So, solvent with appropriate polarity can extract the antioxidant compounds selectively and effectively.

From our result, 50 % methanol and 70 % methanol were used as proper solvents for extracting of antioxidants from wild tamarind and cow pea seed, respectively.

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

2.3.1 Wild tamarind seed.

The effect of extraction time and repetition on the antioxidant activity and total phenolic content of wild tamarind seed is shown in Figure 17 and 18.

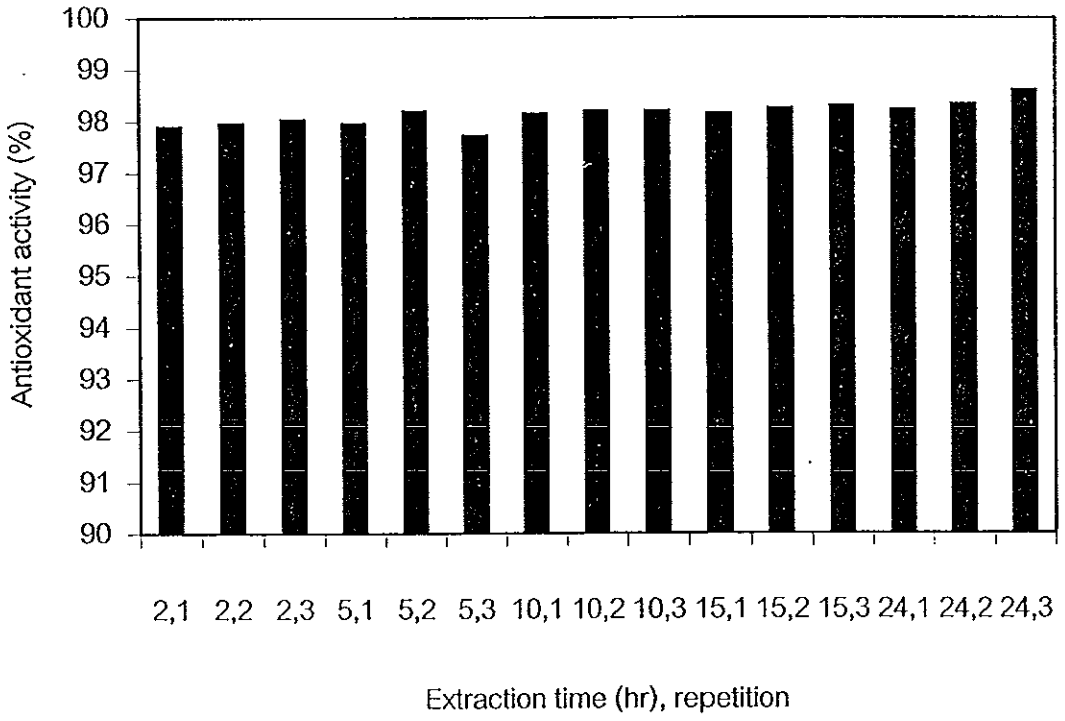

Figure 17 Effect of extraction time and repetition on the antioxidant activity of wild tamarind seed extract.

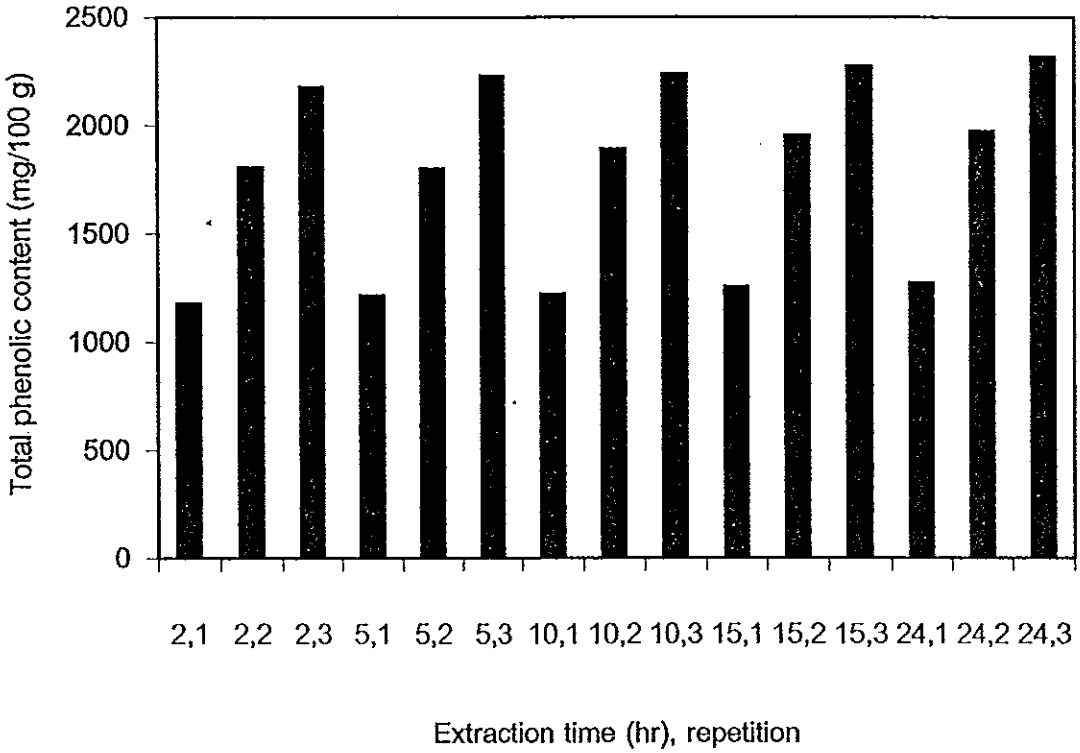

Figure 18 Effect of extraction time and repetition on total phenolic content of wild tamarind seed.

Extraction time and repetition had no significant effect on antioxidant activity ($p > 0.05$). However, an increase in total phenolic content was obtained with the increased repetition ($p < 0.05$), while extraction time did not affect total phenolic content ($p > 0.05$). Total phenolic content of extracts prepared with one repetition ranged from 1176 to 1273 mg/100 g. Extracts prepared with two and three repetitions contained total phenolic content of 1866 - 1974 and 2179 - 2320 mg/100 g, respectively. The extract with extracting time of 24 hrs and three repetitions rendered highest total phenolic content ($p < 0.05$). This result is in contrast with Alonso *et al.* (1991) who reported that the catechins and proantrocyanidins from grape seed increased with increasing extraction

time from 3 to 72 hrs. However, this result was in agreement with Tian and White (1994) who observed that phenolic content in oat extract increased with increasing repetition.

Generally, no differences in antioxidant activity were observed with increased extraction time and repetition. Moreover, longer extraction times increased the possibility of oxidation of phenolic unless reducing agents were added to the solvent system (Khanna *et al.*, 1968). Therefore, the optimum condition for extracting of antioxidant from wild tamarind seed involved extracting seed powder with 50 % methanol for 2 hrs.

2.3.2 Cow pea seed

Antioxidant activity and total phenolic content in cow pea seed extracts prepared with different extraction times and repetitions is shown in Figure 19 and 20. No significant differences ($p > 0.05$) in antioxidant activity were found with different repetitions (Figure 19). 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 ($p > 0.05$) (Figure 20). This result was similar to that observed in wild tamarind seed. Total phenolic content of extracts prepared with one, two, and three repetitions was 172 - 218, 243 - 265, and 256 - 291 mg/100 g seed, respectively.

Since no significant differences in antioxidant activity were found with increased repetition and extraction time, the optimum condition for preparing of cow pea seed extract involved extracting seed powder with 70 % methanol for 5 hrs.

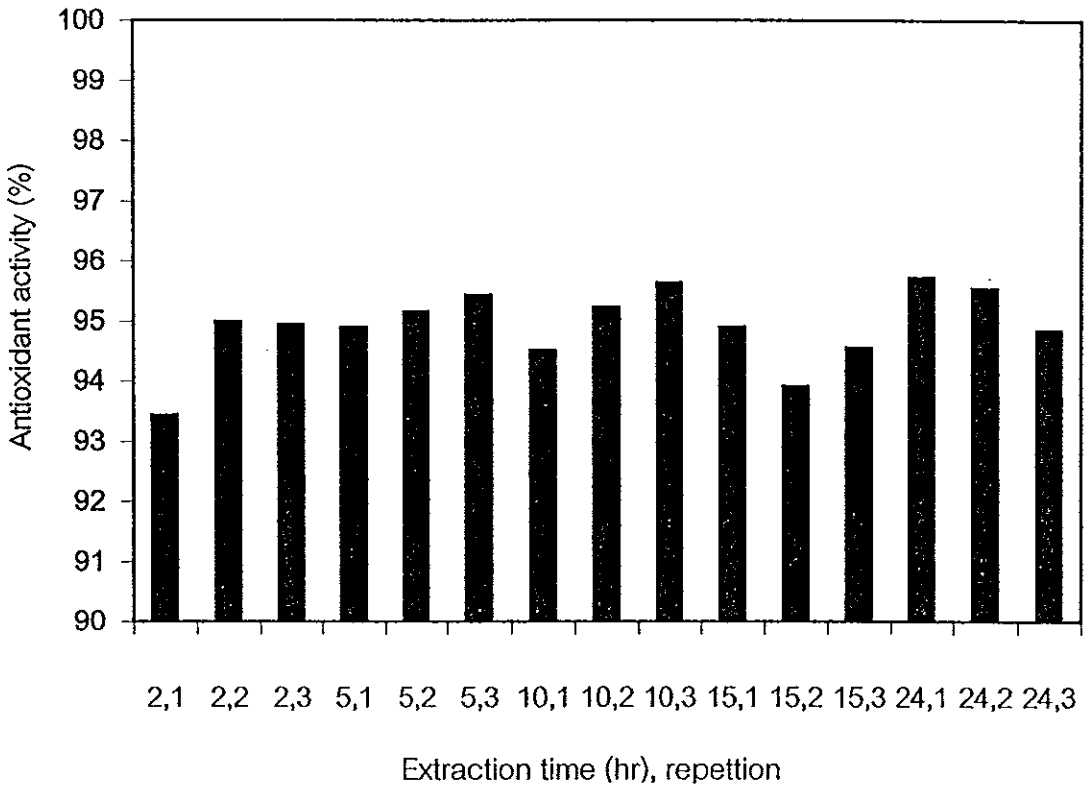

Figure 19 Effect of extraction time and repetition on the antioxidant activity of cow pea seed extract.

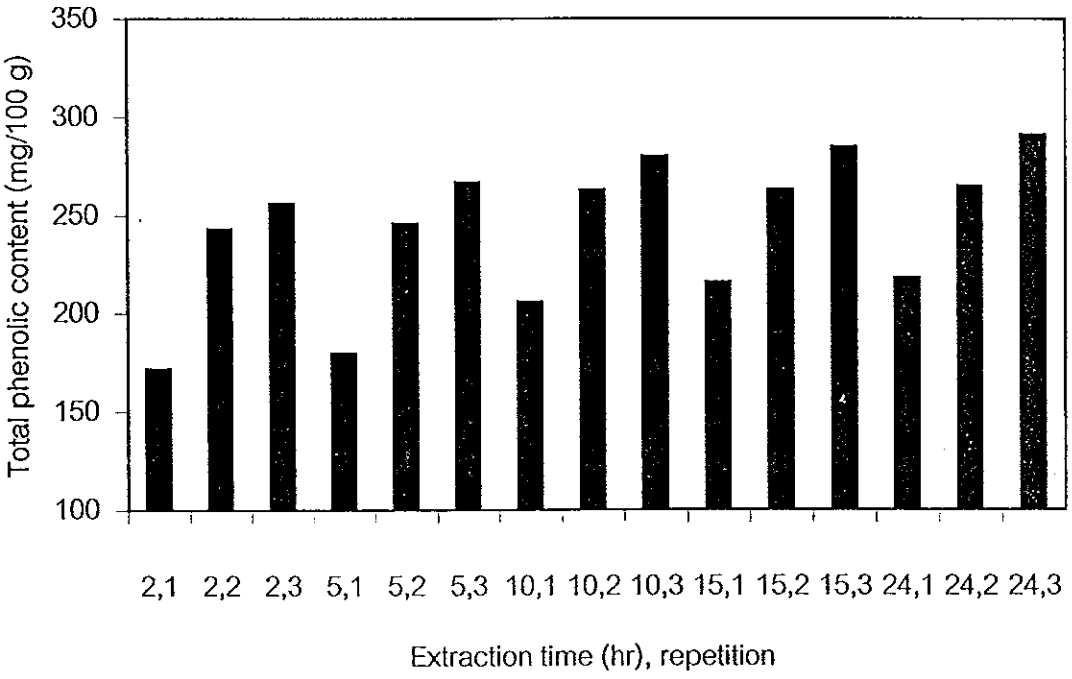

Figure 20 Effect of extraction time and repetition time on total phenolic content of cow pea seed extract.

After extraction of antioxidant under the optimum condition, the extracts of wild tamarind and cow pea seeds were subjected to vacuum distillation at 40 $^{\circ}$C to remove the methanol and then freeze-dried to obtain the antioxidants powder. Yields of extract powder from wild tamarind and cow pea seed were 7.30 $\pm$ 1.27 % (w/w) and 5.10 $\pm$ 1.25 % (w/w), respectively. Figure 21 and 22 illustrate the wild tamarind and cow pea seed extract powder.

Figure 21 Wild tamarind seed extract powder.

Figure 22 Cow pea seed extract powder.

3. Some properties of wild tamarind and cow pea seed extracts.

Wild tamarind and cow pea seed extracts may have a potential use in the food system. If the wild tamarind and cow pea seed extract are used in food system, their effectiveness will depend on various factors such as the concentration of the antioxidants, pH of food, pH stability of antioxidant, the extent of thermal processing applied to the food, or the synergistic substance in the food, etc. Therefore, these factors must be evaluated to more thoroughly understand the feasibility of using the wild tamarind and cow pea seed extracts in the food system.

3.1 Effect of wild tamarind and cow pea seed extract concentration on antioxidant activities.

Table 10 displays the antioxidant activity of the wild tamarind and cow pea seed extracts at different concentrations. The antioxidant activity of both seed extracts was compared with three commercial antioxidants including BHA, BHT and α-tocopherol.

Table 10 Antioxidant activity of wild tamarind and cow pea seed extracts at different concentrarions.

Concentration (mg/ml)	Antioxidant activity (%)	
	Wild tamarind	Cow pea
0.02	41.2 ± 0.29 a[a]	36.03 ± 0.05 a[b]
0.05	65.89 ± 0.49 b	58.66 ± 0.62 b
0.1	80.01 ± 0.52 c	75.86 ± 0.74 c
0.3	90.46 ± 0.83 e	80.72 ± 0.05 d
0.6	94.83 ± 0.29 f	87.47 ± 0.50 e
0.9	96.68 ± 0.65 g	93.99 ± 0.85 f
1.2	97.00 ± 0.55 g	95.25 ± 0.82 g
1.5	97.40 ± 0.51 g	95.89 ± 0.12 g
BHA 0.02	97.54 ± 0.50 g	97.54 ± 0.50 h
BHT 0.02	97.54 ± 0.47 g	97.54 ± 0.47 h
α-tocopherol 0.02	86.93 ± 0.73 d	86.93 ± 0.73 e

[a] Mean ± standard deviation from triplicate determinations.

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

The results indicated that antioxidant activity of wild tamarind seed extract increased with increasing concentration. No significant differences in antioxidant activity of the wild tamarind seed extract with concentration from 0.90 to 1.5 mg/ml, BHT and BHA at a level of 0.02 mg/ml were observed. However, the activity of the extract with concentration ranging from 0.3 to 1.50 mg/ml was higher than that of α-tocopherol at a level of 0.02 mg/ml ($p < 0.05$).

For cow pea seed extract, the same result was observed with wild tamarind seed extract. The antioxidant activity of cow pea seed extract increased with increasing concentration ($p < 0.05$) (Table 10). Cow pea seed extract at every concentration tested had significantly lower antioxidant activity than BHA and BHT ($p < 0.05$). However, no significant differences in antioxidant activity between cow pea seed extract at a concentration of 0.30 mg/ml and α-tocopherol were observed.

At the same concentration, the extract of wild tamarind seed exhibited stronger antioxidant activity than cow pea seed extract. This was postulated to be due to different antioxidantive compounds in both extracts. The concentration of the wild tamarind and cow pea seed extracts needed for β-carotene protection in emulsion was higher than that of BHA, BHT and α-tocopherol to obtained the same antioxidant activity. BHA, BHT and α-tocopherol possibly had high affinity for the lipid phase, whereas wild tamarind and cow pea seed extracts were soluble in the aqueous phase. As a result, their effective concentration in the lipid was lower (Shimoni *et al.*, 1994). In addition, extracts used were crude extracts, which contained a variety of compounds. At the same concentration, pure compounds isolated from susabinori exhibited strong antioxidant activity as α-tocopherol (Nakayama *et al.*,1999). Tsuda *et al.* (1994b) reported the extent of antioxidant activity of the purified compound in tamarind seed coat extracts including 2-hydroxy-3′,4′-dihydroxyacetophenone, methyl 3,4-dihydroxybenzoate, and 3,4-dihydroxyphenyl acetate at a level of 20 μM was the same as α-tocopherol at the same concentration. However, pure compound isolated from young green barley leaves showed less activity than the crude extract (Osawa *et al.*, 1992).

From this result, antioxidant activities of both seed extract depended on concentrations used. This result was in agreement with Yen and Lee (1997)

who found that antioxidant activity of the extract from *Aspergillus candidus* broth filtrate increased with an increasing concentration, but it reached maximum after 200 ppm. Similarly, Duh *et al.* (1997) observed that antioxidant activity of mung bean hull extract increased with increasing concentration up to 100 ppm and no significant differences in activity were observed with concentration ranging from 100 to 500 ppm ($p < 0.05$).

3.2 Effect of heat treatment on antioxidant activity of wild tamarind seed and cow pea seed extracts.

The wild tamarind seed and cow pea seed extracts were heated at 80°C and 100°C for 0, 10, 20, 30, 40, 50, 60, 90 and 120 min, and the residual antioxidant activity was determined by the β-carotene bleaching method. Table 11 and 12 show the antioxidant activity and remaining antioxidant activity of wild tamarind and cow pea seed extract as a function of heating time at 80°C and 100°C.

After heat treatment at 80°C and 100°C, the antioxidant activity of wild tamarind seed extract was slightly reduced with the heating time (Table 11). The antioxidant activity of wild tamarind seed extract was slightly decreased by heating for 40 min or longer. Similarly, extract heated at 100°C for 20 min was not significantly different in antioxidant activity, compared to the control ($p > 0.05$). However, a decrease in antioxidant activity of wild tamarind seed extract was observed when heated at 100°C for longer time. After heating at 80°C and 100°C for 120 min, the remaining antioxidant activities were 97.19 % and 96.05 %, respectively. At higher temperature (100°C), antioxidant activity of wild tamarind seed extract was decreased at higher extent, compared to a lower temperature (80°C).

Table 11 Antioxidant activity of wild tamarind seed extract as a function of heating time at 80°C and 100°C.

Heating time (min)	Antioxidant activity (%)		Remaining antioxidant activity (%)	
	80°C	100°C	80°C	100°C
0	96.82 ± 0.20 d[a]	96.82 ± 0.20 f[b]	100 d	100 f
10	96.80 ± 0.13 d	96.76 ± 0.11 ef	99.98 ± 0.16 d	99.94 ± 0.14 ef
20	96.70 ± 0.10 d	96.67 ± 0.07 ef	99.88 ± 0.13 d	99.85 ± 0.09 ef
30	96.60 ± 0.25 d	96.06 ± 0.75 de	99.78 ± 0.32 d	99.22 ± 0.94 de
40	95.98 ± 0.71 cd	95.93 ± 0.30 d	99.15 ± 0.88 cc	99.09± 0.38 d
50	95.25 ± 0.80 bc	94.58 ± 0.35 c	98.40 ± 1.00 bc	97.71 ± 0.44 c
60	94.44 ± 0.40 ab	94.22 ± 0.15 bc	97.68 ± 0.41 ab	97.34 ± 0.18 bc
90	94.19 ± 0.19 a	93.72 ± 0.13 b	97.31 ± 0.24 a	96.83 ± 0.15 b
120	94.07 ± 0.11 a	92.96 ± 0.34 a	97.19 ± 0.13 a	96.05 ± 0.42 a

[a] Mean ± standard deviation from triplicate determinations.

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

For the cow pea seed extract, the similar result was observed with wild tamarind seed extract. The antioxidant activity of seed extract was slightly decreased with the heating time (Table 12). Heating at both temperatures (80 and 100°C) for 10 min did not significantly reduce antioxidant potency ($p > 0.05$). However, heating at 80°C and 100°C for 20 or longer further reduced the activity seed extracts. After heating at 80°C and 100°C for 120 min, the remaining antioxidant activities were 93.87 % and 91.51 %, respectively. At

the same heating time, cow pea seed extracts exposed to 100°C showed lower antioxidant activity than that exposed to 80°C.

Table 12 Antioxidant activity of cow pea seed extract as a function at 80°C and 100°C.

Heating time (min)	Antioxidant activity (%)		Remaining antioxidant activity (%)	
	80°C	100°C	80°C	100°C
0	94.02 ± 0.45 g[a]	94.02 ± 0.45 g[b]	100 g	100 g
10	93.74 ± 0.10 fg	93.48 ± 0.28 fg	99.70 ± 0.10 fg	99.44 ± 0.28 fg
20	92.36 ± 0.26 ef	92.15 ± 0.20 ef	98.30 ± 0.28 f	98.09 ± 0.21 f
30	92.07 ± 0.03 de	90.85 ± 0.21 e	97.92 ± 0.32 e	96.74 ± 0.22 e
40	91.67 ± 0.14 cd	89.17 ± 0.25 de	97.59 ± 0.14 de	94.83 ± 0.27 d
50	91.38 ± 0.53 bc	88.75± 0.26 cd	97.09 ± 0.55 cd	94.59 ± 0.27 cd
60	91.03 ± 0.04 bc	88.17 ± 0.24 bc	96.83 ± 0.04 c	93.78 ± 0.25 bc
90	89.46 ± 0.34 b	87.82 ± 0.49 b	95.33 ± 0.36 b	93.59 ± 0.51 b
120	88.21 ± 0.29 a	85.83 ± 0.62 a	93.87 ± 0.31 a	91.51 ± 0.65 a

[a] Mean ± standard deviation from triplicate determinations.

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

Decrease in antioxidant activity of the wild tamarind seed and cow pea seed extracts at both temperatures (80°C and 100°C) probably resulted from evaporation of extracts as well as from chemical composition (Hamama and Nawar, 1996). Lee *et al.* (1986) reported that the extract of ginger rhizome was heat stable. Two thirds (relative antioxidant potency = 67.8) of the original

antioxidant activity still remained after 2 hrs at 100°C. Asamarai *et al.* (1996) noted that no significant difference in TBARS of samples treated with wild rice hull extracts exposed to 60°C and 100°C was found, when compared to samples treated with wild rice hull extracts. Furthermore, antioxidant activity of pure compound from the extract of pea bean was completely stable, even after heating for 1 hr at 100°C (Tsuda *et al.*, 1993a).

The results of this study also demonstrated that antioxidant activity of wild tamarind and cow pea seed extract was heat stable.

3.3 Effect of pH on antioxidant activities of wild tamarind and cow pea seed extracts.

While pH should not affect a purely free radical system, it could affect the state of the antioxidant (Marco, 1968). The effect of pH on antioxidant activity of wild tamarind and cow pea seed extracts is shown in Figure 23. The wild tamarind seed extract exhibited strong antioxidant activity at neutral and alkaline pHs and the activity was decreased at acidic pH (Figure 23). At pH 3, the extract from wild tamarind seed exhibited lowest antioxidant activity while the highest antioxidant activity was found at pH 9. Lee *et al.* (1986) found that protection efficiency value of ginger extract depended on pH and concentration of the extract. At low concentration, the protection factor value was decreased at alkaline pH, whereas while at high concentration, the protection factor value was increased when increasing pH values. BHA showed higher antioxidant response value at pH 9.0 than pH 7.0 while 2,4,5-trihydroxybutyrophenone and 3,3'-thiodipropionic acid acted as pro-oxidants at pH 9.0 (Marco, 1968). However, Yen and Duh (1993) and Yen and Lee (1997) reported that the extract from peanut hull and *Aspergillus candidus* broth filtrate exhibited strong antioxidant activity at neutral and alkaline pH but rendered no activity at alkaline pH. The difference in activity of wild tamarind seed extracts at various pH possibly because the change in pH may alter the

charges on the molecules hence altering partitioning of the extracts into the emulsified carotene/linoleic acid globule (Marco, 1968).

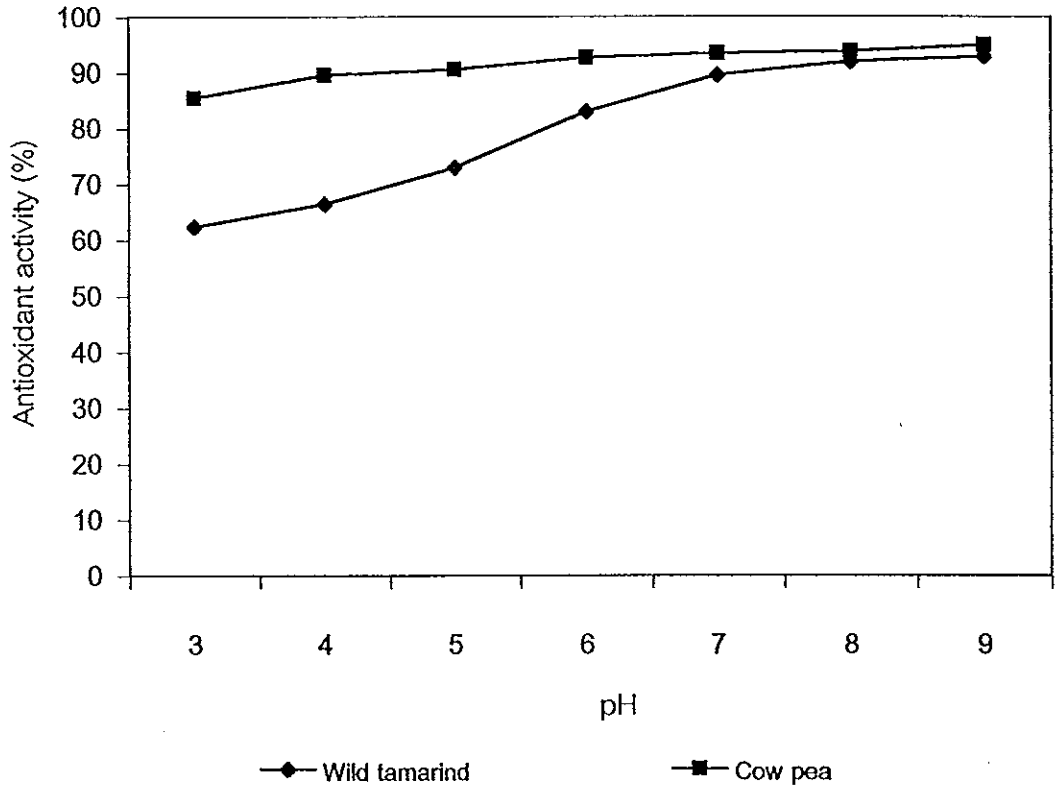

Figure 23 Effect of pH on antioxidant activity of wild tamarind and cow pea seed extracts.

The similar result was obtained in cow pea seed extract when compared to wild tamarind seed extract. The antioxidant activity of cow pea increased with increasing pHs (Figure 23). At neutral and alkaline pHs, the activity of cow pea seed extract was stronger than acidic pH. The extract of cow pea seed had red color in acidic pH, purple in neutral pH and blue in alkaline pH. So, the antioxidant active compound of cow pea seed extract was possibly anthocyanin. Tsuda *et al.* (1994a) reported that anthocyanin from red and black beans showed different antioxidant activity, dependent on pH.

Pelargonidin 3-α-β-D-glucoside and delphapnidin 3-α-β-D-glucoside did not show antioxidant activity in linoleic acid system at pH 7.0, but cyanidin 3-α-β-D-glucoside exhibited strong activity. Pelargonidin 3-α-β-D-glucoside and delphanidin 3-α-β-D-glucoside, which showed no antioxidant activity at pH 7.0, exhibited strong activity at both pH 3.0 and 5.0. From the result, the activity of cow pea seed extract at alkaline pH was stronger than that at acidic pH, possibly due to the different conformation and charges of antioxidant compounds under different pHs.

3.4 pH stability of wild tamarind and cow pea seed extracts.

pH stability of wild tamarind seed and cow pea seed extracts are illustrated in Figure 24. For wild tamarind seed, the result suggested that antioxidants in wild tamarind seed extract had high stability over the wide pH ranges. From the result, the wild tamarind seed extracts had high pH stability. It was presumed that the change of compounds in the extracts was reversible. Thus, the activity still remained after treatment.

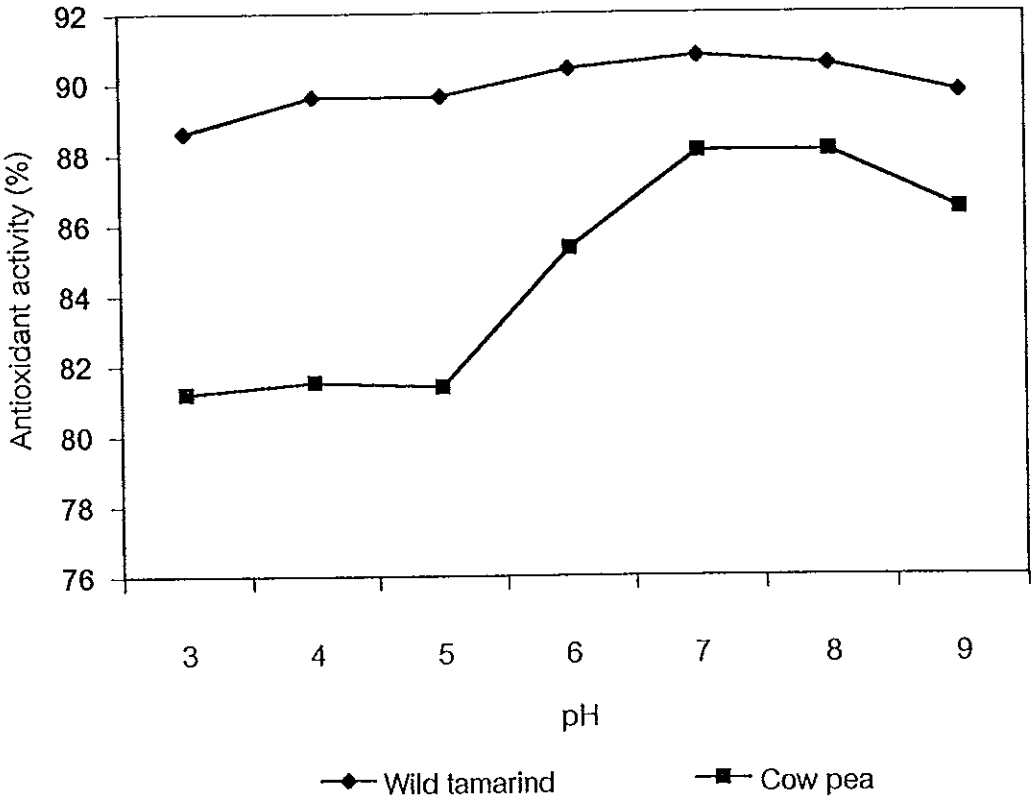

Figure 24 pH stability of antioxidant activity of wild tamarind and cow pea seed extracts.

Cow pea seed extract had high stability at pH 7 and 8 but the stability was slightly decreased in acidic pH ranges and pH 9.0. No significant difference in antioxidant activity at pH 6.0 and 9.0. From the result, it indicated that cow pea seed extract had high pH stability in a neutral pH ranges. Acidic conditions could be able to change the antioxidant property of extract.

3.5 Synergistic effect of wild tamarind and cow pea seed extracts with some compounds.

In general, the action of antioxidants is influenced by the synergistic components in the food system, such as α-tocopherol, citric acid and ascorbic acid. Therefore, the synergistic effects of α-tocopherol, citric acid and ascorbic acid on the antioxidant activity of wild tamarind and cow pea seed extracts were evaluated in emulsion system using β-carotene bleaching method.

3.5.1 Synergistic effect of wild tamarind seed and cow pea seed extracts with α-tocopherol.

Synergistic effect of wild tamarind seed extracts with α-tocopherol is shown in Figure 25. The combination of wild tamarind seed extract (0.02 mg/ml and 0.03 mg/ml) and α-tocopherol (0.02 mg/ml and 0.03 mg/ml) exhibited higher antioxidant activity than only α-tocopherol or wild tamarind seed extract ($p < 0.05$). The synergistic effect of α-tocopherol with wild tamarind seed extract increased with an increasing amount of the extract. However, increased amount of α-tocopherol did not affect synergism between α-tocopherol and seed extract.

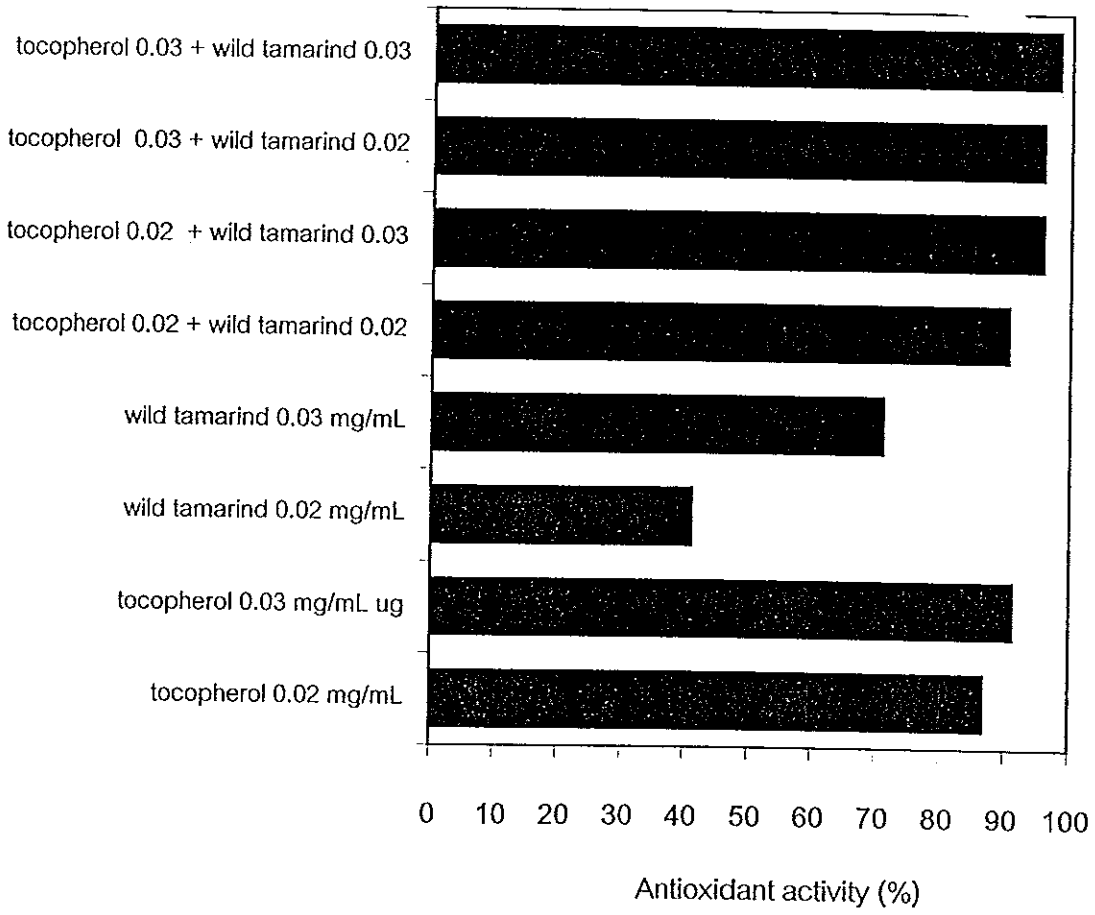

Figure 25 Synergistic antioxidant activity of wild tamarind seed extract with α-tocopherol in β-carotene/linoleic acid system.

Synergistic antioxidant action was also found between the cow pea seed extract and α-tocopherol (Figure 26). Combination of cow pea seed extract (0.02 and 0.03 mg/ml) and α-tocopherol (0.02 or 0.03 mg/ml) rendered stronger antioxidant activity than only α-tocopherol or extract ($p < 0.05$).

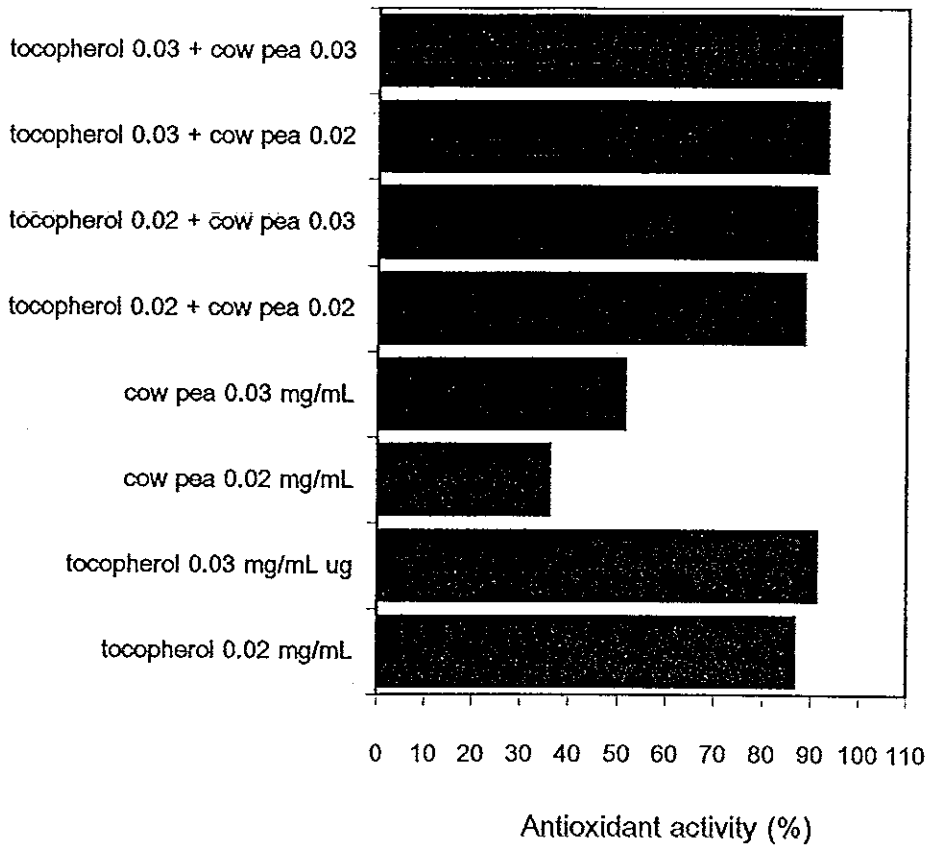

Figure 26 Synergistic antioxidant activity of cow pea seed extract with α-tocopherol in β-carotene/linoleic acid system.

This result was in agreement with Yen and Lee (1997) who reported that the extract from *Aspergillus candidus* broth filtrate had synergistic effect with α- tocopherol in linoleic acid peroxidation system. The extract showed strong free radical scavenging activity on 2,2-diphenyl-1-picrylhydrazyl (DPPH), indicating that the antioxidant substances in the extract had hydrogen-donating ability. Therefore, the synergistic effect of extract with α-tocopherol might be due to a redox mechanism involving reduction of the α-tocopheroxyl radical intermediate by the extract to regenerate α-tocopherol. In this experiment, the extracts from wild tamarind seed and cow pea seed showed strong free radical scavenging activity on 1,1 diphenyl-2-hydrazyl radical (DPPH). This was presumed that synergistic effect of wild tamarind seed or cow pea seed with α-tocopherol was probably due to a redox mechanism, leading to regeneration of α-tocopherol.

3.5.2 Synergistic effect of wild tamarind seed extracts with citric acid.

Figure 27 shows the antioxidant activity of the extract from wild tamarind seed (0.02 and 0.03 mg/ml), citric acid (0.02 and 0.03 mg/ml) and combination of the extract and citric acid. Citric acid exhibited weak antioxidant effect on β-carotene/linoleic acid emulsion system. Citric acid, which has been used in oils as a synergist to antioxidants and chelator, this component is not hydrogen donor (Labuza, 1971). No synergistic action was found between the wild tamarind seed extract and citric acid. Lower antioxidant activity of extract in a presence of citric acid was observed. Citric acid caused an acidic pH. As a result, wild tamarind seed extract exhibited weak antioxidant activity in such an acidic pH.

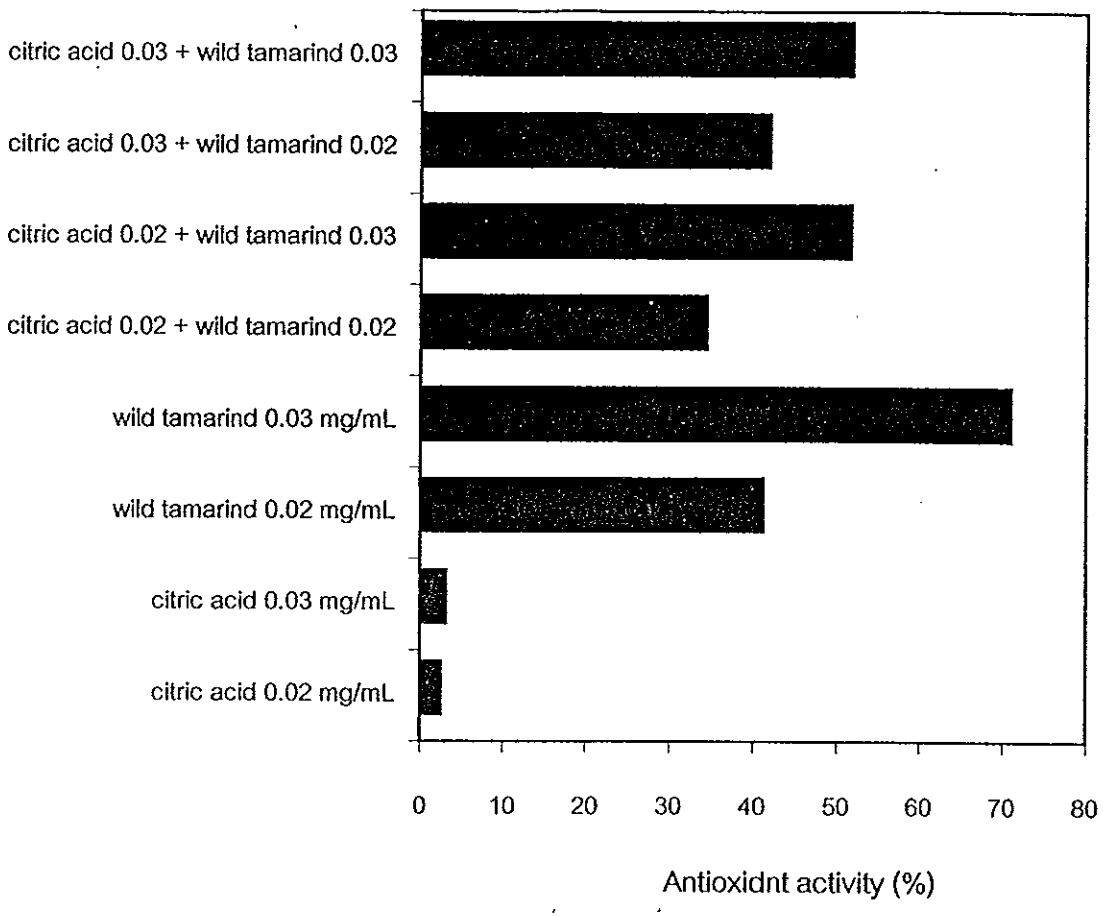

Figure 27 Synergistic antioxidant activity of wild tamarind seed extract with citric acid in β-carotene/linoleic acid system.

Figure 28 presents the antioxidant activity of the extract from cow pea seed (0.02 and 0.03 mg/ml), citric acid (0.02 and 0.03 mg/ml) and combination of the extract and citric acid. The same result was observed with wild tamarind seed extract. Citric acid was not synergistic to the extract. Moreover, a lower antioxidant activity of cow pea seed extract - citric acid combination was found, when compared to only cow pea extract.

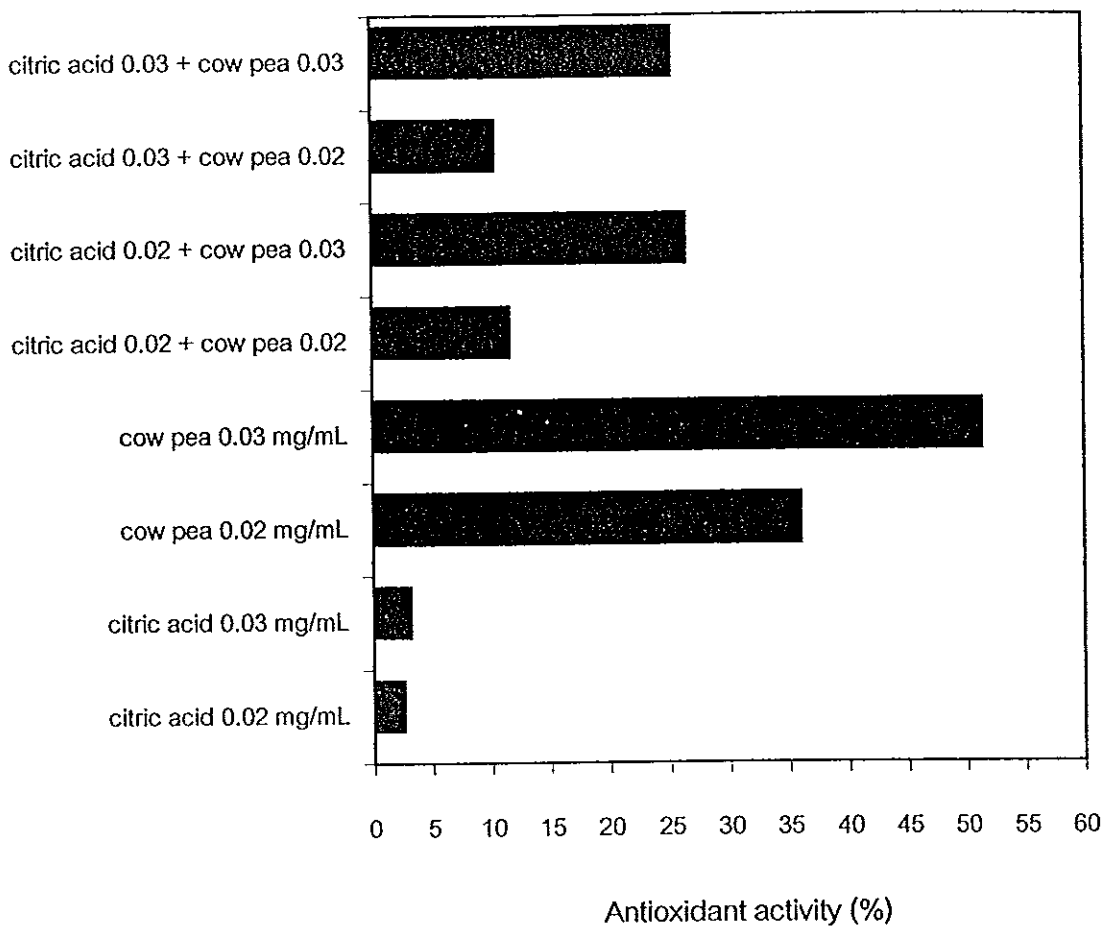

Figure 28 Synergistic antioxidant activity of cow pea seed extract with citric acid in β-carotene/linoleic acid emulsion system.

3.5.3 Synergistic effect of wild tamarind seed and cow pea extract with ascorbic acid.

Synergistic effect of ascorbic acid (0.02 and 0.03 mg/ml) on the antioxidant activity of wild tamarind seed extract (0.02 and 0.03 mg/ml) is shown in Figure 29. Ascorbic acid exhibited weak antioxidant activity and increased amount of ascorbic acid resulted in decreased antioxidant activity. Antioxidant activity of ascorbic acid at higher concentration (0.03 mg/ml) was lower than that at lower concentration (0.02 mg/ml). This was presumed that higher concentration of ascorbic acid promoted bleaching of the β-carotene

emulsion. Storey and Davises (1992) noted that ascorbic acid at 320 μg/ml promoted bleaching of the β-carotene emulsion. Ascorbic acid functioned as an oxygen scavenger, which is particularly useful in canned or bottled products with a headspace of air (Dziezak, 1986). About 3.2 mg of ascorbic acid are required to scavenge the oxygen in one cm^3 of headspace (Cort, 1982). In our experiment, oxygenated water was added in β-carotene emulsion system. Weak antioxidant activity of ascorbic acid was caused by excess oxygen in β-carotene emulsion system. Furthermore, Cort (1982) suggested that at low levels, such as 100 ppm, ascorbic acid acted as pro-oxidant. No synergism of ascorbic acid on the antioxidant effect of wild tamarind seed extract was observed. Moreover, antioxidant activity of ascorbic acid–wild tamarind seed combination was lower than that of only seed extract.

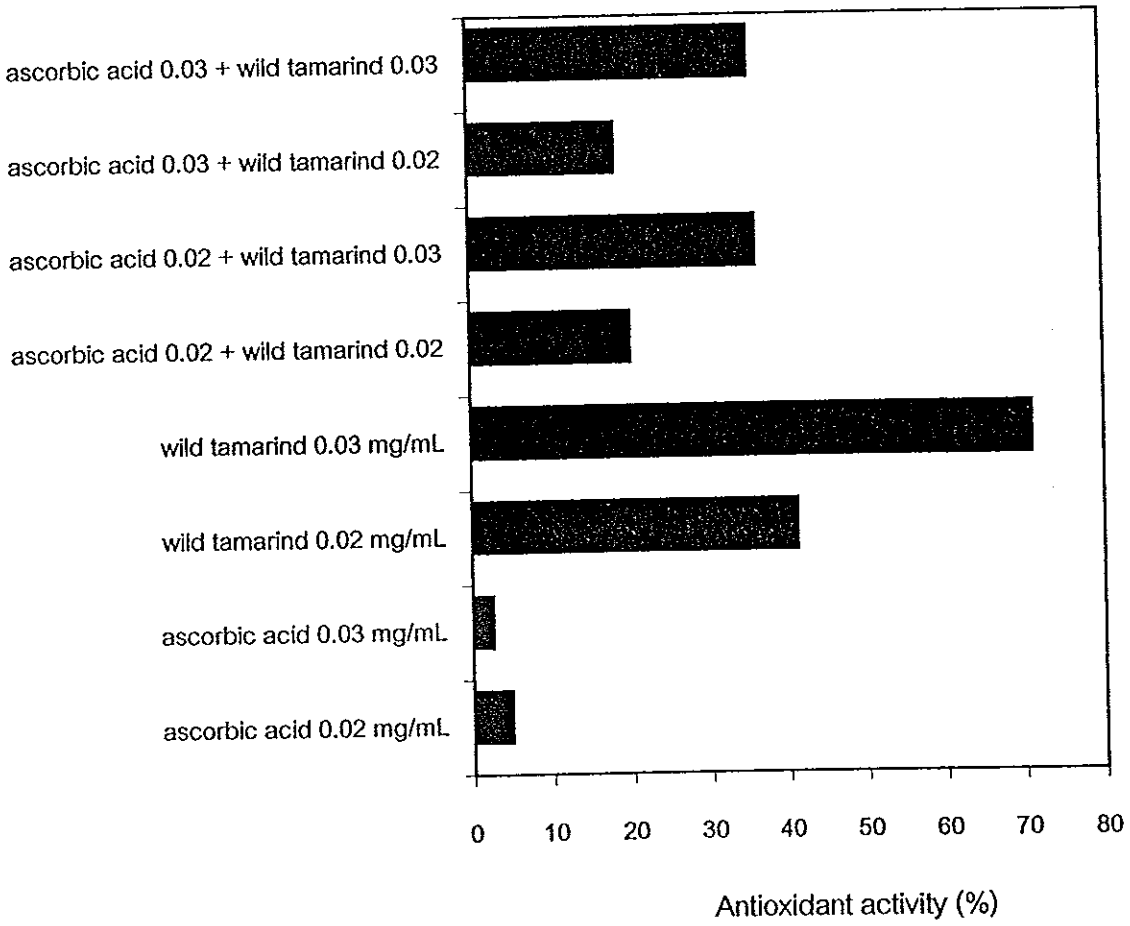

Figure 29 Synergistic antioxidant activity of wild tamarind seed extract with ascorbic acid in β-carotene/linoleic acid system.

Synergistic effect of ascorbic acid (0.02 and 0.03 mg/ml) on the antioxidant activity of cow pea seed extracts (0.02 and 0.03 mg/ml) is presented in Figure 30. The result was similar to those obtained in wild tamarind seed extract. Cow pea seed extract did not act synergistically with ascorbic acid. Antioxidant activity of ascorbic acid–cow pea seed extract combination was lower than that of only seed extract.

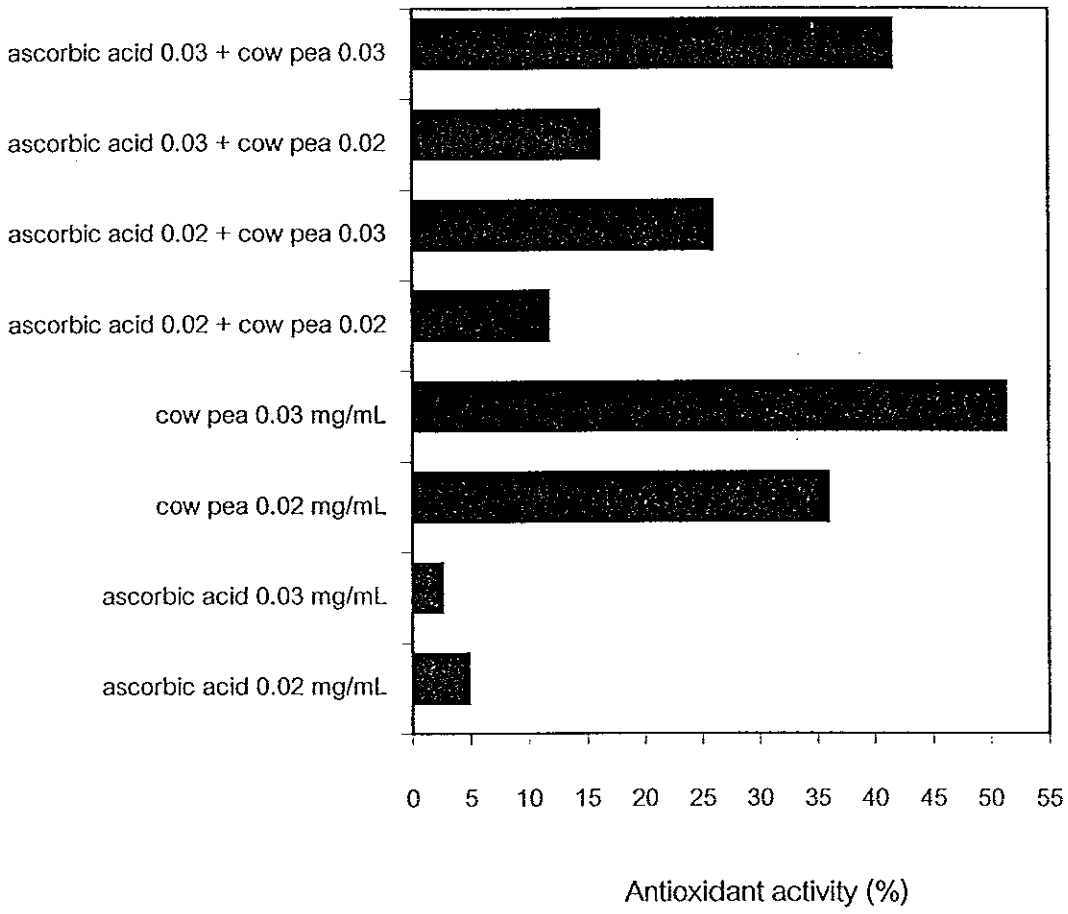

Figure 30 Synergistic antioxidant activity of cow pea seed extract with ascorbic acid in β-carotene/linoleic acid emulsion system.

Either of wild tamarind or cow pea seed extracts did not act synergistically with ascorbic acid and the activity of the combination was lower. This was presumed that ascorbic acid caused acidic pH, where wild tamarind and cow pea seed extracts exhibited weak antioxidant activity. Therefore, weak antioxidant activity of ascorbic acid – wild tamarind or cow pea seed combination was observed.

4. Mode of action

4.1 Radical-scavenging activity of wild tamarind and cow pea seed extracts.

It is well known that free radicals cause autoxidation of unsaturated lipids in food (Kaur and Perkin, 1991). On the other hand, antioxidants are believed to interrupt the free-radical chain of oxidation and to donate hydrogen from phenolic hydroxy group, thereby, forming stable free radicals, which do not initiate or propagate further oxidation of lipids (Sherwin, 1978). Recent reports have described antioxidants and compounds with radical-scavenging activity present in peanut hull, mung bean hull, buckwheat seed and burdock. (Yen and Duh, 1994; Duh *et al.*, 1997; Przybylski *et al.*, 1998; Duh, 1998). Therefore, radical-scavenging of wild tamarind and cow pea seed extracts were determined. Elimination of 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH) is used to indicate the presence of hydrogen donors in a reaction system. Ionized DPPH produces a color which is changed, when radicals are removed from the system and is measured by diminishing absorption at 515 nm (Brand-Wiliams *et al.*, 1995).

Scavenging effects of different amounts of wild tamarind seed extract on the DPPH radical is presented in Table 13. The absorbance at 517 nm of ionized DPPH solution decreased with increasing amount of the extracts. Increased wild tamarind seed extract donated a higher hydrogen atom to DPPH radicals (Blois, 1958; Shimada *et al.*, 1992). Wild tamarind seed extract at 4 mg showed scavenging activity of 95.23 $\pm$ 0.36 %. This was equivalent to the 95.38 $\pm$ 0.49 %, 95.60 $\pm$ 0.89 %, 95.59 $\pm$0.73 % and 95.23 $\pm$ 0.94 % scavenging activity of TBHQ, BHT, BHA and α-tocopherol at a level of 1 mg, respectively. The amount of extract needed for scavenging DPPH radicals was higher than that of BHA, BHT, TBHQ and α-tocopherol to obtain the same radical – scavenging activity.

Table 13 Radical - scavenging activity of wild tamarind seed extract at different amounts.

Amount (mg)	Absorbance at 517 nm	Radical – scavenging activity (%)
0.0	0.384 ± 0.010 i [a]	0.00 a [b]
0.1	0.292 ± 0.004 h	24.35 ± 1.13 b
0.2	0.238 ± 0.003 g	38.26 ± 0.83 c
0.3	0.200 ± 0.001 f	48.27 ± 0.39 d
0.4	0.163 ± 0.000 e	57.86 ± 0.15 e
0.8	0.075 ± 0.005 d	80.66 ± 1.17 f
1.2	0.035 ± 0.001c	90.93 ± 0.26 g
2.0	0.025 ± 0.003 b	93.44 ± 0.97 h
3.0	0.025 ± 0.004 b	93.52 ± 1.13 h
4.0	0.018 ± 0.000 a	95.23 ± 0.19 i
BHA 1.0	0.018 ± 0.000 a	95.37 ± 0.15 i
BHT 1.0	0.018 ± 0.001 a	95.59 ± 0.26 i
TBHQ 1.0	0.018 ± 0.001 a	95.49 ± 0.31 i
α-tocopherol 1.0	0.018 ± 0.000 a	95.25 ± 0.15 i

[a] Mean ± standard deviation from triplicate determinations.

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

Scavenging activity of cow pea seed extracts (0.1–4 mg) on the DPPH radicals was evaluated (Table 14). The same result was observed with wild tamarind seed extract. The extract of cow pea seed was capable of scavenging of DPPH radicals in a concentration-dependent manner. The scavenging activity of cow pea seed extract at 4 mg was significantly lower than that of TBHQ, BHA, BHT and α-tocopherol at a level of 1 mg ($p < 0.05$).

Table 14 Radical - scavenging activity of cow pea seed extract at different amounts.

Amount (mg)	Absorbance at 517 nm	Radical – scavenging activity (%)
0.0	0.384 ± 0.010 i [a]	0.00 a [b]
0.1	0.273 ± 0.002 h	29.36 ± 0.64 b
0.2	0.170 ± 0.001 g	55.96 ± 0.26 c
0.3	0.107 ± 0.000 f	72.28 ± 0.00 d
0.4	0.043 ± 0.000 e	88.95 ± 0.15 e
0.8	0.037 ± 0.003 d	90.07 ± 0.14 f
1.2	0.034 ± 0.002 c	91.10 ± 0.39 g
2.0	0.032 ± 0.003 b	91.62 ± 0.65 h
3.0	0.029 ± 0.000 b	92.57 ± 0.14 hi
4.0	0.026 ± 0.001 a	93.26 ± 0.26 i
BHA 1.0	0.018 ± 0.000 a	95.37 ± 0.15 j
BHT 1.0	0.018 ± 0.001 a	95.59 ± 0.26 j
TBHQ 1.0	0.018 ± 0.001 a	95.49 ± 0.31 j
α-tocopherol 1.0	0.018 ± 0.000 a	95.25 ± 0.15 j

[a] Mean ± standard deviation from triplicate determinations.

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

This result was agreement with Yen and Duh (1994) who reported that radical-scavenging activity of peanut hull extract depended on concentration. Similarly, the extract from mung bean hull and burdock seed exhibited strong radical-scavenging activity in a concentration- dependent manner (Duh *et al.*, 1997; Duh, 1998). Furthermore, Przybylski *et al.* (1998) reported that scavenging activity of buckwheat seed extract increased when more polar solvents were used for extraction. The highest activity was observed for the methanol extract and the radical scavenging effectiveness of the buckwheat extract was concentration dependent.

The data obtained revealed that the extracts of wild tamarind and cow pea seed acted as free-radical inhibitors, possibly as primary antioxidants that reacted with free radicals. This action was possibly the main factor to cause inhibition of peroxidation in β-carotene and linoleic acid system.

4.2 Reducing power of wild tamarind and cow pea seed extracts.

The antioxidant activity has been reported by some investigators to be concomitant with development of reducing power (Yen and Duh, 1993; Duh, 1998). As shown in Table 15, the reducing power and antioxidant activity of wild tamarind seed extract increased with an increasing amount of extract. Correlation between reducing power and amounts of the wild tamarind seed extract was very high (r^2 = 0.9963). In general, antioxidant activity correlated with reducing power (r^2 = 0.9657) (Figure 31). This indicated that reducing power in the extract was contributed to antioxidant activity. A 0.25 mg extract exhibited significantly greater reducing power than 0.05 mg ascorbic acid, which is a reducing agent and potent reductone (Shimada *et al.*, 1992).

Table 15 Antioxidant activity and reducing power of wild tamarind seed extract at different amounts.

Amounts (mg)	Antioxidant activity (%)	Reducing power (Absorbance at 700 nm)
0.05	42.44 ± 0.95 a[a]	0.102 ± 0.002 a[b]
0.10	50.53 ± 0.89 b	0.171 ± 0.005 b
0.15	55.72 ± 0.53 c	0.252 ± 0.013 c
0.20	63.72 ± 0.66 d	0.333 ± 0.010 d
0.25	67.95 ± 0.72 e	0.462 ± 0.009 f
0.30	72.41 ± 0.23 f	0.528 ± 0.002 g
0.35	75.00 ± 0.45 g	0.638 ± 0.010 h
0.40	78.63 ± 0.85 h	0.702 ± 0.005 i
0.45	80.23 ± 0.46 i	0.775 ± 0.002 j
0.50	85.72 ± 0.56 j	0.857 ± 0.013 k
Ascorbic acid 0.05		0.426 ± 0.003 e

[a] Mean ± standard deviation from triplicate determinations.

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

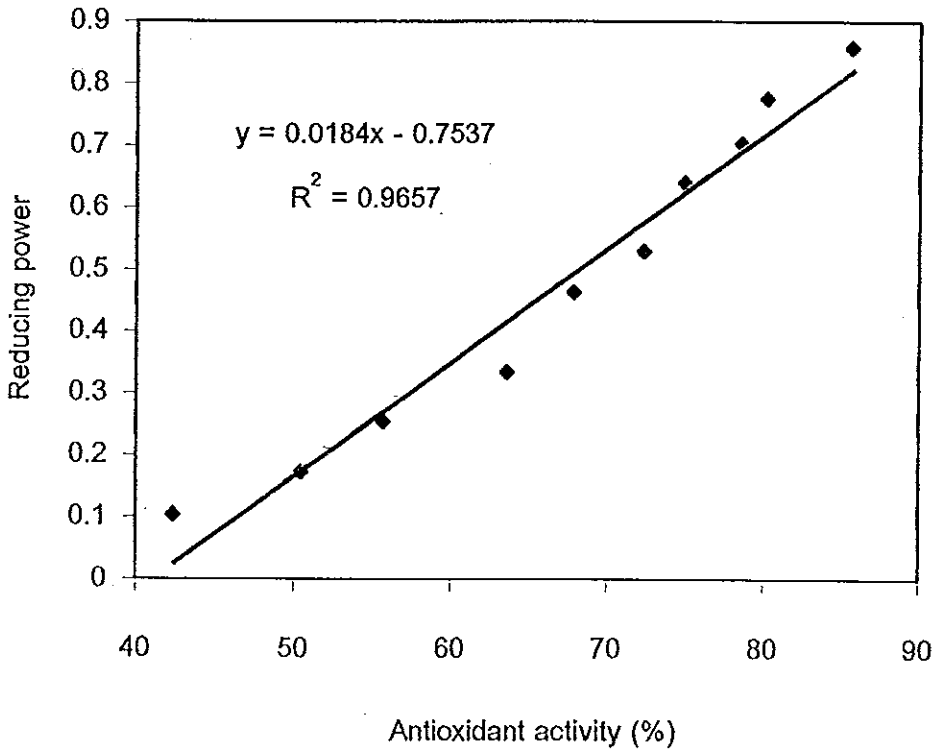

Figure 31 Relationship between antioxidant activity and reducing power of wild tamarind seed extract.

The reducing power and antioxidant activity of cow pea seed extract at different amounts are present in Table 16. Similar result was observed in cow pea seed extract compared to that found in wild tamarind seed. Antioxidant activity and reducing power of seed extract increased with increasing amount of the extract. Reducing power correlated well with amounts of extract (r^2 = 0.9792). Additionally, correlation coefficient between antioxidant activity and reducing power was high (r^2 = 0.8009) (Figure 32), indicating that antioxidant properties in cow pea seed was concomitant with the development of reducing power. No difference in reducing power was found between the extract at 1.00 mg and ascorbic acid at 0.05 mg.

At the same amount, wild tamarind seed extract exhibited higher reducing power than cow pea seed extract. It is postulated that wild tamarind seed extract contained higher reductones than the extract of cow pea seed.

Table 16 Antioxidant activity and reducing power of cow pea seed extract at different amounts.

Amounts (mg)	Antioxidant activity (%)	Reducing power (Absorbance at 700 nm)
0.05	37.15 ± 0.78 a[a]	0.015 ± 0.002 a[b]
0.10	40.72 ± 0.69 b	0.038 ± 0.005 b
0.15	45.33 ± 0.45 c	0.053 ± 0.003 c
0.20	48.62 ± 0.35 d	0.078 ± 0.000 d
0.25	52.33 ± 0.18 e	0.117 ± 0.008 f
0.30	58.63 ± 0.98 f	0.127 ± 0.007 g
0.35	62.52 ± 1.02 g	0.130 ± 0.010 g
0.40	65.82 ± 0.85 h	0.156 ± 0.008 h
0.45	71.53 ± 0.98 i	0.168 ± 0.012 i
0.50	74.65 ± 0.76 j	0.254 ± 0.010 j
1.00	80.40 ± 0.88 k	0.435 ± 0.012 k
Ascorbic acid 0.05		0.426 ± 0.003 k

[a] Mean ± standard deviation from triplicate determinations.

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

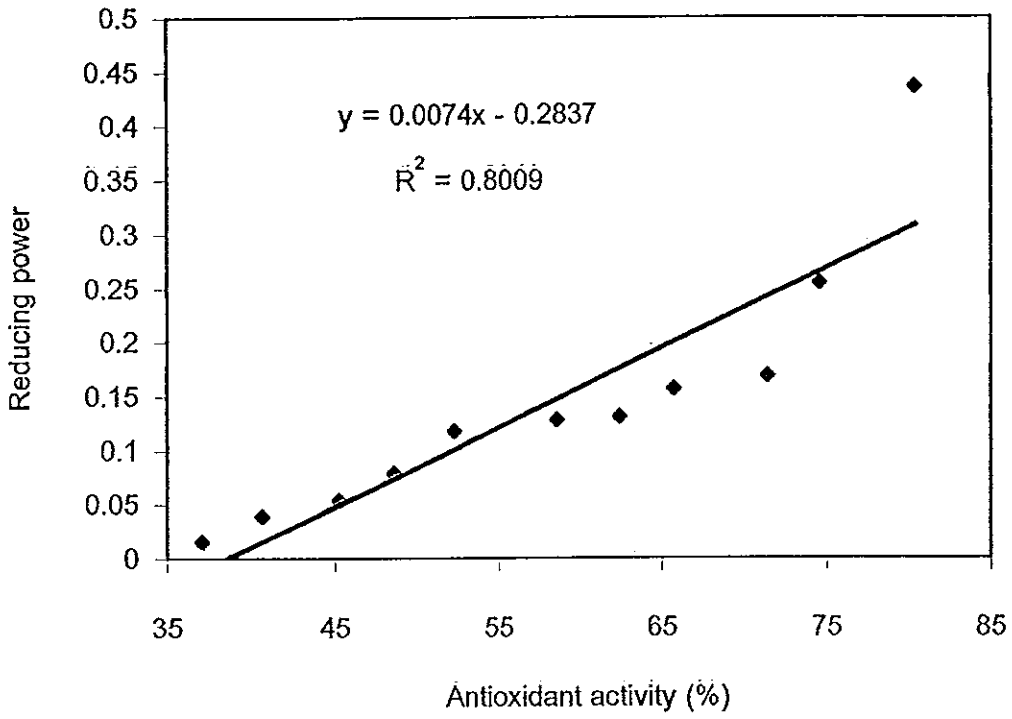

Figure 32 Relationship between antioxidant activity and reducing power of the cow pea seed extract.

This result is in accordance with that of Yen and Duh (1993) who reported that the reducing power of peanut hull extract increased with an increase in concentration and correlated (r^2 = 0.9793) well with the extent of antioxidant activity. Similarly, Duh *et al.* (1997) and Duh (1998) found that the antioxidant properties of mung bean hull and burdock extracts were shown to be concomitant with the development of reducing power. Gordon (1990) reported that the antioxidant action of reductones is based on breaking of the radical chain by donation of a hydrogen atom. Lingnert and Erikson (1981) noted that antioxidant properties were considered to be associated with the presence of reductone. Okuda *et al.*(1983) reported that the reducing power of tannins prevented liver injury by inhibiting the formation of lipid peroxides.

Reductones are believed not only to react directly with peroxides but also prevent peroxide formation by reacting with certain precursors.

Therefore, extracts of wild tamarind and cow seed were suggested to act as electron donors, reacting with free radicals to convert them to more stable products, which can terminate radical chain reaction. Our data indicated that the marked antioxidant action of wild tamarind and cow pea seed extracts was presumed to be a result of their reducing power.

4.3 Chelating activity on metal ions of wild tamarind and cow pea seed extracts.

Lipid peroxidation has been studied extensively in food science, nutrition, and clinical medicine because its products are related to food deterioration, cytotoxicity, and many pathological reactions in degenerative diseases such as cancer (Halliwell and Chirico, 1993). Transition metals can catalyze the generation of reactive oxygen species such as hydroxyl radical ($OH^{\bullet}$) and superoxide (O_2^{-}). Hydroxyl radical and superoxide can also propagate the lipid peroxides into peroxyl and alkoxyl radicals (Halliwell and Chirico, 1993; Minotti and Aust, 1987; 1989). Copper and iron are the most active metals for inducing oxidation in oil and fat-containing foods (Evan *et al.*,1951). Iron (2.2 ppm) is very effective in reducing the antioxidant effect of rosmariquinone with a reduction in induction period from 13.4 to 3.9 hrs. For α-tocopherol, iron reduces the induction period from 10.0 to 5.6 hrs (Weng and Gordon, 1992). Furthermore, List and Erickson (1980) noted that the copper content should be kept below 0.02 ppm in refined, bleached, and deodorized soybean oil to ensure oxidative stability. Although chelating agents are not antioxidants, they play a valuable role in the stabilization of fatty foods against rancidity (Yen and Duh, 1994). Thus, chelating activity of wild tamarind and cow pea seed extracts on two metal ions were determined, compared to citric acid and ethylenediaminetetraacetic acid (EDTA).

4.3.1 Fe^{2+} - binding activity of wild tamarind and cow pea seed extracts.

Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via Fenton type reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^o$). Fe^{3+} ion also produces radicals from peroxides, although the rate is ~ 10-fold less than that of Fe^{2+} ion (Miller, 1996). Fe^{2+} ion is the most powerful pro-oxidant among various species of metal ions (Halliwell and Gutteridge, 1984). Tetramethyl murexide (TMM), a chelating reagent, was used to indicate the presence of chelator in a reaction system. TMM formed a complex with free Fe^{2+} but not with Fe^{2+} bound by the extracts. The TMM- Fe^{2+} complex showed an absorption maximum at 485 nm (Shimada *et al.*, 1992). Measurement of Fe^{2+} - binding activity of wild tamarind seed extract was done with extract ranging from 0.1 to 2 mg (Table 17). The absorbance of reaction mixture decreased with increasing amount of the extracts due to Fe^{2+} complex with the wild tamarind seed extract. Thus, Fe^{2+} - binding activity of the extract was increased with increasing amount of the extract. High positive correlation between Fe^{2+} - binding activity and amount of the extract ($r^2 = 0.9418$) was found. The extract at 2 mg showed chelating activity on Fe^{2+} ion (57.26 $\pm$ 0.88 %). It was significantly lower ($p < 0.05$) than that of citric acid at 0.20 M (86.79 $\pm$ 0.54 %). However, it was higher than that of EDTA at the same concentration used (37.86 $\pm$ 1.05 %) ($p < 0.05$).

Table 17 Fe^{2+} - binding activity of wild tamarind seed at different amounts.

Amounts (mg)	Absorbance at 480 nm	Fe^{2+} - binding activity (%)
0.00	0.684 ± 0.002 l[a]	0.00 a[b]
0.10	0.680 ± 0.004 l	0.63 ± 0.51 b
0.20	0.652 ± 0.003 k	4.68 ± 0.44 c
0.40	0.614 ± 0.010 j	10.28 ± 1.41 d
0.60	0.557 ± 0.004 i	18.57 ± 0.53 c
0.80	0.523 ± 0.012 h	23.58 ± 1.73 f
1.00	0.508 ± 0.005 g	25.78 ± 0.73 g
1.20	0.488 ± 0.003 f	28.60 ± 0.42 h
1.40	0.464 ± 0.002 e	32.16 ± 0.30 i
1.60	0.437 ± 0.002 d	36.06 ± 0.22 j
1.80	0.424 ± 0.010 c	38.01 ± 1.49 k
2.00	0.406 ± 0.002 b	57.26 ± 0.88 l
Citric acid 0.20 M	0.090 ± 0.002 a	86.79 ± 0.42 m
EDTA 0.20 M	0.425 ± 0.012 c	37.86 ± 0.96 k

[a] Mean ± standard deviation from triplicate determinations.

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

Table 18 shows Fe^{2+} - binding activity of cow pea seed extract at different amounts. Cow pea seed extract showed chelating activity on Fe^{2+} ion in a concentration-dependent manner. Fe^{2+} - binding activity of the extract correlated well with amount of the extract (r^2 = 0.9797). Fe^{2+} - binding activity of the extract at 1.40 mg was equal to EDTA at 0.20 M. However, the Fe^{2+} - binding activity of the extract was significantly lower than that of citric acid at the same concentration ($p < 0.05$).

Table 18. Fe^{2+} - binding activity of cow pea seed at different amounts.

Amounts (mg)	Absorbance at 480 nm	Fe^{2+} - binding activity (%)
0.00	0.716 ± 0.002 l[a]	0.00 a[b]
0.10	0.684 ± 0.006 k	4.70 ± 0.727 b
0.20	0.666 ± 0.003 j	6.98 ± 0.37 c
0.40	0.629 ± 0.006 l	12.10 ± 0.777 d
0.60	0.594 ± 0.004 h	17.08 ± 0.58 e
0.80	0.553 ± 0.003 g	22.77 ± 0.42 f
1.00	0.518 ± 0.007 f	27.78 ± 0.91 g
1.20	0.479 ± 0.008 e	33.15 ± 0.09 h
1.40	0.436 ± 0.007 d	39.10 ± 0.96 i
1.60	0.422 ± 0.008 c	41.01 ± 0.99 j
1.80	0.404 ± 0.008 b	43.57 ± 1.09 k
2.00	0.395 ± 0.004 b	44.83 ± 0.74 k
Citric acid 0.20 M	0.090 ± 0.003 a	86.79 ± 0.42 l
EDTA 0.20 M	0.425 ± 0.007 c	37.86 ± 0.96 i

[a] Mean ± standard deviation from triplicate determinations.

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

Some phenolic compounds acted as metal chelators, such as phytic acid, quercetin and luteolin (Graf *et al.*,1984; Crawford *et al.*, 1960; Hudson and Lewis, 1984). Moreover, Chen and Ahn (1998) found that natural phenolics including quercetin, rutin, catechin, and caffeic acid acted as Fe^{2+}-chelators.

The data obtained revealed that wild tamarind and cow pea seed extracts functioned as Fe^{2+}- chelators. Wild tamarind and cow pea seed extract either chelated metal ions or suppressed reactivity by occupying all coordination site on the metal ion (Mahoney and Graf, 1986; Lemon *et al.*, 1950). Therefore, it can be used as an effective agent in retarding Fe^{2+}-catalyzed lipid oxidation.

4.3.2 Cu^{2+}- binding activity of wild tamarind and cow pea seed extracts.

Cu^{2+}- binding activity of wild tamarind seed extract at different amounts is illustrated in Table 19. From the result, it was found that Cu^{2+}-binding activity was increased with an increasing amount of the extracts. High positive correlation (r^2 = 0.89) between Cu^{2+} - binding activity and amount of the extract was obtained. Chelating activity of extract at amount of 1.0 mg was 49.04 $\pm$ 0.53 %. It was significantly lower than that of EDTA at 0.20 M (62.65 $\pm$ 0.68 %), but it was higher than that of citric acid (39.66 $\pm$ 1.05 %) at the same concentration ($p < 0.05$).

Table 19 Cu^{2+} - binding activity of wild tamarind seed at different amounts.

Amounts (mg)	Absorbance at 480 nm	Cu^{2+} - binding activity (%)
0.00	1.021 ± 0. 007 l[a]	0.00 a[b]
0.10	0.986 ± 0.013 k	3.46 ± 1.74 b
0.20	0.974 ± 0.009 j	4.63 ± 1.40 c
0.40	0.892 ± 0.004 i	12.60 ± 0.051 d
0.60	0.779 ± 0.006 h	23.74 ± 0.77 e
0.80	0.653 ± 0.007 g	36.01 ± 0.91 f
1.00	0.520 ± 0.004 e	49.04 ± 0.58 h
1.20	0.442 ± 0.010 d	56.71 ± 1.09 i
1.40	0.427 ± 0.007 c	58.18 ± 0.91 j
1.60	0.420 ± 0.002 bc	58.89 ± 0.30 jk
1.80	0.417 ± 0.003 bc	59.19 ± 0.45 jk
2.00	0.410 ± 0.007 b	59.81 ± 0.95 k
Citric acid 0.20 M	0.616 ± 0.003 f	39.66 ± 0.26 g
EDTA 0.20 M	0.381 ± 0.002 a	62.65 ± 0.15 l

[a] Mean ± standard deviation from triplicate determinations.

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

Cu^{2+}-binding activity of cow pea seed extract at different amounts is illustrated in Table 20. Similar result was observed in cow pea seed extract compared to that obtained for wild tamarind seed. Cu^{2+} - binding activity of cow pea seed extract increased with an increasing amount of the extracts. High positive correlation between Cu^{2+} - binding activity and amount of the extract (r^2 = 0.9567) was noted. Cu^{2+} - binding activity of the extract at 1.6 mg was equal to that of citric acid at 0.20 M but it was significantly lower than that of EDTA at the same concentration ($p < 0.05$). So, cow pea seed extract possessed Cu^{2+} - binding activity.

Table 20 Cu^{2+} - binding activity of cow pea seed at different amounts.

- Amounts (mg)	Absorbance at 480 nm	Cu^{2+} - binding activity (%)
0	1.107 ± 0.012 l[a]	0.00 a[b]
0.10	0.984 ± 0.006 k	11.38 ± 1.04 b
0.20	0.967 ± 0.003 j	12.62 ± 0.23 c
0.40	0.960 ± 0.003 j	13.31 ± 0.23 c
0.60	0.898 ± 0.013 i	18.85 ± 1.18 d
0.80	0.799 ± 0.005 h	27.79 ± 0.42 e
1.00	0.765 ± 0.005 g	30.89 ± 0.48 f
1.20	0.715 ± 0.006 f	35.41 ± 0.50 g
1.40	0.696 ± 0.007 e	37.13 ± 0.64 h
1.60	0.671 ± 0.009 d	39.41 ± 0.84 i
1.80	0.656 ± 0.004 c	40.77 ± 0.32 j
2.00	0.622 ± 0.004 b	43.78 ± 0.34 k
Citric acid 0.20 M	0.616 ± 0.002 b	39.66 ± 0.26 i
EDTA 0.20 M	0.381 ± 0.002 a	62.65 ± 0.15 l

[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 wild tamarind and cow pea seed extracts functioned as Cu^{2+}-chalators. Copper was reported as the most pro-oxidant (Evan *et al.*, 1951). Wild tamarind and cow pea seed extracts formed a complex with free Cu^{2+}, resulting in the retardation of Cu^{2+}-catalyzed lipid oxidation.

4.4 Lipoxygenase inhibitory effects of wild tamarind and cow pea seed extracts.

Lipoxygenase catalyzes the oxygenation of polyunsaturated fatty acid containing a *cis,cis*-1-4 pentadiene system to hydroperoxides (Nawar, 1984). 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 (Huang *et al.*,1991; Belman *et al.*, 1989). Inhibitors of arachidonic acid metabolism also inhibited tumor promotion in animal models (Huang *et al.*,1991; Belman *et al.*, 1989). Moreover, lipoxygenase is the main factor initiating fatty acid oxidation during wort production, resulting in "cardboard off flavor" generated in stored beer (Kobayashi *et al.*, 1994; Angelino, 1991). So, an attempt was made to study the lipoxygenase inhibitory activities of wild tamarind and cow pea seed extract.

Lipoxygenase inhibitory activities of different amounts of wild tamarind seed extract are illustrated in Table 21. The extract inhibited lipoxygenase activity in a concentration-dependent manner. The inhibition of lipoxygenase activity of extract at 1 mg was not significantly different when compared to that of α-tocopherol at 0.10 mg but it was significantly lower than that of BHA, BHT, and TBHQ at the same concentration.

Table 21 Lipoxygenase inhibitory activity of wild tamarind seed extract at different amounts.

Amounts (mg)	Absorbance at 234 nm	Lipoxygenase inhibitory activity (%)
0.00	0.467 ± 0.007 j[a]	0.00 a[b]
0.05	0.434 ± 0.003 i	7.06 ± 0.65 b
0.10	0.421 ± 0.001 h	9.85 ± 0.20 c
0.20	0.419 ± 0.001 gh	10.20 ± 0.25 d
0.30	0.415 ± 0.003 g	11.13 ± 0.60 d
0.50	0.405 ± 0.006 f	13.35 ± 1.18 e
0.80	0.373 ± 0.002 e	20.13 ± 0.45 f
1.00	0.353 ± 0.003 d	24.48 ± 0.58 g
BHA 0.10	0.216 ± 0.005 b	53.75 ± 0.98 h
BHT 0.10	0.212 ± 0.001 b	54.60 ± 0.22 h
TBHQ 0.10	0.166 ± 0.003 a	64.53 ± 0.57 i
α - tocopherol 0.10	0.344 ± 0.004 c	26.34 ± 0.94 g

[a] Mean ± standard deviation from triplicate determinations.

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

Lipoxygenase-inhibitory activity of different amounts of cow pea seed extract is present in Table 22. Similar results were observed, compared to those obtained in wild tamarind seed. Lipoxigenase-inhibitory activity of the extract was dependent on concentration. However, the activity of extract at 1.00 mg was significantly lower than that of BHA, BHT, TBHQ, and α-tocopherol at a level of 0.10 mg.

At the same concentration, the wild tamarind seed extract showed lipoxigenase inhibitory activities higher than cow pea seed extract, presumably due to the differences in structure of antioxidant components in each extracts. Richard-Forget *et al.* (1995) reported that phenolic compounds including kaempferol, quercetin, myricetin, quercitrin, isoquercitrin, rutin, astragalin, fisetin, dihydroquercetin, (-)-epicatechin, (+)-catechin and epigallocatechin showed inhibitory effect on lipoxygenase oxidation of linoleic acid. Inhibition constants were strongly affected by structure of antioxidants.

Table 22 Lipoxygenase inhibitory activity of cow pea seed extract at different amounts.

Amounts (mg)	Absorbance at 234 nm	Lipoxygenase inhibitory activity (%)
0.00	0.514 ± 0.021 g[a]	0.00 a[b]
0.05	0.501 ± 0.006 f	2.46 ± 1.26 b
0.10	0.500 ± 0.006 f	2.66 ± 1.17 b
0.20	0.494 ± 0.004 ef	3.83 ± 0.79 bc
0.30	0.489 ± 0.007 ef	4.93 ± 1.38 c
0.50	0.487 ± 0.003 e	5.34 ± 0.60 c
0.80	0.461 ± 0.005 d	10.24 ± 0.92 d
1.00	0.452 ± 0.003 d	12.13 ± 0.59 e
BHA 0.10	0.216 ± 0.005 b	53.75 ± 0.98 g
BHT 0.10	0.212 ± 0.001 b	54.60 ± 0.22 g
TBHQ 0.10	0.166 ± 0.003 a	64.53 ± 0.66 h
α - tocopherol 0.10	0.344 ± 0.004 c	26.34 ± 0.94 f

[a] Mean ± standard deviation from triplicate determinations.

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

This result was in agreement with Shimoni *et al.* (1994) who reported that deferoxamine showed inhibitory effect on lipoxygenase in a concentration dependent – manner. Lipoxygenase, an enzyme that specially introduces oxygen into free fatty acid, contains iron within its molecular structure (Shimoni *et al.*, 1994). From our result, wild tamarind and cow pea seed extract acted as metal-chelator. Therefore, wild tamarind and cow pea seed extracts inhibited lipoxygenase activity, possibly via interaction with a ferric ion at the active site, leading to inactivation of enzyme.

5. Separation of antioxidants from wild tamarind and cow pea seed extracts.

Thin-layer chromatography (TLC) was used for separation of components in wild tamarind seed extract. Wild tamarind seed extract was applied and developed with dichloromethane : methanol : water (5:3:2). Two bands were found, after drying and spraying with different reagents (Table 23) at R_f 0.80 (band A) and 0.74 (band B) (Figure 33). Spray 1 revealed the presence of phenolic compound in wild tamarind seed extract. Spray 2 indicated that phenolic compound without free *ortho-* and *para-* hydroxy groups were present. Reducing compounds were identified by the appearance of a gray color after chromatograms were sprayed with an ammonical silver nitrate solution (spray 3).

Table 23 Identification of antioxidant compounds in wild tamarind seed extract.

Sprays	Band[a]	Color	Compound identified
$FeCl_3$-$K_3Fe(CN)_6$	A,B	blue	Phenolics
$FeCl_3$	A,B	brown	Phenolics wihtout *o*-/*p*-OH
NH_4OH-$AgNO_3$	A,B	gray	Reducing compound

[a] The bands corresponded to positive identification of compounds at R_f values of 0.80 (band A) and 0.74 (band B).

After the bands were located by different reagents, the silica gel scrapings, which contained the interested compounds were soaked in excess 50 % methanol for 30 min, filtered and evaporated to remove methanol, then freeze-dried to obtain each antioxidant fraction. The residue was redissolved in 1 ml of 50 % methanol and antioxidant activity was determined.

The decrease in absorbance of β - carotene in the presence of band A and B extract is shown in Figure 34. The rate of decrease was reduced with extract from band A and B. This result reconfirmed that band A and B contained the antioxidant components, particularly phenolic compounds.

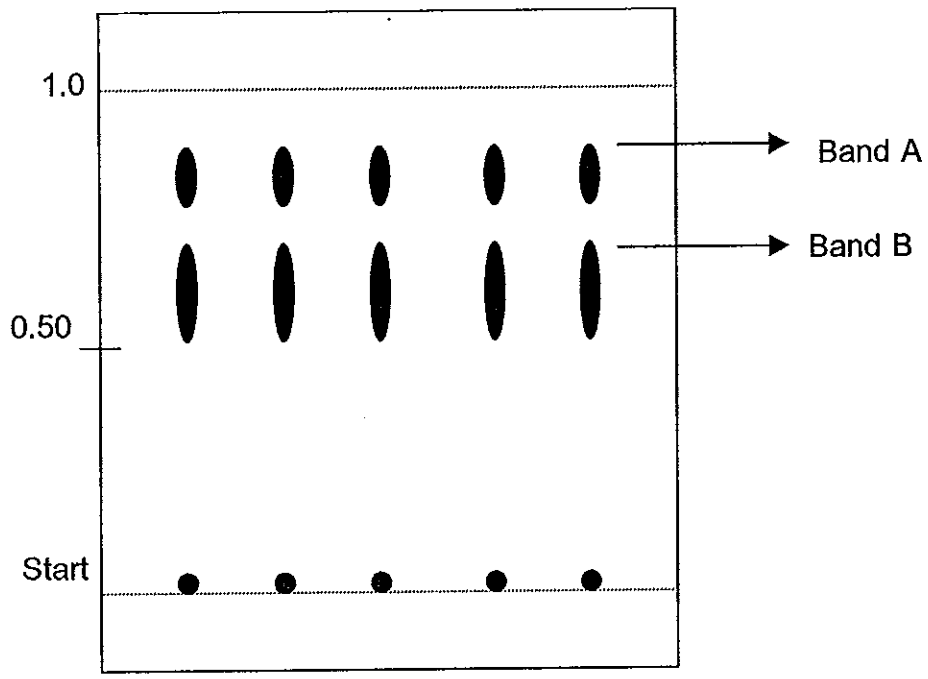

Figure 33 Thin – layer chromatography (TLC) pattern of wild tamarind seed extract.

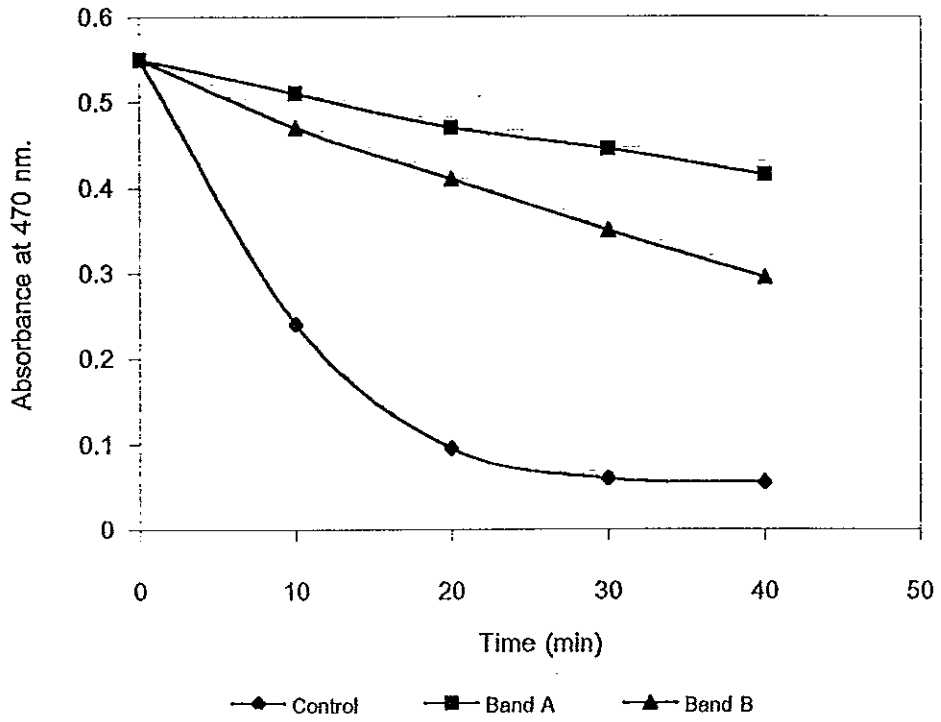

Figure 34 Antioxidant activity of components extracted from bands located on thin – layer chromatography.

Thin-layer chromatography (TLC) was also used for separation of components in cow pea seed extract. Cow pea seed extract was developed with butanol: acetic acid: water (4:1:5). After drying and spraying with different reagents, five bands were observed. The bands corresponded to positive identification of compounds were found at R_f values of 0.10 (band A), 0.45 (band B), 0.59 (band C), 0.68 (band D), and 0.95 (band E) (Figure 35). Spray 1 revealed the presence of phenolic compound in cow pea seed extract (Table 24). Phenolic compounds with free ortho- and para- hydroxy groups were found, as indicated by spray 2. Reducing compounds were identified by the appearance of a black color after spraying with an ammonical silver nitrate

solution (spray 3). Antioxidant activities of five bands were determined in β-carotene emulsion system (Figure 36). Band A (70.68 ± 0.65 %) and D (54.97 ± 0.35 %) exhibited strong prevention of β-carotene bleaching. Band B (13.47 ± 0.21 %), C (16.27 ± 0.10 %) and E (21.39 ± 0.10 %) exhibited weaker antioxidant activities than band A and D. Band A had highest antioxidant activity when compared to other bands separated from cow pea seed extract.

Table 24 Identification of antioxidant compounds in cow pea seed extract.

Sprays	Band[a]	Color	Compounds identified
$FeCl_3$-$K_3Fe(CN)_6$	A,B,C,D,E	blue	Phenolics
$FeCl_3$	A,B,C,D,E	brown	Phenolics wihtout *o*-/*p*-OH
NH_4OH-$AgNO_3$	A,B,C,D,E	black	Reducing compound

[a] The bands corresponded to positive identification of compounds at R_f values of 0.10 (band A), 0.45 (band B), 0.59 (band C), 0.68 (band D), and 0.95 (band E).

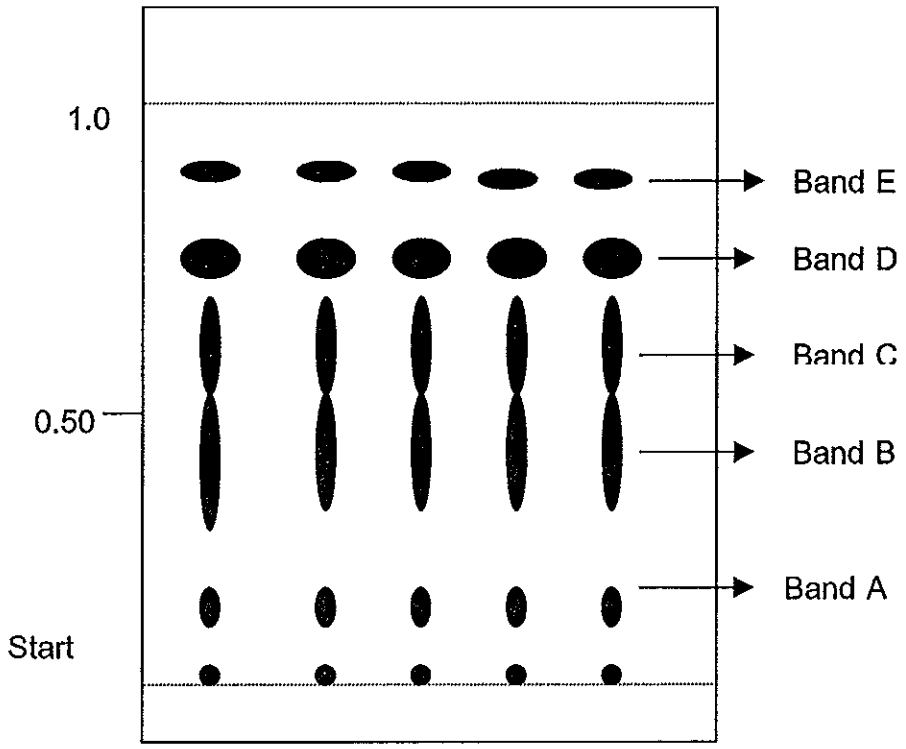

Figure 35 Thin – layer chromatography (TLC) pattern of cow pea seed extract.

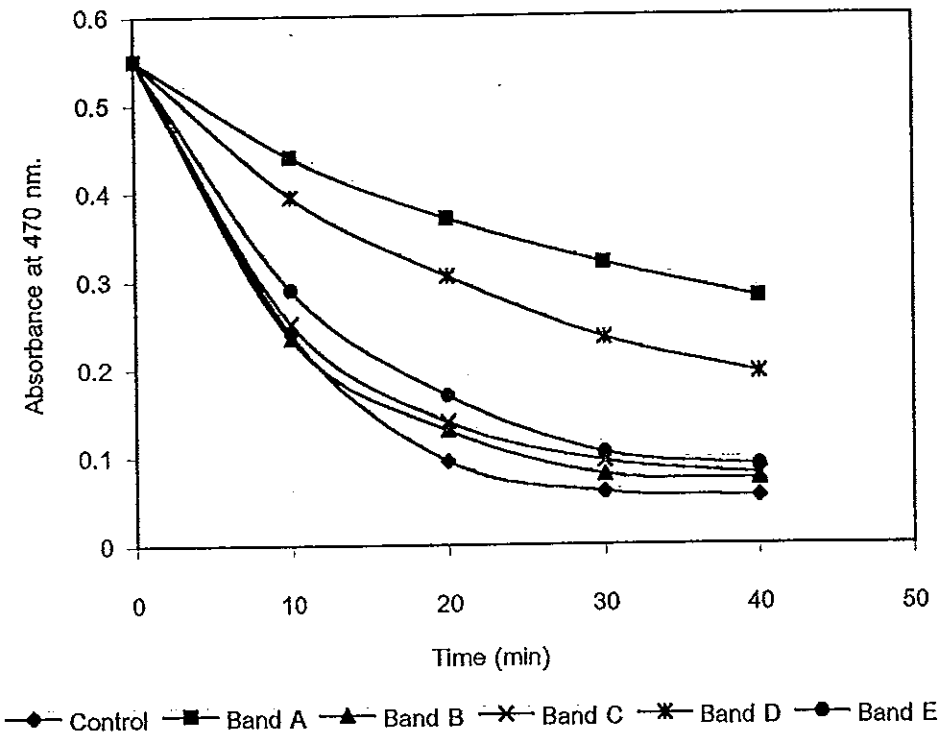

Figure 36 Antioxidant activity of components extracted from bands located on thin – layer chromatography.

6. Application of wild tamarind and cow pea seed extracts in cooked ground pork.

6.1 Wild tamarind seed extract.

The antioxidant effect of wild tamarind seed extract at different concentrations on lipid oxidation of cooked ground pork was determined. Lipid oxidation of cooked ground pork was assessed by TBARS, peroxide value and conjugated diene. TBARS of cooked ground pork treated with different concentrations of wild tamarind, BHA, BHT and α-tocopherol is shown in Figure 37. All samples treated with wild tamarind seed extracts and other antioxidants had significantly lower TBARS than the control ($p < 0.05$). No significant differences in TBARS between sample treated with the extracts 0.05 or 0.1 %, BHA 0.02% and BHT 0.02 % were observed. Moreover, TBARS of sample treated with 0.02 % wild tamarind seed extract was significantly lower than those treated with α-tocopherol at 0.02%. Peroxide values of cooked ground pork treated with different concentrations of wild tamarind seed extract (0.02, 0.05 and 0.1 % w/w), BHA (0.02 % w/w), BHT (0.02 % w/w) and α-tocopherol (0.02 % w/w) are illustrated in Figure 38. All samples treated with wild tamarind seed extracts, BHA, BHT and α-tocopherol had significantly lower peroxide value than the control ($p < 0.05$). No significant difference in peroxide value was found between the treatment with wild tamarind seed extract at 0.05 or 0.1 %, BHA 0.02% and BHT 0.02 % stored at 4 ^{o}C for 15 days ($p > 0.05$). Moreover, treatment with wild tamarind extract at 0.02 % showed the lower peroxide value than the treatment with α-tocopherol at 0.02 %. All treatment treated with different concentration of wild tamarind seed extract and other antioxidants had lower conjugated diene formation than the control (Figure 39). Wild tamarind extract inhibited conjugated diene formation in a concentration-dependent manner. After 15 days of storage, wild tamarind extracts at 0.05 and 0.1 % were a better

inhibitor of conjugated diene formation than α-tocopherol but not significantly different ($p > 0.05$) when compared to BHA and BHT at 0.02 %.

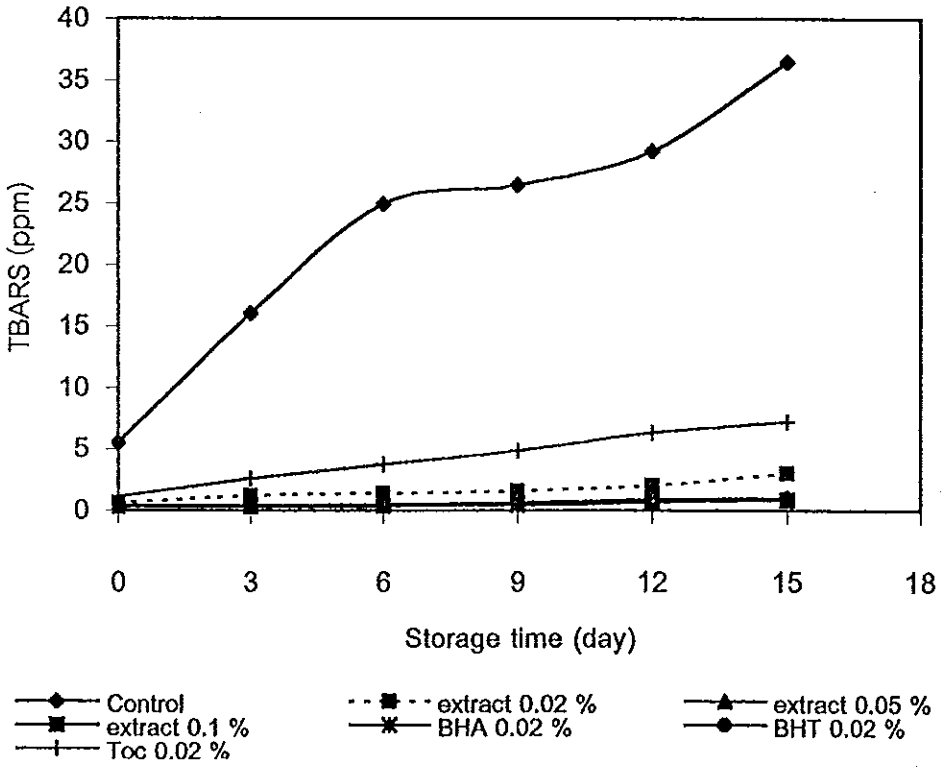

Figure 37 TBARS of cooked ground pork treated with wild tamarind seed extract and other commercial antioxidants during storage at 4 °C for 15 days.

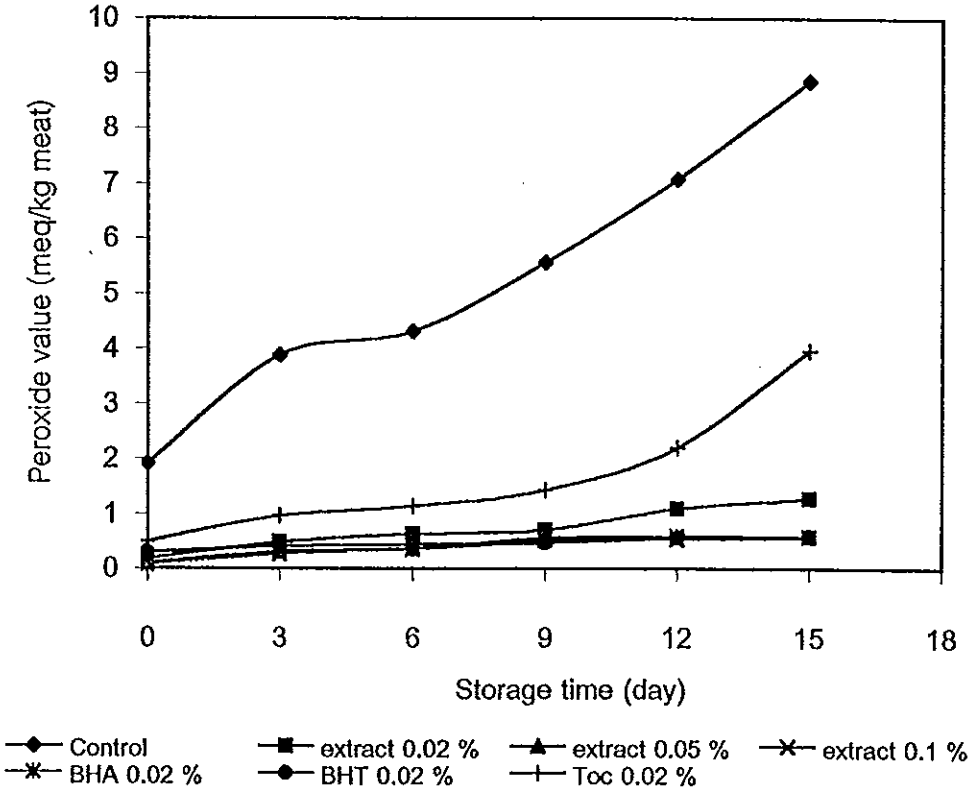

Figure 38 Peroxide value of cooked ground pork treated with wild tamarind seed extract and other commercial antioxidants during storage at 4 °C for 15 days.

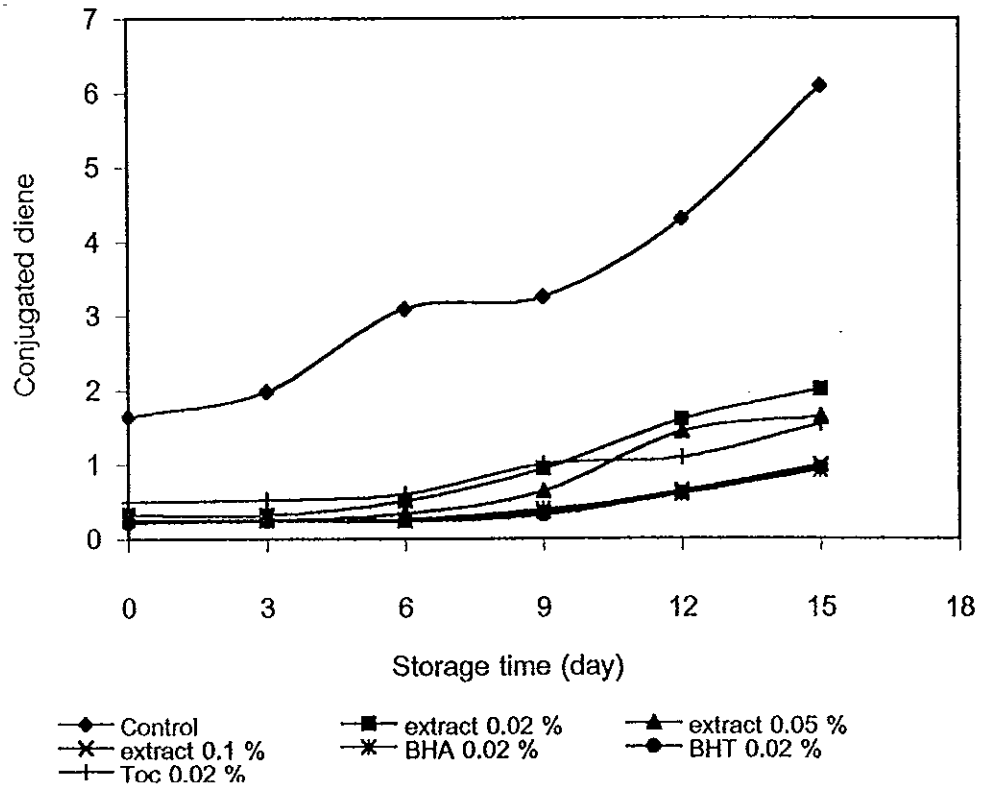

Figure 39 Conjugated diene of cooked ground pork treated with wild tamarind seed extract and other commercial antioxidants during storage at 4 ^{o}C for 15 days.

The results revealed that wild tamarind seed extracts inhibited oxidation in cooked ground during storage at 4 ^{o}C for 15 days. The extract at 0.02, 0.05 and 0.1 % were more active than α-tocopherol at 0.02 %, the best well - known natural antioxidant (Dziezak, 1986). The data in this study demonstrated that wild tamarind seed extract acted as antioxidant in cooked ground pork. However, toxicological tests are necessary before the extract of wild tamarind seed can be used in food products.

6.2 Cow pea seed extract.

Retardation of oxidation of cooked ground pork by different concentrations of cow pea seed extract was investigated. The results of TBARS, peroxide value, and conjugated diene of cooked ground pork treated with different concentrations of cow pea seed extract, BHA, BHT, and α - tocopherol are shown in Figure 40, 41, and 42. All samples treated with extracts, BHA, BHT, and α-tocopherol had significantly lower TBARS, peroxide values, and conjugated diene than the control ($p < 0.05$). TBARS, peroxide value, and conjugated diene in all samples increased as storage time increased. No significant difference ($p<0.01$) in TBARS of samples treated with BHA and BHT at 0 day and 15 day of storage was noted ($p > 0.05$). Samples treated with the extract at 0.02, 0.05 or 0.1 % had significantly higher TBARS, peroxide value, and conjugated diene than those treated with BHA, BHT and α-tocopherol ($p < 0.05$).

The data obtained showed that cow pea seed extract inhibited oxidation in cooked ground pork to some extent. The lower efficiency of the extract in oxidation inhibition of cooked ground pork was observed, compared to BHA, BHT, and α - tocopherol.

Generally, wild tamarind seed extract exhibited much higher antioxidant activity in cooked ground pork compared to cow pea seed extract. This is postulated to be due to the different compounds in both extracts, which had different efficiency in oxidation prevention in cooked ground pork.

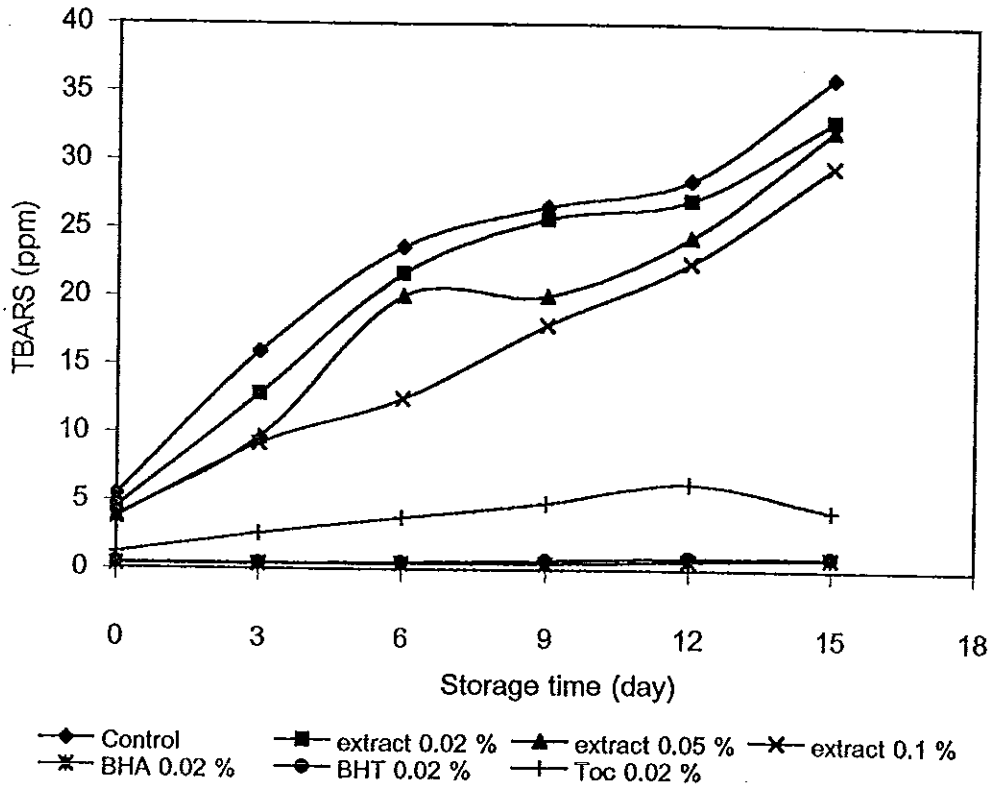

Figure 40 TBARS of cooked ground pork treated with cow pea seed extract and other commercial antioxidants during storage at 4 °C for 15 days.

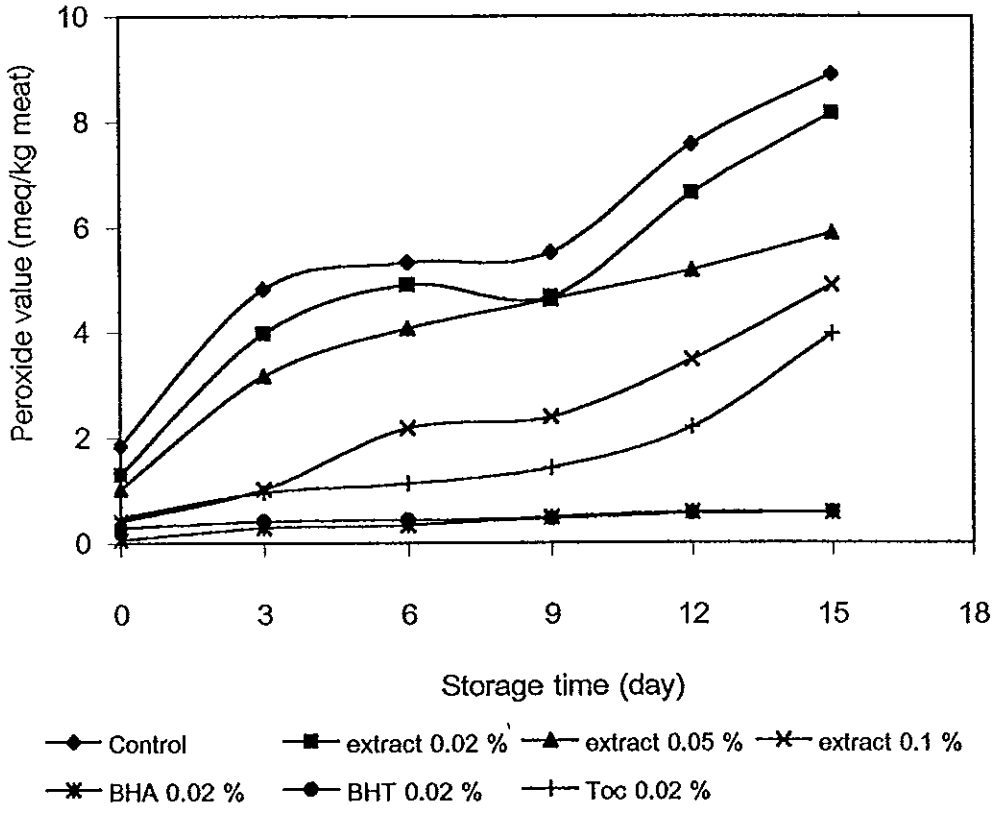

Figure 41 Peroxide value of cooked ground pork treated with cow pea seed extract and other commercial antioxidants during storage at 4 ^{o}C for 15 days.

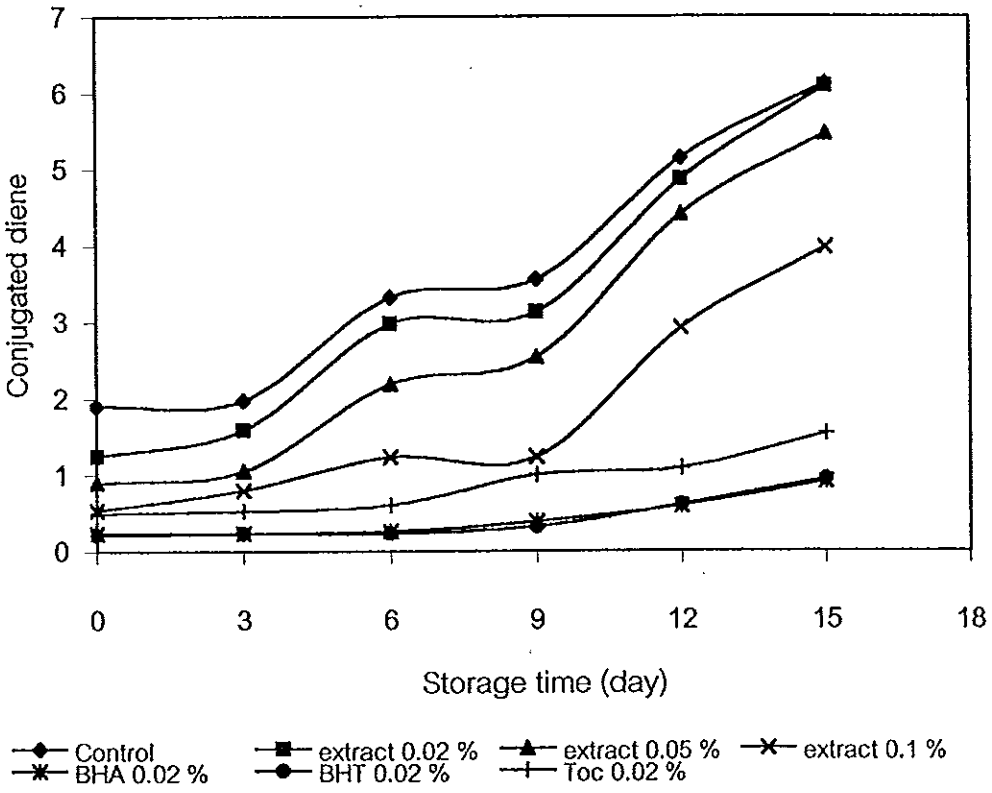

Figure 42 Conjugated diene of cooked ground pork treated with cow pea seed extract and other commercial antioxidants during storage at 4 ^{o}C for 15 days.

6.3 Synergistic effect of citric acid or ascorbic acid with wild tamarind or cow pea seed extracts on lipid oxidation of cooked ground pork.

The combinative effect between citric acid or ascorbic acid with wild tamarind seed extract in retarding oxidation of cooked ground pork was determined. Figure 43, 44, and 45 show TBARS, peroxide value, and conjugated diene of samples treated with different compounds. TBARS, peroxide value, and conjugated diene formation of all samples increased as storage time increased. No significant differences in TBARS, peroxide value, and conjugated diene between samples treated with 0.005 % or 0.01 % ascorbic acid and 0.005 % or 0.01 % citric acid ($p < 0.05$) were obtained. Moreover, samples treated with ascorbic acid 0.005 and 0.01 % or citric acid 0.005 % had significantly higher TBARS, peroxide value, and conjugated diene of the control ($p < 0.05$). Ascorbic acid has been widely used as a food ingredient for its reducing and antioxidant activity (Bendich *et al.*, 1986) but it is highly susceptible to oxidation, especially when catalyzed by metal ions such as Cu^{2+} and Fe^{3+} (Khan and Martell, 1967). Muscle foods contain high amounts of the metal ions, possibly resulting in rapid oxidation of ascorbic acid when ascorbic acid is added to meats. Bendict *et al.* (1975) reported that addition of 50 ppm (0.005 %) ascorbic acid to ground beef caused increased lipid peroxidation. However, ground beef treated with 500 ppm ascorbic acid had reduced lipid peroxidation (Mitsumoto, 1991). Citric at 0.01 % prevented oxidative rancidity in fresh pork sausage (Dziezak, 1986). From the result, sample treated with 0.005 % citric acid had an increased oxidation in cooked ground pork, possibly due to improper mixing at low concentration of citric acid. Synergistic effect of wild tamarind seed extract with ascorbic acid and citric acid (0.01%, w/w) was observed. TBARS, peroxide value, and conjugated diene of these samples were significantly lower than sample

treated with only wild tamarind seed extract. Combination of wild tamarind seed extract - ascorbic acid had significant lower TBARS, peroxide value, and conjugated diene than wild tamarind seed extract – citric acid combination ($p < 0.05$).

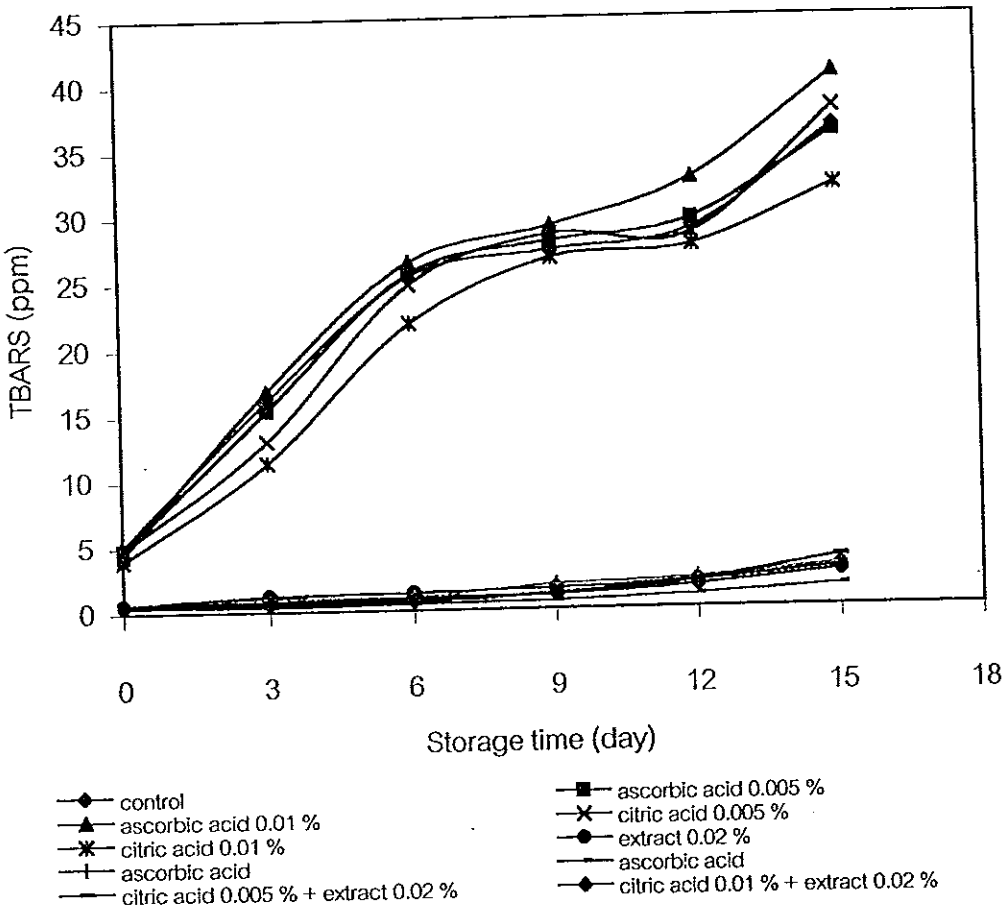

Figure 43 Synergistic effects of wild tamarind seed extract with ascorbic acid or citric acid on TBARS formation of cooked ground pork during storage at 4 ^{o}C for 15 days.

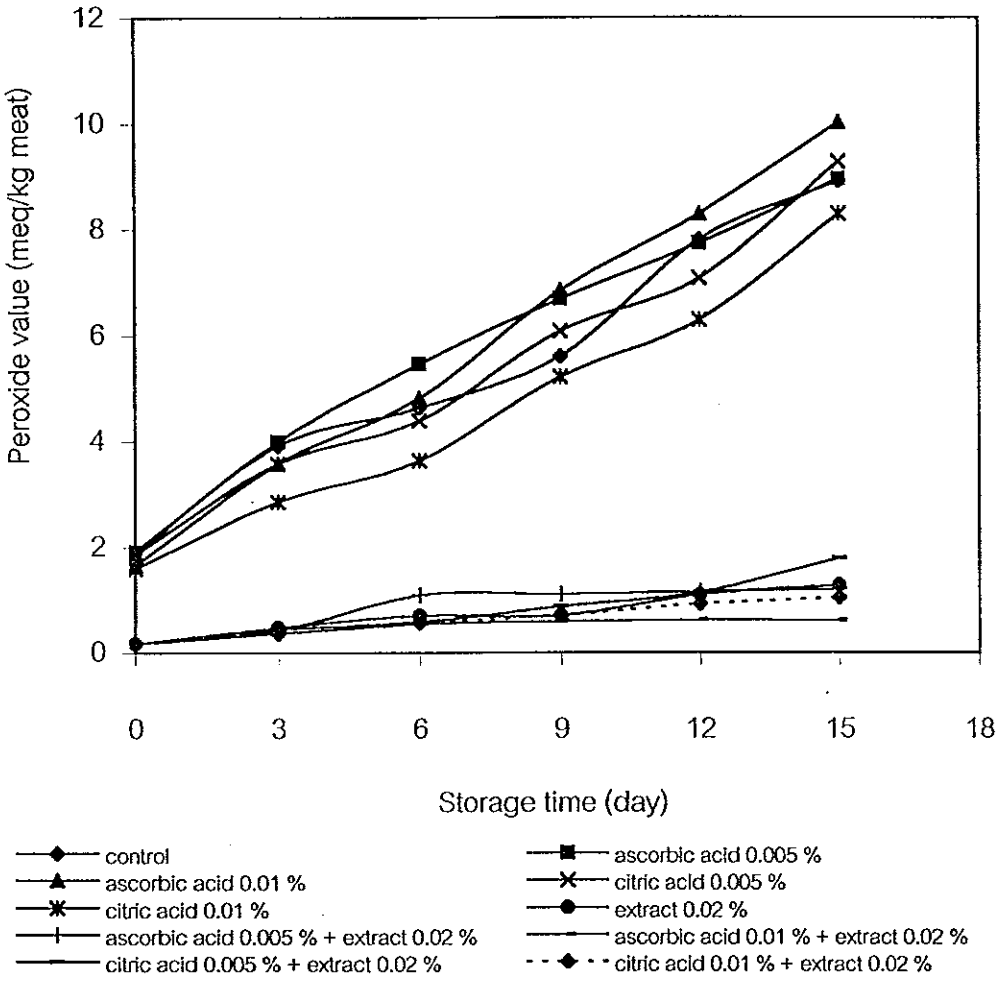

Figure 44 Synergistic effects of wild tamarind seed extract with ascorbic acid or citric acid on peroxide formation of cooked ground pork during storage at 4 ^{o}C for 15 days.

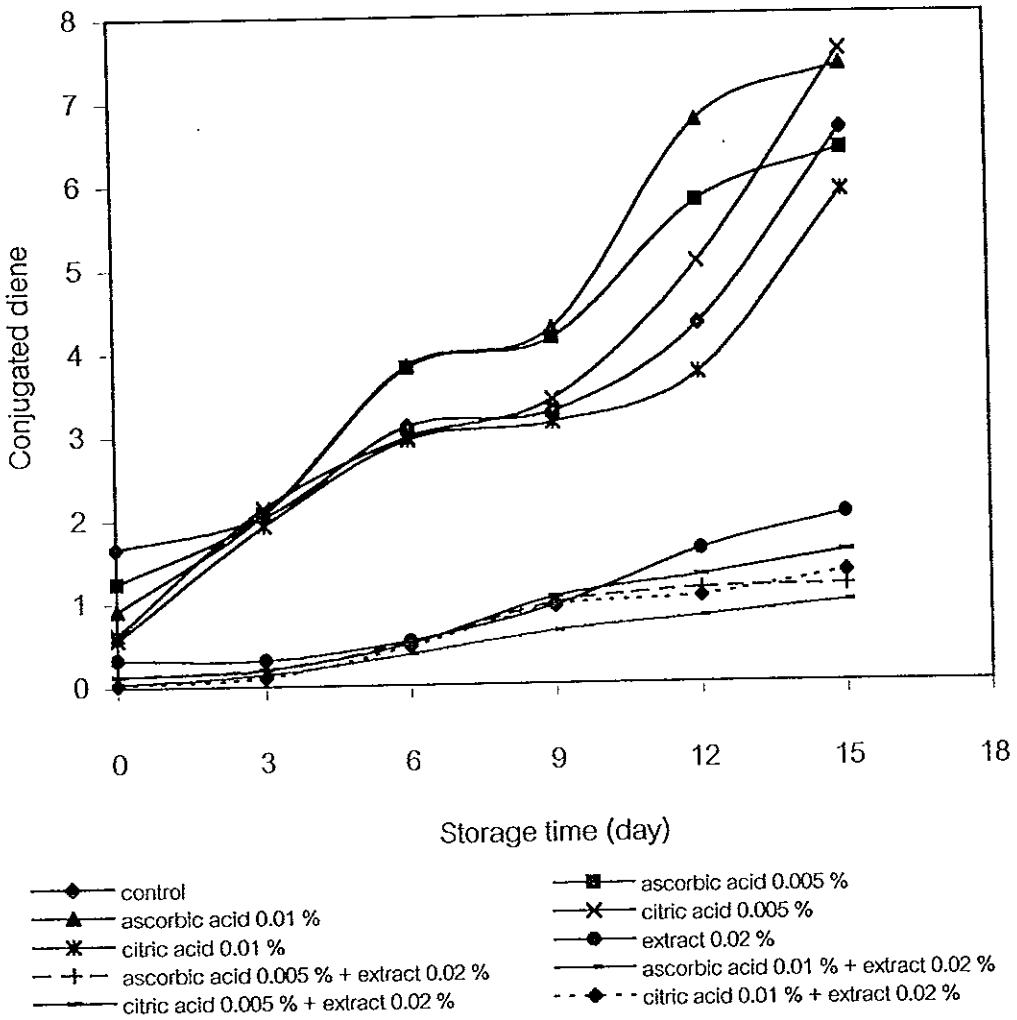

Figure 45 Synergistic effects of wild tamarind seed extract with ascorbic acid or citric acid on conjugated diene formation of cooked ground pork during storage at 4 ^{o}C for 15 days.

The combined effect between citric acid or ascorbic acid with cow pea seed extract in retarding oxidation of cooked ground pork was determined. From the result, combination of cow pea seed extract and ascorbic acid or citric acid at a level of 0.005 or 0.01% had highly significantly ($p<0.01$) lower TBARS, peroxide value and conjugated diene formation than samples treated with only cow pea seed extract (Figure 46, 47, and 48) ($p < 0.05$). The synergistic effect of ascorbic acid or citric acid with cow pea seed extract increased with an increasing concentration of ascorbic acid or citric acid. Treatment with the combination of cow pea seed extract and citric acid had significantly lower TBARS, peroxide value and conjugated diene than those with the combination of cow pea seed extract and ascorbic acid.

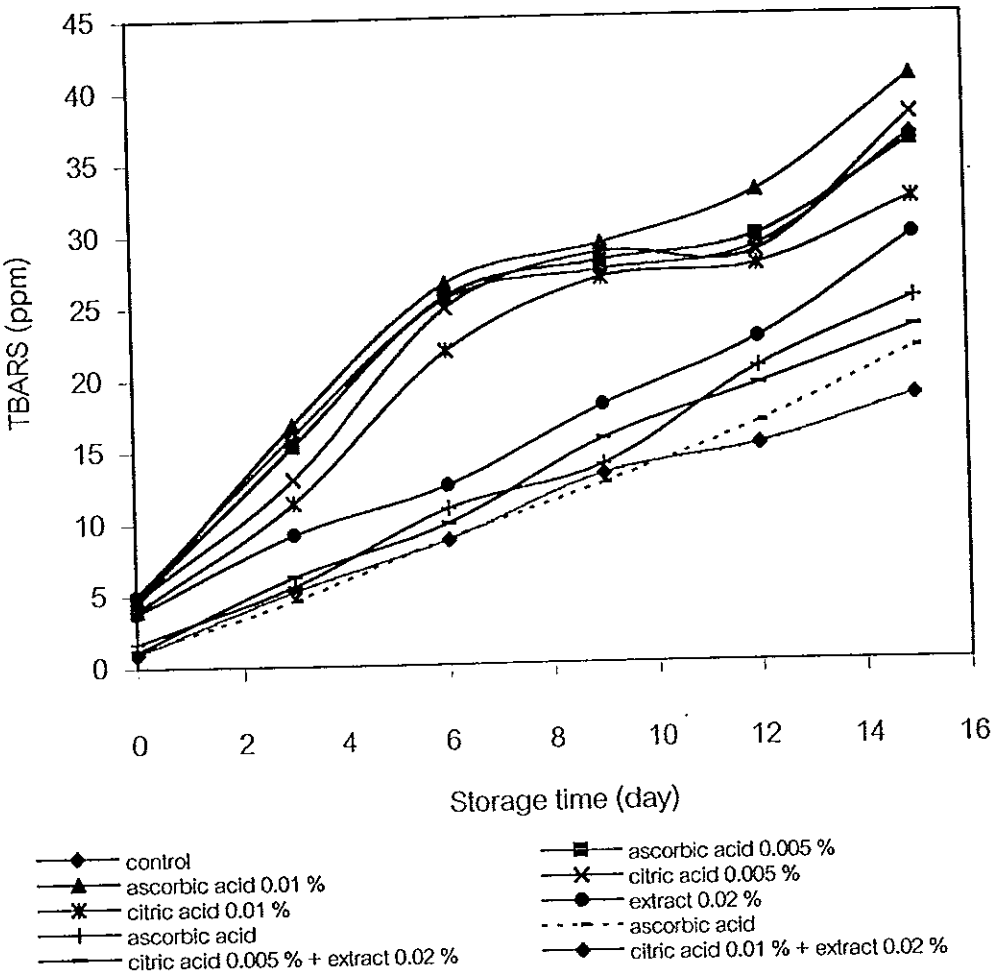

Figure 46 Synergistic effects of cow pea seed extract with ascorbic acid or citric acid on TBARS formation of cooked ground pork during storage at 4 ^{o}C for 15 days.

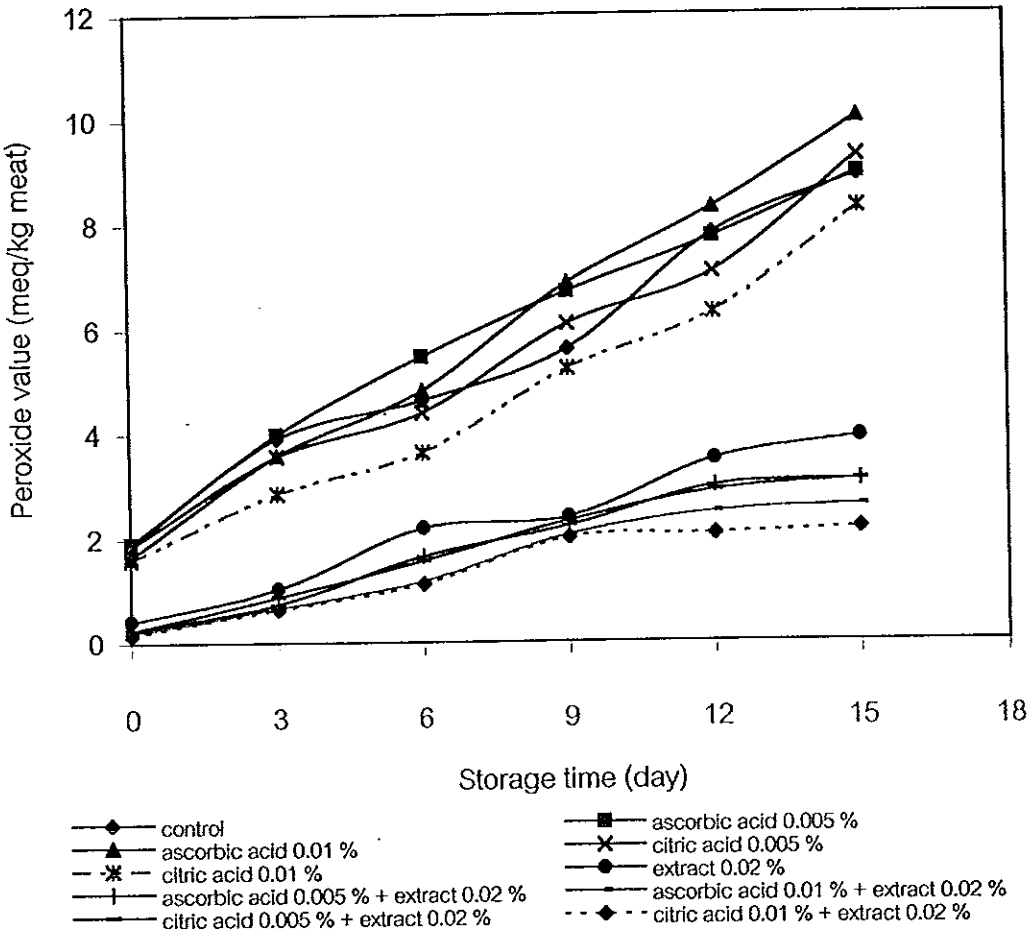

Figure 47 Synergistic effects of cow pea seed extract with ascorbic acid or citric acid on peroxide formation of cooked ground pork during storage at 4 ^{o}C for 15 days.

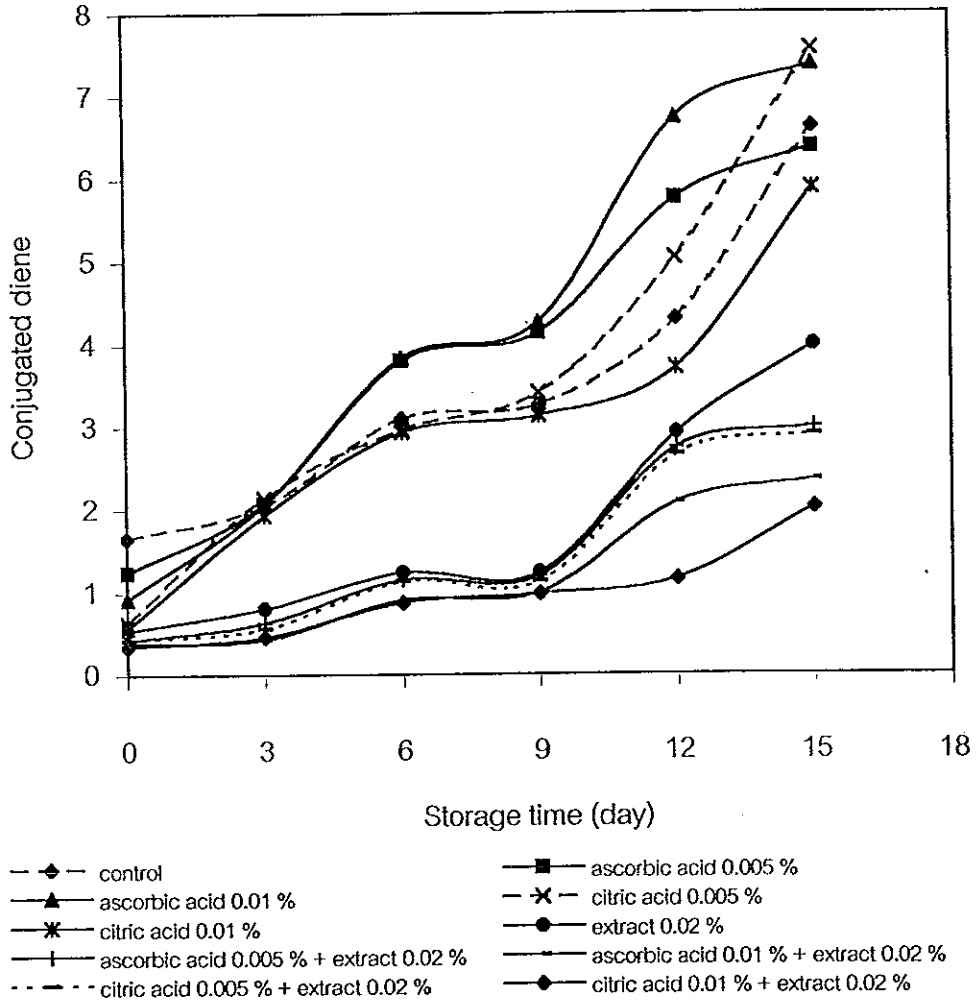

Figure 48 Synergistic effects of cow pea seed extract with ascorbic acid or citric acid on conjugated diene formation of cooked ground pork during storage at 4 °C for 15 days.

Ascorbic acid functioned as an oxygen scavenger and regenerated phenolic antioxidant by contributing hydrogen atom to phenoxyl radicals produced by lipid oxidation (Lindsay, 1976). In this study, we found that wild tamarind and cow pea seed extract contained phenolic compounds. Therefore, synergistic effect of wild tamarind and cow pea seed extract with ascorbic acid probably resulted from regeneration of extract antioxidants by donated hydrogen atoms from ascorbic acid.

Citric acid functioned as chelating pro-oxidant metal ion such as iron and copper (Dziezak, 1986). Ground pork contains high amount of metal ion, particulary iron. Therefore, the synergistic effect of wild tamarind seed and cow pea seed extract was possibly due to the chelating of metal ion in cooked ground pork, resulting in promotion of antioxidant efficacy of the seed extracts to inhibit the oxidation of cooked ground pork.

Chapter 4

Conclusions

1. Among 19 different varieties of legume seed extracts, wild tamarind seed extract exhibited highest antioxidant activity, followed by cow pea seed extract.
2. Methanol extracts of wild tamarind seed and cow pea seed provided higher antioxidant activities, total phenolic content and reducing power than ethanol, ethyl acetate, and hexane extracts, respectively.
3. The optimum condition for preparing wild tamarind and cow pea seed extract involved extracting seed powder with 50 % methanol for 2 hrs, and 70 % methanol for 5 hrs., respectively.
4. Wild tamarind and cow pea seed extracts had good thermal and pH stability.
5. Antioxidant activities of wild tamarind and cow pea seed extracts were higher at neutral and alkaline pHs, compared to acidic pHs.
6. Synergistic action of wild tamarind and cow pea seed extracts with α-tocopherol in β-carotene/linoleic acid system was observed. However, no synergism of ascorbic acid and citric acid on the antioxidative effect of both extracts was obtained.
7. Antioxidant activities of wild tamarind and cow pea seed extracts increased with an increasing concentration and were concomitant with the development of reducing power.
8. Wild tamarind and cow pea seed extracts acted as radical scavenger in a concentration-dependent manner.

9. Wild tamarind and cow pea seed extracts functioned as Fe^{2+} and Cu^{2+} chelators. The chelating activities of both extracts were increased with increasing amount of the extracts.
10. Wild tamarind and cow seed extracts showed inhibitory effect on lipoxygenase activity in a concentration - dependent manner.
11. Phenolic compounds with free *ortho*- and *para*- hydroxy groups, which were reducing compounds, were present in wild tamarind and cow pea seed extracts.
12. Wild tamarind and cow pea seed extracts inhibited oxidation in cooked ground pork during storage at 4 $^{\circ}C$ for 15 days. Synergistic action was found in seed extracts with citric acid and ascorbic acid for retardation of oxidation in cooked ground pork.

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Appendix 1

Analytical methods

1. Determination of total phenolic content (Weurman and Swain, 1955).

Chemicals

- Folin and Ciocalteu's Phenol Reagent
- Saturated sodium bicarbonate solution

Method

The extract (0.5 ml) was added with 5 ml of distilled water, followed by 1 ml of Folin and Ciocalteu's Phenol Reagent. 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 by using a UV 1601 spectrophotometer. 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
- 10 % Trichloroacetic acid (TCA)
- 1 % Potassium ferricyanide
- 0.1% ferric chloride

Method

The extract (0.5 ml) was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1 %). The mixture was then incubated at 50 ^{o}C for precisely 20 min. An aliquot (2.5 ml) of TCA (10 %) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 2.5 ml of ferric chloride (0.1 %), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

3. Determination of scavenging effect on 1,1-diphenyl-2-picryhydrazyl (DPPH) radical (Blois, 1958).

Chemicals

- Methanol solution of DPPH (at the final concentration of 0.2 mM)

Method

The extract (4.0 ml) was mixed vigorously with 1.0 ml of DPPH and incubated for 30 min. The absorbance of the resulting solution was measured at 517 nm using a 1601 UV spectrophotometer. Values reported are mean from triplicate determinations of the experiment.

4. Measurement of chelating activity on Fe^{2+} and Cu^{2+} (Shimada *et al.*, 1992).

Chemicals

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

Method

The extract (2.0 ml) was added to 2.0 ml of hexamine buffer, and 0.2 ml of tetramethylmurexide was added. Absorbance at 480 nm was measured. The measurement was carried out at 20 ^{o}C to prevent oxidation of Fe^{2+}.

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

Chemicals

- Substrate solution : Sonication 0.05 ml of linoleic acid and 0.05 ml tween 20 in 10 ml of borate buffer (0.2 M, pH 9.0) by sonicator.
- Lipoxygenase solution : 1.2 mg of lipoxygenase was dissolved in 20 ml of borate buffer (0.2 M, pH 9.0).

Method

The extract (0.2 ml) was added to 8.0 ml of substrate solution, then the enzyme solution (0.5 ml) was added. The mixture was shaken at room temperature for 15 min. A 1.0 ml, aliquots from these mixture was transferred to test tube containing 2.0 ml ethanol and 60 % ethanol was added to make a total final volume of 10 ml. Absorbance at 234 nm was measured.

6. Determination of thiobarbituric acid substance (TBARS) (Buege and Aust, 1978).

Chemicals

- TBA solution : 0.375 g of thiobarbituric acid, 15 g of trichloroacetic acid, and 0.875 ml of hydrochloric acid were mixed thoroughly in 100 ml of distilled water.

Method

Sample (0.5 g) was mixed with 2.5 ml of TBA solution. The mixture was heated for 10 min in a boiling water bath (95-100 $^{\circ}$C) to develop pink color, cooled with tap water, centrifuged at 5500 rpm for 25 min, and absorbance of the supernatant was measured at 532 nm. A standard curve was prepared with malonaldehyde bis(dimethyl acetal) (MDA) at concentration ranging from 0-10 ppm. The quantity of TBARS in each sample was calculated as mg MDA/kg sample using standard curve.

7. Determination of peroxide value (IUPAC, 1979).

Chemicals

- Acetic acid: chloroform (3:2, v/v)
- Saturated potassium iodide solution
- 0.01 N Sodium thiosulphate solution
- 1 % Starch solution

Method

Sample (≈ 1 g) was mixed with a mixture of acetic acid and chloroform, follow by addition of 1.0 ml of patassium iodide. The reaction mixture was left to stand for 5 min in the dark. Distilled water (75.0 ml) was added to the mixture. The mixture was titrated with sodium thiosulphate solution and shaken vigorously until color of mixture was light yellow, then 0.5 ml of starch solution was added. The mixture had blue color. Sodium thiosulphate was used to titrate until clear solution was obtained. Peroxide value was expressed as meq / kg oil using:

$$\text{Peroxide value (meq/kg oil)} = \frac{(a-b) \times N \times 1000}{W}$$

Where a = Volume (ml) of sodium thiosulphate of blank

b = Volume (ml) of sodium thiosulphate of sample

N = Concentration of sodium thiosulphate (Normal)

W = weight of sample

8. Measurement of conjugated diene (Frankel and Huang, 1996).

Chemicals

- Iso-octane

Method

Sample (0.1 g) was dissolved in 5.0 ml of iso-octane and the absorbance was measured at 234 nm. Conjugated diene was calculated as an increase in absorbance per 0.2 mg oil.

Appendix 2

Analysis of variance

Table 1-A. Analysis of variance for antioxidant activities of 19 different varieties of legume seed.

SV	DF	SS	MS	F
Treat	18	332.9117	18.4950	36.03 **
Error	38	19.5087	0.5134	
Total	56	352.4204		

cv = 0.8 %

** = Significant at 1 % level

Table 2-A. Analysis of variance for total phenolic content 19 different varieties of legume seed.

SV	DF	SS	MS	F
Treat	18	410322.7452	22795.7081	17988.79 **
Error	38	48.1543	1.2672	
Total	56	410370.8995		

cv = 1.3 %

** = Significant at 1 % level

Table 3-A. Analysis of variance for reducing power of 19 different varieties of legume seed.

SV	DF	SS	MS	F
Treat	18	2.6552	0.1475	651.78 **
Error	38	0.0086	0.0002	
Total	56	2.6638		

cv = 4.1 %

** = Significant at 1 % level

Table 4-A. Analysis of variance for radical-scavenging activities of 19 different varieties of legume seed.

SV	DF	SS	MS	F
Treat	18	323.3294	17.9627	27.73 **
Error	38	28.7667	0.7570	
Total	56	352.0962		

cv = 0.9 %

** = Significant at 1 % level

Table 5-A. Analysis of variance for antioxidant activity of wild tamarind seed prepared with different solvents.

SV	DF	SS	MS	F
Treat	3	13158.6156	4386.2052	4464.86 **
Error	8	7.8591	0.9824	
Total	11	13166.4746		

cv = 1.5 %

** = Significant at 1 % level

Table 6-A. Analysis of variance for total phenolic content of wild tamarind seed prepared with different solvents.

SV	DF	SS	MS	F
Treat	3	280232.3004	93410.7668	140855.64 **
Error	8	5.3053	0.6632	
Total	11	280237.6057		

cv = 0.7 %

** = significant at 1 % level

Table 7-A. Analysis of variance for reducing power of wild tamarind seed prepared with different solvents.

SV	DF	SS	MS	F
Treat	3	1.0953	0.3651	14603.89 **
Error	8	0.0002	0.00002	
Total	11	2.6638		

cv = 1.4 %

** = Significant at 1 % level

Table 8-A. Analysis of variance for antioxidant activities of cow pea seed prepared with different solvents.

SV	DF	SS	MS	F
Treat	3	12293.5769	4097.8590	10670.81 **
Error	8	3.0722	0.3840	
Total	11	12296.6491		

cv = 0.9 %

** = Significant at 1 % level

Table 9-A. Analysis of variance for reducing power of cow pea seed prepared with different solvents.

SV	DF	SS	MS	F
Treat	3	1.09529167	0.36509722	14603.89 **
Error	8	0.00020000	0.00002500	
Total	11	1.09549167		

cv = 1.4 %

** = Significant at 1 % level

Table 10-A. Analysis of variance for total phenolic content of cow pea seed prepared with different solvents.

SV	DF	SS	MS	F
Treat	3	35842.98269	11947.66090	15911.65**
Error	8	6.00700	0.75087	
Total	11	35848.98965		

cv = 2.0 %

** = Significant at 1 % level

Table 11-A. Analysis of variance for antioxidant activities of wild tamarind seed prepared with different ratio of methanol to water.

SV	DF	SS	MS	F
Treat	5	4.09809444	0.81961889	3.55 *
Error	12	2.76780000	0.23065000	
Total	17	6.86589444		

cv = 0.5 %

* = Significant at 5 % level

Table 12-A. Analysis of variance for total phenolic content of wild tamarind seed prepared with different ratio of methanol to water.

SV	DF	SS	MS	F
Treat	5	1482278.3666	296455.6733	3912.86 **
Error	12	1483187.5397	75.7643	
Total	17	1483761554.1122		

cv = 1.1 %

** = Significant at 1 % level

Table 13-A. Analysis of variance for reducing power of wild tamarind seed prepared with different ratio ofmethanol to water.

SV	DF	SS	MS	F
Treat	5	0.1227	0.0245	169.85 **
Error	12	0.0017	0.0001	
Total	17	2.6638		

cv = 1.7 %

** = Significant at 1 % level

Table 14-A. Analysis of variance for antioxidant activities of cow pea seed prepared with different ratio of methanol to water.

SV	DF	SS	MS	F
Treat	5	105.0765	21.0153	20.60 **
Error	12	12.2441	1.0203	
Total	17	117.2441		

cv = 1.1 %

** = Significant at 1 % level

Table 15-A. Analysis of variance for total phenolic content of cow pea seed prepared with different ratio of methanol to water.

SV	DF	SS	MS	F
Treat	5	19200.3743	3840.0749	257.03 **
Error	12	179.2806	14.9400	
Total	17	19379.6550		

cv = 2.1 %

** = Significant at 1 % level

Table 16-A. Analysis of variance for reducing power of cow pea seed prepared with different ratio of methanol to water.

SV	DF	SS	MS	F
Treat	5	0.0140	0.0028	21.07 **
Error	12	0.0016	0.0001	
Total	17	0.0156		

cv = 4.1 %

** = significant at 1 % level

Table 17-A. Analysis of variance for antioxidant activities of wild tamarind seed prepared with different extraction time and repetition.

SV	DF	SS	MS	F
Treat	14	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 ns
T x R	8	0.1417	0.0177	< 1
Error	30	1.7204	0.0573	
Total	44	3.2491		

cv = 0.2 %

** = Significant at 1 % level

ns = not significant

Table 18-A. Analysis of variance for total phenolic content of wild tamarind seed prepared with different extraction time and repetition.

SV	DF	SS	MS	F
Treat	14	8870906.7465	633636.1965	199.690 **
Extraction time (T)	4	348511.4446	87127.8611	27.46 **
Repetition (R)	2	8271637.6888	4135818.8444	1303.32 **
T x R	8	250757.6122	31344.7011	9.88 **
Error	30	95195.1071	3173.1700	
Total	44	8966101.8521		

cv = 3.1 %

** = Significant at 1 % level

Table 19-A. Analysis of variance for antioxidant activities of cow pea seed prepared with different extraction time and repetition.

SV	DF	SS	MS	F
Treat	14	11.02520	0.7859	3.96 **
Extraction time (T)	4	4.4004	1.1001	5.54 **
Repetition (R)	2	0.8246	0.4123	2.08 ns
T x R	8	5.7774	0.7221	3.63 *
Error	30	2.9801	0.1987	
Total	44	13.9827		

cv = 0.5 %

** = Significant at 1 % level

* = Significant at 5 % level

ns = not significant

Table 20-A. Analysis of variance for antioxidant activity of wild tamarind seed extract as a function of heating time at 80 ^{o}C.

SV	DF	SS	MS	F
Treat	8	33.2478	4.1559	17.04 **
Error	18	4.3889	0.2438	
Total	26	37.6367		

cv = 0.5 %

** = Significant at 1 % level

Table 21-A. Analysis of variance for relative antioxidant activity of wild tamarind seed extract as a function of heating time at 80 °C.

SV	DF	SS	MS	F
Treat	8	34.0559	4.2569	17.69 **
Error	18	4.3326	0.2407	
Total	26	38.3885		

cv = 0.5 %

** = Significant at 1 % level

Table 22-A. Analysis of variance for antioxidant activity of wild tamarind seed extract as a function of heting time at 100 °C.

SV	DF	SS	MS	F
Treat	8	50.8282	6.3535	39.31 **
Error	18	2.9096	0.1616	
Total	26	53.7378		

cv = 0.4 %

** = Significant at 1 % level

Table 23-A. Analysis of variance for relative antioxidant activity of wild tamarind seed extract as a function of heating time at 100 °C.

SV	DF	SS	MS	F
Treat	8	53.4131	6.6766	40.69 **
Error	18	2.9533	0.1641	
Total	26	56.3664		

cv = 0.4 %

** = Significant at 1 % level

Table 24-A. Analysis of variance for antioxidant activity of cow pea seed extract as a function of heating time at 80 °C.

SV	DF	SS	MS	F
Treat	8	23.1357	2.8919	21.93 **
Error	18	2.3738	0.1319	
Total	26	25.5095		

cv = 0.4 %

** = Significant at 1 % level

Table 25-A. Analysis of variance for relative antioxidant activity of cow pea seed extract as a function of heating time at 80 °C.

SV	DF	SS	MS	F
Treat	8	25.1159	3.1395	29.51 **
Error	18	1.9152	0.1064	
Total	26	27.0311		

cv = 0.3 %

** = Significant at 1 % level

Table 26-A. Analysis of variance for antioxidant activity of cow pea seed extract as a function of heating time at 100 °C.

SV	DF	SS	MS	F
Treat	8	129.9271	16.2409	80.80 **
Error	18	3.6181	0.2010	
Total	26	133.5453		

cv = 0.5 %

** = Significant at 1 % level

Table 27-A. Analysis of variance for relative antioxidant activity of cow pea seed extract as a function of heating time at 100 °C.

SV	DF	SS	MS	F
Treat	8	144.0089	18.0011	85.03 **
Error	18	3.8105	0.21169	
Total	26	147.8195		

cv = 0.5 %

** = Significant at 1 % level

Table 28-A. Analysis of variance for antioxidant activity of wild tamarind seed extract at different pH.

SV	DF	SS	MS	F
Treat	6	2873.5658	478.9276	1395.61 **
Error	14	4.8043	0.3432	
Total	20	2878.3701		

cv = 0.7 %

** = Significant at 1 % level

Table 29-A. Analysis of variance for antioxidant activity of cow pea seed extract at different pH.

SV	DF	SS	MS	F
Treat	6	193.2138	32.2023	135.38 **
Error	14	3.3301	0.2379	
Total	20	196.5491		

cv = 0.5 %

** = Significant at 1 % level

Table 30-A. Analysis of variance for antioxidant activity of wild tamarind seed extract as a function of pH stability.

SV	DF	SS	MS	F
Treat	6	9.7985	1.6331	4.44 **
Error	14	5.1444	0.3675	
Total	20	196.5491		

cv = 0.7 %

** = Significant at 1 % level

Table 31-A. Analysis of variance for antioxidant activity of cow pea seed extract as a function of pH stability.

SV	DF	SS	MS	F
Treat	6	178.3730	29.7288	42.34 **
Error	14	9.8304	0.7022	
Total	20	188.2033		

cv = 1.0 %

** = Significant at 1 % level

Table 32-A. Analysis of variance for synergistic antioxidant activity of wild tamarind seed extract with α-tocopherol.

SV	DF	SS	MS	F
Treat	7	3860.8435	551.5491	2096.03 **
Error	16	3.0472	0.1904	
Total	23	3863.8907		

cv = 0.5 %

** = Significant at 1 % level

Table 33-A. Analysis of variance for synergistic antioxidant activity of wild tamarind seed extract with citric acid.

SV	DF	SS	MS	F
Treat	7	13873.3655	1981.9094	2280.14 **
Error	16	13.9073	0.8692	
Total	23	13887.2727		

cv = 2.3 %

** = Significant at 1 % level

Table 34-A. Analysis of variance for synergistic antioxidant activity of wild tamarind seed extract with ascorbic acid.

SV	DF	SS	MS	F
Treat	7	13556.9513	1981.9093	2280.14 **
Error	16	4.9784	0.8692	
Total	23	13561.9297		

cv = 2.3 %

** = Significant at 1 % level

Table 35-A. Analysis of variance for synergistic antioxidant activity of cow pea seed extract with α-tocopherol.

SV	DF	SS	MS	F
Treat	7	8270.2579	1181.4654	2419.26 **
Error	16	7.8137	0.4883	
Total	23	8278.0717		

cv = 1.0 %

** = Significant at 1 % level

Table 36-A. Analysis of variance for synergistic antioxidant activity of cow pea seed extract with ascorbic acid.

SV	DF	SS	MS	F
Treat	7	6449.9524	921.422	1557.88 **
Error	16	9.4633	0.5914	
Total	23	6459.4157		

cv = 3.3 %

** = Significant at 1 % level

Table 37-A. Analysis of variance for synergistic antioxidant activity of cow pea seed extract with citric acid.

SV	DF	SS	MS	F
Treat	7	5806.6898	829.5271	1343.58 **
Error	16	9.8784	0.6174	
Total	23	5816.5682		

cv = 3.9 %

** = Significant at 1 % level

Table 38-A. Analysis of variance for radical-scavenging activity of wild tamarind seed extract at different amounts.

SV	DF	SS	MS	F
Treat	12	28633.4231	2386.1186	5078.90 **
Error	26	12.2151	0.4698	
Total	38	28645.6382		

cv = 1.0 %

** = Significant at 1 % level

Table 39-A. Analysis of variance for absorbance at 517 nm of wild tamarind seed extract at different amounts.

SV	DF	SS	MS	F
Treat	13	0.6393	0.0492	3548.89 **
Error	28	0.0003	0.00001	
Total	41	0.6397		

cv = 2.9 %

** = Significant at 1 % level

Table 40-A. Analysis of variance for radical-scavenging activity of cow pea seed extract at different amounts.

SV	DF	SS	MS	F
Treat	12	21722.6740	1810.2228	16064.87 **
Error	26	2.9297	0.1127	
Total	38	21725.6037		

cv = 0.4 %

** = Significant at 1 % level

Table 41-A. Analysis of variance for absorbance at 517 nm of cow pea seed extract at different amounts.

SV	DF	SS	MS	F
Treat	13	0.5759	0.0443	4961.24 **
Error	28	0.0003	0.0000	
Total	41	0.5761		

cv = 2.9 %

** = Significant at 1 % level

Table 42-A. Analysis of variance for Fe^{2+} - binding activity of wild tamarind seed extract at different amounts.

SV	DF	SS	MS	F
Treat	12	18635.9184	1552.9932	24.09 **
Error	26	1676.4011	64.4770	
Total	38	20312.3194		

cv = 2.6 %

** = Significant at 1 % level

Table 43-A. Analysis of variance for absorbance of wild tamarind seed extract at different amounts.

SV	DF	SS	MS	F
Treat	13	0.8975	0.0690	2116.59 **
Error	28	0.0009	0.0000	
Total	41	0.8984		

cv = 1.2 %

** = Significant at 1 % level

Table 44-A. Analysis of variance for Fe^{2+} - binding activity of cow pea seed extract at different amounts.

SV	DF	SS	MS	F
Treat	12	16722.4432	1393.5369	2128.91 **
Error	26	17.0190	0.6546	
Total	38	16739.4622		

cv = 2.5 %

** = Significant at 1 % level

Table 45-A. Analysis of variance for absorbance of cow pea seed extract at different amounts.

SV	DF	SS	MS	F
Treat	13	1.0233	0.0787	2537.26 **
Error	28	0.0008	0.0000	
Total	41	1.0242		

cv = 1.1 %

** = Significant at 1 % level

Table 46-A. Analysis of variance for Cu^{2+} - binding activity of wild tamarind seed extract at different amounts.

SV	DF	SS	MS	F
Treat	12	17812.4482	1484.3707	3637.73 **
Error	26	10.6092	0.4081	
Total	38	17823.0574		

cv = 1.6 %

** = Significant at 1 % level

Table 47-A. Analysis of variance for absorbance at 480 nm of wild tamarind seed extract at different amounts.

SV	DF	SS	MS	F
Treat	13	2.3289	0.1792	4.113.89 **
Error	28	0.0012	0.0000	
Total	41	2.3301		

cv = 1.0 %

** = Significant at 1 % level

Table 48-A. Analysis of variance for Cu^{2+} - binding activity of cow pea seed extract at different amounts.

SV	DF	SS	MS	F
Treat	12	7945.0290	662.0858	1800.86 **
Error	26	9.5589	0.3676	
Total	38	7954.5879		

cv = 1.9 %

** = Significant at 1 % level

Table 49-A. Analysis of variance for absorbance at 480 nm of cow pea seed extract at different amounts.

SV	DF	SS	MS	F
Treat	13	1.4393	0.1107	2494.74 **
Error	28	0.0012	0.0000	
Total	41	1.4405		

cv = 0.9 %

** = Significant at 1 % level

Table 50-A. Analysis of variance for lipoxygenase inhibitory of wild tamarind seed extract at different amounts.

SV	DF	SS	MS	F
Treat	10	13053.5396	1305.3540	4080.89 **
Error	22	7.03710	0.3199	
Total	32	13060.5768		

cv = 2.1 %

** = Significant at 1 % level

Table 51-A. Analysis of variance for absorbance at 234 nm of wild tamarind seed extract at different amounts.

SV	DF	SS	MS	F
Treat	11	0.3279	0.0298	2997.78 **
Error	24	0.0002	0.0000	
Total	35	0.3282		

cv = 0.9 %

** = Significant at 1 % level

Table 52-A. Analysis of variance for lipoxygenase inhibitory of cow pea seed extract at different amounts.

SV	DF	SS	MS	F
Treat	10	17366.2855	1736.6286	2040.45 **
Error	22	18.7242	0.8511	
Total	32	17385.0097		

cv = 4.2 %

** = Significant at 1 % level

Table 53-A. Analysis of variance for absorbance at 234 nm of cow pea seed extract at different amounts.

SV	DF	SS	MS	F
Treat	11	0.5737	0.0522	949.78 **
Error	24	0.0013	0.0000	
Total	35	0.5751		

cv = 1.8 %

** = Significant at 1 % level

Table 54-A. Analysis of variance for TBARS of cooked ground pork treated with wild tamarind seed extract and other commercial antioxidants.

SV	DF	SS	MS	F
Treatment	41	165332.6713	403.2359	1516.56 **
Treat (T)	6	10226.5241	1704.4207	6410.30 **
Day (D)	5	3908.9797	791.7960	2977.93 **
T x D	30	2347.1676	78.2389	294.26 **
Error	84	22.3346	0.2659	
Total	125	16555.0040		

cv = 4.3 %

** = Significant at 1 % level

Table 55-A. Analysis of variance for peroxide value of cooked ground pork treated with wild tamarind seed extract and other commercial antioxidants.

SV	DF	SS	MS	F
Treatment	41	468.7478	11.4329	1759.82 **
Treat (T)	6	349.7090	58.2848	8971.59 **
Day (D)	5	47.6422	9.5284	1466.68 **
T x D	30	71.3966	2.3799	366.33 **
Error	84	0.5457	0.0065	
Total	125	469.2935		

cv = 6.0 %

** = Significant at 1 % level

Table 56-A. Analysis of variance for conjugated diene of cooked ground pork treated with wild tamarind seed extract and other commercial antioxidants.

SV	DF	SS	MS	F
Treatment	41	180.0899	4.3924	473.51 **
Treat (T)	6	121.6660	20.2777	2185.98 **
Day (D)	5	36.5197	7.3039	787.38 **
T x D	30	21.9041	0.7301	78.71 **
Error	84	0.7792	0.0093	
Total	125	180.8691		

cv = 9.3 %

** = Significant at 1 % level

Table 57-A. Analysis of variance for TBARS of cooked ground pork treated with cow pea seed extract and other commercial antioxidants.

SV	DF	SS	MS	F
Treatment	41	175332.6713	403.2359	1616.56 **
Treat (T)	6	12226.5241	1704.4207	6410.30 **
Day (D)	5	4908.9797	791.7960	2977.93 **
T x D	30	2347.1676	78.2389	294.26 **
Error	84	22.3346	0.2659	
Total	125	16555.0040		

cv = 4.3 %

** = Significant at 1 % level

Table 58-A. Analysis of variance for peroxide value of cooked ground pork treated with cow pea seed extract and other commercial antioxidants.

SV	DF	SS	MS	F
Treatment	41	754.2332	18.3959	261.95 **
Treat (T)	6	487.4350	81.2392	1156.82 **
Day (D)	5	181.0015	36.2003	515.48 **
T x D	30	85.7967	2.8598	40.72 **
Error	84	5.8990	0.0702	
Total	125	760.1322		

cv = 9.6 %

** = Significant at 1 % level

Table 59-A. Analysis of variance for conjugated diene of cooked ground pork treated with cow pea seed extract and other commercial antioxidants.

SV	DF	SS	MS	F
Treatment	41	381.1331	9.2959	362.03 **
Treat (T)	6	202.6421	33.7737	1315.31 **
Day (D)	5	125.5364	25.1072	977.80 **
T x D	30	52.9547	1.7652	68.74 **
Error	84	2.1569	0.0257	
Total	125	383.2900		

cv = 8.4 %

** = Significant at 1 % level

Table 60-A. Analysis of variance for TBARS of cooked ground pork treated with wild tamarind seed extract and citric acid combination or wild tamarind seed extract and ascorbic acid combination.

SV	DF	SS	MS	F
Treatment	59	31440.9268	532.8971	1896.16 **
Treat (T)	9	21268.6053	2363.1784	8408.68 **
Day (D)	5	5645.6329	1129.1266	4017.67 **
T x D	45	4526.6886	100.5931	357.93 **
Error	120	33.7248	0.2810	
Total	179	31474.6516		

cv = 4.3 %

** = Significant at 1 % level

Table 61-A. Analysis of variance for peroxide value of cooked ground pork treated with wild tamarind seed extract and citric acid combination or wild tamarind seed extract and ascorbic acid combination.

SV	DF	SS	MS	F
Treatment	59	1552.5610	26.3146	470.46 **
Treat (T)	9	1010.4081	112.2676	2007.14 **
Day (D)	5	337.8979	67.5796	1208.20 **
T x D	45	204.2550	4.5390	81.15 **
Error	120	6.7121	0.0559	
Total	179	1559.2731		

cv = 7.7 %

** = Significant at 1 % level

Table 62-A. Analysis of variance for conjugated diene of cooked ground pork treated with wild tamarind seed extract and citric acid combination or wild tamarind seed extract and ascorbic acid combination.

SV	DF	SS	MS	F
Treatment	59	765.0145	12.9663	276.21 **
Treat (T)	9	403.4332	44.8259	954.90 **
Day (D)	5	249.9189	49.9838	1064.77 **
T x D	45	111.6624	2.4814	52.86 **
Error	120	5.6332	0.0469	
Total	179	31474.6516		

cv = 9.9 %

** = Significant at 1 % level

Table 63-A. Analysis of variance for TBARS of cooked ground pork treated with cow pea seed extract and citric acid combination or cow pea seed extract and ascorbic acid combination.

SV	DF	SS	MS	F
Treatment	59	20636.1851	349.7659	640.03 **
Treat (T)	9	5484.8104	609.4234	1115.17 **
Day (D)	5	14235.0987	2847.0197	5209.70 **
T x D	45	916.2760	20.3617	37.26 **
Error	120	65.5781	0.5465	
Total	179	20701.7632		

cv = 4.2 %

** = Significant at 1 % level

Table 64-A. Analysis of variance for peroxide value of cooked ground pork treated with cow pea seed extract and citric acid combination or cow pea seed extract and ascorbic acid combination.

SV	DF	SS	MS	F
Treatment	59	1246.8608	21.1332	319.41 **
Treat (T)	9	624.8561	69.4284	1049.35 **
Day (D)	5	516.0293	103.2059	1559.86 **
T x D	45	105.9754	2.3550	35.59 **
Error	120	7.9396	0.0662	
Total	179	1254.8004		

cv = 7.2 %

** = Significant at 1 % level

Table 65-A. Analysis of variance for conjugated diene of cooked ground pork treated with cow pea seed extract and citric acid combination or cow pea seed extract and ascorbic acid combination.

SV	DF	SS	MS	F
Treatment	59	668.2092	11.3256	211.76 **
Treat (T)	9	250.7790	27.8643	521.00 **
Day (D)	5	344.6093	68.9219	1288.68 **
T x D	45	72.8209	1.6182	30.26 **
Error	120	6.4179	0.0534	
Total	179	674.6270		

cv = 9.2 %

** = Significant at 1 % level

Vitae

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