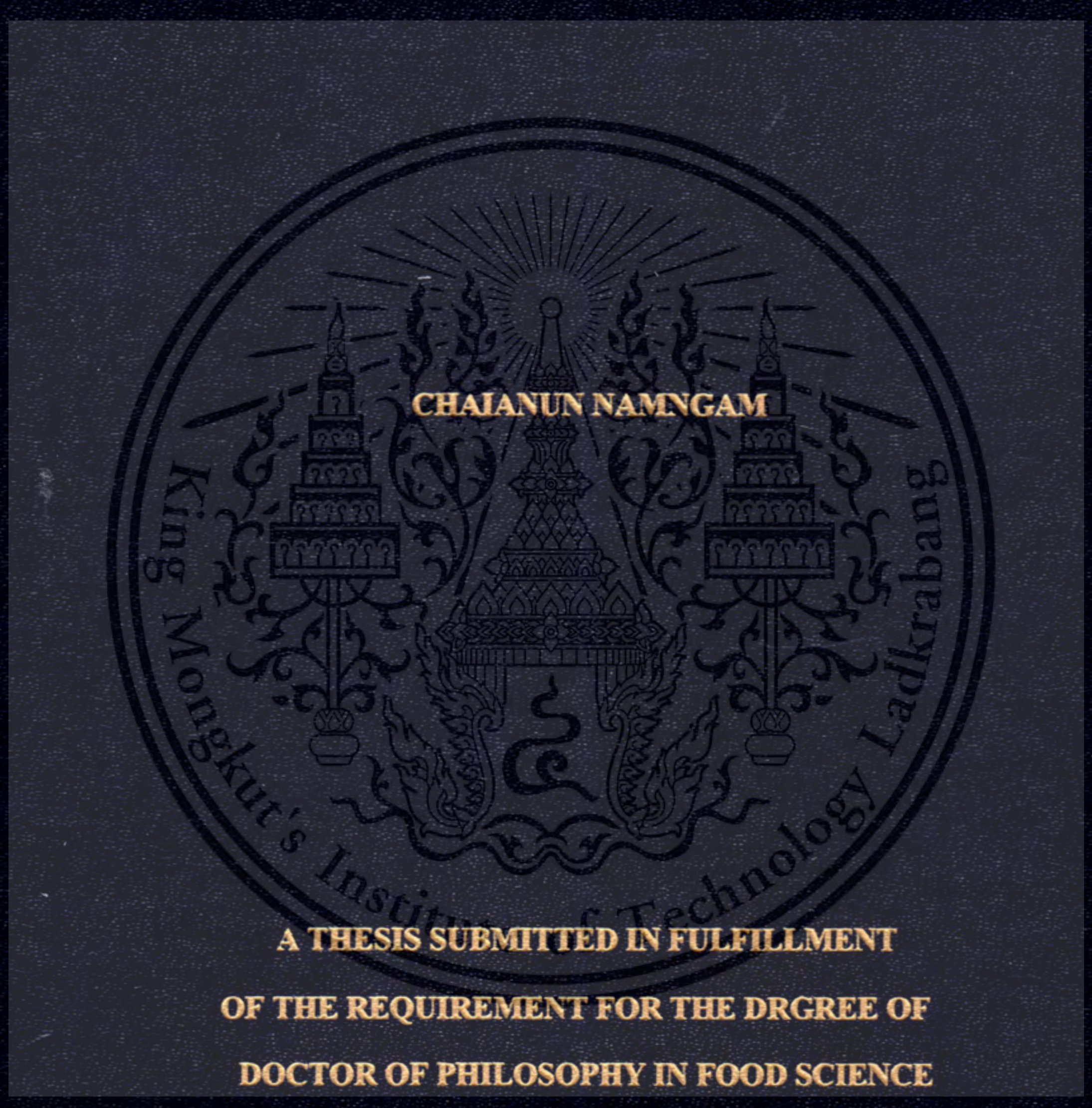
ANTIOXIDANT ACTIVITY AND *in-vitro* INHIBITORY POTENTIAL AGAINST SELECTED ENZYMES OF THAI MANGO SEED KERNEL EXTRACTS AND THEIR APPLICATION IN FOOD AND COSMETIC



CHAIANUN NAMNGAM

A THESIS SUBMITTED IN FULFILLMENT

OF THE REQUIREMENT FOR THE DRGREE OF

DOCTOR OF PHILOSOPHY IN FOOD SCIENCE

FACULTY OF AGRO-INDUSTRY

KING MONGKUT'S INSTITUTE OF TECHNOLOGY LADKRABANG

2018

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Thesis Certification

Faculty of Agro-Industry

King Mongkut's Institute of Technology Ladkrabang

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Date : July 23, 2018

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Thesis	Antioxidant activity and *in-vitro* inhibitory potential against selected enzymes of Thai mango seed kernel extracts and their application in food and cosmetic
Student	Mr. Chaianun Namngam
Student ID	53680152
Degree	Doctor of Philosophy
Program	Food Science
Year	2018
Thesis Advisor	Assoc. Prof. Dr. Praphan Pinsirodom

ABSTRACT

Thailand has a viable mango processing industry from which a major waste product is the seeds. The research described in this thesis evaluated the antioxidant properties and inhibition of selected enzymes activities (tyrosinase, lipoxygenase, hyaluronidase and α-glucosidase) of seed kernel extracts (MSKEs) using ultrasonic assisted extraction from 2 cultivars Kaew and Choke-Anan commonly used in processing in Thailand. MSKEs from Kaew and Choke-Anan were fractionated using Sephadex LH-20 column chromatography. Antioxidant activity and the selected enzymes inhibitory effects of MSKE fractions were evaluated. The key components of polyphenol, found in the most active fractions, were identified using liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS). The effects of 0, 1, 2 and 3% of MSKE, extracted from the mango cultivar Choke-Anan were investigated, on retarding lipid and protein oxidation in mince fish during frozen storage at -18 ^{o}C for 0, 3, 6, 9, 12 and 15 weeks. The pH, color peroxide value (PV), thiobarbituric acid reactive substances (TBARS), conjugated diene (CD), protein carbonyl, sulfphydryl groups (SH), total volatile base nitrogen (TVB-N) of samples were determined. Cosmetic cream contained 1, 2, and 3 % MSKE from Choke-Anan cultivar was prepared and physical stability and skin irritation were evaluated. The variables observed were

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phase separation, pH, viscosity, TPC and the difference of color changes.

The yield percentage and total polyphenols content (TPC) of MSKE from both cultivars were affected by the duration time of ultrasonication. Within the scope of this study the optimum time for extraction of MSKE was 45 min, but since this was also the maximum, further work is suggested. The MSKEs prepared by ultrasonic assisted extraction of both cultivars exhibited greater antioxidant activities (DPPH, FRAP, H_2O_2 scavenging and ABTS). The yield, TPC, antioxidant properties and inhibition of 5-lipoxygenase, hyaluronidase and α-glucosidase were significantly higher ($P \leq 0.05$) for MSKE from Choke-Anan than from Kaew. The MSKE from both cultivars showed no significant difference ($P > 0.05$) in tyrosinase inhibition activity compared to arbutin but a slightly lower α-glucosidase inhibition activity than acarbose was observed. Three major isolates were obtained from both cultivars of the MSKE with the absorbance being higher for Choke-Anan than Kaew. Fraction 3 of MSKE from both cultivars showed significantly ($P \leq 0.05$) higher antioxidant activity and 5-lipoxygenase, hyaluronidase and α-glucosidase inhibitory activity with Choke-Anan being more effective than Kaew. Six different polyphenols were found in fraction 3 of both cultivars. These were tri-*o*-galloyl-glucoside, tetra-*o*-galloyl-glucoside, maclurin tri-*o*-galloyl-glucoside, penta-*o*-galloyl-glucoside, hexa-*o*-galloyl-glucoside, and hepta-*o*-galloyl-glucoside. In the MSKE from Choke-Anan, hexa-*o*-galloyl-glucoside, and tetra-*o*-galloyl-glucoside were the two major components, whereas in the MSKE from Kaew tetra-*o*-galloyl-glucoside was the only major component.

Mince fish samples treated with different concentrations of MSKE from Choke-Anan and BHT had significantly ($P \leq 0.05$) increased pH values after storage at -18 ^{o}C for 12 to 15 weeks. After storage for 15 weeks the samples treated with 3% MSKE and BHT had significantly ($P \leq 0.05$) lower lightness (L) and redness (a) changes with no significant effects on yellowness (b). Samples treated with 3% MSKE had the lowest formation of protein carbonyl and decreased SH groups, PV, TBARS, TVB-N value and CD value compared to the non-treated samples and the samples treated with only BHT.

The cosmetic cream that contained 1% of MSKE from Choke-Anan was stable, did not irritate even mild skin and appeared to be acceptable in terms of consumer safety. The results

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indicate that MSKE is a suitable by-product that could be utilized for adding value to the mango processing industry and could represent a valuable input into functional foods and pharmaceutical productions.

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Chaianun Namngam

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CHAPTER I

INTRODUCTION

The use of synthetic ingredients in food, cosmetics and pharmaceutical products have raise concerns about toxicity and safety (Maqsood and Benjakul, 2013). Moreover, in countries such as Japan and some European countries synthetic antioxidants have banned (Tang et al., 2001; Wanasundara and Shahidi, 1998). Numerous studies of natural bioactive compounds from plants including fruits and vegetables have been evaluated as replacements for synthetic antioxidants, not only to meet legislative requirements but also because many people prefer natural products for a healthy life style.

Among the bioactive phytochemicals found in plants, polyphenols have been extensively studied for their antioxidant, anti-inflammatory, anti-carcinogenic and cardio-protective properties (Cao and Cao, 1999). Polyphenols are secondary metabolites found in higher plants holding one or more phenolic rings (Parr and Bolwell, 2000). They have strong antioxidant activities *in-vitro*, being able to scavenge a wide range of reactive oxygen species (ROS), reactive nitrogen species (RNS), chlorine species, such as superoxide, hydroxyl, peroxyl radicals, peroxynitrous acid and hypochlorous acid (Hernandez et al., 2009).

Mango (*Mangifera indica* L.) belongs to the family of *Anacardiaceae* and polyphenols are commonly found in fruit pulp, peel and seeds as well as leaves, flowers and stem bark. Polyphenols found in various parts of the mango include mangiferin, catechin, quercetin, kaempferaol, rhamnetin, anthocyanin, gallic and ellagic acid, propyl and methyl gallate, benzoic acid, and protocatechuic acid (Masibo and He, 2008). In terms of world production mango is second to banana (FAOSTAT, 2011). And mango fruit and its processed products are in increasing demand on the international markets (Jahurul et al., 2014). Mango seed and peel are the main by-products of the processing industry representing depending on cultivar 35%-60% of the fruit (Ayala-Zavala et al., 2011). The main bioactive compound of mango seed kernel are polyphenols. Ahmed et al., (2007) reported that tannin, gallic acid, coumarin, cafeic acid, vanillin,

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mangiferin, ferulic acid and cinamic acid are found in mango seed kernel. Kittiphoom (2012) claimed that mango seed kernel could be used as a potential source for functional food ingredients, antimicrobial compounds and cosmetic. Soong and Barlow (2004) reported that mango seed has a strong antioxidant activity due to its high phenolic compounds. Moreover, mango seed kernel extracts have been reported to have anti-tyrosinase, anti-inflamatory, hepatoprotective activities (Nithitanakool et al., 2009a,b) and anti-enzymatic activities against snake venom (Leanpolchareanchai et al., 2009).

Ultrasonic assisted extraction (ultrasonication) has become an established method in the processing of plant material (Vilkhu et al., 2008) and was reported to be an inexpensive, simple and time saving method that is highly efficient and has low energy consumption (Herrera and Luque, 2005). The technique enhances the extraction of polyphenols and offers a mechanical effect allowing better penetration of solvents into the sample matrix, increasing the contact surface area from the solid and liquid phases, as a result the solute rapidly diffuses from the solid to the liquid phase (Rostagno et al., 2003).

From the above it was therefore determined that the utilization of mango by-products as a natural ingredient in food and cosmetic industry required further study and evaluation. The objective of this research was therefore to investigate whether ultrasonic assisted extraction could be successfully used for the extraction of polyphenols from the seed kernels of two mango cultivars commonly used in the Thai processing industry. The bioactivities (antioxidant properties, the inhibition of tyrosinase, 5-lipoxygenase, hyaluronidase and α-glucosidase), fractonationation and the key components of mango seed kernel extract were evaluated. The ability of the extract was evaluated on stabilizing the lipid and protein oxidative parameters of mince fish and its application in cosmetic cream.

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OBJECTIVES

1. To investigate the suitable ultrasonication time for ethanolic extraction of mango seed kernel.
2. To evaluate the *in-vitro* antioxidant activities and selected enzyme inhibitory potentials of mango seed kernel extracts of two cultivars used in processing.
3. To identify the key component in the mango seed kernel extract fractionated by Liquid chromatography mass spectrometer (LC-MS)
4. To investigate the effect of mango seed kernel extract on oxidative stability of mince fish during frozen storage -18 ^{o}C for up to 15 weeks.
5. To investigate the effect of mango seed kernel extract as an ingredient in cosmetic cream.

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CHAPTER II

LITERATURE REVIEWS

2.1 Mango (*Mangifera indica* L.)

Mangoes belong to the genus *Angifera*, consisting of numerous species of tropical fruiting trees in the flowering plant family *Anacardiaceae*. It is cultivated and grown vastly in many tropical regions and widely distributed in the world including Southeast Asia and Central America (Morton, 1987). It is one of the most extensively exploited fruits for food, juice, flavor, fragrance and color and a common ingredient in new functional foods often called super fruits.

There are several mango varieties grown in Thailand. The well known cultivars are Choke-Anan, Ok-Long Khaew, Nam-Dorkmai, Rad and Keow-Savoey. The mango can be eaten both ripe, unripe or processed. Nam-Dorkmai and Ok-Long are the favorite choice as desert fruit. Keow-Savoey is sweet and has a powdery texture, while Rad is predominantly sour with a hint of sweet, both are good for eating as green mango. The Choke-Anan and Khaew cultivar are popular for processing in factories.

2.2 Polyphenols in mango parts

All a part of mango fruit contains various polyphenol that well known strong antioxidant activity. Previous studies have been reported polyphenol in different mango part (Table 2.1).

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Table 2.1 Polyphenol in mango parts

Mango part	Polyphenols	References
Pulp	Mangiferin, Gallic acids, Gallotannins, Quercetin, Isoquercetin, Ellagic acid, β-glucogallin, Tannin, p-OH-benzoic acid, m-coumaric acid, p-coumaric acid, Ferulic acid.	Schieber et al., 2000) Kim et al., (2007)
Peel	Mangiferin, Quercetin, Rhamnetin, Ellagic acid, Kaempferol, 3-0-galactoside, Anthocyanins	Berardini et al., (2005 a,b)
Seed kernel	Tannin, Gallic acid, Coumarin, Caffeic acid, Vanillin Mangiferin, Ferulic, Cinnamic	Ahmed et al., (2007)

2.3 Bioactivities of mango polyphenols and human health

As with other fruit, mangoes are high in polyphenolic compounds, which make their consumption, as part of a regular diet, highly beneficial to human health. The important phenolic compounds that mangoes contain are described in the following.

2.3.1 Mangiferin

Mangiferin (C 2-β-D -glucopyranosyl-1, 3, 6, 7-tetrahydroxyxanthone) (Figure 2.1) which is a polyphenolxanthone with strong antioxidant activity. Mangiferin is widely found in higher plants (Sanchez et al., 2000) where it gives protection against different forms of static and dynamic stresses including the ingress of pathogenic microorganisms (Muruganandan et al., 2002). Previous studies reported that mangiferin from fruit and extracts from mango leaves, stem bark and flowers, showed a wide range of pharmacological effects including antioxidant, anticancer, antimicrobial, antiatherosclerotic, antiallergenic, anti-inflammatory, analgesic, immunomodulatory and Alzheimer's disease (Sethiya and Mishra, 2014). Antioxidant activities,

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immune stimulating and antiviral properties of mangiferin were reported by Rouillard et al., (1998) and Zheng and Lu, (1990). Muruganandan et al., (2002) showed that mangiferin gave protection against hepatocytes, lymphocytes, neutrophils and macrophages from oxidative stress, reduced atherogenicity in streptozotocin diabetic rats and reduced the streptozotocin-induced oxidative damage to cardiac and renal tissues in rats.

Figure 2.1 Chemical structure of mangiferin (C 2- β-D- glucopyranosyl-1, 3, 6, 7-tetrahydroxyxanthone). (Masibo and He, 2008)

Mangiferin has also been reported to inhibit colon tumorigenesis in rats. Bhattacharya et al., (1972) reported that in the central nervous system the mechanism of mangiferin related to the inhibition activity of monoamine oxidase. The reduction of malignant cell proliferation in the colonic mucosa of male F344 rats by 75% was observed after treated 0.1 % of mangiferin to the diet. In addition, Yoshimia et al., (2001) suggested that mangiferin may be expressed as a naturally chemo-preventive agent in colon cancer. Moreover, tumor studies of rats given mangiferin showed that it also inhibited their body weight gain (Yoshimia et al., 2001).

The potential utilization of mangiferin in food products for special dietary has been discussed. Muruganandan et al., (2005) reported that in diabatic rats mangiferin significantly reduced plasma total cholesterol, triglycerides and LDL-C, which was associated with a concomitant increase in HDL-C levels and a decrease in atherogenic index, indicating its potential antihyperlipidemic and antiatherogenic activity.

The lowering the triglyceride levels of mangiferin could indirectly contribute to the overall antihyperglycemic activity through the glucose–fatty acid cycle mechanism (Randle et

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al., 1963). According to the Randle glucose–fatty acid cycle, an increased supply of plasma triglycerides could constitute a source of increased free fatty acid availability and oxidation that can impair insulin action as well as glucose metabolism and utilization leading to development of hyperglycemia. Therefore, the reduction of triglycerides level using mangiferin would also facilitate glucose oxidation and utilization, subsequent to the reduction of hyperglycemia (Muruganandan et al., 2005).

2.3.2 Flavonoids

Flavonoids are the most abundant polyphenols found in the human diet. Flavonoids were once thought to be vitamins andwere given such names as vitamin P and vitamin C2. Flavonoids are classified according to the degree of oxidation of the oxygen heterocycle and include flavones, isoflavones, flavanones, flavonols, flavanols, anthocyanins and proanthocyanidins. The occurrence of some of these flavonoids is restricted to a few foodstuffs. Hollman et al., (1996) reported that flavonoids with a diphenylpropane skeleton (C_6–C_3–C_6) showed antioxidative properties as well as, antimutagenic, anticarcinogenic, anti-inflammatory and anti-allergic effects.

Harborne (1994) found that mangoes contained several flavonoids including catechin, epicatechin, quercetin, isoquercetin (quercetin-3-glucoside), fisetin, and astragalin (kaempferol-3-glucoside). Flavonoid glucosides are commonly found in mango leaves, whereas fisetin is confined to twigs and quercetin has been reported to be found in unripe fruits, but both of quercetin and glucoside have been previously found in both immature and mature fruits but disappears during ripening (Elansari et al.,1969).

The skin of the mango cultivar Haden was found peonidin-3-galactoside. In some mango cultivars the there is a red pigment in the skin, which is due to anthocyanins. Anthocyanins are a group of phenolic compounds with good antioxidant properties that are higher than that of phenolic acids (Rice-Evans et al., 1997).

Flavonoid from the group of catechins including (+)-Catechin, (–)-epicatechin, (–)-epigallocatechin, (–)-epicatechingallate, and (+)-gallocatechin. Several studies also reported

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that catechins have been shown to have beneficial effects on human health due to their free radical scavenging and antioxidant activities (Augustyniak et al., 2005) and serve to protect against congestive heart failure (Ishikawa et al., 1997), cancer (Yamanaka et al., 1997), myoglobin-uric acute renal failure (Chander et al., 2003) and to reduce the incidence of myocardial ischemia as well as supporting anti-aging. Rastraelli et al., (2002) showed that mango stem bark extract contained (+) catechin and (–) epicatechin 1308 and 807.4 mg/100 g dry matter, respectively (Figure 2.2 and 2.3), which is used as an ingredient in the formulation of a anthocyanins food supplement in Cuba called Vimang. These high concentrations indicate the potency of mango stem bark extract as an antioxidant for medicinal use.

Moreover, catechin, epicatechin and mangiferin may react with H_2O_2 directly or prevent the Fenton reaction, between Fe^{2+} and H_2O_2, to form hydroxyl radicals (Sanchez and et al., 2000) reducing H_2O_2, induced by T-cell receptor activation, and thus controlling the reactive oxygen species-pathway against activation-induced cell death (Hernandez et al., 2007). These reactions result in the protection of human T-lymphocytes from *in vitro* activation-induced cell death (AICD) in a concentration dependent manner.

Figure 2.2 Chemical structure of (+)-catechin (Masibo and He, 2008)

Figure 2.3 Chemical structure of (–)-epicatechin (Masibo and He, 2008)

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Quercetin is responsible for the color of many fruits, flowers and vegetables. They often occur in plants as glycosides such as rutin (quercetinrutinoside). Schieber et al., (2000) demonstrated that mango pulp contains flavonol glycosides with quercetin 3-galactoside at 22.1 mg/kg, followed by quercetin 3-glucoside at16.0 mg/kg and quercetin 3-arabinoside at 5.0 mg/kg (Figure 2.4).

The amount of the quercetin aglycon was 3.5 mg/kg and flavonol glycosides (kaempferol) were present in the trace amounts. In another study of mango peel, Berardini et al., (2005b) found higher quantities of quercetin and its related glycosides. Numerous studies have reported bioactivity of quercetin extracted from mango. Lamson and Brignall, (2001) found that quercetin decreased the expression of mutant breast cancer cells, arrested human leukemic T-cells, inhibited tyrosine kinase and inhibited heat shock proteins. Moreover, Peng and Kue, (2003) also found that quercetin protects Caco-2 cells from lipid peroxidation induced by hydrogen peroxide and Fe^{2+}. Molina et al., (2003) shwed that mouse liver was protected from oxidative damage by the activity of quercetin by decreasing lipid oxidation and increasing glutathione. Woude et al., (2003) demonstrated the use of quercetin in the inhibition of cancer cell. They showed that high doses of quercetin inhibited cell proliferation in colon carcinoma cell lines and in mammary adenocarcinoma cell lines, but at low doses quercetin increased cell proliferation in colon and breast cancer cells. The inhibition property of quercetin of cell proliferation in Mol-4 human leukemia cells and induces apoptosis was reported by Mertens-Talcott et al., (2003). They showed that the modulation of platelet activity may help prevent cardiovascular disease. It has also been demonstrated that quercetin showed anti-histamine and anti-inflammatory effects associated with various forms of arthritis, with quercetin working mainly as an antioxidant.

In mango fruit Kaempferol and its related conjugates are found of kaempferol-3-0 glucoside form and were reported to be at 36 mg/kg (Berardini et al., 2005b). In addition, in mango peel Kowalski et al., (2005) found kaempferol to show a strong antioxidant activity since it inhibited monocyte chemoattractant protein (MCP-1) that plays a role in the initial steps of atherosclerotic plaque formation. Elsewhere, Ackland et al., (2005) demonstrated that kaempferol

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inhibited cancer in cultured human cancer cell lines by reducing the resistance of cancer cells to anticancer drugs. Kaempferol was observed to induce apoptosis in human glioblastoma cells (Sen et al., 2007). Another study also found that it was absorbed more efficiently than quercetin in humans at low oral doses (Kroon et al., 2004). The chemical structure of kaempferol is shown in Figure 2.5. Berardini et al., (2005b) found that mango peel contained 94.4 mg/kg of rhamnetin 3-0-galactoside/glucoside (Figure 2.6).

Igarashi et al., (2008) also demonstrated that pure compounds of, rhamnetin affected serum and liver cholesterol concentrations, liver lipoperoxide content and antioxidative enzyme activities. Currently the emphasized on anthocyanins is due to their possible health benefits as dietary antioxidants. They have a basic structure as shown in Figure 2.7. Elsewhere, the peel of the mango cultivar Tommy Atkins was identified as a novel anthocyanin (7-*O*-methylcyanidin 3-*O*-β-D-galactopyranoside) by (Berardini et al., 2005b). The daily intake of anthocyanins in humans has been recommended as up to 200 mg/day (Kuhnau, 1976).

Anthocyanins have been used to effect for therapeutic activities in humans to control such as coronary heart disease, cancer, protect DNA damage, anti-inflammation, subsequent blood vessel damage, dampen allergic reactions, prevent tyrosine nitration, protect neurological diseases, manage diabetes, prevent abnormal protein proliferation and improve eye sight (Nakaishi 2000; Duthie et al., 2000; Lazze et al., 2003; Bertuglia, 1995; Perossini et al., 1987).

Figure 2.4 Chemical structure of quercetin (Masibo and He, 2008)

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Figure 2.5 Chemical structure of kaempferol (Masibo and He, 2008)

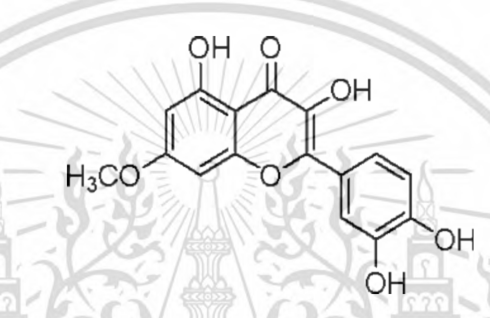

Figure 2.6 Chemical structure of rhamnetin (Masibo and He, 2008)

2.3.3 Phenolic Acids

Phenolic acids were already identified in mangoes included gallic acid, 3,4-dihydroxybenzoic acid, benzoic acid, gallic acid methyl ester, gallic acid propyl ester, and benzoic acid propyl ester (Rastraelli et al., 2002). The phenolic acids are either gallic acid in gallotannins (mango fruit) or other phenolic acids derived from the oxidation of galloyl residues in ellagitannins (Scalbert and Williamson, 2000).

Gallic acid (3, 4, 5-trihydroxybenzoic acid) (Figure 2.8) and its derivative ellagic acid, exist either in the free form or bound as gallo-tannins and/or ellagitannins, respectively. Gallic acid was identified as the main polyphenolic compound in mangoes, followed by 6 hydrolyzable tannins (Kim et al., 2007). Soong and Barlow (2006) also reported that the amount of gallic acid in mango seed extract ranged of 23 to 838 mg/100 g (on dry matter basis) depending on the method of extraction. Rastraelli et al., (2002) found 226.2 mg/100 g (of dry matter) of gallic acid in mango stem bark extract and Schieber et al., (2000) reported that gallic acid was the

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major compound (6.9 mg/kg) found in the mango pulp. Gallic acid has been demonstrated, through *in vivo* and *in vitro* evaluation, to have antioxidant and radical scavenging activity and was shown to be anti-inflammatory, antimicrobial, antimutagenic and anticancer (Madsen and Bertelsen, 1995). In addition, Matsuo et al., 1997 found that gallic acid decrease histamine release in rat basophilic leukemia cells and Shin et al., (2005) showed that it inhibited inflammatory allergic reactions.

Ellagic acid is a fused 4-ring polyphenol (Figure 2.9). In mangoes it is present in the form of ellagitannin (ellagic acid bound to a sugar molecule). It is water soluble and easily absorb by animals. Soong and Barlow (2006) obtained ellagic acid from mango seed extract and found it was in range of 3 to 156 mg/100 g (GAE). Ellagic acid has been shown to inhibit DNA binding and DNA adduct formation of N-nitrosobenzylmethylamine (NBMA) in cultured explants of rat esophagus (Mandal et al., 1988). Moreover, Khanduja et al., (1999) demonstrated that it can prevent N-nitrosodiethylamine induced lung tumorigenesis in mice and exhibit antimutagenic, antiviral and antioxidant properties, and stimulate the activity of detoxifying enzymes (Mandal et al., 1988). Application of small amounts of ellagitannins derived from natural sources has been shown to be more effective in the human diet than large doses of purified ellagic acid.

Propyl gallate and methyl gallate (Figure 2.10 and 2.11) are derivatives of gallic acid. Kane et al., (1988) showed that methyl and propyl gallate exhibited *in vitro* inhibition activity of the herpes simplex virus. The adhesion of human leukocytes, adhesion of cancer cells with vascular endothelial cells, human collagenase, growth of intestinal bacteria was reported by Chung et al., (1998).

Benzoic acid and related conjugates are the simplest aromatic carboxylic acid containing a carboxyl group bonded directly to a benzene ring (Figure 2.12). In studies of the conjugation of benzoic acid in man showed that the body has no store of preformed glycine and that benzoic acid acts as a stimulus for the synthesis of this amino acid. Quick (1931) reported that glycine production was observed to increase when increasing amounts of benzoic acid were applied. Conjugation of glycine with glucuronic acid occurs due to oxidation because benzoic

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acid shows inhibition activity of fatty oxidation in the liver.

Protocatechuic acid (3, 4 dihydroxybenzoic acid) (Figure 2.13) has several forms that have been identified and quantified in mango stem bark extract at about 226.2 mg/100 g of dry matter (Rastraelli et al., 2002). Dihydroxybenzoic acids are used as intermediates for antipyetic, analgesic, antirheumatism drugs and other organically synthesized drugs in pharmaceutical products. Wang et al., (2007) reported that many researches have undertaken evaluation of these phenolic acids and their derivatives and have shown that they are antimutagenic, anticarcinogenic, antifungal, antibacterial, antioxidant and neuroprotective properties.

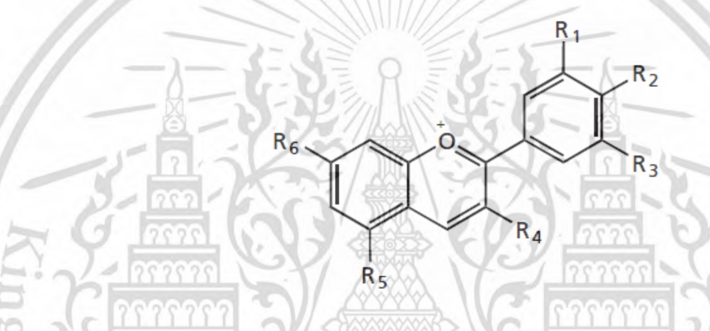

Figure 2.7 Chemical structure of anthocyanins (Masibo and He, 2008)

Figure 2.8 Chemical structure of gallic acid (Masibo and He, 2008)

Figure 2.9 Chemical structure of ellagic acid (Masibo and He, 2008)

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Figure 2.10 Chemical structure of propyl gallate (Masibo and He, 2008)

Figure 2.11 Chemical structure of methyl gallate (Masibo and He, 2008)

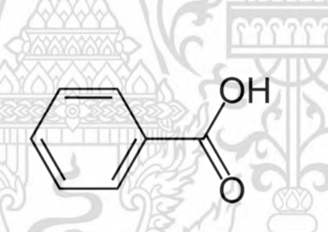

Figure 2.12 Chemical structure of benzoic acid (Masibo and He, 2008)

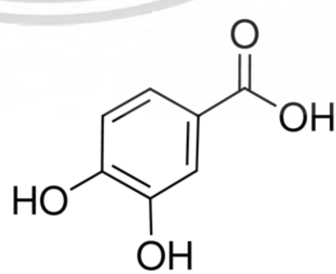

Figure 2.13 Chemical structure of protocatechuic acid (Masibo and He, 2008)

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2.4 Mango seed kernel utilization

Mango, a tropical fruit, is among the most important in world trade. From the processing of mango into juices and other products, their seed kernels are a waste that constitutes some 17-22% of the fruit. Numerous studies also reported the utilization mango seed by-product. The main components of mango seed kernels are starch, fat and protein, which could be utilized as a potential source for food ingredients, antimicrobial compounds, antioxidants and cosmetics due to the high quality of their fat and protein. The most important properties of the oil from mango seed kernels are free fatty acids. Peroxide values (PV) are an index of fat and oil quality and the low free fatty acid content of mango seed kernel oil indicated that the mango seed was almost free from hydrolytic rancidity brought almost by lipases (Arogba, 1997).

Sims et al., (1972) showed that mango seed kernel oil had a low level of PV, with the degree of unsaturation contained in fat and oil, expressed as iodine value (IV), ranged of 39 to 53. They explained that saponification value represents the average molecular weight (or chain length) of all the fatty acids. Unsaponifiable matter is a component of an oil mixture which does not form soap when NaOH is added. The composition of unsaponifiable matter of vegetable oils that contains tocopherols, sterols and squalene is of great importance for the characteristics and stability of oils. The main saturated fatty acids in mango seed kernels oil included stearic and palmitic acids and the major unsaturated fatty acids are oleic and linoleic acids.

Nzikou et al., (2010) reported that the fatty acid in mango seed kernel oil consisted of palmitic 6.48 %, stearic 37.94 %, oleic 45.76 %, linoleic 7.45 % and linolenic 2.37 %. The comparison of the composition in fatty acids of mango seed kernel oil with that of vegetable oils indicates that it is rich in stearic and oleic acids. Kittiphoom (2012) also reported that mango seed kernel oil is more stable than many other vegetable oils rich in unsaturated fatty acids and had a potential to be suitable for blending with other vegetable oils in the confectionery and manufacturing, industries and in the soap manufacturing industry.

Cocoa butter, extracted from the *Theobroma* cacao seeds, are a commonly ingredient used in confectionery products, especially, of course, in chocolate. Cocoa butter consists mainly of palmitic acid, stearic acid and oleic acid and a trace amount of lauric and myristic acids

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(Kheiri, 1982; Pease, 1985). Cocoa butter is expensive so manufacturers are constantly seeking an alternative vegetable oil that has similar chemical and physical properties to cocoa butter. Kaphueakngam et al., (2009) and Kittiphoom (2012) reported the mango seed kernel oil could be an alternative source and when blended 80/20 % (w/w) between mango seed kernel oil and palm oil could be used as cocoa butter substitute.

In recent years, many countries including Japan and some European countries have banned the use of the synthetic antioxidants butylatedhy-droxytoluene (BHT) and butylated hydroxyanisole (BHA), due to their toxicity and carcinogenicity (Wanasundara and Shahidi, 1998). Many studies have focused on natural antioxidant that could be used in the food industry to replace BHT and BHA, particularly those that give healthy benefits to consumers such as the reduction in the incidence of cardiovascular diseases and cancer. Several studies have shown that mango seed kernels contain various phenolic compounds and can be a good source of natural antioxidants (Puravankara et al., 2000; Abdalla et al., 2007; Namngam and Pinsirodom, 2017). Moreover, polyphenols contain tannins, gallic acid, coumarin, ellagic acid, vanillin, mangiferin, ferulic acid, cinammic acid were found in mango seed kernels (Arogba, 1997). This suggests that the mango seeds kernel by-products should be further utilized as natural antioxidant ingredients in food and cosmetic.

The addition to antimicrobial agents in food and cosmetics has been particularly effective for controlling microbial contamination but they need to be acceptable to consumers. Previous studies showed that phenolic compounds, tannins and flavonoids in mango seed kernel exhibited antimicrobial properties and prevented microorganisms by inhibiting extracellular growth and by avoiding oxidative phosphorylation (Schieber et al., 2003).

Phenolic compounds have also been developed as whitening, sunscreen and anti-wrinkle agents in cosmetics (González, 2008). Mango seed kernel was shown to be a good source of phenolic compounds (Soong and Barlow, 2004) including microelements like selenium, copper and zinc (Schieber et al., 2003). In addition, the mango seed kernel extract exhibited the highest degree of free-radical scavenging and tyrosinase inhibition activities compared to methyl gallate (Maisuthisakul and Gordon 2009). Moreover, Miasuthisakul (2011) reported that phenolic

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compounds from the mango seed kernel and methyl gallate in emulsions affected the stability of the cosmetic emulsion systems. Therefore, mango seed kernel has potential to be used as active ingredient in cosmetics and pharmaceuticals.

2.5 Ultrasonication assisted extraction of plant polyphenol

Plants contain substatial amounts of polyphenolic compounds that are useful in a wide range of applications due to their biological activity. The toxicity of using organic solvents in conventional technique phytochemical extraction have been raised. Moreover, prolonged extraction is required, which indicates high energy consumption resulting an incremental cost. Thus, the implementation of ultrasonication assisted extraction (UAE) has been promoted. UAE is based on the phenomenon of acoustic cavitation, which is its ability to break down the cell walls of the plant matrix thereby favoring the release of bioactive compounds (Tiwari, 2015). The propagation of the ultrasonic waves through a liquid medium causes destruction of the cell walls and improvement in solvent penetration and the subsequent release of the polyphenolic compounds. UAE can be used in the extraction of different phytochemicals but particularly for phenolic compounds. Phenolic compounds are constantly used by various fields of industry, such as the food and pharmaceutical industries because it is easy to handle, is safe, economical and reproducible. This is due to UAE being able to be used under conditions of atmospheric pressure and at ambient temperatures. UAE is an effective way to extract and analyzes from different matrices in shorter times than with other extraction techniques (Dobias et al., 2010). In addition, UAE is a technique that can be used in implementing the substitution of organic solvents with solvents that do not have toxic effects.

UAE is simple extraction techniques due to its performed using only common laboratory equipment (i.e. an ultrasonic bath). The frequency of ultrasound is higher than the audible range to humans. Thus the lowest ultrasonic frequency is accepted as 20 kHz (Luque-Garcia and Luque de Castro, 2003). In addition, since these frequencies of extraction do not affect the stability of bioactive compounds (Soria and Villamiel, 2010).

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The ultrasound extraction trials have demonstrated improvements in extraction yield ranging from 6 to 35% (Vilkhu et al., 2008). Ledesma-escobar et al. demonstrated that UAE presented a higher extraction yield of polyphenol when tested on lemon fruit compared to the Microwave-Assisted Extraction (MAE), particularly for neosperidin (16%) and eriodictiol (13%). They concluded that UAE was a faster process than MAE. Yildiz-ozturk et al., (2015) also evaluated the comparison among MAE and UAE during the extraction of phenolic compounds from leaves of Stevia (*Stevia rebaudiana*) and reported that the phenolic compounds obtained by UAE exhibited antioxidant activity using the DPPH method. UAE is depended on the destructive effects of ultrasonic waves. According to Vinatoru et al., (1997) the possible advantages of UAE are as follows: intensification in mass transfer, cell disruption, enhanced penetration and capillary effects. Rodriguez-rojo et al., (2012) observed that theimprovements with UAE was due to the fact that this technique improves the transport of internal solvents by disruption of the cells through cavitation.

2.6 Tyrosinase and inhibitor

Tyrosinase (polyphenol oxidase, EC 1.14.18.1) is a metalloprotein that has a bi-functional catalytic mechanism consisting of the hydroxylation of monophenols to *o*-diphenols (monophenolase or cresolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity). It is widely distributed in human, animals, bacteria, fruits, vegetables and sea foods where it catalyzes the oxidation of tyrosine. It is a key enzyme in melanin synthesis, where it is involved in determining the color of mammalian skin and hair, where tyrosinase polymerization leads to melanin formation (Ben-Yosef et al., 2010). Tyrosinase is also important in browning in plants and wound healing and cuticle formation in arthropods (Yi et al., 2011).

Melanins are heterogeneous polyphenolic polymers with colors ranging from yellow through brown to black and are widely distributed in plants and animals. Melanogenesis fulfils a number of physiological roles in different organisms. In humans melanins are synthesized in membranous organelles called melanosomes, which are located in the dendrites of melanocytes.

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The color of mammalian skin, hair and eyes is determined by a number of factors, the most important of which is the degree and distribution of melanin pigments. In mammals, melanin pigments play several diverse and important roles, including thermoregulation, camouflage and sexual attraction. In humans there are three main types of melanin: Eumelanin (black and brown), Neuromelanin and Pheomelanin (red and yellow). The main role of the melanins is skin photoprotection by absorbing UV radiation that causes DNA damage and the formation of ROS. Dermal pigmentation is either dependent on the number, size, composition and distribution of melanocytes or activity of melanogenic enzymes.

The first, and rate-limiting, step of melanin synthesis is the conversion of tyrosine to dopa and dopaquinone by tyrosinase. This step is common to both eumelanin and pheomelanin synthesis (Prota, 1980). From there, synthesis of pheomelanin requires the addition of cysteine to dopaquinone, forming 5-S-cysteinyl-dopa or 2-S-cysteinyl-dopa. Alternatively, dopaquinone can enter the eumelanin-specific pathway by oxidation to dopachrome, which is further processed to form the two building blocks of eumelanin: DHI (5,6-dihydroxyindole) and DHICA (6-dihydroxyindole- 2-carbolic acid) (Ito and Wakamatsu, 2008). The formation of eumelanin requires additional enzymes beyond tyrosinase: TYRP1 (tyrosinase-related protein 1) and TYRP2 (tyrosinase-related protein 2, or dopachrome tautomerase). Mice, with mutations in any of the enzymes involved in melanin synthesis, develop variable amounts of defects in pigmentation. Mutations in tyrosinase produce entirely white mice (albino), while loss of function mutations in TYRP1 lead to the production of a brown rather than a black pigment (Jackson and Bennett, 1990; Bennett et al., 1990). Many tyrosinase null mutations affect one of the protein's two copper binding regions that are necessary for catalytic activity (Schweikardt et al., 2007).

Recently the global market demand for skin whitening agents has developed because some dark skinned individuals in many countries prefer lighter a skin color. In some Oriental countries, such as China, Korea and Japan, a female beauty criterion since ancient times has been a face with fair skin. Melanin is a human skin pigment that gives a darker color and tyrosinase isa key enzyme in the melanin biosynthetic pathway. Abnormal deposition of melanin pigment is causes of hyperpigmentary disorders. Plant extracts are a major source for development of

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potential new ingredients in food, cosmetic and pharmaceutical products. Thus there is a need for new potent tyrosinase inhibitors from natural source with acceptable human safety limits. Since plants are a rich source of bioactive chemicals, and are mostly free of harmful side effects, there is an increasing interest in using them as a source of natural tyrosinase inhibitors.

A number of ingredients, from natural sources, with an inhibitory effect on melanin hyperpigmentation have been identified and developed as cosmetic products to prevent hyperpigmentary disorders such as melasma, freckles and age spots including tyrosinase inhibitors from plant polyphenols. Polyphenols are useful secondary metabolites with abundant biological activity. Some phenolic compounds, such as ellagic acid, tannic acid and quercetin, act as potent tyrosinase inhibitors (Rompel et al., 1999; Shimogaki, Tanaka, Tamai and Masuda, 2000). Thai mango seed kernel extracts from the cultivars Choke-Anan, Kaew, and Fahlun have tyrosinase inhibitory activity (Maisuthisakul and Gordon, 2009; Nithitanakool et al., 2009a; Namngam and Pinsirodom, 2017). Van Acker et al., (1996) reported that flavonoids act as metal chelators of the copper at the tyrosinase active site forming the copper-flavonoid complexes. Some flavonoids, such as kaempferol, quercetin and morin, show inhibitory activity of tyrosinase, while others, e.g. catechin and rhamnetin, act as cofactors or substrates of tyrosinase (Gomez-Cordoves et al., 2001). The most active flavonol, quercetin, showed only 20% of the inhibitory activity compared to kojic translocate to the nuclear envelope where arachidonic acid was released from acid with regard to the diphenolase activity on mushroom tyrosinase. All flavanoids inhibit enzyme activity due to their ability to chelate copper at the active site. However, complexation with copper is only possible if the 3-hydroxy group is not functionalized (Kubo and Kinst-Hori, 1999).

2.7 5-lipoxygenase and inhibitor

Inflammatory reactions can be induced by a variety of mediators including eicosanoids derived from the cyclooxygenase (COX) and lipoxygenase (LO) pathways. Lipoxygenases are oxidative enzymes activate in metabolism that are recognized for their pro-inflammatory effects. 5-lipoxygenase (5-LOX, EC 1.13.11.34, 78 kDa) is a non-heme iron dioxygenase that is found in

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the soluble parts of cells and expressed primarily in leukocytes (LTs). 5-LOX is correlated with inflammatory and allergic reactions due to the formation of LTs. High levels of LTs could be observed in the case of asthma, psoriasis, allergic rhinitis, rheumatoid arthritis and colitis ulcerosa (Funk, 2001).

5-LOX catalyzes the oxidation of AA to 5-hydroperoxy eicosatetraenoic acid (5-HPETE) and then to LTA_4 by LTA_4 hydrolase into the potent phagocyte chemo-attractant LTB_4.. Alternatively, LTC_4 synthase synthesizes the conjugation of LTA_4 with glutathione to form LTC_4 , which can be modified further to LTD_4 and LTE_4 (Samuelsson et al., 1987). In resting neutrophils, 5-LO is detected in the cytosol and, upon cell activation by different stimuli, undergoes a calcium-dependent translocation to the nuclear envelope and endoplasmic reticulum, where it associates with the 5-LO activating protein (FLAP). In addition, production of LTs is dependent upon the availability of FLAP that would facilitate the transfer of AA to initiate LTs catalyzed (Dixon et al., 1990) (Figure 2.14).

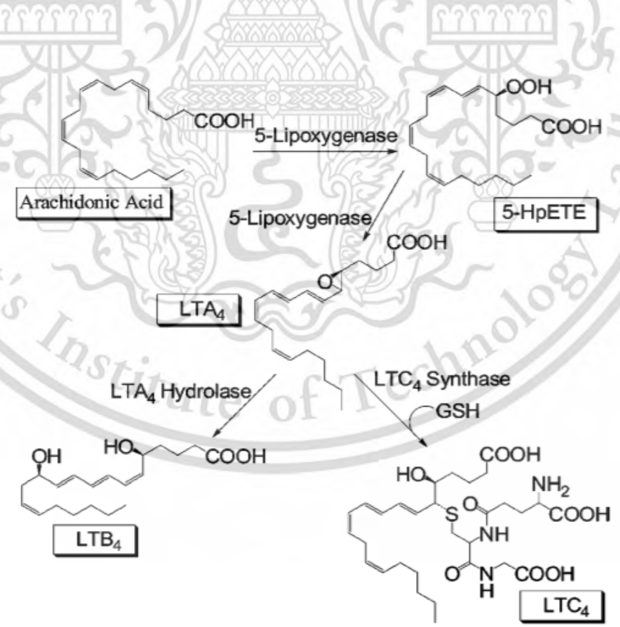

Figure 2.14 Biochemical pathway for the conversion of arachidonic acid into biologically active leukotrienes (Robert et al., 2007).

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Njenga and Viljoen (2006) emphasized that the evaluation of natural extracts to inhibit 5-LOX activity may provide important information about the potential activity of anti-inflammatory drugs. Henderson (1994) demonstrated that abnormal activity of 5-LOX has been implicated in several disorders related to allergy and inflammation, such as asthma, rheumatoid arthritis and psoriasis. Products of the 5-LOX pathway have also been associated with carcinogenesis (Sethi et al., 2012) and atherosclerosis in humans (Jawien and Korbut, 2010). Carter et al., (1991) reported that these pathological effects of 5-LOX make it an attractive candidate for drug targeting but, at that time, the only drug specifically targeting 5-LOX that has made to commercial distribution was Zileuton.

In fact, plant extracts that are used as anti-inflammatory remedies were shown to delay the formation of LTs, mostly by inhibiting 5-LOX activity. Mechanistically, many plant extract containing flavonoids, polyphenols and coumarins act to interfere with 5-LOX activity due to their antioxidant activity, that is, by uncoupling of the redox cycle of the 5-LOX active-site iron (Werz, 2007). Phenolic compounds also possess an array of potentially beneficial LOX inhibitory and anti-oxidant properties and have been used for the treatment of inflammatory diseases (Sreejayan and Rao, 1996). Yff et al., (2002) reported that the methanol extract of the roots of *Pentanisia prunelloides* showed 5-lipoxygenase inhibition activity with an IC_{50} of 32.71 ± 9.06 ppm. Napagodaa et al., (2014) demonstrated that the extracts of *Plectranthus zeylanicus* prepared with n-hexane or dichloromethane expressed the inhibition of 5-LOX activity in stimulated human neutrophils with anIC_{50} of 6.6 and 12mg/ml and inhibited isolated human recombinant 5-LOX at IC_{50} 0.7 and 1.2mg/ml. In South Africa the methanolic extracts from 8 plants of the 18 plants tested showed significant inhibition of 5-LOX activity. Among the plants tested *Bidens pilosa* showed maximum anti-inflammatory activity (IC_{50} 21.8 μg/mL) (Akula and Odhav, 2008). Benrezzouk et al., (2001) observed potent inhibitory effects on human neutrophil LTB_4 production without affecting COX or NOS activities. They explained that aethiopinone is an *in-vitro* inhibitor of 5-LO from human neutrophils (IC_{50}=0.11mM). In addition, aethiopinone

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isolated from *Salvia aethiopis* reduced leukocyte accumulation and showed *in vivo* inhibitory activity on 5-LOX.

2.8 Hyaluronidases and inhibitor

Hyaluronidases are ubiquitously found in the animal kingdom and have been identified and/or isolated from liver, kidney, spleen, testis, uterus and placenta, bacteria and pathogenic fungi as well as the venom of lizards, fish, bees, wasps, scorpions and spiders. The enzymes that were discovered were found to differ in their molecular weight, substrate specificity or optimum pH (Kreil, 1995). Based on biochemical experiments and the characterization of degradation products, Meyer et al., (1940) first classified the hyaluronidases into three groups

EC 3.2.1.35 represented by the mammalian or testis type hyaluronidases is an important enzyme that degrade hyaluronic acid and can hydrolyse the (1,4)-linkages between N-acetyl-beta-D-glucosamine and D-glucouronate residues in hyaluronate/hyaluronan, resulting in tetrasaccharide products. Hyaluronan falls under category of GAGs and can be found in significant quantities in skin, brain and central nervous system. In mammalian system especially human, the hyaluronic acid concentration depends primarily on its enzymatic synthesis and enzymatic degradation that occurs via hyaluronate synthase and hyaluronidase enzymes, respectively (Stern and Jedrzejas, 2006; Girish et al., 2009)

EC 3.2.1.36 is commonly termed leech-hyaluronidases and represents hyaluronate-3-glycanohydrolases that cleave the β-1$\longrightarrow$3 linkages of the substrate hyaluronan. These enzymes from leeches and hook worms (Hotez et al., 1992), in contrast to the mammalian hyaluronidases, do not cleave other GAGs. The products of hyaluronan degradation are again tetrasaccharides and hexasaccharides but with glucuronic acid at the reducing end.

EC 4.2.2.1, microbial hyaluronidases (also called β-eliminases or lyases) catabolize Hyaluronic acid (HA) is a mucopolysaccharide and found primarily in the extracellular matrix and pericellular matrix. The polysaccharide hyaluronan is a linear poly-anion, with a poly repeating disaccharide structure [(1$\longrightarrow$3)-β-d-GlcNAc-(1$\longrightarrow$4)-β-d-GlcA-]. The uronic acid and amino sugar in the disaccharide are d-glucuronic acid and d-N-acetyl- glucosamine that are linked

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together through alternating β-1,4 and β-1,3 glycosidic bonds. Its function in the body is, amongst other things, to bind water and to lubricate movable parts of the body, such as joints and muscles. Its consistency and tissue-friendliness allows it to be used in skin-care products as an excellent moisturizer. HA is among the most hydrophilic molecules in nature and has been described as nature's moisturizer.

HA an essential structural element, elicits its key role in tissue architecture via immobilization of specific proteins in desired locations within the body. Its degradation by hyaluronidase induces angiogenesis and show inflammatory effects as allergies. High level of HA deposition enhances tumor growth and even degradation of HA by hyaluronidase can cause depletion of the extracellular matrix and support cancer cells by leaving primary tumors and forming metastasis. So a balance in the HA concentration is of the utmost importance (Olgen et al., 2011).

As mentioned above, hyaluronidase inhibitors can be used as anti-ageing and anti-wrinkling agents in cosmetics; anti-allergic or anti-inflammatory cases; as prophylactic measure to prevent spreading of HIV and other sexually transmitted diseases, also as anti-tumor agents etc. (Oztekin et al., 2008). Several phytochemicals were reported to be potent hyaluronidase inhibitors (Machiah et al., 2006). Tannic acid and quercetin have been shown to bring about 94 and 100% inhibition respectively of hyaluronidase purified from Indian cobra (*Najanaja*) venom (Girish and Kemparaju, 2005). Meyer and Rapport (1951) reported that hyaluronidase are inhibited by iron, copper and zinc salts, heparin, polyphenols and flavonoids. Due to the structural similarity of heparin and heparan sulfate to hyaluronic acid, these oligosaccharides were investigated as inhibitors of hyaluronidase, but the inhibition was achieved only at concentrations much higher than occurs at the physiological levels. Asada et al., (1997) reported the effect of various types of alginic acid consisting of L-glucuronic acid and D-mannuronic acid on bovine testicular hyaluronidase. The inhibition by sodium alginate was dependent on the molecular weight with the higher the molecular weight the stronger the inhibition. Based on these results, Toida et al., (1999) investigated *O*-sulfated glycosaminoglycans of which the fully sulfated compounds showed the highest inhibitory effect. Also some flavones and flavone analogues including

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apigenin and kaempferol were shown to inhibit hyaluronidase (Kakegawa et al., 1992), but not selectively and only at millimolar concentrations.

2.9 α-glucosidase and inhibitor

α-glucosidase (EC.3.2.1.20, maltase) is an enzyme located in the brush border surface membrane of intestinal cells thathydrolyzes α-1,4 glycosidic bond of carbohydrates such as starch, glycogen and disaccharides to glucose by residues to release a single α-glucose molecule (Chiba, 1997). α-glucosidase converts glucose to glycogen in muscle and liver for storage.

Defects in insulin secretion, insulin action or both can result in diabetes mellitus (DM), which is a metabolic disorder of multiple aetiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism (WHO, 1999). Type II DM, also referred to as maturity-onset diabetes, results from defective insulin secretion, mostly accompanied by insulin resistance. It accounts for over 90% of all diabetic cases (ADA, 2005). The majority of patients with Type II DM tend to be obese and have an increased percentage of body fat distributed predominantly in the abdominal region. Obesity itself causes or aggravates insulin resistance (Campbell et al., 1993). Genetic predisposition is a high risk factor to its development (Kahn et al., 2001). There is no β-cell antibody activity in patients affected with Type II DM and insulin is not required for survival. This form of diabetes is frequently undiagnosed for many years because the hyperglycaemia is often not sufficiently severe to provoke noticeable symptoms of diabetes. Nevertheless, such patients are at increased risk of developing macrovascular and microvascular complications.

The α-glucosidase inhibitors are also anti-hyperglycemic, as they lower postprandial hyperglycemia. The inhibition α-glucosidase may reduce or delay the absorption of glucose in the digestive track and hence, its level in the blood stream. Such delayed rates can aid in the management or regulation of Type 2 diabetes (Sheliya et al., 2015). The most commonly used drugs for inhibiting α-glucosidase, and consequently for treating Type 2 diabetes, are acarbose, miglitol and voglibose. However, these drugs may cause side-effects (Dabhi et al., 2013).

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There is renewed interest in functional foods and plant-based medicines modulating physiological effects in the prevention and cure of diabetes and obesity. This is particularly beneficial in Type II diabetes patients, as glucose release from dietary sources is the primary cause of post-prandial hyperglycemia which worsens glycemic control. The side-effects associated with the use of insulin and oral hypoglycemic agents have also led to an increase in the demand for alternative approaches to treat diabetes (Marles and Farnsworth, 1994). The World Health Organization has recommended that traditional treatments based on plant extracts for diabetes control should be further evaluated (Singh et al., 2000), with the provision of scientific proof for the basis of their activity. Previous studies also showed the α-glucosidase and amylase inhibition as mechanisms of anti-diabetic activity by various plant extracts. The inhibition of these enzymes leads to a decrease in blood glucose level following a meal. Compounds responsible for anti-diabetic activity in plants include complex carbohydrates, alkaloids, glycopeptides, terpenoids, peptides, steroids, flavonoids, lipids, coumarines, sulphur compounds and inorganic ions (Marles and Farnsworth, 1996). Recent reports have shown that polyphenols can affect glucose utilization (Bhandari et al., 2008), even though the health benefits of polyphenols have been attributed mainly to their antioxidative properties, including scavenging free radicals, chelating redox metals, inhibiting oxidative enzymes and/or activating antioxidative enzymes (Madhujith et al., 2005; Oomah et al., 2010; Kim et al., 2000; McDougall et al., 2005). Moreover, previous studies have found that polyphenols and flavonoids are among the natural active antidiabetic agents (Andrade-Cetto et al., 2008). These compounds have been reported to exert various biological effects, including inhibition of carbohydrate hydrolyzing enzymes. Polyphenolic compounds have also been shown to inhibit the activity of digestive enzymes due to their ability to bind with proteins. The inhibitory activities of plant phytochemicals, including polyphenols, against carbohydrate hydrolyzing enzymes contribute to the lowering of postprandial hyperglycemia in the management of diabetes, as observed *in vivo* (Mai et al., 2007). The methanolic extracts of Cyperus rotundus L. tubers exhibited a potent inhibition of α-glucosidase activity with IC_{50} value of 3.98 μg/mL which was more active than voglibose (Bachhawat et al., 2011). Moreover, Tunsaringkarn et al., (2009) also reported that the aqueous

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extract from the branches of *Albizia myriophylla* exhibited 9% inhibitory effect on the α-glucosidase at a concentration of 1 mg/mL.

2.10 Lipidoxidation

Lipids containing high levels of polyunsaturated fatty acids are highly susceptible to oxidation, including photo-oxidation, during exposure to light. They are also susceptible to enzymatic oxidation, when exposed to lipooxygenases, and auto-oxidation, which is the direct reaction of molecular oxygen with organic compounds (Frankel, 2005). The composition of fatty acids, oxygen concentration, temperature, surface area, water activity, antioxidants and pro-oxidants are affected to the degree of lipid oxidation. (Fennema et al., 2007a). Auto-oxidation is the primary oxidation of lipids, since photo-oxidation only occurs when lipids are exposed to direct sunlight or fluorescent light (List et al., 2005) and enzymatic oxidation is also of less concern in lipids because lipooxygenases are inactivated by heating during refining or processing (Fennema et al., 2007a). The process of lipid oxidation can be described in three general steps: initiation, propagation and termination.

Initiation: In lipids the initiation step involves abstraction of a hydrogen atom from a fatty acid or acylglyserol, to form an alkyl radical ($R^{\bullet}$). Stabilization through delocalization of the double bond occurs once the alkyl radical has been formed. This leads to changes in the conformation of the double bonds in *cis*- and *trans*-configurations, with the more stable *trans*-configuration predominating. In the case of polyunsaturated fatty acids, conjugated double bonds are rapidly formed upon the abstraction of hydrogen in the initiation step (Fennema et al., 2007a). Chaiyasit et al., (2007) summerized the initiation step of lipidoxidation in equation 2.1

$$RH \longrightarrow R^{\bullet} + H^{\bullet} \qquad (2.1)$$

Propagation: The oxidation process proceeds to the propagation step as the alkyl radical reacts with oxygen. The oxygen molecule can exist in several states and both the singlet and the triplet state are involved in the oxidation of lipids.

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The singlet oxygen ($^1O_2$) has an empty outer anti-bonding orbital. The $^1O_2$ is seeking to fill this empty orbital, which makes it a highly reactive electrophile capable of reacting directly with unsaturated fatty acids. The $^1O_2$ is a substrate in the oxidation process and promotes the formation of a fatty acid radical, one type of alkyl radicals. In the triplet state ($^3O_2$) the two outer anti-bonding orbitals contain a single electron each, with the same spin direction. The low energy state and the oxygen will not be able to abstract a hydrogen atom directly. However, one of the available electrons in $^3O_2$ can interfere with the alkyl radical at a diffusion-limited rate and form a covalent bond. Interference of the $^3O_2$ electron and alkyl radical leads to the formation of a high-energy peroxyl radical ($ROO^{\cdot}$). The high-energy radical promotes abstraction of a hydrogen atom from a proximate fatty acid. When a hydrogen atom is gained, a fatty acid hydroperoxide (ROOH) is formed. However, at the same time a new alkyl radical will be formed. In this way the propagation step proceeds through repetitive chain reactions (Fennema et al., 2007b). Chaiyasit et al., (2007) summarized the propagation step of lipidoxidation in equation 2.2 and 2.3.

$$R^{\cdot} + {}^3O_2 \longrightarrow ROO^{\cdot} \qquad (2.2)$$

$$ROO^{\cdot} + RH \longrightarrow ROOH + R^{\cdot} \qquad (2.3)$$

Termination. In the termination step two radicals are formed a non-reactive unit. During conditions of oxygen excess, peroxyl radicals will join to make the termination product. This is a result of oxygen being added to the alkyl radical and leaves peroxyl as the main radical in the reaction. In conditions of low oxygen levels, the termination products are a result of interference between alkyl radicals, creating fatty acid dimers (Fennema et al., 2007b). Chaiyasit et al., (2007) summarized the termination step of lipidoxidation in equations 2.4 and 2.5.

$$ROO^{\bullet} + R^{\bullet} \longrightarrow ROOR \qquad (2.4)$$

$$R^{\bullet} + R^{\bullet} \longrightarrow RR \qquad (2.5)$$

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2.10.1 Polyphenol compounds in retarding lipidoxidation in fish product

Fish muscle is susceptible to lipid oxidation because of its unsaturated long chain polyunsaturated fatty acids (Frankel, 2005). The quality of fish muscle including flavour, odour, colour, texture and the nutritional value are decreased by the lipidoxidation process during processing and storage (Tichivangana and Morrissey, 1985). Lipid oxidation in fish occurs rapidly after death and continues during the processing, giving the fish a rancid smell.

Numerous studies have emphasized the potential of polyphenols from plants as antioxidant and as well as in the control of microbial growth and the improvement of the shelf-life of various foods, since polyphenols may show some inhibition of the enzyme activity involved in food alteration (Banerjee, 2006).

Antioxidant property is dependent on their ability to scavenge free radicals and/or to chelate metals of polyphenols (Galati et al., 2006). Iron binds to polyphenols via the ortho-dihydroxy-(catechol) or trihydroxy-benzene (galloyl) groups. Lopes et al., (1999) demonstrated that tannins can also form complexes with Fe^{+2} for example $(Fe^{+2})_n$-tannic acid. It has also previous reported that the effectiveness of flavonoids in retarding lipid oxidation is related to their free-radical scavenging (Jovanovic et al., 1994) or metal-chelating ability (Ramanathan and Das, 1993). Various food products, including unsaturated marine oils, meat and fish muscle, have been evaluated for their antioxidant activities from a wide range of plant polyphenols (He and Shahidi, 1997; Wanasundara and Shahidi, 1998).

Plant polyphenol such as tea catechins, olive oil hydroxytyrosol, rosemary extracts and grape procyanidins have been observed to prevent lipid oxidation in fish muscle-based food products (Tang and et al., 2001a; Pazos and et al., 2006). Grape polyphenols have been reported to delay lipid oxidation in minced fatty fish muscle during frozen storage (Pazos et al., 2005a) and preserving endogenous antioxidant systems (e.g. vitamin E) in fish muscle (Pazos et al., 2005b). Tang et al., (2001) demonstrated that the antioxidant activity of tea catechins was double that of α-tocopherol at the same concentration when added to mackerel or whiting minced fish. Tea catechins at concentrations over than 300 mg/kg were observed to reduce the oxidation in mackerel patties containing high levels of lipids and unsaturated fatty acids. Tea catechins

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show radical scavenging capacities and have high affinity for the lipid bilayers of muscle (Tang and et al., 2001). Tannic acid showed superior radical scavenging activity because of its reducing power and effectively inhibited the lipid oxidation in fish mince and fish oil-in-water emulsion (Maqsood and Benjakul 2010).

Maqsood and Benjakul (2010) showed that phenolic compounds including ferulic acid, caffeic acid and tannic acid have preventive effects on lipid oxidation of fish mince during iced storage. Also caffeic acid showed good antioxidant activity in preserving fish muscle against oxidation and could retard off-flavor development and production of peroxides and TBARS (Medina and et al., 2007).

2.11 Protein oxidation

Proteins in food product are subject to oxidation reactions. During food processing and storage, proteins can be modified via oxidation, glycation and glycoxidation reactions. Stadtman et al., (2003) reported that protein oxidation occurs by reactive oxygen species (ROS) generated from metabolic processes or by external factors such as oxygen availability, heating, fermentation, chemicals, light (photochemical reactions), air pollutants and irradiation (γ-irradiation, x-rays and UV radiation). Free radical species can react directly with the protein or they can react with other molecules such as lipids and carbohydrates, forming products that subsequently react with the protein (Figure 2.15). Thus, protein oxidation affects to physicochemical and functional properties are changed and may even result in formation of toxic compounds (Rice-Evans et al., 1993).

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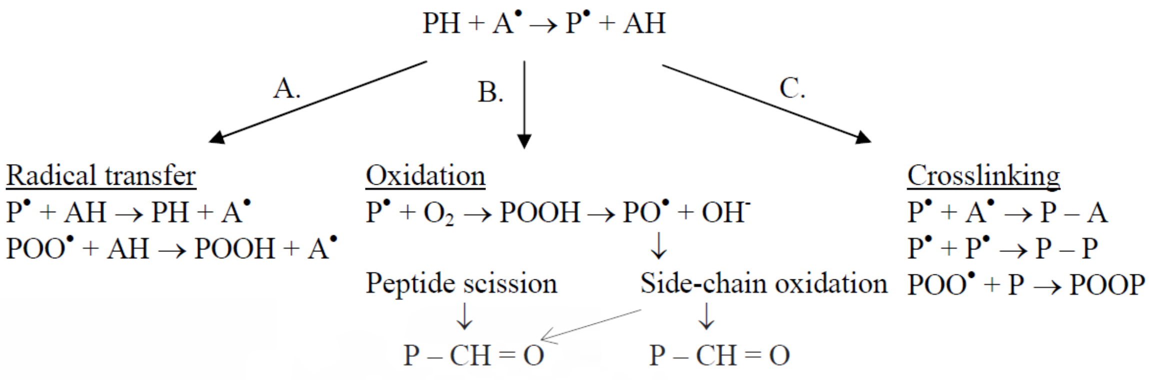

Figure 2.15 Protein oxidation pathways via A) free radical transfer, B) oxidation, and C) crosslinking = any molecule with abstractable hydrogens, PH = protein, $P^{\bullet}$ = protein radical, AH $A^{\bullet}$ = non-protein radical, $PO^{\bullet}$ = alkoxyl radical, $POO^{\bullet}$ = peroxyl radical, POOH = hydroperoxide, P-CH=O = secondary products such as aldehydes (Adapted from Karel et al., 1975 and Schaich, 2008).

Protein radicals ($P^{\bullet}$) are formed when lipid peroxyl and alkoxy radicals arise from lipid hydroperoxides and transfer free radicals to proteins by abstracting hydrogen (Karel et al., 1975) (Figure 2.17A). Protein hydroperoxides ($POO^{\bullet}$) and other protein radicals ($P^{\bullet}$) are highly reactive, and thus oxidize to secondary compounds (Davies et al., 1995). The peptide bond in the backbone of the protein or the side-chains of the amino acids may be the target for amino acid modifications. The oxidative modification can cause cleavage of the protein backbone and crosslinking of the side-chains. These reactions are usually strongly influenced by redox cycling metals such as iron and copper. In addition, protein radicals can also transfer radicals to other proteins, lipids, carbohydrates, vitamins and other molecules, especially in the presence of metal ions. Radical transfer occurs early in lipid oxidation and this process underlies the antioxidant effect for lipids. Consequently, it may appear that lipid oxidation is not proceeding whereas the radical transfer to proteins is in its highest (Schaich, 2008).

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The general reaction for free radical crosslinking usually generates polymers of intact protein monomers, with or without oxygen bridges (Figure 2.17C) (Schaich, 2008). Stadtman (2006) reported that oxidative modifications of proteins generating intramolecular and intermolecular crosslinks and can occur by different mechanisms:

1. Direct interaction of two carbon-centered radicals

2. Interaction of two tyrosine radicals

3. Oxidation of cysteine sulfhydryl groups

4. Interactions of the carbonyl groups of oxidized proteins with the primary amino groups of lysine side-chains in the same or different protein

5. Reactions of both carbonyl groups of malonaldehyde with two different lysine side-chain in the same or two different protein molecules

6. Interactions of glycation/glycoxidation derived protein carbonyls with either a lysine or an arginine side-chain of the same or a different protein molecule

7. Interaction of a primary amino group of lysine side-chain with protein aldehydes obtained via Michael addition reactions with the lipid aldehydes such as 4-hydroxy-2-alkenal (HNE).

2.11.1 Effect of polyphenols to retarding protein oxidation

Polyphenols act as antioxidants by donating electrons and terminating radical chain reactions (Dangles et al., 2006) and by binding metals as metal chelators (Fernandez et al., 2002). The role of polyphenol antioxidants on protein oxidation has been reported in different oxidation models such as oil-in-water emulsions, meat, liposomes and low-density lipoproteins (LDL) (Almajano et al., 2007; Yeomans et al., 2005). Berry phenolics have been shown to be antioxidants in oil-in-water emulsions. Viljanen et al., (2005a) explained that anthocyanins isolated from black currants, raspberries and lingonberries exhibited protection toward protein oxidation in whey protein stabilized rapeseed oil-in-water emulsion. Also, the antioxidant activity of raspberry and blackberry juices were evaluated in whey protein stabilized rapeseed oil-in-water emulsions by Viljanen et al., (2005b). Raspberry juice gave better overall antioxidant protection

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towards lipid and protein oxidation compared to blackberry juice. The different berry phenolics such as anthocyanins, ellagitannins, and proanthocyanidins from raspberries, bilberries, lingonberries and black currants, as well as pure compounds of procyanidins, anthocyanins and their aglycons were found to inhibit protein oxidation in α-lactalbumin-lecithin liposome system (Viljanen et al., 2004a; 2004b).

Chen et al., (2007) showed that, depending on the concentration, polyphenolics extracted from the testa of almond seeds exhibited 6.7-76% inhibition toward tryptophan oxidation in human LDL. Red wine phenolics and phenolic compounds with a catechin structure (catechin, epicatechin and quercetin) prevented LDL tryptophan from oxidation by 30-40% under peroxynitrite-mediated oxidation of LDL. The combination of quercetin and caffeic acid inhibited tryptophan oxidation and formation of protein carbonyls in copper-induced oxidation of human LDL (Yeomans et al., 2005).

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CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

- Gallic acid (Sigma-Aldrich, USA)

- 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Trolox ® (Sigma-Aldrich, USA)

- 2,2-diphenyl-1-picryhydrazyl radical (Sigma-Aldrich, USA)

- Acetic acid (Merck, Germany)

- Ethanol 95 % (LAB-Scan, Island)

-Methanol (LAB-Scan, Island)

- Sodium carbonate (Merck, Germany)

- Folin-Ciocateau reagent (Sigma-Aldrich, USA)

- Tyrosinase (Fluka, Swizerland)

- 5-Lipoxygenase (Bioscense, Norway)

- Hyaluronidase (Sigma-Aldrich, USA)

-α-Glucosidase (Sigma-Aldrich, USA)

- Sodiumhyaluronate (Sigma-Aldrich, USA)

- Dimethyl sulfoxide, DMSO (Merck, Germany)

-2,2-azino-bis(3-ethylbenzothiazoline (Sigma-Aldrich, USA)

-6-Sulfonic acid (Sigma-Aldrich, USA)

- Arbutin (Sigma-Aldrich, USA)

- L-DOPA (Sigma-Aldrich, USA)

- Rutin (Merck, Germany)

- Sodiumlinoleate (Merck, Germany)

- Calciumchloride (Sigma-Aldrich, USA)

- Sodiumborate (Sigma-Aldrich, USA)

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- Potassium chloride (Sigma-Aldrich, USA)
- Trichloroacetic acid (Merck, Germany)
- Hydrochloric acid (Sigma-Aldrich, USA)
- 2,4-dinitrpphenylhydrazine (DNPH) (Merck, Germany)
- Ethyl acetate (Sigma-Aldrich, USA)
- Sodium phosphate, dibasic (Sigma-Aldrich, USA)
- Guanidine hydrochloride (Sigma-Aldrich, USA)
- Bovine serum albumin (BSA) (Merck, Germany)
- Sulfanilamide (Sigma-Aldrich, USA)
- 6 2-thiobarbituric acid (Sigma-Aldrich, USA)
- Sodiumhydroxide (Sigma-Aldrich, USA)
- Sephadex LH 20 (Sigma-Aldrich, USA)
- Ferric chloride (Sigma-Aldrich, USA)
- Ferrozine (Sigma-Aldrich, USA)
- BHT (Sigma-Aldrich, USA)

3.2 Equipments

- UV-Vis spectrophotometer (Shimadzu UV-1601, Japan)
- Ultrasonication (JAC Ultrasonic 2010P; Jinwoo Engineering Co., Ltd., Hwasung, Korea)
- Colormeter (Minolta CR-400, Japan)
- pH meter couple with Mettler LE438 probe (Mettler Toledo, delta 320, USA)
- Centrifuge (Harmle Z233 MK-2, Germany)
- Water activity meter (AquaLab™ Series3TE, USA)
- Rotary evaporator unit (RII, Büchi Rotavapor, USA)
- Homogenizer (Ultra-Turrax® T25Bbasic, Germany)
- Meat grinder (SevenFive, Thailand)
- Microplate Reader (Multimode detector, USA)
- Moulinex (Mexico

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3.3 Raw materials

- Mango (*Mangifera indica L.*); Kaew and Choke-Anan
- Clown featherback; (*Chitala ornate*)
- Striped catfish *(Pangasianodon hypophthalmus*)

3.3.1 Mango varieties

The 2 varieties of mango Used in this study Kaew and Choke-Anan were obtained from a local orchard in Nakornratchasima province, Thailand during March to May, 2013-2017. The samples were randomly selected based on size (4-5 mangoes/kg) and all were judged to be mature green maturity. Mango peel and fresh pulp were removed from the fruits by hand and the seeds were washed and stored at -18°C for no longer than 4 months. Before using, the kernels were separated from their shells.

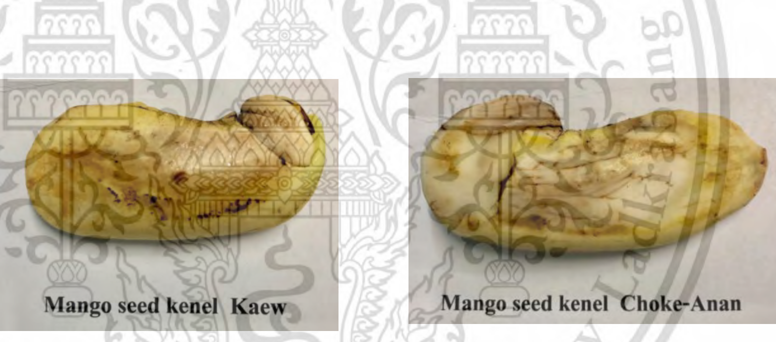

Figure 3.1 Mango seed kernel from 2 varieties Kaew and Choke-Anan

3.4 Methodology

3.4.1 Antioxidant properties sand selected enzyme inhibition capacities of mango seed kernel extracts

3.4.1.1 Preparation of mango seed kernel extracts (MSKEs)

Preparation of crude MSKEs by ultrasonic assisted extraction was described by Ghafoor and Choi, (2009). Briefly, samples were ground. 1 g of samples were blended with 95% ethanol (100 ml) in a blender (Moulinex, Mexico) for 5 min. The samples were

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incubated in a sonication water bath (JAC Ultrasonic 2010P; Jinwoo Engineering Co., Ltd., Hwasung, Korea), with the frequency fixed at 20 KHz, at a temperature of 25 ^{o}C for 15-60 min. Samples were further incubated in a water bath at 80°C for 1 hour. The mixtures were cooled at room temperature and the supernatant passed through No.4 Whatman filter paper. All filtrates were evaporated in a rotary evaporator (RII, BüchiRotavapor, USA) at 50 ^{o}C under vacuum until dry (70% solid) and the extracts were weighed to determine the yield of soluble components.

3.4.1.2 Determination of Total Polyphenol Contents (TPC)

The total polyphenol contents were determined by using FolinCiocalteu's phenol reagent (modified from Singleton and Lamuela, 1999). Briefly, each crude extract 0.5 g was dissolved in 20 ml dimethylsulfoxide (DMSO). The sample 0.5 ml was mixed with 0.5 ml of FolinCiocalteu's phenol reagent and adjusted to 10 ml with distilled water. After mixing for 5 min, 0.8 ml of 10% (w/v) sodium carbonate was added. The mixtures were agitated and incubated for 10 min at room temperature in the dark. The absorbance was measured at 765 nm on a spectrophotometer (Shimadzu UV-1601, Japan). The total polyphenol contents of the samples were expressed as milligram of gallic acid equivalent (GAE) per gram of MSKE (mg GAE/g MSKE) by using a linear equation. All determinations were performed in triplicate.

3.4.1.3 DPPH radical scavenging assay

The radical scavenging activity of MSKEs was measured by using the stable free radical, DPPH (2, 2-diphenyl-2-picrylhydrazyl), described by Murakami et al., (2004). DPPH (0.8 mM) in ethanol was prepared and this solution 0.6 ml was added to the 4.4 ml of sample solutions in ethanol. After 30 min incubation in the dark at room temperature, the absorbance was measured at 517 nm with a spectrophotometer (Shimadzu UV-1601, Japan). The DPPH scavenging ability of the samples was expressed as milligram of Trolox® equivalent per gram of MSKE (mgTrolox/gMSKE) by using a linear equation. All determinations were performed in triplicate.

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3.4.1.4 Ferric reducing antioxidative power assay (FRAP)

Ferric reducing antioxidative power from MSKEs was measured by using FRAP reagents as described by Benzie et, al. (1999). FRAP reagent was freshly prepared by mixing 25 ml acetate buffer (3 mM, pH 3.6), 2.5 ml 2,4,6-tris (2-pyridyl)-S-triazine (TPTZ) solution (10 mM TPTZ in 40 mMHCl) and 2.5 ml $FeCl_3$ (20 mM) water solution. Each sample (150 µl, 0.5 mg/ml dissolved in ethanol) was added in 4.5 ml of freshly prepared FRAP reagent and stirred and after 5 min, the absorbance was measured at 593 nm, by a spectrophotometer (Shimadzu UV-1601, Japan). A calibration curve of Trolox® in various concentrations was used and results were expressed as milligram of Trolox® equivalent per gram of MSKE (mg Trolox/g MSKE). All determinations were performed in triplicate.

3.4.1.5 Hydrogen peroxide scavenging assay

Hydrogen peroxide scavenging assay of MSKEs was measured by using the method of Yen and Chen (1995). H_2O_2 solution 4 mM was prepared in phosphate buffer (0.1 M, pH 7.4). The 4 ml sample was mixed with 0.6 ml of H_2O_2 solution. The mixture was incubated for 10 min. The absorbance of the solution was taken at 230 nm by a spectrophotometer (Shimadzu UV-1601, Japan) against a blank solution containing the plant extract in phosphate buffer without H_2O_2. The H_2O_2 scavenging activity was expressed as milligram of trolox® equivalent per gram of MSKE (mg Trolox/g MSKE) by using a linear equation. All determinations were performed in triplicate.

3.4.1.6 ABTS radical scavenging assay

The ABTS radical cation-scavenging activity of MSKEs was measured by using 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as described by Re et, al. (1999). The ABTS radical cat-ion was produced by reacting 7 mM ABTS stock solution with 2.45 mMpostassiumpersulphate solution (1:1) and allowed the mixture to stand in the dark at room temperature for 12 hours before use. The mixture of ABTS radical cat-ion solution was diluted with deionized water and 95 % ethanol (1:1) to retain absorbance of 0.7$\pm$0.02 at 734 nm

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by spectrophotometer (Shimadzu UV-1601, Japan). The 20 µl extract was mixed with 6 ml of diluted ABTS radical cat-ion solution. The decrease of absorbance was recorded at 6 min after mixing. Trolox® was used as a standard. The ABTS scavenging activity of the samples was expressed as milligram of Trolox® equivalent per gram of MSKE (mg Trolox/g MSKE) by using a linear equation. All determinations were performed in triplicate.

3.4.1.7 Determination of tyrosinase inhibition activity

The tyrosinase inhibition activity of MSKEs was measured using the modified dopachrome method with Mushroom tyrosinase and L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate (Masuda et al., 2005) and their effect on the enzyme was determined based on dopachrome formation at 450 nm using a 96-well reader. Samples were dissolved in 50% dimethylsulphoxide (DMSO) at various concentrations. Each well contained 40 µl of sample with 80 µl of phosphate buffer (0.1 M, pH 6.8), 40 µl of tyrosinase (100 units/ml) and 40 µl of L-DOPA (2.5 mM). Each sample was accompanied by a blank that had all the components except L-DOPA. Results were compared to arbutin as a reference standard. The percent inhibition of tyrosinase was calculated according to formula % inhibition activity = [{Abs control - Abs sample}/Abs control] × 100. The inhibitory effect of the samples was expressed as the inhibitor concentration causing 50% loss of enzyme activity (IC_{50}). All determinations were performed in triplicate.

3.4.1.8 Determination of 5-Lipoxygenase inhibition activity

5-Lipoxygenase inhibition activity of MSKEs was studied using linoleic acid as substrate as described by Shinde et al., (1999). The reaction was initiated by the addition of aliquots (50 µl) of a soybean lipoxygenase (1000 units/ml) solution (prepared daily in potassium phosphate buffer 1 M pH 9.0) and 2.0 ml of sodium linoleate (100 µM) in phosphate buffer. The samples (30 µl) which dissolved in DMSO at various concentrations were added. The mixture was incubated for 5 min. The absorbance of the solution was taken at 234 nm on a spectrophotometer (Shimadzu UV-1601, Japan). Results were compared to rutin as reference

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standard. The percentage inhibition of 5-lipoxygenase was calculated according to the formula; % inhibition activity = [{Abs control - Abs sample}/Abs control] × 100. The inhibitory effect (%) of the samples was expressed as the inhibitor concentration causing 50% loss of enzyme activity (IC_{50}). All determinations were performed in triplicate.

3.4.1.9 Determination of hyaluronidase inhibition activity

Hyaluronidase inhibition activity was determined by measuring the amount of N-acetyl glucosamine that was split from sodium hyaluronate as described by Lee et al., (2001). The assay medium consisted of 50 µl of hyaluronidase (1,000 units/ml prepared in 0.1 M acetate buffer, pH 3.5), 50 µl of various concentrations of MSKE dissolved in 5% dimethylSulfoxide (DMSO) and 12.5 mM calcium chloride. The medium was incubated for 10 min at 37°C. The reaction was then initiated by the addition of 250 µl of sodium hyaluronate as substrate (1.2 mg/ml dissolve in 0.1 M acetate buffer, pH 3.5) and then added 1.5 ml of p-Dimethylaminobenzaldehyde (4 g PDMAB dissolved in 350 ml of Glacial acetic acid and 50 ml of 10 N HCl) to the reaction mixture and incubated for 20 min at 37°C. The absorbance was measured at 585 nm by a spectrophotometer (Shimadzu UV-1601, Japan). Results were compared to vitamin C as reference standard. The percent inhibition of hyaluronidase was calculated according to formula; % inhibition activity = [{Abs control - Abs sample}/Abs control] × 100. The inhibitory effect (%) of the samples was expressed as the inhibitor concentration causing 50% loss of enzyme activity (IC_{50}). All determinations were performed in triplicate.

3.4.1.10 Determination of α-glucosidase inhibition activity

The alpha-glucosidase inhibition activity of MSKEs was measured according to Apostolidis et al., (2011). The reaction contained dilutions of the MSKE (0-200 µL) and 100 µL of α -glucosidase (1000 units/ml 0.5 mg/mL) in phosphate buffer (0.1 M, pH 6.9) solution and incubated at 25°C for 10 min. Then, 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside in phosphate buffer (0.1M, pH 6.9) solution was added. The mixtures were incubated at 25°C for 5 min, before reading the absorbance at 405 nm in the spectrophotometer

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(Shimadzu UV-1601, Japan). Arcabose was used as reference standard. The alpha-glucosidase inhibition activity was expressed as percentage inhibition; % inhibition activity = [{Abs control - Abs sample}/Abs control] × 100. The inhibitory effect (%) of the the samples was expressed as the inhibitor concentration causing 50% loss of enzyme activity (IC_{50}). All determinations were performed in triplicate.

3.4.2 Fractionation, antioxidant and inhibitory activities of selected enzymes of fractions from mango seed kernel extracts (MSKEs) and identification by LC-ESI-MS

3.4.2.1 Fractionation of mango seed kernel extract

The MSKE. Samples (1 g) were dissolved in 5 ml methanol and applied to a Sephadex LH-20 column chromatography (35 mm diameter x 450 mm high). The flow rate was 1 ml/min. Methanol fractions (10 ml) were collected in test tubes. Eluted fractions were then pooled based on their elution profiles. Their absorbance was measured at 280 nm, using a spectrophotometer (Shimadzu UV-1601, Japan). After evaporation of methanol, the samples were weighed to determine the yield of different fractions. Each fraction was used to determined the total polyphenol content (TPC), antioxidant activity, inhibitory activity of tyrosinase, 5-lipoxygenase, hyaluronidase and α-glucosidase activity acording to methods as mention above.

3.4.2.2 Identification of polyphenols by LC-ESI-MS

The fractions with the highest bioactivities were identified as the main polyphenol components by LC-ESI-MS. Electrospray ionization mass spectrometric analysis of polyphenol compounds in fractions of MSKE was performed to identification using an applied TSQ Quantum Ultra-LCMS (Thermo Fisher, San Diego, CA, USA) based the method described by Abdullah et al., (2015). The mass spectra were operated in positive electrospray ionization (ESI) modes with high resolution up to 3000 Daltons. The spray voltage used was 3500 V. The sheath/auxiliary/sweep gas was 99% nitrogen and sheath gas pressure was 30 psi with 5 psi for auxiliary gas pressure. The capillary temperature was 270 °C. The injection volume was 10 μL

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and the flush speed was100 μL/s. In the MS analysis (full scan), data were collected over a mass of 50 to 2000 m/z.

3.4.3 Application of mango seed kernel extract on retarding lipid oxidation and protein oxidation of mince fish during Frozen storage

3.4.3.1 Preparation of mince fish

Fresh Clown featherback (*Chitala ornate*) and Striped catfish (*Pangasianodon hypopthalmus*) weighing between 300g and 500g were purchased from the Fish Marketing Organization in Bangkok within 12 hours of being killed and stored with ice. These samples were washed with cold water, gutted, the skin removed, de-boned and minced at room temperature in a mincer and mixed 70:30 (w/w) of Clown featherback:Striped catfish. The minced fish were divided into 500 g samples and each sample was mixed with 1, 2 or 3% w/w crude MSKE, water as a control (0% MSKE) or 0.01% w/w BHT (butylated hydroxytoluene) as standard reference. There were 18 samples prepared for each treatment, giving 6 sampling dates (0, 3, 6, 9, 12 or 15 weeks) and 3 replicates for each treatment. The samples were then packed individually in polyethylene bags and stored at -18°C. Samples were taken, after each time period, for analysis as follows:

3.4.3.2 Determination of pH

Each 1 g of sample was homogenized with 10 ml distilled water for 1 min and then pH was determined using a digital pH meter (Digi-Sense 5938-10, Chicago, USA)

3.4.3.3 Determonation of color

Color parameters were measured in a colormeter (Minota CR 400, Japan) at room temperature and operated in the CIE system, *L* is lightness (0 = black; 100 = white), *a* is redness and *b* is yellowness. The colorimeter was standardized using a white tile

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(Illuminant D65) and the measurements were made through an 8 mm port viewing area on the surface of the samples.

3.4.3.4 Lipid extraction

Lipids were extracted according to the method of Bligh and Dyer (1959). The mince (25 g) was mixed in an homogenizer (Ultra-Turrax T25, Germany) with 200 ml of chloroform:methanol:distilled water (1:2:1) at 9,5000 rpm for 2 min at 4 $^{\circ}$C. 50 ml of chloroform was then added and homogenized at 9,500 rpm for 1 min and then 25 ml of distilled water was added and homogenized again at 9,500 rpm for 30 s. Each sample was centrifuged at 14,500xg at 4 $^{\circ}$C for 15 min. The chloroform phase was drained off into an Erlenmeyer flask containing about 5 g of anhydrous sodium sulfate, shaken well and filtered into a round bottom flask through a Whatman No. 4 filter paper. The solvent was evaporated at 25 $^{\circ}$C in a rotary evaporator at 50 $^{\circ}$C.

3.4.3.5 Determination of peroxide value

Peroxide value of the minces were determined according to the method of Grunwald and Richard, (2006). Samples were mixed with 50 μl, 2.35 ml of 75% ethanol, 50 μl of 30% ammonium thiocyanate and 50 μl of 20 mM ferrous chloride solution in 3.5 % HCl. After 3 min, the absorbance of the colored solution was measured at 500 nm using a spectrophotometer (Shimadzu UV-1601, Japan). The increasing absorbance at 500 nm indicated the formation of peroxide.

3.4.3.6 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS was measured using the method described by Maqsood et al., (2012). Samples (0.5 g) were mixed with 2.5 ml of a TBA solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl. The mixture developed a pink color when heated in a boiling water for 10 min. It was then cooled with running tap water and

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sonicated for 30 min using an Elma (S 30 H) Sonicator (Kolpingstr, Singen, Germany) and centrifuged at 5000g at 25 °C for 10 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared at the concentrations ranging from 0 to 10 ppm using 1,1,3,3-tetramethoxypropane (MAD). TBARS were expressed as mg of MAD equivalents/kg sample.

3.4.3.7 Determination of Conjugated diene (CD)

CD was measured according to the method of Frankel et al., (1996). The extracted oil (0.1 ml) was dissolved in 5.0 ml of methanol and the absorbance then measured at 234 nm using a spectrophotometer. The CD was measured as the increase in absorbance at 234 nm.

3.4.3.8 Determination of protein oxidation (total carbonyls) by 2,4 dinitrophenyl-hydrazones of mince fish

Protein carbonyls were measured using the method described by Levine et al., (1994). A sample of mince (0.5 g) was homogenised in 10 ml of tris-buffer (pH: 7.4, 50 mM, 1 mM EDTA) containing 0.01% BHT and 100 ml of the homogenate was precipitated with TCA. After centrifugation (12,000g, 3 min) the pellet was incubated in the dark for 30 min with dinitrophenylhydrazine in 2 M HCl. The samples were precipitated with TCA and the pellets were washed three times with 1 ml ethanol : ethyl acetate (1:1 v/v). The pellet was re-dissolved in 6 M guanidine chloride in 20 mM KH_2PO_4. The carbonyl content was determined by measuring the absorbance at 370 and 280 nm. Results are expressed in µmoles carbonyl per mg of soluble protein.

3.4.3.9 Determination of sulfphydryl groups of mince fish

Total sulphydryl contents were determined using 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) according to the method of Ellman et al., (1959). Sample 0.5 g was homogenised in 10 ml of 0.05 M phosphate buffer pH 7.2 with a Polytron PT1200CL (Kinematica AG, Lucerne, Switzerland) for 30 s. 1 ml of the homogenate was mixed with 9 ml of

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0.05 M phosphate buffer (pH 7.2) containing 0.6 M NaCl, 6 mM EDTA, and 8 M urea. The mixture was centrifuged for 15 min at 14,000g at 5 °C (Sorvall RC 5B Plus, SM34 rotor, Dupont, Norwalk, CT, USA). To 3 ml of the supernatant 0.04 ml of 0.01 M DTNB solution in 0.05 M sodium acetate were added and incubated at 40 °C for 15 min. A blank was prepared replacing the homogenate with 0.05 M phosphate buffer pH 7.2 containing 0.6 M NaCl, 6 mM ethylenediaminetetraacetic acid (EDTA), 8 M urea. The absorbance was measured at 412 nm (Shimadzu UV 160A, Japan) and the SH content was calculated using a molar extinction coefficient of 13,600 M^{-1} cm^{-1}. Results were expressed in micromoles of SH per g of mince.

3.4.3.10 Determination of Total volatile base nitrogen of mince fish

TVB-N value was determined according to Ingridy et al., (2014). 10 g samples were homogenized with 60 mL of 10% trichloroacetic acid (TCA) solution for 1 min and set to rest for 2 h. The sample was then filtered through Whatman filter paper (no.4), 25 mL of the filtrate was added to 1 g of magnesium oxide and transferred to a flask and fitted into the nitrogen distillation apparatus. The contents were distilled with 15 mL of methyl red plus bromocresol green mixed indicator and titrated with 0.02 N HCL. The TVB-N values were expressed as mg 100/g.

3.4.4 Application of mango seed kernel extract in cosmetic cream

3.4.4.1 Preparation of cosmetic MSKE cream

The oil-in-water emulsion creams used in this study were prepared according to the method of the Cosmaprof Co. Ltd., Thailand. The cream samples were prepared respectively by melting the lipophilic phase including Emulium Delta (5.0%), stearic acid (1.5%), stearyl alcohol (2.0%), Captex 300 (3%) and cetyl alcohol (1.5%) in a water bath at 80 °C and separately mixing the hydrophilic phase; propylene glycol (3%), xanthan gum (0.1%), EDTA (0.1%) and water in a water bath at 70 °C. The 2 phases were mixed by homogenizer (Dragon Lab, D500, China) at 20,000 rpm for 3 min and cooled down at room temperature. The MSKE (0%,

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1%, 2%, 3% w/w) and phenoxy ethanol as a preservative 0.1% were added and mixed by homogenizer (Dragon Lab, D500, China) at 10,000 rpm for 3 min.

3.4.4.2 Determination of the resistance to centrifugation of MSKE cream

The resistance to centrifugation study was based on the method from the Cosmaprof Co., Ltd. The samples were stored at ambient temperature and humidity for 48 hours. The 10 ml sample was conducted in a centrifuge (Harmle Z233 MK-2, Germany) at 3000 rpm for15 min. Samples were evaluated the phase separation by measuring the supernatant after centrifugation.

3.4.4.3 Determination of physical stability study of MSKE cream

The freeze-thaw cycling tested in order to predict the long term stability and in particular the shelf life of the cosmetic emulsion products was investigated. The samples were submitted under temperature cycles from -4 °C for 24 h and 25 °C within 24 h for a period 6 cycles. The samples were sampled every 48 h to evaluate pH (Ino Lab, Germany), viscosity (Brookfield Engineering Laboratories, USA), the phase separation, color (Minolta CR-400, Japan), total polyphenol contents andABTS radical scavenging. The toatal polyphenol content and ABTS radical scavenging in samples were determined by centrifugation at 15000xg for 30 min at 4 °C in a refrigerated centrifuge (HettichZentrifugen EBA 20, Germany). The supernatant was analyzed usingthe method as mentioned above. The color data of samples were expressed as *L* (degree of lightness), *a* (degree of redness) and *b* (degree of yellowness) values. The total color difference (ΔE) was calculated as follows :

$$\Delta E = \sqrt{(L - L')^2 + (a - a')^2 + (b - b')^2} \quad (3.1)$$

where *L, a, b* are colors of the samples; L', a' and b' are colors of the base time zero.

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3.4.4.4 The clinical *in-vivo*skin irritation test of MSKE cream

The MSKE cream was subjected to the Clinical *in -vivo* skin irritation test by the Skinova Lab Co. Ltd., Thailand in January, 2015. The total of 33 volunteers, Thai men and women, age between 18 and 65 years, participated in this study. The Finn chamber on scanpor tape (Finn Chamber, Epitest Ltd., Finland) was used for the patch testing (Figure 3.2). Each of aluminum disheswith 8 mm diameter of Finn chamber contained 25 µg MSKE cream without dilution, 1% of sodium lauryl sulfate was used as a positive control and distilled water as a negative control. The Finn chamber was occluded on the back of each volunteer and removed after 24 hours. The visual assessment of skin irritation was observed after 24 and 48 hours after the removal of the Finn chamber. This study, which had no direct therapeutic purpose, was undertaken according to the COLIPA the European Cosmetic, Toiletry and Perfumery Association, guideline for assessment of skin compatibility of cosmetic finished products under controlled conditions in man.

The visual assessment of skin irritation was scored for irritation throughout the test, by the investigator, in increasing severity using a grading scale (Table 3.1).

Figure 3.2 Finn Chamber for skin irritation patch test

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Table 3.1 Assessment of reaction for skin irritation

Grading	Description of skin response
0	No reaction
+/- (0.5)	Doubtful reaction : glazed appearance of site or barely perceptible erythema
1	Weak reaction : single erythema or dryness across most of treatment site
2	Moderate reaction : moderate erythema with generalized edema
3	Strong reaction : strong erythema with generalized edema
4	Severe reaction : severe erythema with severe edema, with or without vesicle, pustule or ulcer

3.4.5 Statistical Analysis

Differences of irritation between the MSKE cream and 1% sodium lauryl sulfate were tested for statistical significance using analysis of variance, T test and Tukey'sStudentize Range (HSD) test. A *p*-value of less than 0.05 was considered a statistically significant difference.

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CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Antioxidant properties sand selected enzyme inhibition capacities of mango seed kernel extracts

4.1.1 Extraction yield and total polyphenol contents

The longer ultrasonication time (up to 45 min) resulted in a significantly higher yield and total polyphenol contents (TPC) of the mango seed kernel extracts (MSKEs) from the two cultivars (Table 4.1). However, the values were not significantly different ($p \geq 0.05$) after 45 min, indicating that the optimum duration of the ultrasonication time for the extraction of mango seed kernels under the condition used in this experiment was at least 45 min. There was a cultivar difference in that the yield and TPC from the MSKE of Choke-Anan was higher than that from Kaew. Moreover, the MSKE obtained by ultrasonic-assisted extraction at 45 min gave a 55 % and 63 % increasing of extraction yield and 92 % and 55 % increasing of TPC for the Kaew and Choke-Anan cultivars, respectively compared to conventional ethanol extraction. The typical appearance of the MSKEs is shown in Figure 4.1. The crude extracts were yellow-brown color oleoresin.

Similar results were reported by Cho et al., (2006), who found that ultrasonic assisted extraction of grape peel had up to 30% higher yield than conventional solvent extraction. Higher TPC of MSKE was also observed by Yuntao et al., (2012) with the ultrasonic assisted aqueous two phase extraction of mango seed kernel. Falleh et al., (2012) also reported 32 % higher TPC of the extracts from *Mesembryanthemum edule* L. shoots obtained by ultrasonic assisted extraction.

The higher yield and TPC observed when the ultrasonication was applied in the extraction of plant tissues can be explained by the disruption of cell membrane and cell wall structure increasing the solvent diffusion through the membrane and hence facilitates the release of cell contents (Roastagno et al.,2003; Betancourt, 2008). From these results, ultrasonic assisted extraction with the duration time of 45 min at 20KHz was used to prepare the MSKE for the subsequent experiments.

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Figure 4.1 Apearance of crude MSKEs from Kaew and Choke-Anan cultivars

4.1.2 Antioxidant activities of MSKEs

The MSKEs prepared by ultrasonication assisted extraction of the TPC from both the varieties was about two times greater antioxidant activities for all methods studied (Table 4.2). The higher content of polyphenols in the MSKEs obtained by ultrasonication assisted extraction (Table 4.1) is likely to have contributed to their greater antioxidant activities. In addition, the MSKE from Choke-Anan showed higher antioxidant activities over the sample than the MSKE from Kaew. The results corresponded to the content of total polyphenols which was also higher in MSKE from Choke-Anan (Table 4.1).

The highest antioxidant activities accessed by DPPH and ABTS methods of MSKE prepared from Choke-Anan has been previously reported among several varieties of Thai mangoes by Khammuang and Sarnthima (2011). Moreover, Maisuthisakul (2008) has also reported that extracts from mango seed kernel of Kaew and Choke-Anan showed the highest antioxidant properties among the eleven mango varieties studied.

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Table 4.1. Effect of ultrasonication duration time on the yield and total polyphenol contents of mango seed kernel extracts

Mango varieties	Extraction time (min)	% Yield	Total polyphenol (mg of GAE/g)
Keaw	0	1.60±0.06^{g}	71.74±0.97^{f}
	15	1.71±0.03^{f}	70.93±0.63^{f}
	30	2.10±0.07^{e}	91.64±0.80^{e}
	45	2.48±0.03^{c}	137.49±1.15^{b}
	60	2.49±0.06^{c}	138.71±2.52^{b}
Choke-Anan	0	2.16±0.02^{e}	110.02±1.06^{d}
	15	2.29±0.04^{d}	110.32±0.93^{d}
	30	2.73±0.03^{b}	124.36±1.69^{c}
	45	3.52±0.03^{a}	170.12±1.89^{a}
	60	3.56±0.02^{a}	170.63±0.93^{a}

Values are means ± standard deviations; n=3. The different superscript letters in the same column indicate significant differences ($P \leq 0.05$).

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Table 4.2 Antioxidant capacities of mango seed kernel extracts (MSKEs) using different methods

MSKE Extractions	**Antioxidant capacity** (EC_{50} µg/mL)			
	DPPH	**FRAP**	**H_2O_2**	**ABTS**
Kaew Ethanolic	20.54±0.02[a]	18.24±0.04[a]	37.15±0.96[a]	24.65±0.17[a]
Kaew Ethanolic + Ultrasonication	10.24±0.83[c]	9.32±0.06[c]	18.79±1.05[c]	12.40±0.55[c]
Choke-Anan Ethanolic	15.07±0.12[b]	16.03±2.55[b]	33.54±2.86[b]	22.74±0.96[b]
Choke-Anan Ethanolic+ Ultrasonication	7.64±0.15[d]	8.76±0.26[d]	17.24±1.26[d]	11.13± 0.34[d]

Values are means ± SD; (n=3); values in the same column that are followed by different superscript letters are significantly different ($P \leq 0.05$).

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4.1.3 Tyrosinase inhibitory activity of MSKEs

The ability of MSKE from Kaew and Choke-Anan to inhibit the tyrosinase activity was evaluated and compared to arbutin, a commercial cosmetic whitening agent with tyrosinase inhibitory activity. The two samples of MSKE exhibited no significantly difference in activity compared to arbutin ($P>0.05$) and the IC_{50} values were in the range of 19.86 ± 1.2 to 20.64 ± 0.5 μg/ml (Table 4.3), which is similar to the results reported by Maisuthisakul and Gordon (2009). They demonstrated that the extract prepared from Choke-Anan mango seed kernel under different conditions showed tyrosinase inhibitory activity with ID_{50} about 1.58 times than arbutin.

Also Nititanakool et al., (2009 a) reported the tyrosinase inhibitory activity for Fahlun mango seed kernel extract compared to gallic acid, methyl gallate, pentagalloylglucopyranose and kojic acid with IC_{50} 98.63 ± 1.62, 6.44 ± 14.0, 62.50 ± 0.50, 42.65 ± 1.85 and 2.21 ± 0.05 μg/ml, respectively. The experimental results confirm those from the literatures and indicate that MSKEs can be a good source of phytochemicals with tyrosinase inhibitory activity. The MSKE from Kaew and Choke-Anan exhibited higher activity than that from the cultivar Fahlun. Many plant polyphenols have shown to exhibit tyrosinase inhibitory activity. Specifically, some phenolic compounds such as ellagic acid, tannic acid and quercetin can inhibit the tyrosinase activity (Rompel et al., 1999; Shimogaki et al., 2000).

The tyrosinase inhibition ability of plant polyphenols might due to the hydroxyl group of polyphenols that form the hydrogen bond at a site of the enzyme (Prasad et al., 2010); or dependent of the scavenging of free radicals involved in the tyrosinase reaction and in the formation of melanins (Fatiha et al., 2015). Besides, the similarity in chemical structure of polyphenols and tyrosine, which is a substrate of tyrosinase that may react as a competitive inhibitor binding site at the same site as the substrate tyrosine (Victor et al., 2004). In addition, as tyrosinase is an enzyme containing copper. Another possible mechanism for tyrosinase inhibition of polyphenols may involve the chelation of copper atoms which are required for the catalytic activity of tyrosinase (Kim et al., 1998). This was supported by the finding that MSKEs were shown to be a good chelators of copper (Maisuthisakul and Kordon, 2009). Nithitanakool et al.,

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(2009b) have explained the binding orientation of the polyphenols to the tyrosinase hydrophobic binding pocket around the binuclear copper active site, which could cause the inhibition of tyrosinase activity.

Tyrosinase is the main enzyme in the melanin synthetic pathway in melanocytes. The inhibition activity of tyrosinase could be an important strategy for blocking melanogenesis (Park et al., 2004). Tyrosinase inhibitors have been widely used as ingredients in skin whitening cosmetics. As polyphenols are key phytochemicals found in the MSKEs (Table 4.xx), it is most likely that the contribution of tyrosinase inhibitory activity of MSKEs in the present study is due to the polyphenols. Therefore, MSKEs can be a promising natural cosmetic whitening agent.

4.1.4 5-Lipoxygenase inhibitory activity of MSKEs

5-Lipoxygenase has been known to catalyze the conversion of arachidonic, linoleic and other polyunsaturated fatty acids into biologically active metabolites, which are active mediators in a variety of inflammation process (Alitonou et al., 2006). Therefore, evaluation of 5-Lipoxygenase inhibitory activity of MSKEs may provide information for potential development of a new anti-inflammatory drug.

The MSKE prepared from Chock-Anan exhibited higher 5-Lipoxygenase inhibitory activity over that from Keaw cultivar ($P \leq 0.05$) (Table 4.3). However, the IC_{50} of the MSKEs was about 3.2-4.3 times higher than that of standard reference, rutin. This indicates that lower inhibitory ability of MSKEs was observed when compared to standard rutin. The lipoxygenase inhibition effect of antioxidant compounds can be explained by their involvement in inhibition of lipid hydroperoxide formation and consequently less availability of substrate needed for lipoxygenase (Rackova et al., 2007). Inaddition, many phytochemicals of plant origin (flavonoids, polyphenols, coumarins) interfere with 5-LOX activity due antioxidant activities, that is, by uncoupling of the redox cycle of the 5-LOX active-site iron (Werz, 2007).

Since the 5-Lipoxygenase inhibitory activity of MSKE has not been reported elsewhere, comparative discussion will be done with other plant extracts. Akula and Odhav (2008) reported 5-Lipoxygenase inhibition properties of eighteen plant extracts. Among those, the

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extract from *Bidenspilosa* showed highest inhibitory activity with IC_{50} of 21.8 μg/ml, while the extract from *Emexaustralis* had lowest activity with IC_{50} of 81.4 μg/ml. In addition, Albano et al., (2012) reported the IC_{50} values for 5-Lipoxygenase inhibitory activity of eight plant extracts ranged from 27.4$\pm$0.6 to 66.7$\pm$0.6 μg/ml.

The result of 5-lipoxygenase inhibition properties infers that MSKE has a high potential to be developed as natural an anti-inflammatory drug compared to other plant extracts from the previous studies.

4.1.5 Hyaluronidase inhibitory activity of MSKEs

Hyaluronidase is an enzyme that catalyzes the degradation of hyaluronic acid which is a naturally occurring polyanionic polysaccharide that consists of N-acetyl-d-glucosamine and β-glucoronic acid. Hyaluronic acid is found in the extra cellular matrix (ECM) of most vertebrate connective tissues that helps to holds water and keeps the body smooth, moist and lubricated. (Sahasrabudhe and Deodhar, 2010). Degradation of hyaluronic acid by the action of hyaluronidase can lead to diminishing amounts of hyaluronic acid in the skin and the skin consequently becomes dried and wrinkled. (Sahasrabudhe and Deodhar, 2010).

The inhibitory effect of mango seed kernel extracts on hyaluronidase activity as presented in Table 4.3, Chock-Anan MSKE exhibited no significant difference (P>0.05) of the hyaluronidase inhibitory activity compared to the standard reference, vitamin C with the IC_{50} 37.28$\pm$1.6 and 39$\pm$0.2 μg/ml, respectively. However, MSKE from Keaw cultivar showed about 1.3 times lower inhibitory activity.

Many previous studies have shown that plant polyphenols can inhibit hyaluronidase activity. Including Girish and Kemparaju (2005) who reported that blackberry polyphenols, including quercetin, gallotanins and tannic acid exhibited good hyaluronidase inhibition.

Additionally, Satardekar and Deodhar (2010) reported that the extract from the bark of *Terminaliaarjuna* (250 μg/ml) and dried fruit rinds of *Terminaliachebula* (500 μg/ml) resulted in 90.40$\pm$5.30% and 89.65$\pm$3.90% hyluronidase inhibition, respectively.

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Lee et al., (2001) also reported 57% and 82% inhibition of hyaluronidase activity at concentrations of 250 and 500 μg/ml, respectively of purified *Areca catechu* extracts. In the present study, the MSKEs of Kaew and Choke-Anan gave up to 96.35% and 97.61% inhibition respectively at the concentration of 70 μg/ml. The results indicate that the MSKEs especially from Choke-Anan has a potential to be used as anti-wrinkle cream in the cosmetic industry.

4.1.6 α-glucosidase inhibitory activity of MSKEs

Diabetes mellitus is a common found chronic disease caused by inherited or acquired deficiency in insulin secretion and by decreased sensitivity of the target organs to secreted insulin (Bhandari et al., 2008). These shortcoming results in postprandial hyperglycemia and attendant diabetic complications such as hypertension, cardiovascular disease and diabetic neuropathy.

The hydrolyzed dietary carbohydrates are the major source of blood glucose. The dietary carbohydrates are hydrolyzed by pancreatic α-amylase with absorption aided by α-glucosidases in order to be absorbed by the small intestine (Elsenhans and Caspary, 1987). However, one practical therapeutic approach to the management of diabetes is by controlling postprandial hyperglycemia. It is considered that the progression of diabetes mellitus can be stopped by inhibiting the absorption of dietary carbohydrates in the small intestine (Kumar et al., 2011).

The ability of MSKEs to inhibit the α-glucosidase activity was investigated compared to the standard acarbose. Chock-Anan MSKE exhibited significantly higher inhibitory capacity over Keaw MSKE ($P \leq 0.05$) (Table 4.3). However, the MSKE from Chock-Anan showed about 1.1 times higher IC_{50} values compared with acarbose, indicating slightly lower α-glucosidase inhibitory activity.

These plants are typically rich in polyphenolic compounds, which are known to interact with proteins and can inhibit enzymatic activity (Suryanarayana et al., 2004). Various studies also showed that many plant polyphenols possessed *in vitro* inhibitory activity toward carbohydrate hydrolyzing enzymes (Takashi et al., 2015; Luis et al., 2015). The problems of

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naturalproducts from plants being used for the treatment of diabetes are relate to their affordability. Therefore, MSKE from Chock-Anan as a by-productmay have potential as dietary anti-diabetic agents for the control of postprandial hyperglycemia.

Table 4.3 Enzymes inhibition activities of mango seed kernel extracts (MSKEs) compared to the standard reference

Sample	Enzyme inhibition capacity, IC_{50} (μg/ml)			
	Tyrosinase	5-Lipoxygenase	Hyaluronidase	α-glucosidase
Kaew MSKE	20.64±0.3[a]	39.77±2.4[a]	47.61± 2.9[a]	163.19±2.3[a]
Choke-Anan MSKE	19.86±1.2[a]	30.94±0.3[b]	37.28 ± 1.6[b]	113.51±5.8[b]
Arbutin	20.46±0.3[a]	-	-	-
Rutin	-	9.31±0.5[c]	-	-
Vitamin C	-	-	39.29± 0.2[b]	-
Acarbose	-	-	-	104.42±5.5[c]

Values are means ± SD; (n=3); values in the same column that are followed by different superscript letters are significantly different ($P<0.05$), IC_{50} = the inhibitor concentration causing 50 % loss

4.2 Fractionation, antioxidant and inhibitory activities of selected enzymes of fractions from mango seed kernel extracts (MSKEs)

4.2.1 Fractionation of Kaew and Choke-Anan mango seed kernel extracts

From the results of antioxidant properties and inhibition activity of selected enzymes including tyrosinase, 5-lipoxygenase, hyaluronidase and α-glucosidase of MSKEs from

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both cultivars. The identification of key component found in MSKEs were performed using fractionation and liquid chromatography mass spectrophotometer (LC-MS).

The fractionation of MSKEs from both cultivars, Kaew and Choke-Anan using Sephadex-LH20 as column chromatography and methanol as mobile phase are showed in Figure 4.2. All of crude extracts from both cultivars were yellow. The profile at 280 nm of the fractions from MSKEs showed three major fractionisolates that were separated with the absorbance generally higher for the Choke-Anan than those of Kaew. These results differed from Maisuthisakul (2011) for Choke-Anan MSKE and were obtained 5 and 2 fractions from MSKE using shaking extraction and acid hydrolysis extraction, respectively.

4.2.2 The recovery yield, total polyphenol (TPC) and antioxidant activities of different fractions

The measurement of % yield and TPC of all the different fractions from Kaew and Choke-Anan MSKEs are shown in Table 4.4. The results showed that the % recovery yield of fractions from Kaew and Choke-Anan were in the range of 6.41-17.11 % and 9.49-27.90 % and TPC value were in the range of 130.30±0.87 and 263.28±1.50 mg GAE/g, respectively. All of fractions from both cultivars fraction 3 from Kaew and Choke-Anan and MSKE exhibited a significantly increase ($P \leq 0.05$) in TPC when compared to crude MSKE by 75.75 and 34.85 % increase, respectively. The results were similar to Maisuthisakul (2011) who also reported for Choke-Anan that fraction 3, obtained from MSKE using the shaking extraction had an increasing level of TPC an approximately 160 % when compared to crude extract.

Table 4.4 shows the IC_{50} of antioxidant properties of crude, fractions MSKE from both cultivars using 4 different methods (DPPH, FRAP, H_2O_2 and ABTS). All the crude and fractions of MSKE showed antioxidant activities. Fraction 3 of both cultivars exhibited significantly increased ($P \leq 0.05$) antioxidant activity of all the methods tested compared to crude extracts, which had EC_{50}6.63±0.08, 5.70±0.01, 14.81±0.21 and 8.54±0.06 µg/mL, respectively for Kaew and 4.74±0.14, 3.40±0.09, 13.50±0.34, 6.45±0.04 µg/mL, respectively for Choke-Anan.

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The MSKEs of both Kaew and Choke-Anan exhibited the highest recovery yield in fraction 3. In addition, the highest TPC in the fraction of both cultivars corresponded to the greatest antioxidant activities for all the methods tested. Maisuthisakul (2011) also showed that fraction 3 for Choke-Anan, obtained from MSKE using the shaking extraction and fraction 2, obtained from MSKE using acid hydrolysis extraction had an approximately 43 and 16 % higher antioxidant efficiency when compared to crude extract.

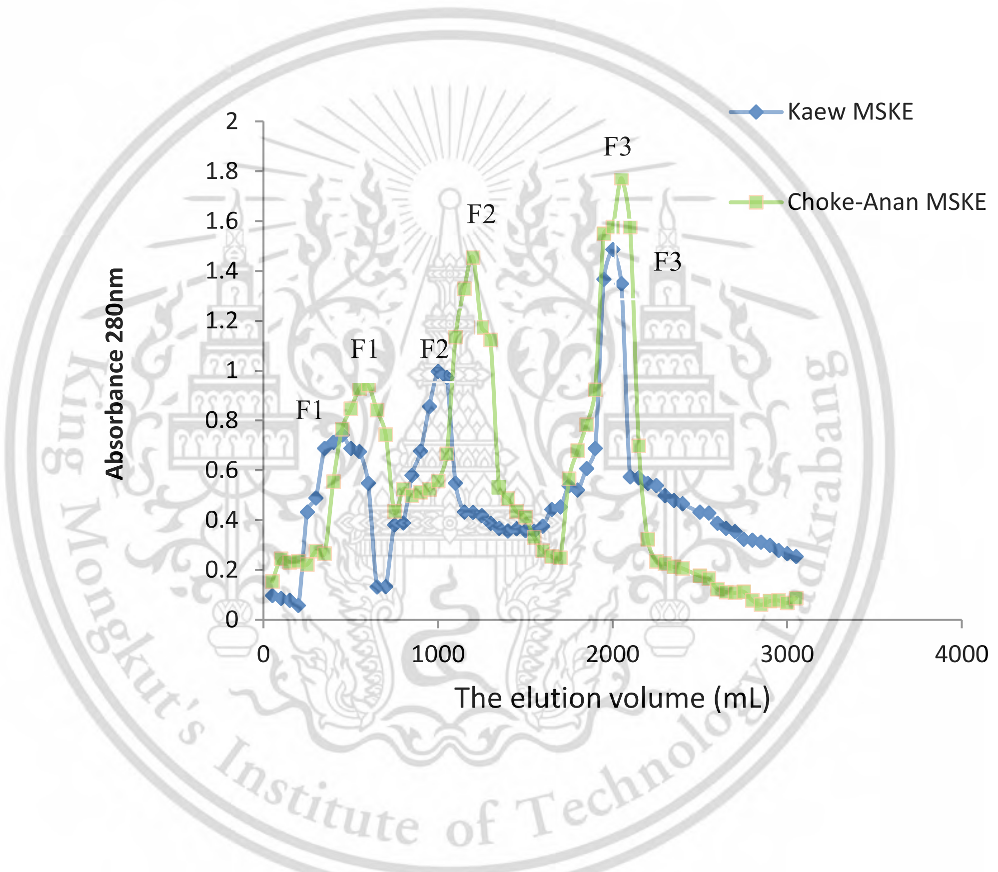

Figure 4.2 Column chromatography profile of the fractions from mango seed kernel extracts (MSKEs)

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Table 4.4 Characteristic of Sephadex LH-20 column chromatography of Kaew and Choke-Anan MSKE; the yield of individual fractions (%) the total polyphenol contents and antioxidant properties

Fractions		% yield	TPC (mg GAE g^{-1})	Antioxidant properties (EC_{50} µg/mL)			
				DPPH	FRAP	H_2O_2	ABTS
Kaew	Crude	ND	132.78±0.77^{g}	10.22±0.05^{a}	9.35±0.02^{a}	19.28±0.15^{a}	12.11±0.09^{a}
MSKE	F1	6.41±0.02^{e}	130.30±0.87^{g}	10.27±0.07^{a}	9.15±0.03^{b}	18.53±0.28^{b}	12.14±0.22^{a}
	F2	9.44±0.01^{d}	184.72±0.59^{d}	8.66±0.32^{b}	7.76±0.01^{d}	15.45±0.03^{e}	10.12±0.09^{c}
	F3	17.11±0.03^{b}	232.00±1.33^{b}	6.63±0.08^{d}	5.70±0.01^{g}	14.81±0.21^{f}	8.54±0.06^{e}
Choke-Anan	Crude	ND	175.06±0.61^{e}	7.17±0.03^{c}	8.89±0.04^{c}	17.63±0.20^{c}	11.33±0.20^{b}
MSKE	F1	9.49±0.16^{d}	150.95±6.79^{f}	6.13±0.15^{e}	7.16±0.02^{e}	16.57±0.61^{d}	11.35±0.04^{b}
	F2	16.30±0.03^{c}	206.01±0.74^{c}	5.46±0.20^{f}	6.53±0.01^{f}	14.12±0.31^{g}	9.56±0.03^{d}
	F3	27.90±0.02^{a}	263.28±1.50^{a}	4.74±0.14^{g}	3.40±0.09^{h}	13.50±0.34^{h}	6.45±0.04^{f}

Values are mean ± SD; (n=3). Values in the same column that are followed by different superscript letters were significantly different ($P \leq 0.05$),

ND means not determine

4.2.3 Selected enzyme inhibitory activities from different fractions

In this investigation, crude MSKE and fractions from Kaew and Choke-Anan were evaluated for inhibition activity of selected enzyme included tyrosinase, 5-lipoxygenase, hyaluronidase and α-glucosidase compared to the reference standard.

The values were expressed as the inhibitor concentration causing 50 % loss of enzyme activity (IC_{50}). The smaller IC_{50} value means the higher enzyme inhibitory activity. Table 4.5 shows the IC_{50} values of enzyme inhibition activities of crude MSKE, fractions and standard reference.

Tyrosinase is the key enzyme in the melanin synthetic pathway in melanocytes and Park et al., (2004) reported that the inhibition of tyrosinase could be an important strategy for prevent of melanogenesis. Tyrosinase inhibitors have been widely used as ingredients of some skin whitening cosmetics. The results showed that fraction 3 of MSKE from Kaew and Choke-Anan showed significant inhibition activity ($P \leq 0.05$) in tyrosinase among all fractions and crude MSKE, the IC_{50} value were 17.10 ± 0.15 and 15.35 ± 0.63 μg/mL, respectively. The results also showed that fraction 3 of both cultivars were more effective in tyrosinase inhibition activity than arbutin, the standard reference, IC_{50} was 20.64 ± 0.75 μg/mL. The results indicate that mango seed by-product is a promising natural ingredient for the whitening cosmetic products to prevent hyperpigmentary disorders such as melasma, freckles and age spots.

Alitonou et al., (2006) explained that 5-Lipoxygenase enzyme catalyze the conversion of arachidonic, linoleic and other polyunsaturated fatty acids into leukotrienes (LTs) metabolites, which are active mediators in a variety of pro-inflammation processes. Therefore, screening of anti 5-lipoxygenase activity from a natural source may provide important information as an anti-inflammatory drug (Njenga and Viljoen, 2006). The inhibitory activity of 5-lipoxygenase from crude MSKE and fractions of MSKE from Kaew and Choke-Anan were evaluated compared to rutin. Fraction 3 of MSKE from Kaew and Choke-Anan showed significant inhibition activity ($P \leq 0.05$) of 5-lipoxigenase among all fractions and crude MSKE. Rutin had significantly higher ($P \leq 0.05$) 5-lipoxygenase inhibition activity than all fractions of both cultivar and the crude MSKE, IC_{50} value was 10.96 ± 0.69 μg/mL.

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Hyaluronidase diminishes amounts of hyaluronic acid in the extra cellular matrix of skin with the result that the skin becomes dried and wrinkled (Sahasrabudhe and Deodhar, 2010). The results of hyaluronidase inhibition activity showed that fraction 3 of Kaew MSKE and fraction 1, 2 and 3 of Choke-Anan MSKE showed significantly higher ($P \leq 0.05$) hyaluronidase inhibitory activity than vitamin C; the reference standard. Fraction 2 from Kaew and crude Choke-Anan MSKE were not significantly different ($P > 0.05$) in hyaluronidase inhibition activity. Moreover, MSKE from Choke-Anan showed significantly higher ($P \leq 0.05$) activity than that of Kaew. From these results it may be confirm that hyaluronidase inhibition activity of MSKE has potential for development of a natural ingredient for anti-ageing and anti-wrinkle cosmetics.

Hydrolyzed dietary carbohydrates are the major source of blood glucose, which are hydrolyzed by pancreatic α-amylase with absorption aided by α-glucosidases to facilitate absorption by the small intestine (Elsenhans and Caspary, 1987). Kumar et al., (2011) described a practical therapeutic approach to the management of diabetes type 2 by controlling postprandial hyperglycemia. They considered that the progression of diabetes mellitus can be stopped by inhibiting the absorption of dietary carbohydrates in the small intestine.

Table 4.5 shows the results of α-glucosidase inhibition activity from crude and fractions of MSKE for both Kaew and Choke-Anan compared to acarbose. Fraction 3 of both cultivars were significantly higher ($P \leq 0.05$) in α-glucosidase inhibitin activity among crude and other fractions with IC_{50} 110.45$\pm$4.01 and 108.00$\pm$2.51 μg/mL, respectively. However, acarbose, a reference standard, had significantly higher ($P \leq 0.05$) α-glucosidase inhibition activity than all crude and fractions of MSKE.

Fraction 3 of MSKE from both cultivars exhibited significantly higher ($P \leq 0.05$) tyrosinase, 5-lipoxygenase, hyaluronidase and α-glucosidase inhibition activities than crude MSKE and other fractions. The results can be explained by the corresponding of the TPC and antioxidant activity shown in Table 4.4. Moreover, fraction 3 of both cultivars showed higher tyrosinase and hyaluronidase inhibition activities than arbutin and vitamin C, respectively.

The antioxidant activity mechanism may also be one of the main reasons for tyrosinase inhibition activity (Kim et al., 2008). Some phenolic compounds such as ellagic acid,

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tannic acid (or gallotannins) and quercetin have been shown to inhibit tyrosinase activity (Shimogaki et al., 2000) and hyaluronidase activity (Girish and Kemparaju, 2005). Moreover, some flavonoids, such as kaempferol, quercetin and morin, show inhibitory activity of tyrosinase (Gomez-Cordoves et al., 2001). All flavanoids inhibit enzyme activity due to their ability to chelate copper in the active site. The experimental results demonstrated that fraction 3 of MSKE from both cultivars contained a key component that had stronger enzyme inhibition activities.

Table 4.5 Enzyme inhibition activities of crude and different fractions MSKE from Kaew and Choke-Anan cultivars

Fractions		Enzyme inhibition activities (IC_{50}, μg/ml)			
		Tyrosinase	5-lipoxygenase	Hyaluronidase	α-glucosidase
Kaew MSKE	Crude	21.11 ± 0.50^{cd}	39.36 ± 0.61^{bc}	41.39 ± 0.85^{a}	154.42 ± 3.64^{b}
	F1	37.10 ± 0.22^{a}	41.63 ± 0.52^{a}	42.69 ± 0.82^{a}	131.75 ± 2.51^{d}
	F2	36.59 ± 0.21^{a}	35.32 ± 0.57^{d}	38.03 ± 0.90^{b}	118.99 ± 3.02^{e}
	F3	17.10 ± 0.15^{e}	29.79 ± 0.74^{f}	32.80 ± 0.84^{d}	110.45 ± 4.01^{g}
Choke-Anan MSKE	Crude	21.29 ± 0.81^{c}	38.75 ± 0.41^{c}	39.19 ± 0.91^{b}	157.83 ± 3.32^{a}
	F1	27.48 ± 0.46^{b}	40.16 ± 0.51^{b}	35.42 ± 0.82^{c}	133.85 ± 5.24^{c}
	F2	27.18 ± 0.46^{b}	32.71 ± 0.69^{e}	35.80 ± 0.81^{c}	116.08 ± 5.15^{f}
	F3	15.35 ± 0.63^{f}	21.35 ± 0.65^{g}	29.56 ± 1.23^{e}	108.00 ± 2.51^{h}
Standard references	Arbutin	20.64 ± 0.75^{d}	ND	ND	ND
	Rutin	ND	10.96 ± 0.69^{h}	ND	ND
	Vitamin C	ND	ND	38.67 ± 0.78^{b}	ND
	Acarbose	ND	ND	ND	105.00 ± 0.51^{i}

Values are mean±SD; (n=3). Values in the same column that are followed by different superscript letters were significantly different ($P\leq0.05$), ND means not determine

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4.2.4 Identification of polyphenols by LC-ESI-MS

Table 4.4 and 4.5 show fraction 3 of MSKE from both cultivars had significantly higher ($P \leq 0.05$) of antioxidant activities, tyrosinase, 5-lipoxygenase, hyaluronidase and α-glucosidase inhibition activities than crude MSKE and other fractions. The identification of the main components of fraction 3 of both cultivars needs to be evaluated.

The results of identification of the main polyphenols in fraction 3 of MSKE from both Kaew and Choke-Anan, using LC-ESI-MS mass spectrometry at the retention time 0.0-8.3 min, are shown in Table 4.6. These results were consistent with previous studies (Luo et al., 2014; Resoanaivo et al., 2014; Berardini et al., 2004) and were all detected in fraction 3 of MSKE from both cultivars. The mass spectrum analyses were: Peak 1 showed an $(M\text{-}Na)^+$ ion of m/z 662.3 analyzed as tri-o-galloyl-glucoside. Peak 2 showed an $(M\text{-}Na)^+$ ion of m/z 813.5 analyzed as tetra-o-galloyl-glucoside. Peak 3 showed an $(M\text{-}Na)^+$ ion of m/z 902.4 analyzed as maclurintri-o-galloyl-glucoside. Peak 4 showed an $(M\text{-}Na)^+$ ion of m/z 961.5 analyzed as penta-o-galloyl-glucoside. Peak 5 showed an $(M\text{-}Na)^+$ ion of m/z 1113.2 analyzed as hexa-o-galloyl-glucoside. Peak 6 showed an $(M\text{-}Na)^+$ ion of m/z 1265 analyzed as hepta-o-galloyl-glucoside.

The retention time of the mass spectrum of the fraction 3 samples from both Kaew and Choke-Anan MSKE showed them to be in the range of 2.90-4.44 and 2.10-3.15, respectively and exhibited different ratios. Their relative abundance at peak 1-5 of the mass spectrum analysis were 5>2>4>3,6>1 and 2>1>4>3>5>6, respectively. Fraction 3 of MSKE from Choke-Anan has hexa-o-galloyl-glucoside and tetra-o-galloyl-glucoside as the two major components, whereas, fraction 3 from Kaew had only tetra-o-galloyl-glucoside as its major component. The key components of fraction 3 of MSKE from Kaew and Choke-Anan contained 6 main polyphenols:tri-o-galloyl-glucoside, tetra-o-galloyl-glucoside, maclurintri-o-galloyl-glucoside, penta-o-galloyl-glucoside, hexa-o-galloyl-glucoside, and hepta-o-galloyl-glucoside.

These results were similar to those previous reported on the NMR spectra of ethanolic extracts of by-products from Hiesy mangoes in Madagascar where they classified ten gallotannins, which consisted of glucose and three to nine gallic acids (Resoanaivo et al., 2014). Berardini et al. (2004) reported that the polyphenols from the MSKE of Tommy Atkins was

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contained 21 gallotannins. A similar study reported gallotannins in seed kernel of from the seed kernels of three Chinese mango cultivars (Maqiesu, Tainong-1, Zihuamang) using LC-ESI-MS/MS, where MSKE contained five major gallotannins, i.e. penta-o-galloyl-glucoside, hexa-o-galloyl-glucoside, hepta-o-galloyl-glucoside, octa-o-galloyl-glucoside and nona-o-galloyl glucoside (Luo et al., 2015).

Table 4.6 Characterization of main polyphenol compositions in fraction 3 of MSKE from Kaew and Choke-Anan cultivars by LC-ESI-MS

Peak	Name	% Relative abundance		Retention Time (min)		$(M\text{-}Na)^+$ (m/z)
		Kaew	Choke-Anan	Kaew	Choke-Anan	
1	Tri-o-galloyl-glucoside	56	13	2.10	2.90	662.3
2	Tetra-o-galloyl-glucoside	100	97	2.60	3.76	813.5
3	Maclurintri-o-galloyl-glucoside	28	50	2.96	2.81	902.4
4	Penta-o-galloyl-glucoside	34	88	3.48	3.99	961.5
5	Hexa-o-galloyl-glucoside	24	100	4.16	3.21	1113.2
6	Hepta-o-galloyl-glucoside	8	50	3.15	4.44	1265.0

The previous results (4.1 and 4.2) clearly showed that the MSKE from Choke-Anan exhibited significantly higher antioxidant activities and selecte enzyme inhibition properties compared to MSKE from Kaew. Therefor, Choke-Anan MSKE was used for the experiment in application to mince fish product and cosmetic cream.

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4.3 Application of mango seed kernel extract (MSKE) to mince fish products on retarding lipid oxidation and protein oxidation

4.3.1 Effect of MSKE on pH of mince fish during frozen storage

The pH of the control progressively increased during storage and was significantly higher ($P \leq 0.5$) than the other treatments from 6 to 12 weeks storage (Table 4.7). Samples treated with MSKE and BHT had relatively stable pH levels with slight but significant increases ($P \leq 0.5$) after 12 weeks storage in all cases (Table 4.7). The results confirm those of Viji et. al., (2014) who found increases in pH during chilled and iced storage of sutchi catfish steaks from an initial value of 6.35 and 6.21 to a final value of 6.64 and 6.62, respectively. Ayse et al., (2017) also reported that the pH of minced Nile Perch patties increased from 6.2 to 7.2 after storage for 15 days but when the patties were treated with red cabbage the increase was only to 6.50. This increase in pH may be related to the production of alkaline compounds that can occur in fish postmortem (Manju et al., 2007).

4.3.2 Effect of MSKE on color of mince fish during frozen storage

There were no significant changes in lightness (*L*) from 0 to 6 weeks storage at -18 ^{o}C (Table 4.7), but the control sample became significantly ($P \leq 0.5$) darker from 9 weeks to the end of storage. Samples treated with BHT or MSKE 3% had no significant changes in their lightness during storage. The reduction in redness (*a*) was lowest with BHT and 3% MSKE compared to the control and the other samples. This effect may be due to BHT and MSKE 3% prevented the oxidation of the heme proteins, haemoglobin and myoglobin, which change from brown in their oxidized ferric form to red in their reduced state (Tesorier et al., 2007). The control sample and those treated with MSKE 1% and 2% showed a significant ($P \leq 0.5$) increase in the yellowness (*b*) at the end of frozen storage, with the control sample being significant ($P \leq 0.5$) more yellow. The increase in yellowness in control can be due to increased lipid oxidation, and these data also correlated well with PV values (Figure 4.4). Richards et al., (2002) explained that fish fillet is more sensitive to oxidation than bovine meat because of hemoglobin increase after

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harvest. Moreover, Sanchez-Alonso et al., (2008) explained that *a* and *b* values of horse mackerel mince with added white grape pomace did not change significantly during frozen storage. However, control sample was found *b* value increased in which was thought to be related with brown oxidation pigments (met-heame proteins). Hamre et al., (2003) stated that lipid oxidation often causes yellow fluorescent pigments to accumulate in the fillet and determined a weak correlation between oxidation indicator parameters and *b* value of herring. The aldehydes produced from lipid oxidation can very rapidly modify some amino acid residues, such as lysine, producing pyrroles which, by means of a polymerization reaction, are responsible for the color changes (Zamora et al., 1999).

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Table 4.7 Changes of pH values during frozen storage of mince fish (MF) that had been treated under different conditions during storage time up to 15 weeks at -18 °C

Samples	Storage Time (weeks)					
	0	3	6	9	12	15
Control MF	6.33±0.03^{Ac}	6.34±0.03^{Ac}	6.98±0.02^{Ab}	6.84±0.07^{Ab}	7.45±0.01^{Aa}	7.89±0.01^{Aa}
MF + BHT	6.31±0.04^{Ab}	6.32±0.17^{Ab}	6.34±0.05^{Bb}	6.374±0.03^{Bb}	6.57±0.02^{Ba}	6.57±0.05^{Ba}
MF + 1% MSKE	6.36±0.05^{Ab}	6.38±0.02^{Ab}	6.33±0.05^{Bb}	6.37±0.03^{Bb}	6.54±0.01^{Ba}	6.55±0.02^{Ba}
MF + 2% MSKE	6.35±0.02^{Ab}	6.36±0.15^{Ab}	6.35±0.05^{Bb}	6.33±0.05^{Bb}	6.54±0.02^{Ba}	6.56±0.05^{Ba}
MF + 3% MSKE	6.37±0.02^{Ab}	6.31±0.04^{Ab}	6.35±0.01^{Bb}	6.32±0.02^{Bb}	6.53±0.02^{Ba}	6.57±0.02^{Ba}

Values are means ± SD; (n=3); values in the same column that are followed by different superscript capital letters are significantly different ($P \leq 0.05$), values in the same row that are followed by different superscript small letters are significantly different ($P \leq 0.05$)

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Table 4.8 Instrumental color scores of mixed mince fish model that were treated under different conditionsduring storage time up to 15 weeks at -18 °C

Color Analysis	Treatments	Storage time (weeks)					
		0	3	6	9	12	15
L value	Control	55.19+0.65^{Aa}	55.16+0.66^{Aa}	55.18+0.35^{Aa}	52.26+0.35^{Cb}	48.99+0.81^{Cc}	45.15+0.30^{Dd}
	BHT	55.24+0.72^{Aa}	55.23+0.73^{Aa}	55.21+0.45^{Aa}	55.22+0.26^{Aa}	53.90+0.65^{Ab}	53.10+0.33^{Ab}
	1% MSKE	55.21+0.39^{Aa}	55.20+0.55^{Aa}	55.23+0.42^{Aa}	54.25+0.31^{Bb}	51.97+0.63^{Bc}	48.18+1.20^{Cd}
	2% MSKE	55.24+0.78^{Aa}	55.15+0.24^{Aa}	55.14+0.29^{Aa}	55.18+0.40^{Aa}	53.90+0.69^{Ab}	48.14+0.19^{Cc}
	3% MSKE	55.17+0.40^{Aa}	55.23+0.43^{Aa}	55.23+0.22^{Aa}	55.21+0.49^{Aa}	53.97+0.42^{Ab}	53.17+0.34^{Ac}
a value	Control	10.71+0.68^{Aa}	10.79+0.31^{Aa}	10.70+0.32^{Aa}	7.67+0.28^{Bb}	5.07+0.623^{Cc}	5.08+0.46^{Cc}
	BHT	10.72+0.33^{Aa}	10.78+0.44^{Aa}	10.63+0.24^{Aa}	10.68+0.22^{Aa}	8.85+0.24^{Ab}	8.81+0.29^{Ab}
	1% MSKE	10.72+0.25^{Aa}	10.76+0.71^{Aa}	10.57+0.37^{Aa}	10.65+0.44^{Aa}	7.57+0.47^{Bb}	7.55+0.45^{Bc}
	2% MSKE	10.70+0.27^{Aa}	10.72+0.28^{Aa}	10.68+0.28^{Aa}	10.66+0.44^{Aa}	7.67+0.21^{Bb}	7.52+0.48^{Bc}
	3% MSKE	10.74+0.31^{Aa}	10.71+0.29^{Aa}	10.74+0.27^{Aa}	10.66+0.26^{Aa}	7.61+0.30^{Bb}	7.64+0.48^{Bc}
b Value	Control	5.66+0.45^{Ad}	5.61+0.43^{Ad}	6.15+0.27^{Ac}	7.72+0.31^{Ab}	8.95+0.67^{Aa}	9.83+0.31^{Aa}
	BHT	5.69+0.33^{Aa}	5.68+0.29^{Aa}	5.71+0.37^{Ba}	5.68+0.12^{Ba}	5.72+0.15^{Ca}	5.74+0.24^{Ca}
	1% MSKE	5.62+0.47^{Ab}	5.68+0.48^{Ab}	5.70+0.11^{Bb}	5.65+0.12^{Bb}	6.47+0.41^{Ba}	6.48+0.16^{Ba}
	2% MSKE	5.64+0.22^{Ab}	5.68+0.23^{Ab}	5.65+0.40^{Bb}	5.70+0.11^{Bb}	6.48+0.18^{Ba}	6.45+0.13^{Ba}
	3% MSKE	5.69+0.21^{Aa}	5.69+0.51^{Aa}	5.63+0.27^{Ba}	5.71+0.36^{Ba}	5.67+0.30^{Ca}	5.70+0.58^{Ca}

Values are means ± SD; (n=3); values in the same column that are followed by different superscript capital letters are significantly different ($P \leq 0.05$), values in the same row that are followed by different superscript small letters are significantly different ($P \leq 0.05$)

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4.3.3 Effect of MSKE on peroxide values (PV) of mince fish during frozen storage

PV shows the degree of oxidation in the substance and measures the amount of total peroxides as a product of primary lipid oxidation in fish products. PV is used to determine the quality of oil sample (Kaya et al., 1993) and indicates the concentrations of peroxides and hydro-peroxides that are produced during the early stages of lipid oxidation. Sharp increases in PV have been taken as a measure of the end of the shelf-life of fish since they correlate with the development of off-flavors, changes in color and nutritional value (Fagan et al., 2003; Sahari et al., 2009). In this study mince fishs treated with BHT and MSKE 1, 2 and 3% and control were evaluated. The effect of MSKE 1, 2 and 3 % on PV of mince fishs during storage at 18 ^{o}C were evaluated compared with control sample and BHT.

The result showed that PV progressively increased in all mince fish samples during storage at -18°C with the lowest increases for BHT and the highest for the control sample with MSKE in between with 3% generally the lowest and 1% the highest (Figure 4.3). The results were similar to Alghazeer et al., (2008) and Tang et al., (2001) who found that the incorporation of green tea catechin in frozen mackerel was effective in lowering PV and TBARS values when compared to the control during frozen storage. Polyphenols such as tea catechins, olive oil hydroxytyrosol, rosemary extracts, and grape procyanidins have been found to retard lipid oxidation in fish muscle based food products (Tang et al., 2001; Pazos et al., 2006). Treatment of fish products with plant extracts has previously been shown to delay changes in PV. For examples Banani and Suchandra (2015) found that minced Tilapia fish treated with different spices (fennel, pepper or cinnamon) showed significantly ($P \leq 0.5$) lower PV changes compared to control sample during refrigerated storage for 28 days. They also found that during storage, chilled minced horse mackerel had low PV when they had been treated with ethanol extracts of potato peel compared to controls. Sarabi et al., (2017) reported that treatment of fried escolar (*Lipidocybium flavobrunium*) fish fillet with rosemary extract at 0.1 %, 0.2 %, 0.3 % or BHT at 0.1 % slowed PV development during 5 months storage. Papuc et al., (2012) found that the highest value of PV was recorded in control samples from dark muscle of Carp Muscle (*Cyprinus Carpio*) (40.3±3.9 O_2/kg), and the lowest in samples from dorsal side white muscle (24.1±2.0 Meq O_2/kg). Peroxide

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value was significantly lower in muscle tissue samples with incorporated sea buckthorn fruits polyphenols (with 72.20 % for dorsal side white muscle, 63.23 % for ventral side white muscle, and 61.04 % for dark muscle) during frozen storage. The results indicate that the MSKE especially 3 % from Choke-Anan has a potential to be used as retard PV formation in mince fish.

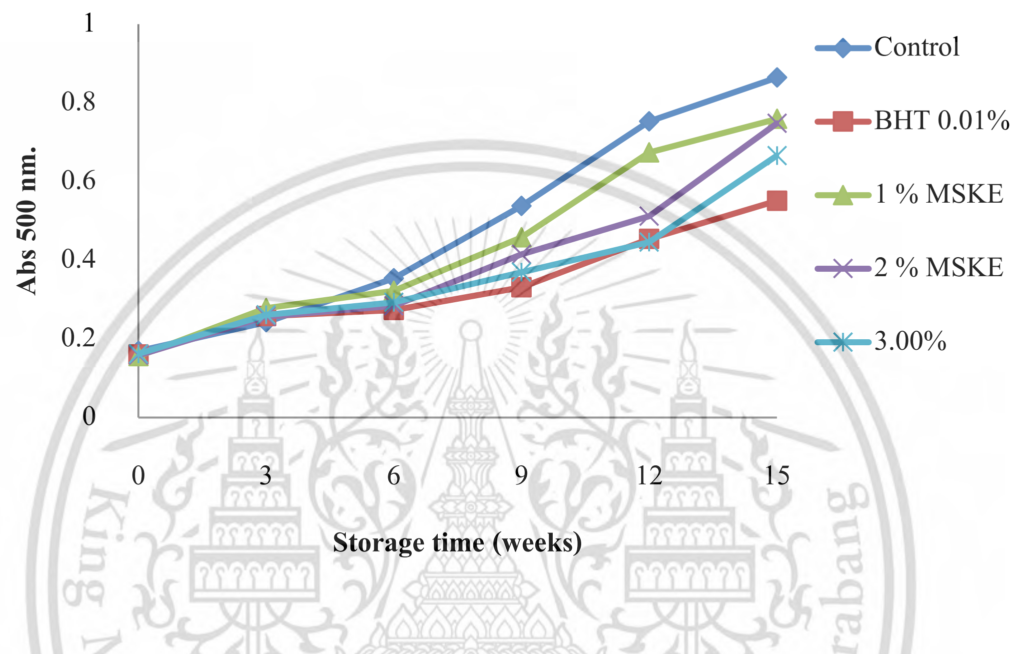

Figure 4.3 Changes in peroxide value (PV) of the mince fishs that were treated under different conditions during storage time up to 15 weeks at -18 °C storage

4.3.4 Effect of MSKE on thiobarbituric acid reactive substances (TBARS) of mince fish during frozen storage

The secondary lipid oxidation was evaluated based on the TBARS value, which is an index of malonaldehyde (MDA) concentration. MDA is formed by multiple scissions of cyclic internal hydroperoxides originating from fatty acids with three or more double bonds during lipid oxidation (Schaich et al., 2013). MDA is one of the main end products of lipid oxidation.

The effect of MSKE 1, 2 and 3 % on TBARS of mince fishs during storage at -18 °C were evaluated compared with control sample and BHT. The results showed that all

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samples showed increases in TBARS during storage with the control sample showed highest increases especially after 12 weeks. No significantly differences in TBARS were found between mince fish treated MSKE 3% and BHT 0.1 % after 15 weeks storage (Figure 4.4). The results indicate that the control samples were more rancid than samples treated with MSKE and BHT throughout storage. Ana et al., (2011) also found that Bologna-type mortadella containing BHT 0.01%, MSKE 0.1%, and 0.2% stored at 2 °C showed no significant increases in TBARS during storage. Ahmadi et al., (2014) also showed that there was no significant difference between two concentrations 200 and 400 ppm of hydroalcoholic and water extracts of nettle leaf (*Urtica dioica*) on changes in TBARS of minced kilka fish (*Clupeonella cultriventris*) samples during storage at -2 °C and showed lowest TBARS value when compared to control. The presence of TBARS in a sample of mince fish indicates that lipid peroxidation has taken place. (Lukaszewicz et al., 2004). Many polyphenol extracts obtained from plants have been reported to retard lipid oxidation when applied in fish muscle. Maqsood and Benjakul (2010) reported that in a comparison between ferulic acid, caffeic acid and tannic acid was most efficient in retarding the lipid oxidation in minced mackerel as showed by its lower PV and TBARS values. Papuc et al., (2012) showed that TBARS level had the highest values in carp muscle (*Cyprinus Carpio*) not treated with polyphenols from sea buckthorn fruits. Incorporation of polyphenols from sea buckthorn in fish muscle evaluated the decrease of TBARS value with 37.55% in dorsal side white muscle, with 44.91% in ventral side white muscle, and with 51.05% in dark muscle. Moreover, the inhibitory effect of polyphenols from the oxidation process of lipids in carp muscle subject to freezing is due to the capacity of these compounds to scavenge free radicals, and to chelate Fe^{2+} ions, which are very important in preventing the development of rancidity in fish. The results indicate that the MSKE especially 3% from Choke-Anan has a potential to be used as prevent MDA formation in mince fish.

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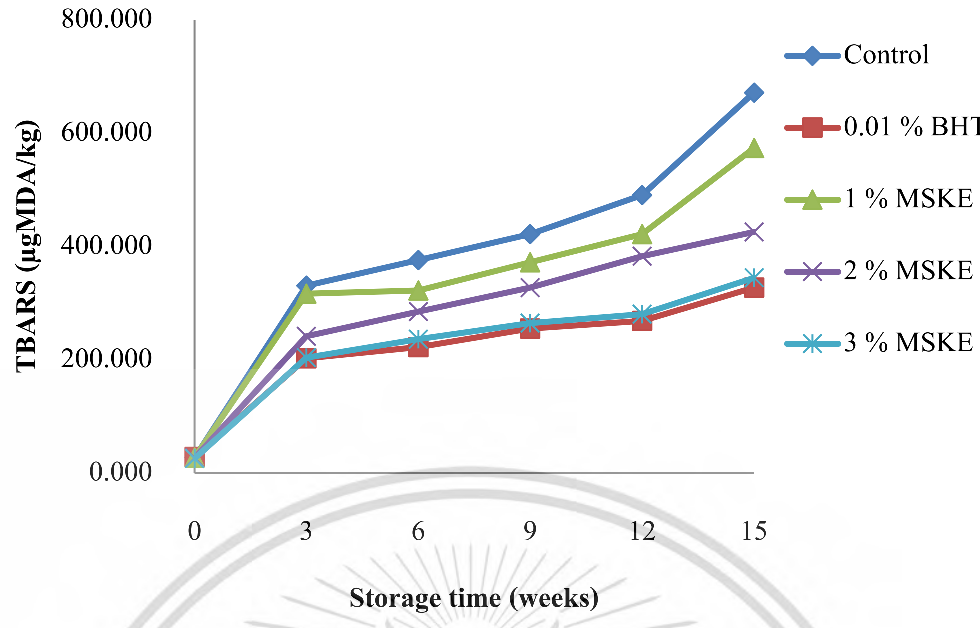

Figure 4.4 Changes in thiobarbituric acid reactive substances (TBARS) of the mince fishes that were treated under different conditions during storage time up to 15 weeks at -18 °C storage

4.3.5 Effect of MSKE on conjugated diene (CD) of mince fish during frozen storage

The effect of MSKE 1, 2 and 3 % on CD of mince fishs during storage at -18 °C were evaluated compared with control sample and BHT. Conjugated dienes are formed as intermediates through a shift of a double bond of polyunsaturated Acids (PUFA). Houhoula et al., (2002) explained that CD is a representative oxidation index that can be used during frying to characterize the status of oxidation of cotton seed oil.

The results showed that CD of all samples increased gradually during 15 weeks of storage. The control samples showed the highest CD formation among all samples tested. The samples treated MSKE 3% and BHT showed the highest activity in preventing formation of CD (Figure 4.5). Formation of high contents of CD may be related to the presence of higher contents of polyunsaturated fatty acids (Liu and White, 1992). Lipids containing methylene- interrupted dienes or polyenes show a shift in the position of the double bond during the oxidation due to isomerization and the formation of CD. The formation of CD was reported to increase with the uptake of oxygen during the early stages of oxidation and to result in the formation of primary

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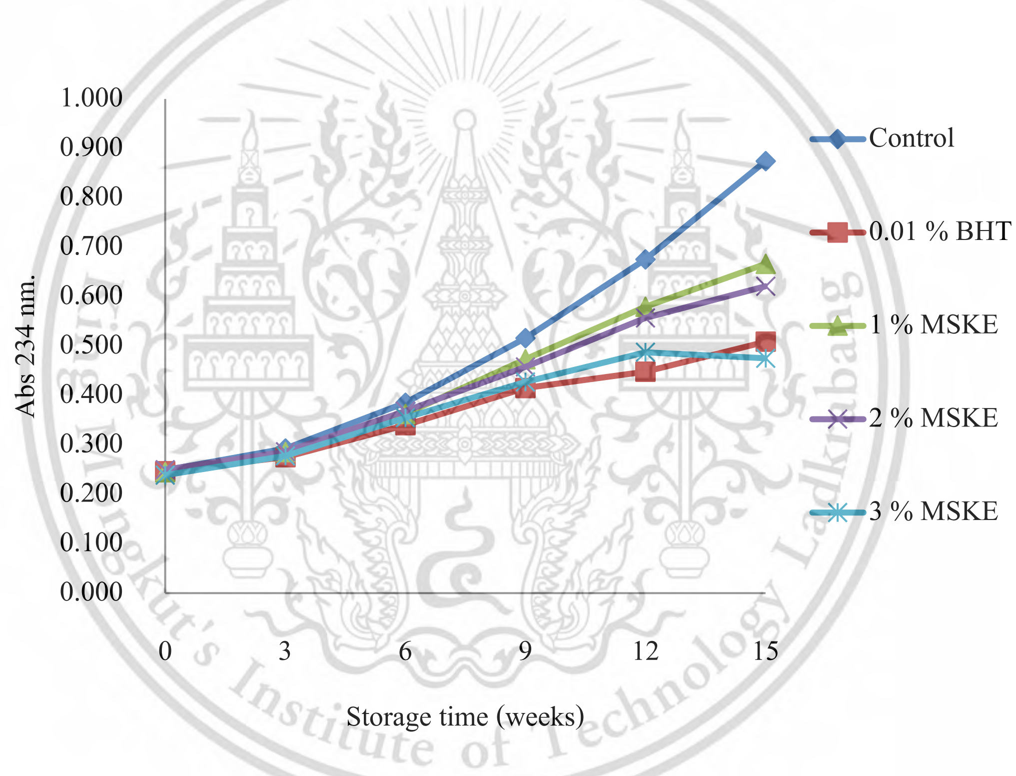
oxidation products, along with the peroxides (Halliwell and Gutteridge, 1989).

Banani and Suchandra (2015) demonstrated that CD value of mince Tilapia fish, which had been mixed with extracts from spices (fennel seeds, black pepper or cinnamon) or BHT, has significantly ($P \leq 0.5$) lower increases in CD than control during refrigerated storage 35 days. Maqsood and Benjakool (2010) reported that among the different phenolic compounds, tannic acid showed the highest activity in preventing CD formation in mackerel mince (*Rastrelliger kanagurta*) during 15 days of iced storage. Papuc et al., (2012) also showed that the level of CD of carp muscle (*Cyprinus carpio*) was lower for those that had been treated with



extracts from sea buckthorn fruits (*Hippophae rhamnoides*) during frozen storage for 6 weeks.

Figure 4.5 Changes in conjugated diene (CD) of the mince fishes that were treated under different conditions during storage time up to 15 weeks at -18 °C storage

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4.3.6 Effect of MSKE on protein carbonyl of mince fish during frozen storage

There was no significant (*P*>0.5) differences in protein carbonyl formation between sample treated BHT or MSKE during storage, but control samples showed a sharp increase after 6 weeks of storage (Figure 5). These results can be explained by the BHT and MSKE having a protective role against oxidation of proteins. It had been previously reported that phenolic acids, phenolic diterpenes (Estevez et al., 2005), flavonols (Salminen et al., 2008) and tea catechins (Rababah et al., 2004) inhibited the formation of protection protein carbonyl in meat products as measured by the DNPH method. In fish Baron et al. (2007) found a significant increase in carbonyl content of rainbow trout after storage at -20 °C for up to 13 months and Sabeena et al., (2012) found that mince horse mackerel (*Trachurus trachurus*) treated with both ethanol and water potato peel extract showed a significantly (*P*≤0.5) lower increased protein carbonyl forming compared to control at the end storage at 5 °C. Siebet et al., (1996) explained that polyphenol compounds can inhibit the oxidation of proteins either by retarding the lipid oxidative reaction, by binding to the protein, or by forming a complex with them.

Proteins are the major components of muscle tissue and therefore modification of the native structure and/or integrity of muscle proteins are known to affect muscle food quality including texture, aroma, water holding capacity, color and functionality (Popova et al., 2009). Two processes lipid and protein oxidation can interact with each other. It is thus well known that redicals, hydroperoxides and secondary compounds resulting from lipid oxidation react with protein leading to the loss of protein functionality (Gardner, 1979). The remarkable changes of protein oxidative modifications in fish muscle consists of generation of protein carbonyls. Some major amino acids, such as lysine, histidiene, proline and arginine yield carbonyl compounds and therefore the concentration of such compounds is a meaningful indicator of the oxidative status of muscle proteins (Delle-Done et al., 2003). Oxidative reactions involving the side chains of amino acids can lead to the formation of carbonyl groups as this conversion may ultimately result in a loss of catalytic activity and increased susceptibility to protein degradation (Stadtman, 1990) or protein aggregation and loss of solubility.

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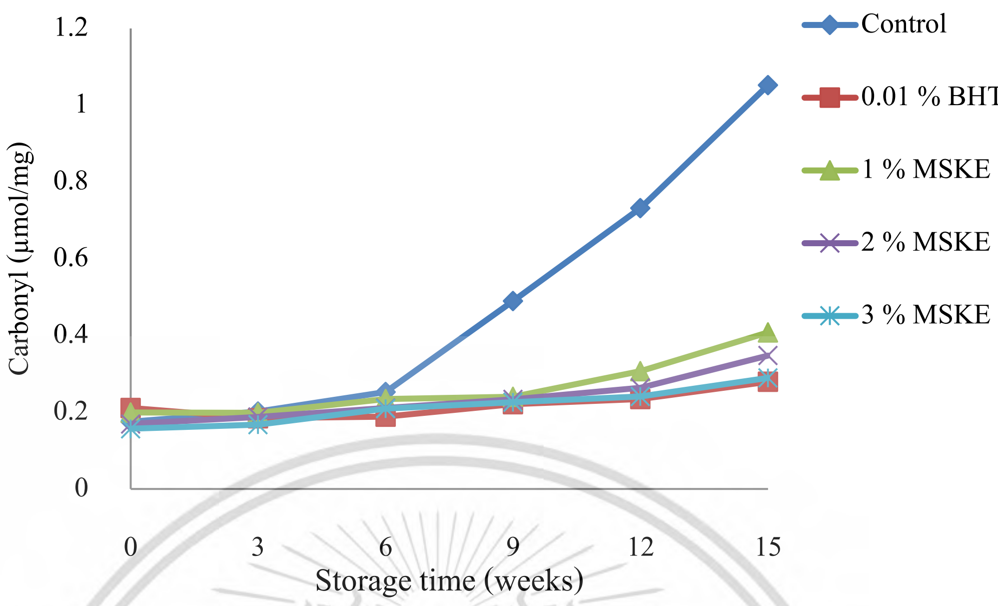

Figure 4.6 Changes in protein carbonyl of the mince fishes that were treated under different conditions during storage time up to 15 weeks at -18 °C storage

4.3.7 Effect of MSKE on sulfhydryl groups (SH) of mine fish durring frozen storage

Changes in SH value of mince fish treated MSKE, BHT and control sample during frozen storage at -18 °C for 15 weeks are shown in Figure 5. SH groups decreased during storage in all the samples, with the controls decreasing most followed by 1 % MSKE, then 2 % MSKE and finally with 3 % MSKE and BHT about the same. During frozen storage, the myofibrillar proteins undergo conformational changes partly resulting in the decrease of SH groups due to the formation of disulphide bonds through oxidation of SH groups or disulphide interchanges (Hayakawa and Nakai, 1985). Therefore, this reduction in the rate of decrease of SH groups by adding BHT and MSKE might be due to lower oxidation. Treating processed fish with organic by-products including ethanol potato peel extract (Sabeena et al., 2012) and brown seaweed (Wang et al., 2016) has previously been shown to decrease their SH groups during storage.

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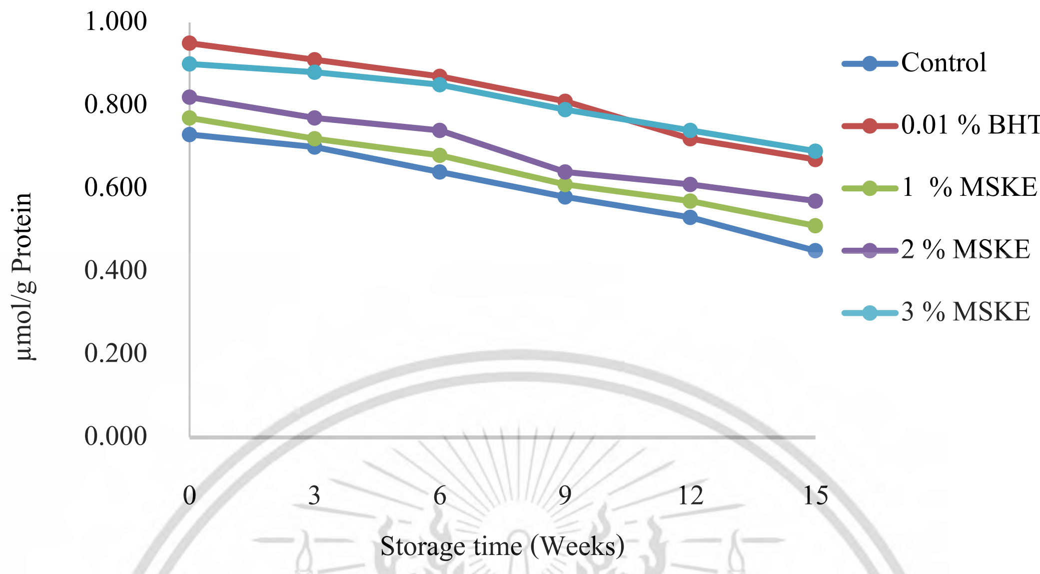

Figure 4.7 Changes in Sulfhydryl groups (SH) of the mince fishes that were treated under different conditions during storage time up to 15 weeks at -18 °C storage

4.3.8 Effect of MSKE on total volatile base nitrogen (TVB-N) of mince fish during storage

The TVB-N was low, as would be expected, for the fresh minced fish, but it rose rapidly during storage, especially for the control. The rate of increase of the MSKE treated sample was significantly (P≤0.5) lower, beginning after 6 weeks storage. The effects of the MSKE were proportional to its concentration with MSKE being more effective the higher the concentration used (Table 4.9). However, the BHT was totally ineffective in reducing this increase in TVB-N and was not significantly different to the control throughout storage. Sarabi et al. (2017) reported a similar effect of BHT on fried escolar, where rosemary extract was significantly (P≤0.5) more effective than BHT. However, in that case BHT was significantly (P≤0.5) more effective than controls in reducing the rate of increase in TVB-N. TVB-N has been shown to be related to microbial growth so a possible explanation could be that MSKE was more effective in inhibiting microorganisms than BHT at the levels tested. A positive correlation

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between total viable bacterial count and changes in TVB-N in chicken marinate has been previously shown (Baltic et al., 2017). Evaluation of TVB-N is a commonly used method to monitor the freshness of fish. Fresh fish must have TVB-N values in the range of 5-10 mg/100 g of muscle, the satisfactory level in the range of 15 to 25 mg/100 g and decayed fish over 50 mg/100 g (Ogawa and Maia, 1999). Ingridy et al., (2014) found the TVB-N value for mince Tilapia fish treated with Nori 50 µg GAE/g and Hijiki Seaweed 50 µg GAE/g was 6.12 and 6.89 mg/100 g, respectively after 180 days of frozen storage. In carp (*Cyprinus carpio*) fillet Shirin and Marayam, (2014) found the lowest TVB-N value was 20.7±0.37 mg/100 g when treated with grape extract compared to 31.40±0.75 mg/100 g for those not treated after storage at 4 °C.

Table 4.9 Changes of total volatile base nitrogen (TVB-N) (mg/100 g) after different treatments of mixed mince fish (MF) during storage time up to 15 weeks at -18 °C storage

Samples	Storage Time (weeks)					
	0	3	6	9	12	15
Control MF	3.17±0.60^{Af}	5.24±0.51^{Ae}	7.59±0.02^{Ad}	11.46±0.07^{Ac}	15.19±0.01^{Ab}	16.85±0.23^{Aa}
MF + BHT	3.31±0.04^{Af}	5.32±0.17^{Ae}	7.94±0.05^{Ad}	11.33±0.03^{Ac}	15.57±0.02^{Ab}	16.57±0.05^{Aa}
MF + 1% MSKE	3.36±0.05^{Af}	5.38±0.02^{Ae}	7.73±0.05^{Ad}	10.37±0.03^{Bc}	13.54±0.01^{Bb}	14.52±0.02^{Ba}
MF + 2% MSKE	3.35±0.02^{Af}	5.36±0.15^{Ae}	6.35±0.05^{Bd}	8.33±0.05^{Cc}	10.54±0.02^{Cb}	12.56±0.05^{Ca}
MF + 3% MSKE	3.47±0.02^{Af}	5.31±0.04^{Ae}	6.35±0.01^{Bd}	7.32±0.02^{Dc}	9.53±0.02^{Db}	10.57±0.02^{Da}

Values are means ± SD; (n=3); values in the same column that are followed by different superscript capital letters are significantly different ($P \leq 0.05$), values in the same row that are followed by different superscript small letters are significantly different ($P \leq 0.05$)

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4.4 Application mango seed kernel extract in cosmetic cream

4.4.1. Physical Stability Study of MSKE cream

An emulsion cream containing MSKE was suggested by the Cosmaprof Co., Ltd., Thailand (Figure 4.8). After centrifugation, the phase separation was not observed in all samples of MSKE cream and control base (data not shown). The physical stability evaluation of MSKE creams are described in Table 4.10. The freeze-thaw cycling test of samples also showed that pH value and viscosity of the samples from each cycle did not exhibit a significant change, and. were in the range of 5.43-5.74 and 15536.22-15558.45cPs, respectively. The phase separation of both MSKE and control base cream samples was not observed. The results indicated that the pH, viscosity and appearance of MSKE creams were stable during storage, and that MSKE did not affect their pH and viscosity of the cosmetic emulsion cream.

The pH is an important parameter for the effectiveness of cosmetic creams. The total polyphenol contents of all MSKE creams were decreased by around 12% after the 4th cycle tested. The phenolics of the MSKE cream reduced toward the end of the thermal cycling test. Maisuthisakul and Harnsilawat, (2011). Furthermore, the phenolic compounds can interact with the emulsifier in the cosmetic formulation. Stockmann et al., (2000)

The color changes of the MSKE creams were also evaluated for their physical stability (Figure 4.2). In particular, the total color different (ΔE) of the 1% MSKE and base cream was not significantly different with increasing storage time. While the (ΔE) of the 2% and 3% MSKE creamswere significantly (p<0.05) different between the beginning and the end of the storage time. Therefore, our results indicate the color of the 1% MSKE cream was stable. A previous study demonstrated that a cosmetic micro emulsion containing 1% MSKE from Fahlun mango cultivar showed good physicochemical stability during a 6 month storage period at 25 ± 2 °C with 60 ± 5% RH (Leanpolchareanchai et al., 2014).

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Figure 4.8 MSKE and control cosmetic cream

4.4.2. The Clinical *in-vivo* skin irritation test of MSKE cream

The skin irritation test of new ingredients for use in cosmetics is important for the safety of these products. In the evaluation all 33 volunteers were present for the complete duration of the study and there were no changes, other than those expected, were observed during the study. The average age of the volunteers was 33.9 years and sex ratio male/female was 12/21.

The minimum age was 18 years and the maximum age was 58 years. The 24 and 48 hours patch test results of the 33 volunteers are shown in Table 5. None of the volunteers exposed to MSKE cream had an adverse reaction. The positive control 1% of sodium lauryl sulfate showed a cutaneous irritant response in 1 volunteer (3%) after 24 hours and 12 volunteers (36.4%) after 48 hours. The skin exposed to the MSKE cream did not respond differently from the skin exposed to the negative control (deionized water). Therefore, in the experimental condition, MSKE cream could be acceptable for consumer safety and was shown to be a non-irritant.

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Table 4.10 pH, viscosity, phase separation and TPC of MSKE creams and base control from the freeze-thaw physical stability evaluation

Samples	Parameters	Freeze-thaw cycle						
		0	**1**	**2**	**3**	**4**	**5**	**6**
Control base	pH^{NS}	5.74±0.02	5.67±0.06	5.63±0.06	5.43±0.38	5.70±0.10	5.63±0.06	5.70±0.17
	Viscosity (cPs)^{NS}	15558.45±18.53	15545.04±14.73	15546.33±3.21	15536.22±4.04	15550.34±8.50	15547.14±10.11	15549.33±11.01
	Phase separation	N	N	N	N	N	N	N
	TPC (mg of GAE/ml)	ND	ND	ND	ND	ND	ND	ND
1% MSKE	pH^{NS}	5.72±0.05	5.63±0.06	5.70±0.10	5.67±0.12	5.70±0.10	5.67±0.06	5.63±0.06
	Viscosity (cPs)^{NS}	15540.43±7.61	15546.33±5.85	15540.66±6.11	15549.21±10.44	15551.06±10.14	15538.66±4.04	15547.66±6.35
	Phase separation	N	N	N	N	N	N	N
	TPC (mg of GAE/ml)	1.64±0.32^{a}	1.65±0.40^{a}	1.66±0.33^{a}	1.62±0.33^{a}	1.48±0.15^{b}	1.47±0.09^{b}	1.47±0.12^{b}
2% MSKE	pH^{NS}	5.69±0.03	5.67±0.06	5.67±0.12	5.73±0.12	5.63±0.06	5.70±0.10	5.70±0.10
	Viscosity (cPs)^{NS}	15543.56±11.15	15542.66±11.05	15549.66±2.50	15539.66±6.31	15547.33±12.42	15549.66±9.60	15541±5.58
	Phase separation	N	N	N	N	N	N	N
	TPC (mg of GAE/ml)	3.32±0.06^{a}	3.34±0.14^{a}	3.27±0.21^{a}	3.23±0.17^{a}	2.84±0.03^{b}	2.79±0.13^{b}	2.79±0.11^{b}
3% MSKE	pH^{NS}	5.70±0.06	5.67±0.06	5.70±0.10	5.73±0.12	5.67±0.12	5.67±0.06	5.63±0.06
	Viscosity (cPs)^{NS}	15527.45±5.80	15529.33±5.50	15539.33±8.32	15535.66±6.69	15539.04±5.19	15538.55±6.03	15540.66±10.60
	Phase separation	N	N	N	N	N	N	N
	TPC (mg of GAE/ml)	4.92±0.12^{a}	4.90±0.21^{a}	4.89±0.14^{a}	4.88±0.21^{a}	4.23±0.13^{b}	4.24±0.31^{b}	4.20±0.11^{b}

Values are mean ± SD (n = 3); values in the same row that are followed by different superscript letters are significantly different ($P \leq 0.05$).

NS means non-significantly different, N means not observed, ND means not determined

Table 4.11 Number of volunteers and grade of irritant response after 24 and 48 hours exposure to the creams

Time	Test material	Number (%) grade of skin reaction (n=33)					Mean±SD of severity grade
		Negative	Positive	0.5	1+	2+	
24 hr.	MSKE Cream 1%	33(100)	0(0)	-	-	-	
	Distilled water	33(100)	0(0)	-	-	-	
	Sodium lauryl sulfate	32(97.0)	1(3.0)	1(3.0)	-	-	0.02±0.09
48 hr.	MSKE Cream 1%	33(100)	0(0)	-	-	-	
	Distilled water	33(100)	0(0)	-	-	-	
	Sodium lauryl sulfate	21(63.6)	12(36.4)	11(33.4)	1(3.0)	-	0.2±0.28

Figure 4.9 Color changes of cosmetic formulations cream containing MSKE and the control (containing no MSKE) at ambient temperature and humidity.

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CHAPTER V

CONCLUSION

The seed kernel of two Thai mango varieties, Kaew and Choke-Anan, were extracted using ultrasonic assisted extraction (ultrasonication). The duration time of ultrasonication affected the yield and total polyphenol content (TPC) of both ccultivars. In this study the optimum ultrasonication time for the extraction was 45 min. The ultrasonication assisted extraction of MSKE from both cultivars had about twice the antioxidant activity compared to the other methods tested (DPPH, FRAP, H_2O_2 and ABTS). There was a cultivar difference since the MSKE from Choke-Anan showed higher antioxidant activity than that from Kaew. The MSKE from both cultivars showed no significant difference (P>0.05) in tyrosinase inhibition activity compared to arbutin. The inhibitory activity of the enzymes 5-lipoxygenase, hyaluronidase and α-glucosidase was generally higher in Choke-Anan than Kaew. However, a slightly lower α-glucosidase inhibition activity than acarbose was observed.

MSKE from both Kaew and Choke-Anan were fractionated using Sephadex LH-20 column chromatography and the antioxidant activity and inhibitory activity on tyrosinase, 5-lipoxygenase, hyaluronidase and α-glucosidase of MSKE fractions were evaluated. The key components of polyphenol, found in the most active fraction, were identified using LC-ESI-MS. Three major isolates were obtained from the MSKE of both cultivars with the absorbance being higher for Choke-Anan than Kaew. Fraction 3 of MSKE from both cultivars showed significantly ($P \leq 0.05$) higher antioxidant activity and 5-lipoxygenase, hyaluronidase and α-glucosidase inhibitory activity with Choke-Anan more effective than Kaew. Six different polyphenols were found in fraction 3 of both cultivars: tri-*o*-galloyl-glucoside, tetra-*o*-galloyl-glucoside, maclurin tri-*o*-galloyl-glucoside, penta-*o*-galloyl-glucoside, hexa-*o*-galloyl-glucoside, and hepta-*o*-galloyl-glucoside. In the MSKE from Choke-Anan, hexa-*o*-galloyl-glucoside and tetra-*o*-galloyl-glucoside were the two major components, whereas in the MSKE from Kaew tetra-*o*-galloyl-glucoside was the only major component.

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The comparative effects of 0, 1, 2 and 3% of MSKE, extracted from 'Choke-Anan' were tested on retarding lipid and protein oxidation in mince fish during storage at -18°C for 0, 3, 6, 9, 12 and 15 weeks. BHT (0.01%) was added as a second control because of its established antioxidant properties. Samples treated with different concentration MSKE and BHT had significantly ($P \leq 0.05$) increased pH values after 12 weeks storage. After 15 weeks storage the sample treated with MSKE 3% and BHT had significantly ($P \leq 0.05$) lower lightness (L) and redness (a) changes with no significant effects on yellowness (b). Samples treated with 3% MSKE had the lowest formation of protein carbonyl and decreased SH groups, PV, TBARS, TVB-N value and CD values compared to the non-treated samples and the samples treated with BHT. The results indicate that the MSKE can be used effectively as a natural ingredient to retard the lipid and protein oxidation in mince fish products.

After accelerated stability studies, the results showed alteration of both 2 and 3% MSKE formulations had similar colorimeter results (ΔE). The pH value and viscosity of cosmetic formulations were not significantly different ($P > 0.05$) during the completion of the testing cycle, while at the end of the testing cycle TPC of all the formulations had decreased. The results from a clinical skin irritation study of the MSKE cream indicated that the product was safe for consumer usage even on non-irritant to mild skin. Thus this study suggests that MSKE from Choke-Anan may be used as a cosmetic supplementary ingredient in whitening and anti-aging creams.

It can be finally concluded that these findings demonstrate that Thai mango seed by-products Kaew and Choke-Anan can be a source of natural ingredients for functional foods, pharmaceutical and cosmetic products.

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APPENDIX

PUBLISHCATION

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Original Research Article

Antioxidant properties, selected enzyme inhibition capacities, and a cosmetic cream formulation of Thai mango seed kernel extracts

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Abstract

Purpose: *To investigate the antioxidant properties, the inhibition of selected enzyme activities of ultrasonication-assisted mango seed kernel extract (MSKE), and to evaluate the physical stability and skin irritation properties of a cosmetic cream formulated with MSKE.*
Methods: *Choke-Anan MSKE and a Kaew cultivar of Thai mangoes were prepared by ultrasonication-assisted extraction. Antioxidant activities (DPPH, FRAP, H_2O_2 scavenging assay, ABTS), antityrosinase, anti 5-lipoxygenase, antihyaluronidase and anti α-glucosidase were determined. Cosmetic creams containing 0, 1, 2 and 3 % of MSKE were prepared and evaluated for physical stability. The most stable formulation was subjected to the clinical skin irritation test.*
Results: *The yield, total polyphenol content, antioxidant properties and inhibition of 5-lipoxygenase, hyaluronidase and α-glucosidase were higher ($p < 0.05$) for MSKE from Choke-Anan than from Kaew cultivar. The MSKE from both cultivars showed no significant difference ($p > 0.05$) in tyrosinase inhibition activity compared to arbutin. However, a slightly lower α-glucosidase inhibition activity than acarbose was observed. The cosmetic cream containing 1 % Choke-Anan MSKE had good physical stability with no skin irritation.*
Conclusion: *MSKE exhibits good antioxidant and enzyme inhibitory activity. Thus, it is a potentially natural functional ingredient for use in food and cosmetic industries.*

Keywords: *Mango, Antioxidant, Enzyme inhibitory activities, Cosmetic product stability, Skin irritation*

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INTRODUCTION

Polyphenol phytochemicals have been extensively studied and are the most well-known bioactive compounds found in plants. Polyphenols are secondary metabolites found in higher plants that contain one or more phenol units [1]. They have strong antioxidant activity, and can scavenge a variety of free radicals including reactive oxygen species (ROS) and reactive nitrogen species (RNS) [2]. Previous studies have shown the protective action of polyphenols on human health and indicate their potential use as key components of a healthy and balanced diet [3]. Currently, the use of natural antioxidants in cosmetics is of increasing interest. Previous studies show that oxidative stress, is the major cause of skin ageing and is an over production of ROS and a reduction of antioxidant activity with age [4]. Moreover, plant polyphenols have been reported to be used as sunscreens, whitening and anti-ageing agents in cosmetic products [5].

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The mango (*Mangifera indica* L) fruit belongs to the *Anacardiaceae* family and is a good source of various polyphenols, which are found in the pulp, peel and seed [6]. The mango fruit and its processed products are in increasing demand in the world market. Consequently, mango seed and peel, which account for 35 - 60 % of the fruit depending on the variety, are the main by-products [7]. There are several mango varieties grown in Thailand. The most well-known cultivars are *Choke-Anan, Ok-Long, Kaew, Nam-Dorkmai, Rad and Keow-Savoey*. The *Choke-Anan* and *Kaew* cultivars are commonly used for processing in factories, and represent for 29.5 % and 27.9 %, respectively, for all mango varieties. It has been reported that industrial mango seed waste generation is as high as 1 ton annually [8].

The bioactive compounds in the mango seed kernel are tannin, gallic acid, coumarin, cafeic acid, vanillin, mangiferin, ferulicacid and cinamic acid [9]. Mango seed kernel extracts have been reported to have anti-tyrosinase, anti-inflammatory and hepatoprotective activities [10]. Therefore, mango seed kernels could be a potential source of ingredients for functional foods and cosmetics [11].

The present study aimed to determine a suitable ultrasonication time for the extraction of two cultivars of Thai mango seed kernel. The bioactivity, antioxidant activity and the effect on tyrosinase, 5-lipoxyganase, hyaluronidase and α-glucosidase activities were also studied. The application of the mango seed kernel extract in a cosmetic cream was evaluated.

EXPERIMENTAL

Plant materials

Two mango cultivars (*Mangifera indica* L.) were studied. The cultivars, *Kaew* and *Choke-Anan* were obtained from a local orchard in Nakornratchasima Province, Thailand between March and May, 2015. Mature green mangoes were selected by weight (200 - 250 g / kg). The peel and pulp were removed from the fruits using a fruit peeler and a knife, and the seeds were kept at -18 °C (Ultra Cold Freezer -80 °C, CTL 821, Thailand) for no longer than 1 month. Before use, the frozen kernels were separated from their shell with scissors.

Preparation of mango seed kernel extract (MSKE)

Crude MSKE were prepared by ultrasonic-assisted extraction as previously described [12]. All of the samples were ground and blended with 95 % ethanol (100 ml) in a blender for 5 min. The samples were incubated in a sonication water bath, at a frequency of 20 KHz and a temperature of 25 °C for 15 - 60 min. The samples were further incubated in a water bath at 80 °C and stirred every 10 min for 1 h. The mixtures were cooled at room temperature and the supernatant from each mixture was passed through Whatman filter paper no. 4. All filtrates were evaporated in a rotary evaporator at 50 °C under a vacuum until dry, and the extracts were weighed to determine the extraction yield of the soluble components.

Determination of total polyphenol content (TPC)

TPC was analysed as previously described [13]. The TPCs of the samples were expressed as mg of gallic acid equivalents per gram of MSKE.

Determination of antioxidant activities

The antioxidant activity of MSKE was evaluated using four different methods; the 2, 2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, the ferric reducing antioxidative power assay (FRAP), the hydrogen peroxide scavenging assay and the 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assay. The assays were performed according to previous reports [14]. The results were expressed as mg of trolox equivalents per gram of MSKE.

Determination of enzyme inhibition activity

The tyrosinase inhibition activity was measured using a modified dopachrome method with mushroom tyrosinase and L-3, 4-dihydroxyphenylalanine as the substrate [15]. The 5-lipoxygenase inhibition activity was studied using sodium linoleate as the substrate according to a previous study [16]. The hyaluronidase inhibition activity was determined using sodium hyaluronate as substrate following a previously described method [17]. The α-glucosidase inhibition activity was measured using p-nitrophenyl-α-D-glucopyranoside as substrate [18]. All

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inhibitory effects of the samples were expressed as the inhibitor concentration causing a 50 % loss of enzyme activity (IC_{50}).

Preparation of cosmetic cream

The oil-in-water emulsion creams used in this study were prepared, respectively by melting the lipophilic phase including Emulium Delta (5.0 %), stearic acid (1.5 %), stearyl alcohol (2.0 %), Captex 300 (3 %) and cetyl alcohol (1.5 %) in a water bath at 80 °C and separately mixing the hydropholic phase; propylene glycol (3 %), xanthan gum (0.1 %), EDTA (0.1 %) and water in a water bath at 70 °C.

The 2 phases were mixed by homogenizer at 20,000 rpm for 3 min and cooled down at room temperature. MSKE (0, 1, 2, and 3 % w/w) and 0.1 % phenoxyethanol as preservative were added and mixed again at 10,000 rpm for 3 min.

Test for resistance to centrifugation

The resistance to centrifugation study was based on a previously described method [19]. The samples were stored at ambient temperature and humidity for 48 h. A 10 ml sample was centrifuged at 3000 rpm for 30 min. The Samples were evaluated for phase separation by measuring the supernatant after centrifugation.

Assessment of physical stability

The samples were stored at -4 °C for 24 h and then 25 °C for another 24 h and this was repeated for 6 cycles. Samples were taken every 48 h for the evaluation of pH, viscosity, phase separation, colour and TPC. The samples were centrifuged at 15000 g for 30 min at 4 °C and the supernatant was analysed for TPC using a previously described method [13].

The colour of the samples was expressed as *L* (degree of lightness), *a* (degree of redness) and *b* (degree of yellowness) values. The total colour difference (ΔE) was calculated by Eq 1.

$$\Delta E = \sqrt{(L - L')^2 + (a - a')^2 + (b - b')^2} \quad \text{........ (1)}$$

where *L*, *a* and *b* are the sample colours, and *L´*, *a´ and b´* are the colours at time zero.

Skin irritation test

MSKE cream was subjected to *in-vivo* skin irritation assessment. A total of 20 Thai men and women with normal skin, 18 years old and older, volunteered to participate in this study. Patch tests were performed on a part of the back (5x4 cm) of all volunteers. After 24 h, the patch was removed and the skin was observed for redness/ irritation after 30 min and 24 h. The method used in this study was approved by the Ethical Committee for *in vivo* Studies of Mae Fah Luang University, Thailand (reference no. REH-50005).

Statistical analysis

The results are expressed as the mean ± standard deviation (SD, n = 3) Statistical analyses were carried out by a one-way ANOVA using SPSS version 16.0. Significant differences were at $p \leq 0.05$.

RESULTS

Extraction yield and TPC

The effect of duration of ultrasonication on the extraction yield and TPC of the MSKEs from the two cultivars are shown in table 1. The results showed that a longer ultrasonication duration (up to 45 min) gave a significantly higher ($p \leq 0.05$) extraction yield and TPC of the extracts. However, the values were not significantly different ($p > 0.05$) after 45 min, indicating that the optimum duration of ultrasonication for the extraction of MSKEs under the conditions used in this experiment was 45 min. The *Choke-Anan* cultivar had a higher extraction yield and TPC than the *Kaew* cultivar. The MSKE obtained by ultrasonic-assisted extraction at 45 min gave a 55 and 63 % higher extraction yield and 92 and 55 % higher TPC for the *Kaew* and *Choke-Anan* cultivars, respectively compared to conventional ethanol extraction.

Antioxidant activity of MSKE

MSKEs prepared by the ultrasonication-assisted extraction of the two cultivars had approximately two-fold greater antioxidant activity for all the methods studied (Table 2). The higher polyphenol content in the MSKEs obtained by ultrasonication-assisted extraction (Table 1) likely contributed to the greater antioxidant activity. In addition, the *Choke-Anan* MSKE showed a higher antioxidant

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activity than the *Kaew* MSKE for all methods evaluated. The results corresponded to the content of TPC, which was also higher in the *Choke-Anan* MSKE (Table 1).

Enzyme inhibition capacity of MSKEs

As shown in Table 3, the *Kaew* and *Choke-Anan* MSKE were not significantly different ($p > 0.05$) for the tyrosinase inhibitory activity compared to arbutin and the IC_{50} values were in the range of 19.86 ± 1.2 to 20.64 ± 0.3 µg / mL. The *Choke-Anan* MSKE had significantly higher ($p \le 0.05$) 5-lipoxygenase inhibitory activity than that the *Kaew* MSKE. However, the inhibitor concentration that caused a 50 % loss of the enzyme activity (IC_{50}) of the MSKEs was approximately 3.2 - 4.3 fold higher than rutin. The hyaluronidase inhibitory activity of the *Choke-Anan* MSKE was not significantly different ($p > 0.05$) compared to vitamin C. However the *Kaew* MSKE showed an approximately 1.3 fold lower inhibitory activity. The *Choke-Anan* MSKE had a significantly higher ($p \le 0.05$) α-glucosidase inhibitory capacity than the *Kaew* MSKE. The *Choke-Anan* MSKE had an approximately 1.1 fold higher IC_{50} than acarbose, indicating a slightly lower α-glucosidase inhibitory activity.

Table 1: Effect of the ultrasonication duration on the extraction yield and TPC of MSKEs

Mango cultivars	Extraction time (min)	Yield (%)	TPC (mg GAE/g)
Kaew	0 (Ethanol extraction)	1.60±0.06[g]	71.74±0.97[f]
	15	1.71±0.03[f]	70.93±0.63[f]
	30	2.10±0.07[e]	91.64±0.80[e]
	45	2.48±0.03[c]	137.49±1.15[b]
	60	2.49±0.06[c]	138.71±2.52[b]
Choke-Anan	0 (Ethanol extraction)	2.16±0.02[e]	110.02±1.06[d]
	15	2.29±0.04[d]	110.32±0.93[d]
	30	2.73±0.03[b]	124.36±1.69[c]
	45	3.52±0.03[a]	170.12±1.89[a]
	60	3.56±0.02[a]	170.63±0.93[a]

Values are the mean ± standard deviation; (n=3); values in the same column followed by different superscript letters are significantly different ($p \le 0.05$)

Table 2: Antioxidant capacity of MSKE by different methods

MSKE extract	Antioxidant capacity (mg Trolox/ g MSKE)			
	DPPH	FRAP	H_2O_2	ABTS
Kaew Ethanolic	92.61±3.72[d]	91.24±3.34[d]	49.15±4.96[d]	82.66±1.17[d]
Kaew Ethanolic + ultrasonication	197.00±5.83[b]	206.32±2.46[b]	108.79±3.05[b]	92.40±1.55[c]
Choke-Anan ethanolic	117.07±4.12[c]	123.43±2.55[c]	60.54±2.86[c]	166.74±0.96[b]
Choke-Anan ethanolic + ultrasonic-cation	254.64±1.15[a]	289.47±3.26[a]	110.24±2.26[a]	198.68± 0.44[a]

Values are the mean ± standard deviation; (n = 3); values in the same column followed by different superscript letters are significantly different ($p \le 0.05$)

Table 3: Enzyme inhibition capacity

Sample	IC_{50} (µg/mL)			
	Tyrosinase	***5-Lipoxygenase***	***Hyaluronidase***	***α-Glucosidase***
Kaew MSKE	20.64±0.32[a]	39.77±2.41[a]	47.61±2.92[a]	163.19±2.33[a]
Choke-Anan MSKE	19.86±1.22[a]	30.94±0.32[b]	37.28±1.67[b]	113.51±5.85[b]
Arbutin	20.46±0.33[a]	-	-	-
Rutin	-	9.31±0.55[c]	-	-
Vitamin C	-	-	39.29±0.25[b]	-
Acarbose	-	-	-	104.42±5.54[c]

Values are the mean ± SD (n=3); values in the same column followed by different superscript letters are significantly different ($p \le 0.05$). IC_{50} = inhibitor concentration causing 50 % loss of enzyme activity

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Physical stability of MSKE cream

After centrifugation, phase separation was not observed in any sample of MSKE cream or the control base (data not shown). A physical stability evaluation of the MSKE creams is shown in Table 4. A freeze-thaw cycling test showed that the pH and viscosity of the samples from each cycle did not exhibit significant changes ($p > 0.05$) and no phase separation was observed in any sample. The results indicate that the pH, viscosity and appearance of the MSKE creams were stable and that the MSKE did not affect the physical properties tested. In particular, the total colour (ΔE) of the 1% MSKE and the base cream was not significantly different ($p > 0.05$) for any cycle (data not shown), while the (ΔE) of the 2 and 3 % MSKE creams were significantly different ($p \leq 0.05$).

Clinical skin irritation

No volunteer exposed to MSKE cream showed an adverse reaction. Skin exposed to the MSKE cream did not respond differently than skin exposed to the negative control (data not shown).

DISCUSSION

This study found that the *Choke-Anan* and *Kaew* MSKEs had higher extraction yields and TPC when ultrasonication was applied during extraction. A higher TPC of MSKE had previously been observed when an ultrasonic-assisted aqueous two phase extraction of mango seed kernel was used [20]. Ultrasonication was shown to disrupt the cell membrane and the cell wall structure, increasing solvent diffusion through the membrane, thus facilitating the release of the cell contents [21].

MSKEs from the two cultivars prepared by ultrasonication-assisted extraction showed greater antioxidant activity for all methods studied and the *Choke-Anan* MSKE had higher antioxidant activity than the *Kaew* MSKE. The results agree with reports of concurrent antioxidant activities indicated by DPPH and ABTS methods for *Choke-Anan* MSKE when compared to several other varieties of Thai mangoes [22]. Moreover, the *Choke-Anan* and *Kaew* MSKEs had the highest antioxidant properties of eleven mango varieties studied [23].

The results showed that tyrosinase inhibitory activity of the *Kaew* and *Choke-Anan* MSKEs were similar to previously reported values. *Choke-Anan* MSKE was previously shown to inhibit tyrosinase activity up to 1.58 fold higher than arbutin [25]. These results indicated that MSKE can be a good source of phytochemicals with tyrosinase inhibitory activity.

5-Lipoxygenase catalyses the conversion of polyunsaturated fatty acids to biologically active metabolites, which are active mediators in a variety of inflammation processes [26]. A previous study showed that the IC_{50} values for the 5-lipoxygenase inhibitory activity of eight plant extracts ranged from 27.4 ± 0.6 to 66.7 ± 0.6 μg / mL [27]. These 5-lipoxygenase inhibition activities imply that MSKE has a high potential for use as natural anti-inflamatory drug compared to other plant extracts reported in previous studies.

It is well understood that the degradation of hyaluronic acid by hyaluronidase can diminish amount of hyaluronic acid in the skin, which consequently becomes dry and wrinkled [28]. An extract of the bark of *Terminaliaarjuna* (250 μg / mL) and dried fruit rinds of *Terminaliachebula* (500 μg / mL) have been reported to have 90.40 ± 5.30 % and 89.65 ± 3.90 % hyaluronidase inhibition, respectively [29]. In the present study, the *Kaew* and *Choke-Anan* MSKEs respectively gave up to 80.35 % and 97.61 % inhibition at concentrations of 70 μg / mL. These results indicate that the MSKEs, and especially *Choke-Anan* MSKEs, have a potential for cosmetic use as an anti-wrinkle agent.

The progression of diabetes mellitus can be controlled by inhibiting the absorption of dietary carbohydrates in the small intestine [30]. The α-glucosidase inhibitory activity of the *Kaew* and *Choke-Anan* MSKEs compared to acarbose showed IC_{50} values of 163.19 ± 2.3, 113.51 ± 5.8, and 104.42 ± 5.5 μg / mL, respectively. A methanol extract of mango seed from Nigeria has been reported to inhibit α-glucosidase with an IC_{50} of 340 μg / mL [31]. The results in this study revealed that MSKEs potently inhibit α-glucosidase activity.

Due to the higher bioactivity of the *Choke-Anan* MSKE, it was further evaluated for its potential use as a cosmetic ingredient. The results revealed that addition of MSKE at 1 % in a cosmetic cream caused no significant difference ($p > 0.05$) of the physicochemical

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Table 4: pH, viscosity, phase separation and TPC of the MSKE creams and the base control from the freeze-thaw physical stability evaluation

Sample	Parameter	Freeze-thaw cycle						
		0	1	2	3	4	5	6
Control base	pH^{NS}	5.74±0.02	5.67±0.06	5.63±0.06	5.43±0.38	5.70±0.10	5.63±0.06	5.70±0.17
	Viscosity (cPs)^{NS}	15558.45±18.53	15545.04±14.73	15546.33±3.21	15536.22±4.04	15550.34±8.50	15547.14±10.11	15549.33±11.01
	Phase separation	N	N	N	N	N	N	N
	TPC (mg of GAE/ ml)	ND	ND	ND	ND	ND	ND	ND
1% MSKE	pH^{NS}	5.72±0.05	5.63±0.06	5.70±0.10	5.67±0.12	5.70±0.10	5.67±0.06	5.63±0.06
	Viscosity (cPs)^{NS}	15540.43±7.61	15546.33±5.85	15540.66±6.11	15549.21±10.44	15551.06±10.14	15538.66±4.04	15547.66±6.35
	Phase separation	N	N	N	N	N	N	N
	TPC (mg of GAE/ ml)	1.64±0.32^{a}	1.65±0.40^{a}	1.66±0.33^{a}	1.62±0.33^{a}	1.48±0.15^{b}	1.47±0.09^{b}	1.47±0.12^{b}
2% MSKE	pH^{NS}	5.69±0.03	5.67±0.06	5.67±0.12	5.73±0.12	5.63±0.06	5.70±0.10	5.70±0.10
	Viscosity (cPs)^{NS}	15543.56±11.15	15542.66±11.05	15549.66±2.50	15539.66±6.31	15547.33±12.42	15549.66±9.60	15541±5.58
	Phase separation	N	N	N	N	N	N	N
	TPC (mg of GAE/ ml)	3.32±0.06^{a}	3.34±0.14^{a}	3.27±0.21^{a}	3.23±0.17^{a}	2.84±0.03^{b}	2.79±0.13^{b}	2.79±0.11^{b}
3% MSKE	pH^{NS}	5.70±0.06	5.67±0.06	5.70±0.10	5.73±0.12	5.67±0.12	5.67±0.06	5.63±0.06
	Viscosity (cPs)^{NS}	15527.45±5.80	15529.33±5.50	15539.33±8.32	15535.66±6.69	15539.04±5.19	15538.55±6.03	15540.66±10.60
	Phase separation	N	N	N	N	N	N	N
	TPC (mg of GAE/ ml)	4.92±0.12^{a}	4.90±0.21^{a}	4.89±0.14^{a}	4.88±0.21^{a}	4.23±0.13^{b}	4.24±0.31^{b}	4.20±0.11^{b}

Values are the mean ± SD (n = 3); values in the same row followed by different superscript letters are significantly different ($p \leq 0.05$). NS means non-significantly different, N means not observed, ND means not determined

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properties or the skin irritation test result of the cream. Moreover, adding MSKE to the cream contributed to the total polyphenol content of the product.

CONCLUSION

The findings of this study indicate that mango seed by-products can be used as a new ingredient source for the food, pharmaceutical and cosmetic industries. The results demonstrate that *Kaew* and *Choke-Anan* MSKEs exhibit antioxidant activities as well as inhibit tyrosinase, 5-lipoxygenase, hyaluronidase and α-glucosidase activities. A cosmetic cream containing 1 % *Choke-Anan* MSKE is physically stable and appears safe for use on human skin.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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Fractionation, Antioxidant and Inhibitory Activity of Thai Mango Seed Kernel Extracts

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Abstract

Namngam Ch., Boonyuen S., Pinsirodom P. (2018): Fractionation, antioxidant and inhibitory activity of Thai mango seed kernel extracts. Czech J. Food Sci., 36: 8–15.

Mango seed kernel extracts (MSKE) from Kaew and Choke-Anan mango cultivars were fractionated using Sephadex LH-20 column chromatography. Antioxidant activity and the inhibitory effects on tyrosinase, 5-lipoxygenase, hyaluronidase and α-glucosidase of MSKE fractions were evaluated. The key components of polyphenol, found in the most active fraction, were identified using LC-ESI-MS. Three major isolates were obtained from both cultivars of the MSKE with the absorbance being higher for Choke-Anan than Kaew. Fraction 3 of MSKE from both cultivars showed significantly ($P \leq 0.05$) higher antioxidant activity and 5-lipoxygenase, hyaluronidase and α-glucosidase inhibitory activity with Choke-Anan more effective than Kaew. Six different polyphenols were found in fraction 3 of both cultivars. These were tri-*o*-galloyl-glucoside, tetra-*o*-galloyl-glucoside, maclurin tri-*o*-galloyl-glucoside, penta-*o*-galloyl-glucoside, hexa-*o*-galloyl-glucoside, and hepta-*o*-galloyl-glucoside. In the MSKE from Choke-Anan, hexa-*o*-galloyl-glucoside, and tetra-*o*-galloyl-glucoside were the two major components, whereas in the MSKE from Kaew tetra-*o*-galloyl-glucoside was the only major component. The results indicate that MSKE is a suitable by-product that could be utilised for adding value to the mango processing industry and could represent a valuable input into functional foods and pharmaceutical production.

Keywords: tyrosinase; 5-lipoxygenase; hyaluronidase; α-glucosidase galloyl-glucoside; *Mangifera indica* extract

Mango (*Mangifera indica* L.) belongs to the *Anacardiaceae* family and is one of the most important tropical fruits. It is consumed both fresh and processed. Thailand is the third largest producer of mango fruits in the world (DUBE & ZUNKER 2004). Among Thai cultivars, Choke-Anan and Kaew are the two that are most commonly used for processing. Major by-products produced during mango processing are seeds and peels in the range 35–60% depending on the cultivar (LARRAURI *et al.* 1996).

Mango seed kernels contain various polyphenols, including flavonols, xanthones and gallotannins, and can be a good source of natural antioxidants (ABDALLA & DARWISH 2007). The anti-tyrosinase, anti-inflammatory, hepatoprotective and anti-microbial activities of mango seed kernel extracts (MSKE) have previously been reported (MAISUTHISAKUL & GORDON 2009; NITHITANAKOOL *et al.* 2009; KHAMMUANG & SARNTHIMA 2011). In our previous study (NAMNGAM & PINSIRODOM 2017), we found, using different methods (DPPH, FRAP, ABTS, and H_2O_2), that Thai MSKE from Kaew and Choke-Anan cultivars exhibited antioxidant activities and showed inhibitory effects on tyrosinase, 5-lipoxygenase, hyaluronidase and α-glucosidase activities.

In a previous study (MAISUTHISAKUL 2011), the polyphenol compounds of MSKE were fractionated and it was found that fraction 3 of 5 fractions obtained using shaking extraction and fraction 2 of two obtained using acid hydrolysis extraction from Choke-Anan MSKEs exhibited the highest antioxidant efficiency. A previous report showed that MSKE

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is a rich source of gallotannins. Five gallotannins, penta-*o*-galloyl-glucoside, hexa-*o*-galloyl-glucoside, hepta-*o*-galloyl-glucoside, octa-*o*-galloyl-glucoside and nona-*o*-galloyl-glucoside were identified in Chinese mango seed kernels using LC-ESI-MS/MS and NMR (Luo *et al.* 2014). Tri-*o*-galloyl-glucoside, tetra-*o*-galloyl-glucoside, penta-*o*-galloyl-glucoside, hexa-*o*-galloyl-glucoside, hepta-*o*-galloyl-glucoside and octa-*o*-galloyl-glucoside were also identified in Hiesy mango seed kernels (Resoanaivo *et al.* 2014) and 21 gallotannins were identified in Tommy Atkins mango seed kernels (Berardini *et al.* 2004).

The present studies were carried out to investigate the fractionation, antioxidant properties and selected enzyme inhibition activities of different fractions of MSKE and to identify key components using extracts from the mango cultivars Kaew and Choke-Anan. An additional objective was to identify by-products that could be obtained from the mango processing industry and which could add value and be used in functional foods and pharmaceutical products.

MATERIAL AND METHODS

Material. Mushroom tyrosinase and soybean 5-lipoxygenase were purchased from Sigma-Aldrich (USA). Bovine hyaluronidase and yeast α-glucosidase were purchased from Merck (Germany). L-3-4-dihydroxyphenylalanine, arbutin, sodium linoleate, rutin, *p*-nitrophenyl-α-D-glucopyranoside, dimethylaminobenzaldehyde, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), hydrogen peroxide, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, and Sephadex LH-20 were purchased from Sigma-Aldrich (USA). Dimethyl sulfoxide (DMSO) was purchased from Fluka (Switzerland). The other chemicals and solvents used in this study were purchased from Sigma-Aldrich (USA).

The two cultivars of mango used in this study, Kaew and Choke-Anan, were obtained from a local orchard in the Nakornratchasima Province of Thailand between March and May, 2016. The samples were randomly selected based on size (4–5 mangoes/kg) and all were judged to be mature-green. Mango peel and pulp were removed from the fruits by hand and the seeds were washed and stored at −18°C for no longer than four months before being used. The kernels were then separated from their shells for analysis.

Preparation of mango seed kernel extracts. Preparation of crude MSKE using ultrasonic-assisted extraction was as described by Gharfoor & Choi (2009). Briefly, samples were ground and blended with 95% ethanol (100 ml) in a blender (Moulinex, Mexico) for 5 minutes. The samples were then incubated in a sonication water bath (JAC Ultrasonic 2010P; Jinwoo Engineering Co., Ltd., Korea), with the frequency fixed at 20 kHz, at a temperature of 25°C for 15–60 minutes. Samples were then further incubated in a water bath at 80°C for 1 hour. The mixtures were cooled at room temperature and the supernatant was passed through filter paper (Whatman No. 4; Merck, Germany). All filtrates were evaporated in a rotary evaporator (Büchi Rotavapor R II; USA) at 50°C under vacuum (to 70% solids) and the extracts were weighed to determine the yield of soluble components.

Fractionation of MSKE. The samples (1 g) were dissolved in 5 ml methanol and applied to a Sephadex LH-20 chromatography column (35 mm diameter × 450 mm height). The flow rate was 1 ml/min. Methanol fractions (10 ml) were collected in test tubes. Eluted fractions were then pooled based on their elution profiles. Their absorbance was measured at 280 nm using a spectrophotometer (UV-1601; Shimadzu, Japan). After evaporation of methanol, the samples were weighed to determine the yield of different fractions. Each fraction was used to determine the total polyphenol content (TPC), antioxidant activity and inhibitory action on tyrosinase, 5-lipoxygenase, hyaluronidase and α-glucosidase activity.

Determination of total polyphenol contents (TPC). The TPC were determined using Folin-Ciocalteu's phenol reagent and a method modified from Singleton and Lamuela-Raventos (1999). Briefly, each crude extract of 0.5 g was dissolved in 20 ml dimethyl sulfoxide (DMSO). Samples of 0.5 ml were mixed with 0.5 ml of Folin-Ciocalteu's phenol reagent and adjusted to 10 ml with distilled water. After mixing for 5 min, 0.8 ml of 10% (w/v) sodium carbonate was added. The mixtures were agitated and incubated for 10 min at room temperature in the dark. The absorbance was measured at 765 nm on a spectrophotometer. The total polyphenol contents of the samples were expressed as milligrams of gallic acid equivalent per gram of MSKE (mg GAE/g MSKE) using a linear equation.

DPPH radical scavenging assay. The radical scavenging activity of MSKE was measured by using the stable free radical, DPPH (2,2-diphenyl-2-picrylhydrazyl), as described by Nishaa *et al.* (2012). DPPH

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(0.8 mM) in ethanol was prepared and 0.6 ml of this solution were added to 4.4 ml of sample solution in ethanol. After 30 min of incubation in the dark at room temperature, the absorbance was measured at 517 nm with a spectrophotometer. The ability to scavenge DPPH was calculated as percent DPPH scavenging activity using Equation (1) and EC_{50} was calculated.

$$\% \text{ DPPH scavenging} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100 \quad (1)$$

Ferric reducing antioxidative power assay (FRAP). FRAP assays on MSKE were carried out as described by NISHAA *et al.* (2012). FRAP reagent was freshly prepared by mixing 25 ml acetate buffer (3 mM, pH 3.6), 2.5 ml 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) solution (10 mM TPTZ in 40 mM HCl) and 2.5 ml $FeCl_3$ (20 mM) water solution. Each sample (150 µl, 0.5 mg/ml dissolved in ethanol) was added to 4.5 ml of freshly prepared FRAP reagent and stirred, and, after 5 min, the absorbance was measured at 593 nm using a spectrophotometer. A calibration curve of various concentrations of Trolox was used and results were expressed as milligrams of Trolox equivalent per gram of MSKE (mg Trolox/g MSKE).

Hydrogen peroxide scavenging assay. The hydrogen peroxide scavenging ability of MSKE was measured using the method of YEN and CHEN (1995). H_2O_2 solution (4 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). Samples of 4 ml were mixed with 0.6 ml of H_2O_2 solution. The mixture was incubated for 10 min. The absorbance of the solution was determined at 230 nm using a spectrophotometer against a blank solution containing the plant extract in phosphate buffer without H_2O_2. The H_2O_2 scavenging activity was expressed as milligrams of Trolox equivalent per gram of MSKE (mg Trolox/g MSKE) using a linear equation.

ABTS radical scavenging assay. The ABTS 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation scavenging activity of MSKE was measured as described by RE *et al.* (1999). The ABTS radical cation was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate solution (1 : 1) and the mixture was allowed to stand in the dark at room temperature for 12 h before use. The mixture was diluted with deionised water and 95% ethanol (1 : 1) to ensure an absorbance of 0.7 ± 0.02 at 734 nm in spectrophotometric analysis. Extracts of 20 µl were mixed with 6 ml of diluted ABTS radical cation solution. The decrease in absorbance was recorded at 6 min after mixing. Trolox was used as a standard. The ABTS scavenging activity of the samples was expressed as milligrams of Trolox equivalent per gram of MSKE (mg Trolox/g MSKE) using a linear equation. All determinations were performed in triplicate.

Determination of tyrosinase inhibition activity. The inhibition activity of MSKE was measured using the modified dopachrome method with mushroom tyrosinase and L-3,4-dihydroxyphenylalanine (L-DOPA) as substrates (MASUDA *et al.* 2005) and their effect on the enzyme was determined based on dopachrome formation at 450 nm using a 96-well reader. Samples were dissolved in DMSO at various concentrations. Each well contained 40 µl of sample with 80 µl of phosphate buffer (0.1 M, pH 6.8), 40 µl of tyrosinase (100 units/ml) and 40 µl of L-DOPA (2.5 mM). Each sample was accompanied by a blank that had all the components except L-DOPA. Results were compared to arbutin as a reference standard. The percent inhibition of tyrosinase was calculated using Equation (2). The inhibitory effect (%) of the samples was expressed as an inhibitor concentration causing a 50% loss of enzyme activity (IC_{50}).

$$\% \text{ inhibition activity} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100 \quad (2)$$

Determination of 5-lipoxygenase inhibition activity. The inhibition activity of MSKE was measured using linoleic acid as a substrate as described by SHINDE *et al.* (1999). The reaction was initiated by the addition of aliquots (50 µl) of a soybean lipoxygenase (1000 units/ml) solution (prepared daily in 1 M potassium phosphate buffer, pH 9.0) and 2.0 ml of sodium linoleate (100 µM) in phosphate buffer. The samples (30 µl) were dissolved in DMSO at various concentrations and then added. The mixture was incubated for 5 min. The absorbance of the solution was taken at 234 nm on a spectrophotometer. Results were compared to rutin as reference standard. The percentage inhibition of 5-lipoxygenase was calculated according to Equation (2). The inhibitory effect (%) of the samples was expressed as an inhibitor concentration causing a 50% loss of enzyme activity (IC_{50}).

Determination of hyaluronidase inhibition activity. Inhibition of hyaluronidase was determined by measuring the amount of N-acetyl glucosamine that was split from sodium hyaluronate as described by LEE *et al.* (2001). The assay medium consisted of 50 µl of hyaluronidase (1000 units/ml prepared in 0.1 M acetate buffer, pH 3.5), 50 µl of various concentrations of MSKE dissolved in 5% DMSO and 12.5 mM

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calcium chloride. The medium was incubated for 10 min at 37°C. The reaction was then initiated by the addition of 250 µl of sodium hyaluronate as substrate (1.2 mg/ml dissolved in 0.1 M acetate buffer, pH 3.5) and then 1.5 ml of *p*-dimethylaminobenzaldehyde (4 g PDMAB dissolved in 350 ml of glacial acetic acid and 50 ml of 10 N HCl) was added to the reaction mixture followed by incubation for 20 min at 37°C. The absorbance was measured at 585 nm using a spectrophotometer. Results were compared to vitamin C as reference standard. The percent inhibition of hyaluronidase was calculated according to Equation (2). The inhibitory effect (%) of the samples was expressed as the inhibitor concentration causing a 50% loss of enzyme activity (IC_{50}).

Determination of α-glucosidase inhibition activity. The inhibitory effects of MSKE on α-glucosidase was measured according to Apostolidis *et al.* (2011). The reactions contained dilutions of the MSKE (0–200 µl) and 100 µl of α-glucosidase (1000 units/ml 0.5 mg/ml) in phosphate buffer (0.1 M, pH 6.9) solution and were incubated at 25°C for 10 min. Then, 50 µl of 5 mM *p*-nitrophenyl-α-D-glucopyranoside in phosphate buffer (0.1 M, pH 6.9) solution was added. The mixtures were incubated at 25°C for 5 min, before reading the absorbance at 405 nm on a spectrophotometer. Acarbose was used as reference standard. The percent inhibition of α-glucosidase was calculated according to Equation (2). The inhibitory effect (%) of the samples was expressed as an inhibitor concentration causing a 50% loss of enzyme activity (IC_{50}).

Identification of polyphenols using LC-ESI-MS. The fractions with the highest bioactivities were identified to harbour the main polyphenol constituents using LC-ESI-MS. Electrospray ionisation mass spectrometry analysis of polyphenol compounds in fractions of MSKE was performed for identification using an applied TSQ Quantum Ultra-LCMS (Thermo Fisher, USA) based on the method described by Abdullah *et al.* (2015). The mass spectra were acquired in positive electrospray ionization (ESI) modes with high resolutions of up to 3000 Daltons. The spray voltage used was 3500 V. The sheath/auxiliary/sweep gas was 99% nitrogen and sheath gas pressure was 30 psi with 5 psi for auxiliary gas pressure. The capillary temperature was 270°C. The injection volume was 10 µl and the flush speed was 100 µl/s. In the MS analysis (full scan), data were collected over masses of 50 to 2000 *m/z*.

Statistical analysis. The results are expressed as the mean ± standard deviation. All determinations were performed in triplicate ($n = 3$). Statistical analyses were carried out using one-way ANOVA in SPSS version 16.0. Differences were considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Fractionation of mango seed kernel extracts. The crude ethanol extracts of MSKE from the two cultivars were fractionated on Sephadex LH-20 chromatography columns using methanol as the mobile phase. The elution profiles measured by the spectrophotometer at 280 nm are shown in Figure 1. Three major isolates were obtained for the MSKE from both cultivars with the absorbance generally higher for the Choke-Anan MSKE fractions than for those of Kaew.

Recovery yield, total polyphenols and antioxidant activities. The recovery yield and TPC of all the obtained fractions from Kaew and Choke-Anan MSKE are shown in Table 1. The recovery yields were in the range of 6.41–17.11 and 9.49–27.90%, respectively. Crude MSKE from Kaew and Choke-Anan exhibited TPC values of 132.78 ± 0.77 and 175.06 ± 0.61 mg GAE/g, respectively. Fraction 3 of MSKE from Kaew and Choke-Anan had approximately 1.75 and 1.50-fold higher levels of TPC than crude MSKE, respectively, which were significant differences ($P \leq 0.05$). Table 1 shows the antioxidant activities of crude fractions MSKE from Kaew and Choke-Anan and gallic acid as a reference standard using four different methods. Fraction 3 of both cultivars exhibited significantly ($P \leq 0.05$) increased antioxidant activities in all the methods tested compared to crude extracts, with approximately 1.54, 1.54, 1.48, and 1.12-times higher antioxidant activities for Kaew and 1.51, 1.49,

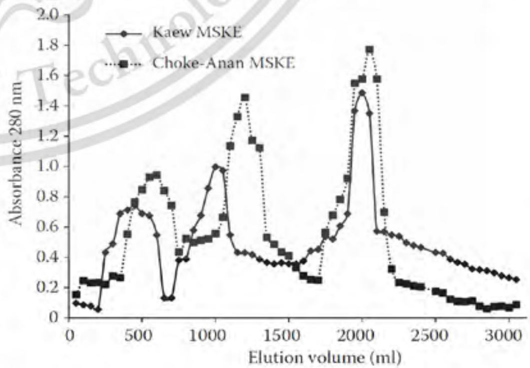

Figure 1. Column chromatography profile of the fractions from mango seed kernel extracts (MSKE)

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Table 1. Results of the Sephadex LH-20 column chromatography

Fractions		Yield (%)	TPC (mg GAE/g)	Antioxidant properties			
				DPPH EC_{50} (μg/ml)	FRAP	H_2O_2	ABTS
					(mg Trolox/g)		
Kaew	crude	nd	132.78 ± 0.77^{g}	10.22 ± 0.05^{a}	209.35 ± 1.52^{f}	95.58 ± 0.25^{h}	163.14 ± 0.89^{g}
	F1	6.41 ± 0.02^{e}	130.30 ± 0.87^{g}	10.37 ± 0.07^{a}	204.45 ± 1.15^{g}	103.13 ± 1.58^{g}	155.59 ± 0.72^{h}
	F2	9.44 ± 0.01^{d}	184.72 ± 0.59^{d}	8.66 ± 0.32^{b}	225.06 ± 0.91^{e}	121.15 ± 0.63^{e}	171.59 ± 1.09^{f}
	F3	17.11 ± 0.03^{b}	232.00 ± 1.33^{b}	6.63 ± 0.08^{d}	322.70 ± 0.91^{b}	141.61 ± 1.21^{c}	184.54 ± 1.76^{c}
Choke-Anan	crude	nd	175.06 ± 0.61^{e}	7.17 ± 0.03^{c}	229.18 ± 0.54^{d}	112.69 ± 0.60^{f}	177.52 ± 0.70^{d}
	F1	9.49 ± 0.16^{d}	150.95 ± 6.79^{f}	6.13 ± 0.15^{e}	232.26 ± 1.23^{d}	113.57 ± 0.81^{f}	175.73 ± 0.34^{e}
	F2	16.30 ± 0.03^{c}	206.01 ± 0.74^{c}	5.46 ± 0.20^{f}	254.41 ± 2.31^{c}	125.29 ± 0.31^{d}	184.56 ± 0.93^{c}
	F3	27.90 ± 0.02^{a}	263.28 ± 1.50^{a}	4.74 ± 0.14^{g}	342.10 ± 1.00^{a}	158.50 ± 0.74^{b}	191.45 ± 1.00^{b}
Gallic acid		nd	nd	4.67 ± 0.24^{g}	344.29 ± 3.04^{a}	180.55 ± 0.74^{a}	200.22 ± 1.40^{a}

Values are mean ± SD ($n = 3$); values in the same column that are followed by different superscript letters are significantly different each other ($P \leq 0.05$); nd – not determined

1.41, and 1.07-times higher antioxidant activities for the Choke-Anan cultivar. Fraction 3 of Choke-Anan MSKE did not have a significantly ($P > 0.05$) different antioxidant activity compared with gallic acid using the DPPH and FRAP methods. Gallic acid clearly exhibited the highest ($P \leq 0.05$) antioxidant activity using the H_2O_2 and ABTS methods. The recovery yields for both Kaew and Choke-Anan MSKE were highest in fraction 3. In addition, the fractions with the highest TPC in both cultivars corresponded to those with the highest antioxidant activities in all the methods tested. Maisuthisakul (2011) also reported that fraction 3 from Choke-Anan, obtained from MSKE using shaking extraction and fraction 2, obtained from MSKE using acid hydrolysis extraction, did not differ significantly ($P > 0.05$) in their antioxidant efficiencies when compared to methyl gallate.

Selected enzyme inhibitory activities. In this investigation, crude MSKE and fractions from Kaew and Choke-Anan were used to determine the inhibition of tyrosinase, 5-lipoxygenase, hyaluronidase and α-glucosidase activities compared to the reference standard. The values are presented as the inhibitor concentration causing a 50% loss of enzyme activity (IC_{50}). A smaller IC_{50} value means a higher enzyme inhibitory activity. Table 2 shows the IC_{50} values of enzyme inhibition activities of crude MSKE, fractions and the standard reference.

Tyrosinase is the main enzyme in the melanin synthetic pathway in melanocytes and Park *et al.* (2004) reported that inhibition of tyrosinase could be an important strategy for blocking melanogenesis. Tyrosinase inhibitors have been widely used as ingredients in some skin whitening cosmetics. Fraction 3 of MSKE from Kaew and Choke-Anan showed significant differences ($P < 0.05$) in tyrosinase inhibitory activity compared to all other fractions and crude MSKE; the IC_{50} values were 17.10 ± 0.15 and 15.35 ± 0.63 μg/ml, respectively. The results also showed that fraction 3 of both cultivars was more effective at inhibiting tyrosinase activity than the standard reference, arbutin. Therefore, MSKE is a promising functional food and natural cosmetic for whitening products.

Alitonou *et al.* (2006) showed that 5-lipoxygenase can catalyse the conversion of arachidonic, linoleic and other polyunsaturated fatty acids into biologically active metabolites, which are active mediators in a variety of inflammatory processes. The inhibitory activity of 5-lipoxygenase from crude MSKE and fractions of MSKE from Kaew and Choke-Anan, compared to rutin, showed a significantly ($P \leq 0.05$) higher 5-lipoxygenase inhibition activity than the other fractions and crude MSKE. The IC_{50} values ranged from 21.35 ± 0.65 μg/ml to 41.63 ± 0.52 μg/ml. The reference standard, rutin, had a lower IC_{50} value (10.96 ± 0.69 μg/ml). It is therefore clear that the inhibitory activity of 5-lipoxygenase from MSKE may hold potential for the potential development of new anti-inflammatory drugs.

Sahasrabudhe and Deodhar (2010) showed that hyaluronidase is a naturally occurring enzyme that catalyses the degradation of hyaluronic acid, whose action can lead to diminishing amounts of hyaluronic acid in the skin with the result that the skin becomes dried and wrinkled. Fraction 3 of Kaew MSKE and fractions 1, 2, and 3 of Choke-Anan MSKE showed

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Table 2. Enzyme inhibition activities of crude extracts and different MSKE fractions

Fractions tyrosinase		Enzyme inhibition activities (IC_{50}, μg/ml)			
		tyrosinase	5-lipoxygenase	hyaluronidase	α-glucosidase
Kaew	crude	21.11 ± 0.50[cd]	39.36 ± 0.61[bc]	41.39 ± 0.85[a]	154.42 ± 3.64[b]
	F1	37.10 ± 0.22[a]	41.63 ± 0.52[a]	42.69 ± 0.82[a]	131.75 ± 2.51[d]
	F2	36.59 ± 0.21[a]	35.32 ± 0.57[d]	38.03 ± 0.90[b]	118.99 ± 3.02[e]
	F3	17.10 ± 0.15[e]	29.79 ± 0.74[f]	32.80 ± 0.84[d]	110.45 ± 4.01[g]
Choke-Anan	crude	21.29 ± 0.81[c]	38.75 ± 0.41[c]	39.19 ± 0.91[b]	157.83 ± 3.32[a]
	F1	27.48 ± 0.46[b]	40.16 ± 0.51[b]	35.42 ± 0.82[c]	133.85 ± 5.24[c]
	F2	27.18 ± 0.46[b]	32.71 ± 0.69[e]	35.80 ± 0.81[c]	116.08 ± 5.15[f]
	F3	15.35 ± 0.63[f]	21.35 ± 0.65[g]	29.56 ± 1.23[e]	108.00 ± 2.51[h]
Standard references	arbutin	20.64 ± 0.75[d]	nd	nd	nd
	rutin	nd	10.96 ± 0.69[h]	nd	nd
	vitamin C	nd	nd	38.67 ± 0.78[b]	nd
	acarbose	nd	nd	nd	105.00 ± 0.51[i]

Values are mean ± SD ($n = 3$); values in the same column that are followed by different superscript letters are significantly different from each other ($P \leq 0.05$); nd – not determined

significantly higher ($P \leq 0.05$) hyaluronidase inhibitory activities than the reference standard, which was vitamin C. Fraction 2 from Kaew and crude Choke-Anan MSKE were not significant different ($P > 0.05$) in their hyaluronidase inhibition activities. Moreover, MSKE from Choke-Anan showed significantly higher ($P \leq 0.05$) activity than that from Kaew. These results suggest that the hyaluronidase inhibitory activity of MSKE has potential as a new ingredient in functional foods and anti-wrinkle cosmetics.

Hydrolysed dietary carbohydrates are the major source of blood glucose, and are hydrolysed by pancreatic α-amylase with absorption by the small intestine aided by α-glucosidases (Elsenhans & Caspary 1987). Kumar *et al.* (2011) described the control of postprandial hyperglycaemia as a practical therapeutic approach for the management of diabetes. They suggested that the progression of diabetes mellitus can be stopped by inhibiting the absorption of dietary carbohydrates in the small intestine. The α-glucosidase inhibitory activities from crude extracts and fractions of MSKE from both Kaew and Choke-Anan compared to acarbose are shown in Table 2. In addition, fraction 3 of both cultivars exhibited significantly higher ($P \leq 0.05$) α-glucosidase inhibitory activity compared to crude extracts and other fractions with IC_{50} values of 110.45 ± 4.01 and 108.00 ± 2.51 μg/ml, respectively. However, acarbose, the reference standard, had significantly higher ($P \leq 0.05$) α-glucosidase inhibitory activity than all crude extracts and fractions of MSKE.

Fraction 3 of MSKE from both cultivars exhibited significantly higher ($P \leq 0.05$) tyrosinase, 5-lipoxygenase, hyaluronidase and α-glucosidase inhibitory activities than crude MSKE and other fractions. These results correspond with the TPC and antioxidant activity values shown in Table 1. Moreover, the IC_{50} values for tyrosinase and hyaluronidase inhibition activities of fraction 3 in both cultivars were lower than those of arbutin and vitamin C, respectively. Antioxidant activity mechanisms may also be one of the main reasons for tyrosinase inhibition activity (Kim *et al.* 2008). Some phenolic compounds such as ellagic acid, tannic acid (or gallotannins) and quercetin have been shown to inhibit tyrosinase activity (Shimogaki *et al.* 2000) and hyaluronidase activity (Girish & Kemparaju 2005). The experimental results indicate that fraction 3 of MSKE from both cultivars contained a key component that had stronger enzyme inhibitory activities.

Identification of key polyphenol constituents. The separation results for the polyphenols in fraction 3 of MSKE from Kaew and Choke-Anan using LC-ESI-MS mass spectrometry with a retention time of 0–8.3 min are shown in Table 3. Consistent with previous studies (Berardini *et al.* 2004; Luo et al. 2014; Resoanaivo *et al.* 2014), six polyphenols were detected in fraction 3 of MSKE from both cultivars and were the following:

Peak 1 showed an $(M\text{-}Na)^+$ ion of m/z 662.3 identified as tri-*o*-galloyl-glucoside.

Peak 2 showed an $(M\text{-}Na)^+$ ion of m/z 813.5 identified as tetra-*o*-galloyl-glucoside.

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Table 3. Characterisation of the main polyphenol constituents in fraction 3 of MSKE

Pea	Polyphenol	Relative abundance (%)		Retention time (min)		(M-Na)$^+$
		kaew	choke-anan	kaew	choke-anan	(*m/z*)
1	tri-*o*-galloyl-glucoside	56	13	2.10	2.90	662.3
2	tetra-*o*-galloyl-glucoside	100	97	2.60	3.76	813.5
3	maclurintri-*o*-galloyl-glucoside	28	50	2.96	2.81	902.4
4	penta-*o*-galloyl-glucoside	34	88	3.48	3.99	961.5
5	hexa-*o*-galloyl-glucoside	24	100	4.16	3.21	1113.2
6	hepta-*o*-galloyl-glucoside	8	50	3.15	4.44	1265.0

Peak 3 showed an (M-Na)$^+$ ion of *m/z* 902.4 identified as maclurintri-*o*-galloyl-glucoside.

Peak 4 showed an (M-Na)$^+$ ion of *m/z* 961.5 identified as penta-*o*-galloyl-glucoside.

Peak 5 showed an (M-Na)$^+$ ion of *m/z* 1113.2 identified as hexa-*o*-galloyl-glucoside.

Peak 6 showed an (M-Na)$^+$ ion of *m/z* 1265 identified as hepta-*o*-galloyl-glucoside.

The retention time of the mass spectra of fraction 3 from both Kaew and Choke-Anan MSKE were in the range of 2.90–4.44 and 2.10–3.15, respectively, and exhibited ratios and a relative abundance of 5 > 2 > 4 > 3.6 > 1 and 2 > 1 > 4 > 3 > 5 > 6, respectively. Hexa-*o*-galloyl-glucoside and tetra-*o*-galloyl-glucoside were the two major components in fraction 3 of MSKE from Choke-Anan, whereas, in Kaew the only major component was tetra-*o*-galloyl-glucoside. The key components of fraction 3 of MSKE from Kaew and Choke-Anan contained the six main polyphenols: tri-*o*-galloyl-glucoside, tetra-*o*-galloyl-glucoside, maclurintri-*o*-galloyl-glucoside, penta-*o*-galloyl-glucoside, hexa-*o*-galloyl-glucoside and hepta-*o*-galloyl-glucoside. These results are similar to those described previously regarding the NMR spectra of ethanolic extracts of by-products from Hiesy mangoes in Madagascar. These authors identified ten gallotannins, which comprised glucose and three to nine gallic acids (Resoanaivo *et al.* 2014). Berardini *et al.* (2004) characterised polyphenols in MSKE Tommy Atkins mangoes and isolated 21 different gallotannins. A similar study identified the gallotannins in the seed kernels of three different Chinese mango cultivars (Maqiesu, Tainong-1, and Zihuamang) using LC-ESI-MS/MS, and found that the MSKE contained five major gallotannins, i.e., penta-*o*-galloyl-glucoside, hexa-*o*-galloyl-glucoside, hepta-*o*-galloyl-glucoside, octa-*o*-galloyl-glucoside and nona-*o*-galloyl glucoside (Luo *et al.* 2015).

CONCLUSIONS

The results of this study are consistent with previous studies on some other mango cultivars and show that fraction 3 of the MSKE from the mango cultivars Kaew and Choke-Anan exhibited the highest antioxidant activities as well as inhibitory effects against the selected enzymes. Moreover, hexa-*o*-galloyl-glucoside and tetra-*o*-galloyl-glucoside were identified as the two major components of fraction 3 from Choke-Anan, whereas tetra-*o*-galloyl-glucoside was found to be the only major component in Kaew. These findings demonstrate that Thai mango seed by-products can be a source of natural ingredients for functional foods, pharmaceutical and cosmetic products and that the type of antioxidant activity can vary between cultivars.

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