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TITLE: Antioxidant Properties of Crude Extracts from Greater Cardamom (*Amomum subulatum* Roxb.) and Siam Cardamom *(Amomum krervanh* Pierre) Seeds and Their Antioxidative Effects in Pork Patties

NAME: Mr. Ramesh Khadka

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR

(Assistant Professor Wannee Jirapakkul, Ph.D.)

THESIS CO-ADVISOR

(Mrs. Kanithaporn Vangnai, Ph.D.)

DEPARTMENT HEAD

(Assistant Professor Wannee Jirapakkul, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON

DEAN

(Associate Professor Gunjana Theragool, D.Agr.)

THESIS

ANTIOXIDANT PROPERTIES OF CRUDE EXTRACTS FROM GREATER CARDAMOM *(Amomum subulatum* Roxb.) AND SIAM CARDAMOM *(Amomum krervanh* Pierre) SEEDS AND THEIR ANTIOXIDATIVE EFFECTS IN PORK PATTIES

RAMESH KHADKA

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Ramesh Khadka 2013: Antioxidant Properties of Crude Extracts from Greater Cardamom *(Amomum subulatum* Roxb.) and Siam Cardamom *(Amomum krervanh* Pierre) Seeds and Their Antioxidative Effects in Pork Patties. Master of Science (Food Science), Major Field: Food Science, Department of Food Science and Technology. Thesis Advisor: Assistant Professor Wannee Jirapakkul, Ph.D. 113 pages.

The objective of this study was to investigate optimum conditions required for extraction of antioxidants from greater cardamom seeds and evaluate the antioxidant activities of crude extracts from the greater cardamom seeds and siam cardamom seeds. The extraction conditions were solvent systems (ethanol and methanol at three concentration levels of 50, 80 and 100% as well as 100% water), extraction temperatures (40-75ºC), extraction time (1-16 hours), solid and solvent ratios (1:10-1:50) and extraction techniques (water bath shaker extraction, ultrasound assisted extraction (USE) at 40±5ºC and maceration (MCN) by soaking in the solvent at room temperature). Based on total phenolic content (TPC) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity measurement, water bath shaker extraction using 50% ethanol with solid and solvent ratio of 1:20 at 75ºC for 8 hours was selected to be an appropriate extraction condition among all the conditions compared. Using the same optimized condition, crude extracts from the seeds of greater cardamom (GCE) and siam cardamom (SCE) were prepared and evaluated for their antioxidant properties. GCE exhibited higher extraction yield and total flavonoid content (TFC) whereas TPC, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and DPPH radical scavenging capacity were higher in SCE with almost equal ferric reducing antioxidant power (FRAP) values. In HPLC analysis, two of the antioxidant compounds in GCE were identified to be protocatechuic acid and protocatechuic aldehyde whereas only one of the compounds in SCE was identified to be protocatechuic acid. Protcatechuic acid concentration was higher in SCE than GCE. Both GCE and SCE at 0.1% (w/w) were more effective than butylated hydroxytolune (BHT) at 0.01% (fat weight basis) for retarding the formation of thiobarbituric acid reactive substances (TBARS) from lipid oxidation in the cooked pork patties. Similarly, compared with control, both GCE and SCE at 0.1% significantly inhibited the protein oxidation and also slowed down the deterioration of red color in raw pork patties. These findings shows that GCE and SCE may be used as the potent natural antioxidants in meat and meat products.

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Student's signature Thesis advisor's signature

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Ramesh Khadka

April, 2013

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LIST OF ABBREVIATIONS

ABTS	=	2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
BHT	=	Butylated hydroxytoluene
BHA	=	Butylated hydroxyanisole
CD	=	Conjugated dienes
DAD	=	Diode-array detector
DNPH	=	Dinitrophenylhydrazine
DPPH	=	1, 1-Diphenyl-2-picryl-hydrazyl
FC	=	Folin-ciocaltau
FRAP	=	Ferric reducing antioxidant power
GAE	=	Gallic acid equivalents
GCE	=	Greater cardamom seeds extract
HCl	=	Hydrochloric acid
HPLC	=	High-performance liquid chromatography
MCN	=	Maceration
MDA	=	Malondialdehyde
mg	=	Milligram
mins	=	Minutes
mL	=	Milliliters
mM	=	Millimolar
nm	=	Nanometers
QE	=	Quercetin equivalents
rpm	=	Rotation per minutes
SCE	=	Siam cardamom seeds extract
TBA	=	Thiobarbituric acid
TBARS	=	Thiobarbituric acid reactive substances
TE	=	Trolox equivalent
TEAC	=	Trolox equivalent antioxidant capacity
TPC	=	Total phenolic content
TFC	=	Total flavonoid content

ANTIOXIDANT PROPERTIES OF CRUDE EXTRACTS FROM GREATER CARDAMOM (*Amomum subulatum* Roxb.) AND SIAM CARDAMOM *(Amomum krervanh* Pierre) SEEDS AND THEIR ANTIOXIDATIVE EFFECTS IN PORK PATTIES

INTRODUCTION

Lipid is an important ingredient of many foods and it is quite susceptible for quality deterioration especially under oxidative stress. Oxidation of lipid is responsible for rancid odors and flavors of the products, with a consequent decrease in nutritional quality and safety caused by the formation of secondary, potentially toxic compounds. Similarly, Oxidative reactions of proteins are another major cause of chemical deterioration in food especially in muscle foods. Protein oxidation affects meat quality, including tenderness, water-holding capacity, and nutritional quality.

Although, protein oxidation in food has been ignored for decades, in recent years, protein oxidation has attracted considerable attention and become an innovative topic of increasing interest among meat researchers. Complex mechanisms and reaction processes are involved in lipid and protein oxidation, while it is generally accepted that both types of oxidation occur mainly via a radical chain reaction including initiation, propagation, and termination stages (Lund *et al.*, 2011;Shahidi and Zhong, 2010).

The oxidation processes of lipid as well as proteins can be retarded by using the antioxidants that effectively break the radical chain reaction and maintain the nutritional and sensory qualities of foods. The synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgallate (PG), and tert-butylhydroquinone (TBHQ) are the most commonly used antioxidants in the present time. Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are both powerful synthetic antioxidants, but they are believed to possess carcinogenic activity. BHA has been removed from GRAS (Generally Recognized as Safe) and TBHQ is not allowed in Japan, Canada and Europe (Mohdaly *et al.*, 2010).

These observations have lead to a demand for antioxidants derived from naturally occurring sources. There is a need to identify new natural antioxidants that the consumer demands for prevention of lipid peroxidation in the food industry. Consumers are willing to pay higher price for the products which are naturally preserved and contain no synthetic food additives (Brewer, 2011).

Herbs and spices, which are important part of the human diet, have been used for thousands of years to enhance the flavor, color and aroma of food. In addition to boosting flavor, herbs and spices are also known for their preservative antioxidative effect. Scientific experiments since the late 19th century have documented the antimicrobial and antioxidative properties of some spices, herbs and their components (Nielsen and Rios, 2000). The antioxidative activity of herbs and spices is mainly due to phenolic compounds. The phenolic compounds have strong H-donating activity (Muchuweti *et al.*, 2007).

There are still many herbs and spices to be investigated more extensively for their antioxidant potential so that they can be used as a natural source of antioxidants and replace the synthetic antioxidants as per the demand of present consumers. One of them may be greater cardamom (*Amomum subulatum* Roxb). Greater cardamom is the dried seed capsules of a small group of species or plants belonging to the family Zingiberaceae which contain seeds possessing a pleasant characteristic aroma and flavour. *Amomum subulatum* Roxb is also known as the large cardamom.

Because of pleasant aromatic odor, large cardamom is used for flavoring various vegetables and meat preparation in Nepalese and Indian dishes. Greater cardamom seed and powder are essential ingredients in mixed preparation and spice masala mixtures. Only few reports are available on antioxidative activity and antioxidant compounds of dried fruit of the greater cardamom. Ghimire *et al*. (2011) has found that methanol crude extract of large cardamom has a significant free radical scavenging activity comparable to synthetic BHA when evaluated using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) and recommended to be used in food. Therefore a detailed study on the antioxidative potential of greater cardamom seeds is needed to

ensure that whether its application as a substitute of synthetic antioxidants is possible or not.

Amomum krevanh Pierre is another species of *Amomum* cardamom commonly called "Kra-Waan" in Thai or "Round Siam Cardamom" in the international spice market and it is the most widespread cardamom species in Thailand (Kamchonwongpaisan *et al.*, 1995). Kra-wan is a common spice and medicinal plant species native to Thailand and Cambodia (Tefera and Wannakrairoj, 2003). To date, no any available literature related to antioxidant properties of siam cardamom has been published.

For the study of antioxidant potential of a natural source, efficient extraction of antioxidant compounds from the source is the first and essential step. As there is no any single extraction protocol for the extraction of antioxidants from natural materials because the different sources have different types of antioxidant compounds and matrix system. Hence the recovery of antioxidant compounds largely depends on extraction conditions such as solvent system, extraction techniques, solid and solvent ratio, extraction time and temperature.

Usually polar solvents such as ethanol, methanol and acetone are the usual solvents used to extract antioxidants from plant material as they provide a high antioxidant yield due to their hydrogen-bonding ability (Rodríguez-Rojo *et al.*, 2012). Green solvents like ethanol, water and their mixtures are more preferable. Various factors such as Ethanol concentration, extraction time and temperature contribute to the efficacy of solvent extraction process and recovery of antioxidants from natural materials and therefore extraction procedure and extraction conditions must be established for each natural source (Thoo *et al.*, 2010).

OBJECTIVES

1. To investigate the effect of extraction conditions and select the appropriate conditions for extraction of antioxidants from greater cardamom seeds.

2. To determine antioxidants (total phenolic content, total flavonoids, protocatechuic acid and protocatechuic aldehyde) and antioxidant capacity (DPPH assay, ABTS assay and FRAP assay) in the crude extracts from greater cardamom and siam cardamom seeds.

3. To evaluate the capacity of the cardamom seeds extracts for inhibiting lipid oxidation in cooked pork patties during refrigerated storage.

4. To evaluate the capacity of the cardamom seeds extracts for inhibiting protein oxidation and color deterioration in raw pork patties during refrigerated storage.

LITERATURE REVIEW

1. Cardamom

Cardamoms are the dried seed capsules or fruits of a small group of species or plants belonging to the family Zingiberacea. The cardamom capsules contain seeds possessing a pleasant characteristic aroma and flavor (Madhusoodanan and Rao, 2001). Although, there has been controversy over the grouping of cardamom, International Standards Organization (ISO) has officially recognized nine species of cardamom under three main groups (Pruthi, 1976):

Group I: *Elettaria cardamomum*

Group II: 4 species of *Aframomum*

(a) *A. augustifolium* (Sonn) K.Schum – Madagascar cardamom

(b) *A. hanburyi* K.Schum – Cameroon cardamom

(c) *A. korarima* (pereira) Engler – Korarima cardamom

(d) *A. melegueta* (Roscol) K.Schum – Grains of paradise or Guinea grains

Group III: 4 species of *Amomum*

(a) *A. aromaticum* Roxburgh

(b) *A. kepulag*a Spraque et

(c) *A. krervanh* pierre

(d) *A. subulatum* Roxburgh

The genus *Amomum* which contains above mention four species of cardamom is the second largest genus under the family Zingiberaceae (formerly known as Scitamineae) with about 150 species (Thomas *et al.*, 2009). The *Aframomum* species of cardamom are found in the African regions of Sierra Leone, Tanzania, Guinea Coast and Madagascar whereas the *Amomum* species of cardamom are known in Nepal, the North East Indian, Bhutan and South East Asian countries. The fruits *Elettaria cardamomum* are much smaller in size in comparison with Amomum and *Aframomum* capsules and it is easy to distinguish them. However, the seed size and anatomy are similar in all the three genera (Madhusoodanan and Rao, 2001).

A. subulatum Roxburgh is commonly known as greater or large cardamom. It is also well known with various names such as Alaichi in Nepali, Sthulaila and Bhadraila in Sanskrit, Bara Ilachi in Bangla, Badi Ilayachi in Hindi, Peralam in Malayalam, Periya elam, Kattelam and Perelam in Tamil, Pedda Yelakaya in Telegu and Didda yelakki in Kannada (Bisht, 2011).The greater cardamom is one of the major cash crops cultivated between elevations of 600 and 2000 m in tropical wet evergreen forests of eastern himalayas in Nepal, India (Sikkim and Darjeeling areas) and Bhutan. The fruit is a trilocular, reddish brown to dark pink, many seeded capsule as shown in Figure 1.

Figure 1 Greater cardamom a) fruits/ capsules and b) seeds

Because of pleasant aromatic odor, greater cardamom is used for flavoring various vegetables and meat preparation in Nepalese and Indian dishes. It is also used as a flavoring ingredient in the preparation of confectionery, hot or sweet pickles and in beverages. Large cardamom seeds and powder are essential ingredients in mixed preparation and spice (masala) mixtures. India has developed various cardamom products such as essential oil, oleoresin, encapsulated flavor, cardamom cola, large cardamom flavored biscuits and large cardamom flavored liquors for diversifying the uses of large cardamom.

Greater cardamom also possesses curative properties in ayurvedic and unani systems of medicine. In medicine, cardamom are fragrant adjuncts to other stimulants, bitters and purgatives, They are used in the conditions like indigestion, vomiting,

enlarged spleen, abdominal pain, throat troubles, rectal disease, mouth infections, inflammation of eye lid, pulmonary tuberculosis and loss of appetite (Gopal *et al*., 2012). It is also well known for its antimicrobial activity, analgesic activity, anti-inflammatory and hypolipidaemic activity (Bisht, 2011).

Only few reports are available on antioxidative activity and antioxidant compounds in the greater cardamom. Ghimire *et al*. (2011) has found that methanol crude extract of large cardamom has a significant free radical scavenging activity comparable to synthetic BHA when evaluated using DPPH assay and recommended to use in foods. Protocatachuic aldehyde and protocatachuic acids (chemical structures shown in Figure 2) are two of the antioxidant compounds that were identified for the first time by Kikuzaki *et al.* (2001) in ethyl acetate fraction of greater cardamom seeds extract. Therefore a detailed study on the antioxidative potential of greater cardamom seeds is needed to ensure that whether its application as a substitute of synthetic antioxidants is possible or not.

Figure 2 Chemical structure of a) protocatechuic acid and b) protocatechuic aldehyde

Amomum krevanh Pierre is another species of *Amomum* cardamom commonly called Kra-wan in Thai or round siam cardamom in the international spice market and it is the most widespread cardamom species in Thailand (Kamchonwongpaisan *et al.*, 1995). Kra-wan is a common spice and medicinal plant species native to Thailand and Cambodia (Tefera and Wannakrairoj, 2003). To date, no any available literature related to antioxidant properties of siam cardamom has been published.

Siam cardamom is used in preparing several Thai dishes. In the northern part of Thailand, it is used as one of the seasonings in the preparation of northern version of larb nuea, a minced beef dish. Similarly, it is used to make kaeng massaman (a rich, mild curry that contains several aromatic spices), kaeng karee (a mild Thai version of an Indian curry), and kaeng phanaeng (a very thick, mild curry rich with coconut cream) in the central and southern regions. In Chinese cooking it is one of the spices used to make phalo dishes, and the pods are simmered in the broth for kui tio nuea (beef noodles) (Sukphisit, 2012).

Siam Cardamom is considered to be a medicinal herb in some parts of Thailand and grows in the Khao Phu Soi Dao area of Chanthaburi province and in certain parts of south. The cardamom plant has the medium-height having tall trunk with long leaves (like those of galangal). The cardamom plant likes light but not direct sunlight. It prefers a moist environment with good drainage the fruits of the cardamom are produced at the base of the plant (Sukphisit, 2012). Siam cardamom fruits are many seeded round capsules as shown in Figure 3 which are smaller in size as compared to greater cardamom.

Figure 3 Siam cardamom a) fruits/capsules and b) seeds

2. Lipid Oxidation

2.1 Mechanism of lipid oxidation

Lipid oxidation is considered to be one of the major chemical changes that occur in lipid containing food stuffs during processing, storage, shipment, and final preparation. Various types of lipids such as triglycerides, phospholipids, and sterols, can be subjected to oxidation in the presence of oxygen. The presence of unsaturated double bonds in the lipid molecules make them susceptible for oxidation. Therefore, polyunsaturated fatty acids containing food stuffs are particularly highly susceptible to lipid oxidation and the degree of unsaturation of fatty acids determines susceptibility of the food stuffs (Brewer, 2011).

Lipid oxidation is a complex phenomenon and occurs *via* three reaction pathways namely; a) non-enzymatic chain autoxidation mediated by free radicals, b) non-enzymatic and non radical photo oxidation, and c) enzymatic oxidation . The first two types of oxidation consist of reactions involving triplet oxygen, the common oxygen that we breathe, and singlet oxygen, the excited form of the common oxygen (Laguerre *et al.*, 2007).

There are many sources of singlet oxygen but it is often produced from triplet oxygen in the presence of photosensitizers such as chlorophyll. A triplet photoosensitizer absorbs photons and becomes converted to singlet transmitting to molecular oxygen, which in turn becomes excited and forms singlet oxygen. The singlet oxygen is around 1500-fold more reactive than triplet and can bind directly to C=C producing hydoperoxide (Laguerre *et al.*, 2007). The hydroperoxides produced in these non radical photo oxidation reactions, undergo break down into free radicals and could initiate autoxidation reactions.

Autoxidation has long been recognized as the most common process of lipid oxidation with free radical chain reaction involving three stages: initiation, propagation, and termination (Shahidi and Zhong, 2005). The mechanism of

autoxidation is conveniently illustrated in the reactions given below (Shahidi and Zhong, 2005).

Initiation: $RH + initiator \rightarrow R^{\bullet}$.....1

$ROOH + initiator \rightarrow ROO^{\bullet}$

Propagation: $R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$ 2

$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$

Termination: $R^{\bullet} + R^{\bullet} \rightarrow R\text{-}R$ 3

$ROO^{\bullet} + R^{\bullet} \rightarrow ROOR$

Initiation occurs as hydrogen is abstracted from unsaturated fatty acids (RH) or a lipid hydro peroxide (ROOH) shown in the initiation step at equation (1). The produced free radical $R^{\bullet}$ then reacts with oxygen to form a peroxy radical ($ROO^{\bullet}$). In the propagation phase of oxidation, there is lipid-lipid interactions, whereby the lipid peroxyl radical abstracts hydrogen from an adjacent lipid molecule to generate a lipid hydro peroxide (ROOH) and another new lipid free radical ($R^{\bullet}$) as shown in equation (2). Interaction of this type may proceed 10 to 100 times causing a cascade mode of chain reactions before two free radicals combine to terminate the process. In the termination phase, two radicals combine into non-free radical products and stop the cascade mode of chain reaction, shown in equation (3) (Erickson, 2002).

At the initial stage, oxidation normally proceeds very slowly but increase suddenly after a certain period of time which is referred to as the induction period (Velasco *et al.,* 2004). At the end of the induction period oxidation is quicker and the oil/fat then deteriorates quickly. The length of this induction period is important in estimating the shelf life of oil and fats and of fatty foods (Brewer, 2011).

Lipid hydroperoxides have been identified as primary products of autoxidation. These hydroperoxides undergo decomposition producing aldehyde, ketones, alcohols, hydrocarbons, volatile organic acids, and epoxy compounds, known

as secondary oxidation products. These oxidation products, together with free radicals, constitute the bases for measurement (Shahidi and Zhong, 2005).

2.2. Measurement of lipid oxidation

Numerous analytical methods are routinely used for measuring lipid oxidation in foods. The available measurement strategies to monitor lipid oxidation in foods can be classified into five groups based on what they measure: i) the absorption of oxygen, ii) the loss of initial substrates, iii) the formation of free radicals, and the formation of iv) primary and/or v) secondary oxidation products (Shahidi and Zhong, 2005). Measurement strategies based on the formation of primary or secondary oxidation seems relatively well adapted for studying all types of system, including model systems, foods or biological samples isolated from their environment (Laguerre, 2007).

The main techniques for assaying formation of primary oxidation products (hydroperoxides) are iodometric hydroperoxide measurement (Peroxide Value, PV)) and ultraviolet measurement of conjugated dienes. Similarly , three tests namely; Thiobarbituric acid reactive substances (TBAR) test, aldehyde measurement by the anisidine test and chromatographic measurement of volatile compounds are the most commonly used in research and industrial condition for assaying the formation of secondary oxidation products (Laguerre, 2007).

2.2.1 Peroxide value: The peroxide value (PV) represents the total hydroperoxide content and is one of the most common quality indicators of fats and oils during production and storage. A number of methods have been developed for determination of PV, among which the iodometric titration, ferric ion complex measurement spectrophotometry, and infrared spectroscopy are most frequently used (Shahidi and Zhong, 2005). Iodometric hydroperoxide measurement is a conventional standardized and the most commonly used methods for quantifying total hydroperoxidesis in lipid substrate (Laguerre, 2007). In this method, a saturated solution of potassium iodide is added to oil samples to react with hydroperoxides. The liberated iodine (I_2) is then titrated with a standardized solution of sodium thiosulfate

and starch as an endpoint indicator. The PV is obtained by calculation and reported as milliequivalents of oxygen per kilogram of sample (meq/kg).

2.2.2 Conjugated dienes: Oxidation of polyunsaturated fatty acids is accompanied by an increase in the ultraviolet absorption of the product. Lipids containing methylene-interrupted dienes or polyenes show a shift in their double bond position during oxidation that is due to isomerisation and conjugated bond formation. The resulting conjugated dienes exhibit intense absorption at 234 nm; similarly conjugated trienes absorb at 268 nm. Theoretically, an increase in absorption at 230–235 nm in UV region indicates the formation of primary oxidation products in fats and oils and good correlations have been found between conjugated dienes and peroxide value (Shahidi and Zhong, 2005).

2.2.3 Anisidine value: Detrmination of the *p*-anisidine value (PAV) is one of the oldest methods for evaluating secondary lipid oxidation (Laguerre, 2007).
It measures the content of aldehydes (principally 2-alkenals and 2, 4-alkadienals) generated during the decomposition of hydroperoxides (Shahidi and Zhong, 2005) and based on the reactiveness of the aldehyde carbonyl bond on the *p*-anisidine amine group, leading to the formation of a Schiff base that absorbs at 350 nm. (Laguerre, 2007). This test is most commonly used in analysis of animal fats and vegetable oils.

2.2.4 TBARS Assay: Thiobarbituric acid reactive substances (TBARS) assay is one of the most extensively used methods to detect oxidative deterioration of fat-containing foods (Kishida *et al.,* 1993). It can be applied for the measurement of antioxidative activity of antioxidants. TBARS method is based on the formation of colored species by reaction of malondialdehyde, an end product of lipid peroxidation, with TBA. This colored complex results in the condensation of 2 moles of TBA and 1 mole of malondialdehyde (Laguerre, 2007). Therefore, the inhibition of lipid peroxidation by antioxidants can be measured by monitoring the decrease in the absorbance at 532 nm of colored species derived from malondialdehyde and TBA.

2.2.5 Chromatographic measurement of volatile compounds: In Chromatography, volatile molecules, mainly aldehydes, ketones, alcohols, short

carboxylic acids and hydrocarbons derived from hydroperoxide decomposition are quantified. Propanal, hexanal and pentanal are more frequently measured volatile compounds to assess the lipid oxidation. Hexanal is one of the major secondary products formed during the oxidation of linoleic and other n-6 fatty acids and used as a reliable indicator of lipid oxidatin in foods rich in n-6 fatty acids (Shahidi and Wanasundara, 2002). Similarly, Propanal is the main marker of oxidation of fatty acids of the n–3 family (Laguerre, 2007). However; GC–MS analysis that involves assessment of large set of volatile compounds has also been promoted and adapted in many new researches.

3. Protein oxidation

Food proteins that may be susceptible to oxidative reactions has been ignored for decades while the oxidation of other food components, namely lipids, was studied in depth in food research field (Lund *et al.*, 2011). The occurrence of protein oxidation in biological systems has been well studied but most of these studies have been focused to examine the role of oxidized proteins in different types of human diseases (Estevez, 2011). Protein oxidation in food systems has remained largely unknown (Elias *et al.*, 2008). In mid nineties, meat scientists took the first approach to study the protein oxidation in meat by using knowledge and techniques from medical research (Decker *et al.*, 1993). Currently, the oxidation of food proteins has become one of the most innovative research topics within the field of food science (Estevez, 2011).

3.1 General mechanism of protein oxidation

Protein oxidation has been defined as the covalent modification of a protein induced either directly by reactive oxygen species (ROS) or indirectly by reaction with secondary byproducts of oxidative stress (Shacter, 2000). Oxidation of proteins is believed to proceed via a free radical chain reaction similar to that of lipid oxidation but, in the former, a higher complexity of the pathways and a larger variety of oxidation products have been reported (Lund *et al*., 2011). Reactive oxygen species (ROS) such as the superoxide (O_2 •-), the hydroperoxyl (HO_2•) and hydroxyl (HO•)

radicals and other nonradical species such as the hydrogen peroxide (H_2O_2) and hydroperoxides (ROOH) have been recognized as potential initiators of protein oxidations (Estevez, 2011). Other agents that lead to protein oxidation includes HOCl, reduced transition metals such as Fe^{2+}, Cu^{+}, γ-irradiation in the presence of O_2, ultraviolet (UV) light, ozone, oxidoreductase enzymes and by-products of lipid and free amino acid oxidation (Shacter, 2000).

In case of muscle tissues, the inherent components such as unsaturated lipids, heme pigments, transition metals and oxidative enzymes are potential precursors or catalysts for the formation of ROS (Estevez, 2011). These ROS are generated during food processing (e.g. heating, fermentation and application of chemicals) and storage (Stadtman and Levine, 2003). ROS commonly reacts with the peptide backbone and the functional groups located in the side chain of amino acid residues (Estevez, 2011).

The abstraction of a hydrogen atom from susceptible target (PH), by an ROS leads to the generation of a protein carbon-centered radical (P•) (Reactions 4) which is consecutively converted into a peroxyl radical (POO•) in the presence of oxygen, and an alkyl peroxide (POOH) by abstraction of a hydrogen atom from another molecule (Reactions 5 and 6). Further reactions with ROS such as the HO_2• radical or with reduced forms of transition metals (Mn+) such as Fe^{2+} or Cu^{+} lead to the formation of an alcoxyl radical ($PO^{\bullet}$) (Reactions 7 and 8) and its hydroxyl derivative (POH) (Reactions 9 and 10) as follows (Estevez, 2011):

$PH + HO^{\bullet} \rightarrow P^{\bullet} + H_2O$ ……4

$P^{\bullet} + O_2 \rightarrow POO^{\bullet}$ ……5

$POO^{\bullet} + PH \rightarrow POOH + P^{\bullet}$ ……...6

$POOH + HO^{\bullet}_2 \rightarrow PO^{\bullet} + O_2 + H_2O$…….....7

$POOH + M^{n+} \rightarrow PO^{\bullet} + HO- + M^{(n+1)+}$ ……..8

$PO^{\bullet} + HO^{\bullet}_2 \rightarrow POH + O_2$……..9

$PO^{\bullet} + H^{+} + M^{n+} \rightarrow POH + M^{(n+1)+}$ ……..10

The common consequence of ROS mediated protein oxidation includes the oxidative modification of the amino acid side chains, the conversion of one amino acid into a different one, the fragmentation of the peptide backbone and the formation of intra- and inter-molecular cross-links (Estevez, 2011). The most common consequences of oxidation of proteins presented in Figure 4.

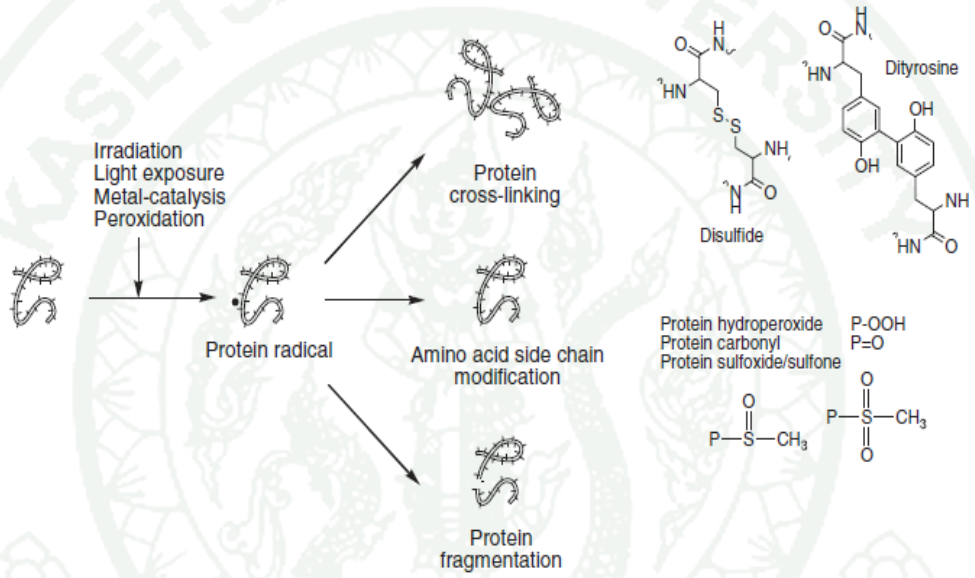


Figure 4 The most common consequences of proteins oxidation

Source: Lund *et al* (2011)

However, chemical nature of the final oxidation products and their specific routes of formation depend on the target, the oxidizing system and the intensity of the oxidation conditions (Estevez, 2011). Among all amino acids, sulfur-containing amino acids such as cysteine and methionine are considered to be most prone to oxidative attack because of susceptible sulfur atoms. All oxidizing species can induce modification of cysteine and methionine while other amino acids may require more stringent conditions for oxidative modification (Shacter, 2000). Cysteine and methionine in the presence of oxidizing lipids are highly susceptible to oxidation and yield varied sulfur-containing compounds such as sulfone, sulfoxide and disulphide derivatives. Oxidative cleavage of the peptide main chain and the oxidation of the side

chains of lysine, proline, arginine, and threonine have been shown to yield carbonyl derivatives (Shacter, 2000; Stadtman and Levine, 2003). Similarly, tryptophan is transformed into kynurenine or N-formylkynurenine, and tyrosine into dityrosine, histidine to oxohistidine and imidazolone derivatives and leucine and valine to hydroxy derivatives (Shacter, 2000).

3.2 Effects of protein oxidation on muscle food quality

Proteins being a major component of muscle tissue play a very important role in muscle foods regarding technological, nutritional and sensory aspects. The impact of protein modification such as denaturation (loss of their native tertiary structure) and hydrolytic degradation (proteolysis) by endogenous and/or exogenous enzymes on muscle food have been well studied and are known to affect the muscle food quality including textural properties, color, aroma, flavor, water holding capacity and biological functionality (Reviewed by, Estevez, 2011). However, the impact of protein oxidation on meat quality is not well reported and is still the subject of multiple studies but in general oxidative reactions are known to affect activity of muscle proteases and the functionality of myofibrillar proteins (Reviewed by, Estevez, 2011).

Oxidative modifications of proteins can lead to the impaired digestibility and biological bioavailability of proteins by destruction of essential amino acids, conversion of amino acids into nonmetabolizable derivatives, and by intra- and intermolecular crosslinking (Lund *et al.*, 2011) with ultimate decrease in the nutritional quality of muscle foods. In addition to the decreased nutritional quality, the oxidation of proteins in muscle-based systems leads to changes in solubility due to damage of amino acids resulting in aggregation of proteins (Schaich, 2008) and other protein functionality such as gelation and emulsifying properties, or water holding capacity (WHC) (Schaich, 2008; Lund *et al.*, 2011). In the review by Lund *et al*., (2011), the authors outlined the deleterious effect of protein oxidation on textural traits (tenderness and juiciness) of muscle foods and also reported that protein oxidation has impact on color and flavor deterioration but the precise chemical mechanisms are not well understood.

3.3 Protein carbonylation and assessment of protein oxidation

Carbonylation has been defined as an irreversible and non-enzymatic modification of proteins that involves the formation of carbonyl moieties induced by oxidative stress and other mechanisms (Estévez, 2011). The formation of carbonyl compounds (aldehydes and ketones) from amino acid side chains is well documented (Lund *et al*., 2011) and principally derives from the oxidation of threonine, proline, arginine and lysine residues by metal catalyzed oxidation systems (Stadtman & Levine,2003).

In the review by Estevez (2011) that focuses on protein carbonylation as an expression of the oxidative damage to meat proteins, reported four different pathways of carbonyl formation, namely, i) direct oxidation of the side chains from lysine, threonine, arginine and proline, ii) non-enzymatic glycation in the presence of reducing sugars, iii) oxidative cleavage of the peptide backbone via the α-amidation pathway or via oxidation of glutamyl side chains and iv) covalent binding to non-protein carbonyl compounds such as 4-hydroxy-2-nonenal (HNE) or malondialdehyde (MDA). But the first pathway has been highlighted as the main route for protein carbonylation.

The assessment of protein oxidation in muscle foods has been carried out based on its multiple chemical manifestations including loss of sulfhydryl groups, loss of tryptophan fluorescence, gain of carbonyl derivatives and formation of intra- and intermolecular cross-links (Estevez, 2011; Lund *et al.*, 2011). However, the formation of carbonyl compounds has been considered as one of the most remarkable modifications in oxidized proteins (Stadtman & Levine, 2003) and used as markers of protein oxidation (Estevez, 2011). The analytical procedures being used in biomedical sciences for quantifying protein carbonyls, have also been extrapolated and commonly used in muscle food systems. Among these, the quantification of protein carbonyls by the dinitrophenylhydrazine (DNPH) method (Oliver *et.al*., 1987) is a widespread and routine procedure for evaluating protein oxidation in meat systems including raw meat, meat emulsions and dry-cured product (Armenteros *et al.*, 2009). The original method (Oliver *et al*., 1987) was developed for analyzing oxidative stress

in biological samples and has been subsequently employed with minor modifications by food scientists.

In DNPH method, the quantification of carbonyl compounds is accomplished through derivatisation with DNPH, which reacts with protein carbonyls to form hydrazones products that display a maximum absorbance peak at around 370 nm. In this procedure, carbonyl derivatives and protein content of the sample are simultaneously determined (Oliver *et al.,* 1987). The concentration of DNP hydrazones is calculated by measuring reacted DNPH spectrophotometrically on the basis of an absorption coefficient of 22,000 M^{-1} cm^{-1} at 370 nm. Concentration of protein is determined in a control sample (without added DNPH) at 280 nm using BSA as standard. Results are usually expressed as nmols DNP hydrazones per mg of protein.

Several authors have developed the methods based on gas chromatography (GC) and high-performance liquid chromatography (HPLC) for the determination of specific carbonyls, namely, α-amino adipic and γ-glutamic semialdehydes (AAS and GGS, respectively) which are considered the main carbonyl products from metal-catalyzed oxidized proteins and used as specific biomarkers of oxidative damage in medical research and human biology (Armenteros *et al.,* 2009). Estevez *et al.* (2009) identified AAS and GGS also in oxidized myofibrillar proteins by using liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS) and highlighted as oxidation markers in food systems. This method is considered as advanced and accurate for the determination of AAS and GGS as protein oxidation markers in meat products (Armenteros *et al.,* 2009; Estevez, 2011) and subsequently used in several researches.

4. Antioxidants

Any substances that delays or prevents oxidation of an oxidizable substrate when present at significantly lower concentrations as compared to that substrate are called antioxidants (Halliwell, 2002). Based on their mechanism of action, antioxidants can be broadly classified as primary antioxidants and secondary antioxidants. Some antioxidants that exhibit more than one mechanism are included into another class called multiple-function antioxidants**.** Antioxidants can also be classified as synthetic and natural antioxidants based on their sources.

4.1 Primary antioxidants

Primary, or type I, antioxidants are free radical scavengers (FRS) that react with lipid and peroxy radicals and convert them to more stable, nonradical products. The term antioxidant is associated with the ability of a compound to scavenge free radicals that participate in lipid peroxidation. Free radical scavengers can slow lipid oxidation by inhibiting the initiation phase of lipid peroxidation (so called preventive primary antioxidants) by scavenging free lipid radicals ($R^{\bullet}$), or by inhibiting the propagation phase of lipid peroxidation by scavenging lipid alkoxyl ($RO^{\bullet}$) and/or lipid peroxyl radicals ($ROO^{\bullet}$) (so called chain-breaking antioxidants) (Nenadis *et al.*, 2007). Primary antioxidants quench free radicals by several mechanisms that include i) Hydrogen-atom transfer (HAT), ii) Single-electron transfer (SET), iii) Termination of Chains and iv) Decomposition of radicals or ROOH (Schaich *et al*., 2013).

Hydrogen atom transfer (HAT) is s the best known antioxidant mechanism to quench the free radiacals (Schaich *et al.*, 2013). In HAT, primary antioxidants donate hydrogen atoms to the lipid radicals and produce non-radical lipid derivatives and stable antioxidant radicals ($A^{\bullet}$). The antioxidant radicals are unreactive or more stable less readily available for further propagation of free radical chain. Affinities of primary antioxidants are higher for peroxy radicals than lipids. Hence, alkoxyl ($RO^{\bullet}$) and/or lipid peroxyl radicals ($ROO^{\bullet}$) formed during the propagation steps of autoxidation are scavenged by primary antioxidants as shown in equation 11 and 12.

Antioxidants may also interact directly with lipid radicals as shown in equation 13 (Reische *et al.*, 2002).

$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$ ……11

$RO^{\bullet} + AH \rightarrow ROH + A^{\bullet}$ ……,12

$R^{\bullet} + AH \rightarrow RH + A^{\bullet}$ ……13

Phenolic compounds have the structures with H-transferring activity. The activity of the antioxidant radical after hydrogen transfer is reduced and stabilized by delocalization of the unpaired electron around a phenol ring to form stable resonance hybrids (Schaich *et al.*, 2013). Furthermore, the antioxidant radicals ($A^{\bullet}$) may participate in termination reactions with other other antioxidant radicals ($A^{\bullet}$) or lipid radicals to form non-radical compounds (14 – 16). The presence of antioxidant dimers in fats and oils indicates that phenolic antioxidant radicals readily undergo termination reactions and autocatalytic free radical chain mechanism is effectively stopped as long as the antioxidant is present in its nonradical form (Reische *et al.*, 2002).

$ROO^{\bullet} + A^{\bullet} \longrightarrow ROOA$.....14

$RO^{\bullet} + A^{\bullet} \longrightarrow ROA$.....15

$A^{\bullet} + A^{\bullet} \longrightarrow AA$.....16

In the SET mechanism, reactive radicals can be reduced or oxidized to unreactive ions (Schaich *et al*., 2013). The hydrogen atom ($H^{\bullet}$) of an antioxidant is transferred as a proton (H^{+})and an electron to the free radical using different sets of orbitals. This means that the electron is transferred to the free radical turning it into an anion while the antioxidant turns itself into a radical cation ($A^{\bullet+}$) (equation 17). In aqueous media, a rapid and reversible deprotonation of the radical cation (equation 18) and a neutralization of the anion (equation 19) occur.

$R^{\bullet} + AH \longrightarrow R^{-} + A^{\bullet+}$.....17

$A^{\bullet+} + H_2O \longleftrightarrow A^{\bullet} + H_3O^+$.....18

$R^- + H_3O^+ \longleftrightarrow RH + H_2O$.....19

It is expected that the SET mechanism prevails in polar solvents (e.g. alcohols) due to solvent stabilization of the charged molecules (antioxidants) and therefore is strongly solvent dependent, whereas HAT mechanism is predominant in non-polar solvents (e.g. hexane) and therefore is only weakly solvent dependent (Ou *et al.*, 2002).

Another mechanism of action is the termination of chains that can occur through direct reaction with $R^{\bullet}$, e.g. quinones, nitro compounds, and quinon imines. The fourth mechanism is the decomposition of radicals or ROOH in which radicals or ROOH may be decomposed without generating new radicals. These types of antioxidants include sulfides and disulfides, phosphates and thiophosphates, carboxylic acids (including phenolic acids and free fatty acids), enzymes (superoxide dismutase, glutathione peroxidase, catalase), amines and phosphatidylcholines (Schaich *et al*., 2013).

4.2 Secondary antioxidants

Secondary, preventive, or type II antioxidants retard the rate of lipid oxidation by several different actions, but they do not convert free radicals to more stable products. They can chelate prooxidant metals and deactivate them or replenish hydrogen to primary antioxidants or decompose hydroperoxide to non-radical species or deactivate singlet oxygen or absorb ultraviolet radiation or act as oxygen scavengers. These antioxidants are often referred to as synergists because they promote the antioxidant activity of primary antioxidants e.g. ascorbic acid, citric acid, lecithin, etc (Reische *et al.*, 2002).

4.3 Synthetic and Natural Antioxidants

The antioxidants are classified into natural and synthetic antioxidants based on the source. The synthetic antioxidants such as butylated hydroxyanisole

(BHA), butylated hydroxytoluene (BHT), propylgallate (PG) and tert butylhydroquinone (TBHQ) are the most commonly used antioxidants in the present time. These all are the phenolic compounds and act as primary antioxidants, as are natural antioxidant tocopherols, flavonoids in fruits, and many herb and spice components (Schaich *et al*., 2013).

Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are both powerful synthetic antioxidants, but they are believed to possess carcinogenic activity. BHA has been removed from GRAS (Generally Regarded as Safe) and TBHQ is not allowed in Japan, Canada and Europe (Mohdaly *et al.*, 2010).

As compared to synthetic antioxidants, natural antioxidants have many advantages (Pokorny, 1991). They are considered to be safe and readily acceptable by the consumers. Furthermore, no safety tests are required by legislation. This natural antioxidant (not as a synthetic chemical antioxidant) is identical to the food which people have taken over a hundred years or have been mixing with food. This antioxidant not only stabilizes the foods but also adds to the nutraceutical value of foods.

4.4. Antioxidant activity of spices and herbs

Due to safety concerns of synthetic antioxidant, food scientists are trying to replace these synthetic antioxidants with natural ones. The natural antioxidants, in general, are supposed to be safer. Spice often contains a high concentration of phenolic compounds which can inhibit free radical formation and/or interrupt propagation of autoxidation. Therefore, spices can be a promising alternative to the synthetic antioxidants (Brewer, 2011).

Spice and herbs are proper materials to search for safe antioxidants. Several types of herbs and spices such as rosemary, sage and clove, allspice, anise seed, basil, cassia, cinnamon, ginger, thyme, nutmeg, oregano, black and white pepper, savory and turmeric have been already assessed for their antioxidant properties (Suhaj, 2006). Antioxidant compounds in some spices such rosemary

extracts have been reported to exhibit antioxidant properties greater than BHA and equal to BHT (Kim *et al.*, 1994). However, antioxidant activity of the spices depends on the substrates of oxidation, the preparation procedure and the oxidation test.

The major antioxidative plant phenolics can be divided into 4 general groups namely I) Phenolic acids (e.g. gallic, protochatechuic, caffeic, and rosmarinic acids), II) Phenolic diterpenes (e.g. carnosol and carnosic acid), III) Flavonoids (e.g. quercetin and catechin) and IV) Volatile oils (e.g. eugenol, carvacrol, thymol, and menthol). Phenolic acids generally act as antioxidants by trapping free radicals and flavonoids can scavenge free radicals and also chelate metals (Brewer, 2011). The flavonoids (flavones, flavonols, flavanols, and flavanones) contain the basic 15-carbon flavan structure ($C_6C_3C_6$). These carbon atoms are arranged in 3 rings (A, B, and C). Classification of flavonoids is based on the level of saturation of the C ring (Brewer, 2011).

The free radical-scavenging potential of natural polyphenolic compounds depend on both number and location of free $^{-}$OH groups on the flavonoid skeleton. Flavonoids containing multiple hydroxyl groups are more effective antioxidants than those with only one. The ortho- 3, 4-dihydroxy structure increases the antioxidative activity (Brewer, 2011).

4.5 Extraction of antioxidants from spices and herbs

The natural materials usually contains very low amount of antioxidants so that large additions would be necessary to obtain a significant improvement in stability against oxidation. But the addition of large amount could have a negative effect on the flavor or functional properties of the product. Therefore, preparation of more concentrated extract is often useful. The easiest way is to remove water by a suitable drying procedure and the next optimal procedure is extraction. There are various methods available to extract antioxidants from the spices and Selection of an appropriate extraction procedure is the most important which can increase the concentration of the antioxidant compounds (Pokorny and Korczak, 2001). Three

procedures may be used, extraction using fats and oils, extraction using organic solvents and supercritical fluid carbon dioxide extraction.

4.5.1 Extraction of antioxidants with fats and oils

A very simple method of extraction is using edible oil or fat in which natural material containing antioxidants, such as herbs and spices, is mixed with fats and/ or oils. Then, the mixture is left at room temperature or at a moderately increased temperature (in case of solid fats, such as pork, lard, beef tallow or cooking fats) for a defined time, for example overnight, with or without stirring. After filtering, the fat or oil containing dissolved antioxidants is used directly in food preparation. This type of trials has been already used in spices like rosemary, sage, paprika, nutmeg or cocoa (Suhaj, 2006).

4.5.2 Extraction of antioxidants with organic solvents

Extraction with organic solvents is more commonly used method. However the choice of a solvent depends on the particular material and on the stabilised substrate. The solvents of intermediary polarity seemed to be preferable to either non-polar or highly polar solvents. (Ghasemzadeh *et al.*, 2011) have used methanol, ethanol and chloroform to isolate antioxidants from stems, leaves and rhizomes of two varieties of zingers namely *Halia bentang* and *Halia bara*. Then the extracts were analyzed for total phenol content, total flavonoid content and DPPH radical scavenging capacity separately and methanol extract has been found to have highest total phenolics, total flavonoids content and DPPH radical scavenging capacity. That showed methanol as a suitable solvent to extract antioxidant from zingers.

There are various novel techniques which include ultrasound-assisted extraction, microwave-assisted extraction and accelerated solvent extraction which can be applied for the extraction of nutraceuticals from plants in relatively shorter period of time. These extraction techniques are considered to be more effective and decrease the solvent consumption, increase the extraction yield, and enhance the

quality of extracts. These techniques are fast and efficient and can also be used at elevated temperatures and/or pressures which greatly decrease the time of extraction (Wang and Weller, 2006).

In many researches, the extraction solvent system and techniques used have been reported to influence largely in the total content of natural antioxidants (Grigonis *et al.*, 2005; Hussain *et al.*, 2012; Michiels *et al.*, 2012). Hussain *et al*. (2011) observed significant variation in the antioxidant potentials of various extracts of pea nut hulls obtained by different methods in different solvents system. Various solvent systems are employed for the recovery of bioactive compounds from plant sources. Usually polar solvents such as methanol, acetone and ethanol are commonly used for the recovery of polyohenols. A pure single solvent have been found to be less effective as compared to the combination of the different solvents or aqueous -organic solvents (Hussain *et. al*., 2012). Michiels *et al*. (2012) have reported that the properties of extracting solvents significantly affected the measured total phenolics content (±25% variation) and antioxidant capacity (up to 30% variation) in fruits and vegetables.

However, consumers don't prefer the use of methanol or acetone extracts of natural antioxidants because of fear to remain residue which may contaminate the food. Therefore, ethanol, water and their binary mixture are considered to be safe solvents for the extraction of antioxidants from plant materials.

4.5.3 Extraction of antioxidants with supercritical fluid carbon dioxide

Extraction of antioxidants with supercritical fluid carbon dioxide is a modern method in which gases, usually carbon dioxide, is used under supercritical conditions. Co-solvents like propane/butane, methanol, ethanol and other substances may be used for improving yield or selectivity. Extraction with carbon dioxide is generally better than that of organic solvents and is relatively selective. This treatment was proposed for removal of volatiles, preceding extraction with organic solvents (both polar and non-polar). The safety aspect should be considered because of the high pressure used although carbon dioxide, being a gas at atmospheric pressure, is

easily removed so that solvent residues present no risk factor. The main disadvantage of supercritical extraction is the high operation pressure, which requires expensive equipment (Pokorny and Korczak, 2001). Its application in the preparation of natural antioxidants is very limited.

4.6 Assessment of antioxidant activity

By definition, the antioxidant activity (AOA) is the capability of a compound (or a mixture) to inhibit oxidative deteriorations, e.g. lipid peroxidation. An increased interest in information about antioxidant potentials of phenolic rich matrices has led to the development of a wide array of assays for determination of antioxidant capacity. There are two main approaches to AOA evaluation that are generally applied in these methods/assays: direct, and indirect (Roginsky and Lissi, 2005).

a) Indirect Methods**:** These methods include assays that evaluate the ability of antioxidants to scavenge some stable colored synthetic free-radicals. This has little in common with real biological oxidative degradation mediated by highly reactive radical oxygen species, or by the effects of transition metals. The ability to donate an electron or a hydrogen proton under conditions that are very different to those *in vivo* is usually measured

b) Direct methods are based on studying the antioxidant effect of antioxidants on the oxidative degradation of foods or other substrates of biological relevance (individual lipids, lipid mixture, oils, lipid membranes, low density lipoprotein, DNA, blood, plasma, etc.). The direct approach of evaluation that utilizes various lipid model systems has been suggested as being superior to the indirect approach where the antioxidant activity is evaluated more or less artificially by means of so called one-dimensional AOA assays.

It is expected that the closer the conditions are to the real lipid systems, both *in vivo* (such as cell biomembranes) and *in vitro* (lipid-containing foods) the more valid information about antioxidant potentials is likely to be achieved. Preferences should be given to the direct methods, however these methods are often

time consuming, which does not fulfill the demand for quick and easy assessments important mainly for food and nutraceutical industry. The use of more than only one and combination of various analytical methods for evaluation of antioxidant activity has been recommended to obtain more objective information about antioxidant potentials of various compounds (Huang *et al.*, 1996).

The indirect assays can be roughly divided into two categories namely, a) Hydrogen atom transfer (HAT) reaction based assays, and b) Single electron transfer (SET) reaction based on mechanisms of the chemical reaction. In general, the SET-based assays measure an antioxidant's reducing capacity, and the HAT-based assays quantify hydrogen atom donating capacity (Huang *et al.*, 2005).

4.6.1 HAT-based assays

In HAT-based assays, the ability of an antioxidant to quench free-radicals by hydrogen atom donation is measured. HAT-based assays are generally composed of a synthetic free radical generator, an oxidizable probe, and an antioxidant. In most HAT-based methods, antioxidants and a probe compete for thermally generated peroxyl radicals ($ROO^{\bullet}$) and the quantification is derived from the kinetic curves after monitoring the competitive reaction kinetics (Sun and Tanumihardjo, 2007).

The most biologically relevant HAT-based assays are namely, oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP) and inhibition of autoxidation of induced low-density lipoprotein (LDL) oxidation. It has been assumed that the antioxidant capacity measured by the HAT-based in vitro assays may more closely reflect in vivo action, because hydrogen atom transfer is a key reaction mechanism in the radical chain reactions (Huang *et al.*, 2005).

4.6.2 Oxygen Radical Absorption Capacity

In this assay, 2, 2'-azobis (2-amidino-propane) dihydrochloride (AAPH) radicals are produced by the loss of nitrogen. AAPH radicals so formed react

with oxygen (O_2) and this reaction results in the formation of stable peroxy radicals ($ROO^{\bullet}$). Peroxy radicals react with fluorescein (FL-H) causing loss of fluorescence (equation 20). In the presence of biological antioxidants (AH), the peroxy radicals are scavenged thus protecting FL-H (equation 21). Therefore, the loss of fluorescence is less. The loss in fluorescence is monitored using spectrofluorometer (Ou *et al.*, 2002).

$ROO^{\bullet} + FL\text{-}H \longrightarrow ROOH + FL^{\bullet}$ 20

$ROO^{\bullet} + A\text{-}H \longrightarrow ROOH + A^{\bullet}$ 21

The ORAC assay was recommended as a standard method for a routine quality control and measurement of food antioxidant capacity because it is a method that uses a controllable source of peroxyl radicals and can detect both hydrophobic and hydrophilic antioxidant (Prior *et al.*, 2005).

4.6.3 SET-based assays

SET-based assays measure the ability of a compound (antioxidant) to transfer one electron to reduce radicals, metals or carbonyls (oxidant). The oxidant serves also as a probe for monitoring the reaction and as an indicator of the reaction end point (Sun and Tanumihardjo, 2007). SET-based assays resemble the redox titration in classical chemical analysis and can be described by the following electron-transfer (redox) reaction

Oxidant (probe) + e (from antioxidant) $\longrightarrow$ reduced probe + oxidized antioxidant

The oxidant (probe) itself is a substance of a specific color which has the ability to absorb light in the visible spectrum (VIS) with a specific wavelength. When abstracting an electron from the antioxidant the color characteristically changes. The degree of the color change is proportional to the antioxidant concentration. The reaction endpoint is reached when the color change stops. Typically, the change of absorbance is plotted against the antioxidant concentration to give a linear curve. The slope of the curve reflects the antioxidant's reducing capacity, which is mostly expressed as equivalents of a chosen standard

compound (trolox, gallic acid, etc.). Because there is not a competitive reaction involved and there is no oxygen radical in these assays, it has often been argued how the results relate to the radical scavenging capacity of a sample. It has therefore been assumed that the AOC expressed by these assays is equal to the reducing capacity (Huang *et al.*, 2005).

The most popular SET-based methods include a) 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, b) 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) assay, c) Ferric reducing antioxidant power (FRAP) assay and d) Folin-Ciocaltau (FC) assay to estimate total phenol (Prior, *et al.*, 2005).

a) 2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

The ABTS assay is also known as TEAC (Trolox Equivalent Antioxidant Capacity) assay and is one of the most popular among other indirect assays. This method measures the ability of antioxidants to neutralize or decay the preformed ABTS radical monocation ($ABTS^{\bullet+}$) which has a strong absorption in the range of 600–750 nm and can be easily determined spectrophotometrically (Roginsky and Lissi, 2005). This assay is a generally used method for the determination of total antioxidant capacity. This method involves the generation of ABTS radical cation ($ABTS^{\bullet+}$) by oxidation. In ABTS assay, a sample containing antioxidants is added to initially prepared $ABTS^{\bullet+}$ radical solution. Antioxidants donate electrons to $ABTS^{\bullet+}$ to form ABTS that does not absorb at 734 nm leading to the decrease in absorbance. The drop in absorbance is directly proportional to the amount of $ABTS^{\bullet+}$ converted into ABTS, and this depends on the antioxidant capacity of the sample. The change in the absorbance i.e. the difference between initial absorbance and final absorbance is used to calculate the total antioxidant capacity of the sample (Prior *et al.*, 2005; Stratil *et al.*, 2006).

The advantage of the ABTS assay is that the $ABTS^{\bullet+}$ is soluble in both aqueous and organic solvents, so it can be used to determine both hydrophilic and lipophilic antioxidants in various matrices (Arnao *et al.*, 2001). Changeable

mechanism of $ABTS^{\bullet+}$ deactivation (HAT or SET) has been considered to be one of the most important weaknesses of the assay: the mechanism may shift with pH and may change during reactions of slowly reacting antioxidants (Prior *et al.*, 2005).

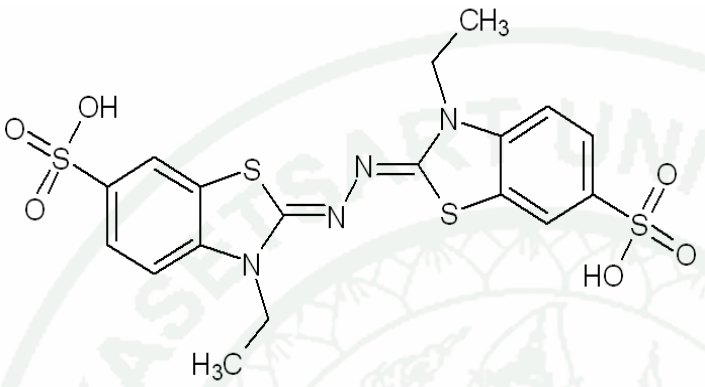


Figure 5 Chemical structure of 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)

Poor selectivity of $ABTS^{\bullet+}$ to H-atom donors is another limitation of the assay. It has been found that $ABTS^{\bullet+}$ reacts with OH-groups of hydroxylated aromatics which do not contribute to the antioxidation. A short incubation time (usually 4 – 6 min) has been the most criticized aspect in methodology of the assay because it may not provide long enough periods for the reaction to be completed. However, the ABTS assay is operationally very simple which makes it a popular and routine test for antioxidant capacity assessment (Prior *et al.*, 2005; Roginsky and Lissi, 2005).

b) DPPH radical scavenging assay

DPPH radical (as shown in Figure 6) scavenging assay is another method that is used widely for the determination of TAC. DPPH radical is stable organic nitrogen-radical having a deep purple color with a UV-VIS absorption maximum at 517 nm (Isono *et al.*, 2005). It is easily soluble in organic solvents like methanol and commercially available. The radical does not have to be generated before the assay as for example the ABTS radical monocation. The principle is the same as that of ABTS radical scavenging assay. The antioxidants (AH) reduce $DPPH^{\bullet}$

into DPPHH (Equation 22). This reaction brings change in color, from violet to yellow and TAC can be measured by monitoring the decrease in absorbance (Prior *et al*., 2005).

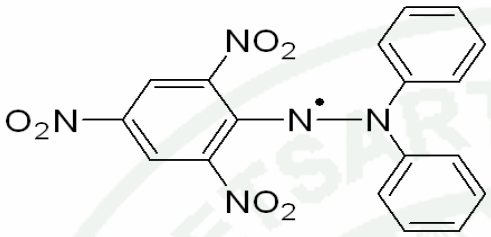


Figure 6 Chemical structure of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radica1

$$DPPH^{\bullet} + AH \rightarrow DPPHH + A^{\bullet} \quad \text{.....22}$$

Where $A^{\bullet}$ represents the antioxidant radical

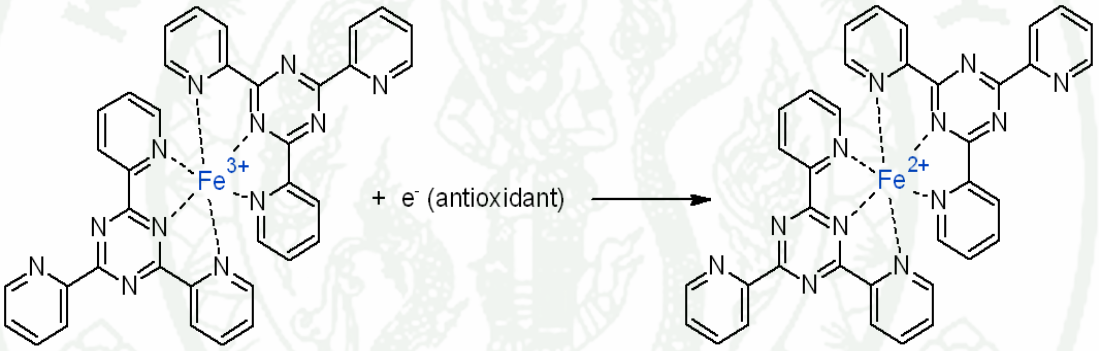
Although the DPPH assay is technically very simple and rapid and only a UV-VIS spectrophotometer is needed to perform it, many drawbacks of the assay have been found which limits the application of the assay making it less valid for antioxidant capacity measurements. The assay is not a competitive reaction because DPPH radical is both radical probe and oxidant. DPPH radical can be decolorized either by reducing agents (SET) or H-donation (HAT) as well as by some unrelated reactions. DPPH radical is stable nitrogen radical that bears no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH radical. Interpretation is complicated when the test compounds have spectra that overlap DPPH radical e.g. carotenoids (Huang *et al*., 2005; Prior *et al*., 2005).

c) Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric Reducing / Antioxidant Power method developed by Iris and Strain (1996) involves the preparation of a solution, containing Fe^{3+} -TPTZ complex in acetate. When a sample containing antioxidants is added to FRAP reagent

the Fe^{3+} -TPTZ complex is reduced to Fe^{2+} TPTZ. The spectrum of Fe^{2+} -TPTZ has absorbance maxima at 593 nm. The reaction involves the donation of an electron from an antioxidant (AH) to Fe^{3+} -TPTZ complex and thereby reducing it into ferrous form (Equation 23), which is blue in color. The increase in absorbance at 593 nm is monitored to find out the reducing ability of the sample (Iris and Strain., 1996). The change in absorbance is proportional to the total ferric reducing / antioxidant power. This is generally expressed in trolox equivalents.

$$Fe\ (III) + A\text{-}H \longrightarrow Fe\ (II) + A\text{-}\ H^{+} \quad \text{.....23}$$



[Fe (III) (TPTZ) $_2$]$^{3+}$ **[Fe (II) (TPTZ)$_2$]$^{2+}$**

Yellow Intense blue at 593 nm

Figure 7 Reaction for the FRAP assay

The FRAP assay cannot detect compounds that act by radical quenching (H atom transfer), particularly thiols (such as glutathione) and proteins. This may lead to underestimation of antioxidant activity of some complex matrices. The assay measures only the reducing capability based upon the ferric ion in the environment of a polar solvent, which has poor relationship to the radical quenching process mediated by most antioxidants via the HAT mechanism in a lipid (non-polar) substrate. The advantage of the FRAP assay is that it is a simple, rapid, inexpensive and robust assay, and it does not require specialized equipment. It can be performed using automated, semiautomatic, or manual methods (Prior *et al.*, 2005).

d) Total phenol assay by Folin-Ciocaltau reagent

The total phenol (ics) assay by Folin-Ciocaltau reagent (further referred to as FC assay) belongs to the oldest and commonly accepted assays in food research laboratories. The basic mechanism of the assay is an oxidation-reduction reaction between the Folin-Ciocaltau reagent (FCR) containing molybdenum (Mo), and a phenolic compound, thus reducing capacity of a sample is measured. Dissociation of a phenolic proton leads to a phenolate anion, which is capable of reducing FCR. Basic conditions (pH ~ 10) are required for the proton dissociation; this is facilitated by the use of a sodium carbonate solution (Huang *et al.*, 2005; Prior *et al.*, 2005).

$$MoVI\ (yellow) + e^{-} \longrightarrow MoV\ (blue) \quad \text{.....24}$$

The total phenols assay by FCR is carried out in water (aqueous phase), thus for lipophilic antioxidants this assay is not applicable. A significant weakness of this method is that the FCR is nonspecific to phenolic compounds and it can be reduced by many non-phenolic compounds (e.g. vitamin C, Fe^{2+}, Cu^{+}).

MATERIALS AND METHODS

Materials

1. Sample collection

Greater cardamom and siam cardamom dried fruits were purchased in a local market, Bangkok, Thailand. The insect infested, damaged, immature and shrivelled capsules (capsules which were not fully developed) of the cardamom were discarded.

2. Chemicals

2.1 Gallic acid: $(HO)_3C_6H_2CO_2H$, Analytical grade (Sigma-Aldrich, St. Louise, MO, U.S.A.)

2.2 Folin-Ciocalteu reagent: Analytical grade (Sigma-Aldrich, St. Louise, MO, U.S.A.)

2.3 Sodium carbonate: Na_2CO_3, Analytical grade (Ajax Finechem, Auckland, New Zealand)

2.4 Potassium di-hydrogen phosphate: KH_2PO_4, Analytical grade (Ajax Finechem, Auckland, New Zealand)

2.5 Potassium acetate: CH_3CO_2K, Analytical grade (Loba chemie, India)

2.6 Di-potassium hydrogen phosphate; K_2HPO_4, Analytical grade (Ajax Finechem, Auckland, New Zealand)

2.7 Sodium hydroxide: NaOH, Analytical grade (Merck, Germany)

2.8 Aluminium chloride: $AlCl_3$, Analytical grade (Ajax Finechem, Auckland, New Zealand)

2.9 Methanol: CH_3OH, Analytical grade (Mallinckrodt Baker Inc., Phillipsburg, NJ, U.S.A.)

2.10 Methanol: CH_3OH, HPLC grade (Mallinckrodt Baker Inc., Phillipsburg, NJ, U.S.A).

2.11 Ethanol; C_2H_5OH, Analytical grade (Mallinckrodt Baker Inc., Phillipsburg, NJ, U.S.A.)

2.12 Acetone; $(CH_3)_2CO$, Analytical grade (Merck, Germany)

2.13 2,2,{-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS): $C_{18}H_{24}N_6O_6S_4$, HPLC grade (Sigma-Aldrich, Canada)

2.14 2,2-Diphenyl-1-picrylhydrazyl (DPPH): $C_{18}H_{12}N_5O_6$ (Aldrich, Steinheim, Germany)

2.15 Acetic acid: CH_3CO_2H, Analytical grade (Merk, Germany)

2.16 Trolox: $C_{14}H_{18}O_4$ (Aldrich, Steinheim, Germany)

2.17 Protocatecheuic aldehyde: $C_7H_6O_3$, HPLC grade (Sigma-Aldrich, St. Louise, MO, U.S.A.)

2.18 Protocatechuic acid: $C_7H_6O_4$, HPLC grade (Sigma-Aldrich, St. Louise, MO, U.S.A.)

2.19 Dinitrophenyl hydrazine (DNPH): $(O_2N)_2C_6H_3NHNH_2$, Analytical grade (Him Media Laboratories Pvt. Ltd., India)

2.20 Quercetin: $C_{15}H_{10}O_7$, HPLC grade (Sigma-Aldrich, St. Louise, MO, U.S.A.)

2.21. 2-Thiobarbituric acid: $C_4H_4N_2O_2S$ (Fisher Scientific, U.K.)

2.22 Guanidine hydrochloride: $NH_2C(=NH)NH_2 \cdot HCl$ (Sigma-Aldrich, St. Louise, MO, U.S.A.)

2.23 Trichloro acetic acid: $C_2HCl_3O_2$, Analytical grade (Fisher Scientific, U.K.)

2.24 Hexane: C_6H_{14}, HPLC grade (Fisher Scientific, U.K.)

2.25 Isopropanol: $(CH_3)_2CHOH$, HPLC grade (Fisher Scientific, U.K.)

2.26 Butylated hydroxytolune (BHT): $C_{15}H_{24}O$ (Fluka, Germany)

2.27 2, 4, 6- tripyridyl-s-triazine (TPTZ): $C_{18}H_{12}N_6$ (Fluka, Switzerland)

3. Equipment

3.1 Spectrophotometer (Spectronic Genesys, 10 UV Scanning Thermo Electron Corporation, U.S.A.)

3.2 Grinder (MX J210GN, Panasonic)

3.3 Homogenizer (IKA, T10 basic, Australia)

3.4 Sonicator (360 T, frequency-45, Crest ultrasonic, Malaysia)

3.5 Vortex mixer (Genie II, U.S.A.)

3.6 High Performance Liquid Chromatography (HPLC Waters 996, Waters Corporation, Massachusetts, U.S.A.)

3.7 Rotary evaporator (Rotavopor, Buchi, Switzerland)

3.9 Colorimeter for color measurement (Chroma meter, CR-210, Minolta, Japan)

3.10 Meat mincer (MANCA, PM-82, Equipamientos Carnicos S.L., Spain)

3.11 Water bath shaker (2000W, National Labnet Company)

3.12 Refrigerator (Sanden intercooler, SEC-1000SBD, Thailand)

Methods

1. Optimization of conditions for the extraction of antioxidants from greater cardamom seeds

Optimization of extraction conditions was carried out by investigating their effects on total phenolic content (TPC) and DPPD radical scavenging capacity of liquid extracts from greater cardamom seeds.

1.1 Sample preparation

The seeds and husk of greater cardamom capsules were separated manually and the seeds sample was grounded in a grinder until the particles could pass through 1mm mesh. Moisture content in the ground sample was determined by official method 986.21 (AOAC, 2006). Then, the ground sample was packed in an air tight polyethylene bag and stored at -18ºC until used for extraction.

1.2 Experimental design and extraction

Single-factor experiments were designed for the optimization of four factors (extraction conditions), namely solvent systems, extraction temperatures, extraction time and solid: solvent ratios using a water bath shaker at 100 rpm speed. Each factor was varied for different levels at a time while keeping other factors at constant levels as mention below

a) To study the effect of solvent systems, extraction was carried by using different solvent systems (ethanol and methanol at three concentration levels of 50, 80 and 100% and 100%water) with the solid solvent ratio of 1:20 at 40ºC for 12 hours.

b) To study the effect of extraction temperatures, extraction was carried out at different temperatures from 40 to 75 ºC using the solvent system selected in step 1.2a) and other conditions were kept constant (solid: solvent ratio of 1:20, extraction time of 12 hours).

c) To study the effect of extraction time, extraction was carried out by varying extraction time from 1 to 16 hours and using the solvent system and temperature selected in 1.2a) and b) with solid: solvent ratio of 1:20.

d) To study the effected of solid and solvent ratios, extraction was carried out by varying solid and solvent ratio from 1:10 to 1:50 and using conditions selected in above mention steps.

After selecting appropriate conditions for water bath shaker extraction (WBE), it was compared with other extraction techniques which were namely;

i) Maceration (MCN): Extraction at room temperature (30±7) ºC in dark for 12, 24, 48, 72, 96, 120 and 144 hours with manual shaking in every 12 hours.

ii) Ultrasound assisted extraction (USE) at 40±5ºC for 5, 15, 30, 45, 60 and 120 minutes using a sonicator.

For each of the above mention extraction, the ground sample (1gm) prepared in method 1.1 was extracted in 20 ml of solvent in 125 ml capacity identical conical flasks. But, to study the effect of solid and solvent ratio, weight of ground sample was varied from 0.4-2 gm so as to maintain the solid solvent ratio from 1:50 to 1:10 in 20 mL of solvent. After extraction, each extract was filtered through Whatman No. 1 filter paper, and the residues were washed with the corresponding solvents to maintain 50 ml final volume of the filtrate.

All the above mentioned extraction trials were carried out in triplicate and each sample was analyzed for total phenolic content (TPC) and 1, 1-diphenyl-2-picrylhydrazyl free radical scavenging capacity.

1.3 Determination of total phenolic content (TPC)

The total phenolic content in different extracts from the greater cardamom seeds was determined using the Folin-Ciocalteu reagent method as described by Hinneburg *et al.*(2006) with slight modification. The cardamom extracts (250 µL) was transferred into a test tube and then mixed with 150 µL of Folin-Ciocalteu reagent and 1.1 mL DI water thoroughly and allowed to stand for 3 minutes. Then, 2.5 mL of 7.5% (w/v) sodium carbonate was added. The mixtures was agitated with a vortex mixer and allowed to stand for a further one hour in the dark. The absorbance of samples and a prepared blank were measured at 760 nm using a UV visible spectrophotometer. A standard curve was prepared using different concentration of gallic acid. The results were expressed as milligrams (mg) gallic acid equivalents (GAE) per 100 gm of the cardamom seeds powder (dry weight basis).

1.4 2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH assay was carried out according to the method of Brand-Williams *et al*. (1995) as described by Nguyen *et al*. (2012) with some modifications. Briefly, 0.75 mL of the greater cardamom extract was mixed with 3.25 mL of DPPH solution (prepared by dissolving 5.4 mg DPPH in 100 mL methanol) and mixed thoroughly. Then, the mixtures were kept at room temperature in the dark for 30 min. The absorbance of the mixtures was measured at 517 nm against a blank without DPPH using a spectrophotometer. The standard curve of trolox was prepared and the results were expressed in milligrams (mg) trolox equivalent (TE)/ 100 gm of the cardamom seeds powder (dry weight basis).

2 Evaluation of antioxidant properties of crude extracts from greater cardamom and siam cardamom seeds

2.1 Preparation of crude extracts from cardamom

Both the greater cardamom and siam cardamom samples were prepared according to the method described in 1.1. The ground cardamom samples were extracted using the optimum conditions (water bath shaker extraction at 75ºC using 50% ethanol with the solid solvent ratio of 1:20 for 8 hours) selected from the first part of the study. After extraction, the liquid extracts from each cardamom sample were collected and filtered through Whatman No. 1 filter paper. The filtrates were evaporated under reduced pressure using a rotary evaporator at 50ºC. After evaporating the solvent, the weights of the crude extracts were measured to calculate extraction yield. Then, the crude extracts were dissolved in 50% ethanol for chemical analysis of antioxidant properties. For application to meat samples, the crude extracts were dissolved in distilled water. The final volume of the crude extracts were made up 25 mL and filled into a brown bottle. The head space in the bottle was flushed with nitrogen gas and stored at -40ºC until used.

2.2 Determination of extraction yield of the cardamom crude extracts

The yield of evaporated extracts on a dry weight basis was calculated from equation (25) shown below:

$$\text{Yield (\%)} = (W_1 \times 100)/W_2 \quad \ldots\ldots 25$$

Where W_1 was the weight of extract after evaporation of ethanol and W_2 was the dry weight of cardamom seeds powder.

2.3 Determinations of antioxidant in the cardamom crude extracts

2.3.1 Total phenolic content (TPC)

The total phenolic content (TPC) in extracts obtained in 2.1 from the greater cardamom and siam cardamom seeds was determined according to method described in 1.3. The results were expressed as milligrams (mg) gallic acid equivalents (GAE) per gm dry extract.

2.3.2 Total flavonoid content (TFC)

The total flavonoid content in the cardamom extracts was determined according to the aluminum chloride colorimetric method described by Chang *et al.*, (2002). The diluted cardamom extracts (1 mL) were separately mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a UV visible spectrophotometer. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Total flavonoid values were expressed in terms of quercetin equivalents (QE) per gram of dry extract. A standard curve was prepared using different concentration of quercetin.

2.4 Antioxidant capacity of the crude extracts by indirect chemical assays

2.4.1 2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH was carried out according to the method described in 1.4. The results were expressed in milligrams (mg) trolox equivalent (TE)/gm dry extract.

2.4.2 2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

The ABTS assay, also known as TEAC (Trolox Equivalent Antioxidant Capacity) assay was carried out according to method of Re *et al.* (1999) as described by Lu *et al*. (2011). ABTS was dissolved in water to a concentration of 7 mM. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution

with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark room for 16 h before use. The $ABTS^{\bullet+}$ solution was diluted with 60% ethanol to an absorbance of 0.700±0.02 at 734 nm. The $ABTS^{\bullet+}$ solution (3.9 mL; absorbance of 0.700±0.02) was added to 0.1 mL of the tested samples and mixed thoroughly. The reactive mixture was allowed to stand at room temperature for 6 min, and the absorbance was immediately recorded at 734 nm using a UV visible spectrophotometer. The standard curve of trolox was prepared and results were expressed as milligrams (mg) trolox equivalents (TE)/gm dry extract.

2.4.3 Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was done according to Iris and Strain (1996). The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2$.$3H_2O$ and 16mL $C_2H_4O_2$, pH 3.6), 10 mM TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3$.$6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5mL TPTZ solution, and 2.5 mL $FeCl_3$.$6H_2O$ solution and then warmed at 37ºC before using. The extracts (150µL) appropriately diluted, was allowed to react with 2850 µL of the FRAP solution for 30 mins in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) was then taken at 593 nm. The standard curve of trolox was prepared and the results were expressed in milligrams (mg) trolox equivalents (TE)/gm dry extract.

2.5 HPLC determination of antioxidant compounds in the cardamom crude extracts

Protocatechuic acids and protocatachuic aldehyde, two of the antioxidant compounds present in greater cardamom seeds (Kikuzaki *et al.*, 2001) were determined in ethanolic crude extracts from greater cardamom and siam cardamom seeds by the method described by Sakakibara *et al.*(2002) with slight modification. High performance liquid chromatography (HPLC) consisting of a binary pump and a diode-array detector (DAD), and equipped with a Waters Symmetry C18 column (5µm, 4.6 × 150 mm) was used. Gradient elution was performed with Solution A (50 mM sodium phosphate in 10% methanol; pH 3.3) and Solution B (70% methanol) in

the following gradient elution program: 0 min, 100% A; 15 min, 70% A; 45 min, 65%; 65 min, 60% A; 70 min, 50% A. Flow rate was 0.8 ml/min and injection volume was 20 μL. Protocatechuic acids and protocatechuic aldehyde in the cardamom extracts were identified by comparing retention time and spectra with those of standard compounds. Detection was monitored at 259 nm for protocatechuic acid and at 280 nm for protocatechuic aldehyde.

3 Evaluation of antioxidative effects of the cardamom crude extracts in pork patties

3.1 Antioxidant activity of the cardamom crude extracts to inhibit the lipid oxidation in cooked pork patties

3.1.1 Preparation of cooked pork patties and storage

Freshly prepared minced pork meat sample was purchased from a reputed local processor. The minced meat was homogeneously mixed with hand and again passed through a meat mincer having a plate with 3 mm holes. Crude fat content in the meat sample was determined by solvent extraction (Submersion) official method 991.36 (AOAC, 2006). The crude fat content in the meat sample was found to be 18.34%. Then, the minced meat was divided into 100 gm portions. Each portion was mixed with salt (1.5 gm) and the antioxidants (cardamom extracts and BHT) according to the following formulation: 1) negative control (no antioxidant); 2) positive control, commercial antioxidant BHT (0.01% of fat) 3) greater cardamom extract (GCE) 0.01%; 4) GCE 0.1%; 5) GCE 0.25%; 6) siam cardamom seeds extract (SCE) 0.01%; 7) SCE 0.1% and 8) SCE 0.25%.

BHT was pulverized and added to the meat sample by mixing with salt as described by Gonzalez *et al.* (2008). Then, all the samples were added with distilled water so that each sample contained 3 mL of externally added distilled water. The ingredients in each sample were hand mixed for five minutes and homogenized by blending for two minutes with a kitchen aid mixer. Each meat sample was molded into identical circular shape using petri dish. All the samples were cooked one at a

time in a microwave oven at 850 watts for four minutes. The optimum cooking time was determined by heat and trial methods. After cooling down to room temperature, each cooked ground pork was divided into identical four smaller portions for analysis in 1, 3, 6, and 9 days. The cooked pork patties were then packed into polystyrene trays and stored under refrigerated condition (4 ±1ºC) for 9 days. Samples were taken out of refrigerator after storage period of 1, 3, 6 and 9 days and analyzed for conjugated diens (CD) and thiobarbituric acid reactive substances (TBARS).

3.1.2 Assay of conjugate dienes

The formation of conjugated dienes was determined according to the procedure described by Juntachote *et al*. (2006). Meat samples (1.00 gm) were suspended in 10 ml of distilled water and homogenized to form smooth slurry. A 0.5 ml aliquot of this suspension was mixed with 5 ml of extracting solution (3:1 hexane: isopropanol) for 1 min. After centrifugation at 2000g for 5 min, the absorbance of the supernatant was read at 233 nm.

3.1.3 Measurement of thiobarbituric acid reactive substances (TBARS)

Lipid oxidation was assessed by the 2-thiobarbituric acid reactive substances (TBARS) method of Witte *et al.* (1970) as described by Juntachote *et al.* (2006) with some modification. In brief, 5 g of sample were homogenized in 25 ml of 10% (w/w) trichloroacetic acid. After homogenization the mixture was transferred to a measuring flask and adjusted to 25 ml with distilled water. The dispersion was filtered through a folded filter paper (whatman no 1). The supernatant (5 ml) was mixed with 5 ml 2-thiobarbituric acid (2.88 g $L^{-1}H_2O$) and heated in a boiling water bath for 10 min to develop the rose-pink color by reaction between malondialdehyde and 2-thiobarbituric acid and cooled to room temperature. The absorbance was measured at 532 nm, against a blank prepared with 5 ml distilled water and 5 ml TBA-reagent, using a UV visible spectrophotometer. The TBARS value, expressed as mg of malonaldehyde/kg of the meat sample, was calculated using a molar extinction coefficient (156,000 M^{-1} cm^{-1}).

3.2 Antioxidant activity of the cardamom crude extracts to inhibit the protein oxidation and color deterioration in raw pork patties

3.2.1 Preparation of raw pork patties and storage

Freshly prepared minced pork meat sample was purchased from the same processor and analyzed for fat content using the official method 991.36 (AOAC, 2006). The crude fat content in the meat sample was found to be 16.98%. Then, pork patties were again prepared using the same method used for lipid oxidation measurement as described in 2.6.1. But instead of three increasing concentrations of each cardamom extract, only one concentration that was found to be comparable to and even more effective than 0.01% BHT to inhibit lipid oxidation was chosen and added to the meat samples. The patties were kept uncooked. Two identical sets of raw pork patties were prepared, one set for color measurement and other for measurement of protein oxidation. After molding into circular shape, the patties were put on the polystyrene trays, wrapped with polyvinyl chloride cling film and stored under refrigeration at (4±1) ºC. The color of the raw pork patties was measured immediately after preparation and then after 1, 3, 6 and 9 days of storage. Similarly, the protein oxidation in the samples was monitored by measuring protein carbonyls in 1, 3, 6, and 9 days.

3.2.2 Measurement of protein oxidation

Protein oxidation was measured according to the method outlined by Oliver et al. (1987) as described by Vuorela *et al.* (2005). Two different measurements were made for protein oxidation: (a) carbonyl quantification and (b) protein quantification. Meat samples of about 1 gm were homogenized with 10 mL of 0.15 M KCl with an ultra turrax homogenizor for 60 s. One hundred microliters of homogenate was transferred into a 2 mL eppendorf vial, where 1 mL of 10% trichloroacetic acid was added. The sample was centrifuged for 5 min at 5000 rpm, and the supernatant was removed. For sample (a) 1 mL of 2 M HCl with 0.2% 2, 4-dinitrophenyl hydrazine (DNPH) was added, and for sample (b) 1 mL of 2 M HCl was added. After an incubation of 1 h (shaken every 20 min), 1 mL of 10% trichloroacetic

acid was added. The sample was vortexed and centrifuged for 5 min at 5000 rpm. Supernatant was removed carefully without damaging the pellet with the Pasteur pipet. The pellet was washed with 1 mL of ethanol/ethyl acetate (1:1), shaken, and centrifuged for 5 min at 10000 rpm; this procedure was repeated three times. After this, the pellet was completely dried with nitrogen. The pellet was dissolved in 1.5 mL of 20 mM sodium phosphate buffer with 6 M guanidine hydrochloride, final pH 6.5, shaken, and centrifuged for 2 min at 5000 rpm. Carbonyls (sample a) and protein concentration (sample b) were measured spectrophotometrically at 370 and 280 nm, respectively.

For protein quantification a standard solution of bovine serum albumin (BSA) in 20 mM sodiumphosphate buffer with 6 M guanidine hydrochloride (pH 6.5) was prepared, and the protein concentrations were determined according to a standard curve. Concentration (nanomolar) of carbonyls was calculated as [Abs370nm/21.0 mM-1 cm-1) x1000], where 21.0 mM^{-1} cm^{-1} is the molar extinction coefficient of carbonyls.

3.2.3 Color measurement

Color measurements were taken with a colorimeter which was calibrated with white standard plate provided with the instrument using an 8 mm diameter measuring area and a 50 mm diameter illumination area. The instrumental colors were expressed as L* (lightness), a* (redness) b* (yellowness) units. Three different areas on the surface of each patty and three pork patties per treatment were analyzed to obtain an average color value.

4 Statistical analysis

Statistical analysis was performed using Microsoft Excel 2007 and SPSS version 12.00. For each measurement, three replicates samples were prepared and the results are expressed as the mean of three replicate analysis ± SD. The comparison of total phenolic content and DPPH radical scavenging capacity of different extracts were carried out using one-way analysis of variance (ANOVA) and Duncan's test. An 8(treatments) ×4 (storage days) factorial design was employed for the measurement of lipid oxidation using TBARS and CD whereas a 4(treatments) x 4 (storage days) factorial design was employed for the measurement of protein oxidation by measuring carbonyls and color deterioration by L* a* and b*values. The results of TBARS, CD, proteins carbonyls and color values (L* a* and b*), for the different treatments on the same day and the same treatment for the different days, were analyzed using one-way analysis of variance (ANOVA) and Duncan's test. Differences were considered significant at $P \leq 0.05$. The standard curves and linear correlation between TPC and DPPH radical scavenging capacity were calculated using Microsoft Excel 2007.

RESULTS AND DISCUSSIONS

1. Effect of extraction conditions

1.1 Effect of solvent systems

In this study, the effect of solvent systems on the efficient extraction of antioxidants from greater cardamom (*Amomum subulutum* Roxb) seeds was investigated. Total phenolic content (TPC) and DPPH free radical-scavenging capacity of the liquid extracts from greater cardamom seeds in different solvent systems namely; methanol and ethanol at three different concentrations (50%, 80%, and 100%) and 100% distilled water are presented in Table 1. The results showed that solvent systems used for extraction of antioxidants had significant effects ($P \leq 0.05$) on both TPC and DPPH radical scavenging capacity. TPC and DPPH radical scavenging capacity of the different solvent extracts ranged from 135.78±11.64to 304.87±23.27mg GAE/100gm sample (DW) and from 69.15±6 to 407.77±08.07 TE/100gm sample (DW) respectively for the extraction time of 12 hours at 40ºC.

The greater cardamom extract in 50 % ethanol gave the highest TPC whereas 50% methanol extract showed the highest DPPH radical scavenging capacity but there was no significant ($P > 0.05$) difference in both TPC and DPPH radical scavenging capacity obtained in 50 % ethanol and 50% methanol extracts. Water extract had the lowest values for both TPC and DPPH radical scavenging capacity. Similarly, absolute ethanol and absolute methanol showed the significantly ($P \leq 0.05$) lower TPC and DPPH radical scavenging capacity than their corresponding aqueous mixtures. This showed that aqueous organic solvents are more efficient than pure solvents for the extraction of antioxidants from greater cardamom and it was in accordance with previous reports suggesting that a binary solvent system was superior to a mono-solvent system in the extraction of phenolic antioxidant compounds in regard to their relative polarity (Musa *et al.*, 2011; Turkmen *et al.*, 2006; Wang *et al.*, 2008).

Methanol extracts are not well accepted by consumer for food application and ethanol is considered to be safer as compared to methanol. Therefore, among the

aqueous solvents of ethanol and methanol which did not show any significance difference in the extraction efficacy, ethanol 50% was selected to be the best one.

Table 1 Effect of different extraction solvents on total phenolic content (TPC) [1] and DPPH radical-scavenging capacity [2] of extracts from greater cardamom seeds obtained by using water bath shaker at 40 ºC

Solvent systems	TPC	RSD% [3]	DPPH radical scavenging capacity	RSD% [3]
Absolute ethanol	143.47±13.72c	9.57	75.06±07.32d	9.68
80% ethanol	270.10±16.24a	6.02	279.44±12.75c	4.56
50% ethanol	304.87±23.27a	7.63	390.37±18.17a	4.65
Absolute methanol	186.29±15.38b	8.26	280.15±15.43c	5.51
80% methanol	294.26±15.37a	5.22	372.72±05.86b	1.57
50% methanol	280.35±24.16a	8.62	407.77±08.07a	1.98
Water	135.78±11.64c	8.57	69.15±06.56d	9.49

Values in each column marked by the different letters are significantly different at $P \leq 0.05$. Results showed mean ± SD

[1] Milligrams of Gallic acid equivalent (GAE) per 100 gm of the cardamom seeds powder (DW: dry weight basis)

[2] Milligrams of trolox equivalent (TE) per 100 gm of the cardamom seeds powder (DW)

[3] Relative standards deviation

Variations in TPC and DPPH radical scavenging activity of extracts in the different solvent systems might be attributed to the change in relative polarity of different solvents used. Polarity indices of water, ethanol and methanol are 10.2, 4.3,

5.1 and respectively (Ahuja, 2003). Thus, water has higher polarity index as compared to pure organic solvents and when water is added to a pure organic solvent the polarity index of the mixture, Pm, increases with the increase in the ratio of water according to following equation 25 (Musa *et al.*, 2011).

$P_m = Ø_1 P_1 + Ø_2 P_2$ ……26

Where $Ø_1$ and $Ø_2$ are the volume fractions of solvents 1(pure water), and solvent 2, respectively, and P_1 and P_2 are polarity indices of solvent 1 and solvent 2, respectively. Therefore, the different mixtures of organic solvents have the different polarity indices and solubilize the compatible compounds according to the ‘‘like dissolves like” principle (Zhang *et al.*, 2007). Thus, in the crude extracts, effective phenolic compounds which contribute to the antioxidant capacities might be intermediately polar and their solubility was very sensitive to the solvent polarity (Thoo *et al.*, 2010).

1.2 Effect of extraction temperature

The impact of extraction temperature on the phenolic content and DPPH radical scavenging capacity were investigated in the range from 40 to 75ºC and presented in Figure 8 and 9**,** respectively. Both TPC and DPPH radical scavenging capacity of the extracts increased significantly with increase in temperature from 40 to 75ºC. The highest values for TPC and DPPH radical scavenging capacity were found for the extract at 75ºC and the same temperature was chosen as optimum temperature. The results showed that extraction temperature has strong influence on the extraction of antioxidants from greater cardamom seeds. From this result, it can be believed that the phenolic compounds present in greater cardamom are thermally stable.

Higher temperatures from 40 to 75ºC enhanced the recovery of phenolic compounds as described by Durling *et al.* (2007) and Silva *et al.* (2007). The solubility and diffusion coefficients of polyphenol get increased with increased temperature promoting extraction of polyphenols in the solvents (Al-Farsi and Lee, 2008). Wang *et al.* (2008) also reported the increased solubility of phenolic

compounds with increasing temperature. But in many studies, losses in antioxidant capacity of plant samples are often reported following a thermal treatment, likely due to the degradation of polyphenols which were previously mobilized at lower temperatures (Chan *et al.*, 2009; Liyana-Pathirana and Shahidi, 2005).

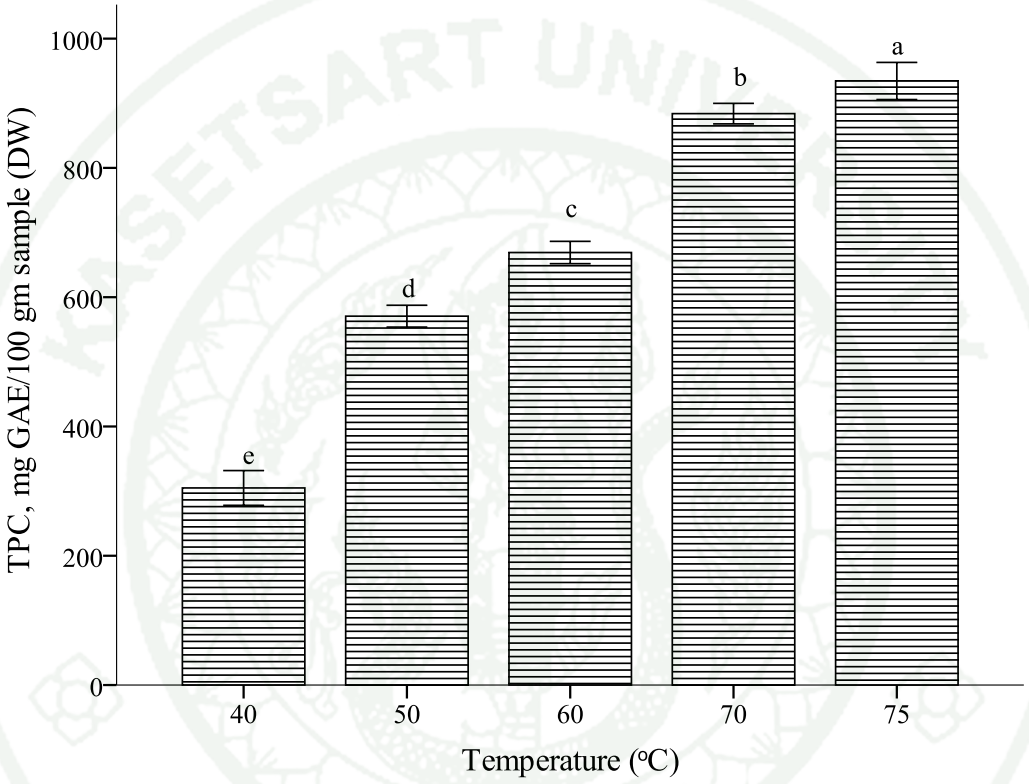


Figure 8 Effect of extraction temperatures (40-75 °C) on total phenolic content (TPC) of the extracts from greater cardamom seeds obtained by using 50% ethanol with solid: solvent ratio of 1:20 in a water bath shaker for 12 hours.
Values are the mean ± standard deviation of at least 3 determinations from independent extractions. Different lower case letters means significantly ($P \leq 0.05$) different. The error bars represent the standard deviation.

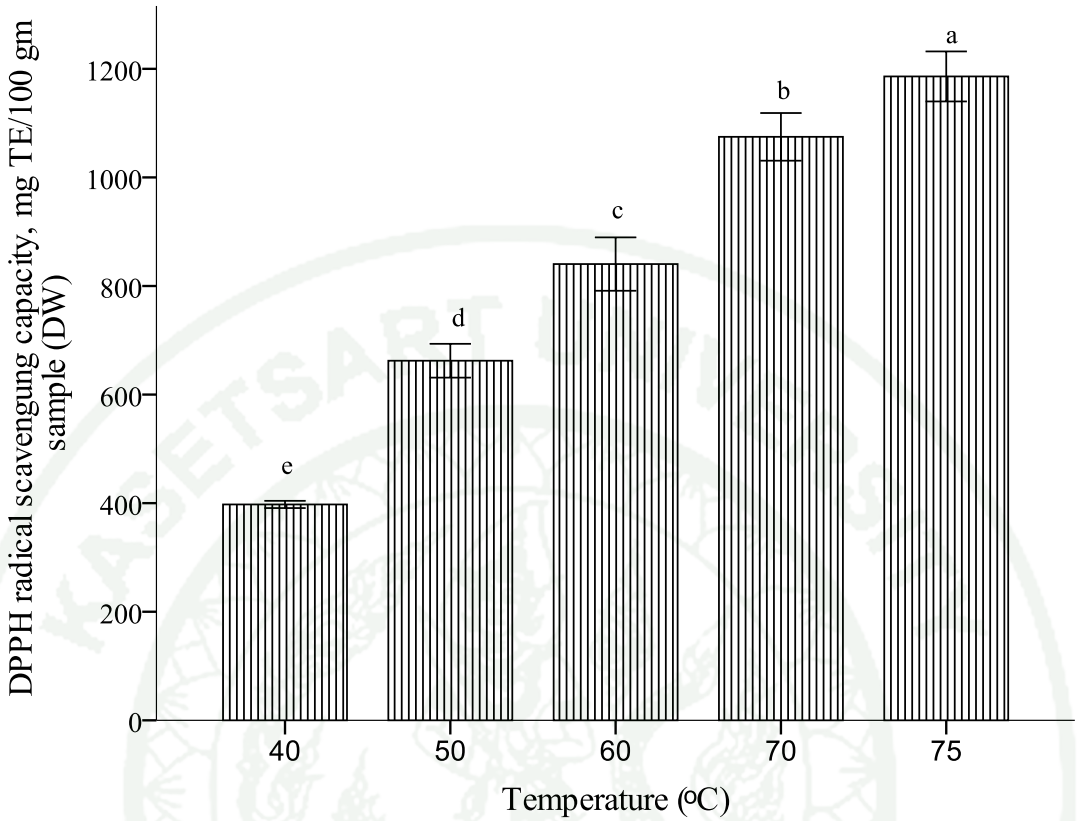


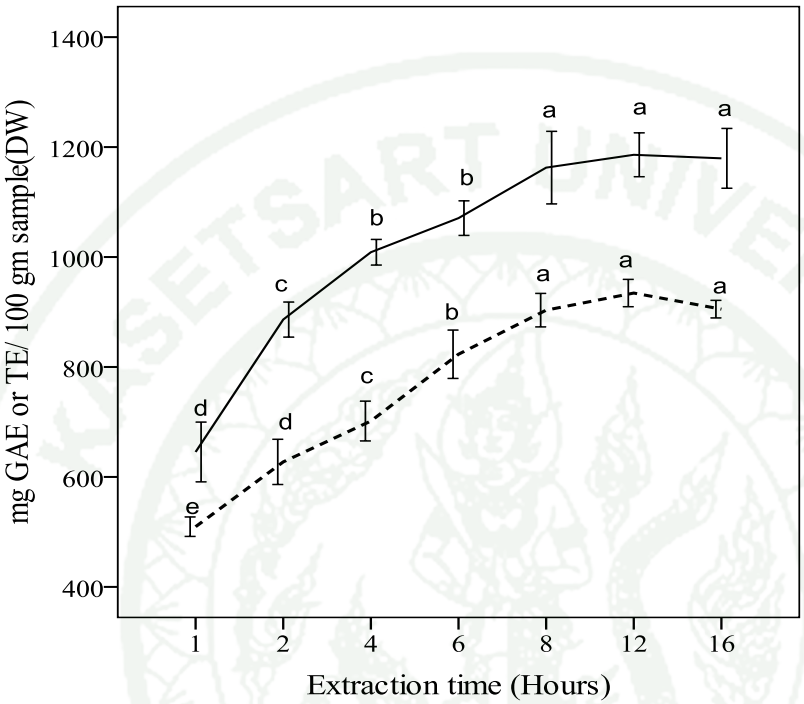
Figure 9 Effect of extraction temperatures (40-75ºC) on DPPH radical scavenging capacity of extracts from greater cardamom seeds obtained by using 50% ethanol with solid: solvent ratio of 1:20 in a water bath shaker for 12 hours.

Values are the mean ± standard deviation of at least 3 determinations from independent extractions. Different lower case letters means significantly ($P \leq 0.05$) different. The error bars represent the standard deviation.

1.3 Effect of extraction time

Figure 10 shows the effects of extraction time on total phenolic content (TPC) and DPPH radical scavenging capacity of the extracts obtained at different extraction time from 1 hour to 16 hours. There were significant ($P \leq 0.05$) increase in both TPC and DPPH radical scavenging capacity of the extracts obtained from 1 hour to 8 hours but no significant difference in both TPC and DPPH radical scavenging capacity were observed in the extracts obtained after extraction time of 8, 12 and 16

hours. Therefore, the extraction time of 8 hours was selected as optimum extraction time.



.....Total phenolic content, mg gallic acid equivalent (GAE)/100 gm sample (Dry Weight)

——DPPH radical scavenging capacity, mg trolox equivalent (TE)/100 gm sample (Dry Weight)

Figure 10 Effect of extraction time (1-16 Hours) on total phenolic content and DPPH radical scavenging capacity of extracts from greater cardamom seeds obtained by using 50% ethanol (solid: solvent ratio 1:20) in a water bath shaker at 75ºC.

Values are the mean ± standard deviation of at least 3 determinations from independent extractions. Values marked with the different lower case letters in each line are significantly ($P \leq 0.05$) different. The error bars represent the standard deviation.

Extraction time is crucial in solvent extraction for phenolic compounds, where phenolic compounds may be governed by the equilibrium concentrations for phenolic compounds reached before their corresponding apparent reduction (Spigno *et al.*, 2007). Hence, excess extraction time indeed reduced the yield of phenolic compounds. However in the present study, there was no significant reduction in TPC and DPPH radical scavenging capacity up to the extraction time of 16 hours.

1.4 Effect of solid and solvent ratio

The impact of solid and solvent ratio on the phenolic content (TPC) and DPPH radical scavenging capacity were investigated in the range from 1:10 to 1:50 (w/v) and presented in Figure 11 and 12 respectively. The extract obtained in solid-to solvent ratio of 1:10 showed lowest values of TPC and DPPH radical scavenging capacity. However, there was no significant ($P > 0.05$) difference in both TPC and DPPH radical scavenging capacity of the extracts obtained by using the solid solvent ratio 1:20 to1:50. Therefore the solid solvent ratio of 1:20 was selected as optimum ratio for the extraction of antioxidants from the greater cardamom.

A high solid-to-solvent ratio could promote an increasing concentration gradient, resulting in an increase of diffusion rate that allows greater extraction of solids by solvent (Al-Farsi and Lee, 2008;Tan *et al.*, 2011). In addition, the chance of bioactive components coming into contact with extracting solvent expanded with increase amount of extraction solvent, leading to higher leaching-out rates (Zhang *et al.*, 2007).

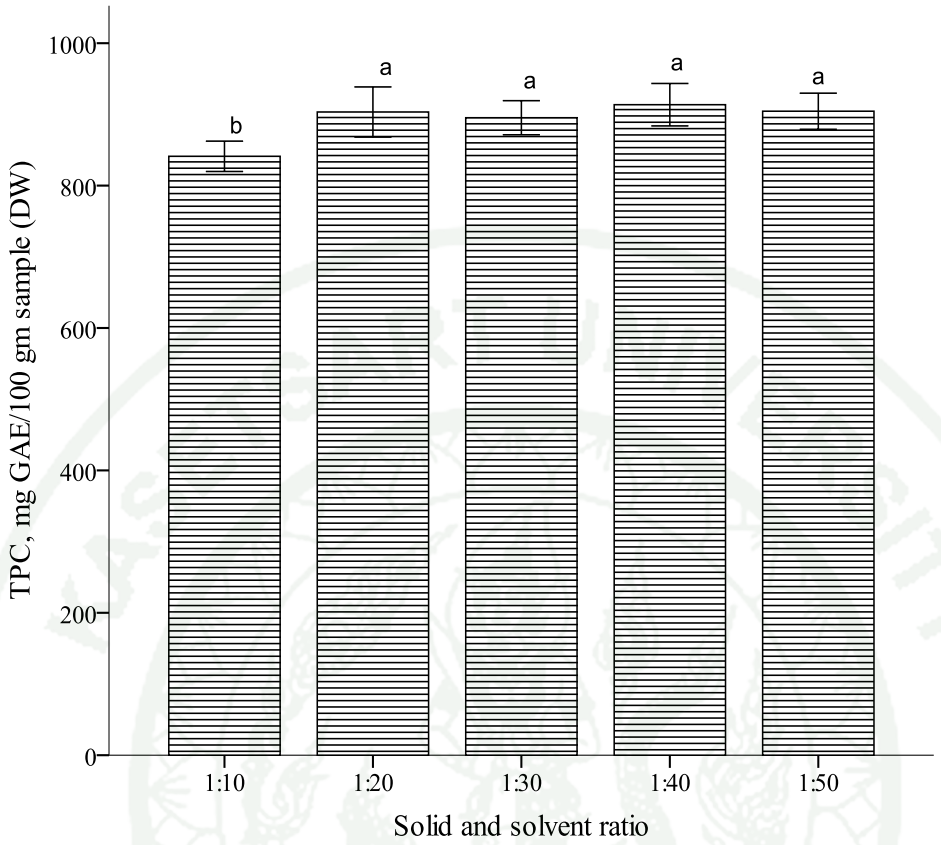


Figure 11 Effect of solid: solvent ratio (1:10 to 1:50) on total phenolic content (TPC) of extracts from greater cardamom seeds obtained by using 50 % ethanol in a bath shaker at 75ºC for 8 hours.

Values are the mean ± standard deviation of at least 3 determinations from independent extractions. Different lower case letters in the columns means significantly ($P \leq 0.05$) different. The error bars represent the standard deviation.

However, active component yields will not continue to increase once equilibrium is reached (Herodež *et al.*, 2003). Similar result was reported by Tan *et al.* (2011) who studied the effects of solid-to-solvent ratio (1:5, 1:10, 1:15and 1:20) on antioxidant compounds and capacities of Pegaga (*Centella asiatica*) and found the solid to solvent ratio of 1:15 as an optimum ratio.

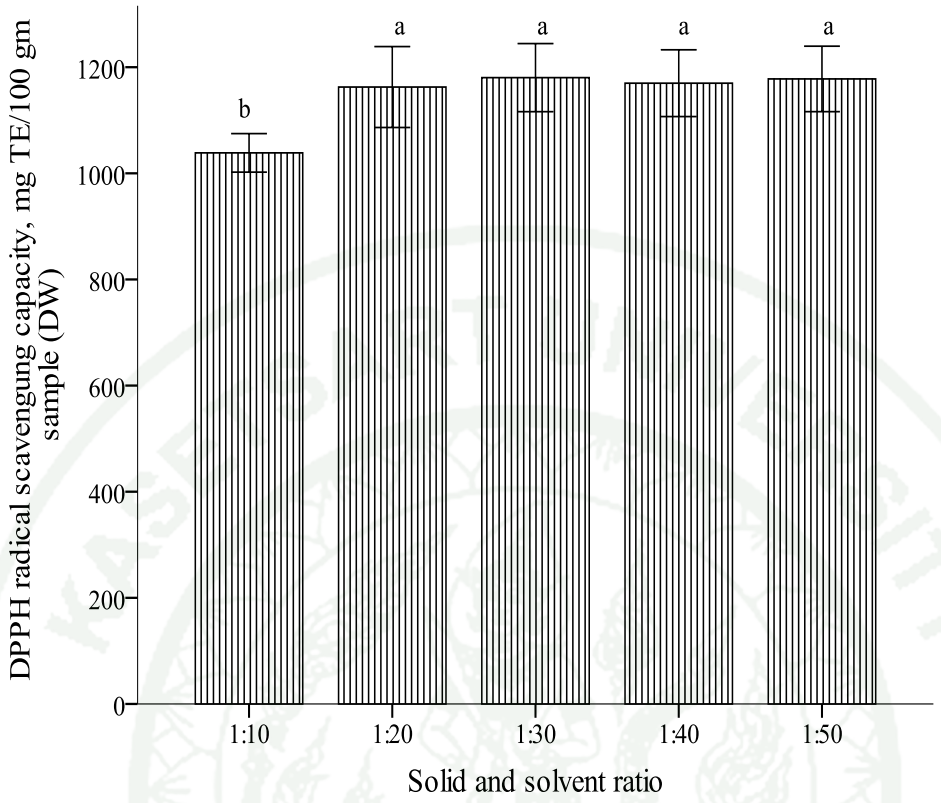
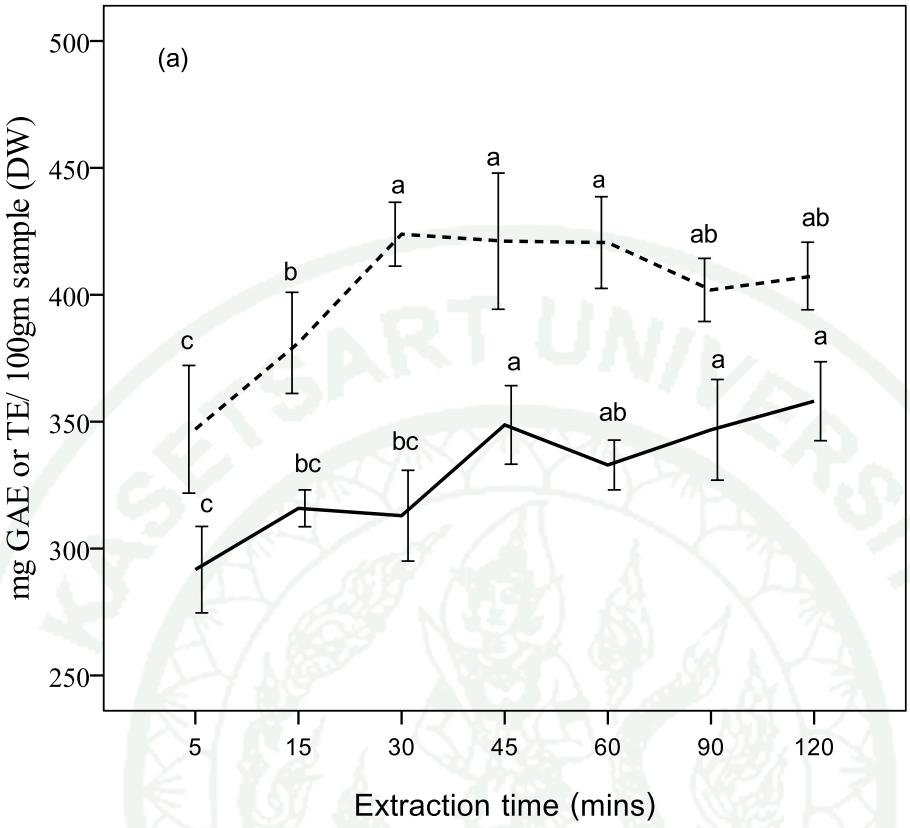


Figure 12 Effect of solid: solvent ratio (1:10 to 1:50) on DPPH radical scavenging capacity of extracts from greater cardamom seeds obtained by using 50% ethanol in a bath shaker at 75ºC for 8 hours.

Values are the mean ± standard deviation of at least 3 determinations from independent extractions. Different lower case letters means significantly ($P \leq 0.05$) different. The error bars represent the standard deviation.

1.5 Effect of extraction techniques

After selecting the optimum conditions for the water bath shaker extraction techniques (WBE), it was compared with other two extraction techniques namely; 1) ultrasound assisted extraction (USE, 45 kHz Frequency**)** at 40±5ºC and 2) maceration (MCN) at room temperature. Figure 13and 14 showed the effect of extraction time on total phenolic content and DPPH radical scavenging capacity of the extracts obtained by USE and MCN, respectively.

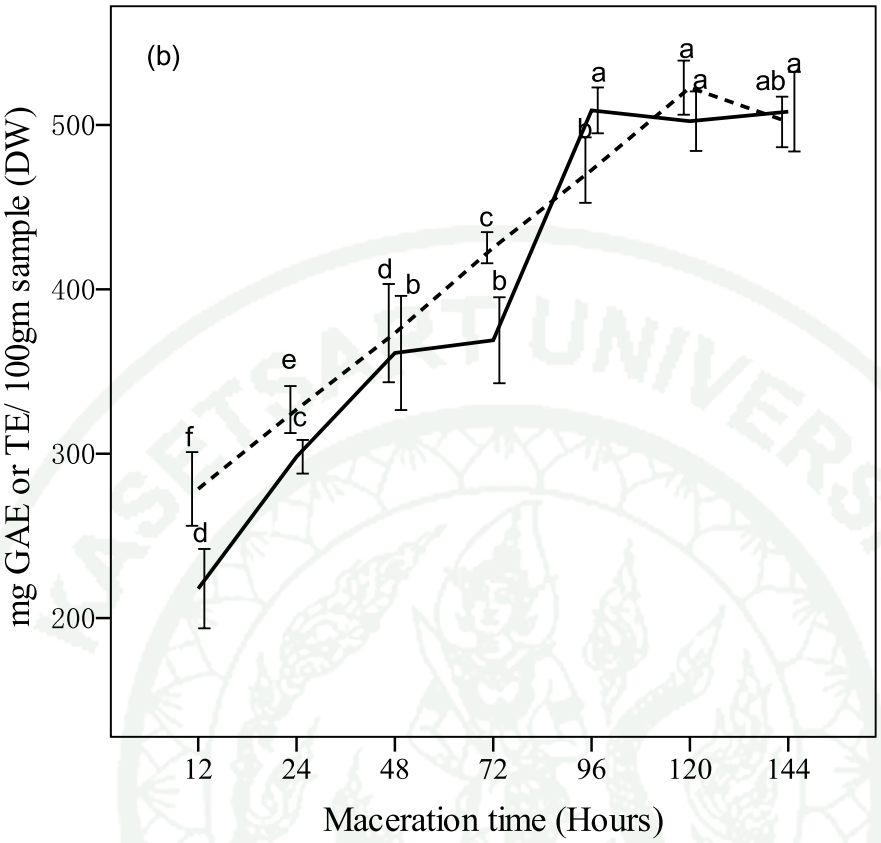


·····Total phenolic content, mg gallic acid equivalent (GAE)/100 gm sample (Dry Weight)

——DPPH radical scavenging capacity, mg trolox equivalent (TE)/100 gm sample (Dry Weight)

Figure 13 Changes in total phenolic content and DPPH radical scavenging capacity of extracts from greater cardamom seeds with extraction time during ultrasound assisted extraction (USE) at 40±5ºC.

Values are the mean ± standard deviation of at least 3 determinations from independent extractions. Values marked with the different lower case letters in each line are significantly ($P \leq 0.05$) different. The error bars represent the standard deviation.



..... Total phenolic content, mg gallic acid equivalent (GAE)/100 gm sample (Dry Weight)

—— DPPH radical Scavenging capacity, mg trolox equivalent (TE)/100 gm sample (Dry Weight)

Figure 14 Changes in total phenolic content and DPPH radical scavenging capacity of extracts from greater cardamom seeds with extraction time during maceration extraction (MCN) by soaking at room temperature

Values are the mean ± standard deviation of at least 3 determinations from independent extractions.

Values marked with the different lower case letters in each line are significantly ($P \leq 0.05$) different. Note: The error bars represent the standard deviation.

Overall, the experimental results showed that extraction time in all the extraction techniques had significant effect on both TPC, and DPPH radical scavenging capacity. Based on TPC and DPPH radical scavenging capacity, the optimum extraction time for USE and MCN were found to be 45 minutes and 120 hours respectively. The lowest optimum extraction time was for USE. The values of TPC and DPPH radical scavenging capacity at the optimum extraction time of each extraction technique was chosen for the comparison of these techniques

Table 2 Effect of different extraction techniques on total phenolic content (TPC) [1] and DPPH radical scavenging capacity[2] of the extracts from greater cardamom seeds.

Extraction Techniques	TPC	%RSD[3]	DPPH radical scavenging Capacity	%RSD[3]
WBE[4]	903.37±30.47a	3.37	1162.6±66.02a	5.68
USE[5]	421.12±26.83c	6.37	348.72±15.48c	4.44
MCN[6]	522.70±16.47b	3.15	502.29±18.11b	3.61

Values in each column marked by the different letters are significantly different at $P \leq 0.05$. Results showed mean ± SD

[1]Milligrams of Gallic acid equivalent (GAE) per 100 gm sample (cardamom seeds powder in dry weight basis, DW)

[2] Milligrams of trolox equivalent per 100 gm sample (DW),

[3] Relative standards deviation

[4]Water bath shaker extraction at 75ºC for 8 hours,

[5] ultrasound assisted extraction at 45 kHz frequency for 45 minutes,

[6]Maceration extraction at room temperature for 120 hours

The highest TPC and DPPH radical scavenging capacity of the extracts in 50% ethanol obtained by above mentioned techniques are shown in Table 2. There was significant effect ($p \leq 0.05$) of extraction techniques on both TPC and DPPH

radical scavenging capacity. The highest TPC and DPPH radical scavenging capacity were observed for SWE at 75ºC and the lowest for USE. The effectiveness of the extraction techniques based on the TPC and DPPH radical scavenging capacity were in the order of WBE at 75ºC for 8 hours > MCN at room temperature for 120 hours > USE at 40±5ºC and 45 kHz frequency for 45 minutes.

Among the techniques used, MCN at room temperature produced the extract with the second highest TPC and DPPH radical scavenging capacity but the optimum extraction time was of 120 hours which is very long time period and impractical to be used. For this technique, both TPC and DPPH radical scavenging capacity have been found to increase up to 120 hours of extraction time. However, the maximum values for TPC and DPPH radical scavenging capacity were lower than that of WBE at 75ºC. Furthermore, very long time period for extraction of phenolic antioxidants may lead to apparent degradation of the phenolic compounds and lower the actual level of extracted phenolic compounds and their antioxidant capacity. The process of degradation can be triggered by many factors such as light, together with air and temperature. The extraction temperature usually needs to be high in order to minimize the duration of the process (Mahugo Santana *et al.*, 2009). For these reasons, WBE at 75ºC in more effective and efficient as compared to MCN for the extraction of phenolic compounds from greater cardamom.

In our study, the optimum extraction time for USE at 40±5ºC was only 45 minutes which was the lowest among all other techniques used, but extract obtained from USE had lower TPC and DPPH radicals compared to SWE at 75ºC and MCN at room temperature. USE is one of the simplest extraction techniques that require laboratory equipment (*i.e.* ultrasonic bath) (Klejdus *et al.*, 2009) and considered to be an effective way to extract phytochemicals and phenolics from different matrices in shorter times than with other extraction techniques (Dobiáš *et al.*, 2010). However, USE was found to be less effective for the maximum recovery of phenolic antioxidants from greater cardamom.

1.6 Repeatability

The repeatability of solvent systems for TPC and DPPH radical scavenging capacity was performed by calculating the relative standards deviations (RSD %). RSD % lower than 10%, indicates good repeatability (Musa *et al*., 2011). All the solvents showed good repeatability for both TPC and DPPH radical scavenging capacity assays. RSD% of the solvent systems for TPC and DPPH radical scavenging capacity ranged from 5.22 to 9.57% and from 1.57 to 9.68 %, respectively. Table 1 shows that organic aqueous solvents mixture had lower RSD% for both assays as compared to pure solvents indicating the better repeatability of aqueous solvents. Musa *et al*. (2011) reported similar higher repeatability of the aqueous solvents as compare to the pure solvents for different antioxidant assays in the solvent extracts from pink-flesh guava. Similarly the RSD% of TPC and DPPH radical scavenging capacity at the optimum temperature, time and solid solvent ratio were calculated (data not shown in the results) and found to be below 10 indicating good repeatability.

The RSD% of the extraction techniques used for TPC ranged from 3.15 to 6.37% whereas for DPPH radical scavenging capacity it was in between 3.61% and 5.68%. MCN had the lowest RSD% for both TPC and DPPH radical scavenging capacity. As in the solvent systems, all extraction techniques used showed good repeatability since all RSD % were lower than 10% for both assays

1.7 Correlation between TPC and DPPH radical scavenging capacity

It is considered that the phenolic compounds contribute to overall antioxidant activities or radical scavenging activity of extracts (Sultana *et al.*, 2007). Regression and correlation analysis were performed to determine relationship between the total phenolic content and DPPH radical scavenging capacity as shown in Figure 15. A strong statistical correlation was found between TPC and DPPH radical scavenging capacity of the greater cardamom extracts obtained by using different extraction conditions with R^2 values of 0.9195. Therefore, phenolic compounds in greater cardamom may be the major contributor to their antioxidant capacity. The

results are in compliance with the result of Lu *et al.* (2011) who found the excellent linear correlation between the "total phenolic content" and antioxidant activity in several types of spices.

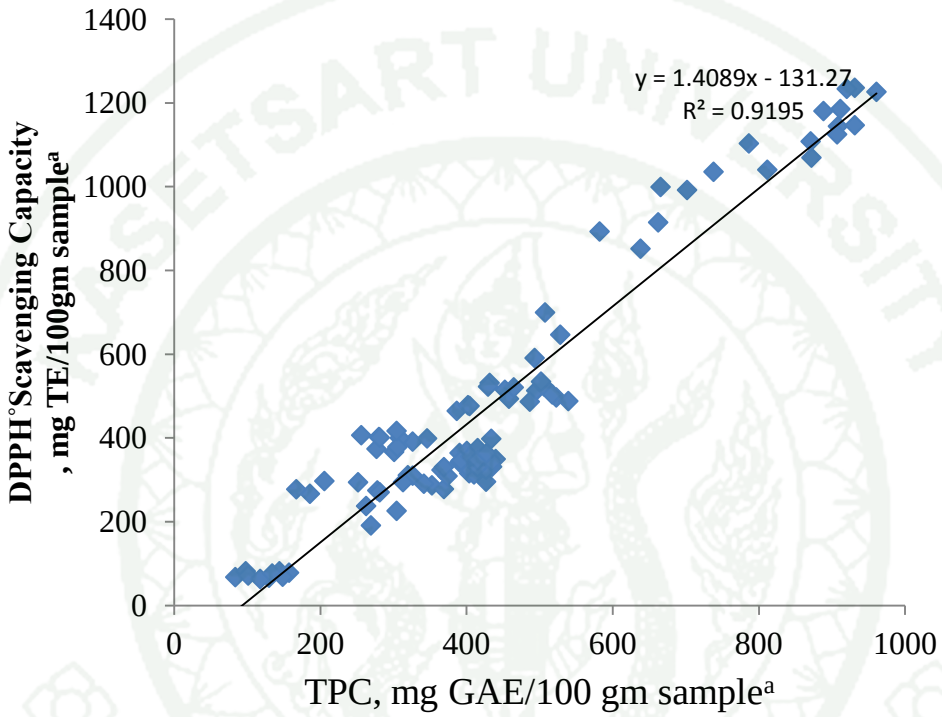


Figure 15 Correlation between (total phenolic content) TPC and DPPH radical scavenging activity of greater cardamom seed extracts obtained by using different extraction conditions

[a] Dry Weight (DW)

2. Antioxidant properties of the crude extracts from cardamom seeds

2.1 Extraction yield and antioxidants in the crude extracts from greater cardamom and siam cardamom seeds.

Table 3 shows extraction yields, total phenolic content, total flavonoids content, protocatechuic acid and protocatechuic aldehyde contents in ethanolic extracts from the greater cardamom and siam cardamom seeds. The results showed

that the extraction yield (dry weight basis) of extracts from greater cardamom seeds is higher than that of siam cardamom seeds. It is known that plant antioxidants are the phenolics constitute that forms one of the major groups of compounds acting as primary antioxidants or free radical terminators (Juntachote *et al.*, 2006). Therefore, it was reasonable to determine their content in the extracts. The antioxidants in the extracts from greater cardamom and siam cardamom seeds were determined by the contents of total phenolics and total flavonoids expressed as mg of gallic acid and quercetin, respectively, per gram of extract. Comparatively, total phenolic content was higher in the extract from siam cardamom seeds but total flavanoid content was higher in the extract from the greater cardamom seeds.

Table 3 Yields and antioxidants in crude extracts from greater cardamom seeds (GCE) and siam cardamom seeds (SCE)

Samples	Extraction yield (%)	TPC[1] ,GAE/ gm extract	TFC[2], mg QE/ gm extract	P. aldehyde[3] µg/gm extract	P. acid[4], µg /gm extract
GCE[5]	13.49±0.62	65.39±1.25	9.75±0.34	1114.24±1.76	334.64±3.36
SCE[6]	9.59±0.5	73.90±3.41	5.95±0.14	ND[7]	812.74±2.46

[1]Total Phenolic content (TPC)

[2]Total Flavonoid content (TFC)

[3]Protocatechuic aldehyde

[4] Protocatechuic acid

[5] Greater cardamom seeds extract (GCE)

[6] Siam cardamom seeds extract (SCE)

[7] Not detected (ND)

Among many peaks in HPLC chromatogram of crude extract from greater cardamom (Appendix Figure 3a and 4a), only two of them namely protocatechuic acid and proptocatechuic aldehyde could be identified and quantified. These two

compounds were identified for the first time as two of the major antioxidant compounds present in greater cardamom by Kikuzaki *et al.* (2001). Similarly, HPLC chromatogram of the extract from siam cardamom (Appendix Figure 3b and 4b) had also many peaks and one of them was identified to be protocatechuic acid. Other peaks could not be identified due to lack of standard compounds.

The ethanolic extract of the greater cardamom seeds was found to contain 334.64 µg of protocatechuic acid and 1114.24 µg of protocatechuic aldehyde per gm of extract respectively. Ethanolic extract from siam cardamom contained 812.74 µg of protocatechuic acid per gram extract. To date, no any research publication related to the antioxidant compounds in the siam cardamom seeds has been found.

2.2 Antioxidants capacity of the crude extracts from greater cardamom and siam cardamom seeds.

Table 4 Antioxidant capacities of crude extracts from greater cardamom seeds (GCE) and siam cardamom seeds (SCE) measured by FRAP, ABTS and DPPH assays.

Samples	Antioxidant capacity, mg trolox equivalent per gm dry extract		
	FRAP assay	ABTS assay	DPPH assay
GCE	125.52±1.19	55.85±2.67	87.05±1.24
SCE	121.82±2.55	65.15±1.77	104.61±2.27

FRAP: Ferric Reducing Antioxidant Power
ABTS: 2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
DPPH: 2,2-Diphenyl-1-picrylhydrazyl radical scavenging assays.

There are various methods and their modification available for evaluation of antioxidant capacity natural sources. Of these, antioxidant activity, reducing power, and DPPH radical scavenging activity are most widely used for determination of antioxidant activity of extracts (Chang *et al*., 2002). The reducing power of extracts

from greater cardamom and siam cardamoms was measured by FRAP assay and free radical scavenging capacity of these extracts was assessed by DPPH radical scavenging assay and ABTS assay and expressed as mg trolox equivalent per gm extract as presented in Table 4.

In FRAP assay, a solution containing Fe^{3+} -TPTZ complex in acetate is reduced to Fe^{2+} TPTZ which is blue in color and measured at 593 nm. The increase in absorbance at 593 nm is monitored to find out the reducing ability of the sample (Iris and Strain, 1996). DPPH and ABTS assays are commonly used for the determination of antioxidant capacity by measuring radical scavenging capacity of the antioxidants (Chang *et al*. 2002, Prior *et al*., 2005). DPPH radical is stable having a deep purple colored organic nitrogen-radical which is scavenged and transformed into DPPH-H by antioxidants changing the color from purple to yellow and the degree of change can be detected by the decrease in absorbance at 517 nm (Isono *et al.*, 2005). In ABTS assay, ABTS radical cation ($ABTS^{\bullet+}$) which has maximum absorbance at 734 nm is initially prepared by oxidation and antioxidants donate electrons to $ABTS^{\bullet+}$ to form ABTS that does not absorb at 734 nm leading to the decrease in absorbance.

Both, DPPH and ABTS values were higher in SCE but FRAP values were found to be almost equal in both extracts. Comparatively higher ABTS and DPPH radical scavenging capacity of siam cardamom seeds extract might be due to higher total phenolic content per gram extract as compared to greater cardamom seeds extract. However, free radical-scavenging activity is greatly influenced by the phenolic composition of the sample (Lee *et al.*, 2010).

3 Antioxidative effects of the cardamom crude extracts in pork patties

3.1 Antioxidant activity of the cardamom crude extracts on lipid oxidation in cooked pork patties

3.1.1 Changes in conjugated dienes of cooked pork patties during refrigerated storage

During lipid peroxidation after the peroxides are formed, the nonconjugated double bonds (C=C–C– C=C), existing naturally in unsaturated lipids, are converted to conjugated double bonds (C=C–C=C) and are measured spectophometrically at 232–234 nm (Kulås and Ackman, 2001). The conjugated dienes (CD) measured as absorbances at 233 nm in cooked pork patties treated with cardamom extracts and BHT as compared to negative control (without antioxidant) are shown in Table 4. For each day of analysis, CD of patties samples treated with GCE and SCE were found to decrease with increased concentration of the extracts and all the treatments except 0.01% GCE and SCE were able to significantly ($P \leq 0.05$) decrease the formation of conjugated dienes as compared to the control. The absorbance of CD increased in all treatments until day 6 and decreased thereafter.

Table 5 Absorbance at 233 nm of conjugated dienes in cooked pork patties treated with different levels of crude extracts from greater cardamom seeds (GCE) and siam cardamom seeds (SCE) and BHT during different period of storage at 4 ±1ºC for 9 days.

Treatments	Storage days			
	1	3	6	9
NC	$_{a}0.713\pm0.02^{z}$	$_{a}0.778\pm0.01^{y}$	$_{a}0.963\pm0.02^{w}$	$_{a}0.853\pm0.04^{x}$
GCE 0.01%	$_{b}0.651\pm0.04^{z}$	$_{ab}0.750\pm0.03^{y}$	$_{a}0.924\pm0.03^{w}$	$_{ab}0.831\pm0.02^{x}$
GCE 0.1%	$_{c}0.553\pm0.04^{z}$	$_{bc}0.700\pm0.05^{y}$	$_{bc}0.838\pm0.02^{w}$	$_{cd}0.772\pm0.03^{x}$
GCE 0.25%	$_{c}0.512\pm0.03^{z}$	$_{c}0.634\pm0.03^{y}$	$_{d}0.713\pm0.03^{x}$	$_{e}0.708\pm0.02^{x}$
SCE 0.01%	$_{a}0.714\pm0.04^{z}$	$_{a}0.775\pm0.02^{y}$	$_{a}0.946\pm0.04^{x}$	$_{abc}0.827\pm0.03^{y}$
SCE 0.1%	$_{b}0.627\pm0.04^{z}$	$_{ab}0.733\pm0.03^{y}$	$_{b}0.869\pm0.03^{x}$	$_{bcd}0.781\pm0.02^{y}$
SCE 0.25%	$_{c}0.534\pm0.03^{z}$	$_{c}0.657\pm0.02^{y}$	$_{c}0.786\pm0.02^{x}$	$_{de}0.729\pm0.05^{x}$
BHT 0.01%	$_{c}0.565\pm0.02^{z}$	$_{c}0.645\pm0.03^{y}$	$_{bc}0.829\pm0.04^{x}$	$_{bc}0.795\pm0.02^{x}$

NC: Negative control without antioxidants

^{abcde} Means with different letters within a column are significantly different at $P \leq 0.05$.

^{wxyz} Means with different letters within a row are significantly different at $P \leq 0.05$.

The results were in agreement with the previous researchers who reported that the CD increased until day 7 when it peaked and followed by a decrease thereafter for the refrigerated cooked pork patties treated with ethanolic extracts of kimchi (Lee *et al.,* 2011). Saha *et al.* (2010) also reported the increased in absorbance of CD values up to 6 days refrigerated storage of cooked chicken patties.

Similar trends of significant initial increment ($P \leq 0.05$) in CD followed by a decrease thereafter were observed in cooked pork containing holy basil and galangal (Juntachote *et al.,* 2006), and also in the samples containing whey and soy protein hydrolysates (Pena-Ramos & Xiong, 2003). The formation of conjugated dienes is related to the production of hydroperoxides and occurs in the early stages of lipid oxidation (Lee *et al.*, 2010). The CD hydroperoxides are relatively stable in the early reaction phase but are expected to decompose subsequently into secondary products (Kulås and Ackman, 2001) with increasing formation of TBARS and hexanal. Therefore, the decreases in CD were due to the fact that the decomposition rate of the hydroperoxides was higher than the formation rate of CD (Choe *et al.*, 2011).

3.1.2 Changes in thiobarbituric acid reacting substances (TBARS) of cooked pork patties during refrigerated storage

TBARS analysis measures the formation of secondary products of lipid oxidation, mainly malondialdehyde, which may contribute off flavor to meats and meat products. Table 6 shows the effect of different treatments on TBARS values of cooked pork patties during storage for 9 days at 4 °C. Both the treatments and storage period have significant effects ($P \leq 0.05$) on TBARS formation from lipid oxidation in the cooked pork patties. Among all the treatments on each day of analysis, negative control without antioxidants had the highest value of TBARS and all the treatments except 0.01% SCE were able to significantly ($P \leq 0.05$) decrease the formation of TBARS as compared to the negative control. Similarly, on each day of analysis, TBARS values of patties samples treated with greater cardamom crude extract (GCE) and siam cardamom crude extract (SCE) decreased with increased concentration of the extracts.

Table 6 Thiobarbituric acid reactive substances (TBARS**,** mg of malonaldehyde/kg) of cooked pork patties treated with different levels of crude extracts from greater cardamom seeds (GCE) and siam cardamom seeds (SCE) and BHT during different period of storage at 4 ±1ºC for 9 days.

Treatments	Days of Storage			
	1	3	6	9
NC	$_{a}0.861\pm0.06^{z}$	$_{a}1.014\pm0.08^{y}$	$_{a}1.36\pm0.04^{x}$	$_{a}1.523\pm0.06^{w}$
GCE 0.01%	$_{c}0.524\pm0.05^{z}$	$_{c}0.706\pm0.03^{y}$	$_{c}0.965\pm0.03^{x}$	$_{c}1.215\pm0.09^{w}$
GCE 0.1%	$_{f}0.186\pm0.03^{z}$	$_{e}0.241\pm0.02^{y}$	$_{e}0.293\pm0.016^{x}$	$_{f}0.349\pm0.03^{w}$
GCE 0.25%	$_{g}0.097\pm0.08^{z}$	$_{f}0.154\pm0.01^{y}$	$_{f}0.190\pm0.017^{x}$	$_{g}0.202\pm0.02^{x}$
SCE 0.01%	$_{b}0.714\pm0.03^{z}$	$_{b}0.871\pm0.04^{y}$	$_{a}1.339\pm0.06^{x}$	$_{b}1.407\pm0.05^{x}$
SCE 0.1%	$_{e}0.289\pm0.02^{z}$	$_{d}0.410\pm0.02^{y}$	$_{d}0.721\pm0.03^{x}$	$_{e}0.850\pm0.06^{w}$
SCE 0.25%	$_{f}0.176\pm0.01^{y}$	$_{ef}0.196\pm0.01_{xy}$	$_{f}0.209\pm0.01^{x}$	$_{g}0.223\pm0.02^{x}$
BHT 0.01%	$_{d}0.462\pm0.03_{z}$	$_{c}0.763\pm0.04^{y}$	$_{b}1.082\pm0.04^{x}$	$_{d}1.123\pm0.04^{x}$

NC: Negative control without antioxidants

^{abcde} Means with different letters within a column are significantly different at $P \leq 0.05$.

^{wxyz} Means with different letters within a row are significantly different at $P \leq 0.05$.

The TBARS value of 1.0 mg malondialdehyde per kg sample was found to threshold value beyond which the rancid flavor would be noticed (Jia *et al.*, 2012; Juntachote *et al.*, 2006). The results showed that all the treatments except control with no antioxidant have the TBARS values below 1.0 mg MDA/Kg up to the storage period of three days indicating the antioxidant capacity of the different antioxidant treatments to inhibit the lipid oxidation. However the extents of inhibition were vary for different treatments, lower for the samples treated with lower concentration of the antioxidants. On the 6th day of the storage period, the pork patties sample with 0.01% BHT, control with no antioxidant and sample with 0.01% SCE showed their TBARS values above the threshold limit. The TBARS values of the

patties samples with 0.1% and 0.25% GCE and 0.25% SCE were found to be below 1.0 mg MDA/Kg sample up to the storage period of 9 days.

The efficiency of the cardamom extracts and BHT in inhibiting lipid oxidation in cooked pork patties throughout refrigerated storage for 9 days was in the following order: greater cardamom seeds extract (GCE) 0.25% and siam cardamom seeds extract (SCE) 0.25% > GCE 0.1% and SCE 0.1% > commercial BHT 0.01% (fat weight basis). From the results, extension the shelf life of cooked pork patties by inhibiting the lipid oxidation would be possible by the addition of antioxidants coming from ethanolic extracts from both the greater cardamom and siam cardamom seeds.

3.2 Antioxidant activity of the cardamom crude extracts on protein oxidation and color deterioration in raw pork patties

3.2.1 Effect on protein oxidation in raw pork patties during refrigerated storage

In addition to loss in nutritional quality, the oxidation of meat proteins leads to changes in solubility and other protein functionality such as gelation and emulsifying properties, or water holding capacity (WHC) or textural traits (tenderness and juiciness) (Decker *et al.*, 1993; Lund *et al.*, 2011). Therefore, in this study, raw pork meat was selected for the study of protein oxidation.

Protein oxidation in raw pork patties samples was assessed by measuring the formation of carbonyls and followed for 9 days at refrigerated temperature (4±1) ºC. The effects of greater cardamom seeds extract (GCE, 0.1%), siam cardamom seeds extract (SCE, 0.1%) and BHT (0.01%) on formation of carbonyl as compared to negative control (without antioxidant) are shown in Figure 16. The carbonyl content in all the patties samples with different treatments was found to be significantly ($P \leq 0.05$) increased with storage time. The patties samples containing BHT showed a slight, but not significant ($P > 0.05$), decrease in the carbonyl content throughout the storage period compared to that of the control, while

the patties with GCE and SCE significantly ($P \leq 0.05$) retarded carbonyl formation compared to the control during the storage period of 6 and 9 days.

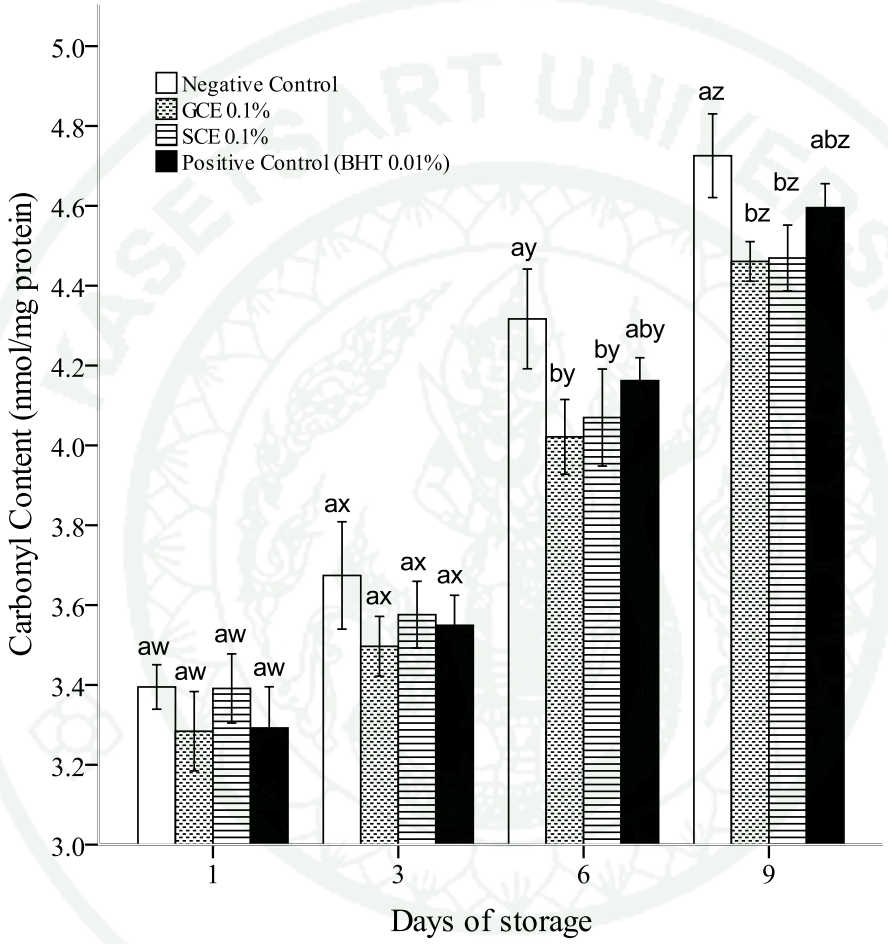


Figure 16 Effect of crude extracts from greater cardamom seeds (GCE, 0.1%) and siam cardamom seeds (SCE, 0.1%) and BHT (0.01% of fat) on the formation of carbonyl in raw pork patties stored for 9 days at 4±1°C.

Error bars refer to the standard deviations obtained from triplicate sample analysis.

$^{(a\text{-}b)}$Means (For the different treatments at the same day) with different letters indicate significant differences ($P \leq 0.05$).

^{wxyz} Means (For the same treatment at different days) with different letters indicate significant differences ($P \leq 0.05$).

The effect of the cardamom seeds extracts on the protein oxidation in raw meat samples was found to be moderate and inconsistent whereas a strong and more consistent effect on lipid oxidation was observed in cooked meat samples. The result is in compliance with Jia *et al.* (2012) who reported similar inconsistent and less intense inhibitory effect of black currant (*Ribes nigrum* L.) extract on protein oxidation as compared to lipid oxidation in raw pork patties. Vuorela *et al*. (2005) also reported that rapeseed and pine bark phenolics were effective antioxidants toward protein oxidation, although the level of inhibition was lower compared to that of lipid oxidation.

There are many researches that studied the effects of plant extracts on protein oxidation in muscle foods and have been shown to have inhibitory effect against protein oxidation (Jia *et al.*, 2012; Jongberg *et al.*, 2011; Vaithiyanathan *et al.*, 2011; Vuorela *et al.*, 2005). However, there are other similar researches with conflicting results which have shown no effect or even promoting effect in protein oxidation. Sun *et al*. (2010) found that apple polyphenols were ineffective against protein oxidation in both pork and beef sliced cooked hams. Similarly, the rosemary extract did not inhibit protein oxidation in beef patties and, instead, the ascorbate/citrate promoted protein oxidation (Lund *et al.*, 2007). The different chemical structure of phenolic compounds and the nature and conformation of the proteins might be attributed for the variable results (Estévez *et al.*, 2008).

3.2.2 Effect of cardamom crude extracts on color deterioration in raw pork patties during refrigerated storage

Color is a very important quality attributes involved in consumer perception of acceptable meat quality. Discoloration of fresh / raw meat is an indication of meat quality deterioration and may reduce consumer acceptance. Both the greater cardamom seeds extract (GCE) and siam cardamom seeds extract (SCE) have their own dark reddish and dark brownish color respectively. Therefore, in addition to 1, 3, 6 and 9 days, the color of the raw minced pork meat were also measured at 0 day (after sample preparation) to observe the impact of inherent color

in the extract. Changes in instrumental color values L*, a* and b* values of raw minced pork meat samples during refrigerated storage are depicted in Figures 17-19.

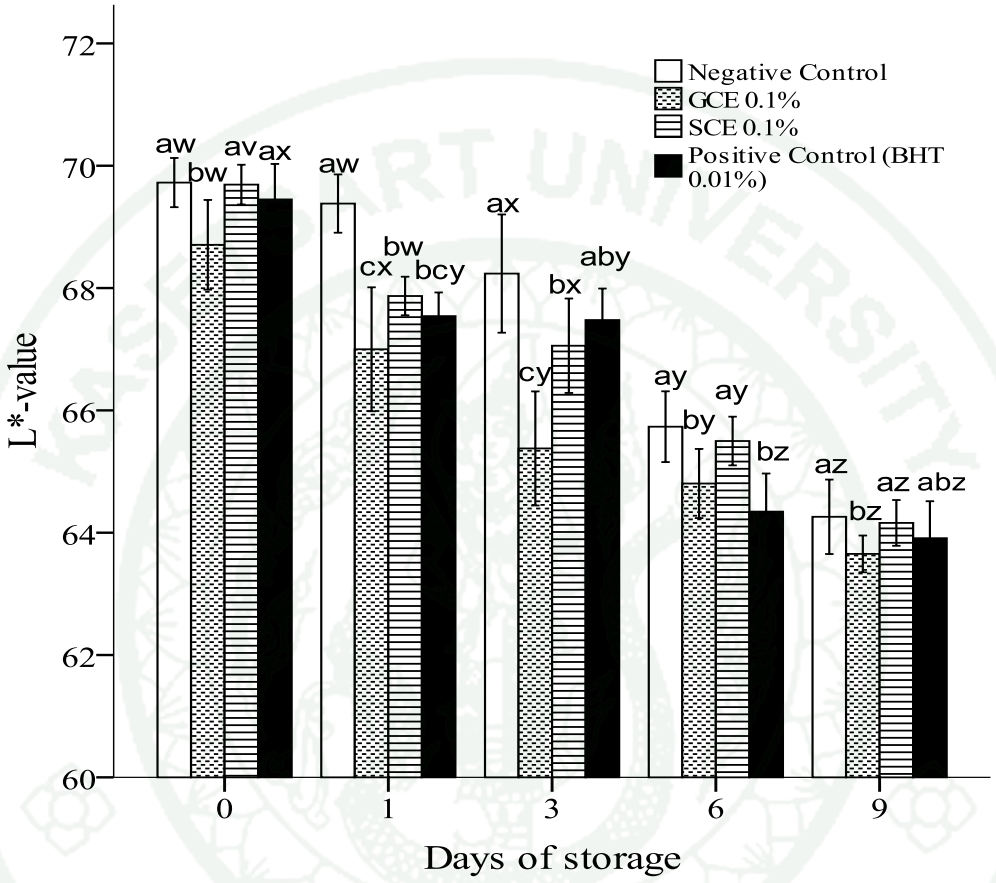


Figure 17 Effect of crude extracts from greater cardamom seeds (GCE, 0.1%) and siam cardamom seeds (SCE, 0.1%) and BHT (0.01% of fat) on instrumental color (L*-value) of raw pork patties stored for 9 days at 4±1°C.

Error bars refer to the standard deviations obtained from triplicate sample analysis.

[abc]Means (For the different treatments at the same day) with different letters indicate significant differences ($P \leq 0.05$).

[vwxyz] Means (For the same treatment at different days) with different letters indicate significant differences ($P \leq 0.05$).

As compared to negative control, the L* value (lightness) decreased significantly ($P \leq 0.05$) with the addition of the dark reddish colored greater cardamom extract (GCE. 0.1%) but siam cardamom extract (SCE. 0.1%) BHT (0.01% of fat) did not significantly ($P > 0.05$) alter L* (lightness) as shown in Figure 17. In a research by Choi *et al.* (2012), also reported the decreased lightness values upon addition of powdered *L. japonica* (1%, 3,%and 5%) in raw pork patties due to the brown and yellow pigments such as chlorophylls, phycophine, and xanthophylls present in the extract. Similar result of darkening effect was reported by Lee and Ahn (2005) on adding dark colored plum extract puree (3%) in turkey rolls.

The redness (a* values) also decreased ($P \leq 0.05$) with the addition of the dark reddish colored greater cardamom extract (GCE. 0.1%) as well as dark brownish colored siam cardamom extract (SCE. 0.1%) as compared to BHT (0.01% of fat) and negative control (without antioxidant). SCE treated sample had the lowest a* value as shown in Figure 18. Lee *et al.* (2010) noted the similar decrease in a* value in raw ground pork meat treated with darker mustard leaf (*Brassica juncea*) kimchi extracts (0.1% and 0.2%) due to the original darker color of mustard leaf kimchi and also found the similar trend of decreasing a* value with the storage time. In Contrast, Jia *et al*. (2012) reported the increased redness in pork patties because of dying effect of black currant (*Ribes nigrum* L.) extract rich anthocyanins but found the similar trend of decreasing a* value during the chilled storage of 9 days period.

The a* values of all the samples with different treatment decreased significantly ($P \leq 0.05$) with the storage time but the rate of decrease with storage time was different depending on the treatments. At 0 day, the redness of samples was in the order of BHT (0.01% of fat) > negative control > GCE (0.1%) > SCE (0.1%) with no significant ($P > 0.05$) difference between negative control and BHT treated samples. But after 9 days storage period, the redness order of the samples changed to be GCE (0.1%) > SCE (0.1%) > negative controls > BHT (0.01%) with no significant ($P> 0.05$) difference between GCE (0.1%) and SCE (0.1%) as well as SCE (0.1%) and negative control. The result showed that although SCE (0.1%) and GCE (0.1%) significantly reduced redness upon addition to raw pork patties, these extracts were

effective to slow down the rate of loss in redness during the refrigerated storage of 9 days.

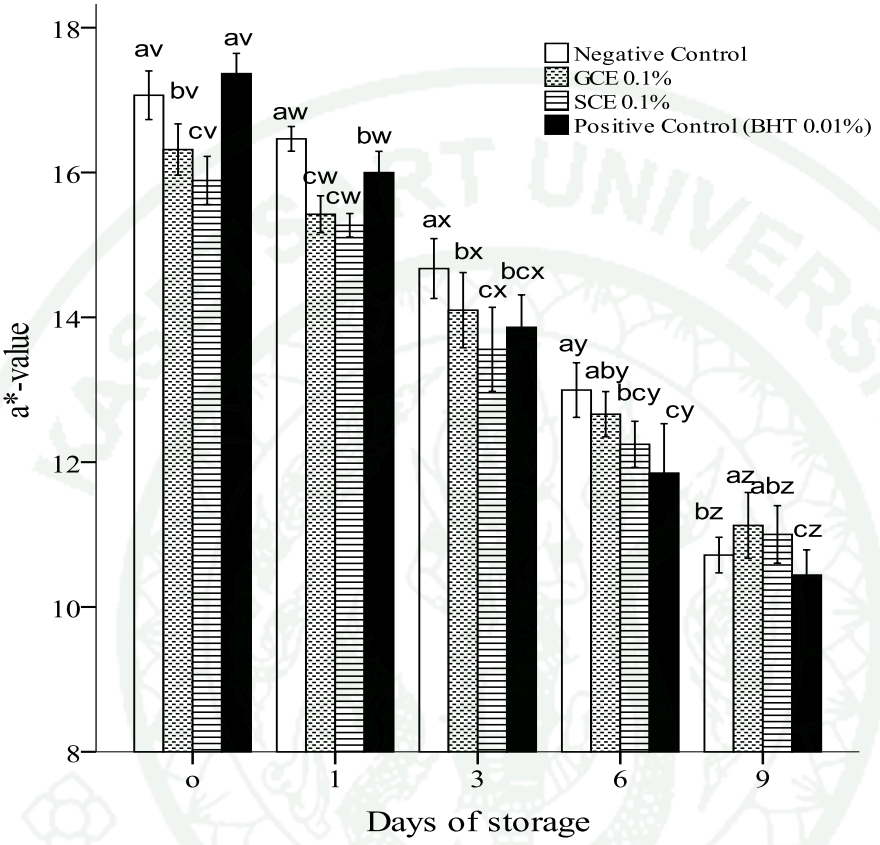


Figure 18 Effect of crude extracts from greater cardamom seeds (GCE, 0.1%) and siam cardamom seeds (SCE, 0.1%) and BHT (0.01%) on instrumental color (a*-value) of raw pork patties stored for 9 days at 4±1 °C.

Error bars refer to the standard deviations obtained from triplicate sample analysis.

[abc]Means (For the different treatments at the same day) with different letters indicate significant differences ($P \leq 0.05$).

[vwxyz] Means (For the same treatment at different days) with different letters indicate significant differences ($P \leq 0.05$).

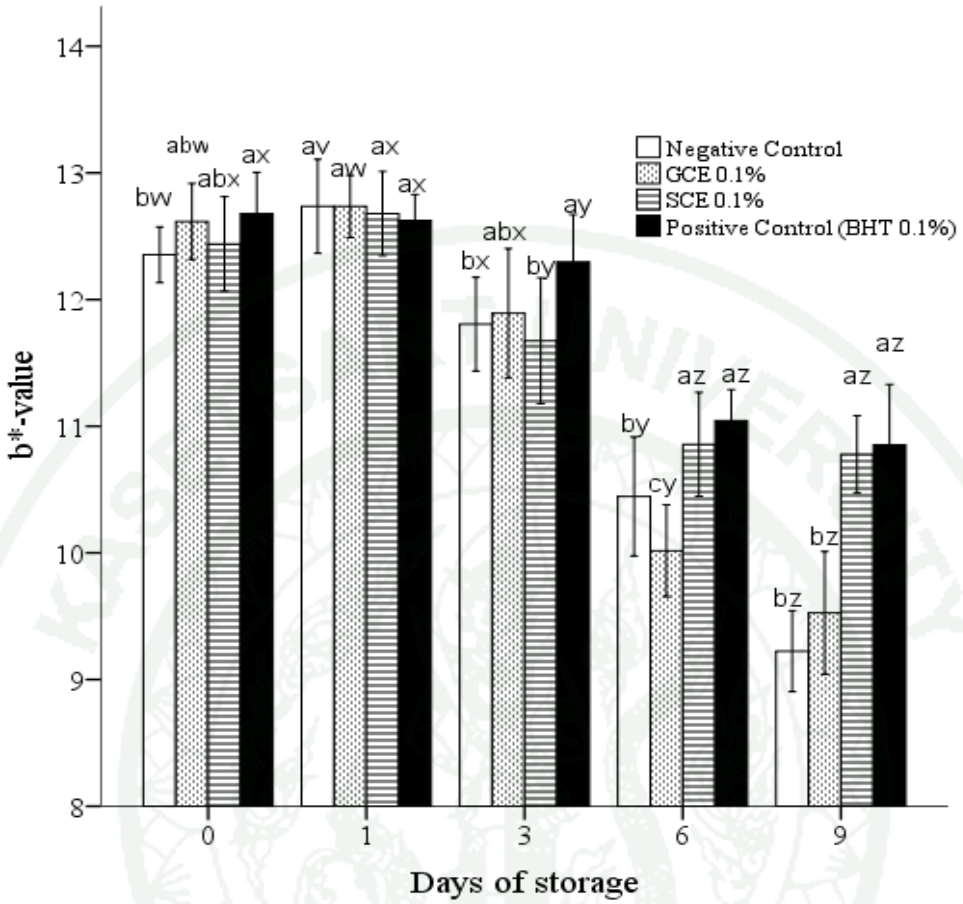


Figure 19 Effect of crude extracts from greater cardamom seeds (GCE, 0.1%) and siam cardamom seeds (SCE, 0.1%) and BHT (0.01%) on instrumental color (b*-value) of raw pork patties stored for 9 days at 4±1 °C.

Error bars refer to the standard deviations obtained from triplicate sample analysis.

[abc]Means (For the different treatments at the same day) with different letters indicate significant differences ($P \leq 0.05$).

[vwxyz] Means (For the same treatment at different days) with different letters indicate significant differences ($P \leq 0.05$).

Before storing under refrigerated condition or on 0 day of storage, the b* value (yellowness) of the samples treated with both GCE and SCE were not significantly different ($P > 0.05$) from that of negative control (without antioxidant) and BHT treated samples as shown in Figure 1**9.** During one day storage, there was

no any significant ($P > 0.05$) change in b* values of all samples except negative control as compared to 0 day. However, after one day storage, the storage time had significant ($P \leq 0.05$) effect on the b* values with decreasing trend. For one day storage, all the samples had b* values with no significant ($P > 0.05$) difference whereas b* values were significantly ($P \leq 0.05$) different on 9^{th} day of analysis. Lee *et al.* (2010) also reported the decreasing trend in yellowness during refrigerated storage of raw ground pork meat treated with mustard leaf (*Brassica juncea*) kimchi extracts.

The bright red color of meat is mainly due to presence of myoglobin protein. Oxidation of myoglobin is most significant factor affecting color stability and heme and non-heme ions released from disrupted cell membrane during mincing/grinding serve as oxidation catalysts ⁄ promoters (Yu *et al.*, 2010). On the other hand, increased levels of lipid oxidation had also been reported to promote oxidation of oxymyoglobin (Moroney *et al.*, 2013;O'grady *et al.*, 2001) thereby resulting in decrease redness. Therefore, lowering color deterioration rate by GCE and SCE during the refrigerated storage may be attributed to their potent antioxidant activity. The phenolic compounds present in GCE and SCE may retard the oxidation of oxymyoglobin by inhibiting the lipid oxidation. Phenolic compounds might also act as metal-chelating agents that form the stable complex with heme and non-heme ions rendering them unavailable for catalysis of oxidation reactions (Yu *et al.*, 2010).

CONCLUSION AND RECOMMENDATION

Conclusion

Based on total phenolic content (TPC) and DPPH radical scavenging capacity, water bath shaker extraction using 50 % ethanol with solid: solvent ratio of 1:20 at temperature of 75ºC for 8 hours of extraction time were selected to be optimum conditions required for extraction of antioxidants from greater cardamom *(Amomum subulatum* Roxb.). Therefore, it can be believed that the antioxidant compounds in greater cardamom might be intermediately polar and thermally stable.

Both the crude extracts of greater cardamom and siam cardamom seeds obtained by using the optimized conditions showed strong and comparable antioxidant properties. The antioxidant properties were measured by chemical assays such as total phenolic content (TPC), total flavonoid content (TFC), ferric reducing antioxidant power (FRAP), 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2'-diphenyl-1-picrylhydrazyl (DPPH). Similar results were obtained when these extracts were applied in cooked pork patties. Both the extracts at concentration of 0.1% were more effective than butylated hydroxy tolune (BHT), 0.01% (fat weight basis) for retarding the formation of thiobarbituric acid reactive substances (TBARS) from lipid oxidation in the pork patties up to 9 days storage period at 4±1ºC. Similarly, these cardamom extracts also retarded the protein oxidation and slowed down the red color deterioration in raw pork patties.

Recommendation

Based on these findings, the natural antioxidants examined may be recommended to be used in the development of synthetic antioxidant free meat products with extended shelf life. Both of the cardamom extracts slowed down the deterioration of red color of the raw pork patties during refrigerated storage. However, the inherent color of the cardamom extracts significantly decreased the redness upon addition to the raw pork patties. Furthermore, other sensory effects of the cardamom extracts have not been covered in this study. Therefore, further study is needed to purify the extracts so as to make it more effective and acceptable.

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APPENDIX

Methods of Analysis

1. Determination of moisture in spices (AOAC, 2006 Official Method 986.21)

1.1 Apparatus

Moisture distillation apparatus that consists of a 500 ml short neck round bottom flask and provided with a reflux condenser discharging into a trap connected to the flask. The trap serves to collect and measure the condensed water and to return the condensed solvent to the flask and a copper wire long enough to extend throughout the condenser with one end twisted into a spiral. The diameter of the spiral should be such that it fits snugly within the graduated portion of the receiver and yet it can be moved up and down

1.2 Reagents

Toluene

1.3 Procedure

1.3.1 Clean the entire apparatus with chromic acid cleaning solution to minimize adherence of water droplets to the sides of the condenser and the receiver.

1.3.2 Rinse thoroughly with water and dry completely before use.

1.3.3 Place 40 g spice (or enough to yield 2–5 mL H_2O) in distilling flask. Add enough solvent to cover test portion completely (never <75 mL).

1.3.4 Fill receiving tube with solvent, pouring through top of condenser.

1.3.5 Insert loose cotton plug in top of con denser to prevent condensation of atmospheric moisture in tube.

1.3.6 Bring to boil and distil slowly, about 2 drops/s, until most of H_2O distils over; then increase rate of distillation to about 4 drops/s.

1.3.7 Continue distilling until 2 consecutive readings 15 minutes apart show no change.

1.3.8 Dislodge any H_2O held up in condenser with brush or wire loop. Rinse condenser carefully with 5 mL toluene. Continue distillation 3 to 5 min.

1.3.9 Cool receiver to room temperature (about 25°C), allowing it to stand in air or immersing it in H_2O. Solvent and H_2O layers should now be clear; if not, let stand until clearing occurs.

1.3.10 Read volume of H_2O, estimating to nearest 0.01 mL, and calculate to percent.

Moisture Content (% by weight) = 100 V/M

Where,

V = Volume in ml of water collected,

M = Weight of sample

2. Determination of fat (crude) in meat sample by solvent extraction (submersion) method (AOAC, 2006 Official Method 991.36)

2.1 Apparatus

2.1.1 Extraction system (Soxtec System, Analytical/Tecator, Inc)

2.1.2 Thimbles and stand (26 X 60 mm, cellulose thimbles, and stand to hold thimbles).

2.1.3 Extraction cups (A1, 44 id, 60 mm height).

2.1.4Glass beads (3–4 mm diameter).

2.1.5 Mechanical convection oven (maintaining 125° ± 1°C).

2.2 Reagents

2.2.1 Petroleum ether.

2.2.2 Sand (<0.004 g extractable/5 g).

2.2.3 Cotton (defatted).

2.3 Procedure

2.3.1 Accurately weigh about 3 gm test portion into thimble and add sand to test portion and mix with glass rod.

2.3.2 Place thimble in thimble stand and dry 1 h in 125°C oven. Remove from oven and let cool.

2.3.3 Loosen test portion/sand mixture using glass rod and wipe glass rod with small amount of cotton and place cotton in top of thimble.

2.3.4 Transfer thimble to extraction unit.

2.3.5 Accurately weigh extraction cup containing a few glass beads.

2.3.6 Extract thimble with dried mixture with 40 mL petroleum ether in boiling position for 25 min and in rinsing position for 30 min.

2.3.7 Adjust temperature of extraction unit to ensure condensation rate 5 drops/s. At completion of extraction, close condenser valves and recover ether.

2.3.8 Dry cup and contents 30 min in 125°C oven. Cool and weigh.

2.4 Calculations

Calculate percent fat in test sample as follows:

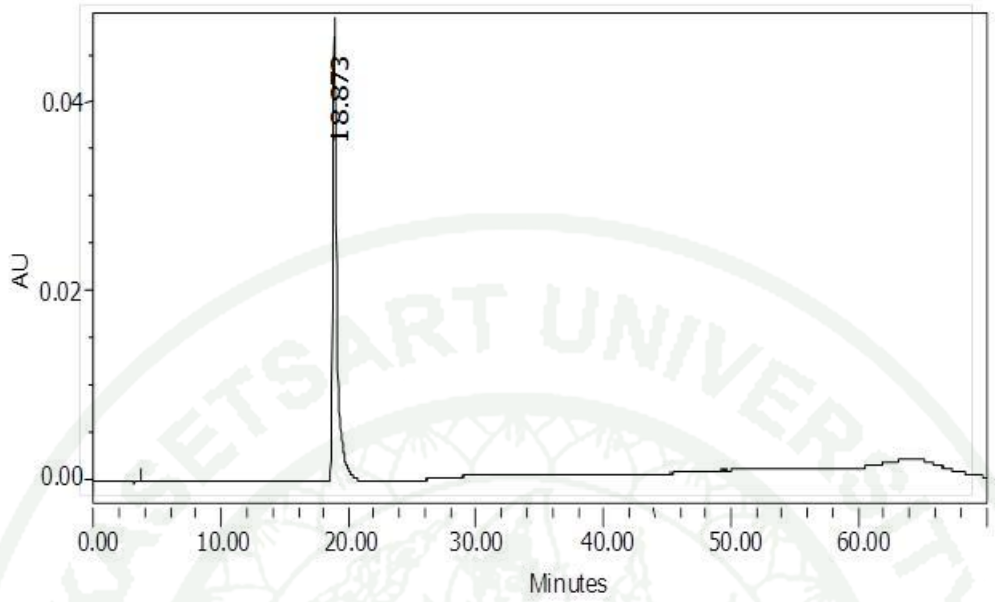
Fat content, % = (B-C) x 100/A

Where,

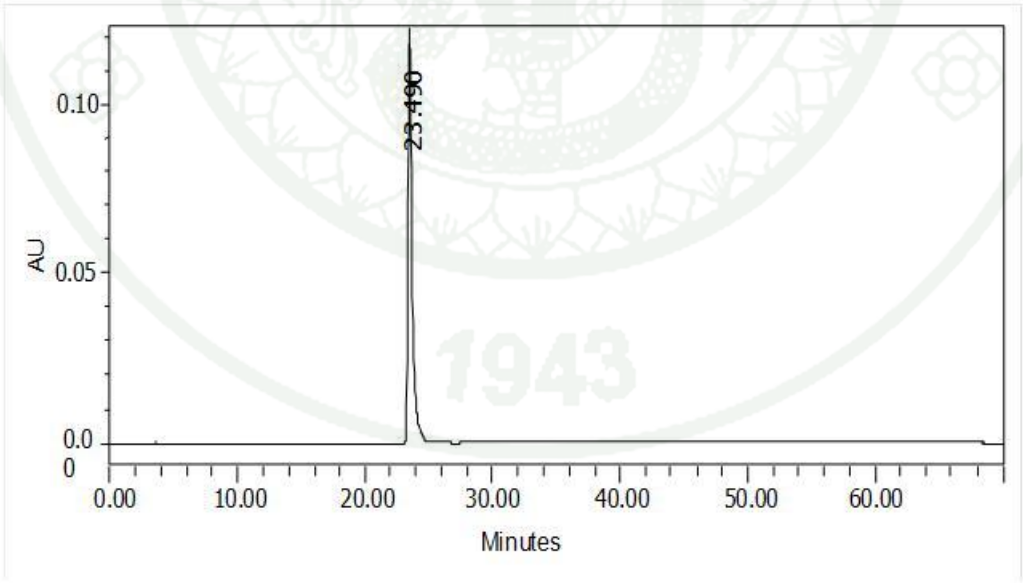
A = g test portion weight,

B = g weight of extraction cup after drying and

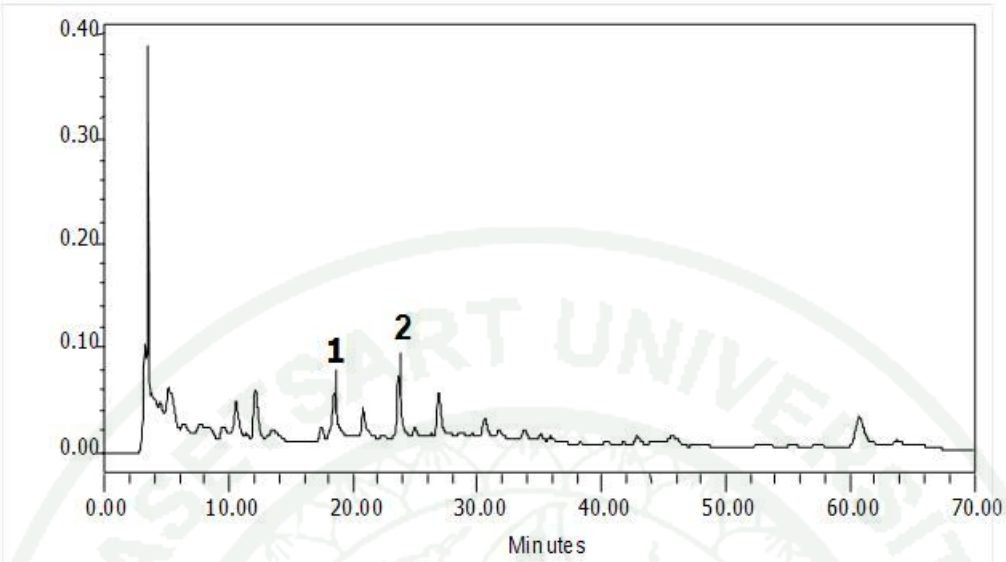
C = g weight of extraction cup prior to extraction.



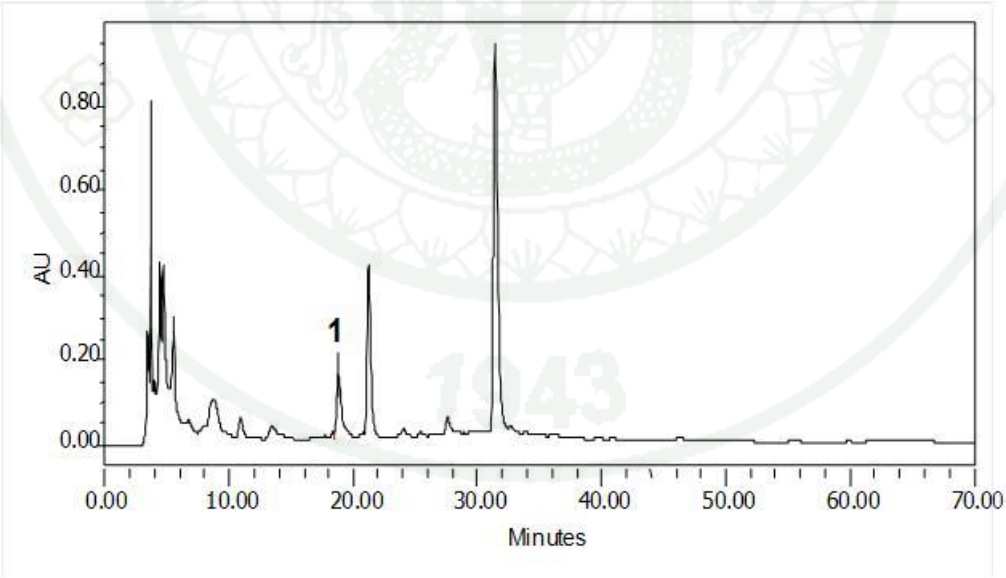
Appendix Figure 1 HPLC Chromatogram of standard protocatechuic acid at 259 nm.



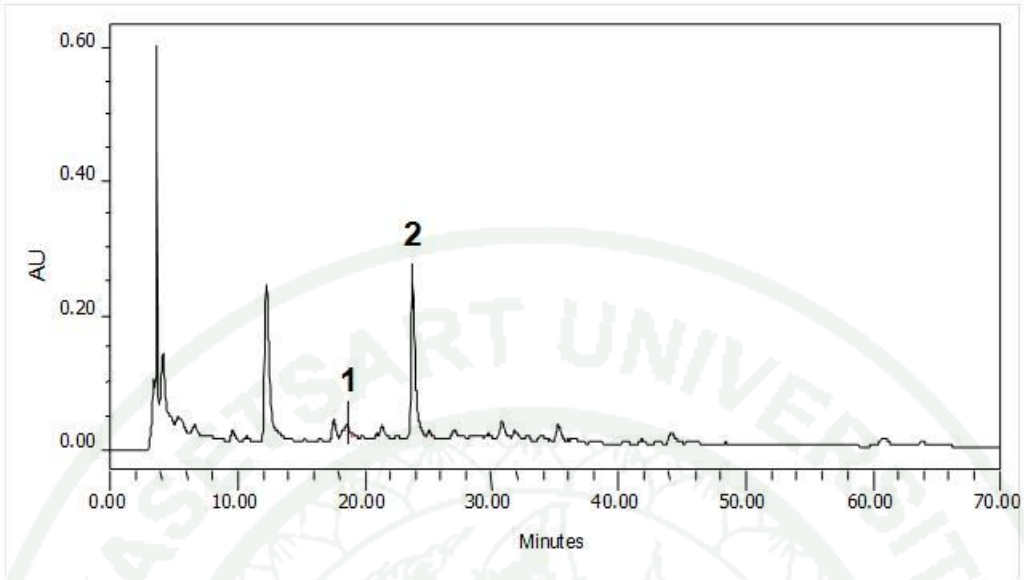
Appendix Figure 2 HPLC Chromatogram of standard protocatechuic aldehyde at 280nm.



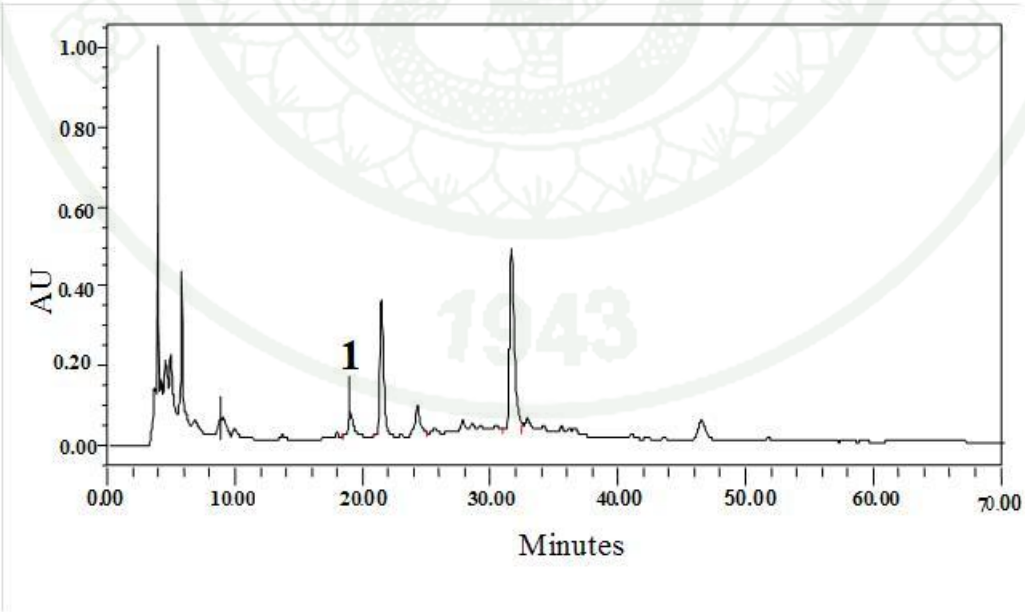
Appendix Figure 3 HPLC Chromatogram of crude extract from greater cardamom *(Amomum subulatum* Roxb.) seeds at 259 nm. Identification of protocatechuic acid (1) and protocatechuic aldehyde (2)



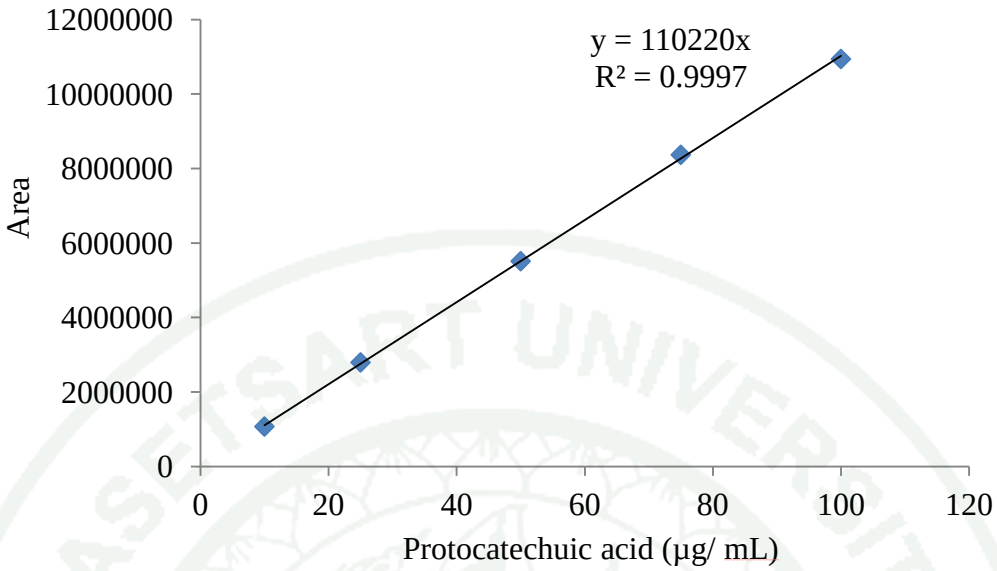
Appendix Figure 4 HPLC Chromatogram of crude extract from siam cardamom (*Amomum krevanh* Pierre) seeds at 259 nm. Identification of protocatechuic acid (1)



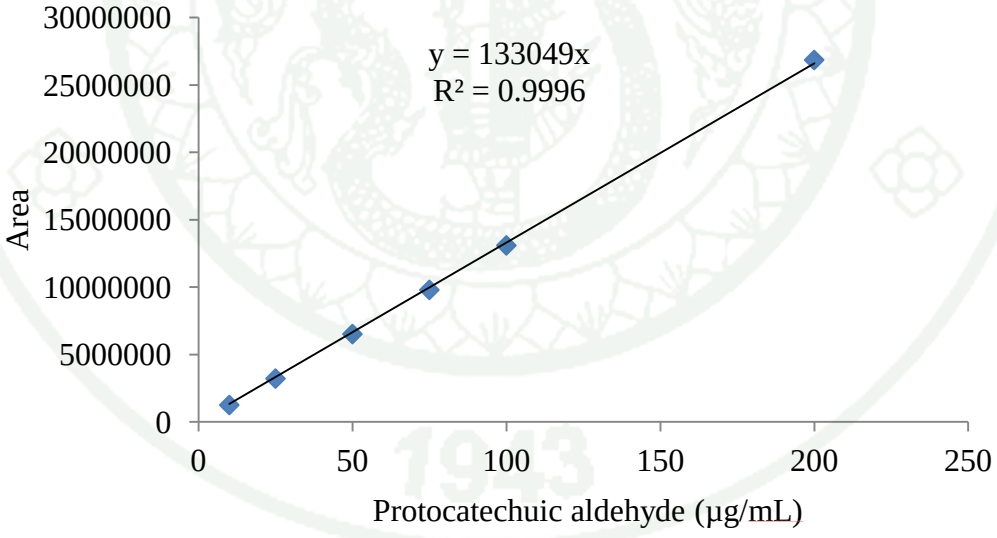
Appendix Figure 5 HPLC Chromatogram of crude extracts from a) greater cardam (*Amomum subulatum* Roxb.) seeds at 280 nm. Identification of protocatechuic acid (1) and protocatechuic aldehyde (2).



Appendix Figure 6 HPLC Chromatogram of crude extracts from siam cardamom (*Amomum krevanh* Pierre) seeds at 280 nm. Identification of protocatechuic acid (1).



Appendix Figure 7 Standard curve of protocatechuic acid.



Appendix Figure 8 Standard curve of protocatechuic aldehyde.

Appendix Table 1 ANOVA for comparison of absorbances of conjugated dienes at 233 nm of cooked pork patties treated with different levels of crude extracts from greater cardamom seeds and siam cardamom seeds as well as BHT (0.01% of fat) during storage period of 1 day, 3 days, 6 days and 9 days.

Days of storage		Sum of Squares	df	Mean Square	F	Sig.
1 Day	Between Groups	0.132	7	0.019	17.18	0.00
	Within Groups	0.018	16	0.001		
	Total	0.15	23			
3 Days	Between Groups	0.072	7	0.010	11.82	0.00
	Within Groups	0.014	16	0.001		
	Total	0.086	23			
6 Days	Between Groups	0.151	7	0.022	22.73	0.00
	Within Groups	0.015	16	0.001		
	Total	0.166	23			
9 Days	Between Groups	0.053	7	0.008	8.18	0.00
	Within Groups	0.015	16	0.001		
	Total	0.068	23			

Appendix Table 2 Duncan test for comparison of absorbances of conjugated dienes at 233 nm of cooked pork patties treated with different levels of crude extracts from cardamom seeds and BHT (0.01% of fat) during storage period of 1 day.

Treatments	N	Subset for alpha = 0.05		
		1	2	3
GCE0.25%	3	0.512		
SCE0.25%	3	0.53367		
GCE0.1%	3	0.553		
BHT0.01%	3	0.56533		
SCE0.1%	3		0.62667	
GCE0.01%	3		0.65067	
NC	3			0.713
SCE0.01%	3			0.714
Sig.		0.087	0.388	0.971

Means for groups in homogeneous subsets are displayed.

Harmonic Mean Sample Size = 3.000.

GCE: Greater cardamom seeds extract

SCE: Siam cardamom seeds extract

NC: Negative control without antioxidant

BHT: Butylated hydroxytoluene

Appendix Table 3 Duncan test for comparison of absorbances of conjugated dienes at 233 nm of cooked pork patties treated with different levels of crude extracts from cardamom seeds and BHT (0.01% of fat) during storage period of 3 days.

		Subset for alpha = 0.05			
Treatments	N	1	2	3	4
GCE0.25%	3	0.63367			
BHT0.01%	3	0.64467			
SCE0.25%	3	0.65733	0.65733		
GCE0.1%	3		0.70000	0.70000	
SCE0.1%	3			0.73267	0.73267
GCE0.01%	3			0.74967	0.74967
SCE0.01%	3				0.77533
NC	3				0.77767
Sig.		0.365	0.095	0.067	0.103

Means for groups in homogeneous subsets are displayed.

Harmonic Mean Sample Size = 3.000.

GCE: Greater cardamom seeds extract

SCE: Siam cardamom seeds extract

NC: Negative control without antioxidant

BHT: Butylated hydroxytoluene

Appendix Table 4 Duncan test for comparison of absorbances of conjugated dienes at 233 nm of cooked pork patties treated with different levels of crude extracts from cardamom seeds and BHT (0.01% of fat) during storage period of 6 days.

Treatments	N	Subset for alpha = 0.05			
		1	2	3	4
GCE0.25%	3	.71333			
SCE0.25%	3		.78633		
BHT0.01%	3		.82933	.82933	
GCE0.1%	3		.83833	.83833	
SCE0.1%	3			.86933	
GCE0.01%	3				.92433
SCE0.01%	3				.94567
NC	3				.96233
Sig.		1.000	.067	.151	.171

Means for groups in homogeneous subsets are displayed.

Harmonic Mean Sample Size = 3.000.

GCE: Greater cardamom seeds extract

SCE: Siam cardamom seeds extract

NC: Negative control without antioxidant

BHT: Butylated hydroxytoluene

Appendix Table 5 Duncan test for comparison of absorbances of conjugated dienes at 233 nm of cooked pork patties treated with different levels of crude extracts from cardamom seeds and BHT (0.01% of fat) during storage period of 9 days.

		Subset for alpha = 0.05				
Treatments	N	1	2	3	4	5
GCE0.25%	3	.7083				
SCE0.25%	3	.7293	.7293			
GCE0.1%	3		.7723	.7723		
SCE0.1%	3		.7810	.7810	.7810	
BHT0.01%	3			.7953	.7953	
SCE0.01%	3			.8277	.8277	.8277
GCE0.01%	3				.8313	.8313
NC	3					.8530
Sig.		.412	.066	.056	.080	.350

Means for groups in homogeneous subsets are displayed.

Harmonic Mean Sample Size = 3.000.

GCE: Greater cardamom seeds extract

SCE: Siam cardamom seeds extract

NC: Negative control without antioxidant

BHT: Butylated hydroxytoluene

Appendix Table 6 ANOVA for comparison of thiobarbituric acid reactive substances (TBARS, mg MDA/ kg) of cooked pork patties treated with different levels of crude extracts from cardamom seeds and BHT (0.01% of fat) during storage period of 1 day, 3 days, 6 days and 9 days .

Days of storage		Sum of Squares	df	Mean Square	F	Sig.
1 day	Between Groups	1.587	7	.227	182.680	.000
	Within Groups	.020	16	.001		
	Total	1.607	23			
3 days	Between Groups	2.354	7	.336	223.283	.000
	Within Groups	.024	16	.002		
	Total	2.378	23			
6 days	Between Groups	5.074	7	.725	566.901	.000
	Within Groups	.020	16	.001		
	Total	5.094	23			
9 days	Between Groups	6.118	7	.874	321.558	.000
	Within Groups	.043	16	.003		
	Total	6.162	23			

Appendix Table 7 Duncan test for comparison of thiobarbituric acid reactive substances (TBARS, mg MDA/ kg) of cooked pork patties treated with different levels of crude extracts from cardamom seeds and BHT (0.01% of fat) during storage period of 1 day.

Treatments	N	Subset for alpha = 0.05						
		1	2	3	4	5	6	7
GCE 0.25%	3	0.096						
SCE0.25%	3		0.175					
GCE 0.1%	3		0.186					
SCE 0.1%	3			0.288				
BHT 0.01%	3				0.462			
GCE 0.01%	3					0.525		
SCE0.01%	3						0.714	
Negative control	3							0.861
Sig.		1	0.713	1	1	1	1	1

Means for groups in homogeneous subsets are displayed.

Harmonic Mean Sample Size = 3.000.

GCE: Greater cardamom seeds extract

SCE: Siam cardamom seeds extract

NC: Negative control without antioxidant

BHT: Butylated hydroxytoluene

Appendix Table 8 Duncan test for comparison of thiobarbituric acid reactive substances (TBARS, mg MDA/ kg) of cooked pork patties treated with different levels of crude extracts from cardamom seeds and BHT (0.01% of fat) during storage period of 3 days

Treatments	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
GCE 0.25%	3	0.1539					
SCE0.25%	3	0.1963	0.1963				
GCE 0.1%	3		0.2409				
SCE 0.1%	3			0.4095			
GCE 0.01%1	3				0.706		
BHT 0.01%	3				0.7629		
SCE0.01%	3					0.871	
Negative control	3						1.014
Sig.		0.2	0.178	1	0.091	1	1

Means for groups in homogeneous subsets are displayed.

Harmonic Mean Sample Size = 3.000.

GCE: Greater cardamom seeds extract

SCE: Siam cardamom seeds extract

NC: Negative control without antioxidant

BHT: Butylated hydroxytoluene

Appendix Table 9 Duncan test for comparison of thiobarbituric acid reactive substances (TBARS, mg MDA/ kg) of cooked pork patties treated with different levels of crude extracts from cardamom seeds and BHT (0.01% of fat) during storage period of 6 days.

Treatments	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
	3	0.1902					
SCE0.25%	3	0.2094					
GCE 0.1%	3		0.2933				
SCE 0.1%	3			0.7206			
GCE 0.01%1	3				0.9647		
BHT 0.01%	3					1.082	
SCE0.01%	3						1.347
Negative control	3						1.356
Sig.		0.519	1	1	1	1	0.756

Means for groups in homogeneous subsets are displayed.

Harmonic Mean Sample Size = 3.000.

GCE: Greater cardamom seeds extract

SCE: Siam cardamom seeds extract

NC: Negative control without antioxidant

BHT: Butylated hydroxytoluene

Appendix Table 10 ANOVA for comparison of carbonyl content (nmol/mg protein) of raw pork patties treated with 0.1% (w/w) of crude extracts from greater cardamom seeds (GCE) and siam cardamom seeds (SCE) and BHT (0.01% of fat) during storage period of 1 day, 3 days, 6 days and 9 days.

Days of storage		Sum of Squares	df	Mean Square	F	Sig.
1 Day	Between Groups	.033	3	.011	1.421	.306
	Within Groups	.062	8	.008		
	Total	.095	11			
3 Days	Between Groups	.050	3	.017	1.830	.220
	Within Groups	.073	8	.009		
	Total	.123	11			
6 Days	Between Groups	.152	3	.051	4.784	.034
	Within Groups	.085	8	.011		
	Total	.237	11			
9 Days	Between Groups	.140	3	.047	7.823	.009
	Within Groups	.048	8	.006		
	Total	.188	11			

Appendix Table 11 Duncan test for comparison of Carbonyl content (nmol/mg protein) of raw pork patties treated with 0.1% (w/w) of crude extracts from greater cardamom seeds (GCE) and siam cardamom seeds (SCE) and BHT (0.01% of fat) during storage period of 6 days

Treatments	N	Subset for alpha = 0.05	
		1	2
GCE 0.1%	3	4.0214	
SCE 0.1%	3	4.0698	
BHT 0.01%	3	4.1622	4.1622
Negative Control	3		4.3168
Sig.		.147	.103

Means for groups in homogeneous subsets are displayed.

Appendix Table 12 Duncan test for comparison of carbonyl content (nmol/mg protein) of raw pork patties treated with 0.1% (w/w) of crude extracts from greater cardamom seeds (GCE) and siam cardamom seeds (SCE) and BHT (0.01% of fat) during storage period of 9 days.

Treatments	N	Subset for alpha = 0.05	
		1	2
GCE 0.1%	3	4.4608	
SCE 0.1%	3	4.4693	
BHT 0.01%	3	4.5955	4.5955
Negative Control	3		4.7255
Sig.		0.075	0.073

Means for groups in homogeneous subsets are displayed. GCE: Greater cardamom seeds extract, SCE: Siam cardamom seeds extract, NC: Negative control without antioxidant, BHT: Butylated hydroxytoluene

Appendix Table 13 ANOVA for comparison of redness (a* values) of raw pork patties treated with 0.1% (w/w) of crude extracts from greater cardamom seeds (GCE) and siam cardamom (SCE) seeds and BHT (0.01% of fat) during storage period of 0 day, 1 day, 3 days, 6 days and 9 days.

Days of storage		Sum of Squares	df	Mean Square	F	Sig.
0 day	Between Groups	12.364	3	4.121	38.612	.000
	Within Groups	3.416	32	.107		
	Total	15.780	35			
1 day	Between Groups	8.120	3	2.707	52.339	.000
	Within Groups	1.655	32	.052		
	Total	9.775	35			
3days	Between Groups	6.016	3	2.005	8.208	.000
	Within Groups	7.819	32	.244		
	Total	13.835	35			
6days	Between Groups	6.703	3	2.234	11.079	.000
	Within Groups	6.454	32	.202		
	Total	13.157	35			
9days	Between Groups	2.533	3	.844	6.193	.002
	Within Groups	4.363	32	.136		
	Total	6.896	35			

Appendix Table 14 Duncan test for comparison of redness (a* values) of raw pork patties treated with 0.1% (w/w) of crude extracts from greater cardamom seeds (GCE) and siam cardamom seeds (SCE) as well as BHT (0.01% of fat) during storage period of 0 day.

Treatments	N	Subset for alpha = 0.05		
		1	2	3
SCE 0.1%	9	15.89		
GCE 0.1%	9		16.3167	
Negative control	9			17.0664
BHT 0.01%	9			17.3656
Sig.		1	1	0.061

Means for groups in homogeneous subsets are displayed. NC: Negative control without antioxidant, BHT: Butylated hydroxytoluene

Appendix Table 15 Duncan test for comparison of redness (a* values) of raw pork patties treated with 0.1% (w/w) of crude extracts from greater cardamom seeds (GCE) and siam cardamom seeds (SCE) as well as BHT (0.01% of fat) during storage period of 1 day.

Treatments	N	Subset for alpha = 0.05		
		1	2	3
SCE 0.1%	9	15.2711		
GCE 0.1%	9	15.4233		
BHT 0.01%	9		15.9989	
Negative control	9			16.4644
Sig.		0.165	1	1

Means for groups in homogeneous subsets are displayed. NC: Negative control without antioxidant, BHT: Butylated hydroxytoluene

Appendix Table 16 Duncan test for comparison of redness (a* values) of raw pork patties treated with 0.1% (w/w) of crude extracts from greater cardamom seeds (GCE) and siam cardamom seeds (SCE) as well as BHT (0.01% of fat) during storage period of 3 days.

Treatments	N	Subset for alpha = 0.05		
		1	2	3
SCE 0.1%	9	13.558		
BHT 0.01%	9	13.862	13.8622	
GCE 0.1%	9		14.0989	
Negative control	9			14.6733
Sig.		0.201	0.317	1

Means for groups in homogeneous subsets are displayed. NC: Negative control without antioxidant, BHT: Butylated hydroxytoluene

Appendix Table 17 Duncan test for comparison of redness (a* values) of raw pork patties treated with 0.1% (w/w) of crude extracts from greater cardamom seeds (GCE) and siam cardamom seeds (SCE) as well as BHT (0.01% of fat) during storage period of 6 days.

Treatments	N	Subset for alpha = 0.05		
		1	2	3
BHT 0.01%	9	11.8489		
SCE 0.1%	9	12.2467	12.2467	
GCE 0.1%	9		12.6622	12.6622
Negative control	9			12.9956
Sig.		.069	.058	.125

Means for groups in homogeneous subsets are displayed. NC: Negative control without antioxidant, BHT: Butylated hydroxytoluene

Appendix Table 18 Duncan test for comparison of redness (a* values) of raw pork patties treated with 0.1% (w/w) of crude extracts from greater cardamom seeds (GCE) and siam cardamom seeds (SCE) as well as BHT (0.01% of fat) during storage period of 9 days.

Treatments	N	Subset for alpha = 0.05		
		1	2	3
BHT 0.01%	9	10.4411		
Negative control	9	10.7167	10.7167	
SCE 0.1%	9		11.0022	11.0022
GCE 0.1%	9			11.1267
Sig.		.123	.111	.480

Means for groups in homogeneous subsets are displayed. NC: Negative control without antioxidant, BHT: Butylated hydroxytoluene

CURRICULAM VITAE

NAME: Ramesh Khadka

BIRTH DATE: August 14, 1977

EDUCATION:

YEAR	INSTITUTE	DEGREE
2002	T.U. Nepal	B. Tech. (Food)

POSITION / TITILE: Food Research Officer

WORK PLACE: Ministry of Industry, Government of Nepal

SCHOLARSHIP: Thailand International Development Co-operation Agency (TICA) Scholarship (2011 – 2013)