Extraction, Characterization and Application of Antioxidant from Cashew (*Anacardium occidentale* L.) Leaves

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Food Science and Technology

Prince of Songkla University

2017

Thesis Title	Extraction, Characterization and Application of Antioxidant from Cashew (*Anacardium occidentale* L.) Leaves
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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ การสกัด คุณลักษณะและการประยุกต์ใช้สารต้านออกซิเดชันจากใบมะม่วงหิมพานต์

ผู้เขียน นางสาวลลิตา โชติพฤฒิพงศ์

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ปีการศึกษา 2559

บทคัดย่อ

จากการศึกษาสภาวะที่เหมาะสมในการสกัดสารประกอบฟีนอลิกที่มีฤทธิ์การต้านออกซิเดชันจากใบมะม่วงหิมพานต์โดยใช้เอทานอลเข้มข้นร้อยละ 80 เป็นตัวทำละลาย โดยใช้วิธีพื้นผิวตอบสนอง (RSM) และวางแผนการทดลองแบบ Central composite design (CCD) ตัวแปรต้นที่ศึกษา คือ อุณหภูมิของการสกัด (30-50 °ซ) ระยะเวลาในการสกัด (60-180 นาที) และอัตราส่วนของเอทานอลต่อของแข็ง (15:1-35:1 ปริมาตร/น้ำหนัก) พบว่าสภาวะที่เหมาะสมของการสกัดสารคือ อุณหภูมิ 34.7 °ซ ระยะเวลาในการสกัด 64 นาที โดยใช้อัตราส่วนเอทานอลต่อของแข็งเท่ากับ 18:1 (ปริมาตร/น้ำหนัก) ภายใต้สภาวะการสกัดดังกล่าวให้ผลผลิตการสกัดสูงสุดเท่ากับร้อยละ 8.64 ปริมาณสารประกอบฟีนอลิก (TPC) เท่ากับ 564.60 มิลลิกรัมสมมูลของกรดแกลลิกต่อกรัมสารสกัดแห้ง ฤทธิ์การต้านอนุมูลอิสระ DPPH และ ABTS และ FRAP มีค่าเท่ากับ 11.74 5.56 และ 8.11 มิลลิโมล สมมูลโทรล็อกซ์ต่อกรัมสารสกัดแห้งตามลำดับ ไอโซควอซิทินและเคเทชินเป็นสารประกอบฟีนอลิกหลักที่พบในสารสกัด

จากการศึกษาการสกัดสารประกอบฟีนอลิกที่มีฤทธิ์การต้านออกซิเดชันจากใบมะม่วงหิมพานต์โดยใช้คลื่นอัลตร้าซาวด์ช่วยสกัด โดยใช้วิธี RSM และวางแผนการทดลองแบบ CCD ตัวแปรต้นที่ศึกษา คือ แอมพลิจูด (30-77%) ระยะเวลาในการสกัด (7-31 นาที) โดยใช้อัลตร้าซาวด์ของเอทานอลต่อของแข็งเท่ากับ 18:1 (ปริมาตร/น้ำหนัก) พบว่าสภาวะที่เหมาะสมของการสกัดคือ แอมพลิจูด 77% และระยะเวลาการสกัด 31 นาที ภายใต้สภาวะการสกัดดังกล่าวให้ผลผลิตการสกัดสูงสุดเท่ากับร้อยละ 23.61 โดยมีค่ามากกว่าการสกัดโดยใช้ตัวทำละลายประมาณ 3 เท่า ปริมาณสารประกอบฟีนอลิก (TPC) มีค่าเท่ากับ 579.55 มิลลิกรัมสมมูลของกรดแกลลิกต่อกรัมสารสกัดแห้ง ฤทธิ์การต้านอนุมูลอิสระ DPPH และ ABTS และ FRAP มีค่าเท่ากับ 11.85 6.04 และ 10.28 มิลลิโมลสมมูลโทรล็อกซ์ต่อกรัมสารสกัดแห้งตามลำดับ ไอโซควอซิทินและเคเทชินเป็นสารประกอบฟีนอลิกหลักที่พบในสารสกัด นอกจากนี้ยังพบไฮโดรควินิน กรดแกลลิก กรดแทนนิก รูติน อีริโอดิคทิออล เควอซิทิน และอะพิจีนินในสารสกัด

เมื่อศึกษาฤทธิ์การต้านออกซิเดชันของสารสกัดจากใบมะม่วงหิมพานต์โดยใช้วิธีการสกัดที่แตกต่างกัน (วิธีการสกัดตัวทำละลาย และการใช้คลื่นอัลตร้าซาวด์ช่วยสกัด) ที่ระดับความเข้มข้นที่ต่างกัน (0-0.2 มิลลิกรัม/มิลลิลิตร) พบว่าฤทธิ์การต้านอนุมูลอิสระ DPPH ABTS และ FRAP ของสารสกัดที่สกัดด้วยกระบวนการทั้งสองเพิ่มขึ้นเมื่อเพิ่มความเข้มข้นจนถึง 0.1 มิลลิกรัม/มิลลิลิตร (P<0.05) สารสกัดที่สกัดด้วยทั้งสองกระบวนการมีฤทธิ์ที่สูงกว่า BHT แต่ต่ำกว่าเคเทชินสำหรับทุกวิธีการที่ใช้ทดสอบ (P<0.05) นอกจากนี้สารสกัดที่สกัดด้วยทั้งสองกระบวนการมีความคงตัวต่อความร้อนและความเป็นกรด-ด่าง หลังจากให้ความร้อนที่อุณหภูมิ 60 ถึง 100 °ซ เป็นเวลา 60 นาที พบว่ายังคงมีฤทธิ์การต้านอนุมูลอิสระ โดยทั่วไปฤทธิ์ต้านออกซิเดชันของสารสกัดมีค่าสูงในช่วงความเป็นกรด-ด่าง 6 ถึง 8 (P<0.05) เมื่อเติมสารสกัดทั้งสองในระบบเบต้าแคโรทีน-ลิโนเลอิกและระบบเลซิทินไลโพโซม พบว่าค่า conjugated diene (CD) ค่าเปอร์ออกไซด์ (PV) และค่า thiobarbituric acid reactive substances (TBARS) ต่ำกว่าเมื่อเทียบกับตัวอย่างชุดควบคุม (P<0.05) แต่ทั้งนี้ผลการป้องกันการเกิดปฏิกิริยาออกซิเดชันต่ำกว่า BHT ในทั้งสองระบบที่ทดสอบ

เมื่อศึกษาความคงตัวต่อการเกิดปฏิกิริยาออกซิเดชันของมายองเนสที่เสริมน้ำมันปลาโดยเติมสารสกัดจากใบมะม่วงหิมพานต์ (CE) เปรียบเทียบกับ BHT ที่ระดับความเข้มข้น 100 ppm และ 200 ppm ในระหว่างการเก็บรักษาเป็นเวลา 30 วัน ที่อุณหภูมิ 30 °ซ พบว่าการเติม CE หรือ BHT โดยเฉพาะที่ความเข้มข้น 200 ppm สามารถชะลอการเกิดปฏิกิริยาออกซิเดชัน ซึ่งบ่งชี้โดยค่า PV TBARS และ พาราอะนิซีดีน (AnV) ที่ต่ำ เมื่อสิ้นสุดการเก็บรักษาตัวอย่างมายองเนสที่เติม CE (200 ppm) มีปริมาณสารระเหยต่ำกว่าตัวอย่างชุดควบคุม อย่างไรก็ตามการเติม CE (200 ppm) ส่งผลให้ค่าความสว่าง (L*) ลดลง แต่ค่าความเป็นสีแดง ค่าความเป็นสีเหลือง (b*) และค่าความแตกต่างของสีทั้งหมด (ΔE^*) เพิ่มขึ้น (P<0.05) โดยภาพรวม CE ทั้งสองความเข้มข้นมีประสิทธิภาพในการชะลอการเกิดกลิ่นหืนและกลิ่นคาวในมายองเนสที่เสริมน้ำมันปลาในระหว่างการเก็บรักษาเป็นเวลา 30 วัน ที่อุณหภูมิ 30 °ซ

ดังนั้น สารสกัดจากใบมะม่วงหิมพานต์โดยเฉพาะที่เตรียมโดยใช้วิธีคลื่นอัลตร้าซาวด์ช่วยสกัดอุดมไปด้วยสารฟีนอลิก สามารถใช้เป็นสารต้านออกซิเดชันจากแหล่งธรรมชาติในการป้องกันการเกิดปฏิกิริยาออกซิเดชันของอาหารที่มีไขมันเป็นองค์ประกอบรวมทั้งอาหารประเภทอิมัลชัน

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Major Program Food Science and Technology

Academic Year 2016

ABSTRACT

Optimization of extraction of antioxidative phenolic compounds from cashew (*Anacardium occidentale* L.) leaves was performed using response surface methodology (RSM) with 80% ethanol as the solvent. The central composite design (CCD) was used to establish treatments based on three independent variables, including extraction temperature (30-50 ˚C), time (60-180 min) and ethanol-to-solid ratio (15:1-35:1 v/w). Optimal conditions for extraction were: extraction temperature at 34.7˚C for 64 min with an ethanol-to-solid ratio of 18:1 (v/w). The highest extraction yield was 8.64% under the following optimized condition. Total phenolic content (TPC) was 564.60 mg GAE/g dry extract and DPPH, ABTS radical scavenging activities and Ferric reducing antioxidant power (FRAP) were 11.74, 5.56 and 8.11 mmol TE/g dry extract, respectively. Isoquercetin and catechin were predominant in the extract.

Phenolic compounds with antioxidative activity from cashew leaves were also extracted using ultrasound-assisted process. RSM and CCD were used to optimize the extraction, based on two independent variables, including amplitude (30-77%) and time (7-31 min) when ethanol-to-solid ratio of 18:1 (v/w) was used. The highest extraction yield was 23.61 ± 0.06 % when the optimal extraction condition (77% amplitude for 31 min) was implemented. This was approximately 3 fold higher than that obtained from typical solvent extraction method. TPC was 579.55 ± 6.82 mg GAE/g dry extract and DPPH, ABTS radical scavenging activities and FRAP were 11.85, 6.04 and 10.28 mmol TE/g dry extract, respectively. Isoquercetin and catechin were the major phenolics in the extract. Additionally, hydroquinin, gallic acid, tannic acid, rutin, eriodictyol, quercetin and apigenin were also found in the extract.

Antioxidative activities of cashew leaf extracts with different extraction methods (solvent extraction and ultrasound-assisted methods) at different concentrations (0-0.2 mg/ml) were investigated. DPPH, ABTS radical scavenging activities and FRAP of both extracts increased when the concentrations increased up to 0.1 mg/ml (P<0.05). Both extracts had higher activities tested by all assays than BHT (P<0.05), but lower than catechin. Furthermore, both extracts had high thermal and pH stabilities. After heating at 60-100 ˚C for 60 min, the antioxidative activities were still remained. High antioxidant activities of the extracts were generally observed at the pH ranging from 6 to 8. When both extracts (50 and 100 ppm) were added into β-carotene-linoleic acid and lecithin liposome systems, the formation of conjugated diene (CD), peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) were lowered, compared to the control (P<0.05). Nevertheless, their preventive effect toward oxidation was lower than BHT in both tested systems.

When cashew leaf extract (CE) was used as antioxidant in fish oil enriched mayonnaise in comparison with BHT at levels of 100 and 200 ppm, oxidative stability during storage of 30 days at 30 ˚C was monitored. With addition of CE or BHT, the lipid oxidation of mayonnaise was retarded, especially at 200 ppm, as indicated by lowered PV, TBARS and AnV values. At the end of storage, a lower abundance of selected volatile compounds was found in mayonnaise added with 200 ppm CE, compared to that of the control. However, the addition of CE (200 ppm) decreased L* value but increased a*, b* and ΔE^* values (P<0.05). Overall, CE at both levels were effective in retard the formation of rancid odor and fishy odor in the mayonnaise enriched with fish oil during the storage of 30 days at 30 ˚C.

Therefore, cashew leaf extract, especially prepared by ultrasound-assisted process, was rich in phenolics. It could be used as natural antioxidant to prevent the oxidation of foods containing lipids including food emulsion.

ACKNOWLEDGEMENT

I would like to express my deepest appreciation and sincere gratitude to my advisor, Prof. Dr. Soottawat Benjakul of the Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, for his kindness, guidance and assistance during my study. I also would like to thank for his invaluable suggestion and lessons for my academic knowledge and personal life. His determination and perseverance to train me to be a good researcher with responsibility, vigilance and honesty are always acknowledged.

I would like to express my profound gratitude to my co-advisor, Asst. Prof. Dr. Kongkarn Kijroongrojana of the Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, for her kindness and helpful guidance in consultation.

I am using this opportunity to record my sincere and deepest thanks and regards to chairpersons and members of my examining committee, Asst. Prof. Dr. Mutita Meenune of the Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University and Assoc. Prof. Dr. Sappasith Klomklao, Department of Food Technology, Faculty of Technology and Community Development, Thaksin University, Phatthalung Campus for their critical and very useful comments, kindness, patience and helpful suggestions.

From the depth of my heart, I take the right to express my deepest humbleness, faithfulness and affection to my father, mother, auntie and all my relatives and friends for their love, affection, ample faith and confidence in me, unrelenting support and inspiration to move always on the right direction, which helped my dreams come true.

I would like to express my special appreciation and thanks to Dr. Thanasak Sae-leaw, Dr. Supatra Karnjanapratum, Dr. Sitthichoke Sinthusamran, Dr. Naveen Kumar, Mr. Natchapol Buamard, Mr. Krisna Nilsuwan, Mr. Avtar Singh, Ms. Jaksuma Pongsetkul, Ms. Tanyamon Petcharat and all other Thai and International friends and colleagues of Seafood Chemistry and Biochemistry

Laboratory (2205) for their kindness, unfailing support and invaluable suggestions.

I would like to thank all faculty members and staffs of Agro-industry for their kind co-operation during my study period. Dedicate, active and accurate services of all professional scientist and technical personnel in Scientific Equipment Center, Prince of Songkla University, are extremely appreciated. My research would not be possible without their helps.

Finally, this study could not be successful without the financial support from the Graduate School, Prince of Songkla University.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Cashew (*Anacardium occidentale* L.) is the member of Anacardiaceae family including the mango, *Mangifera indica* L. and the Spondias genus (Purseglove, 1974). It is commonly grown in many tropical areas of the world. Cashew nut has been popular for consumer and its young leaves have been consumed as fresh in some region of America and ASEAN countries. It contained a large amount of phytochemicals, especially phenolic compounds (Kamath and Rajini, 2007). Phenolic compounds present typically in plants exhibit various biological properties such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Puupponen-Pimia¨ *et al*., 2001). Phenolic compounds, the secondary metabolites of plants, have the aromatic ring bearing one or more hydroxyl substituents (Muchuweti *et al*., 2007; Naczk and Shahidi, 2004). They act as reducing agents, hydrogen donators and singlet oxygen quenchers (Proestos *et al*., 2006).

Nowadays, plant extracts are of increasing interest in the food industry because they are able to retard the oxidative degradation of lipids, thereby improving the quality and nutritional value of foods as well as prevent the occurrence of diseases (Lo¨-liger, 1991). Extraction method is the first important step in isolation of interested compound, in which the target compounds are extracted with high recovery and without interferences (Dobiáš *et al*., 2010). Many techniques, especially conventional method, is limited due to the low yield and long extraction time. To tackle these problems, ultrasound-assisted process has been employed to improve the extraction efficiency with short extraction time, simplicity and low cost (Wang *et al*., 2013).

Optimization of extraction is the procedure that can be achieved by either empirical or statistical methods, which are essential for commercial extraction of the target compounds. Response surface methodology (RSM) has been successfully

used to model and optimize biochemical and biotechnological processes related to food systems (Kaith *et al*., 2014).

Lipid oxidation is one of the major causes for deterioration of many food products, especially food containing long-chain polyunsaturated fatty acids. Lipid oxidation can cause the changes in texture, flavor as well as odor, associated with the loss in food quality. Moreover, it also causes some health hazards in human beings such as cardiovascular disease, cancer and neurological disorders and aging process (Gulcin, 2012). Lipid oxidation commonly occurs in foods rich in oil, such as emulsion. Mayonnaise is an oil-in-water (o/w) emulsion, in which lipid oxidation is initiated at the interface between oil and water and then progresses in the oil phase. To retard lipid oxidation of food or oil rich in polyunsaturated fatty acids (PUFAs), some synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ) are commonly used in food systems. Nevertheless, the use of synthetic antioxidants is limited in many countries. Thus, natural antioxidants from plants, especially from cashew leaves, which are abundant in the southern part of Thailand, can be an alternative antioxidant with safety.

To increase the extraction yield, the extraction condition should be optimized. Characterizations and mode of action of extract should be elucidated. Thus, the extract could be applied effectively, particularly in food containing PUFAs. The information gained will be beneficial to bring about the natural extract for further applications. Also, cashew leaves can be exploited and the undesirable lipid oxidation in foods can be prevented.

1.2 Review of literature

1.2.1 Cashew (*Anacardium occidentale* L.)

Cashew (*Anacardium occidentale L.*) is a member of the family *Anacardiaceae* (Razali *et al*., 2008). Cashew is a perennial-flowering plant and is a local plant from the northeast of the Federative Republic of Brazil. Currently cashew plants are cultivated in various tropical areas to make use from their fruits and seeds.

Cashew is an evergreen plant (10-12 meters height) with short and disorganized branches. Leaves are considerably glazed with a curve to oval shape and arranged in spiral pattern. Their leaves are 4-22 cm long and 2-15 cm in width (Sitthiwarongchai, 2015). Various parts of the plant have been used as a folk medicine in many countries (Konan *et al*., 2007) due to antidiabetic, antibacterial, antiinflammatory, purgatives, hypotensive and diuretic activities. Cashew skin extract contained a large amount of phytochemicals, especially phenolic compounds (Kamath and Rajini, 2007). Those phenolic compounds included gallic acid, flavonoids, tannins, glucosides and glucose (Swarnalakshni *et al*., 1981; Arya *et al*., 1989).

1.2.1.1 Biological properties of cashew (*Anacardium occidentale* L.)

Several parts of cashew tree possess phenolics with bioactivities. Razali *et al*. (2008) investigated total phenolic content and antioxidant activities of cashew shoot (*Anacardium occidentale*) extracts using methanol, hexane and ethyl acetate as solvents. Methanolic extract had the highest activities in scavenging DPPH and ABTS radicals, superoxide anion and nitric oxide as well as in reducing ferric ions whereas the hexane extract contained the weakest antioxidants. In addition, the antioxidant activity of the methanolic extract was comparable to rutin and quercetin. In addition, Omojasola and Awe (2004) reported that cashew leaf extracts had properties in antimicrobial activity against bacteria such as *Escherichia coli*, S*higella dysenteriae, Salmonella* Typhimurium, *Stapphylococcus aureus* and *Pseudomonas auroginosa*. The presence of flavonoids, tannins, glycosides, terpenoids etc., in cashew leaf extract exhibited inhibitory activities against the test organisms (Mustapha *et al*., 2015). Ayepola and Ishola (2009) investigated the antimicrobial activity of extracts from the leaves and stem bark of cashew (*Anacardium occidentale*) using methanol and water as the solvents. The methanolic extracts showed a higher activity than the aqueous extracts. Also the leaf extracts showed greater activity than the stem bark extracts.

1.2.2 Plant extract

Phenolic compounds are secondary plant metabolites, which are important determinants in the sensory and nutritional quality of fruits, vegetables and

other plants (Tomas-Barberan *et al*., 2000; Lapornik *et al*., 2005). These compounds possess an aromatic ring bearing one or more hydroxyl groups and their structures may range from a simple phenolic molecule to a complex high-molecular mass polymer (Balasundram *et al*., 2006).

1.2.2.1 Bioactive compounds in plant extract

Many plants and herbs contain a wide variety of phenolic compounds such as flavonoids, which act potentially as antioxidants to scavenge free radicals and inhibit lipid peroxidation (Brewer, 2011). The major role of phenolic compounds in both plants and humans is to act as antioxidants to protect the systems against oxidative stress promoted by free radical species (Rice-Evans *et al*., 1997). Some natural sources of phenolics are shown in Table 1. Phenolics have ideal structural chemistry for free radical scavenging activities. Several studies reported that the antioxidant ability of phenolics resides was mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (Rice-Evans *et al*., 1997), thereby scavenging the free radicals generated during lipid peroxidation. Plant extracts have been reported to exhibit antioxidative activity in several food systems. Chen *et al*. (2007) found that the addition of guava leaf and guava tea extracts at a concentration of 100 µg/ml inhibited linoleic acid oxidation by 94.4-96.2%. The guava leaf extracts displayed a significant scavenging ability toward peroxyl radicals. Extracts containing phenolic acids, such as ferulic acid, appeared to be responsible for their antioxidant activity (Tachakittirungrod *et al*., 2007). Ethanolic extracts of spice *Limnophila aromatica* showed high total antioxidant activity, reducing power and DPPH radical scavenging activity. This activity was attributed to the high phenolic contents and flavonoids present in the extract (Do *et al.,* 2014). Different solvent extracts of plant *Merremia borneensis* had DPPH radical scavenging activity and reducing power. Extracts were able to inhibit oxidation in β-Carotene model system. Positive correlation was observed between the antioxidant activity potential and total flavonoid levels of the extracts (Hossain and Shah, 2015). Methanolic extract of *Terminalia chebula* Retzius (Combretaceae) fruits had ability in scavenging DPPH radical, nitric oxide radical and hydrogen peroxide and showed reducing power (Saha and Verma, 2016).

Table 1. Antioxidants from natural sources

Sources	Phenolic compounds
Clove	Phenolic acids (gallic acid), flavonol glucosides, phenolic volatile oils (eugenol, acetyl eugenol), tannins
Ginger	Shogoal, gingerol
Mace	Myristphenone
Marjoram	Beta-carotene, beta-sitosterol, caffeic-acid, carvacrol, eugenol, hydroquinone, linalyl-acetate plant 3-17, myrcene, rosmarinic acid, terpinen-4-ol
Nutmeg	Myristphenone, phenolic volatile oils, phenolic acid (caffeic acid), flavanols (catechin)
Oregano	Caffeic acid, *p*-coumaric acid, rosmarinic acid, caffeoyl derivatives, cavacrol, flavonoids
Red pepper	Beta-carotene fruit, beta-sitosterol plant, caffeic acid campesterol, camphene fruit, capsaicin fruit, capsanthin fruit, chlorogenic-acid fruit, eugenol fruit, gamma-terpinene fruit, hesperidin fruit, myristic acid
Black pepper	Kaempferol, rhamnetin, quercetin
Rosemary	Carnosol, 12-O-methylcarnosic, rosmanol, caffeic acid, rosmarinic acid, caffeoyl derivatives, phenolic diterpenes (carnosic acid), carnosol, epirosmanol, flavonoids
Black grapes	Anthocyanins and flavonols
Sage	Rosmanol, epirosmanol, phenolic acids (rosmarinic acid), phenolic diterpenes (carnosic acid), flavonoids
Sesame seed	Sesaminol, α-tocopherol, sesamol
Turmeric	Curcumin, 4-hydroxycinnamoylmethane
Thyme	Phenolic acids (gallic acid, caffeic acid, rosmarinic acid), thymol, phenolic diterpenes, flavonoids
Cumin	Cuminal, γ-terpinene, pinocarveol, linalool, 1-methyl-2-(1methylethyl) benzene, carotol
Olives	Hydroxytyrosol, luteolin 7-O-glucoside, oleuropein, rutin, apigenin, 7-O-glucoside, and luteolin
Berries	Flavanols, hydroxycinammic acids, hydroxybenzoic acids, and anthocyanins
Oats, wheat, rice	Caffeic and ferulic acids
Black, green, Australian fresh tea	Flava-3-ols, flavonols, 5 catechin, 6 catechin gallates, caffeine and theobromine, 5 flavonol glycosides and 6 phenolic acids, including quinic acids and esters

Source: Maqsood *et al*. (2014); Embuscado (2015)

Plant phenolics have been known to have antimicrobial activities, can be used as food additives. Ethanolic extracts from black currant berries showed antimicrobial activity toward *Escherichia coli*, *Aspergillus niger* and *Candida albicans* (Paunović *et al*., 2017). Cavanagh *et al*. (2003) suggested that several fresh berries, including raspberry and blackcurrant, inhibited the growth of gram-negative and gram-positive bacteria. Orue *et al*. (2013) reported ethanolic extracts from oregano leaves and from peel and pulp of limes had similar effectiveness to chlorine or citrol in reducing *Salmonella*, *E. coli* O157:H7, and *S. sonnei* by >2.0 log cycles on leafy greens such as cilantro, parsley, and spinach. Moreover, Tekwu *et al*. (2012) revealed that the methanolic extract of *F. exasperate* leaves showed high activity against *E. coli*, *S. dysenteriae*, *S. Typhi* and *P. aeruginosa* with MIC values of 128 mg/ml. Antibacterial activity is most likely due to the combined effects of adsorption of polyphenols to bacterial membranes with membrane disruption and subsequent leakage of cellular contents (Ikigai *et al*., 1993; Otake *et al*., 1991), and the generation of hydroperoxides from polyphenols (Akagawa *et al*., 2003). Davidson and Branen (2005) also suggested that the outer membrane surrounding the cell walls of gram negative bacteria may restrict the diffusion of hydrophobic compounds through the bacterium's lipopolysaccharide cell wall coverings. Thus, gram positive bacteria are more sensitive to plant extracts than gram-negative bacteria (Rahman and Kang, 2009).

1.2.2.2 Classification of polyphenolic compounds

Phenolic compounds comprise a wide variety of molecules that have a polyphenol structure (i.e. several hydroxyl groups on aromatic rings), and also molecules with one phenol ring, such as phenolic acids and phenolic alcohols. Polyphenols are divided into several classes according to the number of phenol rings and the structural elements that bind these rings to one another. The main groups of polyphenols (Figure 1) are: flavonoids, phenolic acids, tannins (hydrolyzable and condensed), stilbenes and lignans (D'Archivio *et al*., 2007).

1.2.2.2.1 Flavonoids

Flavonoids are the most abundant polyphenols in our diets. The basic flavonoid structure is the flavan nucleus, containing 15 carbon atoms arranged in three rings (C6-C3-C6), which are labeled as A, B and C (Youdim *et al*., 2004). Flavonoids are themselves divided into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins, according to the oxidation state of the central C ring. Their structural variation in each subgroup is partly due to the degree and pattern of hydroxylation, methoxylation, prenylation, or glycosylation (Hollman and Katan, 1997). Some of the most common flavonoids include quercetin, a flavonol abundant in onion, broccoli, and apple; catechin, a flavanol found in tea and several fruits; naringenin, the main flavanone in grapefruit; cyanidin-glycoside, an anthocyanin abundant in berry fruits (black currant, raspberry, blackberry, etc.); and daidzein, genistein and glycitein, the main isoflavones in soybean (D'Archivio *et al*., 2007).

1.2.2.2.2 Phenolic acids

Phenolic acids can be divided into two classes: derivatives of benzoic acid such as gallic acid, and derivatives of cinnamic acid such as coumaric, caffeic and ferulic acid (Teixeira *et al*., 2013). Caffeic acid is the most abundant phenolic acid in many fruits and vegetables, most often esterified with quinic acid as in chlorogenic acid, which is the major phenolic compound in coffee. Another common phenolic acid is ferulic acid, which is present in cereals and is esterified to hemicelluloses in the cell wall (D'Archivio *et al*., 2007).

1.2.2.2.3 Tannins

Tannins are another major group of polyphenols in our diets and usually subdivided into two groups: (1) hydrolyzable tannins and (2) condensed tannins. Hydrolyzable tannins are compounds containing a central core of glucose or another polyol esterified with gallic acid, also called gallotannins, or with hexahydroxydiphenic acid, also called ellagitannins. The great variety in the structure of these compounds is due to the many possibilities in forming oxidative linkage.

Intermolecular oxidation reactions give rise to many oligomeric compounds having a molecular weight between 2,000 and 5,000 Daltons (Khanbabaee and Ree-van, 2001). Condensed tannins are oligomers or polymers of flavan-3-ol linked through an interflavan carbon bond. They are also referred to as proanthocyanidins because they are decomposed to anthocyanidins through acid-catalyzed oxidation reaction upon heating in acidic alcohol solutions (Dai and Mumper, 2010).

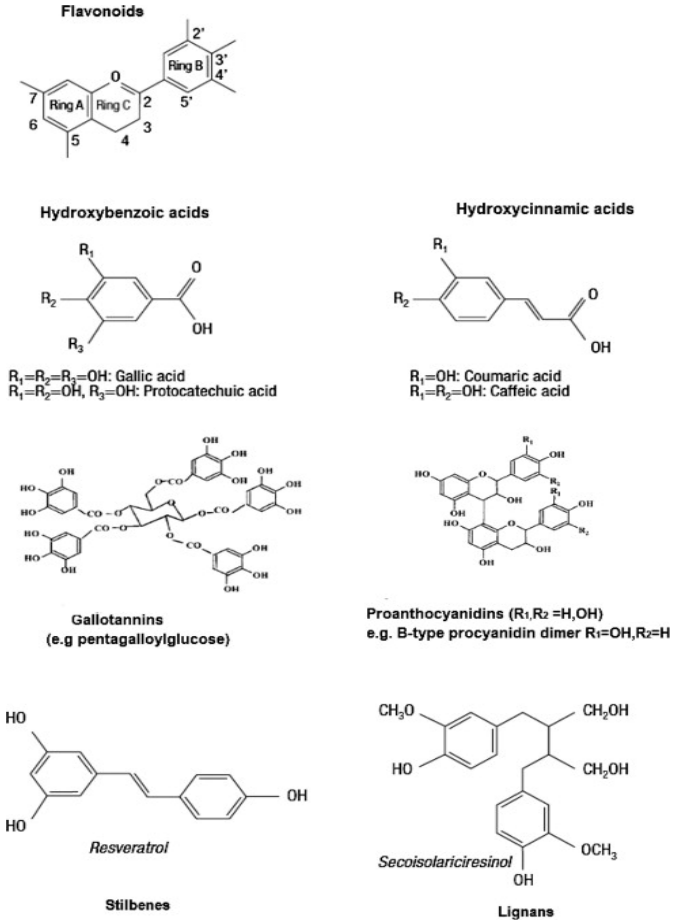

Figure 1 Chemical structures of phenolic compounds.
Source: Ignat *et al*. (2011)

1.2.2.2.4 Stilbenes and lignans

Silbenes are present at low content in the human diet. Resveratrol is the major component, existing in both cis and trans isomeric forms and glycosylated forms are dominant (Delmas *et al*., 2006). They are synthesized by plants in response to infection by pathogens or to a variety of stress conditions (Bavaresco, 2003). Those

compounds could be found in more than 70 plant species, including grapes, berries and peanuts. Lignans are produced by oxidative dimerization of two phenylpropane units. They are generally present in the free form, whereas their glycoside derivatives are only a minor form. Lignans and their synthetic derivatives have gained increasing interest due to potential applications in cancer chemotherapy and various other pharmacological effects (Saleem *et al*., 2005).

1.2.3 Extraction of antioxidants from plants

Extraction method is one of the most important step in isolation of interested compound, in which the target compounds are extracted with high recovery and without interferences (Dobiáš *et al*., 2010). Solvent extractions are the most commonly used methods for preparation of plant extracts because of their ease of use, efficiency, and wide applicability. The yield of extracts depends on the type and polarity of solvents, extraction time and temperature and sample-to-solvent ratio. Chemical composition and physical characteristics of the samples are also determining the yield (Dai and Mumper, 2010).

1.2.3.1 Factors affecting extraction of plant phenolics

Extraction of phenolic compounds from plant materials generally depends on their native structure, which may contain varying quantities of phenolic acids, phenylpropanoids, anthocyanins, tannins, etc. (Dai and Mumper, 2010). There is a possibility of interaction between phenolics and other plant components such as carbohydrates and proteins. These interactions may lead to the formation of complexes (Dai and Mumper, 2010).

1.2.3.1.1 Solvent

Solubility of phenolics is governed by the polarity of solvents used, degree of polymerization of phenolics as well as the interaction of phenolics with other plant constituents (Cvetanović *et al*., 2014). Many organic solvents have been used for extraction of phenolic compounds from plant materials. Those solvents include methanol, ethanol, propanol, acetone, ethyl acetate and dimethylformamide

(Antolovich *et al*., 2000; Kozlowska *et al*., 1983; Luthria *et al*., 2006; Naczk *et al*., 1992; Robbins, 2003; Zadernowski *et al*., 2005). Different organic solvents have been used for the extraction of antioxidative compounds from plants (Table 2). Belwal *et al.* (2016) studied the extraction of phenolic compounds from *Berberis asiatica* fruits using different solvents. The results indicated that 70% methanol yielded the extract with the highest content of total phenolics, total anthocyanins and FRAP, as compared to ethanol, acetone, propanol and distilled water at the same concentration used (P<0.05). Metrouh-Amir *et al*. (2015) reported that the methanolic and ethanolic extracts of *Matricaria pubescens* at the levels of 50% showed the highest total phenolic contents as compared to those using other solvents (P<0.05). A lower value of IC_{50} in scavenging DPPH and ABTS radicals was found in 50% ethanol, followed by 50% acetone, 50% methanol, pure methanol and pure ethanol, respectively. Hussain *et al*. (2012) found that the extraction of peanut hull using 80% methanol provided the higher extraction yield than 80% ethanol. Yingngam *et al*. (2015) reported that the increase in yield of *Cratoxylum formosum* spp. *formosum* leaf extract was found as the concentration of ethanol increased from 0 to 30%. However, the decrease in yield was observed as the concentration of ethanol increased from 30% to 90%. These might be attributed to different solvent polarity and viscosity (Gong *et al*., 2012). Water is the most polar solvent that acts as the plant swelling agent, while ethanol can disrupt the bonding between the solutes and plants matrices (Wang and Weller, 2006). Furthermore, the addition of ethanol into water also reduces the viscosity of solvent when compared to pure water, leading to increased mass transfer of solutes (Sahin and Samlí, 2013).

1.2.3.1.2 Temperature

Temperature is one of the main factors in extraction processes. Both kinetics of phenolics release from the solid matrix and the antioxidant activity of the extract are effected by temperature (Mussatto *et al*., 2011). In general, the efficiency of the extraction can be improved by high temperature. Shi *et al.* (2003) suggested that increased temperature might lead to softening of the plant tissue, disrupting the interactions between phenolic compounds and protein or polysaccharides. In addition, the elevation in temperature could enhance the phenolic solubility, diffusion rate,

mass transfer rate, extraction rate and reduce solvent viscosity and surface tension, which promote the extraction of phenolics from plant matrix (Richter *et al*., 1996; Saha *et al*., 2011). However, the rise in temperature above a certain level might lead to undesirable consequences, such as vaporization of solvent and degradation of phenolic compounds (Chan *et al*., 2009). Excessive temperature can degrade polyphenolic compounds (Escribano-Bailón and Santos-Buelga, 2003). Ho *et al.* (2015) reported that an increase in extraction temperature resulted in a large increment in the TPC and its antioxidant activities of the extract from artichoke leaves using 20% ethanol as extracting medium for 100 min. When temperature was elevated from 30 to 60˚C, TPC and FRAP absorbance values of extract increased from 516 to 905 mg GAE/100 g and from 0.699 to 1.329, respectively. Similar result was reported by Wardhani *et al*. (2010) who found that 65.3˚C is the optimum temperature for the extraction of phenolic compounds from soybeans fermented by *Aspergillus oryzae*. Ereifej *et al*. (2016) also reported that increasing temperature of extraction from 20 to to 60˚C for 60 min resulted in the increases in total phenolic contents of the methanolic extracts from cloves and turmeric.

1.2.3.1.3 Extraction time

Extraction time is another factor determining the efficiency for preparing plant extracts. Gan and Latiff (2011) reported that an extended extraction time from 100 to 150 min enhanced the extraction of polyphenolic compounds from *Parkia speciosa* pod powders. In addition, Saha *et al.* (2011) suggested that the extraction of phenolics from a soy bean increased as extraction time increased from 30 to 150 min. Yingngam *et al*. (2015) investigated the effects of extraction time on the yields of total phenolics from *Cratoxylum formosum* ssp. *formosum* leaves using 30 % ethanol at 60 °C. The yields of total phenolics increased when extraction time increased from 1 to 5 h. However, a slight decrease was observed with a further extension of extraction times. Kua *et al*. (2015) reported that an increase in extraction time from 15 min to 60 min enhanced the extraction of phenolic compounds from palm kernel cake, whereas phenolic yield was slightly decreased when the extraction was extended to 120 min. Shorter extraction time was generally insufficient to completely extract the bound-phenolic compounds (Vasco *et al*., 2008). However,

prolonged extraction time plausibly favored the exposure of phenolics to environmental factor such as light, oxygen and heat, thus contributing to lower antioxidant activities (Lafka *et al*., 2007; Naczk and Shahidi, 2006; Thoo *et al*., 2010; Wong *et al*., 2014). Polymerization of antioxidant compounds also occurred with longer extraction time (Toh *et al*., 2016).

Table 2. Organic solvents used for extraction of polyphenolic compounds

Polyphenolic compounds	Solvents	References
phenolic acids, flavonols and anthocyanins	ethyl acetate	Pinelo *et al*. (2005); Russell *et al*. (2008)
anthocyanins, phenolic acids, catechins, flavanones, flavones, flavonols, procyanidins, ellagic acids, rutin and chlorogenic acids	methanol and different aqueous forms (50-90%, v/v)	Bleve *et al*. (2008); Caridi *et al*. (2007); Ross *et al*. (2009); Mattila; Kumpulainen (2002)
anthocyanins, flavonols and free phenolic acids	ethanol and different aqueous forms (10-90%, v/v)	Balas and Popa (2007); Wang *et al*. (2009); Bleve *et al*. (2008), Bucic´-Kojic´ *et al*. (2006); Corrales *et al*. (2009) ; Ross *et al*. (2009)
flavonols and free phenolic acids	chloroform	Sharififar, Dehghn-Nudeh, and Mirtajaldini (2009)
favonols and phenolic acids	dietyl ether	Ross *et al*. (2009)
proantocyanidins and phenolic acids	hot water (80-100˚C)	Diouf, Stevanovic, and Cloutier (2009)
tannins and bound phenolic acids	NaOH (2 N-10 N)	Nardini *et al*. (2002); Popa *et al*. (2008); Ross *et al*. (2009)
phenolic acids	petroleun ether	Zhang *et al*. (2009)
flavonols, phenolic acids, hydroxycinamic acids, coumarins, flavonols and xanthones	acetone/water 10-90% (v/v)	Altiok *et al*. (in press); Naczk and Shahidi (2006); Sharififar *et al*. (2008); Schieber *et al*. (2003)
flavonols, quercetin 3,4´-diglucoside and quercetin 4´-monoglucoside	methanol/water 70% v/v	Caridi *et al*. (2007)

Source: Ignat *et al*. (2011)

1.2.3.1.4 Solid-to-solvent ratio

Extraction efficacy is also governed by solid to solvent ratio. Yingngam *et al*. (2015) studied the extraction of phenolic compounds from *Cratoxylum formosum* ssp. formosum leaves at different solid-to-ethanol ratios (1:10 to 1:70 g/ml). An increase in solid-to-solvent ratio decreased the extraction yields. The lowest yield of phenolic compounds was observed at 1:10 g/ml, while a higher yield was found at 1:30 g/ml. This indicated that more volume of extractant was required to sufficiently solubilize the target phenolic compounds since the higher driving force of molecules was supposed to be higher (Şahin and Şamlí, 2013). However, there was no significant difference in yields of phenolic compounds as the ratio of plant material-to-solvent decreased from 1:30 to 1:70 g/ml (P>0.05). A high amount of solvent could promote an increasing concentration gradient, resulting in an increase of diffusion rate that allows greater extraction of solids by solvent (Al-Farsi and Chang, 2007). Liu *et al.* (2013) reported that the total phenolic contents (TPC) of the extract from *Euryale ferox* seed shells increased with increasing ethanol to solid ratio up to 30:1 ml/g. Thereafter, TPC was slightly decreased after the ratio of ethanol to solid exceeded 30:1 ml/g. This result was similar to the extraction of polysaccharides from *Acanthopanax senticosus* (Zhao *et al*., 2013). This phenomenon may be attributed to the mass transfer principle (Sahin and Samli, 2013). Xu *et al*. (2016) reported that there was difference in the antioxidative activities of the extract from *Limonium sinuatum* flower when ethanol to solid ratio varied from 40:1 to 70:1 ml/g (P>0.05). An increase in the ratio of ethanol to solid resulted in the increased contact area between material and solvent. This led to enhancement of solubility of antioxidant components in the plants cells (Yang *et al*., 2010).

1.2.4 Ultrasound-assisted extraction (UAE)

Ultrasound is a sound wave with a frequency of 20 kHz to 100 MHz (Azmir, 2013). The application of ultrasounds in industrial processes has two main requirements: a liquid medium and a source of high energy vibrations (Patist and Bates, 2008). Nowadays, there are numerous ultrasound applications in food, agricultural and biotechnological systems. It can be used for the improvement of food

properties such as emulsification ability, solubility and texture, etc. It is also applied for homogenization, viscosity alteration, extraction, drying, crystallization and defoaming (Soria and Villamiel, 2010). Khan *et al*. (2010) reported that the use of UAE (25 kHz, 90 W) enhanced the efficiency of the extraction of total phenolic contents from orange peel and antioxidative activity was also increased up to 30-40%. UAE (20 kHz, 300 W) resulted in a higher yields of both total anthocyanins and total phenolic contents than the conventional method when blueberry (*Vaccinium ashei*) wine pomace was used as raw material as reported by He *et al*. (2016). Xu *et al*. (2016) also suggested that UAE (40 kHz, 400 W) provided higher antioxidant activities of flower of *Limonium sinuatum* extracts as compared with those of the conventional method and soxhlet extraction method. Virot *et al*. (2010) also compared UAE (25 kHz, 150 W, with an ultrasonic bath) and conventional extraction (with mechanical stirring, no specified conditions). Phenolic, proanthocyanidins and flavan-3-ols contents of apple pomace extracts were 1.2, 1.4 and 1.3-fold higher when UAE was used.

1.2.4.1 Principle of ultrasound technology

Extraction by ultrasonic has been attributed to the propagation of ultrasound pressure waves. These waves are generated during the sonication process. The waves pass through a medium by creating compression and expansion (Figure 2). This process produces a phenomenon called cavitation, which leads to implosion (Butz and Tauscher, 2002). The implosion of cavitation bubbles generates macro-turbulence, high-velocity inter-particle collisions and perturbation in micro-porous particles of the biomass, which accelerates the eddy diffusion and internal diffusion (Vilkhu *et al*., 2002).

The mechanism of disruption of cell wall due to cavitation is depicted schematically in Figure 3. Cavitation, the cracks developed in the cell wall, increases permeability of plant tissues, facilitating the entry of the solvent into the inner part of the material as well as leaching the extracts. However, beyond a certain limit, there is a wastage of ultrasonic energy as no further increase can be seen owing to the limitations of equilibrium extraction. Consideration of these facts is very important in

making the whole extraction process more economically feasible (Romdhane and Gourdon, 2002).

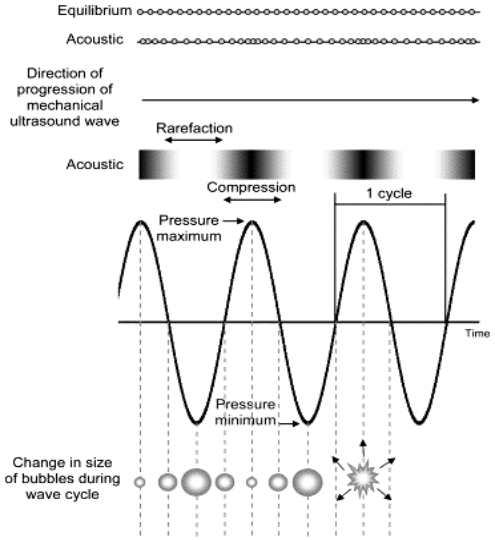

Figure 2 Ultrasonic cavitation.

Source: Soria and Villamiel (2010)

Apart from the effects of ultrasound induced cavitation phenomena on the disruptions of leaf tissues and cell walls, the enhanced quantum of solvents can penetrate into the inner areas. The turbulence and acoustic streaming can significantly increase the solid liquid mass transfer coefficients due to microscale effects in the system. The mechanical effects of ultrasound can also increase the contact surface area between solid and liquid phases due to the possibility of size reduction in the solid matrix (Romdhane and Gourdon, 2002). The enhanced rates of mass transfer are related with the enhanced rate of the solvent brought to the solid surface. The transfer of the soluble constituents into the solvents is also enhanced (Shirsath *et al.*, 2012).

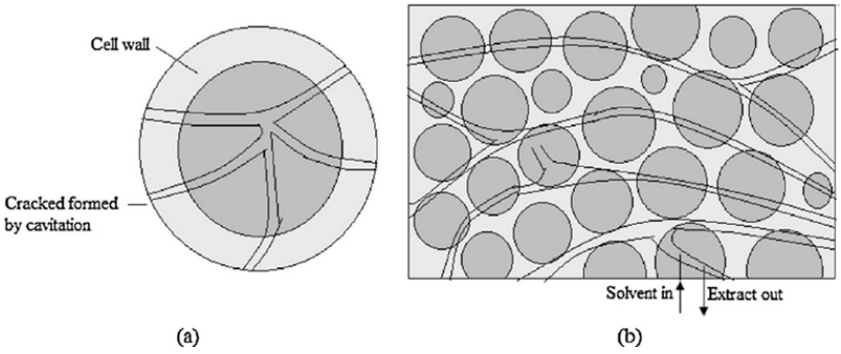

Figure 3 The mechanism of cell wall disruption (a) breaking of cell wall due to cavitation. (b) diffusion of solvent into the cell structure.

Source: Shirsath *et al.* (2012)

1.2.4.2 Factors affecting the cavitation phenomenon

1.2.4.2.1 Pressure applied

External pressure and solvent vapor pressure are two major influencing factors of the cavitation phenomenon. Increasing external pressure increases the cavitation threshold by dissolving the suspended gas molecules, and thus the number of cavitation bubbles required are reduced (Muthukumaran *et al*., 2006). More importantly, the increase in applied pressure increases the pressure in bubbles, thereby increasing the energy during their collapse. Thus, cavitation bubble collapse is enhanced, which ultimately increases the sonochemical effect (Lorimer and Mason, 1987). The sonochemical effects are caused by free radicals produced by symmetric bubble oscillation when the pressure and temperature become extremely high inside a bubble. OH radicals are produced by the pyrolysis of water molecules, and H_2O_2, which is formed by recombination of OH radicals (Riesz, 1992). The energy required to produce cavitation in a solvent of low vapor pressure is relatively high because there is less mass transfer into the bubble. Thus a more volatile solvent will support cavitation at lower acoustic energy and produce vapor filled bubbles (Lorimer and Mason, 1987).

1.2.4.2.2 Intensity

Ultrasonic intensity is the power dissipated per unit of surface area of the sonotrode and is defined in W/cm^2 (Patist and Bates, 2008). Ultrasound energy is of the integral of the dissipated power as a function of time. The time of ultrasonic exposure is inversely proportional to the flow rate through the ultrasonic devices (l/h). In general, an increase in ultrasonic intensity increases the sonochemical effect (Luque De Castro and Capote, 2007). However, the amount of energy that can be applied to any system is limited. M'hiri *et al.* (2015) studied the effect of ultrasonic power on the total phenolic content (TPC) and total flavonoid content (TFC) of orange peel extract using 80% ethanol as the solvent. Extraction was performed at 35˚C for 35 min. The increase in ultrasonic power from 100 W to 200 W increase TPC (P<0.05). However, no difference in TFC was found when power increased from 125 to 200 W (P>0.05).

1.2.4.2.3 Temperature

The vapor pressure, surface tension and viscosity of liquid are affected by the temperature of liquid (Muthukumaran *et al*., 2006). An increase in temperature decreases the cavitation threshold. Increase in temperature also facilitates the cavitation and increases the number of cavitation bubbles at a low acoustic intensity. However, the sonochemical effects are reduced under these conditions because the higher number of cavitation bubbles will act as a barrier to sound transmission (Muthukumaran *et al*., 2006). Thus, in order to maximize sonochemical effects, experiments should be conducted at low temperature or using a solvent of low vapor pressure. The extraction of flavonoid from bamboo can be improved using ultrasound assisted extraction at low temperature rather than hot-water extraction at 80 °C (Xu *et al*., 2001).

1.2.4.2.4 Frequency

It is known that cavitation bubbles require an extremely short but definite time to form at sites of rarefaction that have nucleation sites. Thus, as ultrasonic frequency is increased, the duration of low pressure phase shortens and intensity of ultrasound has to be increased to maintain an equivalent amount of cavitation energy in the system. For example, the duration of the rarefaction cycle reduces from 25 μs to 0.025 μs when frequency is increased from 20 kHz to 20 MHz (Luque De Castro and Capote, 2007).

1.2.5 Lipid oxidation

Lipid oxidation is a major cause of deterioration of food products, especially those containing lipids with long-chain polyunsaturated fatty acids. Those lipids can react with molecular oxygen via a free radical chain mechanism, forming fatty acyl hydroperoxides as well as non-volatile and volatile hydroperoxide breakdown products (Medina *et al*., 2012; Shahidi and Zhong, 2010). This phenomenon can be influenced by both intrinsic and extrinsic factors such as fatty acid composition, the concentration of prooxidants, endogenous ferrous iron, myoglobin, enzymes, pH, temperature, ionic strength and oxygen consumption

reaction (Andreo *et al*., 2003). Lipid oxidation also leads to discoloration, drip losses, off-odor and off-flavor development, texture defects and the production of potentially toxic compounds in foods (Morrissey *et al*., 1998; Richards *et al*., 2002).

1.2.5.1 Mechanisms of lipid oxidation

Lipid peroxidation is a free-radical chain reaction that causes a total change in the sensory properties and nutritive value of food products (Rajapakse *et al*., 2005). Decomposition of hydroperoxides by heating or by transition metal ion catalysis can produce both peroxyl and alkoxyl radicals. The formation of peroxyl radical is the major chain-propagation step in lipid peroxidation (Headlam and Davies, 2003; Niki, 1987). Lipid oxidation is a chain reaction that consists of initiation, propagation, and termination reactions, and involves the production of free radicals (Nawar, 1996; Renerre, 2000; Underland, 2001).

Initiation

At this step, a fatty acid radical is produced. The most notable initiators in living cells are reactive oxygen species (ROS), such as OH• and H_2O_2, which combines with a hydrogen atom to make water and a fatty acid radical (Chan, 1987; Belitz *et al*., 2004).

Propagation

The fatty acid radical is not a very stable molecule and reacts readily with molecular oxygen, thereby creating a peroxyl-fatty acid radical. This is an unstable species that reacts with another free fatty acid, producing different fatty acid radicals and lipid peroxide, or cyclic peroxide if it reacts with itself. This cycle continues, as the new fatty acid radical reacts in the same way (Berton *et al*., 2012).

Termination

This step is the final step of reaction. When a radical reacts with a nonradical, it produces another radical. This process is related with a "chain reaction mechanism". The radical reaction stops when two radicals react and produce a non-

radical species. This happens only when the concentration of radical species is high enough for collision of two radicals (Chan, 1987; Belitz *et al*., 2004).

1.2.5.2 Lipid oxidation in emulsions

Lipid oxidation in different systems can be varied and determined by different factors. Lipid oxidation in emulsions is very complex reaction, in which oxidation or electron transfer in different phases of the system make the mechanisms of lipid oxidation in emulsions different and more complex than those found in bulk oil systems (Jacobsen *et al*., 2008). Although the same basic oxidation reactions of lipids in the emulsions are reported, compared to those of lipids in bulk lipids, the factors affecting lipid oxidation in emulsions can be different. Intrinsic factors such as transition metal and pH, which affect lipid oxidation, are different between emulsions and bulk lipid systems (Jacobsen *et al*., 2008).

1.2.5.2.1 Factors influencing lipid oxidation in emulsions

Multiphase system

In O/W emulsions, the highest prooxidant concentrations are found in the aqueous phase. Therefore oxidation may be initiated in the aqueous phase or at the interface rather than in the oil phase ‘‘inside’’ the oil droplets (Grigoriev and Miller 2009). Oxygen, prooxidant metals, and water soluble antioxidants can diffuse through the aqueous phase and reach the oil droplets or the O/W interface. Solubility, mobility and mass transfer rates of transition metals and antioxidants are varying through different phases, thereby affecting oxidation in emulsions (Jacobsen *et al*., 2008). Oxygen is more soluble in oil than in water (Battino, 1987; Weast *et al*., 1987) and this may also influence oxidation rates. In bulk oils, the oxidation reactions take place at the interface between the oil and the air in the headspace above the oil or at the interface between the oil and air dissolved in the oil (Jacobsen *et al*., 2008). In general, oxidation rate is faster in emulsions than in bulk oil. This is owing to the large interfacial area in emulsions, compared with bulk oils. The total interfacial area in any emulsion obviously depends on the size distribution of the oil droplets (Jacobsen *et al*., 2008).

Emulsifier and pH

In emulsions, transition metals are the major prooxidants in oil-in-water emulsions and primarily promote the oxidation by decomposing lipid hydroperoxides located at the droplet surface into free radicals (Nuchi *et al*., 2001). Ferrous ($Fe^{2+)}$ is a stronger prooxidant than ferric (Fe^{3+}), owing to its higher solubility and reactivity (Halliwell and Gutteridge, 1990). The emulsifiers have been used to build the interfacial layer and stabilize emulsion. Generally, emulsifiers are surface active molecules with amphiphilic properties, and are able to interact with oil and water interface and lower surface tension (Claire *et al*., 2014). Nevertheless, several emulsifiers are able to interact with other components of the food product. The choice of emulsifier can therefore be of significant importance for both physical and oxidative stability of the food product (Jacobsen *et al*., 2008). Emulsifiers used in foods can be either macromolecules, such as proteins, or smaller surfactant molecules, including phospholipids, free fatty acids, di- and monoacylglycerols and synthetic surfactants. Emulsifiers affect lipid oxidation by influencing the thickness of the interface. For emulsions stabilized by proteins, pH will be either below or above the *p*I of the protein in order to avoid coalescence of droplets. Emulsified droplets may have either a positive or negative surface charge. The surface charge of emulsion droplets is essential for lipid oxidation accelerated by trace metal ions, such as Fe^{2+}. With a negative surface charge, emulsion droplets will attract the potentially highly prooxidative positively charged trace metals. This brings them into closer proximity to the n-3 PUFA oil, thus enhancing lipid oxidation (Mei *et al*., 1999). Milk proteins have the ability to reduce the lipid oxidation rate when present in the emulsion continuous phase due to their ability to scavenge free radicals through their sulfhydryl groups (Elias *et al*., 2005). Li *et al*. (2016) showed the addition of zein hydrolysate reduced the peroxide values and thiobarbituric acid reactive substances values of oil-in-water (o/w) emulsions prepared by myofibrillar protein during 10 days of storage. Protein hydrolysates were investigated to have strong antioxidant activity (Kong *et al*., 2006). Protein hydrolysates are ability to form a high degree of continuity and rigidity fat-protein interfacial layer, which would be expected to inhibit the

penetration and diffusion of lipid oxidation initiators into the interior of emulsion droplets in the o/w emulsions (Wong *et al*., 2012).

In emulsions, transition metals are the major prooxidants in oil-in-water emulsions. The solubility of trace metals generally increases at decreasing pH (Elias *et al*., 2005). The increased trace metal solubility then potentially promotes oxidation. Since different emulsifiers are used in different food emulsions, and pH values also vary in different types of food emulsions, the significance of the emulsifier and the pH varies in different systems (Jacobsen *et al*., 2008). Charoen *et al*. (2011) reported that the oxidative stability of rice bran o/w emulsions depends on pH and the nature of emulsifiers. Rice bran oil emulsions containing modified starch and whey protein isolate coated lipid droplets were relatively more stable to lipid oxidation, than those containing gum arabic at both pH 3 and 7. The electrical charge on lipid droplets plays a key role in determining their stability to lipid oxidation (McClements and Decker, 2000). Hu *et al*. (2003) showed the rate of lipid oxidation in salmon oil-in-water emulsions stabilized with whey protein isolate, sweet whey, β-lactoglobulin or α-lactoglobulin was slower at pH 3 (protein have positive charge) than at pH 7 (negative charge). This is due to the repulsive forces at pH 3 between the positively charged droplets and the positively charged prooxidants in the continuous phase. Combrinck *et al*. (2014) found that increase in pH of whey protein emulsions led to a decrease in the mean droplet size. When the pH approaches the isoelectric point of the protein, the electrostatic repulsion of the protein adsorption layers decreases, which allows more compact packing and stronger attractive interfacial interactions so that coalescence and flocculation occur (Dickinson, 2008; Guzey and McClements, 2006).

1.2.6 Antioxidants

Antioxidants are a group of chemicals effective in extending the shelf-life of a wide variety of food products. The use of antioxidants has been extended to a wide variety of food products including high-fat foods, cereal and even products containing very low levels of lipids. The addition of antioxidants is one effective way to retard lipid oxidation. The most widely used antioxidants include free radical

scavengers (also known as chain-breaking antioxidants) that inactivate free radicals formed in the initiation and propagation steps of lipid oxidation, and metal chelators (Shahidi and Zhong, 2010). Antioxidants can break this chain reaction by reacting with peroxyl radicals to form unreactive radicals which are more stable or form non-radical products and that cannot propagate the chain reaction.

1.2.6.1 Classification of antioxidants

Antioxidants can be classified according to the mechanism of action into two groups.

1.2.6.1.1 Primary antioxidants

Primary antioxidants (chain-breaking antioxidants) are free radical acceptors that delay or inhibit the initiation step or the interrupt the propagation step of autoxidation through reaction with lipid and peroxyl radicals and convert them to more stable, non-radical products (Gil, 2011). Antioxidant radicals are stable due to delocalization of the unpaired electron around a phenol ring and cannot easily react with fatty acids. They are able to terminate radical chain process by reacting with radicals (Reische *et al*., 2002). The most commonly used primary antioxidants in food are synthetic compounds such as phenolic antioxidants including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tertiary butylhydroquinone (TBHQ) (Sun *et al*., 2011).

1.2.6.1.2 Secondary antioxidants

Secondary antioxidants or preventive antioxidants do not break free radical chain but are able to act through various mechanisms other than converting free radicals to more stable products to slow the rate of oxidation. They can hinder reactive oxygen species (ROS) formation or scavenge species responsible for oxidation initiation (O^{-2},$^1O_2$ etc.) (Sun *et al*., 2011). There are many different preventive antioxidation pathways including reducers and chelators of metals (e.g. citric acid, phosphoric acid, EDTA), oxygen scavengers and reducing agents (e.g.

ascorbic acid, ascorbyl palmitate, sulfites), singlet oxygen quenchers (carotenoids) (Reische *et al*., 2002).

1.2.7 Natural antioxidants/plant extracts

The use of natural antioxidants is emerging as an effective methodology for controlling rancidity and limiting its deleterious consequences. Natural phenolic compounds with antioxidant activity such as rosemary extract, tea catechin, tannins, etc. have been gaining increasing attention for the preservation of food, due to their potential use in food for two principal reasons: (1) safety considerations regarding the potentially harmful effects of the chronic consumption of synthetic compounds in food and beverages; and (2) “natural” additives are perceived as beneficial for both quality and safety aspects and also possible beneficial effects on human health (Viuda-Martos *et al*., 2010; Chang, 1997).

1.2.7.1 Heat stability

Heat stability is important for antioxidant known as carry through. Different compounds have varying thermal stability, which affect their functions, particularly in thermally processed foods. Francisco and Resurreccion (2009) investigated the effect of heat treatment on total phenolic compounds and antioxidant capacities of peanut skins from Runner, Virginia and Spanish cultivars. Skin of Runner had higher total phenolics and TEAC values than Virginia and Spanish types (P<0.05). Mild heat treatments (<135 ˚C, 15 min) could further enhance the antioxidant capacity of peanut skins. Klompong and Benjakul (2015) evaluated the antioxidative activities of the ethanolic extracts from the seed coat of Bambara groundnut (*Voandzeia subterranean*). DPPH, ABTS radical scavenging and metal chelating activities of the extract slightly decreased as the temperature increased up to 80 °C and then slightly increased as the temperature increased to 100 ˚C, regardless of the temperature used (P<0.05). However, FRAP slightly increased as the temperature increased (P<0.05). Phenolic compounds with antioxidative activities could be generated while heating. Phenolic compound was more likely stable to heat. Choi *et al*. (2006) reported that after heating, the polyphenolic contents and antioxidant activities in the extracts from Shiitake mushroom increased as heating temperature

and time increased. The free polyphenolic content in Shiitake mushroom (*Lentinus edodes*) extract heated at 121 ˚C for 30 min was increased by 1.9-fold, compared to the extract from the raw sample. ABTS and DPPH radical scavenging activities were increased by 2.0-fold and 2.2-fold, compared to those of extract from the raw sample.

1.2.7.2 Effect of pH

Efficiency of antioxidant is dependent on an environmental factors such as pH of foods, etc. Lee *et al*. (1986) reported that the antioxidant effectiveness of extract from ginger rhizome was dependent on pH. The activity increased when pH increased from 5 to 7. Mansour and Khalil (2000) observed that the antioxidant activity of freeze-dried extracts from ginger rhizomes and fenugreek seeds was maximum at pH 7. For potato peel extract, the maximal activity was observed at pH ranging from 5 to 6, however it was decreased at neutral and alkaline pHs. Arabshahi-D *et al*. (2007) reported that the antioxidant activity of the ethanolic extracts from mint leaves and carrot was higher at pH 9 than pH 4, while that of drumstick extract remained the same under both pH conditions. Ruenroengklin *et al*. (2008) reported that an ethanolic extract from litchi fruit pericarp tissue had a higher antioxidant activity at pH 3-4, than at neutral pH.

1.2.7.3 Effect of plant extracts on prevention of lipid oxidation in different model systems

1.2.7.3.1 Lecithin liposome system

Liposome is phospholipid forming bilayer vesicles with amphiphilic structure (Arseki, 2002). Liposome system is used as a model system of biological membranes for examining the lipid peroxidation, since the highly unsaturated fatty acids membrane lipid are particularly susceptible to oxidation by free radicals (Kaurinovic *et al*., 2011). Antioxidative properties of phenolic compounds generally vary depending on many factors such as the intrinsic property, the molecular size, the location and orientation of the targeted substrate as well as the interactions with other food ingredients (Decker *et al*., 2005; Kathirvel and Richards, 2009). In liposome systems, the polar heads are exposed toward the aqueous phase.

Phenolic compounds and other antioxidative compounds, which are most likely hydrophilic in nature, orient themselves at the bilayers of liposomes and act as effective antioxidants. Antioxidative capacity of phenolic compounds in heterogonous systems is governed by their interaction with the environment, especially their partitioning behavior between the different phases (Frankel *et al*., 1994) and their diffusibility toward reactive centers (Laguerre *et al*., 2007). Benjakul *et al*. (2013) investigated the antioxidant activities of lead seed extract without prior chlorophyll removal and mimosine in a lecithin liposome system. The rate of increase in PV was lower in the system containing 200 ppm mimosine ($P<0.05$). No differences in PV were observed amongst samples added with 100 and 200 ppm of lead seed extract and 100 ppm mimosine ($P>0.05$). The result indicated that lead seed extract and mimosine could inhibit the formation of primary lipid oxidation products. Moreover, samples containing lead seed extract or mimosine at 200 ppm had the lowest increase in TBARS value, compared with other samples ($P<0.05$). Klompong and Benjakul (2015) investigated the antioxidant activity of 60% ethanolic extracts from seed coat of Bambara groundnut (*Voandzeia subterranean*) ($BGSE_{60}$) in lecithin liposome system. $BGSE_{60}$ at 60 mg/l showed the stronger antioxidative activity than the lower concentrations (6 and 30 mg/l) and the control ($P<0.05$) as indicated by the lower conjugated diene and TBARS. However, at the same level (6 mg/l), Trolox showed stronger antioxidative activity than α-tocopherol and $BGSE_{60}$ ($P<0.05$).

1.2.7.3.2 β-carotene-linoleic acid system

β-carotene-linoleic acid is the method measured the ability of an antioxidant to inhibit lipid peroxidation. In this method, a model system made of β-carotene and linoleic acid undergoes a rapid discoloration in the absence of an antioxidant. The free linoleic acid radical formed upon the abstraction of a hydrogen atom from one of its methylene groups attacked the β-carotene molecules. As a result the double bonds and its characteristic orange color are lost (Kanatt *et al*., 2011; Matthäus, 2002). The rate of bleaching of the β-carotene solution was measured by monitoring the absorbance at 470 nm (Juntachote and Berghofer, 2005). Lai and Lim (2011) studied antioxidant activities of the methanolic extracts from selected ferns in Malaysia. Among all samples tested, *D. linearis* extract exhibited the highest β-

carotene bleaching antioxidant activity, compared to the extracts from other ferns tested. Extracts of *A. raddianum*, *C. latebrosa*, *D. quercifolia*, *B. orientale* and *C. barometz* also showed strong DPPH radical scavenging activity, FRAP and inhibition of lipid peroxidation. Extract of *Pteris vittata* and *Pteris venulosa* had the strong chelating activity. The potential antioxidant capabilities of the extracts of *C. latebrosa, C. barometz, D. quercifolia, B. orientale* and *D. linear* were attributed to the phenolic compounds. Benjakul *et al*. (2013) investigated the antioxidant activity of lead seed extracts and mimosine in β-carotene-linoleic acid system. The control showed the highest rate of decrease in A_{470}, indicating the highest oxidation of β-carotene in the system, more likely caused by lipid oxidation. Decrease in A_{470} was effectively retarded when mimosine and lead seed extract at a higher level were incorporated. At the same level of used, mimosine showed more efficiency in lowering the decrease in A_{470}. Thus, mimosine exhibited a higher antioxidant activity than lead seed extracts in the tested system. β-carotene bleaching antioxidative activities of methanolic extracts (2 g/l) of eight *Salvia* species, including *S. aethiopis*, *S. candidissima*, *S. limbata*, *S. microstegia*, *S. nemorosa*, *S. pachystachys*, *S. verticillata*, and *S. virgata*, from Eastern Anatolia in Turkey was tested. Extract of *S. verticillata* showed the highest β-carotene bleaching antioxidant activity (75.8%) as compared to others (P<0.05). However, the antioxidant activity of synthetic BHA was higher than those of all *Salvia* extracts (Tosun *et al*., 2009). Maisarah *et al*. (2013) examined the antioxidative activity of 80 % methanolic extracts from different parts of *Carica papaya* in β-carotene-linoleic acid system. Extract of unripe fruit showed the highest β-carotene bleaching antioxidant activity (90.67%) when compared with the extracts of young leaves, ripe fruit and seed. However, all extracts had the lower antioxidant activities than α-tocopherol. On other hand, extract of young leaves exhibited the higher DPPH radical scavenging activity than other extracts.

1.2.8 Application of natural antioxidants/ plant extracts in food systems

1.2.8.1 Fish, poultry and meat products

Lipid oxidation is one of important reactions, which limits the shelf-life of muscle foods, particularly fish meat rich in PUFA (Chaijan *et al*., 2006; Sohn

et al., 2005) and can initiate the other changes in nutritional quality, color, flavor and texture (Erickson, 1993). Tang *et al*. (2001) reported the higher antioxidant effect of tea catechin in meat, poultry and fish than α-tocopherol at the same concentration (0.3 g/kg) used. Corresponding to the ability of electron donating, caffeic acid showed the most preventive effect on lipid oxidation in minced horse mackerel, compared with other hydroxycinnamic acids and catechins (Medina *et al*., 2007). During low temperature storage, discoloration of tuna meat from red to dark brown proceeds gradually due to oxidation of deoxymyoglobin (deoxyMb) and oxymyoglobin (oxyMb) to metmyoglobin (metMb) (Chaijan *et al*., 2006; Sohn *et al*., 2005). The biological antioxidant, α-tocopherol, has been used both endogenously and exogenously to delay the oxidation of oxyMb and lipid in meat by neutralizing free radicals and terminating free radical chain propagation (Lee *et al*., 2003). In general, antioxidants function by reducing the rate of initiation reaction in the free-radical chain reactions and are used at very low concentrations (Gordon, 2001; Nawar, 1996). Bao *et al*. (2009) investigated the antioxidative effect of mushroom (*Flammulina velutipes*) extract in bigeye tuna (*Thunnus obesus*) meat during ice storage. The addition of 5 ml of mushroom extract to 100 g of minced bigeye tuna meat was more effective than adding ascorbic acid sodium salt (500 ppm) or *α*-tocopherol (500 ppm) with regard to oxidation of lipid in the tuna meat. The color changes were significantly correlated with lipid oxidation as well as metmyoglobin formation in the tuna meat. Therefore, the mushroom extract is a potential antioxidant, which has the ability to prevent lipid oxidation and stabilize fresh color of tuna meat during ice storage. Thiansilakul *et al*. (2013) investigated the effect of phenolic compounds in combination with modified atmospheric packaging on the shelf-life of tuna slices. It was found that tannic acid exhibited a greater preventive effect on metmyoglobin (metMb) formation and lipid oxidation than did caffeic acid. Samples treated with tannic acid and kept under MAP (MT) had the lowest lipid oxidation. After 12 days of storage, changes in unsaturated fatty acids, especially n-3 fatty acids, were lower in MT, compared with tuna slices stored in air. Based on microbiological quality, the shelf-life of tuna kept in air and MAP was estimated to be 6 and 12 days, respectively, regardless phenolic compounds treatment. Therefore, tannic acid exhibited a combined effect with MAP on inhibition of metMb formation, lipid oxidation and

microbial growth, thereby improving the acceptance and increasing the shelf-life of tuna slices during refrigerated storage. The addition of tea catechins at 100 ppm reduced lipid oxidation in chicken nuggets both in the presence and absence of salt. However, when sodium tripolyphosphate (STPP) was incorporated into the same product system, the efficacy of natural extracts was significantly reduced (O'Sullivan *et al*., 2004). In addition, green tea extract can also help improve the color stability of fresh meat products. Moawad *et al*. (2012) reported that the use of tea catechins extract (at 300 ppm) in combination with nitrite was more effective in keeping quality of dry-cured sausages during refrigerated storage as indicated by more attractive red color (a* value) as compared to samples treated with nitrite. Jongberg *et al*. (2013) reported that the addition of the extracts of green tea (500 ppm) and rosemary (400 ppm) in Bologna type sausages prepared from oxidatively stressed meat reduced the TBARS values by ~ 80% or 73%, respectively as compared to the control (without extracts). Bologna type sausages added with either green tea or rosemary extract provided lower values for old smell after storage up to 2 weeks. Bologna type sausages added with green tea extract showed less rancidity after 4 weeks of storage than the two other sausages.

1.2.8.2 Food emulsion and oils

Oil-in-water emulsions such as milk, infant formula, salad dressing, mayonnaise, sauces, soups, beverages, cream, and some desserts are one of the most common forms of lipids in foods (McClements, 2005). Lipid oxidation can occur rapidly in oil-in-water emulsions due to their large surface area that facilitates interactions between the lipids and water-soluble prooxidants. There are many factors that can potentially influence the rate of lipid oxidation in oil-in-water emulsions. Those include fatty acid composition, aqueous phase pH and ionic composition, type and concentration of antioxidants and prooxidants, and oxygen concentration. Furthermore, lipid droplet characteristics such as particle size, concentration and physical state and emulsion droplet interfacial properties such as thickness, charge, rheology, and permeability and also determine the rate of lipid oxidation (Waraho *et al*., 2011). To alleviate lipid oxidation in emulsion, antioxidants have been used. Natural extracts are of interest to prevent lipid oxidation. Gökoğlu *et al*. (2012)

reported that tomato and garlic extracts showed good inhibitory activity against lipid oxidation in anchovy oil (*Engraulis encrasicholus*) enriched emulsions during refrigerated storage. The value of conjugated dienes (3.07±0.15) was markedly low in the emulsions containing garlic extract at the end of the storage period. The emulsions treated with the extracts had lower *p*-Av values than untreated samples ($P<0.01$). Pourashouri *et al*. (2014) showed the fish oil-in-water emulsion containing the rosemary extracts at the levels of 500 and 700 ppm had lower oxidative stability than that containing the wild pistachio and green tea extracts at the same levels during storage at 30 ˚C for 12 days. Additionally, the efficiency of inhibiting oxidation was also depended on type and concentration of plant extract used. Different behaviors of antioxidants, mainly due to the concentrations and their physical locations in phases, could affect their effectiveness in food system. Kishk and Elsheshetawy (2013) investigated the role of ginger powder on oxidative stability of mayonnaise. Ginger powder at concentrations of 1.0% and 1.25% reduced the production of primary and secondary oxidation products (measured by anisidine value) and subsequently retarded oxidation process during storage for 20 weeks. Ginger contains polyphenol compounds such as 6-gingerol and its derivatives, which have a high antioxidant activity (Chen *et al*., 1986). The rheological properties of mayonnaise were not influenced by ginger powder. Interestingly, ginger powder improved the taste, flavor, mouth feel, and overall acceptability of mayonnaise at zero time, and after 20 weeks the overall acceptability of mayonnaise samples at concentration of 1.0% and 1.25% was still high. Altunkaya *et al*. (2013) reported that grape seed extract at concentration of 0.5 mg/ml showed antioxidative activity in mayonnaise during storage for 8 weeks when compared to the control sample (without grape seed extract). Grape seed contained catechin and proanthocyanidins, which have radical scavenging and antioxidative properties. Lagunes-Galvez *et al*. (2002) reported that the addition of rosemary extract to sunflower oil mayonnaise decreased the level of volatile compounds formed from photooxidation in the headspace. Rosemary extracts have a chelating effect in sunflower oil mayonnaise. Also, the antioxidative effect of dried rosemary at a concentration of 1% was reported in fish oil enriched tuna salad (Sørensen *et al*., 2010). Although rosemary inhibited formation of peroxide and showed antioxidative effect, it might cause undesirable taste in tuna salad (Sørensen

et al., 2010). Hermund *et al*. (2015) investigated the antioxidant properties of water extract (WE) and an ethyl acetate fraction (EAF) of *Fucus vesiculosus* on oxidative stability of fish oil mayonnaise during storage of 28 days at 20˚C. WE at the level of 2 g/kg had ability in lowering the formation of primary oxidation products and secondary oxidation products, while EAF was more efficient in decreasing the decomposition of the n-3 PUFA. However, *Fucus vesiculosus* extracted using ethanol and acetone had higher antioxidant efficiency, compared to water extracts as reported by Honold *et al*. (2015).

Cooking oil is also prone to oxidation, especially during or after cooking. Hydrolysis of oil induced by water in foods can accelerate the hydrolysis and oxidation of cooking oil (Iqbal and Bhanger, 2007). Chammem *et al*. (2015) reported that the addition of the ethanolic extract from rosemary (at 0.08% w/v) in the mixture of soybean and sunflower oil of fried potatoes was able to reduce the peroxide value by 38% after 30 h of heating at 180 ˚C. Based on sensorial evaluation, the fried potato with rosemary extract had the higher crispiness and taste likeness as compared to the oil without extract. The antioxidative properties of rosemary was attributed to the presence of phenolic diterpene compounds, such as carnosic acid, carnosol, rosmanol, rosmariquinone and rosmaridiphenol, which break free radical chain reactions by hydrogen atom donation and chelating metal ions (Erkan *et al*., 2008). Pomegranate peel extracts obtained from *Nayana*, *Daya* and *Nimali* varieties (2%, w/w) exhibited strong antioxidant activity in preventing oxidation of white coconut oil during frying at 180 ± 5 °C for 10 min (Bopitiya and Madhujith, 2014).

1.2.9 References

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1.3 Objectives

1.3.1 To optimize the extraction of antioxidative phenolic compounds from cashew (*Anacardium occidentale* L.) leaves using response surface methodology (RSM).

1.3.2 To study the optimization of ultrasound-assisted extraction of antioxidative phenolic compounds from cashew leaves using RSM.

1.3.3 To characterize and examine the antioxidative activity of cashew leaf extract.

1.3.4 To investigate the use of cashew leaves extract for prevention of lipid oxidation in fish oil enriched mayonnaise during storage.

CHAPTER 2

OPTIMIZATION OF EXTRACTION OF ANTIOXIDATIVE PHENOLIC COMPOUNDS FROM CASHEW (*ANACARDIUM OCCIDENTALE* L.) LEAVES USING RESPONSE SURFACE METHODOLOGY

2.1 Abstract

Optimization of extraction of antioxidative phenolic compounds from cashew (*Anacardium occidentale* L.) leaves was performed using response surface methodology (RSM). The central composite design (CCD) was used to establish treatments based on three independent variables, including extraction temperature, time and ethanol-to-solid ratio. Total phenolic content (TPC), antioxidative activities (DPPH, ABTS radical scavenging activities and FRAP) and % yield were monitored. The extraction yield was 8.64% under the following optimized condition: extraction at 34.7˚C for 64 min with an ethanol-to-solid ratio of 18:1 v/w. TPC was 564.60 mg GAE/g dry extract and DPPH, ABTS radical scavenging activities and FRAP were 11.74, 5.56 and 8.11 mmol TE/g dry extract, respectively. The experiment values were in accordance with the predicted values. Isoquercetin, catechin, hydroquinin, gallic acid, tannic acid and rutin were found in the extract. The extract could therefore be used as natural antioxidant for food application or as nutraceutical.

2.2 Introduction

Phenolic compounds are generally found in plants and exhibit various biological properties such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Puupponen-Pimia¨ *et al*., 2001). Phenolic compounds, the secondary metabolites of plants, have the aromatic ring bearing one or more hydroxyl substituents (Naczk and Shahidi, 2004). They play a role as reducing agents, hydrogen donators and singlet oxygen quenchers (Proestos *et al*., 2006).

Nowadays, plant extracts are of increasing interest in the food industry because they retard the oxidative degradation of lipids, thereby improving the quality and nutritional value of food as well as prevent the occurrence of disease (Jianga and Xiong, 2016). Plant extracts have been known to exhibit antioxidant activity, related with their protection of the human body from cancer and heart disease (Scalbert *et al*., 2005). Cashew trees are abundant in the tropical area, especially the southern part of Thailand. Cashew nut has been popular for consumer and its young leaves have been consumed as fresh in some region of America and Asean countries. It contained a large amount of phytochemicals, especially phenolic with antioxidative activity (Kamath and Rajini, 2007). Additionally, cashew leaf extracts had antimicrobial activity against *Escherichia coli, Shigella dysenteriae, Salmonella* Typhimurium, *Stapphylococcus aureus* and *Pseudomonas auroginosa* (Omojasola and Awe, 2004). However, a little information on extraction and antioxidative activity of cashew leaf extract has been reported.

The extraction method is the first important steps in isolation of interested compound, in which the target compounds are extracted with high recovery and without interferences (Dobiás *et al*., 2010). In general, several parameters influence the extraction efficacy of phenolics. Those included temperature, sample-to-solvent ratio, time, polarity of solvent, etc. (Dai and Mumper, 2010). Extraction can be optimized by either empirical or statistical methods. Optimization is essential for commercial extraction of the target compounds. To optimize biochemical and biotechnological processes related to food systems, response surface methodology (RSM) has been widely implemented (Kaith *et al*., 2014). Thus, the present study aimed to investigate the extraction of phenolic from cashew leaves using RSM and to study antioxidative activity of the extracts.

2.3 Materials and methods

2.3.1 Chemicals

Ethanol, ethyl acetate and hydrochloric acid were procured Merck (Darmstadt, Germany). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Aldrich Chemical Company (Steinheim, Germany).

2,4,6-Tri (2-pyridyl)-S-triazine (TPTZ) was obtained from Fluka Chemicals (Buchs, Switzerland). Gallic acid, 2,2-diphenyl-1-picryhydrazyl (DPPH), ferrous sulfate ($FeSO_4 \cdot 7H_2O$), ferric chloride ($FeCl_3 \cdot 6H_2O$), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and potassium persulfate were purchased from Sigma-Aldrich, Inc (St. Louis, MO, USA). Phenolic standards (gallic acid, catechin, isoquercetin, tannic acid, rutin, hydroquinin, eriodictyol and quercetin) were procured from Sigma-Aldrich, Inc. (Chemie GmbH. Steinheim, Germany).

2.3.2 Collection of cashew leaves

Cashew (*Anacardium occidentale*, L.) leaves were collected from an orchard in Songkhla province, Thailand during May and June 2016. The trees were approximately 15-20 years old. Leaves from III (apical) to V (basal) of twig were used in the study. Cashew leaves were prepared as per the method of V´azquez-Torres *et al*. (1992) with slight modifications. The samples were washed with tap water and dried overnight in an air dryer at 50˚C until the moisture content was less than 10%. Dried samples were blended using a blender (Panasonic, Model MX-898N, Berkshire, UK) and sieved through a stainless steel (sieve 80 mesh). Thereafter, chlorophyll was removed from the obtained powder. The sample powder (50 g) was stirred for 30 min with chloroform (1l) and the mixture was filtered using Whatman filter paper No.1 (Whatman International Ltd., Maidstone, UK) (Vovk *et al*., 2005; Row and Jin, 2006). The retentate was dried in a hot air oven (Memmert, Schwabach, Germany) at 105˚C for 1 h. The obtained powder termed ‘cashew leaf powder’ was placed in a polyethylene bag and kept at room temperature until used for extraction.

2.3.3 Preparation of cashew leaf extracts

Firstly, cashew leaf powder was subjected to extraction using ethanol at different concentrations (0, 40, 60, 80 and 100%). The dried powder was mixed with different solvents using a powder/solvent ratio of 1:15 (w/v) for 1 h, followed by centrifugation at 5000xg for 30 min at 27˚C using a RC-5B plus centrifuge (Beckman, JE-AVANTI, Fullerton, CA, USA) and the supernatants were then filtered through a Whatman filter paper No.1 (Jan *et al*., 2013). The filtrates were evaporated at 40˚C by an EYELA rotary evaporator (Tokyo Rikakikai, Co. Ltd., Tokyo, Japan). The residual

ethanol was removed by nitrogen purging. The extracts were subsequently dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). Dried extracts were kept in a desiccator prior to analysis. All extracts were determined for total phenolic content (TPC) and antioxidative activity. Solvent yielding the extract with high TPC and antioxidative activity was selected for further study.

2.3.4 Analyses

2.3.4.1 Extraction yield

Extraction yield (%) of cashew leaf extracts were determined and defined as dry weight of extract, compared to the initial dry weight of raw material. Extraction yield was calculated as follows:

$$\text{Extraction yield (\%)} = \frac{\text{Dry weight of obtained extract (g) x 100}}{\text{Dry weight of initial leaf (g)}}$$

2.3.4.2 Determination of total phenolic content (TPC)

TPC was determined using the Folin-Ciocalteau reagent as described by Sato *et al*. (1996) with a slight modification. The extracts were dissolved in distilled water to obtain a final concentration of 0.2 mg/ml. An aliquot of 100 µl was mixed with 0.75 ml of Folin-Ciocalteu reagent. After 3 min, 0.75 ml of 6% (w/v) sodium carbonate was added. The mixture was allowed to stand for 1 h at room temperature. The absorbance at 760 nm was measured by UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Gallic acid (0-500 mg/ml) was used as the standard. The total phenolic content was expressed as mg gallic acid equivalent (GAE) / g dry extract.

2.3.4.3 Determination of antioxidative activities

2.3.4.3.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Wu *et al.* (2003) with a slight modification. Sample (1 ml) was added with 1 ml of 0.15 mM 2,2-diphenyl-1-picryl hydrazyl (DPPH) in 95% ethanol. The mixture was

vigorously and allowed to stand at room temperature in dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. Sample blank was prepared in the same manner except that 95% ethanol was used instead of DPPH solution. Trolox (10-60 μM) was used as the standard. The activity was expressed as mmol Trolox equivalents (TE)/g dry extract.

2.3.4.3.2 ABTS radical scavenging activity

ABTS radical scavenging activity was measured as per the method of Arnao *et al*. (2001) with a slight modification. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities. The mixture was left for 12 h at room temperature in dark. The solution was then diluted by mixing 1 ml of ABTS solution with 50 ml of methanol in order to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a spectrophotometer. ABTS solution was freshly prepared for each assay. Sample (150 μl) was mixed with 2850 μl of ABTS solution and the mixture was allowed to stand at room temperature for 2 h in dark. The absorbance was then measured at 734 nm. Sample blank was prepared in the same manner except that methanol was used instead of ABTS solution. Trolox (0-500 μM) was used as the standard. The activity was expressed as mmol Trolox equivalents (TE)/g dry extract.

2.3.4.3.3 Oxygen radical absorbance capacity assay (ORAC assay)

The ORAC assay was determined as described by Kittiphattanabawon *et al*. (2012) with a slight modification. Prior to assay, the concentration of samples was adjusted to 0.075 mg/ml with 75 μM phosphate buffer (pH 7.0). Sample (20 μl) was loaded onto a black polystyrene, non-steriled 96-well microplate (Nunc, Denmark), followed by addition of 50 μl fluorescein (0.11 μM). The loaded microplate was equilibrated at 37˚C for 20 min in a microplate reader (FLUOstar Omega, BMG Labtech Multi-mode microplate reader, Ortenberg, Germany) Hundred microliter of AAPH (60 mM) was added to initiate reaction at 37˚C. The fluorescence intensity was measured every 5 min for 90 min with

excitation and emission filters of 485 and 535 nm, respectively. The area under the fluorescence decay curve (AUC) of the samples was calculated by the normalized curves with the following equation:

$$AUC = 0.5 + (f_2/f_1) + (f_3/f_1) + (f_4/f_1) +...+ 0.5\ (f_n/f_1)$$

where f_1 and f_n are the fluorescence reading at the initiation and the last measurements of the reaction, respectively. The net AUC was calculated by subtracting the AUC of the blank from that of a sample or standard. The standard used was Trolox (0-200 μM). The ORAC was expressed as mmol Trolox equivalents (TE)/g dry extract.

2.3.4.3.4 Ferric reducing antioxidant power (FRAP)

FRAP assay was determined according to the method of Benzie and Strain (1996). Stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. A working solution was prepared freshly by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 mL of $FeCl_3 \cdot 6\ H_2O$ solution. The mixed solution was incubated at 37 ºC for 30 min in a water bath (Memmert, D-91126, Schwabach, Germany), referred to as FRAP solution. A sample (150 μl) was mixed with 2850 μl of FRAP solution and kept for 30 min in dark at room temperature. The ferrous tripyridyltriazine complex (colored product) was measured by reading the absorbance at 593 nm. Sample blank was prepared by omitting $FeCl_3$ from FRAP solution and distilled water was used instead. Trolox (0-500 μM) was used as the standard. The activity was expressed as mmol Trolox equivalents (TE)/g dry extract.

2.3.4.3.5 Ferrous ion chelating activity

Ferrous ion chelating activity was measured by the method of Thiansilakul *et al.* (2007). Diluted sample (1 ml) was mixed with 20 μl of 2 mM $FeCl_2$ and 40 μl of 5 mM ferrozine. After 20 min of reaction at room temperature, the absorbance was then read at 562 nm. EDTA with the concentration range of 0-50 μM was used as the standard. Ferrous ion chelating activity was expressed as mmol EDTA equivalents (EE)/g dry extract.

2.3.5 Optimization of extraction of antioxidative phenolic compounds

The selected solvent (80% ethanol) was used for extraction of phenolic compounds from cashew leaves. Response surface methodology (RSM) and central composite design (CCD) were used. The experimental plan consisted of 17 runs with three replicates at the center point. The independent variables studied included the extraction temperature (X_1, ˚C), time (X_2, min) and ethanol-to-solid ratio (X_3) (v/w), in which each was assessed at five coded levels. The central values of all variables were coded as zero. The levels of variables used were obtained from preliminary study, in which extraction temperature above 60˚C resulted in the decrease in TPC and antioxidative activity. The use of extraction longer than 230 min had no effect on increase extraction efficiency. For ethanol-to-solid ratio, the ranges of 10:1 to 35: 1 have been widely used. Both actual and coded forms of the level of variables are present in Table 3. The response values (Y) in each trial were the average of the triplicates. Response surface methodology (RSM) was applied to the experimental data using the Design-Expert Statistical package version 7.0 (Statease, Inc Minneapolis, Minn, USA). The trial data were fitted by a second-order polynomial model and the regression coefficients were obtained by multiple linear regression.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=2}^{3} \beta_{ij} X_i X_j$$

where Y is the dependent variables (%yield, TPC, DPPH, ABTS radical scavenging activities and FRAP); X_i and X_j are the coded forms of the independent variables; β_0, β_i, β_{ii} and β_{ij} are constant, linear coefficient, quadratic coefficient and interaction coefficient of the model, respectively. The fitted polynomial equations were expressed in the form of contour plots, to illustrate the interaction between dependent variables and the independent counterparts. The optimum condition was determined by superimposing maximum areas of all responses from all combinations of independent variables using Design-Expert.

2.3.6 Verification of the optimum condition

All the responses were determined under the optimized condition of the extraction. The experimental errors for the models were determined by comparing observed values with the predicted values as follows:

Error (%) = [(Observed value - predicted value) / Observed value] x 100

Table 3. Central composite design for extraction of antioxidative cashew leaf extracts

Run order	Coded levels			Actual levels		
	Temperature (X_1)	Time (X_2)	Ethanol-to-solid ratio (X_3)	Temperature (˚C)	Time (min)	Ethanol-to-solid ratio (v/w)
1	-1	-1	-1	30	60	15
2	1	-1	-1	50	60	15
3	-1	1	-1	30	180	15
4	1	1	-1	50	180	15
5	-1	-1	1	30	60	35
6	1	1	1	50	60	35
7	-1	1	1	30	180	35
8	1	1	1	50	180	35
9	-1.68	0	0	23.18	120	25
10	1.68	0	0	56.82	120	25
11	0	-1.68	0	40	19.09	25
12	0	1.68	0	40	220.91	25
13	0	0	-1.68	40	120	8.18
14	0	0	1.68	40	120	41.82
15	0	0	0	40	120	25
16	0	0	0	40	120	25
17	0	0	0	40	120	25

2.3.7 LC/DAD/MSD analysis of phenolic compounds

LC/DAD/MSD analysis was determined according to the method of Peñarrieta *et al*. (2007). Phenolic compounds in cashew leaf extract obtained from the

optimized extracting condition was analyzed using a LC/DAD/MSD equipped with a diode array detector (DAD) and a scan mode 100-700 m/z MS detector. Sample was firstly separated on LiChroCART® Purospher® STAR RP-18e column (Merck, USA) (150 x 4.6 mm, i.d., 5 µm) with an Agilent 1100 series (Agilent Technologies, Waldbronn, Germany). Acetonitrile and 10 mM ammonium formate buffer pH 4 with formic acid were used as mobile phase A and mobile phase B, respectively (flow rate: 1.0 ml/min; temperature: 40˚C). The gradient program was as follows: 100% B constant (0-5 min), 0-20% A (5-10 min), 20% A constant (10-20 min), and 20-40% A (20-60 min). The detection was carried out at 270, 330, 350 and 370 nm. MS detection was carried out in the positive ionization mode, where an electrospray ionizing source with nitrogen as drying gas was used. The conditions used were as follows: the capillary voltage: 4000 V; gas temperature: 320˚C; drying gas flow: 13 l/min; nebulizer pressure 60 psi. Quantitative analysis by MSD was done in the SIM (Selected Ion Monitoring) mode. Standards were as follows: 188, 209 m/z for gallic acid; 185, 329, 503, 649 m/z for catechin, isoquercetin, tannic acid and rutin; 289, 327, 341 m/z for hydroquinin, eriodictyol and quercetin ().

2.3.8 Statistical analysis

All experiments were carried out in triplicate. The experimental data were expressed as means ± standard deviation (n = 3). For the study on solvent extraction, the data were subjected to analysis of variance (ANOVA). Comparisons of means were carried out by the Duncan's multiple range test. Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA). For optimization of extraction, CCD was used as mentioned above.

2.4 Results and discussion

2.4.1 Effect of different solvents on extraction and antioxidative activity of phenolic compounds from cashew leaves

Extraction yield, total phenolic contents (TPC) and antioxidative activities of all extracts using different solvents as extracting media are shown in

Table 4. Among all solvents used, 80% ethanol showed the highest yield, TPC, and highest DPPH radical scavenging activity and metal chelating activity, compared with other solvents (P<0.05). It was noticed that the extract prepared using 80% ethanol had no differences in ABTS radical scavenging activity and FRAP, compared with that extracted using 60%. Additionally, no difference in ORAC was observed among all extracts using ethanol with concentrations ranging from 40 to 100% (P>0.05). Degree of polymerization of phenolics, the interaction between phenolics and other plant constituents, as well as polarity of solvent influence the solubility of phenolic compounds (Cvetanovic *et al*., 2014). Ethanol is a low polar solvent, whereas water is a strong polar solvent. Ethanol at an appropriate concentration could extract phenolics with antioxidative activity to a high extent. With the addition of water to ethanol, the polarity of the mixed solvent increased continuously (Zhang *et al*., 2007).

All extracts had ability of providing proton as well as electron, in which the propagated reaction could be terminated. Also, extracts were able to chelate the metal, known as prooxidant. However, the activities were found to vary, depending on the types of solvents used. DPPH radical scavenging activity is used to determine the antioxidant activity in hydrophobic system, while ABTS radical scavenging activity is used to determine the antioxidant activity both hydrophilic and lipophilic system (Neelamegam and Valantina, 2015). ORAC assay is generally used to measure the antioxidant capacity in the inhibition of peroxyl radicals generated by azo-compounds such as AAPH both in hydrophilic and lipophilic systems (Huang *et al*., 2002). FRAP is used to determine the ability of compound in reducing TPTZ-Fe (III) complex to TPTZ-Fe (II) complex (Benzie and Strain, 1996). The chelating activity method is based on ability of tested samples in chelating or deactivating transition metals by inhibiting Ferrozine-Fe^{2+} complex formation (Sofidiya and Familoni, 2012). With the highest TPC and antioxidative activity, especially DPPH radical scavenging activity and metal chelating activity, along with non-significantly higher FRAP and ORAC of the extract using 80% ethanol, compared with other extracts, 80% ethanol was selected as extracting medium for further study.

Table 4. Total phenolic contents and antioxidative activities of cashew leaf extracts using different solvents

Treatments	Extraction yield (%)	Total phenolic content (mg GAE/g dry extract)	DPPH radical scavenging activity (mmol TE/g dry extract)	ABTS radical scavenging activity (mmol TE/g dry extract)	FRAP (mmol TE/g dry extract)	ORAC (mmol TE/g dry extract)	Metal chelating activity (mmol EDTA/g dry extract)
water	7.11 ± 0.16^{c}	350.02 ± 8.99^{d}	7.47 ± 0.31^{c}	5.66 ± 0.04^{c}	7.53 ± 0.41^{c}	0.02 ± 0.01^{b}	0.01 ± 0.00^{d}
E_{40}	7.89 ± 0.12^{b}	487.97 ± 10.42^{b}	9.50 ± 0.50^{b}	5.88 ± 0.05^{a}	8.89 ± 0.38^{b}	0.03 ± 0.01^{ab}	0.01 ± 0.00^{c}
E_{60}	8.15 ± 0.25^{b}	443.58 ± 16.80^{c}	9.88 ± 0.88^{b}	5.89 ± 0.07^{a}	9.07 ± 0.26^{ab}	0.03 ± 0.01^{ab}	0.02 ± 0.00^{b}
E_{80}	10.71 ± 0.17^{a}	518.89 ± 0.71^{a}	12.17 ± 0.55^{a}	5.88 ± 0.02^{a}	9.53 ± 0.12^{a}	0.04 ± 0.01^{a}	0.03 ± 0.00^{a}
E_{100}	7.38 ± 0.11^{c}	495.20 ± 14.42^{b}	9.56 ± 0.27^{b}	5.77 ± 0.05^{b}	8.91 ± 0.28^{b}	0.03 ± 0.00^{ab}	0.01 ± 0.00^{c}

Values are mean ± SD (n=3).

Different lowercase superscripts in the same column indicate significant difference ($P<0.05$)

2.4.2 Optimization of extraction of antioxidative phenolic compounds from cashew leaf powder

Based on CCD experiment, 17 treatments with different three experimental variables including extraction temperature (X_1), time (X_2) and ethanol-to-solid ratio (X_3) are presented in Table 3. The experimental data, % extraction yield, TPC, DPPH, ABTS radical scavenging activity and FRAP fitted with the second order polynomial equation with coefficients of analysis (R^2) of 0.9700, 0.9899, 0.9847, 0.9891 and 0.9903, respectively (Table 5).

Table 5. Regression coefficients of the predicted second order polynomial models for total phenolic contents, antioxidative activities and yield

Regression coefficients (β)	**% Yield**	**TPC**	**DPPH radical scavenging activity**	**ABTS radical scavenging activity**	**FRAP**
Intercept					
β_0	6.82	555.02	13.67	5.72	8.53
Linear					
β_1	-0.94	-8.93	-0.08	-0.015	-0.22
β_2	-0.23	-20.48	-0.49	-0.10	-0.25
β_3	-0.84	-10.53	0.03	-0.046	-0.03
Cross product					
β_{12}	-0.55	-1.95	-0.39	0.024	-0.14
β_{13}	-0.18	-20.76	-0.39	0.076	-0.06
β_{23}	0.33	5.90	-0.24	0.024	0.03
Quadratic					
β_{11}		-21.27	-0.33	0.002	-0.45
β_{22}		-11.10	-0.46	-0.08	-0.31
β_{23}		-14.59	-0.87	0.032	-0.30
R^2	0.9700	0.9899	0.9847	0.9891	0.9903
Lack of fit	0.0978	0.3022	0.4361	0.1687	0.2190
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

The ANOVA result was used to indicate that at least one of the parameters of the model could explain the experimental variation for response variables as shown by significant *P*-value for the model. Non-significant lack of fit also revealed the quality of the fitted models.

2.4.3 Effect of independent variables on extraction yield

Different extraction conditions showed varying yields (Table 6). The extraction yield obtained ranged from 4.54 to 8.71%. The extraction yield was highly influenced by linear term of temperature (X_1) and ethanol-to-solid ratio (X_3) (P<0.0001). Interaction between temperature and time (X_1X_2) was also found (P<0.0001). The results indicated that all factors had significant negative effect on yield (Figure 4A-C). An increase of variable values did not increase the extraction yield. The highest yield was found at temperature of 30˚C, time of 180 min and ethanol-to-solid ratio of 15:1 (v/w). A decrease in yield was noticeable when temperature, time and ratio were above 40˚C, 120 min and 25:1 (v/w), respectively. The R^2 value of the predicted model in yield value was 0.9700 (Table 5). Moreover, *P*-value of the model (<0.0001) and the non-significant lack of fit (0.0978) showed that the model fitted with good prediction. Effect of independent variables on extraction yield of cashew leaf extracts was shown as follows:

$$Y = 6.82 - 0.94X_1 - 0.23X_2 - 0.84X_3 - 0.55X_1X_2 - 0.18X_1X_3 + 0.33X_2X_3$$

where Y is the predicted extraction yield, X_1 is extraction temperature, X_2 is time, X_3 ethanol-to-solid ratio.

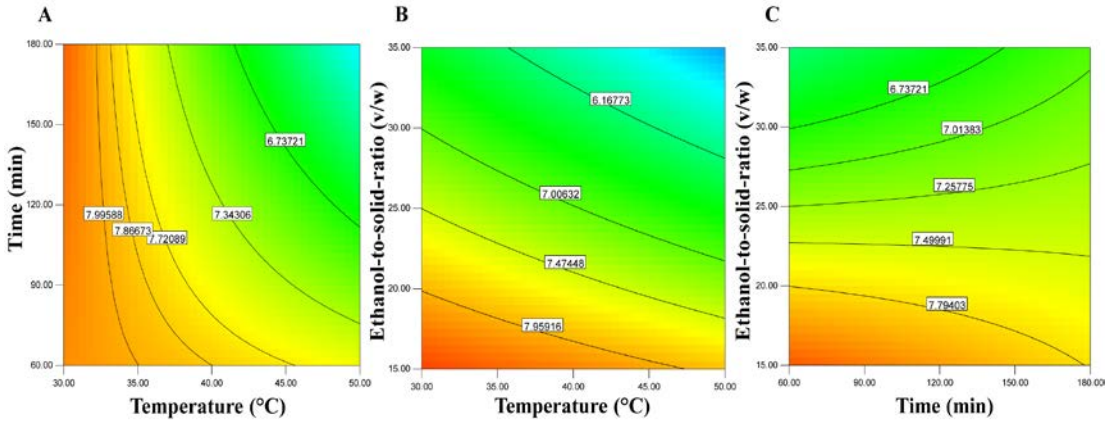

Figure 4 The contour plots of extraction yield of cashew leaf extracts as affected by the selected independent variables.

Table 6. Experimental data for total phenolic compound (TPC), antioxidative activities and yield of cashew leaf extracts under different extraction conditions

Run order	Yield (%)	TPC (mg GAE/ g dry extract)	DPPH radical scavenging activity (mmol TE/g dry extract)	ABTS radical scavenging activity (mmol TE/g dry extract)	FRAP (mmol TE/g dry extract)
1	8.46 ± 0.24	534.08 ± 0.25	11.68 ± 0.57	5.92 ± 0.17	7.78 ± 0.67
2	8.01 ± 0.57	560.20 ± 2.18	13.06 ± 0.36	5.68 ± 0.07	7.74 ± 0.52
3	8.71 ± 0.24	487.86 ± 1.14	11.78 ± 0.04	5.73 ± 0.20	7.58 ± 0.11
4	5.71 ± 0.14	503.98 ± 5.42	11.62 ± 0.12	5.57 ± 0.19	6.96 ± 0.24
5	6.72 ± 0.27	539.08 ± 6.42	12.90 ± 0.17	5.74 ± 0.21	7.74 ± 0.61
6	5.17 ± 0.31	479.99 ± 1.36	12.74 ± 0.20	5.79 ± 0.32	7.42 ± 0.07
7	7.91 ± 0.21	514.26 ± 5.42	12.04 ± 0.67	5.44 ± 0.05	7.62 ± 0.22
8	4.54 ± 0.23	449.53 ± 0.86	10.34 ± 0.23	5.60 ± 0.12	6.80 ± 0.04
9	7.85 ± 0.11	506.10 ± 1.94	12.86 ± 0.57	5.72 ± 0.10	7.64 ± 0.31
10	5.23 ± 0.20	482.10 ± 1.55	12.60 ± 0.65	5.71 ± 0.03	6.90 ± 0.05
11	7.34 ± 0.04	559.14 ± 2.21	12.96 ± 0.41	5.66 ± 0.01	8.18 ± 0.02
12	6.32 ± 0.16	486.59 ± 5.58	11.74 ± 0.06	5.31 ± 0.06	7.18 ± 0.23
13	8.19 ± 0.03	525.03 ± 1.18	11.04 ± 0.13	5.89 ± 0.08	7.68 ± 0.18
14	5.28 ± 0.43	500.96 ± 1.32	11.34 ± 0.15	5.71 ± 0.12	7.70 ± 0.04
15	6.85 ± 0.06	556.95 ± 0.15	13.54 ± 0.23	5.73 ± 0.15	8.50 ± 0.10
16	6.87 ± 0.21	551.05 ± 0.91	13.62 ± 0.79	5.71 ± 0.06	8.58 ± 0.04
17	6.71 ± 0.08	557.34 ± 0.61	13.84 ± 0.32	5.73 ± 0.21	8.50 ± 0.01

Values are mean ± SD (n=3).

2.4.4 Effect of independent variables on total phenolic contents (TPC)

Effects of different extraction conditions on TPC of cashew leaf extracts varied from 449.53 to 559.14 mg GAE/g dry extract (Table 6). Among all variables, TPC were highly influenced by linear term of time (X_2), quadratic term of extraction temperature (X_1^2), ethanol-to-solid ratio (X_3^2) as well as interaction term between extraction temperature and ethanol-to-solid ratio (X_1X_3) (P<0.0001). Effect of independent variables on TPC of cashew leaf extracts was shown as follows:

$Y = 555.02 - 8.93X_1 - 20.48X_2 - 10.53X_3 - 1.95X_1X_2 - 20.76X_1X_3 + 5.90X_2X_3 - 21.27X_1^2 - 11.10X_2^2 - 14.59X_3^2$

where Y is the predicted TPC, X_1 is extraction temperature, X_2 is time, X_3 ethanol-to-solid ratio.

Relationships between different variables in term of contour plot are shown in Figure 5A-C. The results indicated that TPC increased as the function of temperature, time and ethanol-to-solid ratio. TPC of cashew leaf extracts increased when temperature increased from 35 to 40˚C and time increased from 60 to 90 min. The increase of ethanol-to-solid ratio from 15:1 to 25:1 (v/w) also increased TPC of the extract. The decrease in TPC was observed as the independent variables were above the optimal values, 40˚C, 120 min and 25:1 (v/w). These results clearly indicated that three factors had the impact on TPC of resulting extracts. Increasing temperature more likely promoted extraction efficiency by enhancing both solubility and diffusion coefficients of phenolic compounds (Silva *et al.*, 2007). Increasing extraction temperature enhanced the release of bound polyphenols in a sample via disruption of cellular constituents of plant cells (Wang *et al.*, 2008). Furthermore, extraction time also had significant effect on the extraction of TPC (P<0.0001). From Figure 5A and 5C, it was obvious that higher TPC was obtained with a shorter time of extraction. Longer time might enhance the degradation of phenolic compounds to a higher extent. Naczk and Shahidi (2004) found that excess extraction time could reduce the total phenolic content and also increased oxidation of phenolics unless reducing agents were added to solvent system.

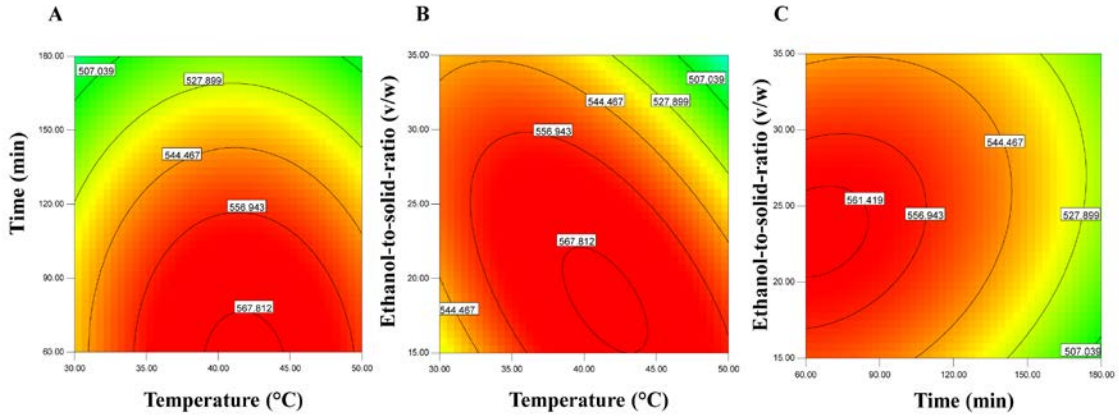

Figure 5 The contour plots of TPC of cashew leaf extracts as affected by the selected independent variables.

The regression equation obtained from analysis of variance (ANOVA) with the R^2 value of 0.9899 (Table 5) revealed that the model was able to explain 98.99% variation in the response, whereas *P*-value of the model (<0.0001) and non-significant lack of fit (0.3022) indicated that the obtained experimental data could fit well with the model.

2.4.5 Effect of independent variables on antioxidant activities

Most of term of variables (linear, quadratic and interaction) significantly affected antioxidative activities tested by all assays (P<0.05). Nevertheless, the linear terms of DPPH radical scavenging activity (X_1 and X_3) and FRAP (X_1) were not significantly affected (P>0.05). The effect of interactive terms of DPPH and ABTS radical scavenging activities (except X_1X_2 and X_2X_3 in the case of FRAP) was significant (P<0.05). Quadratic term of ABTS radical scavenging activity (X_1^2) was not significant (P>0.05), whereas other terms were shown to be significant (P<0.01). Effect of extraction temperature, time and ethanol-to-solid ratio on DPPH and ABTS radical scavenging activity as well as FRAP of cashew leaf extracts are shown with the following equations:

$$Y_{DPPH} = 13.67 - 0.079X_1 - 0.49X_2 + 0.03X_3 - 0.39X_1X_2 - 0.39X_1X_3 - 0.24X_2X_3 - 0.33X_1^2 - 0.46X_2^2 - 0.87X_3^2$$

$$Y_{ABTS} = 5.72 - 0.015X_1 - 0.10X_2 - 0.046X_3 + 0.024X_1X_2 + 0.076X_1X_3 - 0.024X_2X_3 + 0.0016X_1^2 - 0.08X_2^2 + 0.032X_3^2$$

$$Y_{FRAP} = 8.53 - 0.22X_1 - 0.25X_2 - 0.033X_3 - 0.14X_1X_2 - 0.06X_1X_3 + 0.03X_2X_3 - 0.45X_1^2 - 0.31X_2^2 - 0.30X_3^2$$

where Y is the predicted antioxidative activities (DPPH, ABTS radical scavenging activities and FRAP), X_1 is extraction temperature, X_2 is time, X_3 ethanol-to-solid ratio.

The relationship between independent and dependent variables is illustrated in two-dimensional contour plots as shown in Figure 6, 7 and 8. All antioxidative activities of extracts tested by all assays increased with increasing temperature, time and ethanol-to-solid ratio up to the mid-range. Overall, the activities were decreased as temperature, time and ratio were out of the mid-range. The decrease in all activities were found when extraction temperature was above 40-45˚C,

especially after 120 min of extraction (Figure 6A, 7A and 8A). Prolonged extraction time plausibly favored the exposure of phenolics to environmental factor such as light, oxygen and heat, thus contributing to lower antioxidant activities (Wong *et al*., 2014). Polymerization of antioxidant compounds occurred with longer extraction time (Toh *et al*., 2016). However, shorter extraction time was insufficient to completely extract the bound-phenolic compounds. Figure 6B, 7B and 8B show the interaction between X_1 and X_3 on DPPH and ABTS radical scavenging activities and FRAP. Antioxidant activities increased with increasing temperature and ethanol-to-solid ratio. Antioxidant activities were in accordance with TPC in the extracts. Increased mass transfer with higher solvent to material ratio was presumed. Although the increase in TPC was associated with increasing solvent-to-solid ratio, high solvent-to-solid ratio could cause the dilution effect on TPC (Ho *et al*., 2008).

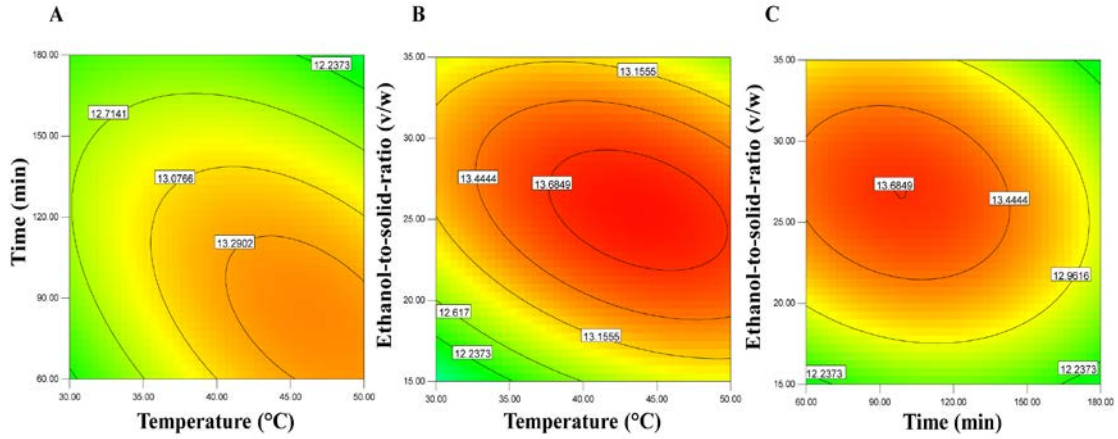

Figure 6 The contour plots of DPPH radical scavenging activity of cashew leaf extracts as affected by the selected independent variables.

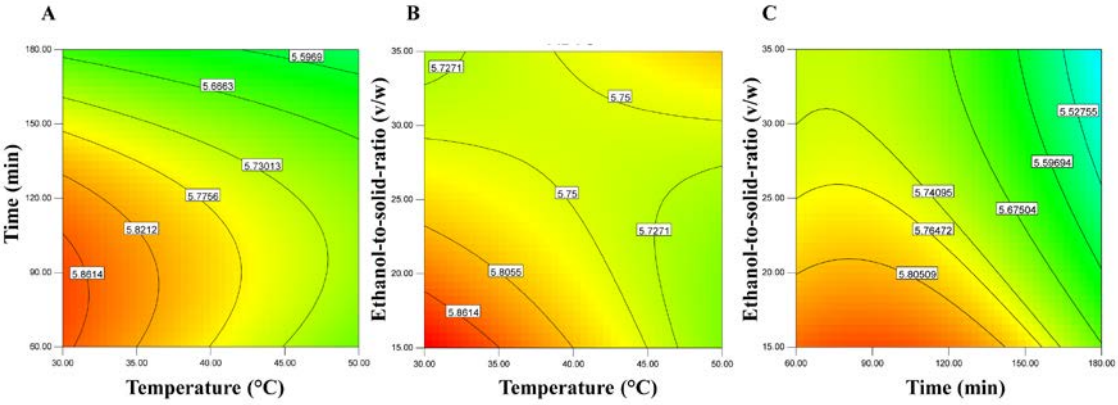

Figure 7 The contour plots of ABTS radical scavenging activity of cashew leaf extracts as affected by the selected independent variables.

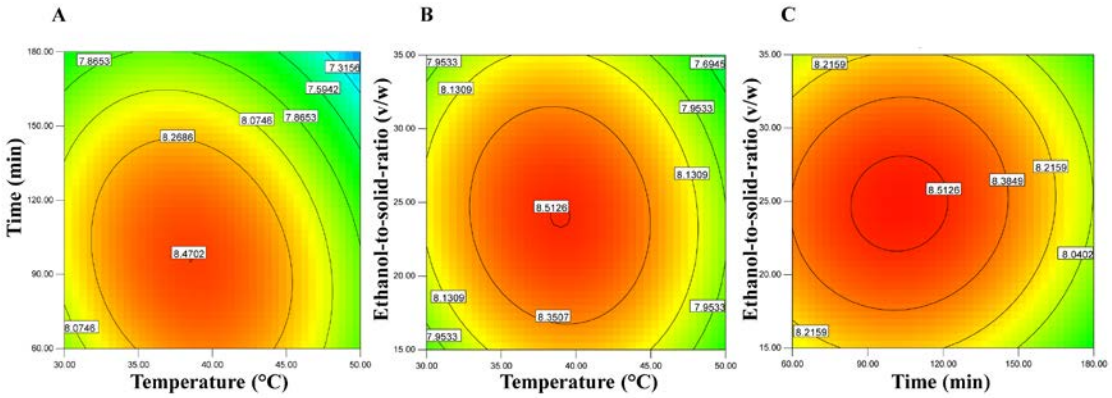

Figure 8 The contour plots of FRAP of cashew leaf extracts as affected by the selected independent variables.

The R^2 value of the predicted models for DPPH and ABTS radical scavenging activities and FRAP were 0.9847, 0.9891 and 0.9903, respectively (Table 5). *P*-value of the model (<0.0001) and non-significant lack of fit (0.4361, 0.1687 and 0.2190, respectively) demonstrated the good fit with the model.

2.4.6 Verification of the optimum condition

The validation of the statistical model and regression equation of optimal condition (34.7˚C, 64 min and ethanol-to-solid ratio of 18:1 v/w) was done. Under the optimized condition, the predicted values were as follows: yield (7.99%), TPC (558.41 mg GAE/g dry extract), DPPH, ABTS radical scavenging activities and FRAP (12.76, 5.83 and 8.30 mmol TE/g dry extract, respectively). The observed values for yield, TPC, DPPH, ABTS radical scavenging activities and FRAP were 8.64 ± 0.03% (error = 7.52%), 564.6 ± 2.13 mg GAE/g (error = 1.09%), 11.74 ± 0.10 mmol TE/g dry extract (error = 8.69%), 5.56 ± 0.06 mmol TE/g dry extract (error = 4.86%) and FRAP (8.11 ± 0.23 mmol TE/g dry extract (error = 2.34%), respectively. Similar predicted and observed values demonstrated the acceptability and validity of the statistical model for the optimization of extraction of antioxidative phenolic compounds from cashew leaves. Therefore, the effective extraction of phenolic compounds from cashew leaves could be achieved under the optimized condition.

2.4.7 LC/DAD/MSD analysis of phenolic compounds

Phenolic compositions of cashew leaf extract prepared under the optimized extracting condition are shown in Table 7. Six phenolic compounds were detected in the extract. Isoquercetin (9842 mg/kg dry extract) was the major phenolic compound, followed by catechin (5286 mg/kg dry extract). Hydroquinin (2361 mg/kg dry extract) and gallic acid (1751 mg/kg dry extract) were also found at high content. Tannic acid and rutin constituted in the extract as minor components. Different phenolic compounds were reported in various plant leaves. Guava leaf extract contained gallic acid, catechin, quercetin and rutin (Chen *et al*., 2009). Konan and Bacchi (2007) reported that quercetin, myricetin, catechin, epicatechin and amentoflavone, tetramer of proanthocyanidin, were major components in *Anarcadium occidentale* leaf extract. The differences in phenolic composition were governed by several factors, including harvesting time, genotype, maturity, climate, soil, extraction methods as well as analysis methods (Gao *et al*., 2011). Phenolic compounds, particularly isoquercetin, more likely played an essential role in antioxidative activity as indicated by DPPH, ABTS radical activity, FRAP as well as metal chelating activity. Isoquercetin was reported to have ability to scavenge hydroxyl, peroxyl and 2,2 diphenyl- 1-picrylhydrazyl (DPPH) radicals as well as have iron chelating activity (Yang *et al*., 2008). Thus, the cashew leaf extract was shown to be a promising source of antioxidative phenolic compounds.

Table 7. Content of phenolic compounds in cashew leaf extract prepared under the optimized extracting condition analyzed by LC/DAD/MSD.

Phenolic compounds	**Retention time (min)**	**Content of phenolic compounds (mg/kg dry extract)**
Gallic acid	6.9	1751
Catechin	12.5	5286
Tannic acid	12.7	189.7
Rutin	15.2	9.1
Isoquercetin	16.4	9842
Hydroquinin	24.1	2361

2.5 Conclusions

Extraction conditions for phenolic compounds with high antioxidant activities from cashew leaves were optimized using RSM. Optimal conditions for extraction were as follows: extraction temperature of 34.7˚C for 64 min with ethanol-to-solid ratio of 18:1 (v/w). Isoquercetin was predominant in the extract. Extract can be used as natural antioxidant for food application and as the nutraceutical.

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CHAPTER 3

EXTRACTION AND CHARACTERIZATION OF ANTIOXIDATIVE PHENOLICS FROM CASHEW (*ANACARDIUM OCCIDENTALE* L.) LEAVES BY ULTRASOUND-ASSISTED PROCESS

3.1 Abstract

Extraction of phenolic compounds with antioxidative activity from cashew (*Anacardium occidentale* L.) leaves by ultrasonic-assisted process was optimized using ethanol as the solvent. Central composite design (CCD) was used to establish treatments based on two independent variables, including amplitude (30-77%) and time (7-31 min). With response surface methodology (RSM), total phenolic content (TPC), antioxidative activities (DPPH, ABTS radical scavenging activities and FRAP) and % extraction yield were determined. First and second-order polynomial model were used for predicting the response. The highest extraction yield was 23.61 ± 0.06 % when the optimal extraction condition (77% amplitude for 31 min) was implemented. TPC was 579.55 ± 6.82 mg GAE/g dry extract and DPPH, ABTS radical scavenging activities and FRAP were 11.85 ± 0.18, 6.04 ± 0.13 and 10.28 ± 0.42 mmol TE/g dry extract, respectively. The values from experiment were in accordance with the predicted values. Isoquercetin (7002 ± 187 mg/kg dry extract) and catechin (4946 ± 99 mg/kg dry extract) were the major phenolics in the extract. Additionally, hydroquinin, gallic acid, tannic acid, rutin, eriodictyol, quercetin and apigenin were also found in the extract. Ultrasound-assisted process could be a potential means to enhance the extraction efficacy of phenolics from cashew leaves.

3.2 Introduction

Cashew (*Anacardium occidentale* L.) is the member of Anacardiaceae family (Saroj *et al*., 2014). It is commonly grown in many tropical areas of the world. Several parts of cashew tree, especially cashew leaves possess phenolics with bioactivities (Kamath and Rajini, 2007). Those phenolic compounds included gallic acid, flavonoids and tannins (Arya *et al*., 1989; Swarnalakshmi *et al*., 1981). Cashew

leaves, which are abundant in the southern part of Thailand, can be an alternative natural antioxidant with safety. Phenolic compounds have been reported for their antioxidative activity by donating electrons and metal chelation (Medina *et al*., 2007). They are able to retard the oxidative degradation of lipids, thereby improving the quality and nutritional value of food as well as prevent the occurrence of diseases (Sindhi *et al*., 2013).

Nowadays, the use of natural antioxidants from plants are of increasing interest in the food industry due to their low toxicity, complete biodegradability, availability from renewable sources and low cost (Yingngamet *et al*., 2015). The extraction process of phenolic compounds is an important step prior to further isolation, purification and application of the extracts. Many techniques, especially conventional method, is limited due to toxicological effect associated with solvent used and long extraction time. To tackle these problems, ultrasonic-assisted process has been employed to improve the extraction efficiency with short extraction time, simplicity and low cost (Wang *et al*., 2013).

Ultrasound is an oscillating sound pressure wave with a frequency greater than the upper limit of the human hearing range (Chandrapala and Leong, 2015). The mechanism of ultrasonic is cavitation phenomena and mechanical effect (Ince *et al*., 2014). When ultrasound passes through a liquid medium, the interaction between the ultrasonic waves, liquid and dissolved gas leads to an exciting phenomenon known as acoustic cavitation (Chandrapala *et al*., 2012). An acoustic cavitation and thermal effects cause disruption of plant cell, reduction of particle size and intensification of mass transfer (Sharmila *et al*., 2016). Thus, the use of non-toxic solvent in combination of ultrasound could be a means to increase the extraction efficiency of plant antioxidant.

Response surface method (RSM) is a statistical tool, which has been used to optimize various parameters in industrial process (Deepak *et al*., 2008). It can evaluate the impact of different multiple parameters, and simultaneously optimize experimental conditions (Shekarchizadeh *et al*., 2009; Xu *et al*., 1998). Therefore, the present study aimed to optimize the extraction of phenolic from cashew leaves by

ultrasound-assisted process using RSM and to study antioxidative activity of the resulting ethanolic extracts.

3.3 Materials and methods

3.3.1 Chemicals

Ferrous sulfate ($FeSO_4 \cdot 7H_2O$), ferric chloride ($FeCl_3 \cdot 6H_2O$), and potassium persulfate were purchased from Sigma (MO, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Aldrich Chemical Company (Steinheim, Germany). 2,4,6-Tri (2-pyridyl)-S-triazine (TPTZ) was procured from Fluka Chemicals (Buchs, Switzerland). Gallic acid, 2,2-diphenyl-1-picryhydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma (MO, USA). Ethanol, ethyl acetate and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Phenolic standards (gallic acid, catechin, isoquercetin, tannic acid, rutin, hydroquinin, eriodictyol, quercetin and apigenin) were purchased from Sigma-Aldrich, Inc. (Chemie GmbH. Steinheim, Germany).

3.3.2 Collection of cashew leaves

Cashew (*Anacardium occidentale* L.) leaves were collected from an orchard in Songkhla province, Thailand during November and December 2016. The trees were approximately 15-20 years old. Leaves from III (apical) to V (basal) of twig were used in the study. Cashew leaves were prepared following the method of Váquez-Torres *et al*. (1992) with slight modifications. The samples were washed with tap water and dried overnight in an air dryer at 50˚C until the moisture content was less than 10%. Dried samples were blended using a blender (Panasonic, Model MX-898N, Berkshire, UK) and sieved using a stainless steel (sieve 80 mesh). Thereafter, the obtained powder was subjected to chlorophyll removal. The sample powder (50 g/l) was mixed with chloroform and the mixture was stirred for 30 min, followed by filtration using Whatman filter paper No.1 (Row and Jin, 2006; Vovk, Simonovska and Vuorela, 2005). The retentate was dried in a hot air oven (Memmert, Schwabach,

Germany) at 105˚C for 1 h. The obtained powder termed ‘cashew leaf powder’ was placed in a polyethylene bag and kept at room temperature until used for extraction.

3.3.3 Preparation of cashew leaf extracts

Ultrasound-assisted extraction was performed using an ultrasonic equipment (Sonics, Model VC750, Sonica & Materials, Inc., Newtown, USA) operating at a frequency of 20 kHz ± 50 Hz with high intensity power of 750 W. The cashew leaf powder (10 g) was placed into a beaker (250 ml) and 180 ml of 80% ethanol were added. The temperature was controlled by the external water from a thermostatic water bath. The sonication time was controlled via the equipment panel. After extraction, the mixtures were centrifuged at 5000 g for 30 min at room temperature using a RC-5B plus centrifuge (Beckman, JE-AVANTI, Fullerton, CA, USA) and the supernatants were then filtered through a Whatman filter paper No.1 (Whatman International Ltd., Maidstone, UK) (Jan *et al*., 2013). The filtrates were evaporated at 40 ˚C using an EYELA rotary evaporator (Tokyo Rikakikai, Co. Ltd., Tokyo, Japan). The extracts were then dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). Dried extracts were kept in a desiccator until further analysis.

3.3.4 Optimization of extraction of antioxidative phenolics by ultrasound-assisted process using RSM

3.3.4.1 Experimental design

Response surface methodology (RSM) was used to investigate the optimal condition for extracting. A CCD with two independent variables including the amplitude (X_1, %) and sonication time (X_2, min) was applied at five coded levels as shown in Table 8. The minimum and maximum ranges of the variables were used, and the full experimental plan consisting of 9 experimental points with regard to their values in actual and coded form is provided in Table 9. Five replicates (run order 9-13) at the center point were used to estimate a pure error sum of squares. The dependent variables include %yield, TPC, DPPH and ABTS radical scavenging activities and FRAP, in which each trial was the average of the triplicates. The

optimal extraction condition was analyzed using the Design-Expert Statistical package version 7.0 (Statease, Inc Minneapolis, Minn, USA). The experimental data were fitted to a first-order polynomial model (1) and second-order model (2). The regression coefficients were obtained by multiple linear regression.

$$Y = \beta_0 + \beta_i X_i + \beta_j X_j + \beta_{ij} X_i X_j \quad (1)$$

$$Y = \beta_0 + \beta_i X_i + \beta_j X_j + \beta_i X_i^2 + \beta_j X_j^2 + \beta_{ij} X_i X_j \quad (2)$$

where Y is the dependent variables (%yield, TPC, DPPH, ABTS radical scavenging activities and FRAP); X_i and X_j are the independent variables; β_0 is the model constant; β_i is the linear coefficient and β_{ij} is the interaction coefficient. The fitted polynomial equations were then expressed in the form of response surface plots, in order to illustrate the relationship between dependent variables and the experimental variables used. The optimum condition was determined by superimposing maximum areas of all responses from all combinations of independent variables using Design-Expert.

Table 8. Independent variables and their coded and actual values used for optimization of extraction of antioxidative phenolics from cashew leaves using ultrasound-assisted process

Independent variables	Units	Symbol	Code levels				
			-α	**-1**	**0**	**1**	**+α**
Amplitude	%	X_1	20.27	30	53.50	77	86.73
Time	min	X_2	2.03	7.00	19.00	31.00	35.97

3.3.4.2 Analyses

3.3.4.2.1 Determination of extraction yield

Extraction yield of cashew leaf extracts was defined as the percentage of dry weight of extract, compared to the initial dry weight of raw material. Extraction yield was calculated according to the following equation:

$$\text{Extraction yield (\%)} = \frac{\text{Dry weight of obtained extract (g) x 100}}{\text{Dry weight of initial leaf (g)}}$$

Table 9. Central composite design for extraction of antioxidative cashew leaf extracts

Run order	Independent variables	
	Amplitude (X_1)	Time (X_2)
1	30 (-1)	7 (-1)
2	77 (1)	7 (-1)
3	30 (-1)	31 (1)
4	77 (1)	31 (1)
5	20.27 (-1.41)	19 (0)
6	86.73 (1.41)	19 (0)
7	53.50 (0)	2.03 (-1.41)
8	53.50 (0)	35.97 (1.41)
9	53.50 (0)	19 (0)
10	53.50 (0)	19 (0)
11	53.50 (0)	19 (0)
12	53.50 (0)	19 (0)
13	53.50 (0)	19 (0)

3.3.4.2.2 Determination of total phenolic content (TPC)

TPC was determined using the Folin-Ciocalteau reagent as described by Sato *et al*. (1996) with a slight modification. The extracts were dissolved in distilled water to obtain a final concentration of 0.2 mg/ml. An aliquot of 100 µl was mixed with 0.75 ml of Folin-Ciocalteu reagent. After 3 min, 0.75 ml of 6% (w/v) sodium carbonate was added. The mixture was allowed to stand for 1 h at room temperature. The absorbance at 760 nm was measured by UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Gallic acid (0-500 mg/ml) was used as the standard. TPC was expressed as mg gallic acid equivalent (GAE) / g dry extract.

3.3.4.2.3 Determination of antioxidative activities

Prior to assay, the samples were appropriately diluted using the distilled water.

3.3.4.2.3.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Wu *et al*. (2003) with a slight modification. Sample (1 ml) was added with 1 ml of 0.15 mM 2,2-diphenyl-1-picryl hydrazyl (DPPH) in 95% ethanol. The mixture was mixed vigorously and allowed to stand at room temperature in dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. Sample blank was prepared in the same manner except that 95% ethanol was used instead of DPPH solution. Trolox (10-60 μM was used as the standard. The activity was expressed as mmol Trolox equivalents (TE)/g dry extract.

3.3.4.2.3.2 ABTS radical scavenging activity

ABTS radical scavenging activity was measured as per the method of Arnao *et al*. (2001). Sample (150 μl) was mixed with 2850 μl of ABTS solution and the mixture was allowed to stand at room temperature for 2 h in dark. The absorbance was then measured at 734 nm. Sample blank was prepared in the same manner except that methanol was used instead of ABTS solution. Trolox (0-500 μM) was used as the standard. The activity was expressed as mmol Trolox equivalents (TE)/g dry extract.

3.3.4.2.3.3 Ferric reducing antioxidant power (FRAP)

FRAP assay was determined according to the method of Benzie and Strain (1996). A sample (150 μl) was mixed with 2850 μl of FRAP solution and kept for 30 min in dark at room temperature. The ferrous tripyridyltriazine complex (colored product) was measured by reading the absorbance at 593 nm. Sample blank was prepared by omitting $FeCl_3$ from FRAP solution and distilled water was used instead. Trolox (0-500 μM) was used as the standard. The activity was expressed as mmol Trolox equivalents (TE)/g dry extract.

3.3.4.3 Verification of the optimum condition

All the responses were determined under the optimized condition for extraction. The experimental errors for the models were determined by comparing observed values with the predicted values as follows:

Error (%) = [(Observed value - predicted value) / Observed value] x 100

3.3.5 LC/DAD/MSD analysis of phenolic compounds

LC/DAD/MSD analysis was determined according to the method of Peñarrieta *et al*. (2007). Phenolic compounds in cashew leaf extract obtained from the optimized extracting condition was analyzed using a LC/DAD/MSD equipped with a diode array detector (DAD) and a scan mode 100-700 m/z MS detector. Sample was firstly separated on LiChroCART®Purospher®STAR RP-18e column (Merck, USA) (150 x 4.6 mm, i.d., 5 µm) with an Agilent 1100 series (Agilent Technologies, Waldbronn, Germany). Acetonitrile and 10 mM ammonium formate buffer pH 4 with formic acid were used as mobile phase A and mobile phase B, respectively (flow rate: 1.0 ml/min; temperature: 40˚C). The gradient program was as follows: 100% B constant (0-5 min), 0-20% A (5-10 min), 20% A constant (10-20 min), and 20-40% A (20-60 min). The detection was carried out at 270, 330, 350 and 370 nm. MS detection was carried out in the positive ionization mode, where an electrospray ionizing source with nitrogen as drying gas was used. The conditions used were as follows: the capillary voltage: 4000 V; gas temperature: 320˚C; drying gas flow: 13 l/min; nebulizer pressure 60 psi. Quantitative analysis by MSD was done in the SIM (Selected Ion Monitoring) mode. Standards were as follows: 188, 209 m/z for gallic acid; 185, 329, 503, 649 m/z for catechin, isoquercetin, tannic acid and rutin; 289, 327, 341 m/z for hydroquinin, eriodictyol and quercetin; 271, 287, 309, 325 m/z for apiginin and kaemferol.

3.3.6 Statistical analyses

Optimization of extraction was analyzed using CCD as mentioned above.

3.4 Results and discussion

3.4.1 Optimization of extraction of antioxidative phenolic compounds by ultrasound-assisted process using RSM

Based on CCD experiment, 17 treatments derived from two independent variables including amplitude (X_1) and sonication time (X_2) are presented in Table 9. The experimental data, %extraction yield, TPC, DPPH radical scavenging activity and FRAP fitted with the first order polynomial equations, while ABTS radical scavenging activity fitted with the second order polynomial equation. The ANOVA result for each response variable indicated that at least one of the parameters of the model could explain the experimental variation for response variables as shown by significant *F*-value and *P*-value for the model (Table 10). Lack of fit was also given in Table 10. Based on the result, lack of fit was not significant, confirming the validation of the fitted models.

3.4.2 Effect of independent variables on extraction yield

Among all dependent variables studied, the extraction yield was highly influenced by linear term of time (X_2) ($P<0.0001$). Although the interaction between two parameters showed no effect on extraction yield, the linear term of amplitude (X_1) was still considered as a significant factor in this model ($P<0.05$). The extraction yield was in the range of 20.15-23.19% (Table 11). The response surface plot of extraction yield is illustrated in Figure 9. The result indicated that both amplitude and sonication time had significantly positive effect on yield. An increase in variable values resulted in higher extraction yield. The yield reached a maximum at amplitude of 77% and sonication time of 31 min. The increased extraction yield was more likely due to the cavitation effect. The phenomenon of cavitation consists of the repetition of three distinct steps: formation (nucleation), rapid growth (expansion) during the cycles until it reaches a critical size, and violent collapse in the liquid (Pang *et al*., 2011). The increased cavitation bubble collision caused further reduction in particle size and promoted the penetration of solvent into the sample matrix as well as increased mass transfer rates (Avhad and Rathod, 2015; Soria and Villamiel, 2010). This led to the increased extraction efficacy as indicated by increased yield. The R^2 value of the

predicted model in extraction yield value was 0.8406. Moreover, *P*-value of the model (<0.001) and the non-significant lack of fit (0.0987) (Table 10) showed that the model fitted with good prediction. Effect of independent variables, amplitude (X_1) and time (X_2), on extraction yield of cashew leaf extracts is shown as follows:

$$Y = 22.01 + 0.32X_1 + 0.97X_2 \qquad (3)$$

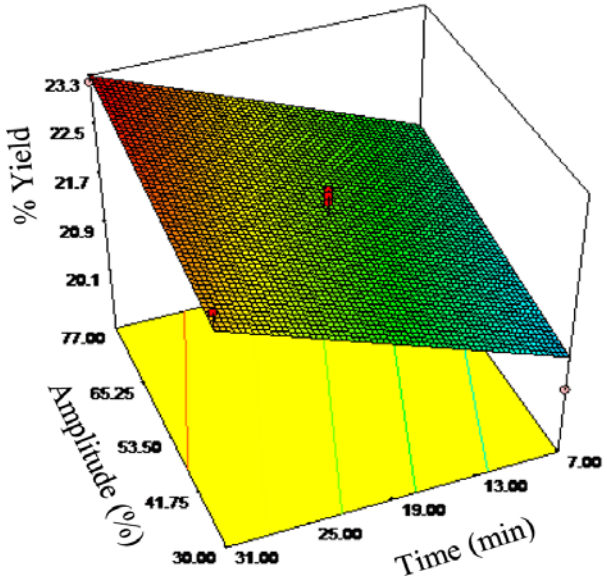

Figure 9 Response surface plot of extraction yield of cashew leaf extracts as affected by the selected independent variables.

3.4.3 Effect of independent variables on total phenolic contents (TPC)

Different extraction conditions showed varying TPC of resulting extracts. TPC obtained varied from 516.15 to 579 mg GAE/g dry extract (Table 11). TPC were highly influenced by linear term of amplitude (X_1) and time (X_2) (P<0.0001). Impact of independent variables on TPC of cashew leaf extracts is demonstrated as follows:

$$Y = 548.72 + 17.41X_1 + 14.14X_2 \qquad (4)$$

Table 10. Analysis of variance (ANOVA) for the fitted linear and quadratic polynomial models for optimization of extraction parameters

Source	DF[a]	SS[b]	MS[c]	*F*-value	*P*-value
% Yield					
Model	2	8.26	4.13	26.36	0.0001[s]
Lack of fit	6	1.34	0.22	4.04	0.0987[ns]
Pure error	4	0.22	0.055		
Total error	12	9.82			
R^2	0.8406				
TPC					
Model	2	4187.70	2093.85	71.75	< 0.0001[s]
Lack of fit	6	252.52	42.09	4.28	0.0903 [ns]
Pure error	4	39.30	9.83		
Total error	12	4479.52			
R^2	0.9713				
DPPH radical scavenging activity					
Model	2	2.19	1.10	24.37	< 0.0001[s]
Lack of fit	6	0.37	0.061	2.94	0.1580 [ns]
Pure error	4	0.083	0.021		
Total error	12	2.64			
R^2	0.8298				
ABTS radical scavenging activity					
Model	5	3.59	0.72	96.77	< 0.0001[s]
Lack of fit	3	0.033	0.011	2.31	0.2182 [ns]
Pure error	4	0.019	0.0005		
Total error	12	3.64			
R^2	0.9857				
FRAP					
Model	3	3.59	1.20	27.67	< 0.0001[s]
Lack of fit	5	0.33	0.066	4.44	0.0869 [ns]
Pure error	4	0.059	0.015		
Total error	12				
R^2	0.9022				

[a] Degree of freedom
[b] Sum of squares
[c] Mean square
[s]Significant
[ns]Non-significant

Table 11. Yield and antioxidative activities of cashew leaf extracts prepared by ultrasound-assisted process under different conditions

Run order	Yield (%)	TPC (mg GAE/ g dry extract)	DPPH radical scavenging activity (mmol TE/g dry extract)	ABTS radical scavenging activity (mmol TE/g dry extract)	FRAP (mmol TE/g dry extract)
1	20.15 ± 0.84	516.15 ± 18.09	10.39 ± 0.14	4.62 ± 0.08	8.87 ± 0.10
2	20.58 ± 0.40	558.87 ± 14.99	11.72 ± 0.07	5.67 ± 0.08	10.40 ± 0.04
3	22.92 ± 2.29	545.69 ± 3.53	11.10 ± 0.13	5.30 ± 0.07	10.07 ± 0.24
4	23.19 ± 0.13	579.00 ± 8.91	12.14 ± 0.01	6.29 ± 0.03	10.28 ± 0.21
5	21.51 ± 1.86	525.73 ± 3.97	10.96 ± 0.21	4.73 ± 0.08	9.22 ± 0.02
6	22.59 ± 0.25	570.47 ± 16.84	11.65 ± 0.07	6.21 ± 0.14	10.57 ± 0.02
7	20.91 ± 2.76	527.07 ± 11.19	11.08 ± 0.27	4.83 ± 0.08	8.95 ± 0.23
8	22.78 ± 0.62	571.94 ± 15.71	12.06 ± 0.14	6.11 ± 0.07	10.63 ± 0.04
9	21.84 ± 0.01	543.14 ± 3.10	11.21 ± 0.01	5.27 ± 0.04	10.20 ± 0.23
10	22.21 ± 1.40	545.85 ± 8.30	11.39 ± 0.03	5.18 ± 0.07	9.89 ± 0.05
11	22.41 ± 2.26	550.55 ± 1.17	11.32 ± 0.06	5.14 ± 0.02	9.97 ± 0.06
12	22.40 ± 2.97	548.73 ± 1.99	11.58 ± 0.43	5.11 ± 0.10	10.10 ± 0.22
13	22.32 ± 3.37	550.13 ± 3.44	11.26 ± 0.13	5.25 ± 0.09	10.10 ± 0.04

Values are mean ± SD (n=3).

Relationship between different variables in term of response surface plot is shown in Figure 10A. The increases in amplitude and time tended to increase TPC of the extract, more likely due to the enhanced acoustic cavitation and thermal effects, which caused disruption of plant cell, reduction of particle size and intensification of mass transfer (Sharmila *et al*., 2016). The increased rate of mass transfer is related with the enhanced rate of the solvent brought to the solid surface. The transfer of the soluble constituents into the solvents is also enhanced when ultrasonic process is applied (Shirsath *et al*., 2012). The highest TPC was observed at amplitude of 77% and time of 31min. Increasing amplitude above 77% had no effect on TPC (P>0.05) (Table 11). The regression equation obtained from analysis of variance (ANOVA) with the R^2 value of 0.9713 revealed that the model could explain 97.13% variation in the

response, whereas *P*-value of the model (<0.0001) and non-significant lack of fit (0.0903) (Table 10) also suggested that the obtained experimental data could fit well with the model.

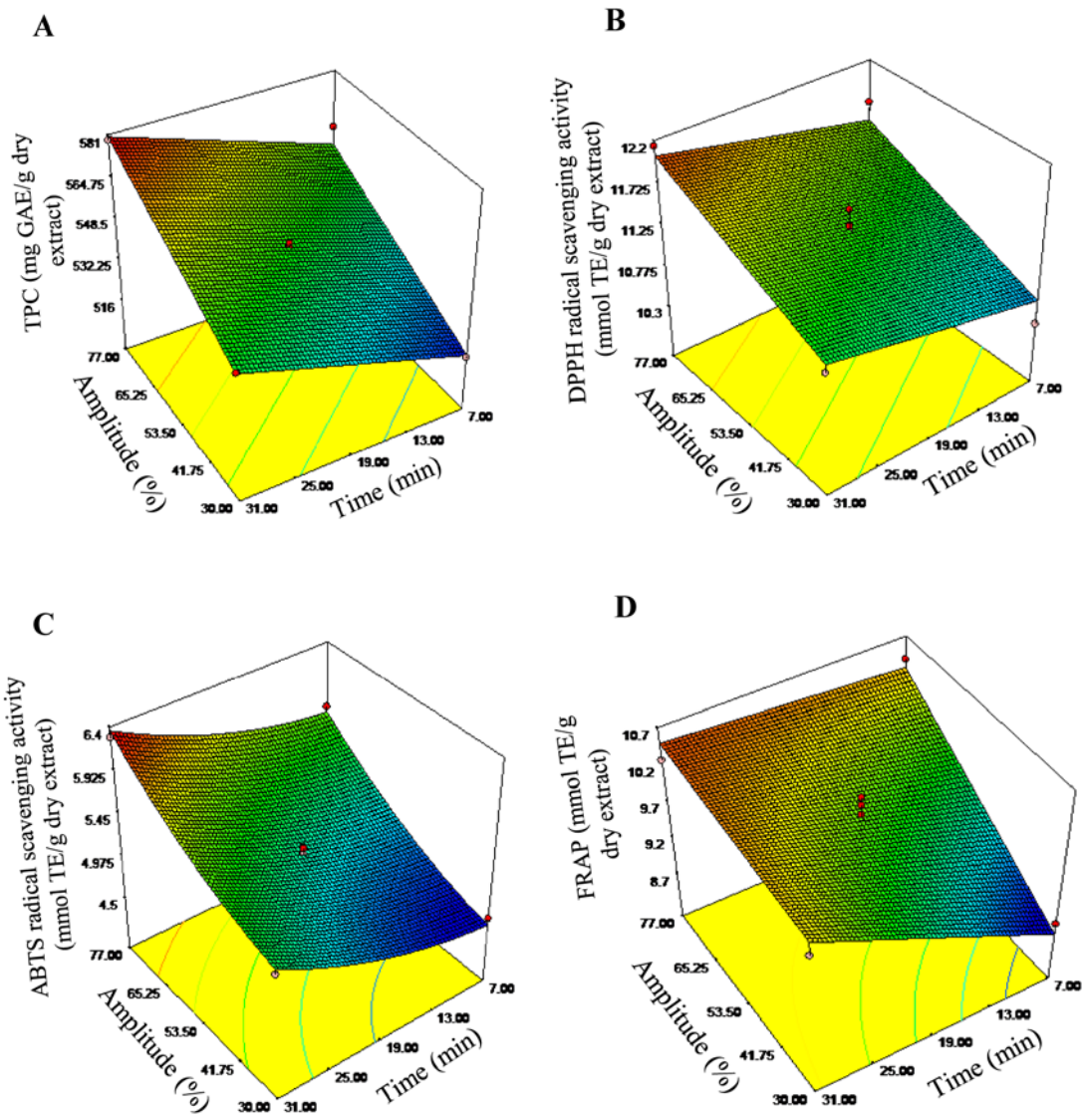

Figure 10 Response surface plots of phenolic compounds and antioxidative activities of cashew leaf extracts as affected by the selected independent variables. TPC (A), DPPH radical scavenging activity (B), ABTS radical scavenging activity (C) and FRAP (D).

3.4.4 Effect of independent variables on antioxidant activities

Most of linear term of variables (X_1 and X_2) significantly affected antioxidative activities tested by all assays (P<0.05). However, linear terms (X_1 and X_2) showed the most significance toward ABTS radical scavenging activity (P<0.0001). Quadratic terms (X_1^2 and X_2^2) were shown to be significant for ABTS radical scavenging activity (P<0.01). When considering interactive terms between variables, the significance was found only for FRAP (P<0.05). Effect of amplitude (X_1) and time (X_2) on DPPH and ABTS radical scavenging activity and FRAP of cashew leaf extracts are shown with the following equations:

$$Y_{DPPH} = 11.37 + 0.42X_1 + 0.31X_2 \quad (5)$$

$$Y_{ABTS} = 5.19 + 0.52X_1 + 0.39X_2 - 0.015X_1X_2 + 0.14X_1^2 + 0.14X_2^2 \quad (6)$$

$$Y_{FRAP} = 9.94 + 0.46X_1 + 0.43X_2 - 0.33X_1X_2 \quad (7)$$

Relationship between variables in term of response surface plots is shown in Figure 10B-D. Overall, antioxidative activities increased with increasing amplitude and sonication time. According to equations (5-7), the amplitude was the higher significant variable on activities (P<0.001) as compared with sonication time (P<0.05). The highest activities of extract were found at amplitude 77% and time 31 min. It was noted that antioxidative activities were in accordance with TPC and extraction yield. The increase in amplitude mostly caused the augmented decomposition of plant cell. As a result, the phenolics with antioxidative activity were more released. However, the higher amplitude might lead to the decrease in activities of phenolics. Pyrolysis during cavitational collapse and the generation of hydroxyl radicals (·OH) caused by cavitational thermolysis resulted in the chemical decomposition by opening ring of phenolic compounds (Alighourchi *et al*., 2013; Tiwari *et al*., 2009). The R^2 value of the predicted models for DPPH and ABTS radical scavenging activities and FRAP were 0.8298, 0.9857 and 0.9022, respectively. *P*-value of the model (<0.0001) and non-significant lack of fit (0.1580, 0.2182 and 0.0869, respectively) (Table 10) also suggested that the obtained experimental data fitted well with the model.

3.4.5 Verification of the optimum condition

The validation of the statistical model and regression equation of optimal condition (77%, and 31 min) was examined (Table 12). Under this optimized condition, the observed experimental value of TPC was 579.55 ± 6.82 mg GAE/g dry extract. DPPH, ABTS radical scavenging activities and FRAP were 11.85 ± 0.18, 6.04 ± 0.13, 10.28 ± 0.42 mmol TE/g dry extract, respectively. The yield was 23.61 ± 0.06% (Table 11). Similar predicted value and observed values revealed the validity and acceptability of the statistical model for the optimization of extraction of antioxidative phenolic compounds from cashew leaves using ultrasound-assisted process. Therefore, the effective extraction of phenolic compounds from cashew leaves with the aid of ultrasound processor could be a promising approach for development of nutraceutical and functional foods from indigenous plant.

3.4.6 LC/DAD/MSD analysis of phenolic compounds

Phenolic compositions of cashew leaf extract prepared under the optimized extracting condition are shown in Table 13. Nine phenolic compounds were detected in the extract. Isoquercetin (7002 mg/kg dry extract) was the major phenolic compound, followed by catechin (4946 mg/kg dry extract). Gallic acid (2692 mg/kg dry extract), quercetin (2660 mg/kg dry extract) and hydroquinin (1291 mg/kg dry extract) were also found at high content. Tannic acid, rutin, apigenin and eriodictyol constituted in the extract as minor components. Recently, Chotphruethipong *et al.* (2017) reported six phenolic compounds including isoquecetin, catechin, hydroquinin, gallic acid, tannic acid and rutin in the extract from cashew leaves using 80% ethanol as the solvent. It was noted that isoquercetin and catechin were the major phenolic compounds in both ethanolic extracts of cashew leaves, regardless of extraction methods used. However, some additional phenolics including quercetin, apigenin and eriodictyol were found in the ethanolic extract when ultrasound-assisted process was used. Coincidentally the extraction yield obtained from ultrasound-assisted process under the optimal extraction condition was higher than that from solvent extraction as reported by Chotphruethipong *et al.* (2017). Ultrasounds have been employed to induce the disruption of plant cell and rinsing the contents of cell after breaking the

walls (Mason *et al.* 1996) via ultrasonication, isoquercetin bound with leaf tissue could be more released. This resulted in the higher efficiency in extraction of phenolics as evidenced by the presence of additional compounds as well as increased yield. Thus ultrasound-assisted process was able to enhance the effectiveness in extraction of phenolics from cashew leaves.

Table 12. Comparison between the predicted and observed values obtained under the optimum conditions based on the combination of responses

Dependent variables	Predicted values	Observed values*	Error (%)
Extraction yield (%)	23.29	23.61 ± 0.06	1.36
TPC (mg GAE/g dry extract)	581.50	579.55 ± 6.82	0.34
DPPH radical scavenging activity (mmol TE/g dry extract)	12.11	11.85 ± 0.18	2.15
ABTS radical scavenging activity (mmol TE/g dry extract)	6.36	6.04 ± 0.13	5.30
FRAP (mmol TE/g dry extract)	10.50	10.28 ± 0.42	2.14

*Values are mean ± SD (n=3).

Table 13. Phenolic compounds in cashew leaf extract prepared under the optimum extraction condition analyzed by LC/DAD/MSD

Phenolic compounds	Retention time (min)	Content of phenolic compounds* (mg/kg dry extract)
Gallic acid	6.70-7.20	2692 ± 420
Catechin	12.40-12.60	4946 ± 99
Tannic acid	12.70-13.00	281 ± 19
Rutin	15.20-15.40	499 ± 50
Isoquercetin	16.40-16.50	7002 ± 187
Hydroquinin	24.00-24.30	1291 ± 133
Eriodictyol	31.20-31.30	5.9 ± 0.5
Quercetin	33.90-34.00	2660 ± 388
Apigenin	41.90-42.00	6.6 ± 0.6

*Values are mean ± SD (n=3).

3.5 Conclusions

Extraction conditions for phenolic compounds with high antioxidant activities from cashew leaves by ultrasound-assisted process were optimized using RSM. Optimal conditions for extraction were: 77% of amplitude and time of 31 min. Isoquercetin was the most predominant in the extract. Cashew leaf extract could be used as natural antioxidant for food application and also serve as the nutraceutical.

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CHAPTER 4

CHARACTERISTICS AND ANTIOXIDATIVE ACTIVITY OF CASHEW (*ANACARDIUM OCCIDENTALE* L.) LEAF EXTRACTS

4.1 Abstract

Antioxidative activities of cashew leaf extracts with different extraction methods (conventional and ultrasound-assisted methods) as affected by concentrations (0-0.2 mg/ml) were investigated. DPPH, ABTS radical scavenging activities and FRAP of both extracts increased when the concentrations increased up to 0.1 mg/ml (P<0.05). However, all activities decreased at higher concentration (0.2 mg/ml). Both extracts had higher activities tested by all assays than BHT (P<0.05), but lower than catechin at the concentration range of 0.025-0.1 mg/ml. ABTS radical scavenging activity and FRAP of extracts from ultrasound-assisted process were higher than those from conventional process when the concentrations of 0.05-0.1 mg/ml were used. When thermal and pH stabilities of both extracts were determined, antioxidative activities were still remained after heating at 60-100 ˚C for 60 min. Both extracts exhibited strong activities at the pH ranging from 6 to 8, while the lowest activity was found in pH range of 2-4. Both extracts (50 and 100 ppm) exhibited antioxidative activities in β-carotene-linoleic acid and lecithin liposome systems in a dose dependent manner as evidenced by the retarded formation of conjugated diene, peroxide value and thiobarbituric acid reactive substances (TBARS) (P<0.05). Extract from ultrasound-assisted method generally showed the higher efficacy than that of conventional method. Nevertheless, their preventive effect toward oxidation was lower than BHT in both tested systems. Thus, cashew leaf extracts could act as natural antioxidant for food application or as nutraceutical.

4.2 Introduction

Lipid oxidation is a deteriorative reaction, where unsaturated fatty acids react with molecular oxygen via a free radical chain mechanism, forming fatty acyl hydroperoxides and non-volatile and volatile hydroperoxide breakdown products (Medina *et al.*, 2012; Shahidi and Zhong, 2010). This reaction induces the changes in

texture, flavor as well as odor, thus leading to the loss in food quality and nutritional value. Moreover, it also causes some disease in human beings such as cardiovascular disease, cancer and neurological disorders and aging process (Gülçin, 2012).

To retard lipid oxidation of food, especially food containing long-chain polyunsaturated fatty acids, some synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ) have been used in food systems. Nevertheless, the use of synthetic antioxidants is limited in many countries. Nowadays, the use of antioxidant from plants are of an increasing attention for food industry, due to the safety concern. Plant extracts rich in antioxidative compounds also gain the interest as nutraceuticals for health benefit. Phenolic compounds, the secondary metabolites of plants, comprise benzene rings with one or more hydroxyl substituents (Velderrain-Rodríguez *et al.*, 2014). They can play a role as antioxidants such as reducing agents, hydrogen donators and metal chelators and their structure have resonance stabilization of the captured electron (Proestos *et al.*, 2006; Shahidi *et al.*, 1992).

The extraction method is the first important step in the recovery and isolation of bioactive compounds from plant materials (Stalikas, 2007). Conventional methods such as maceration and solvent extraction are generally used for bioactive compounds extraction (Azmir *et al.*, 2013). With low yield and toxicological effect associated with inappropriate solvent used as well as the long extraction time, the potential technology, especially ultrasonic-assisted process, has been employed to tackle the drawback. It has been known to improve the extraction efficiency with short extraction time, simplicity and low cost (Wang *et al.*, 2013).

Cashew (*Anacardium occidentale* L.) is a member of the family *Anacardiaceae* (Razali *et al.*, 2008). Their trees are abundant in the tropical area, especially the southern part of Thailand. Cashew nut has been popular for consumer and its young leaves have been consumed as fresh in some region of America and Asian countries. It contained a large amount of phytochemicals, especially phenolic with antioxidative activity (Kamath and Rajini, 2007).

Recently, ethanolic extracts from cashew leaves have been prepared. With the aid of ultrasound, the extraction yield was increased. Additionally, the additional phenolics were extracted in comparison with that prepared by conventional process (Chotphruethipong *et al.*, 2017a). However, the antioxidative activity and properties of extracts from both processes have not been reported.

Thus, the present study aimed to examine the antioxidative activity and some properties of cashew leaf extracts with different extraction methods. The prevention of lipid oxidation by extracts in different model systems was also investigated.

4.3 Materials and methods

4.3.1 Chemicals

Butylated hydrotoluene (BHT), catechin, linoleic acid, 2,2-diphenyl-1-picryhydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), ferrous sulfate ($FeSO_4 \cdot 7H_2O$), ferric chloride ($FeCl_3 \cdot 6H_2O$), thiobarbituric acid (TBA) and potassium persulfate were purchased from Sigma (St Louis, MO, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Aldrich Chemical Company (Steinheim, Germany). β-carotene and 2,4,6-Tri (2-pyridyl)-S-triazine (TPTZ) were procured from Fluka Chemicals (Buchs, Switzerland). Ethyl acetate and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Chloroform, ethanol and methanol were obtained from Lab-Scan (Bangkok, Thailand).

4.3.2 Collection of cashew leaves

Cashew (*Anacardium occidentale* L.) leaves were obtained from trees with 15-20 years old, in an orchard located in Songkhla province, Thailand during November and December 2016. Only leaves from III (apical) to V (basal) of twig were collected. Cashew leaves were prepared as per the method of Váquez-Torres *et al.* (1992) with slight modifications. Leaves were washed with tap water and dried overnight in an air dryer at 50˚C until the moisture content was less than 10%. Dried samples were blended using a blender (Panasonic, Model MX-898N, Berkshire, UK)

and sieved using a stainless steel (sieve 80 mesh). Thereafter, the obtained powder was dechlorophyllized by mixing the powder with chloroform (50 g/l). The mixture was stirred for 30 min, followed by filtration using a Whatman filter paper No.1 (Whatman International Ltd., Maidstone, UK) (Row and Jin, 2006; Vovk *et al.*, 2005). The retentate was dried in a hot air oven (Memmert, Schwabach, Germany) at 105˚C for 1 h. The obtained powder termed 'cashew leaf powder' was packaged in a polyethylene bag and kept at room temperature (28-30 ˚C) until used.

4.3.3 Preparation of cashew leaf extracts

Cashew leaf powder (10 g) was subjected to extraction using conventional and ultrasound-assisted processes. Ethanol (80%, v/v) and the powder/solvent ratio of 1:18 (w/v) were used. For conventional process, the mixture was stirred for 64 min (Chotphruethipong *et al.*, 2017a). For ultrasound-assisted process, the mixture was subjected to ultrasonic treatment using an ultrasonic equipment (Sonics, Model VC750, Sonica & Materials, Inc., Newtown, USA) at the amplitude of 77% for 31 min with the frequency of 20 kHz ± 50 Hz and high intensity power of 750 W. The temperature was controlled at 35 ± 40 ˚C by external water from a thermostatic water bath (Chotphruethipong *et al.*, 2017b). After extraction, the obtained mixtures from both processes were centrifuged at 5000xg for 30 min at room temperature using a RC-5B plus centrifuge (Beckman, JE-AVANTI, Fullerton, CA, USA) and the supernatants were then filtered through a Whatman filter paper No.1. The filtrates were evaporated at 40 ˚C using an EYELA rotary evaporator (Tokyo Rikakikai, Co. Ltd., Tokyo, Japan). The extracts were then dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). Dried extracts from conventional and ultrasound-assisted processes were referred to as 'CE-C' and 'CE-US', respectively.

4.3.4 Effect of cashew leaf extracts with different extraction processes at various concentrations on antioxidative activity

Cashew leaf extracts from both extraction processes were dissolved in deionized water to obtain the concentrations of 0.025, 0.05, 0.1 and 0.2 mg/ml. Antioxidative activities (DPPH and ABTS radical scavenging activities and FRAP) of

all solutions were determined. Catechin and BHT at the same concentrations were also examined for antioxidative activities.

4.3.5 Determination of antioxidative activities

DPPH, ABTS radical scavenging activities and Ferric reducing antioxidant power of the extracts were tested since the extracts showed higher aforementioned activities (chapter 2 and 3).

4.3.5.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Wu *et al.* (2003) with a slight modification. Sample (1 ml) was added with 1 ml of 0.15 mM DPPH dissolved in 95% ethanol. The mixture was mixed vigorously and allowed to stand at room temperature in dark for 30 min. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. Sample blank was prepared in the same manner except that 95% ethanol was used instead of DPPH solution. Trolox (10-60 μM) was used as the standard. The activity was expressed as mmol Trolox equivalents (TE)/g dry extract.

4.3.5.2 ABTS radical scavenging activity

ABTS radical scavenging activity was measured as per the method of Arnao *et al.* (2001). Sample (150 μl) was mixed with 2850 μl of ABTS solution and the mixture was left at room temperature for 2 h in dark. The absorbance was then measured at 734 nm. Sample blank was also prepared, in which ABTS solution was omitted and methanol was used. Trolox (0-500 μM) was used as the standard. The activity was expressed as mmol Trolox equivalents (TE)/g dry extract.

4.3.5.3 Ferric reducing antioxidant power (FRAP)

FRAP assay was determined according to the method of Benzie and Strain (1996). A sample (150 μl) was mixed with 2850 μl of FRAP solution and kept for 30 min in dark at room temperature. The ferrous tripyridyltriazine complex (colored product) was measured by reading the absorbance at 593 nm. Sample blank was prepared by excluding $FeCl_3$ from FRAP solution and distilled water was used

instead. Trolox (0-500 μM) was used as the standard. The activity was expressed as mmol Trolox equivalents (TE)/g dry extract.

4.3.6 Thermal stability of cashew leaf extracts with different extraction processes

Five milliliters of CE-C and CE-US solutions (0.125 mg/ml) were placed in a temperature-controlled water bath (W350, Memmert, Schwabach, Germany) at 60, 80 and 100 ˚C for 15, 30, 45 and 60 min. At the designated time, the treated samples were cooled rapidly in iced water. The sample without heat treatment was used as the control. Residual DPPH and ABTS radical scavenging activities and FRAP were determined.

4.3.7 pH stability of cashew leaf extracts with different extraction processes

pH stability was determined following the method of Klompong and Benjakul (2015). CE-C and CE-US solutions at the concentration of 0.125 mg/ml (5 mL) were prepared using 100 mM acetate buffer (pH 2 and 4) or 100 mM Tris HCl buffer (pH 6, 8 and 10). The extract solutions were allowed to stand for 30 min, followed by adjusting to pH 7 with 1 and 6 M HCl or 1 and 6 M NaOH. The final volumes of all solutions were brought up to 25 ml using deionized water. Residual antioxidative activities were examined.

4.3.8 Effect of cashew leaf extracts with different extraction methods on prevention of lipid oxidation in the selected model systems

4.3.8.1 Lecithin liposome system

Lecithin liposome system was determined as described by Thiansilakul *et al.* (2007). Lecithin was suspended in deionized water at a concentration of 8 mg/ml by stirring with a glass rod, followed by sonicating for 30 min using a sonicating bath (Model Transsonic 460/ H, Elma, Germany). CE-C or CE-US solutions (3 ml) at concentrations of 0, 50 and 100 ppm were added into the

lecithin liposome system (15 ml). The mixtures were sonicated for 2 min. To initiate the assay, 20 µl of cupric acetate (0.15 M) were added. The mixtures were shaken at 120 rpm using a shaker at 37 ˚C in the dark. The control (without extract) and the system containing BHT (100 ppm) were also prepared. Oxidation of the liposome was monitored every 6 h for totally 36 h by determining the conjugated dienes (CD) (Heinonen *et al.*, 1998) and thiobarbituric acid reactive substances (TBARS) (Buege and Aust, 1978).

4.3.8.2 β-carotene-linoleic acid system

β-carotene-linoleic acid assay was carried out as per the method of Taga *et al.* (1984). A stock solution of β-carotene-linoleic acid was prepared by mixing 10 mg of β-carotene with 10 ml of chloroform. Thereafter, the solution (0.2 ml) was added to 20 mg linoleic acid and 200 mg Tween 40. Chloroform was completely removed by purging with nitrogen. Then, 50 ml of oxygenated deionized water were added to β-carotene emulsion and mixed well. Thereafter, 500 µl of CE-C or CE-US solutions were added into 4.5 ml of emulsion to obtain the final concentrations of 50 and 100 ppm. BHT (100 ppm) was also added. The control was prepared in the similar manner except that distilled water was used instead of the extracts. During the incubation at 50 ˚C, samples were taken at various times designated (0, 10, 20, 30, 40, 50 and 60 min). The absorbance at 470 nm was then measured using a spectrophotometer. Retardation of decrease in A_{470} indicates the ability of the extracts in prevention of oxidation.

4.3.9 Statistical analysis

All experiments were run in triplicate. Factorial design with 2 factors was used for experimental design. Data were subjected to analysis of variance (ANOVA), and mean comparisons were carried out using the Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the statistical package for social sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

4.4 Results and discussion

4.4.1 Antioxidative activities of cashew leaf extracts with different extraction methods as affected by concentrations

4.4.1.1 DPPH radical scavenging activity

DPPH radical scavenging activity of CE-C and CE-US at various concentrations is shown in Figure 11A. Both extracts had the increase in DPPH radical scavenging activity as the concentration increased up to 0.1 mg/ml (P<0.05). Nevertheless, the activity of both CE-C and CE-US decreased when the concentration of 0.2 mg/ml (P<0.05) was used. Similar results were observed for BHT and catechin. However, there was no difference in activity of BHT at concentrations of 0.1 and 0.2 mg/ml (P>0.05). It was noted that both extracts and catechin at high concentration (0.2 mg/ml) had the decrease in activity. At high concentration, phenolic compounds plausibly interacted each other, especially via hydroxyl group. As a result, the proton or electron donating ability was lowered. At the same concentration used, similar activity was observed between CE-C and CE-US (P>0.05), except at 0.2 mg/ml, in which the former showed a slightly higher activity. The lowest activity was found for BHT, while catechin showed the highest DPPH radical scavenging activity (P<0.05). The higher DPPH radical scavenging activity of catechin than BHT was probably due to the higher numbers of hydroxyl groups of the former. The substitution of hydroxyl with methyl groups in the aromatic ring more likely contributes to lower antioxidant activities in BHT (Brewer, 2011). Additionally, the polyphenols with a second hydroxyl group in the ortho or para positions such as catechol and hydroquinone had the increased antioxidative activity due to the strong electron donation ability of the hydroxyl group in these positions (Ali *et al.*, 2013). Catechin was found as the dominant phenolic in the ethanolic extract of cashew leaves (Chotphruethipong *et al.*, 2017a; Chotphruethipong *et al.*, 2017b). Furthermore, Chotphruethipong *et al.* (2017b) reported that there were some additional phenolic compounds in the extract, especially quercetin, when ultrasound-assisted process was used. Radical scavenging activity of flavonoids toward DPPH radicals is dependent on the number of hydroxyl groups in the B ring (Loganayaki *et al.*, 2013). Three structural groups are important

determinants for radical scavenging activity. Those include *o*-dihydroxyl groups in the B-ring, the 4-oxo group in conjugation with the 2,3-alkene, and the 3- and 5-hydroxyl groups in *o*-position in B-ring (Hollman and Katan, 1997). These functional groups can donate electrons to the rings, which increase the number of resonance forms available in addition to those created by the benzene structure (Mariani *et al.*, 2008). Thus, the presence of hydroxyl groups in catechin and cashew leaf extracts most likely contributed to DPPH radical scavenging activity. Also, the extraction methods had no marked impact on DPPH radical scavenging activity of resulting extracts.

4.4.1.2 ABTS radical scavenging activity

ABTS radical scavenging activity of CE-C and CE-US at various concentrations in comparison with BHT and catechin is shown in Figure 11B. All samples had the increase in ABTS radical scavenging activity as the concentrations increased and reached the maximum at 0.1 mg/ml (P<0.05). Nevertheless, BHT had the continuous increase in activity with increasing concentrations (P<0.05). Catechin exhibited the highest ABTS radical scavenging activity as compared to other samples at the same concentrations tested (P<0.05). However, catechin exhibited the similar activity to CE-US at 0.1 mg/ml (P<0.05). Similar to the results of DPPH radical scavenging activity, cashew leaf extracts and catechin were more effective in ABTS radical scavenging, compared to BHT (P<0.05) when the concentration range of 0.025-0.1 mg/ml was used. The ability in scavenging free radical of phenolics was related to their active hydrogen donor ability. When comparing activity between CE-C and CE-US, the latter had higher activity at the concentrations of 0.05 mg/ml and 0.1 mg/ml, while no differences in ABTS radical scavenging activity between 0.025 mg/ml and 0.2 mg/ml were observed (P>0.05). The difference in activity between both extracts was plausibly due to the difference in tannic acid content. Higher tannic acid content was reported in CE-US (281 mg/kg dry extract) than CE-C (189.7 mg/kg dry extract) (Chotphruethipong *et al.*, 2017a; Chotphruethipong *et al.*, 2017b). Hagerman *et al.* (1998) reported that tannic acid is able to quench the $ABTS^{\bullet+}$ radical and the effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl groups' substitution. The lower ABTS radical scavenging

activity of both cashew leaf extracts and catechin at higher concentration contributed to the lower efficiency in prevention of lipid oxidation. The result suggested that the concentration of extract was an essential factor affecting antioxidative activity.

4.4.1.3 FRAP

FRAP of different extracts, BHT and catechin at various concentrations is presented in Figure 11C. Among all samples tested, catechin possessed the highest FRAP (P<0.05) at all concentrations tested, indicating that catechin could reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) complex most effectively. Overall, the increases in FRAP were noticeable for CE-C and CE-US as the concentrations increased up to 0.1 mg/ml (P<0.05). FRAP of both extracts decreased when concentration was above 0.1 mg/ml. For catechin, FRAP increased when the concentration increased up to 0.05 mg/ml (P<0.05). Decrease in FRAP was found at higher concentrations (0.1 and 0.2 mg/ml). These results indicated that reducing power of catechin and cashew leaf extracts was decreased at high concentration. However, continuous increases in FRAP were found for BHT with increasing concentrations. When comparing FRAP between both cashew leaf extracts, CE-US had higher FRAP than CE-C, except at 0.2 mg/ml (P<0.05), reflecting that extraction methods influenced FRAP of resulting extracts. CE-US could donate an electron to free radicals more effectively than CE-C, leading to the prevention or retardation of propagation (Klompong *et al.*, 2007).

4.4.2 Thermal stability of cashew leaf extracts with different extraction methods

4.4.2.1 DPPH radical scavenging activity

DPPH radical scavenging activity of the extracts from cashew leaves using different extraction methods subjected to heating at various temperatures and times is shown in Figure 12A. For all temperatures tested, both extracts had the decrease in DPPH radical scavenging activity as heating time increased (P<0.05). At the same heating time, the highest activity was retained when heated at 60 ˚C for both CE-C and CE-US, compared with higher temperatures (P<0.05). The increased

temperature and time led to the higher degradation of phenolic compounds (Wong *et al.*, 2014). Within the first 30 min of heating time, there was no difference in activity of both extracts heated at 80 ˚C and 100 ˚C (P>0.05). Nevertheless, CE-US heated at 80 ˚C for 45 min had higher activity than that heated at 100 ˚C with the same heating time (P<0.05). Thereafter, the activity of CE-US was slightly decreased as heating time increased, while no change in activity of CE-C was found (P>0.05). CE-US had higher activity than CE-C when heated at 80 ˚C for 45 min (P<0.05). The result suggested that extraction methods influenced thermal stability of resulting extracts to some degree.

4.4.2.2 ABTS radical scavenging activity

ABTS radical scavenging activity of the extracts from cashew leaves prepared using different extraction methods after heating under various conditions is shown in Figure 12B. Both extracts, CE-C and CE-US, heated at 60 ˚C showed the highest remaining ABTS radical scavenging activity when heated for 15-60 min. CE-C heated at 60 ˚C had higher remaining activity than that heated at 80 ˚C and 100 ˚C (P<0.05). The result revealed that an increasing temperature negatively affected activity of the extracts as evidenced by lower remaining activity. The sharp decrease in activity was noticeable within the first 15 min of heating, regardless of heating temperature. For CE-US, the continuous decrease in activity was found when heated at all temperatures. Within the first 30 min, no differences in activity of CE-US heated at all temperatures tested (P>0.05). In general, the activity decreased as heating time increased (P<0.05) but the drastic decrease was noticeable within the first 15 min of heating. The decrease in activity of the extracts might be caused by the destruction of antioxidative compounds present in the extracts as heating time increased. This resulted in the loss in antioxidative activity. When comparing CE-C with CE-US, no difference in activity of both extracts heated at 60 ˚C for 15 min was found. Overall, heating with the time between 15 and 60 min had no marked effect on activity reduction. The results showed that antioxidative activity of cashew leaf extracts was reduced by 50% when heating at temperature up to 100 ˚C for 60 min.

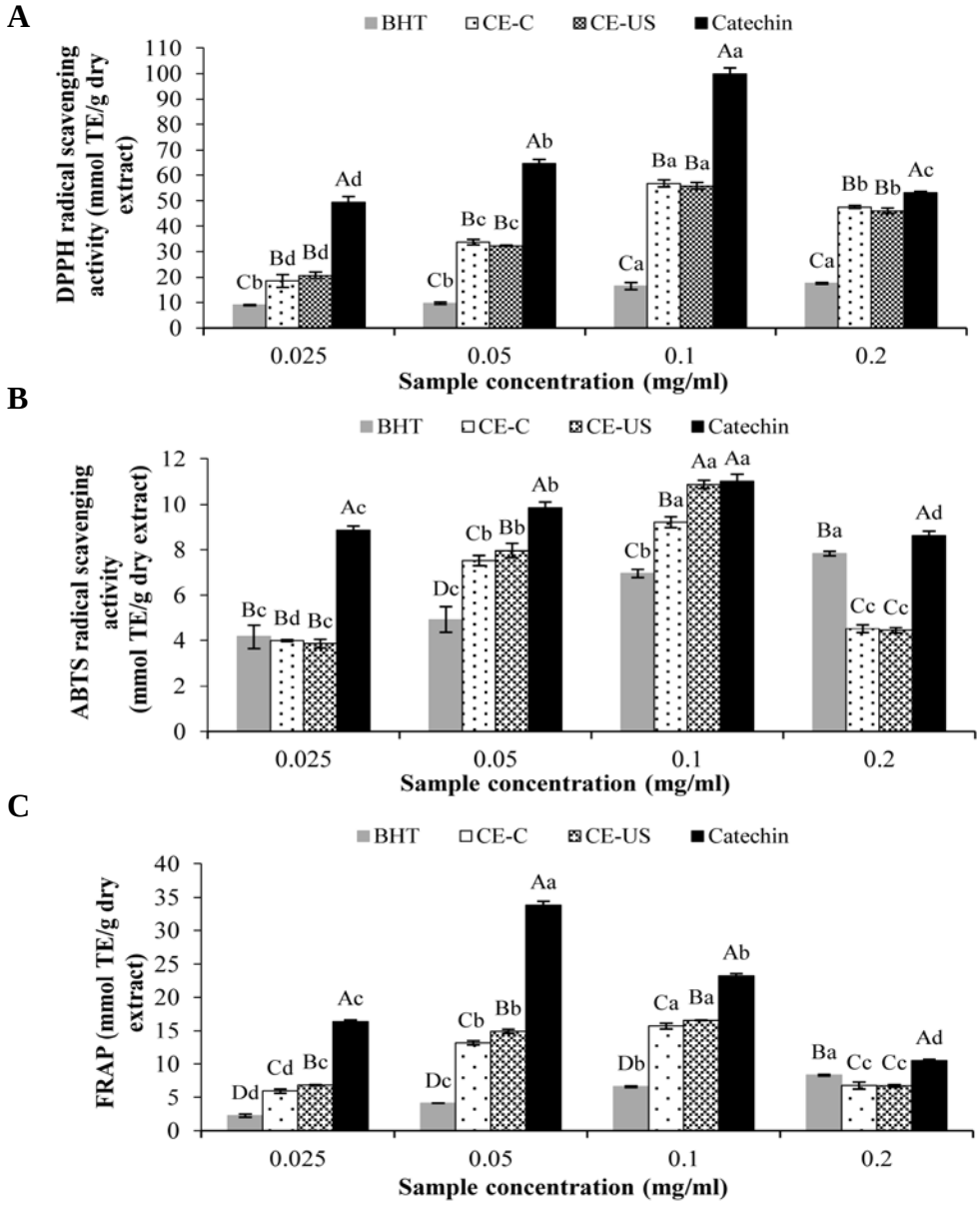


Figure 11 DPPH radical scavenging (A), ABTS radical scavenging (B) and FRAP (C) of the extracts from cashew leaves prepared using different processes at various concentrations. Bars represent standard deviation (n = 3). Different capital letters on bars within the same concentration indicate significant differences (P<0.05). Different small letters on bars within the same type of antioxidant indicate significant differences (P<0.05). CE-C and CE-US represent cashew leaf extract extracted using conventional extraction and ultrasound-assisted methods, respectively. BHT: Butylated hydroxytolulene

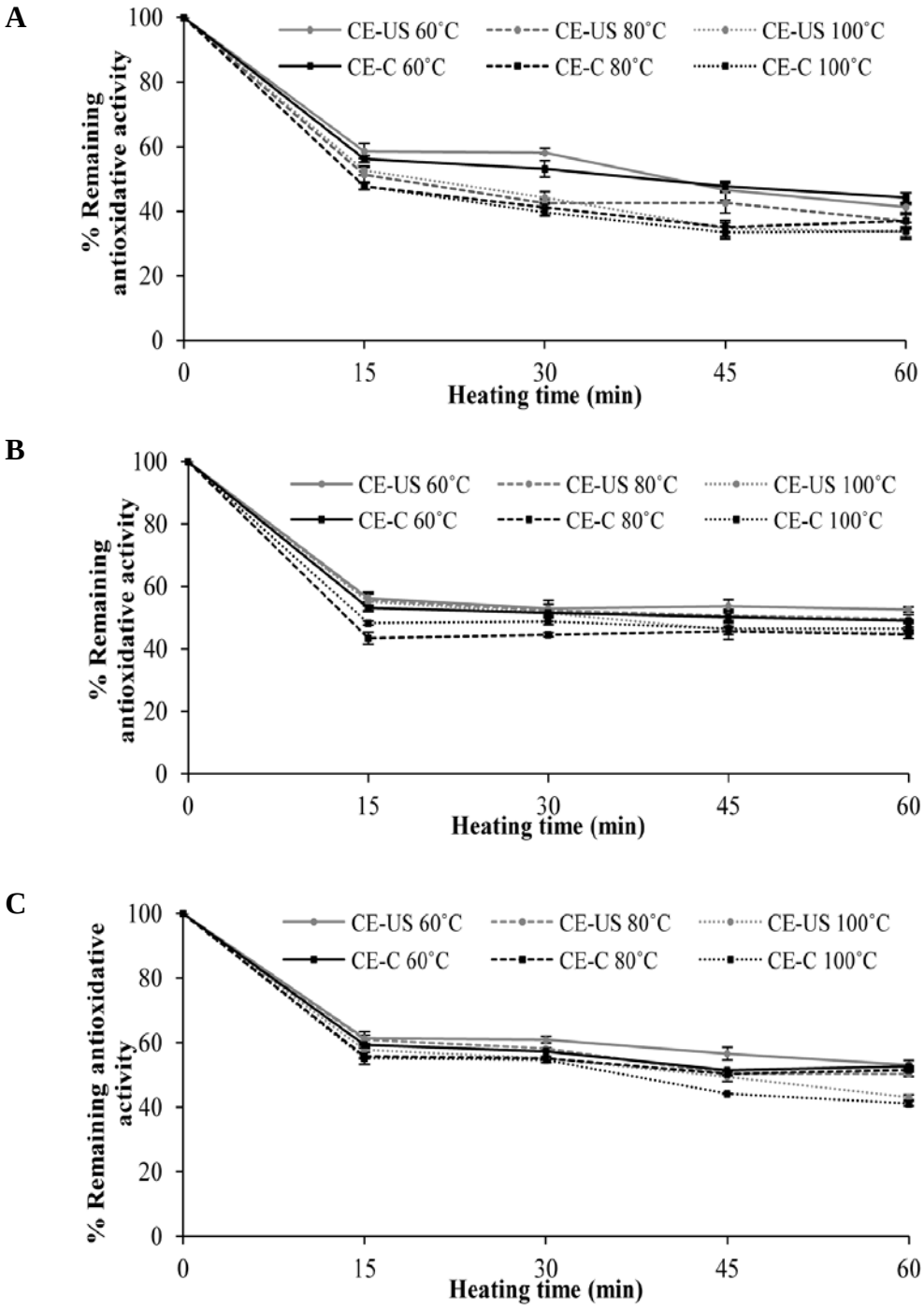


Figure 12 Thermal stability of the extracts from cashew leaves prepared using different processes as measured by DPPH radical scavenging (A), ABTS radical scavenging (B) and FRAP (C). Bars represent standard deviation (n = 3). CE-C and CE-US represent cashew leaf extract extracted using conventional extraction and ultrasound-assisted methods, respectively.

4.4.2.3 FRAP

After being heated under different conditions, FRAP of the extracts from cashew leaves using different extraction methods was monitored as shown in Figure 12C. Similarly, the decrease in FRAP was found as the heating time and temperature increased, especially within the first 15 min. Within the first 15 min of the heating, no difference in FRAP of both extracts was found, irrespective of heating temperature used (P>0.05). For the heating time above 15 min, both extracts heated at 60 ˚C had higher remaining activity than those heated at 80 ˚C and 100 ˚C (P<0.05). However, no difference in FRAP between CE-C heated at 80 ˚C and 100 ˚C was found (P>0.05). Moreover, similar in FRAP between both extracts heated at 80 ˚C and 100 ˚C for 45 min was obtained (P>0.05).When comparing CE-C with CE-US, the latter had a higher activity than the former when heated at 60 ˚C (30 and 45 min), 80 ˚C (15 and 30 min) and 100 ˚C (45 and 60 min) (P<0.05). Nwanguma and Eze (1996) found that an increase of mashing temperature from 65 to 75 °C resulted in a significant reduction in the polyphenolic content of wort. A decreased contents of total proanthocyanidins in cassava and Leucaena leaves (10.1 and 21.4%, respectively) were reported when heated at 90 °C for 24 h (Makkar and Singh, 1991). Thus, cashew leaf extracts could be used as natural antioxidants in thermally processed foods due to the remaining antioxidative activities after heating.

4.4.3 pH stability of cashew leaf extracts with different extraction methods

The effects of pH on antioxidative activities of both cashew leaf extracts are shown in Figure 13A-C. Both extracts exhibited strong activity at the pH ranging from 6 to 8 for all activities tested as examined by all assays. Most of phenolic compounds in both extracts were isoquercetin and catechin (Chotphruethipong *et al.*, 2017a; Chotphruethipong *et al.*, 2017b). The results was accordance with Milovanovic *et al.* (1994) who reported that flavonoids were more stable in the pH range of 7 to 10. The lowest activities were observed when subjected to pH 2 to 4, compared to other pHs (P<0.05). Moreover, the decreases in DPPH and ABTS radical scavenging activities were also found at pH 10 (P<0.05), while FRAP

of both extracts was quite stable. The loss in antioxidative activities of cashew leaf extracts at very acidic and alkaline pHs might be due to the decomposition of phenolic compounds. Phenolic compounds might undergo the conformational changes, leading to the loss in activity in scavenging DPPH and ABTS radicals as well as reducing power. When comparing CE-C with CE-US, the latter had a higher DPPH and ABTS radical scavenging activities than the former in pH range of 2-6 (P<0.05). There was no difference in DPPH radical scavenging activity between both extracts at pH 8 and pH 10 (P>0.05), while ABTS radical scavenging activity of CE-US was higher than CE-C at pH 10. For FRAP, the lower activity was found in CE-US in the pH range of 2-6. However, at pH 8, CE-US had higher FRAP than CE-C. No difference in FRAP at pH 10 was observed between both extracts (P>0.05). The results indicated that the extraction processes had an effect on pH stability to some extent. This might be governed by the different phenolics present in both extracts. Stability of plant extracts toward pH varied. Ruenroengklin *et al.* (2008) reported that an ethanolic extract from litchi fruit pericarp tissue had a higher antioxidant activity at pH 3-5 than at pH 1 or 7. The flavan-3-ols of cocoa were more stable at pH 6.5 than 7.4. (Kosińska *et al.*, 2012). Arabshahi-D *et al.* (2007) reported that the remaining antioxidant activity of the ethanolic extracts from mint leaves and carrot was higher at pH 9 than pH 4, while that of drumstick extract remained the same under both pH conditions. Thus, the stability of phenolic compounds in cashew leaf extracts was determined by pHs.

4.4.4 Effect of cashew leaf extracts on prevention of lipid oxidation in different model systems

4.4.4.1 Lecithin liposome system

Antioxidative activity of the CE-C, CE-US and BHT in lecithin liposome system was monitored during the incubation at 37 ˚C for 36 h by determining CD and TBARS as shown in Figure 14A and B, respectively. The systems containing antioxidants showed lower CD formation as compared to the control (P<0.05) (Figure 14A). Within the first 18 h of incubation, the control sample showed the highest increase in CD. Thereafter, CD formation of the control sample gradually decreased up to the end of the incubation (P< 0.05). In general, CD occurs

at the early stages of lipid oxidation and hydroperoxide was degraded to the secondary products as associated with an increase in TBARS (Frankel *et al.*, 1998). When comparing CD formation of all samples containing antioxidants, the lowest increase in CD was found in the sample containing BHT, indicating the high efficiency in retarding lipid oxidation. For the systems containing CE-C and CE-US, the antioxidative activity of both extracts was in a dose-dependent manner. However, the antioxidative activity of CE-C was lower than that of CE-US at the same concentration used. The differences in ability in inhibiting CD formation of the different antioxidants were probably due to the differences in hydrophobicity/hydrophilicity balance as well as their localization in emulsion system (Medina *et al*., 2007).

Changes in TBARS in lecithin liposome system in the absence and presence of different antioxidants are shown in Figure 14B. Systems containing antioxidants showed the lower TBARS formation as compared to the control ($P< 0.05$). When comparing TBARS value between the systems added with antioxidants, the highest antioxidative activity was found in the system added with BHT, as indicated by lower TBARS value. Between the systems containing CE-C and CE-US at the same concentration, the latter had lower TBARS value than the former ($P<0.05$). Furthermore, the efficiency in inhibiting lipid oxidation was also dependent on the concentration of the extracts used. It was indicated that the addition of antioxidants could retard the occurrence of TBRAS formation in the lecithin liposome system. Liposome is phospholipid forming bilayer vesicles with amphiphilic structure, and has been used to evaluate antioxidant activity of tested compounds in lipid food as well as lipoprotein particles. In liposome systems, the polar heads are exposed toward the aqueous phase. Phenolic compounds, which are most likely hydrophilic in nature, orient themselves at the bilayers of liposomes and act as effective antioxidants (Benjakul *et al.*, 2013). Moreover, antioxidative activity of phenolic compounds is dependent on many factors including the molecular size, location of antioxidant in the food, interactions with other food constituents, and the overall conditions of the food environment (pH, ionic strength as well as hydrophilic/lipophilic balance) (Decker *et al.*, 2005). The result suggested that cashew

leaf extracts could inhibit the early stages of lipid oxidation (CD formation) and retard the propagation of the oxidation process (degradation of hydroperoxide to TBARS) in a dose-dependent manner.

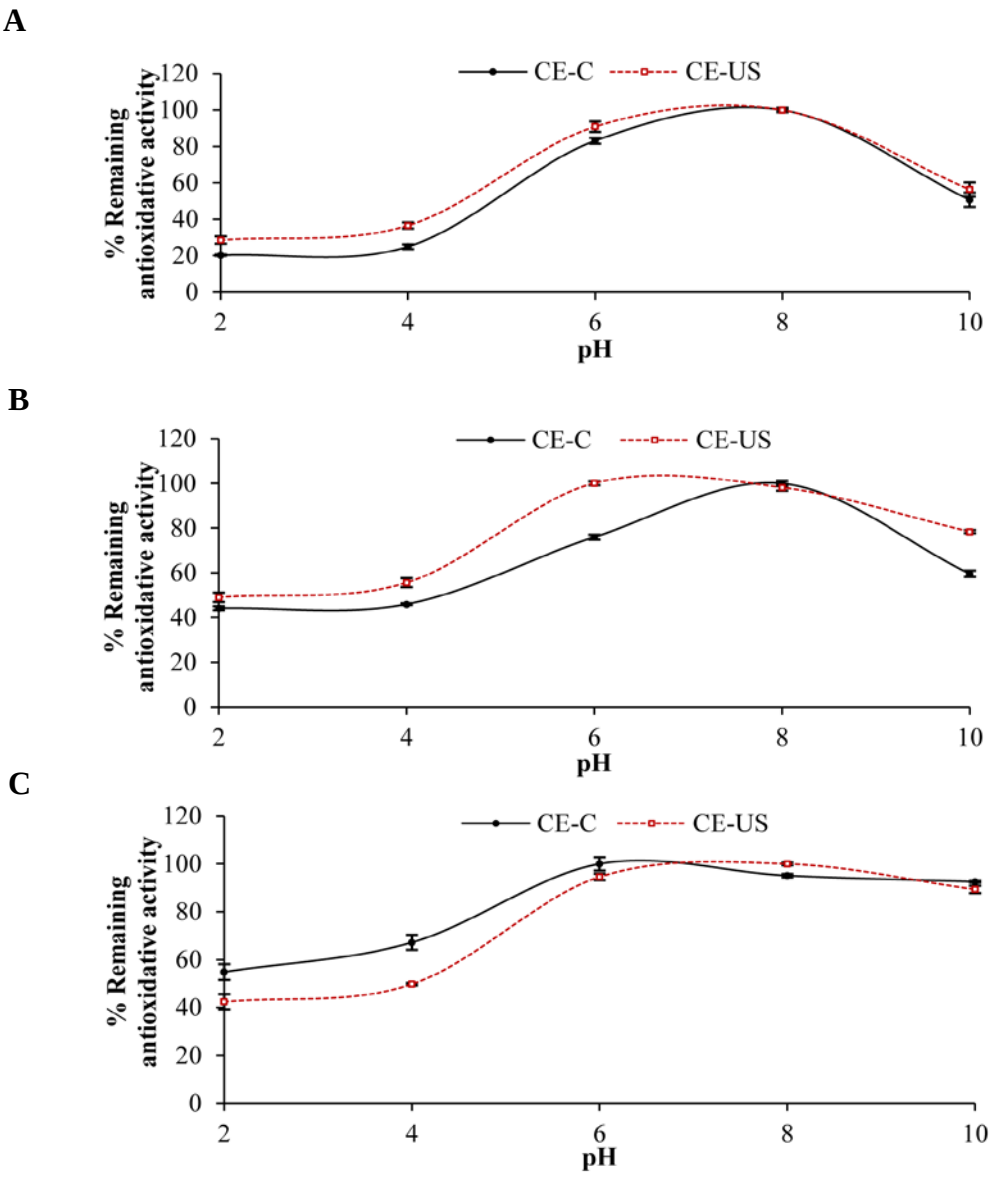
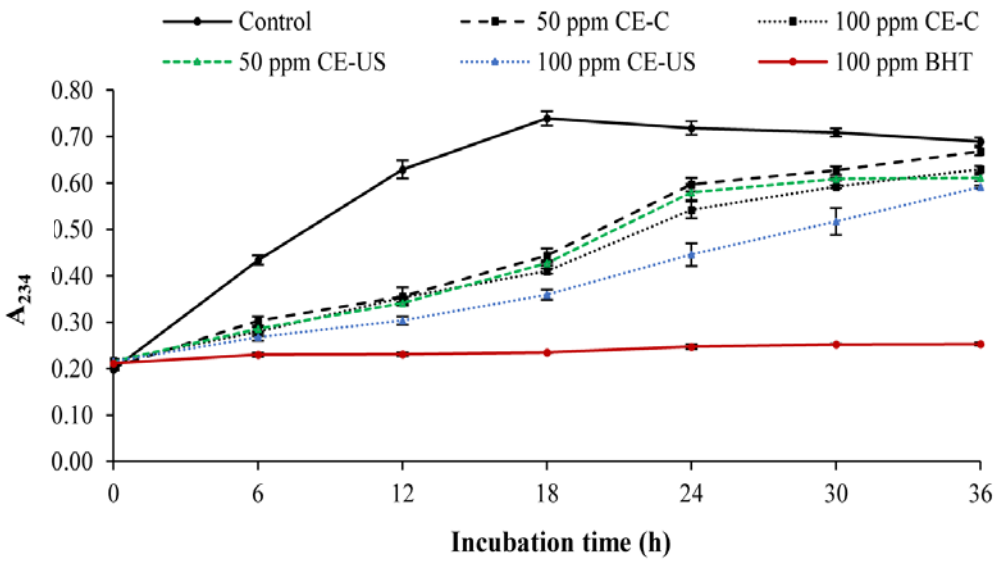


Figure 13 pH stability of the extracts from cashew leaves prepared using different processes as measured by DPPH radical scavenging (A), ABTS radical scavenging (B) and FRAP (C). Bars represent standard deviation (n = 3). CE-C and CE-US represent cashew leaf extract extracted using conventional extraction and ultrasound-assisted methods, respectively.

A



B

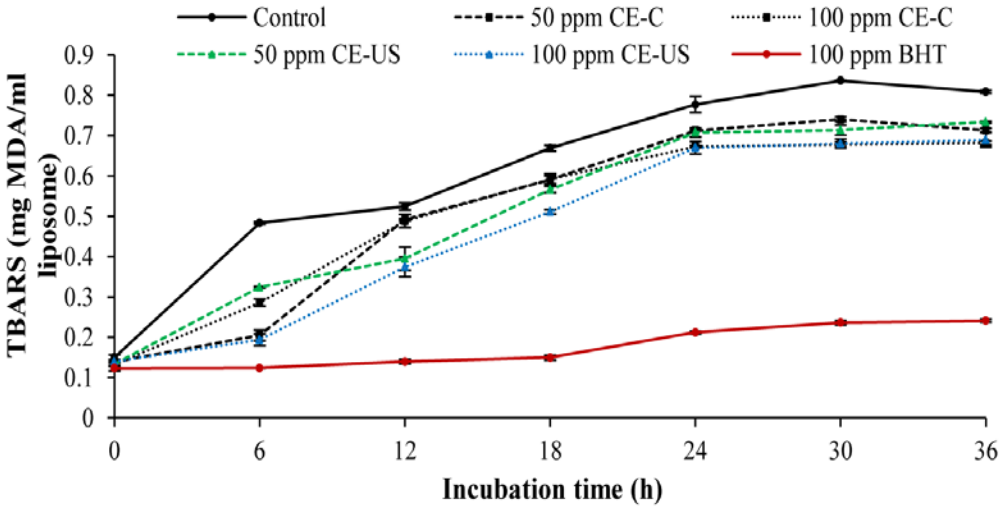


Figure 14 Changes in conjugated diene (CD) (A) and thiobarbituric acid reactive substances (B) of lecithin liposome system in the absence and presence of cashew leaf extracts prepared using different processes at various levels during the incubation at 37 ˚C for 36 h. Bars represent standard deviation (n = 3). CE-C and CE-US represent cashew leaf extract extracted using conventional extraction and ultrasound-assisted methods, respectively. BHT: Butylated hydroxytolulene

4.4.4.2 β-carotene-linoleic acid system

β-carotene-linoleic acid system containing cashew leaf extracts, CE-C and CE-US, or BHT had the lower decrease in A_{470} than the control during the incubation at 50 ˚C for 60 min (P<0.05) (Figure 15). Decrease in A_{470} was caused by lipid oxidation. The β-carotene was oxidized by the free radicals generated from oxidized linoleic acid during incubation at 50 °C. Those radicals could attack the β-carotene molecules. This resulted in the reduction in the absorbance at 470 nm (Kamath and Rajini, 2007). When comparing the ability in prevention of decrease in A_{470}, the higher antioxidative activity was found in BHT, compared with cashew leaf extracts (P<0.05), at the same concentration (100 ppm) used. This was possibly owing to the difference in their polarity and their location at interphase between linoleic acid and water. The hydrophobic antioxidants such as BHT have higher efficiency than hydrophilic antioxidant in the prevention of oxidation in oil-in-water emulsion systems by preferably orienting at oil-water interface (Chen, 1996; Yarnpakdee *et al.*, 2015). Conversely, cashew leaf extracts might have higher polarity than BHT. As a result, cashew leaf extracts were more likely located at the aqueous phase. This might be associated with the lower preventive effect toward the oxidation of emulsion by the extracts. At the same level of cashew leaf extracts tested, CE-US exhibited more efficiency in lowering the decrease in A_{470} than CE-C when the same level was used (P<0.05). However, there were no differences in the decreasing pattern in A_{470} between the system containing CE-C at 100 ppm and CE-US at 50 ppm (P>0.05). The efficiency of inhibition of lipid peroxidation might depend on the hydrogen donating ability of phenolic compounds and subsequent radical stabilization. Thus, the cashew leaf extracts could serve as natural antioxidants for the retardation of lipid oxidation in oil-in-water emulsions. Extraction method also determined the antioxidant activity of both extracts in emulsion system.

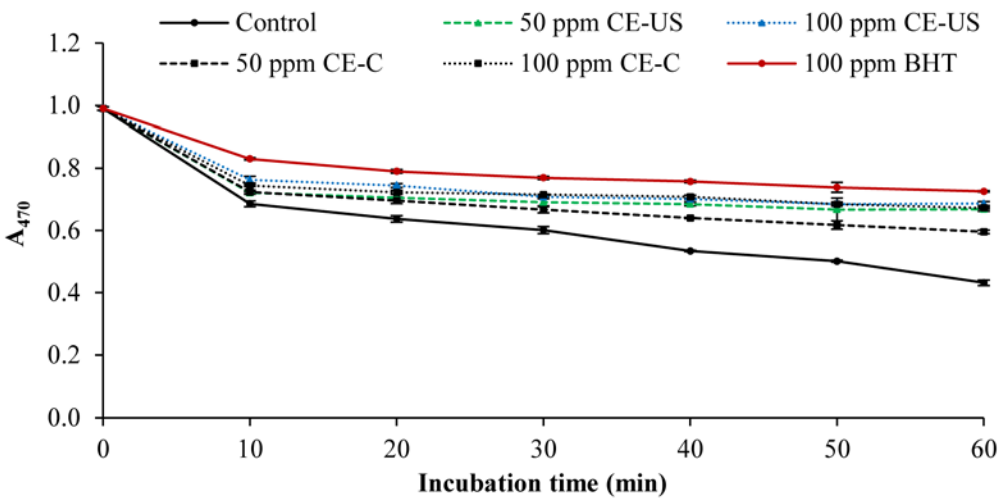


Figure 15 Changes in A_{470} of β-carotene-linoleic acid emulsion system in the absence and presence of cashew leaf extracts prepared using different processes at various levels during the incubation at 50 ˚C for 60 min. Bars represent standard deviation (n = 3). CE-C and CE-US represent cashew leaf extract extracted using conventional extraction and ultrasound-assisted methods, respectively. BHT: Butylated hydroxytolulene

4.5 Conclusion

Antioxidative activities of ethanolic extracts from cashew leaves were governed by extraction methods used. Both CE-C and CE-US exhibited higher antioxidative activities than BHT at the concentrations of 0.025-0.1 mg/ml. Antioxidative activities of both extracts were fairly stable after heating from 60 ˚C up to 100 ˚C for 60 min. Cashew leaf extracts had high stability in pH ranges of 6-8. Cashew leaf extracts were able to retard lipid oxidation in emulsion and liposome systems in a dose-dependent manner. CE-US had higher efficacy than CE-C in prevention of oxidation in all systems. Thus, cashew leaf extract could serve as potential natural antioxidant for food additive or as nutraceutical.

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CHAPTER 5

APPLICATION OF CASHEW (*ANACARDIUM OCCIDENTALE* L.) LEAF EXTRACT PREPARED USING ULTRASOUND-ASSISTED PROCESS FOR PREVENTION OF LIPID OXIDATION IN FISH OIL ENRICHED MAYONNAISE

5.1 Abstract

Effects of cashew leaf extract (CE) or BHT at levels of 100 and 200 ppm on the oxidative stability of fish oil enriched mayonnaise during storage of 30 days at 30 ˚C were investigated. Samples containing either CE or BHT had the lower peroxide value (PV), thiobarbituric acidreactive substances (TBARS) and *p*-anisidine (AnV) values throughout the storage, compared to the control (P<0.05). Among all samples, that containing 200 ppm BHT exhibited the lowest PV, TBARS and AnV values (P<0.05), while those added with 200 ppm CE and 100 ppm BHT showed similar values (P>0.05). At the end of storage, a lower abundance of selected volatile compounds was found in mayonnaise added with 200 ppm CE, in comparison with that of the control. Addition of CE, especially at 200 ppm, decreased L* value but increased a*, b* and ΔE^* values (P<0.05). However, CE at both levels were effective in retard the formation of rancid odor and fishy odor in the mayonnaise. Total viable count of all samples was below 10^3 CFU/g throughout the storage. Thus, the use of CE at the concentration of 200 ppm could retard lipid oxidation and the formation of fishy odor and rancid odor in developments of fish oil enriched mayonnaise during the storage of 30 days at 30 ˚C.

5.2 Introduction

Lipid oxidation is one of the major causes for deterioration of many food products, especially those containing long-chain polyunsaturated fatty acids (PUFA). These fatty acids are known to have several benefits for human health, e.g. to reduce the risks of cardiovascular diseases, carcinogenesis and allergies (Nettleton and Katz, 2005). However, PUFAs are prone to oxidation, which is associated with the changes in taste, odor, texture, appearance and shortened shelf-life. Moreover, it

also causes some diseases in human beings such as cardiovascular disease, cancer and neurological disorders and aging process (Gülçin, 2012).

Mayonnaise is an oil-in-water (o/w) emulsion having high fat content and egg yolk (Li *et al.*, 2014) Lipid oxidation of mayonnaise is generally initiated at the interface between the oil and water and progresses in the oil phase (Sørensen *et al.*, 2010). There are many factors that can potentially influence the rate of lipid oxidation in oil-in-water emulsions. Those include fatty acid composition, aqueous phase pH and ionic composition, type and concentrations of antioxidants, prooxidants, and oxygen (Waraho *et al.*, 2011). To prevent lipid oxidation in emulsion, synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and ethylenediaminetetraacetic acid (EDTA) are commonly used. Nevertheless, the use of synthetic antioxidants is limited in many countries. Natural antioxidants from plants have become the alternative antioxidants in the food industry. They are able to retard the oxidative degradation of lipids, improve the quality and nutritional value of food as well as prevent the occurrence of diseases (Shahidi and Ambigaipalan, 2015).

Cashew (*Anacardium occidentale* L.) is a member of the family *Anacardiaceae* (Razali *et al.*, 2008), which are abundant in the southern part of Thailand. Several parts of cashew tree, especially its leaves, contain phenolics with bioactivities, especially antioxidative activity. Chotphruethipong *et al*. (2017) reported that ethanolic cashew leaf extract showed antioxidative activity in a dose dependent manner. Additionally, it has been reported that cashew leaf extracts had antimicrobial activity against several microorganisms (Omojasola and Awe, 2004). Since cashew leaf extract had high antioxidative activity, it could be used as a natural antioxidant to retard lipid oxidation, especially in food emulsion enriched with PUFA, such as mayonnaise. However, no information regarding the use of cashew leaf extract in prevention of lipid oxidation in fish oil enriched mayonnaise exists. Thus, the present study aimed to examine the effect of cashew leaf extract on the retardation of lipid oxidation of fish oil enriched mayonnaise during storage of 30 days at 30˚C.

5.3 Materials and methods

5.3.1 Chemicals

Butylated hydroxytolulene (BHT) was obtained from (Guangzhou Huangpu Chemical Co., Ltd., Guangzhou, China). 1,1,3,3-tetramethoxypropane and 2-thiobarbituric acid were purchased from Fluka (Buchs, Switzerland). Ammonium thiocyanate, *p*-anisidine, and pyridine were procured from Sigma (St. Louis. MO, USA). Trichloroacetic acid, isooctane and ferrous chloride were obtained from Merck (Darmstadt, Germany). Chloroform, methanol and hydrochloric acid were purchased from Lab-Scan (Bangkok, Thailand).

5.3.2 Collection of cashew leaves and preparation of cashew leaf extract

5.3.2.1 Collection of cashew leaves

Cashew (*Anacardium occidentale* L.) leaves were collected from an orchard in Songkhla province, Thailand during November and December 2016. The trees were approximately 15-20 years old. Leaves from III (apical) to V (basal) of twig were used. After collection, cashew leaves were prepared following the method of Váquez-Torres *et al.* (1992) with slight modifications. The samples were washed with tap water and dried overnight in an air dryer at 50˚C until the moisture content was less than 10%. Dried samples were blended using a blender (Panasonic, Model MX-898N, Berkshire, UK) and sieved using a stainless steel (sieve 80 mesh). Thereafter, the obtained powder was subjected to chlorophyll removal. The sample powder was mixed with chloroform to obtain a concentration of 50 g/l and the mixture was stirred for 30 min, followed by filtration using a Whatman filter paper No.1 (Whatman International Ltd., Maidstone, UK) (Chotphruethipong *et al.*, 2017a). The retentate was dried in a hot air oven (Memmert, Schwabach, Germany) at 105˚C for 1 h. The obtained powder termed ‘cashew leaf powder’ was placed in a polyethylene bag and kept at room temperature until used for extraction.

5.3.2.2 Preparation of cashew leaf extract

The cashew leaf powder (10 g) was placed into a 250 ml beaker and 180 ml of 80% ethanol were added. Ultrasound-assisted extraction was performed using an ultrasonic equipment (Sonics, Model VC750, Sonica & Materials, Inc., Newtown, CI, USA) operating at a frequency of 20 kHz ± 50 Hz with high intensity power of 750 W. The temperature was controlled by the external water from a thermostatic water bath. After extraction for 64 min, the mixtures were centrifuged at 5000xg for 30 min at room temperature using a RC-5B plus centrifuge (Beckman, JE-AVANTI, Fullerton, CA, USA) and the supernatants was then filtered through a Whatman filter paper No.1 (Chotphruethipong *et al.*, 2017b). The filtrate was evaporated at 40 ˚C using an EYELA rotary evaporator (Tokyo Rikakikai, Co. Ltd., Tokyo, Japan). The extract was then dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). Dried extract was placed in a ziplock bag and kept in a desiccator until used.

5.3.3 Collection and extraction of fish oil from visceral depot fat of seabass

5.3.3.1 Collection of fish oil from visceral depot fat of seabass

Depot fat from viscera of seabass, collected from a market in Hat Yai, Songkhla province, Thailand, was dissected manually. The sample was placed in polyethylene bag and then embedded in ice with a fish/ice ratio of 1:2 (w/w), and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within 30 min. The sample was immediately washed with tap water, drained and used for lipid extraction.

5.3.3.2 Extraction of fish oil from visceral depot fat of seabass

Lipid was extracted according to the method of Sae-leaw and Benjakul (2017). Visceral depot fat (100 g) was placed in a bottom flask equipped with a rotary evaporator. The extraction was performed at 70˚C for 20 min under vacuum. After extraction, the oil was transferred into an Erlenmeyer flask containing 2-5g of anhydrous sodium sulfate, shaken well and decanted into a centrifuge tube through a

Whatman No.4 filter paper. The mixture was centrifuged at 10,000xg for 20 min at 4˚C using a refrigerated centrifuge (CR22N, Hitachi, Hitachi Koki Co., Ltd., Tokyo, Japan). The oil was collected using a Pasteur pipette. The oil sample was transferred into the amber bottle and purged with nitrogen gas. The vials were capped tightly and stored at -40˚C until used.

5.3.4 Preparation of mayonnaise

Mayonnaise was prepared using the following ingredients: soy bean oil (60.25%), fish oil (7.8%), egg yolk (6.78%), salt (0.5%), sugar (14.27%), lemon juice (5%), vinegar (3.8%) and water (1.6%). All the mixtures were blended using a blender (Panasonic, Model MK-GB1, New Taipei City, Taiwan) at a speed of 800 rpm for 15 min. Mayonnaise was divided into five portions. One portion was used as the control (without the cashew leaf extract or BHT). Another two portions were added with cashew leaf extract previously dissolved in distilled water to obtain the final concentrations of 100 and 200 ppm. The last two portions were incorporated with BHT at 100 and 200 ppm. After the cashew leaf extract or BHT were added, the mixtures were thoroughly mixed for 5 min in order to ensure uniformity. Different samples were transferred into glass jar and the lid was closed. The samples were kept at room temperature (30 ± 2˚C). At designated times (0, 5, 10, 15, 20, 25 and 30 days), the samples were taken for analyses.

5.3.4.1 Analyses

5.3.4.1.1 Measurement of peroxide value (PV)

PV was determined as per the method of Richards and Hultin (2002) with slight modifications. Mayonnaise (1 g) was homogenized at a speed of 13,500 rpm for 2 min in 11 ml of chloroform/methanol (2:1, v/v) using a homogenizer. Homogenate was filtered using Whatman No. 1 filter paper. To 7 ml of the filtrate, 2 ml of 0.5% NaCl were added. The mixture was vortexed at a moderate speed for 30 s and then centrifuged at 3000xg for 3 min at 4˚C using a refrigerated centrifuge to separate the sample into two phases. Twenty-five microliters of 30% (w/v) ammonium thiocyanate and 25 ml of 20 mM iron (II) chloride were added to 3

ml of lower phase. The reaction mixture was allowed to stand for 20 min at room temperature and the absorbance at 500 nm was read. The blank was prepared in the same manner, except that the distilled water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide at a concentration range of 0-100 ppm. PV was expressed as mg cumenehydroperoxide/kg sample after blank subtraction.

5.3.4.1.2 Measurement of thiobarbituric acid-reactive substances (TBARS)

TBARS was determined according to the method of Hamzeh and Rezaei (2012) with slight modifications. The sample (10 g) was homogenized with 50 ml of distilled water at a speed of 5000 rpm for 2 min. The prepared sample was transferred into distillation flask. Thereafter, 47.5 ml of water and 2.5 ml of 4 N HCl were added into the flask to bring the pH down to 1.5. An antifoaming agent (1 ml) and a few glass beads were added. The sample was distilled for 10 min to obtain 50 ml of the distillate. Five milliliters of the distillate were added with 5 ml of TBA solution containing 0.375% thiobarbituric acid (w/v), 15% trichloroacetic acid (w/v) and 0.25 M HCl. The mixture was heated in a boiling water bath (95-100 ˚C) for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3600xg at 25 ˚C for 20 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane at the concentrations ranging from 0 to 6 ppm. TBARS was calculated and expressed as mg malonaldehyde/kg sample.

5.3.4.1.3 Measurement of *p*-anisidine value

p-Anisidine value of the sample was determined according to the method of AOCS (AOCS, 1990). Sample (2 g) was added in 25 ml of isooctane. After mixing thoroughly, the sample was centrifuged at 5000xg for 10 min. The supernatant (5 ml) was mixed with 1 ml of 0.5% (w/v) *p*-anisidine in acetic acid for 10 min. The absorbance was read at 350 nm. The *p*-anisidine value was calculated using following equation:

$$p\text{-anisidine value} = 25 \times [(1.2 \times A_2 - A_1)/W]$$

where A_1 = A_{350} before adding *p*-anisidine, A_2 = A_{350} after adding *p*-anisidine and W= weight of sample (g).

5.3.4.1.4 Microbiological determination

Total viable count (TVC) was measured. Mayonnaise samples (25 g) were collected aseptically in a stomacher bag. After adding with 10 volumes of 8.5 g/l sterile saline solution, the samples were mixed using a Stomacher blender (Stomacher M400, Seward Ltd., Worthington, England) for 1 min and a series of 10-fold dilutions were made by the same diluent. TVC was determined by plate count agar (PCA) after the incubation at 35 ± 2 °C for 2 days (Fan *et al.*, 2008).

5.3.4.1.5 Color

Mayonnaise samples were subjected to color measurement using a CIE colorimeter (Hunter associates laboratory, Inc., VA, USA) with the port size of 0.75 inch. The color of the mayonnaise was expressed as L*-value (lightness), a*-value (redness/greenness), b*-value (yellowness/blueness) and total difference of color (ΔE^*) were calculated as follows (Ghanbarzadeh *et al.*, 2010):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, $\Delta a^*$and Δb^* are the differences between the corresponding color parameter of the sample and that of white standard (L* = 92.25, a* = -0.99 and b* = 1.78).

5.3.4.1.6 Sensory evaluation

Sensory evaluation of mayonnaise samples was conducted using multisample difference test (Meilgaard *et al.*, 2007). Four attributes evaluated included acetic acid odor, fishy odor, rancid odor and color. Evaluation was carried out at day 0, 10, 20 and 30 of storage. Twelve trained panelists with the age of 20-35 years were used. Prior to the evaluation, the panelists were trained three times a week with two sessions using a scale of 0 - 15, where 0 was no acetic acid, no rancidity, no fishy odor and light-yellow and 15 is the strongest acetic acid, the strongest rancidity, the strongest fishy odor and brown color, respectively. The panelists were asked to

open the sealable cup and sniff the headspace above the samples for odor evaluation (Yarnpakdee *et al.*, 2012).

5.3.4.1.7 Determination of volatile compounds

Sample at day 0 and the sample without the extract or BHT (control) and that added with the cashew leaf extract at a level of 200 ppm at day 30 were collected for analysis of volatiles.

5.3.4.1.7.1 Extraction of volatile compounds by SPME fibre

To extract volatile compounds, the samples (3 g) were homogenized at a speed of 13,500 rpm for 2 min with 8 ml of distilled water. The mixture was centrifuged at 2000xg for 10 min at 4 ˚C. The supernatant (6 ml) was heated at 60 ˚C with equilibrium time of 1 h in a 20 ml headspace vial. Finally, the SPME fiber (75 µm Carboxen^{TM}/PDMS StableFlex^{TM}; (Supelco, Bellefonte, PA, USA) was exposed to the head space of the vial containing the sample extract and the volatile compounds were allowed to absorb in the SPME fiber for 1 h. The volatile compounds were then desorbed in the GC injector port for 10 min at 250 ˚C.

5.3.4.1.7.2 GC-MS analysis

GC-MS analysis was performed using a GC mass spectrometer consisting of an Agilent 7890B gas chromatography equipped with a 7000D triple-quadrupole mass spectrometry detector (Agilent Technologies, Wilmington, DE, USA). Compounds were separated on VF-WAXms capillary column (30 m x 0.25 mm ID; 0.25 µm film thickness, Agilent, USA). Helium was used as the carrier gas, with a constant flow of 1.5 ml/min and the injection was performed in the splitless mode. The GC oven temperature program was: 35 ˚C for 3 min, followed by an increase of 3 ˚C /min to 70 ˚C, then an increase of 10 ˚C /min to 200 ˚C and hold for 5 min and finally an increase of 20 ˚C /min to a final temperature of 250 ˚C and hold for 5 min. Transfer line temperature was maintained at 250 ˚C. Mass spectrometer conditions were: electronic ionization (EI) mode at 70 eV; source temperature: 230 ˚C; quad temperature: 150 ˚C; scanning rate 0.220s/scan; mass range: 10-200 amu.

5.3.4.1.7.3 Analysis of volatile compounds

Identification of volatile compounds was performed, based on their retention times, compared with the mass spectra of reference compounds in the Wiley Mass Spectral Libraries (version 9). Selected volatile compounds identified related to lipid oxidation, included aldehydes, alcohols, ketones, etc., and were expressed as abundance of each identified compound. Analyses were done in duplicate.

5.3.5 Statistical analysis

All experiments were run in triplicate. Factorial design with 2 factors was used for experimental design. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using the Duncan's multiple range test. Statistical analysis was performed using the statistical package for social sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

5.4 Results and discussion

5.4.1 Effect of cashew leaf extract on oxidative stability of fish oil enriched mayonnaise during storage

5.4.1.1 Peroxide value (PV)

Changes in PV of fish oil enriched mayonnaise without and with the addition of cashew leaf extract or BHT at levels of 100 and 200 ppm during storage of 30 days at 30 ˚C are depicted in Figure 16A. At all storage times, the control sample (without antioxidants) had the higher PV than those containing cashew leaf extract or BHT (P<0.05). All samples had the marked increases in PV during the first 15 days of storage (P<0.05). After 15 days of storage, a continuous decrease in PV was found in the control with increasing storage time (P<0.05). On the other hand, other samples had the increase in PV up to 25 days (P<0.05), followed by the slight decrease till the end of storage time (P<0.05). The decrease in PV was due to the decomposition of hydroperoxide to the secondary oxidation products (Chaijan *et al.*, 2006). In general, the mayonnaise is susceptible to lipid oxidation due to their large surface area that

facilities the interactions between the oil and water-soluble prooxidants (Gorji *et al.*, 2016). Moreover, the pH is the main factor affecting lipid oxidation in fish oil enriched mayonnaise. In mayonnaise, egg yolk, used as the emulsifier, contained a large amount of iron (734 µM) (Jacobsen, 1999). The iron forms cation bridges between the protein phosvitin and other components at pH 6. At the low pH (3.8-4) found in the mayonnaise, the iron bridges between phosvitin, lipovilelin and low-density lipoprotein (LDL) are destroyed and the iron is released, leading to the increased lipid oxidation (Jacobsen, 1999). When comparing PV of all samples containing antioxidants, the lowest increase in PV was found in the sample added with BHT at the concentration of 200 ppm, indicating the high efficiency in retarding lipid oxidation. Differences in PV between mayonnaises added with different antioxidants possibly resulted from differences in their polarity as well as localization of antioxidants in emulsion system. Recently, Chotphruethipong *et al.* (2017b) reported that catechin and isoquercetin were found as the dominant phenolic in cashew leaf extracts. These phenolics were more polar antioxidants, as compared to BHT. The hydrophobic antioxidants could have higher efficiency than hydrophilic antioxidant in the prevention of oxidation in oil-in-water emulsion systems by preferably orienting at oil-water interface and functioned as a hydrogen donor or radical scavenger (Yarnpakdee *et al.*, 2015). Moreover, the efficiency in inhibiting lipid oxidation was also dependent on the concentration of the extracts used. The results indicated that BHT and cashew leaf extract at 200 ppm lowered PV more effectively than those at 100 ppm ($P<0.05$). Thus, the concentration of antioxidants used was an essential factor determining the efficiency in inhibiting lipid oxidation, especially at the early stage.

5.4.1.2 TBARS

Changes in TBARS in fish oil enriched mayonnaise in the absence and presence of different antioxidants at various concentrations are shown in Figure 16B. TBARS values of all samples increased when storage time increased ($P<0.05$). The control sample showed the higher TBARS values, compared to the samples containing antioxidants ($P<0.05$). After the first 5 days of storage, all samples had a marked increase in TBARS up to 15 days ($P<0.05$). Subsequently, TBARS values of

all samples were slightly increased up to the end of storage (P<0.05). Increases in TBARS value indicated the decomposition of hydroperoxides into the secondary products (Coupland *et al.*, 1996). Hydroperoxides are decomposed to malonaldehyde, which contributes to off-flavor of oxidized lipids (Zhang *et al.*, 2013). Among all samples containing antioxidants, the samples added with BHT at 200 ppm exhibited stronger oxidative stability than those added with BHT at 100 ppm or cashew leaf extracts at both concentrations (P<0.05). The differences in ability in inhibiting oxidation of the different antioxidants were probably owing to the differences in hydrophobicity/hydrophilicity balance as well as their localization in emulsion system (Yarnpakdee *et al.*, 2015). When comparing TBARS values between the sample added with cashew leaf extract at 100 ppm and 200 ppm, the latter had lower TBARS than the former throughout 30 days of storage (P<0.05). The result suggested that the efficiency in inhibiting lipid oxidation depended on the concentration of extracts used. Chotphruethipong *et al.* (2017a) reported that cashew leaf extract was able to donate hydrogen atom to free radicals, and could inhibit the propagation chain reaction during lipid oxidation process. Also, the extract was able to chelate the metal, known as prooxidant (Chotphruethipong *et al.*, 2017a). Thus, cashew leaf extract and BHT at the levels of 200 ppm were effective in retarding lipid oxidation in fish oil enriched mayonnaise at 30˚C for 30 days.

5.4.1.3 *p*-Anisidine value (AnV)

AnV of fish oil enriched mayonnaise without and with antioxidants at different concentrations during storage of 30 days at 30 ˚C is shown in Figure 16C. Overall, a similar trend was observed in comparison with TBARS values. AnV of all samples increased as the storage time increased (P<0.05). The control sample had the highest AnV, as compared to the samples containing antioxidants throughout 30 days of storage (P<0.05). At day 0, there was no differences in AnV between the samples containing antioxidants (P>0.05). Moreover, there were no differences in AnV between the samples added with BHT and cashew leaf extract at the concentration of 200 ppm at day 30 (P>0.05). Cashew leaf extract showed the antioxidative activity in mayonnaise in a dose dependent manner (P<0.05). The increase in AnV indicated the formation of the secondary lipid oxidation products, mainly non-volatile compounds

(principally 2-alkenals and 2,4-alkadienals) in lipids (Choe and Min, 2006). When comparing AnV of the samples containing antioxidants, the lowest AnV was observed for the sample added with BHT at the concentration of 200 ppm (P<0.05), whereas the highest AnV was found in the sample added with cashew leaf extract at 100 ppm (P<0.05). During emulsification, oxidation could take place, especially in the absence of antioxidant. This was shown by the higher PV, TBARS and AnV values in the control at day 0. Thus, cashew leaf extract, especially at 200 ppm, could be used as a natural antioxidant to retard the formation of non-volatile lipid oxidation products in fish oil enriched mayonnaise during the extended storage.

5.4.2 Effect of cashew leaf extract on microbial load of fish oil enriched mayonnaise during storage

TVC of all samples was below the recommended values of the Thai Industrial Standard (1997) (TVC $<10^3$ CFU/g) during 30 days of the storage (data not shown). Organic acids used as ingredients in mayonnaise including acetic acid and citric acid could lower the pH and acted as antimicrobial agents (Hinton Jr, 2006). The concentration of acids used was more likely sufficient to inhibit the microbial growth. In general, organic acids are effective food preservatives by lowering the pH of food products to levels that can inhibit bacterial growth (Beales, 2004). Organic acids are able to penetrate cell membranes of microorganisms and intracellular dissociation, resulting in acidification of the cytoplasm and intracellular acid anion accumulation to toxic levels (Hinton Jr, 2006). Moreover, the addition of cashew leaf extract in mayonnaise might result in the reduction of microbial count. It has been reported that cashew leaf extract had antimicrobial activity against *Porphyromonas gingivalis*, *Prevotella intermedia*, *Escherichia coli*, *Shigella dysenteriae*, *Salmonella* Typhimurium, *Stapphylococcus aureus* and *Pseudomonas auroginosa* (Varghese, 2013). Thus, the use of organic acids such as citric acid and acetic in combination with cashew leaf extract plausibly inhibited the microbial growth, leading to the extension of shelf-life and quality maintenance of fish oil enriched mayonnaise.

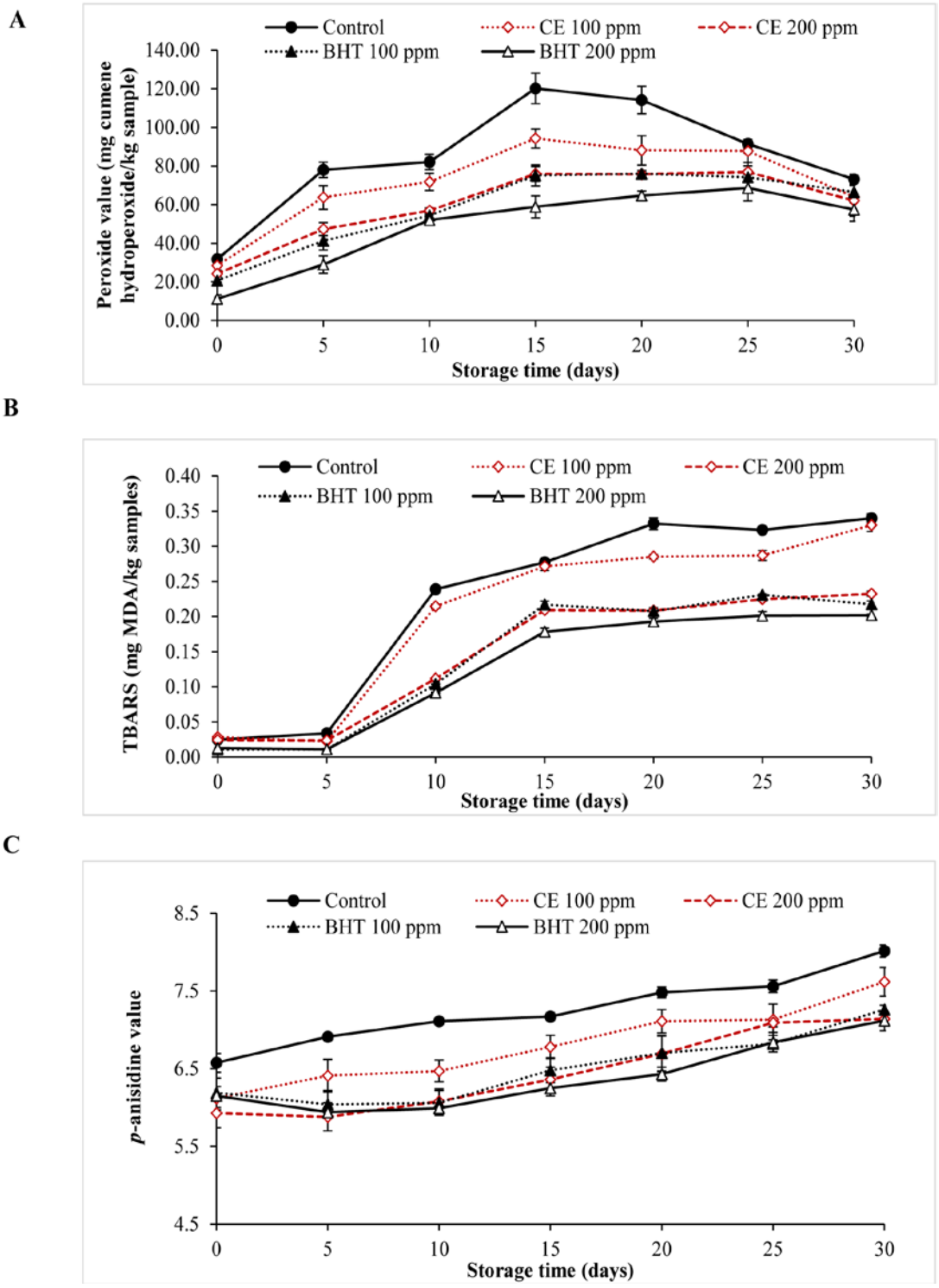

Figure 16 Changes in peroxide value (PV) (A), thiobarbituric acid reactive substances (TBARS) (B) and *p*-Anisidine value (AnV) (C) of fish oil enriched mayonnaise without and with cashew leaf extract or BHT at various levels during the storage at 30±2˚C for 30 days. Bars represent standard deviation (n = 3). CE: cashew leaf extract and BHT: Butylated hydroxytolulene.

5.4.3 Effect of cashew leaf extracts on color and sensory properties of fish oil enriched mayonnaise during storage

Color changes in lightness (L*), redness (a*), yellowness (b*) and total color difference (Δ*E**) of fish oil enriched mayonnaise added without and with antioxidants at various concentrations during storage of 30 days at 30 ˚C are shown in Table 14. An increase in the storage time resulted in the decrease in L* value of all samples tested ($P<0.05$). On the other hand, a*, b*and Δ*E**values of all samples increased when the storage time increased ($P<0.05$). These discolorations were probably associated with lipid oxidation products generated during storage, which might serve as the source of carbonyl compounds for non-enzymatic browning reaction as well as polymerization of oxidized phenolic compounds (Bharate and Bharate, 2014). Moreover, the addition of cashew leaf extract resulted in the decrease in lightness and an increase in redness as well as Δ*E**values ($P<0.05$). Δ*E** values of the sample containing cashew leaf extract were higher than those of other samples ($P<0.05$). This might be due to pigments present in cashew leaf extract, which most likely contributed to the changes in color of mayonnaise during storage. Altunkaya *et al.* (2013) reported that the addition of grape seed extract decreased in lightness and yellowness of the mayonnaise samples, while increased redness. Moreover, the intensity of color might depend on the amount of the extract added. The result revealed that the mayonnaise added with 200 ppm cashew leaf extract had higher decrease in L* value and higher increases in a* and b* values than those of the mayonnaise containing cashew leaf extract at the lower concentration. At the end of storage, the highest L* value and the lowest a* value were observed in mayonnaise added with BHT at the level of 200 ppm ($P<0.05$), while the lowest L* value and the highest a*, b* and Δ*E** values were found in the sample added with cashew leaf extract at 200 ppm ($P<0.05$). No difference in b* and Δ*E** values between the samples containing BHT at both levels was found ($P>0.05$). Although, cashew leaf extract at high concentration could retard the lipid oxidation product as indicated by the lower PV and TBARS formations, the pigments in the extract might impact on color changes of mayonnaise, especially with extended storage. Chlorophyll underwent pheophytin at acidic condition.

Changes in sensorial characteristics including rancid, fishy and acetic acid odors as well as color of fish oil enriched mayonnaise without and with antioxidants at different concentrations are shown in Table 15. Within the first 10 days of storage, there were no differences in scores between samples for all odors evaluated (P>0.05). However, the sample containing cashew leaf extract at the level of 200 ppm showed the highest score of color, compared to other samples (P<0.05). The result indicated the changes in color of sample, in which brown color was developed. This was in accordance with the decrease in L* value of sample (Table 14). After day 20 of storage, the highest score of rancidity was observed in the control, as compared to other samples (P<0.05), indicating that lipid oxidation proceeded to higher extent. The result was in agreement with the TBARS values (Figure 16B), in which the higher value was found in the control sample (P<0.05). No difference in score of acetic acid odor was found among all samples tested throughout 30 days of storage (P>0.05). At the end of storage, the sample containing BHT or cashew leaf extract at both levels had lower intensity of fishy and rancid odors than the control (P<0.05). Also, there was no difference in both attributes among samples containing BHT and cashew leaf extracts at all levels added (P>0.05). Rancid odor was generally in accordance with rancidity odor. It was reported that lipid oxidation of fish lipid contributed to development of fishy odor (Yarnpakdee *et al.*, 2012). Thus, the addition of cashew leaf extracts in mayonnaise could prevent off-odor, especially rancidity. Nevertheless, the addition of cashew leaf extract negatively, affected the color of mayonnaise, particularly when the extract at high level was incorporated.

5.4.4 Effect of cashew leaf extract on volatile compounds of fish oil enriched mayonnaise during storage

Selected volatile compounds in fish oil enriched mayonnaise added without and with cashew leaf extract at 200 ppm at day 0 and day 30 of storage at 30 °C are presented in Table 16. Fish oil enriched mayonnaise had high content of linoleic acid (18:2), linolenic acid (18:3) eicosapentanoic acid (20:5) and docosahexaenoic acid (22:6), which are susceptible to oxidation (Depree and Savage, 2001). Sae-leaw and Benjakul (2017) reported that oil extracted from seabass visceral depot fat contained oleic acid (25.49%), palmitic acid (21.8%), linoleic acid (13.84

%), DHA (6.91%) and EPA (2.09%). In general, volatile compounds detected in mayonnaise at day 0 of storage were lower in abundance than those found at the end of storage (30 days). After storage, aldehydes were the most prominent volatiles detected in all samples. At day 0 of storage, pentanal and hexanal were found as the major compounds in the control sample. The result suggested that oxidation had already taken place before or during mayonnaise preparation. Other aldehydes including benzaldehyde, (*E*)-2-decenal, heptenal and (*E*)-2-hexenal were also detected at the low levels. Hexanal and heptenal were major compounds, which contributed to rancid odor and fishy odor (Yarnpakdee *et al.*, 2012). Moreover, volatile alcohols (1-methyl-4-(1-methylethenyl)-cyclohexanol), ketones (1-penten-3-one) and furans (2-ethyl-furan) were found at low contents at day 0. At the end of storage, lower formation of volatile compounds were observed in the sample containing cashew leaf extract, compared with the control sample. This result was coincidental with lower TBARS formation (Figure 16B), indicating that cashew leaf extract at a level of 200 ppm had high efficiency in preventing the formation of volatile lipid oxidation compounds. In the control, pentanal and hexanal were the predominant aldehydes. 2-hydroxy-3-pentanane and 1-octen-3-ol were the prevalent ketone and alcohol, respectively. Additionally, some additional volatile compounds were detected in the samples after storage. For the sample containing the cashew leaf extract, the lower abundance of aforementioned compounds was noticeable. Aldehydes, ketones and alcohols have been known to be associated with lipid oxidation (Sae-leaw and Benjakul, 2014), which might occur during storage. Several volatiles compounds in both samples have been shown to correlate with increased fishy and rancid off-flavor in fish oil enriched mayonnaise. Those included 3-methyl-butanal, 2-ethyl-furan, 1-penten-3-one, 1-octen-3-ol and *(E, E)*-2,4-heptadienal (Jacobsen *et al.*, 2000). Hexanal was a degradation product of linoleic acid hydroperoxide, which was responsible for grassy and fatty odor (Yu *et al.*, 2016). Unsaturated alcohols including 1-octen-3-ol and 1,5-octadien-3-ol also had impact on off-flavors, mushroom-like odor (Bader *et al.*, 2009). Furans were increased after 30 days of storage, especially for the control. Furans and their derivatives such as 2-ethylfuran and 2-pentylfuran are formed by the decomposition of 12-hydroperoxide of linolenate (18:3n-3), 14-hydroperoxide of eicosapentaenoate (20:5n-3) and 16-hydroperoxide of

docosahexaenoate (22:6n-3) (Maqsood and Benjakul, 2011). Thus, volatile compounds presented in fish oil enriched mayonnaise more likely affected their sensory properties, particularly odors and flavors. Nevertheless, their formation could be retarded by the addition of cashew leaf extract.

Table 14. Changes in L*, a*, b* and Δ*E** values of fish oil enriched mayonnaise without and with cashew leaf extract or BHT at various levels during storage of 30 days at 30 ˚C

Storage time (days)	Samples	L*	a*	b*	Δ*E**
0	Control	73.48 ± 0.01^{aA}	3.37 ± 0.04^{bG}	30.76 ± 0.39^{bcD}	34.81 ± 0.32^{cA}
	$CE_{100\ ppm}$	72.23 ± 0.16^{cA}	3.68 ± 0.04^{aE}	31.41 ± 0.60^{abE}	36.07 ± 0.53^{bE}
	$CE_{200\ ppm}$	72.03 ± 0.06^{dA}	3.66 ± 0.04^{aF}	31.84 ± 0.63^{aE}	36.52 ± 0.53^{aG}
	$BHT_{100\ ppm}$	73.42 ± 0.43^{aA}	3.37 ± 0.03^{bD}	29.97 ± 0.01^{cC}	34.18 ± 0.24^{cE}
	$BHT_{200\ ppm}$	72.95 ± 0.08^{bA}	3.35 ± 0.04^{bE}	30.45 ± 0.47^{cC}	34.84 ± 0.40^{cD}
5	Control	72.24 ± 0.12^{abB}	3.55 ± 0.04^{bF}	31.30 ± 0.01^{cD}	35.95 ± 0.08^{cB}
	$CE_{100\ ppm}$	71.57 ± 0.45^{cB}	3.73 ± 0.06^{aE}	33.65 ± 0.05^{bCD}	38.28 ± 0.29^{bD}
	$CE_{200\ ppm}$	71.59 ± 0.04^{cA}	3.74 ± 0.03^{aE}	34.43 ± 0.31^{aD}	38.92 ± 0.27^{aF}
	$BHT_{100\ ppm}$	72.48 ± 0.01^{aB}	3.51 ± 0.03^{bC}	30.28 ± 0.18^{dBC}	34.98 ± 0.15^{dDE}
	$BHT_{200\ ppm}$	71.91 ± 0.03^{bcB}	3.32 ± 0.06^{cE}	30.32 ± 0.32^{dBC}	$35.31\pm0.28d^{CD}$
10	Control	71.50 ± 0.03^{bC}	3.76 ± 0.02^{aE}	32.32 ± 0.11^{cC}	37.22 ± 0.14^{cC}
	$CE_{100\ ppm}$	70.84 ± 0.22^{cC}	3.86 ± 0.04^{aD}	33.45 ± 0.43^{bD}	38.53 ± 0.45^{bCD}
	$CE_{200\ ppm}$	69.24 ± 1.03^{cB}	3.84 ± 0.02^{aD}	34.86 ± 0.50^{aCD}	40.59 ± 0.25^{aD}
	$BHT_{100\ ppm}$	71.48 ± 0.01^{bC}	3.32 ± 0.19^{cD}	31.12 ± 0.24^{dB}	35.43 ± 0.15^{dD}
	$BHT_{200\ ppm}$	71.96 ± 0.03^{aB}	3.55 ± 0.02^{bD}	30.15 ± 0.61^{dABC}	35.99 ± 0.51^{dBCD}
15	Control	69.08 ± 0.02^{dD}	3.83 ± 0.02^{cD}	34.36 ± 0.14^{bAB}	40.27 ± 0.42^{bB}
	$CE_{100\ ppm}$	71.01 ± 0.05^{bC}	3.91 ± 0.01^{bCD}	34.42 ± 0.27^{bC}	39.25 ± 0.25^{cC}
	$CE_{200\ ppm}$	69.04 ± 0.02^{dC}	4.10 ± 0.01^{aC}	35.73 ± 0.22^{aC}	41.44 ± 0.18^{aE}
	$BHT_{100\ ppm}$	70.56 ± 0.04^{cD}	3.75 ± 0.03^{dB}	31.23 ± 0.61^{cB}	36.88 ± 0.48^{dC}
	$BHT_{200\ ppm}$	71.54 ± 0.37^{aC}	3.61 ± 0.02^{eC}	31.37 ± 0.29^{cABC}	36.41 ± 0.44^{dBC}

Table 14. (continued)

Storage time (days)	Samples	L*	a*	b*	Δ*E**
20	Control	68.16 ± 0.62^{bE}	3.88 ± 0.01^{bC}	33.54 ± 0.36^{bB}	40.16 ± 0.56^{bB}
	$CE_{100\ ppm}$	70.68 ± 0.54^{aC}	3.97 ± 0.02^{bC}	35.78 ± 0.55^{aB}	40.57 ± 0.67^{bB}
	$CE_{200\ ppm}$	68.43 ± 0.03^{bD}	4.03 ± 0.07^{aC}	36.82 ± 0.50^{aB}	42.66 ± 0.40^{aC}
	$BHT_{100\ ppm}$	69.01 ± 0.60^{bE}	3.76 ± 0.03^{bB}	32.42 ± 1.02^{bcA}	38.75 ± 1.16^{cAB}
	$BHT_{200\ ppm}$	70.98 ± 0.01^{aD}	3.71 ± 0.03^{bB}	31.86 ± 0.86^{cAB}	37.10 ± 0.71^{dB}
25	Control	67.49 ± 0.07^{bF}	4.05 ± 0.03^{cB}	33.93 ± 0.79^{bB}	40.89 ± 0.65^{cB}
	$CE_{100\ ppm}$	67.40 ± 0.02^{bD}	4.16 ± 0.03^{bB}	37.05 ± 0.54^{aA}	43.45 ± 0.43^{bA}
	$CE_{200\ ppm}$	65.02 ± 0.03^{cE}	4.53 ± 0.03^{aB}	38.18 ± 0.55^{aA}	45.79 ± 0.42^{aB}
	$BHT_{100\ ppm}$	67.40 ± 0.17^{bG}	3.85 ± 0.03^{dAB}	32.51 ± 0.50^{bcA}	38.35 ± 0.08^{dB}
	$BHT_{200\ ppm}$	68.52 ± 0.08^{aE}	3.82 ± 0.09^{dA}	32.06 ± 1.46^{cA}	38.71 ± 1.18^{dA}
30	Control	66.85 ± 0.04^{cG}	4.37 ± 0.02^{cA}	35.10 ± 0.65^{cA}	42.24 ± 0.49^{cA}
	$CE_{100\ ppm}$	66.14 ± 0.05^{dE}	4.57 ± 0.04^{bA}	37.07 ± 0.53^{bA}	44.25 ± 0.44^{bA}
	$CE_{200\ ppm}$	60.35 ± 0.02^{eF}	7.70 ± 0.06^{aA}	38.64 ± 0.70^{aA}	49.51 ± 0.51^{aA}
	$BHT_{100\ ppm}$	68.36 ± 0.05^{bF}	3.96 ± 0.04^{dA}	33.13 ± 0.59^{dA}	39.65 ± 0.45^{dA}
	$BHT_{200\ ppm}$	68.48 ± 0.02^{aE}	3.85 ± 0.04^{eA}	32.45 ± 0.61^{dA}	39.18 ± 0.50^{dA}

Values are mean ± SD (n=3).

Different lowercase superscripts within the same storage time in the same column denotes the significant difference ($P<0.05$).

Different uppercase superscripts within the same sample in the same column denotes the significant difference ($P<0.05$).

$CE_{100\ ppm}$ and $CE_{200\ ppm}$: mayonnaise added with cashew leaf extracts at the concentration of 100 ppm and 200 ppm, respectively.

$BHT_{100\ ppm}$ and $BHT_{200\ ppm}$: mayonnaise added with butylated hydroxytoluene at the concentration of 100 ppm and 200 ppm, respectively.

Table 15. Changes in sensorial characteristics of fish oil enriched mayonnaise without and with cashew leaf extract or BHT at various levels during storage of 30 days at 30 ˚C

Storage time (days)	Samples	Attributes			
		Rancid odor	Fishy odor	Acetic acid odor	Color
0	Control	3.51±0.92^{aC}	5.58±1.26^{aB}	13.84±1.67^{aA}	2.75±0.64^{aC}
	$CE_{100\ ppm}$	3.64±0.99^{aB}	4.73±0.89^{aB}	13.47±1.09^{aA}	2.94±0.54^{aC}
	$CE_{200\ ppm}$	3.54±0.88^{aA}	4.89±0.90^{aA}	13.46±1.71^{aA}	3.14±1.13^{aD}
	$BHT_{100\ ppm}$	3.34±0.65^{aB}	4.76±1.19^{aA}	13.88±0.86^{aA}	2.83±0.41^{aC}
	$BHT_{200\ ppm}$	3.61±1.09^{aA}	4.64±1.01^{aA}	13.79±0.91^{aA}	2.80±0.66^{aC}
10	Control	4.17±0.90^{aC}	5.68±1.17^{aB}	14.09±1.15^{aA}	2.99±0.45^{cC}
	$CE_{100\ ppm}$	3.90±0.71^{aB}	5.20±1.17^{aAB}	13.56±0.89^{aA}	4.05±1.31^{bcC}
	$CE_{200\ ppm}$	3.77±1.11^{aA}	5.15±1.26^{aA}	13.24±1.25^{aA}	7.36±1.08^{aC}
	$BHT_{100\ ppm}$	3.80±0.90^{aAB}	5.06±0.97^{aA}	13.88±0.86^{aA}	3.38±1.04^{bcC}
	$BHT_{200\ ppm}$	3.54±0.88^{aA}	4.71±0.92^{aA}	13.63±1.27^{aA}	4.50±1.30^{bB}
20	Control	6.44±1.33^{aB}	5.86±1.25^{aAB}	14.14±0.78^{aA}	6.53±1.56^{bcB}
	$CE_{100\ ppm}$	4.50±1.43^{bAB}	6.01±1.11^{aA}	13.78±0.74^{aA}	7.25±2.04^{bB}
	$CE_{200\ ppm}$	4.19±1.05^{bA}	5.95±1.24^{aA}	13.96±0.80^{aA}	11.34±1.16^{aB}
	$BHT_{100\ ppm}$	4.06±0.98^{bAB}	5.06±0.79^{abA}	13.71±0.78^{aA}	5.43±1.84^{bcB}
	$BHT_{200\ ppm}$	3.79±0.58^{bA}	4.68±1.01^{bA}	13.51±0.94^{aA}	5.08±1.60^{cB}
30	Control	7.82±1.39^{aA}	7.34±1.48^{aA}	14.01±0.89^{aA}	8.31±1.24^{bA}
	$CE_{100\ ppm}$	5.37±1.73^{bA}	6.38±1.48^{abA}	13.49±0.84^{aA}	10.34±3.07^{bA}
	$CE_{200\ ppm}$	4.79±1.75^{bA}	6.14±1.81^{abA}	14.07±0.86^{aA}	13.94±0.89^{aA}
	$BHT_{100\ ppm}$	4.69±1.07^{bA}	5.01±0.63^{bA}	13.63±0.78^{aA}	9.29±1.64^{bA}
	$BHT_{200\ ppm}$	4.31±0.85^{bA}	4.86±1.07^{bA}	13.76±1.03^{aA}	9.10±1.56^{bA}

Values are mean ± SD (n=3).

Different lowercase superscripts within the same storage time in the same column denotes the significant difference (P<0.05).

Different uppercase superscripts within the same sample in the same column denotes the significant difference (P<0.05).

CE_{100ppm} and CE_{200ppm}: mayonnaise added with cashew leaf extracts at the concentration of 100 ppm and 200 ppm, respectively.

$BHT_{100\ ppm}$ and $BHT_{200\ ppm}$: mayonnaise added with butylated hydroxytoluene at the concentration of 100 ppm and 200 ppm, respectively.

Table 16. Volatile compounds in fish oil enriched mayonnaise without and with 200 ppm cashew leaf extract at day 0 and day 30 of storage at 30 °C

Volatile compounds	Peak area (Abundance) x10^5		
	Control day 0	day 30	
		Control	CE_{200}
Furans			
2-Ethyl-furan	29	330	143
2-Pentyl-furan	ND	33	ND
Aldehydes			
Propanal	ND	49	50
2-Methyl-butanal	ND	ND	56
3-Methyl-butanal	ND	295	25
Pentanal	119	697	219
Hexanal	162	695	574
(E)-2-Hexenal	5	ND	ND
Heptenal	10	273	70
(E, E)-2,4-heptadienal	ND	125	95
Octanal	ND	52	ND
(E)-2-decenal	15	ND	ND
Benzaldehyde	22	215	50
Ketones			
1-phenyl-1,2-propandione	ND	ND	71
1-Penten-3-one	5	169	ND
2,3-Pentanedione	ND	49	ND
2-Hydroxy-3-pentanone	ND	1940	ND
Alcohols			
1-Cyclobutylcyclopropanol	ND	44	ND
1-Penatanol	ND	107	ND
(Z)-2-pentenol	ND	40	56
(E)-2-hexenol	ND	64	ND
1-Methyl-4-(1-methylethenyl)-cyclohexanol	9	ND	ND
1-Octen-3-ol	ND	2712	ND
(Z)-1,5-octadien-3-ol	ND	42	ND

ND: not detectable.

CE_{200ppm}: mayonnaise added with cashew leaf extracts at the concentration of 200 ppm.

5.5 Conclusion

Cashew leaf extract and BHT at the concentration of 200 ppm effectively inhibited lipid oxidation of fish oil enriched mayonnaise during storage as indicated by lower PV, TBARS and AnV values. However, addition of cashew leaf extract at both levels (100 ppm and 200 ppm) decreased L* but increased a*, b* and ΔE^* values. Rancidity and fishy odor of the samples containing cashew leaf extract at 200 ppm were lower than control sample. This was related with the decrease in volatile secondary lipid oxidation products in mayonnaise. TVC of all samples was below 10^3 CFU/g throughout 30 days of the storage. Thus, cashew leaf extract, especially at the level of 200 ppm, could be used as a natural antioxidant for retardation of the lipid oxidation in fish oil enriched mayonnaise during the extended storage at room temperature.

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CHAPTER 6

SUMMARY AND FUTURE WORKS

6.1 Summary

1. Optimization of extraction of antioxidative phenolic compounds from cashew leaves was achieved by statistical models and experimental design. Solvent, temperature, time and ethanol-to-solid ratio had the impact on extraction of phenolic compounds. The optimal condition for the extraction was as follows: 34.7˚C for 64 min with 80% ethanol-to-solid ratio of 18:1 (v/w). The most predominant compound in the extract was isoquercetin.

2. Ultrasound-assisted process was an effective method to enhance extraction yield and antioxidative activity of the ethanolic extract from cashew leaves via cavitation mechanism. Optimal condition for extraction was as follows: the amplitude of 77% for 31 min with 80% ethanol-to-solid ratio of 18:1 (v/w). Moreover, some additional phenolics, apart from isoquercetin, were quercetin, apigenin and eriodictyol.

3. Different extraction methods and the concentration of extracts determined antioxidative activities of cashew leaf extracts. Activity was increased as the concentration increased up to 0.1 mg/ml. Both extracts exhibited fairly stable when heated at 60-100 ˚C for 60 min and stable at pH ranges of 6-8. Additionally, the extract from ultrasound-assisted method showed the higher efficacy than that of conventional method in preventing oxidation both emulsion and liposome systems.

4. Cashew leaf extract into fish oil enriched mayonnaise was able to prevent lipid oxidation and retarded the development of fishy odor of product in dose-dependent manner. However, its efficacy was lower than BHT at the same level. Furthermore, the addition of cashew leaf extract affected color of product.

6.2 Future works

1. Discoloration in mayonnaise caused by cashew leaf extract should be solved, in which wider and more effective applications can be achieved.

2. The uses of cashew leaf extract should be tested in different food products, especially in combination with other antioxidants or appropriate packaging.

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Publication

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