EXTRACTION AND EVALUATION OF ACTIVE COMPOUNDS FROM *ARECA CATECHU* LINN. FRUIT FOR COSMETIC APPLICATION

SARITA SANGTHONG

MASTER OF SCIENCE
IN
COSMETIC SCIENCE

SCHOOL OF COSMETIC SCIENCE
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Sarita Sangthong

Thesis Title	Extraction and Evaluation of Active Compounds from *Areca catechu* Linn. Fruit for Cosmetic Application
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Degree	Master of Science (Cosmetic Science)
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ABSTRACT

This study was aimed to extract and evaluate phenolic compounds from betel nut (*Areca catechu* L.) in order to develop it as cosmetic multifunctional agent. Stabilities of the betel nut extract obtained from various conditions as well as cosmetic formulation are also investigated. Sample part and ripening stage, extraction method and solvent influencing the extraction efficiency were studied. Extractable phenolics content (EPC), extractable flavonoids (EFC), extractable catechin (ECC) content, DPPH and ABTS radicals scavenging capacities, ferric reducing antioxidant power (FRAP), lipid peroxidation, tyrosinase, and elastase as well as anti-bacteria inhibitory activity assays were employed to assess the extraction achievement. The HPLC elution profiles and UV-absorbability of the extracts were also investigated.

The raw betel nut seed obviously exhibited higher values of all assessments than those of the ripe seed, ripe pericarp and raw pericarp, respectively. Microwave assistance in extraction showed its significantly greater potential than the conventional shaking method. The raw betel nut crude extract from 50% ethanol extraction provided highest EPC, EFC, and ECC of 226.58 mg GAE/g sample, 140.79

mg QE/g sample, and 67.23 mg CE/g sample, respectively. This extract also exhibited the greatest antioxidant capacity of 495.51, 908.39 and 383.17 mg TEAC/g sample when determined by DPPH, ABTS and FRAP assays, respectively. The furthermost lipid peroxidation and tyrosinase inhibitory activity of 60.51 mg BHTE/g sample and 348.81 mg KAE/g sample, respectively, were obtained.

Sequential solid-liquid fractionation of bioactive agent from raw betel nut seed by 6 different polarity of organic solvents, including hexane, ethyl acetate, acetone, 95% ethanol, 50% ethanol and water were accomplished. Amongst all fractions, acetone extract possessed the highest EPC, EFC, and ECC of 82.39 mg GAE/g sample, 12.71 mg QE/g extract, and 1.51 mg CE/g sample, respectively. The most powerful antioxidant capacity was also obtained from the acetone fraction exhibiting 20.76 and 14.31 mg TEAC/g extract when assayed by DPPH and FRAP method, respectively. The first fraction of hexane and the last fraction of water exhibited lowest bioactive compounds and activities. The HPLC chromatogram showed the major components of raw betel nut seed were catechin-like compounds with the remarkable highest peak area in acetone fraction.

Stability of raw betel nut seed was investigated for its application in cosmetic products. The raw betel nut seed extract retained residual EPC and radical scavenging activity at pH 4-6 and 8-10, especially when storing at low temperature. The formula containing 0.5% raw betel nut seed extract retained residual EPC of 93.50 and 96.27% after 3 cycles of freeze/thaw and heating/cooling, respectively. The residual antioxidant activity of 90.78 and 102.35% were investigated. The storage at 4°C and room temperature showed better EPC and antioxidant stabilized than 50°C storage.

Keywords: *Areca* catechu Linn./cosmetic/extraction/multifunctional/stability

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Thai cosmetic market has been raising annually. In 2010, cosmetic marketing gained over 82,747 millions baht. Moreover, the export market was over 164.2 mllions baht (Thai Ministry of Commerce, 2012). Thai cosmetic product market segmentation of the skin care was of 41% for facial product. The lightening and anti-wrinkle products are accounted for 48% and 43%, respectively and another 10% belongs to sun-protection and others (Marketing Oops, 2008). Skin care product in Asian countries also has a decidedly more dominant market share of 40% in both Japan and China market (Global Insight, 2007). Moreover, the consumer demands interested in the development of the product contains natural ingredients and the environmental friendly products. These make the green marketing in Europe, America, and Asia being the main export market of Thailand. Thai cosmetic product, approximately 70%, was recognized to be natural products. The worldwide natural product was investigated to have the market value over 3 trillion baht (Kasikorn Research, 2010).

The study and development of natural bioactive ingredients in cosmetics are expanded. In addition, the new source of natural bioactive compound is being explored. The purposed active properties are, for example, anti-oxidant and anti- tyrosinase which considered to be the active ingredient in the anti-wrinkle or anti-aging cosmetic an in the whitening or lightening cosmetic, respectively. The example of natural ingredients

employed in the cosmetic are yeast extract, ginseng extract, soy-protein, yuzu extract, almond and mango butter. Those ingredients are function to balance, repair, moisture, and firm the skin. The natural active ingredients used in the reducing of melanin pigment production are mulberry extract, licorice extract and algae extract, for example (Bureau of Cosmetics and Hazardous Substances, 2004). The plant sources were had to be imported overseas which increases the production capital. According to that, the development of the native plant extract will increase the import rate and lower the cosmetic cost. Furthermore, this will be alternative for value-adding of Thai agricultural products.

Betel nut is the traditional plant related to Thai culture for long time. The betel nut seed chewing have the helminticide and other traditional medicinal properties (Lee, K.K., Cho, J.J., Park, E.J. & Cho, J.D., 2001). The betel nut seed and leaf were used to threat diarrhea, edema, heat stroke, beriberi, pharyngitis, eczema, lumbago, bronchitis, the urinary system malfunction (Oxenham, M.F., Locher, C., Cuong, N.L. & Thuy, N.K., 2002) decrease the tooth decay bacterium. The betel nut alkaloids have the stimulate affect to the central nervous system (Nelson, B.S. & Heishber, B., 1999) as same as the caffeine and nicotine consumption, but not effected to the appetite (Norton S.A., 1998). The previous studies show that betel nut owns the variety bioactive compounds, the major constituents belong to the polyphenolic and tannin (condensed tannin) which possesses the antioxidant and UV absorbent activities. In addition, flavonoids group of catechin, epicatechin, and cyanidins also found with their bioactive activities such as antioxidant, anti-inflammatory, and anti-bacterial activities (Zhang, W.M., Li, B., Han, L. & Zhang, H.D., 2009; Wetwitayaklung, P., Phaechamud, T., Limmatvapirat, C. & Keokitichai, S., 2006; Lee, K.K. & Choi, J.D., 1999 a,b).

The increment of cosmetic consumer leads to the development of raw materials and active ingredients which own the multifunction in the same time. The multifunctional ingredients are also found to have the synergistic effect and product unique perspective in the market and also lower the cost. As the previous reports, the betel nut contains a number of phenolic substances and offers various biological activities. It would greatly

provide multifunctional properties for cosmetic applications. The obtained betel nut extract and its finished product would represent Thai tradition and culture and also promote the outstanding of Thai betel nut.

This thesis was then purposed to study an extraction and evaluation of bioactive compounds from betel nut (*Areca catechu* L.) fruit for application it as cosmetic multifunctional agent. The results were also purposed to be the novel information for developing of advance natural active ingredient in the future. In according to the betel nut, this thesis could inherit the folk wisdom of consuming betel nut into the valued cosmetic to support and conserve the native plant leads to the agricultural sustainable development.

1.2 Objective of the Study

1.2.1 To study the effect of ripening stage and part of betel nut fruit on cosmetically bioactive compounds extraction

1.2.2 To study the effect of the extraction method on cosmetically bioactive compounds extraction from the betel nut

1.2.3 To study the effect of the extraction solvent on cosmetically bioactive compounds extraction from the betel nut

1.2.4 To evaluate the phenolics, flavonoids, and catechin content, antioxidant, anti-tyrosinase, anti-elastase, antimicrobial activities, and ultraviolet-rays absorbability of the betel nut extracts

1.2.5 To study the biochemical and physiochemical stability of the betel nut extract

1.2.6 To investigate the elution profile of betel nut extracts by high performance liquid chromatography (HPLC)

1.2.7 To study the stability of the formula containing the betel nut extract

1.3 Scope of the Study

1.3.1 Collecting of the betel nut fruit in the age of 3-6 months (raw) and 7-9 months (ripe) from Thasala, Nakorn Sri Thammarat, Thailand

1.3.2 Studying the effect of ripening stage of raw and ripe and part of seed and pericarp of the betel nut

1.3.3 Studying the effect of extraction method of conventional shaking and microwave-assistance

1.3.4 Studying the effect of extraction solvent of ethyl acetate, 95% ethanol, 50% ethanol, water, and propylene glycol

1.3.5 Studying the cosmetic bioactivities of betel nut extract; extractable phenolics, flavonoids, and catechin content, antioxidant, anti-tyrosinase, anti-elastase, antimicrobial activities and ultraviolet-ray absorbability.

1.3.6 Studying the cosmetic bioactivities stability of the betel nut extract; extractable phenolic, anti-oxidant, and anti-tyrosinase

1.3.7 Studying the physical stability of the betel nut extract; pH value and color of the extract

1.3.8 Studying the solid-liquid fractionation extraction using hexane, ethyl acetate, acetone, 95% ethanol, 50% ethanol, and water to obtain the various polar constituents in the betel nut

1.3.9 Investigating the elution profile of the betel nut extract using high performance liquid chromatography (HPLC)

1.3.10 Studying the stability of the formula containing the betel nut extract

1.4 Outcome of the Study

1.4.1 To know some factors influence cosmetic bioactive compounds extraction of betel nut

1.4.2 To obtain the cosmetic bioactivities of the betel nut extract

1.4.3 To obtain the guideline for the application and storage condition of the betel nut extract

1.4.4 To obtain the multi-functional betel nut extract for the application in cosmetic product

1.4.5 To obtain the formulation containing the betel nut extract as the multi-functional active ingredient

1.4.6 To provide the alternative way for value-adding of betel nut, Thai agricultural product

1.4.7 To provide the guideline of inheritance development and conservation of native Thai plant leads to the sustainable development

1.5 Timeline of Research (1st year)

Activity	2011								2012			
	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr
1. Betel nut sample preparation	←	→										
2. Study of sample and extraction method		←	→	→								
3. Study of extraction solvent				←	→	→						
4. Determination of extractable phenolics, flavonoids and catechin content			←	→	→	→	→					
5. Determination of antioxidant activities			←	→	→	→	→					
6. Determination of anti-tyrosinase and anti-elastase inhibitory activities							←	→	→			
7. Determination of antimicrobial activity									←	→	→	→

1.5 Timeline of Research (2nd year)

Activity	2012								2013			
	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr
1. Determination of UV absorbability	←	→										
2. Study of the extract stability		←	→	→	→							
3. Solid-liquid fractionation extraction					←	→	→					
4. HPLC analysis							←	→	→			
5. Study of formula containing betel nut seed								←	→	→		
6. Statistical analysis										←	→	
7. Information accumulation and thesis book preparation									←	→	→	→

CHAPTER 2

LITERATURE REVIEWS

2.1 Betel Nut (*Areca catechu* L.)

Betel nut (*A. catechu*) is belonging to the Palmaceae family, distributed much in the Tropical Pacific, Asia, and parts of East Africa (Staples, G.W. & Bevacqua, R.F., 2006). Betel quid (mixture of areca seed, lime, and piper betel) chewing culture is presented for a long time in Asia. In India and other Southern Asian areas, the seed of betel nut which can be obtained from its fruit is used as one component in betel quid. In Taiwan, fresh-unripe betel nut fruit is the main ingredient of betel quid.

Figure 2.1 Betel Palm Morphology

Tree: Betel nut is a slender, single-stemmed palm tree, up to 30 m high. It has a crown of 8-12 leaves at the top.

Stem: cylindrical stem and about 10-15 cm wide. It is covered with rings, which are the scars of old leaves that have died and dropped off. The flower sheaths were used as a packing material and can also be used to make dishes, small umbrellas or even skull caps. The trunk often formed the roof rafters of poorer houses in Bombay, and wood from the palm was put walls of houses. The hollow stems make a good water channel. The palm wood has sometimes been used to make small items like boxes or pen trays (Swarup). Pieces of the stems of betel nut palms are used as brush handles to apply pigment in Indian mural paintings. Betel nut stem is preferred for this as the wood does not react with the color of the pigments.

Leaves: 1 to 1.5 m long and feather-shaped. The base of each leaf completely encircles the palm stem like a tube. The leaves and leaf sheath can be used as a source of fiber basketry and containers. The leaves and petioles the betel nut palm are woven to make cups, bags and plates for food and water. They can also be written on and are useful for making fans, umbrellas and hats.

Flowers: both male and female flowers appear on the same plant on branched inflorescences below the crown of leaves. Male flowers grow close to the tips of flowering branches, while female flowers grow close to the base. They are tiny, fragrant and creamy-white in color.

Palm hearts: As with most palms, the tender shoot of the palm stem, known as the cabbage or palm heart, is edible. In India it is cooked in syrup.

Fruits: ranging in color from yellow through orange to red, 5cm in diameter and oval. There is one seed (called the nut) inside the fruit and it varies in shape from oval to round. The palm is cultivated for the hard, dried inner layer (endosperm) of its seeds. This is chewed by people in South Asia as a stimulant. The fruits are used as a source of tannin for dyeing.

Seed: The green unripe fruits were used to tan leather in South India. The fruits contain tannin and can be boiled down into a solid brown mass called 'catechu'. Catechu is used as an orange-brown dye and is also used as a source of tannin for preparing fibers before using other dyes.

Figure 2.2 Betel Nut Palms Cultivated in South Asia

Betel pepper: Leaves from the betel pepper plant, *Piper bete*l L. are used a 'wrapping' for betel quid. It is a climbing plant with shiny, green, heart-shaped leaves. It is only known in cultivation but is thought to have originated in Malaysia, Sumatra and possibly Java. Today it is cultivated in all betel chewing areas. The leaves are known as paan in India, and plant is known as tambuli in Hindi.

Betel paan: The most basic betel chew, or paan, consists of the sliced nut or supari, wrapped with slivers of lime (calcium hydroxide) in leaves of the betel pepper vine, together with some catechu (a vegetable extract from an acacia tree). For a more elaborate chew other aromatic condiments would be included. For a stronger effect, a twist of tobacco is frequently utilized in a plain chew.

Figure 2.3 Cultivation of *Piper betel* L. (Betel Pepper Leaf)

All the ingredients combine to stain the saliva red and give a red tinge to the lips and mouth. It has an energizing effect and is often used to stave off tiredness. It has also become part of a ritual symbolizing gracious living. Betel nuts play an important role in south Asia. The fresh or dried endosperm of seeds of the betel nut palm is chewed alone or in combination with a leaf of betel pepper (*Piper betel* L.), lime paste and catechu, which is a scarlet and astringent extract made from boiling chips of wood of the betel nut palm.

Other ingredients may be added to the little package, known as a betel quid, including tobacco, palm sugar and various spices such as cardamom (*Elettaria cardamomum*) and clove (*Syzygium aromaticum*). The rich assortment of paraphernalia involved in making up betel mixtures include receptacles for each of the separate ingredients, mortars, dishes, spittoons and cutters for the betel nuts. The mixture is also taken after meals to sweeten the breath.

Chewing the quid increases the production of saliva, which is blood red in color, and gastric juices and so is believed to aid digestion. After chewing, the quid is spat out. There can be up to thirteen ingredients, including camphor, copra, pepper, betel nut, cardamom, cloves, musk, lime and catechu.

Figure 2.4 Sliced Betel Nut (Supari)

Figure 2.5 Betel Quid Composed of Calcium Hydroxide (Slaked Lime), Betel Pepper Leaf, Sliced Betel Seed and Additional with Tobacco

Spiritual and religion: In parts of India, the offering and chewing of betel nuts fulfills an important religious and social function. It is a symbol of well-being and represents a refined lifestyle. Betel nut forms an integral part of many Indian rituals and ceremonies. Here, paan is being prepared for a wedding. The seeds of betel nut palms are so important that they form an integral part of most rituals from birth to death in Indian society. Offerings of betel nuts are made at social occasions as a lucky token for all relationships. Betel nut is offered to images of gods as part of religious ceremonies. In India betel nut seed chewing is a symbol of the Hindu Trinity, and betel nut seeds are believed to delight Brahma. Sometimes the flowers of the betel nut palm are used in ceremonies such as weddings.

Figure 2.6 Betel Quid Fulfill Indian Ceremony Atmosphere

History: Betel nut palms originally grew wild in Southeast Asia, perhaps in the Philippines or Malaysia. They were brought to the Indian subcontinent by humans in prehistoric times. The betel nut palm is now cultivated across the Asian tropics as a cash crop, as well as in almost every village garden. It is used for its seeds which are chewed as a stimulant.

South Asian history: Betel nut's origins in India cannot be precisely dated but it has been mentioned in ancient literature and texts for almost 2,000 years. In a Tamil classic story, Silappadikaram, the heroine feeds her husband with betel chews. Betel nuts also feature in ancient traditional medicine. Ayurvedic medical texts dating back 2000 years discuss the ingredients of betel quids, and Sanskrit writings list different types of betel quid. More precise references to betel nut chewing were dated from the Gupta period in the 5th century AD. By this time, it appears to have been a widespread practice.

Betel nut chewing is cited as one of eight enjoyments, or bhogas, of royal life in the 11th century text called *Manasollasa* or Splendour of Thought. It was mentioned by Marco Polo in the 13th century, and the 14th century traveler Ibn Battuta wrote of how it was part of all palace meals in the Delhi Sultanate. One of the earliest surviving Indian cookbooks, dating from about 1500, was produced for the Sultan of Mandu. It shows him supervising the preparation of elaborate betel nut paan. In the 15th century, a Persian ambassador called Abdul Razzak visited the south of India and wrote about the invigorating effects of betel nut chewing. By the 17th century, Indian writings noted the best palm varieties to use, and where they were grown. As betel nut chewing evolved, so have the accessories used to make betel quids. They include fine boxes and seed cutters.

Origins: The betel nut palm is only known in cultivation, and its exact origin is not known. It is likely to have evolved in South East Asia where it is thought to be of very ancient cultivation and where diversity of the genus *Areca* is greatest. The chewing of the seeds is an ancient tradition for millions of people across South and Southeast Asia. The practice is known as 'tamboul' in India and may have originated in the region of Vietnam and Malaysia. From here it was probably first introduced to India.

Production and trade: The betel nut palm is widely grown in South Asia, particularly India, Pakistan and Bangladesh, for its stimulating properties. India is the most important betel nut palm growing country in the world. The production of betel nuts has increased from 75,000 tons in 1955 to 330,000 tons in 2003. Betel nut cultivation in India now covers some 716,605 acres.

In Thailand, the main betel nut cultivation areas (Table 2.1) were in the southern of Thailand, Chum-phorn, Nakorn Sri Thammarat, Ranong, Pattalung, Trang, Pang-nga, and Surat Thani, respectively. The eastern of Thailand also cultivate betel palm in Cha Chaerng Sao, Rayong, and Nakorn Prathom, respectively (Thai Department of Agriculture Extension, 2002). The betel palm also widely cultivated in all part of Thailand such as Prae in the northern. The latest report of Thai Ministry of Commerce report 2012 betel nut export value was 1,153.3 million baht which more than 35% raised from 2011 (Thai Ministry of Commerce, 2012).

Cultivation: Seeds of the palm are sown in fields. The palms grow and generally start to bear fruit at about the age of four to eight years. Flowering starts between November and February in India, or slightly earlier in Bangladesh. The flowers are mostly wind pollinated, and the fruits take about 8 months to fully ripen. Each bunch can yield anywhere between 50 and 400 fruits, and each palm can continue to produce fruits for 60 to 100 years.

Harvesting and processing: Betel vine is cultivated on a wooden frame to give it support. The seeds are harvested every year. Harvesters climb the palms that are in close and regularly spaced plantings. They cut the bunch lower them down on a rope and move from one crown to the other. Another method is to harvest bunches with a knife mounted on a bamboo pole. The stage of harvesting the seeds depends on the product wanted. Immature fruits supply 'kalipak', an important form of processed betel nut in India. These are picked when the fruits are 6-7 months old. For the mature nut product, fruits should be harvested fully ripe.

Table 2.1 *A. catechu* L. Cultivation Areas in Thailand

Rank	District	Cultivation area (Rai)	Production (Kg/ Rai)	Total production (Kg)
1	Chum-phorn	24,516	6,700.00	107,849.90
2	Nakorn Sri Thammarat	18,408	6,091.84	99,461.50
3	Ranong	13,556	3,430.74	31,230.04
4	Cha Chaerng Sao	9,583	1,958.40	17,641.30
5	Pattalung	6,451	6,700.00	34,786.40
6	Trang	4,649	6,700.00	28,387.90
7	Pang-nga	4,282	5,362.81	14,683.36
8	Rayong	4,048	2,089.88	7,026.19
9	Surat Thani	3,427	4,705.38	11,509.36
10	Nakorn Prathom	3,409	3,172.30	10,697.01

From Thai Department of Agriculture Extension. (2002). **อนาคตหมากไทยจะเป็นอย่างไร.** Retrieved August 10, 2011, from http://www.doae.go.th/library/ html/detail/

After harvesting, the fruits are husk removed, either while fresh or after drying, and the whole or sliced nuts are dried in the sun or with artificial heat. Ripe and almost ripe nuts are left whole or are sliced. They are sometimes boiled in water, which reduces the tanning content of the nuts, and then dried. The product is graded on the basis of the ripeness at harvesting and on the color, shape and size of the nuts. The most popular form is the dried, ripe, whole nut.

Food: The betel nut palm does not play much of a role in the food of South Asia. The seeds are sucked or chewed, but are not classed as a food. The seeds are added to some foods and sweets to provide an aromatic flavoring. In India and in Indian restaurants in Europe, betel nuts may be added to mukwas. These are mixtures containing aromatic seeds and fragments of sugary sweets. Dishes are often placed on Indian restaurant tables as an after-dinner nibble. Betel nuts may also be used to make

paan masala in India. These are sort of breath-freshening ready-made mixes containing little pebbly little bits of betel nut dusted with powdered betel pepper (*Piper betel*) leaf and lime, and usually flavored strongly with menthol.

Ayurvedic medicine: Ayurvedic practitioners have a range of uses for betel nut. Ripe and unripe seeds are used to treat urinary infections and vaginal discharges. Ayurveda also recognizes betel nut as useful for relieving toothache, to aid digestion and as a laxative. It is reported to sweeten the breath, strengthen the gums and prevent perspiration. Some claim the plant has aphrodisiac properties too. The powdered seeds also feature in Unani medicine. They are prescribed for the treatment of diarrhea and urinary disorders.

Traditional and folk medicine: The seeds of the betel nut palm are best known for their use as a stimulant but traditional medicinal uses include relief from abdominal discomfort and eliminating intestinal parasites like roundworms and tapeworms. Betel nut's use in folk medicine extends to smallpox, cholera and sexually transmitted diseases such as syphilis. One traditional remedy claims that '*immortality can be attained by consuming a decoction of this plant*'. Externally the nuts are used for treating wounds, ulcers and sores.

Recent research: The main medicinal activities of the seed (also known as the nut) are reported to be anti-parasitic, laxative and to promote urination. It is also proven to be a stimulant since it increases both heart rate and blood pressure. The effects of betel nut on mental health are more controversial. By increasing glucose absorption in the brain, betel nut may be able to help those suffering from Alzheimer's disease. There are also hints that betel nut consumption might help sufferers from schizophrenia, but this is not yet demonstrated. Any medicinal use of betel nut will need to be balanced against its known harmful effects. The betel quid causes strong dental health, reduces teeth decay and discolors teeth and mouth, but also implicates as carcinogen since the polyphenols in areca seed can generate oxidative stress under alkaline condition (Huang, P.L., Chi, C.W. & Lui, T.Y., 2010). The tender shoot is also edible and cooked as syrup (Wang, C.W., Lee, W.H. & Peng, C.H., 1997). The seed is used as chewing material anthelmintic and also traditional medicine (Lee et al., 1999a,b) containing polyphenols; alkaloids (arecoline, arecaidine, guvacine, and guvacoline), tannins (phlobatannin and catechin), sugars and lipids (Wetwitayaklung

et al., 2006) The pericarp also contains leukocyanidins, tannins, and lipids that exhibit antibacterial and antifungal properties (Yenjit, P., Issarakraisila, M., Intana, W. & Chantrapromma, W., 2010). Both leaves and seed were used in various preparation including treatment of diarrhea, dropsy, sunstroke, beri-beri, throat inflammations, oedema, lumbago, bronchia catarrh, and urinary disorders (Oxenham et al., 2002).

S*timulating action*: Betel nut is usually chewed with lime (calcium hydroxide) and betel pepper leaves. When mixed with the lime the arecoline in the betel nut is converted to arecaidine. This contributes to the various effects of betel quid on the central nervous system. Both arecoline and arecaidine have been shown to interfere with the action of some of the nerves in the brain, which results in the stimulant effects. The action of these alkaloids might also explain why betel nut affects the appetite. Compounds called tannins are also found in the fruit and it is these compounds that may help treat the symptoms of dysentery.

2.2 Biological Activities of *A. catechu* L.

There was the previous study of phenolics and alkaloid content in *A. catechu* Linn. with the various sample of fresh areca fruits of various maturities, upside-down areca fruit, tender shoot, root, leaf, spike, and vein of the tree (Wang et al., 1997). The phenolics in *A. catechu* Linn. samples were determined by Folin-Ciocaltue method and found mainly distributed in root followed by fresh unripe fruit, leaf, spike, and vein, respectively. The alkaloids content in *A. catechu* Linn. samples were determined by HPLC. The order was highest in root and lower in fresh unripe fruit, spike, leaf, and vein, respectively. Total amount of phenolics in areca fruit were well correlated with the length and maturation, but those alkaloids were only correlated with maturation. Upside-down areca fruit contained a much higher amount of arecaidine alkaloid (4 mg/g of fresh weight) than normal fresh unripe areca fruit (1.5 mg/g of fresh weight). There is no significantly different of phenolic group content (condensed tannins, hydrolyzable tannins, non-tannin flavans, and simple phenolic) between normal unripe fruit and upside-down fruit. Tender shoot contained a small amount of total phenolics (0.58 mg of gallic acid equivalent/g of fresh weight), condense tannin

(0.85 mg of catechin equivalent/g of fresh weight), and total alkaloids (2.38 mg/g of fresh weight).

Biological screening of 100 plant extracts for cosmetic use in the perspective of free radical scavenging activity (Kim B.J., Kim J.H., Kim, H.P. & Heo, M.Y., 1997) stated the methanolic extracts including *A. catechu* (seed and peel) were screened for anti-oxidative activity using Fenton's reagent/ethyl linoleate system (lipid peroxidation system) and for free radical scavenging activity using 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical generating system. *A. catechu* peel showed no inhibition at a concentration of 10 μg/mL or 68% inhibition at 1,000 μg/mL in lipid peroxidation assay using the TBA method, and 9% inhibition at a concentration of 10 μg/mL or more than 24% inhibition at 1,000 μg/mL in DPPH free radical assay. The *A. catechu* seed showed 24% inhibition at a concentration of 10 μg/mL or 73% inhibition at 1,000 μg/mL in lipid peroxidation assay using the TBA method, and 52% inhibition at a concentration of 10 μg/mL or 47% inhibition at 1,000 μg/mL in DPPH free radical assay. The results suggest that *A. catechu* may be potential sources of anti-oxidants.

Free radical scavenging effect of ethanol extract from *A. catechu* L. was studied (Ahn, B.Y., 2009). The antioxidant activities of the ethanol extracts obtained from selected medicinal plants, which are frequently used as food and herb medicine, have been evaluated by measuring their DPPH radical scavenging activities. Among most plant extracts, the medicinal plant which showed the highest DPPH radical scavenging activity are the ethanol extract from semen of *A. catechu* L. follow by radix of *Usmus macrocarpa*, fryctus of *Terminalia chebula* Retz., Flos *santalum album* L., and semen of *Cassia tora* L. at IC_{50} of 4.5, 6.0, 7.0, 8.0, and 8.0 μg/mL, respectively. The ethanol extract of *A. catechu* was also shown to have stronger radical elimination ability than ascorbic acid and BHT.

The effects of *A. catechu* L. extract on anti-inflammation and anti-melanogenesis were studied (Lee & Choi, 1999a). The ethanolic extract from areca seed was prepared and its various biological activities were evaluated, the extract showed potent anti-oxidative (lipid peroxidation system), free radical scavenging (DPPH assay), and anti-hyaluronidase activity (anti-inflammatory inhibition of hyaluronidase). Anti-oxidative effect of the extract (IC_{50}, 45.4 μg/mL) was lower than

butylated hydroxytoluene (IC_{50}, 5 μg/mL), but similar to tocopherol and higher than ascorbic acid, especially, the extract exhibited relatively high free radical scavenging activity (IC_{50}, 10.2 μg/mL) compared to control. The extract inhibited effectively hyaluronidase activity (IC_{50}, 416 μg/mL), showed inhibition *in vivo* on delayed hypersensitivity as well as croton-oil induced ear edema in mice when it was topically applied. These results strongly suggest that the extract may reduce immuno-regulatory/inflammatory skin trouble. Also, from the results, it can be elucidated that the extract showed anti-allergic and anti-cytotoxicity activity. The whitening effect of the extract was shown by the inhibition of mushroom tyrosinase activity with IC_{50} of 0.48 mg/mL and of melanin synthesis in B16 melanoma cells (Lee & Choi, 1999a). They also do the related research on the anti-aging effect of ethanolic extract from areca seed on skin both *in vitro* and *in vivo* (Lee & Choi, 1999b). The extract has a high proportion of proline (13%) of free amino acid content. The inhibitory effect on the elastase exhibited 37 to 90% inhibition by 10 to 250 μg/mL concentration; the IC_{50} values with 40.8 μg/mL for porcine pancreatic elastase (PPE) and 48.1 μg/mL for human leukocyte elastase, respectively. One of the effects of elastase is that it is known to reduce the number of elastin fibers at the level of enzyme deposition. The number of elastin fibers was increased when it was drifted from the deposit number of elastase by 100 μg/mL of the extract. The extract showed protection of elastin fiber against degradation by the enzyme in an *ex vivo* essay. The extract increased proliferation of human fibroblast cell by 85% at 10^{-4} concentration, compared with control, whereas the increase by ascorbic acid was 50%. The collagen synthesis was increased by 40% at 10.4% concentration of the extract. The treatment with the extract improved skin hydration, the skin elasticity and skin wrinkles. The results from (Lee & Choi, 1999b) were strongly suggested that the ethanolic extract from areca seed can be used as a new anti-aging component for cosmetics. Moreover, the anti-elastase and anti-hyaluronidase of phenolic substances from *A. catechu* L. as new anti-aging agent was reported (Lee, K.K., Cho, J.J., Park, E.J. & Cho, J.D., 2001). Purified ethanolic-aqueous extract from areca seed by each fraction of solvents (combination of solvent containing various proportions of ethanol, hexane, chloroform, ethyl acetate, and butanol), siliga gel column chromatography, preparative TLC, and reversed-phase HPLC were studied. The peak in HPLC, which

coincided with the inhibitory activity against elastase, was identified as a phenolic substance by using various colorimetric methods, UV and IR. IC_{50} values of this phenolic substance were 26.9 μg/mL for porcine pancreatic elastase (PPE) and 60.8 μg/mL for human neutrophil elastase (HNE). This phenolic substance showed more potent activity than that of reference compounds, oleanolic acid (76.5 μg/mL for PPE and 219.2 μg/mL for HNE) and ursolic acid (31.0 μg/mL for PPE and 118.6 μg/mL for HNE). According to the Linewaver-Burk plots, the inhibition against both PPE and HNE by this phenolic substance was competitive inhibition with the substrate. The phenolic substance from *A. catechu* effectively inhibited hyaluronidase activity (IC_{50}, 210 μg/mL). These results suggest that the phenolic substance purified from *A. catechu* has an anti-aging effect by protecting connective tissue proteins (Lee et al., 2001).

The study of antioxidant capacity in various parts of *A. catechu* L. (Wetwitayaklung et al., 2006) found that the antioxidant activities in various ages of seed and various plant parts of areca related to their tannin percentage and total phenolic contents, and to find out which kind of solvent giving the highest antioxidant activities by TEAC method. Results showed that the water and methanol extracts of the seeds in various ages presented higher tannin percentage and phenolic content than the other parts of areca tree extract. The methanol extracts of the seeds in various ages gave higher antioxidant activities than the other parts (leaves, crown shafts, fruit shells; 4 and 8 months, root, and adventitious root) of areca tree extracts. The sequence of antioxidant activities of methanol seeds extracts from high to low were 4, 6, 8, 3, 2, and 1 month seed extracts. The 82.05% antioxidant activities assessed from phenolic compounds in areca, and arecoline indicated no antioxidant activity (Wetwitayaklung et al., 2006). *In vitro* antioxidant activity of ethanolic extracts of *Centella asiatica, Punica grenatum*, *Glycyrrhiza glabra*, and *Areca catechu* L. were reported (Ashawat, M.S., Shailendra, S. & Swarnlata, S., 2007). The effects of all ethanolic extracts of the herbs were studied via reducing power estimation method. The antioxidant activities of the extracts were compared with ascorbic acid standard and found that areca nut showed highest activity ($p<0.05$). These studies may suggest that the chemical combination with extract as antioxidant can be utilized in

pharmaceutical and cosmetic formulation or chemical antioxidant replaced by herbal natural antioxidants (Ashawat et al, 2007).

The study of areca nut for its antimicrobial properties evaluated the antibacterial, antifungal, and antiviral properties of the areca nut in vitro using isolated organisms (Anthikat, R.R.N. & Michel, A., 2009). A variety of human veterinary isolates, both gram positive and gram negative, were tested against areca nut extract by measuring the growth of the organisms by spectrophotometric method. It was found that both gram negative and gram positive organisms were susceptible to the areca nut extract. The concentration needed for 100% inhibition of growth was found to be 3.3-7 μg/mL for gram negative bacteria and 16 μg/mL for gram positive bacteria. The extract was also found to inhibit the growth of *Candida albicans* at a concentration of 16 μg/mL and inhibited alfatoxin production by *Aspergillus flavus*. The extract was also found to inhibit the viral growth of the New Castle Disease Virus (NDV) and Egg Drop Syndrome Virus (EDS) grown in embryo cultures. These results indicate that betel nut chewing may have significant disinfective properties (Anthikat & Michel, 2009).

2.3 Free Radicals

Free radicals are known to be the major cause of various chronic and degenerative diseases, including aging, coronary heart disease, inflammation, stroke, diabetes mellitus and cancer (Cheng, H.Y., Lin, T.C., Yu, K.H., Yang, C.M. & Lin, C.C., 2003). Reactive oxygen species (ROS) include free radical such as O_2^- (superoxide anion), OH (hydroxyl radical, H_2O_2 (hydrogen peroxide) and $^1O_2$ (singlet oxygen) can cause cellular injuries and initiate peroxidation of polyunsaturated fatty acids in biological membranes. The tissue injury caused by ROS may include DNA damage, protein damage and oxidation of important enzymes in the human body. These events could consequently lead to the occurrence of various Free radical-related diseases. Any free radical involving oxygen can be referred to as reactive oxygen species (ROS). Oxygen centered free radicals contain two unpaired electrons in the outer shell. When free radicals steal an electron from a surrounding compound or

molecule a new free radical is formed in its place. In turn the newly formed radical then looks to return to its ground state by stealing electrons with anti-parallel spins from cellular structures or molecules. Thus the chain reaction continues and can be thousand of events long. The electron transport chain (ETC), which is found in the inner mitochondrial membrane, utilizes oxygen to generate energy in the form of adenosine triphosphate (ATP). Oxygen acts as the terminal electron acceptor within the ETC. The literature suggests that anywhere from 2 to 5% of the total oxygen intake during both rest and exercise have the ability to form the highly damaging superoxide radical via electron escape. During exercise oxygen consumption increases 10 to 20 fold to 35-70 mL/ kg/ min. In turn, electron escape 4 from the ETC is further enhanced. Thus, when calculated, 6 to 3.5 mL/kg/min of the total oxygen intake during exercise have the ability to form free radicals (Halliwell, B., 2005).

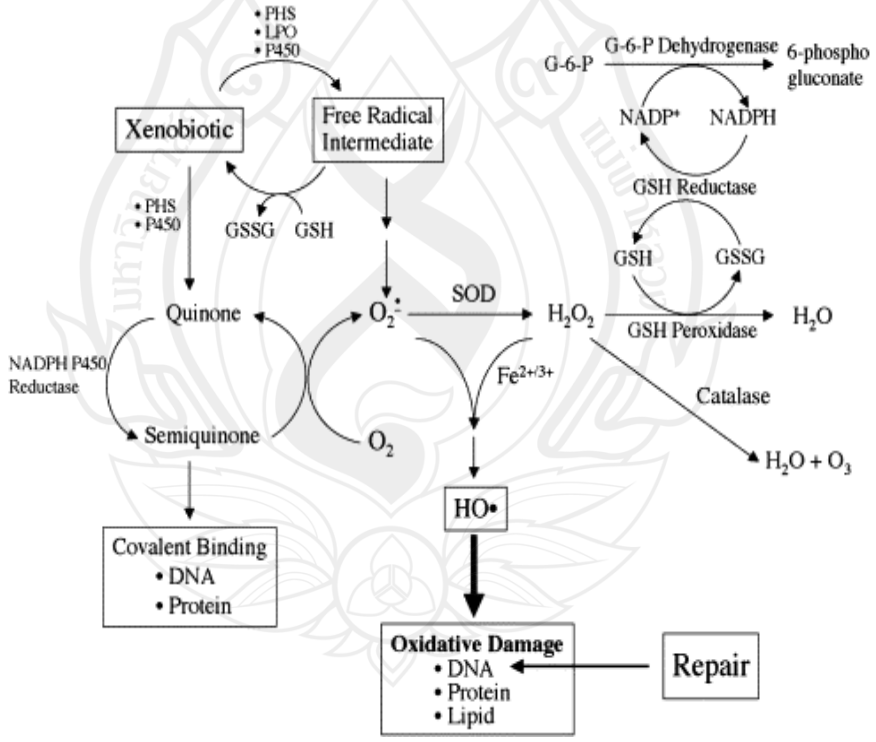


Figure 2.7 Pathways Contributing to the Formation and Detoxification of Reactive Oxygen Species (ROS)

There are numerous types of free radicals that can be formed within the body, especially, oxygen centered free radicals or ROS. The formulation of reactive oxygen species (ROS) is shown in Figure 2.7. In addition to the embryonic enzymatic bioactivation of xenobiotics to free radical intermediates, and the redox cycling of quinone metabolites, ROS also may be produced in the embryo (Wells, P.G., Bhuller, Y., Chen, C.S., Jeng, W., Kasapinavic, S., Kennedy, J.C., Kim, P.M., Laposa, R.R., McCallum, G.P., Nicol, C.J., Parman, T., Wiley, M.J. & Wong, A.W., 2005).

The most common ROS were shown in the equations below (Flora, S.J.S., 2009) including the hydroperoxyl radical ($HO_2^{\cdot}$), superoxide anion ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$).

$$O_2 + e^- + H^+ \text{ ---------- } H0_2^{\cdot} \quad (2.1)$$

$$HO_2^{\cdot} \text{ ---------- } H^+ + 0_2^{-\cdot} \quad (2.2)$$

$$O_2^{-\cdot} + 2H^+ + e^- \text{ ------- } H_2O_2 \quad (2.3)$$

$$H_2O_2 + e^- \text{ ---------- } OH^- + OH^{\cdot} \quad (2.4)$$

$$OH + e^- + H^+ \text{ --------- } H_2O \quad (2.5)$$

The superoxide anions are formed when oxygen (O_2) acquires an additional electron, leaving the molecule with only one unpaired electron. They are derived from molecular oxygen under reducing conditions. However, because of their reactivity, these same free radicals can participate in unwanted side reactions resulting in cell damage. Excessive amounts of these free radicals can lead to cell injury and death, which results in many diseases such as cancer, stroke, myocardial infarction, diabetes and major disorders. Within the mitochondria O_2^- is continuously being formed. The rate of formation depends on the amount of oxygen flowing through the mitochondria at any given time.

$$O_2^{-\bullet} \longrightarrow H_2O_2 \longrightarrow OH^{\bullet} \text{ (Harber Weiss Reaction)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^- \text{ (Fenton Reaction)}$$

Hydroxyl radicals are short-lived, but the most damaging radicals within the body. This type of free radical can be formed from O_2^- and H_2O_2 via the Harber-Weiss reaction. The interaction of copper or iron and H_2O_2 also produce $OH^\cdot$ as first observed by Fenton. Singlet oxygen is not a free radical, but can be formed during radical reactions and also cause further reactions. When oxygen is energetically excited one of the electrons can jump to empty orbital creating unpaired electrons. Singlet oxygen can then transfer the energy to a new molecule and act as a catalyst for free radical formation. The molecule can also interact with other molecules leading to the formation of a new free radical.

2.4 Antioxidants

Antioxidant is increasingly popular in modern society as it gains publicity through mass media coverage of its health benefits. The dictionary definition of antioxidant is rather straightforward but with a traditional annotation (Huang, D., Ou, B. & Prior, R.L., 2005) a substance that opposes oxidation or inhibits reactions promoted by oxygen or peroxides, many of these substances (as the tocopherols) being used as preservatives in various products (as in fats, oils, food products, and soaps for retarding the development of rancidity, in gasoline and other petroleum products for retarding gum formation and other undesirable changes, and in rubber for retarding aging) (Huang et al., 2005). A more biologically relevant definition of antioxidants is synthetic or natural substances added to products to prevent or delay their deterioration by action of oxygen in air. In biochemistry and medicine, antioxidants are enzymes or other organic substances, such as vitamin E or β-carotene, that are capable of counteracting the damaging effects of oxidation in animal tissues (Huang et al., 2005). Antioxidants are widely used as ingredients in dietary supplements and have been investigated for the prevention of diseases such as cancer, coronary heart disease and even altitude sickness. Reactive oxygen species are produced through inflammation, the normal body process by which the body attacks foreign invaders and repairs itself. The body has a built-in ability to eventually neutralize these compounds, once they have done their work. When inflammation

persists, the body's own antioxidants may become depleted, allowing reactive oxygen species to accumulate and damage normal healthy cells. A common example of chronic inflammation is longstanding allergic dermatitis while a well-known and much feared consequence of reactive oxygen species is damage to DNA that results in the formation of cancer cells (Shalini, S., 2012). To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals.

There are 3 main types of antioxidant:

1) Nutrient-derived antioxidants: ascorbic acid (vitamin C), tocopherols (vitamin E), carotenoids, and other low molecular weight compounds such as glutathione, lipoic acid and other polyphenols compounds.

2) Antioxidant enzymes: superoxide dismutase, glutathione peroxidase, and glutathione reductase, which catalyze free radical quenching reactions.

3) Metal binding proteins: ferritin, lactoferrin, albumin, and ceruloplasmin that sequester free iron and copper ions those are capable of catalyzing oxidative reactions.

Kinetically antioxidant is classifieds into 6 categories as below (Flora, 2009):

1) Antioxidants that break chains by reacting with peroxyl radicals having weak O-H or N-H bonds: phenol, napthol, hydroquinone, aromatic amines and aminophenols.

2) Antioxidants that break chains by reacting with alkyl radicals: quinones, nitrones, iminoquinones.

3) Hydroperoxide decomposing antioxidants: sulphide, phosphide, thiophosphate.

4) Metal deactivating antioxidants: diamines, hydroxyl acids and bifunctional compounds.

5) Cyclic chain termination by antioxidants: aromatic amines, nitroxyl radical, variable valence metal compounds.

6) Synergism of action of several antioxidants: phenol sulfide in which phenolic group reacts with peroxyl radical and sulphide group with hydro peroxide.

2.5 Phenolic Compounds

Phenolic compounds in plants are essential to the human diet, and are of considerable interest due to their antioxidant properties.

Table 2.2 Main Classes of Phenolic Compounds in Higher Plants

Classes and sub-classes	**Examples of specific compounds**
Non-flavonoids compounds	
Phenolic acids	
Benzoic acids	Gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid
Hydroxycinnamic acids	Coumaric acid, caffeic acid, ferulic acid
Hydrolyzable tannins	Pentagalloylglucose
Stilbenes	Resveratrol
Lignans	Secoisolaiciresinol, matairesinol, lariciresinol, pinoresinol
Flavonoid compounds	
Flavonols	Kaempferol, quercitin, myricetin
Flavones	Apigenin, luteolin
Flavanones	Naringenin, hesperetin
Flavanols	Catechins, gallocatechins
Anthocyanidins	Pelargonidin, cyaniding, malvidin
Condensed tannins or proanthocyanidins	Trimeric procyanidin, prodelphinidins
Isoflavones	Daidzein, genistein, glycitein

From Farah, A. & Donangelo, C. M. (2006). Phenolic compounds in coffee. **Brazil. J. Plant Physiol., 18**(1), 23-36.

These compound posses an aromatic ring bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule to that of a complex high-molecular weight polymer. Flavonoids, which bear the C6-C3-C6 structure, account for more than half of the over eight thousand different phenolic compound. The antioxidant activity of phenolic compound depends on the structure, in particular the number and positions of the hydroxyl groups and the nature of substitutions on the aromatic rings. Fruits, vegetables and beverages are the major sources of phenolic compound in the human diet. The food and agricultural products processing industries generate substantial quantities of phenolics-rich by-products, which could be valuable natural sources of antioxidants. Some of these by-products have been the subject of investigations and have proven to be effective sources of phenolic antioxidants. When tested in edible oils, and in fish, meat and poultry products, phenolic-rich extracts have shown antioxidant activities comparable to that of synthetic antioxidants. Practical aspects of extraction and production of sufficient amounts of natural antioxidants from most of these sources remain to be elucidated (Balasundram, N., Sundram, K. & Samman, S., 2006).

2.6 Phenolic Antioxidants Determinations

The multifaceted aspects of antioxidants and the basic kinetic models of inhibited autoxidation and analyzes the chemical principles of antioxidant capacity assays. Depending upon the reactions involved, these assays can roughly be classified into two types; assays based on hydrogen atom transfer (HAT) reactions and assays based on electron transfer (ET). The majority of HAT-based assays apply a competitive reaction scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds. These assays include inhibition of induced low-density lipoprotein autoxidation, oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP), and crocin bleaching assays. ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced. The degree of color change is correlated with the sample's antioxidant

concentrations. ET-based assays include the total phenols assay by Folin−Ciocalteu reagent (FCR), Trolox equivalence antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), total antioxidant potential assay using a Cu (II) complex as an oxidant, and DPPH. In addition, other assays intended to measure a sample's scavenging capacity of biologically relevant oxidants such as singlet oxygen, superoxide anion, peroxynitrite, and hydroxyl radical are also summarized. On the basis of this analysis, it is suggested that the total phenols assay by FCR be used to quantify an antioxidant's reducing capacity and the ORAC assay to quantify peroxyl radical scavenging capacity. To comprehensively study different aspects of antioxidants, validated and specific assays are needed in addition to these two commonly accepted assays (Huang et al., 2005).

2.6.1 Extractable Phenolic Content (EPC) Assay

The extractable content phenolic content of extracts was determined using to Folin-Ciocalteu method (Singelton, V.L., Orthofer, R. & Lamune-Raventos, R.M., 1999). The EPC were determined by the Folin-Ciocalteu reagent which contains molybdenum on superior oxidation state (Mo^{+6}) with a yellow color. Polyphenols compounds which contain at least one hydroxyl group (-OH) was determined its reducing activity of Mo^{6+} to inferior states of oxidation (Mo^{4+} or Mo^{5+}) which have a blue color and can be monitored spectrophotometry at 765 nm. Using gallic acid as standard total phenolic content and expressed as mg GAE/g dried weight sample.

2.6.2 Extractable Flavonoids Content (EFC) Assay

The EFC was determined by the theory of aluminium colorimetric assay. The principle of aluminum chloride colorimetric method is that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminium chloride forms acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids (Chang, C.C., Yang, M.H., Wem, H.M. & Chern, J.C., 2002). Using quercetin as standard total flavonoids content and expressed as mg QE/g dried weight sample.

2.6.3 DPPH Radical Scavenging Activity

Free radical scavenging is the generally accepted mechanism for how antioxidants inhibit lipid oxidation. The newly formed radical can mainly follow radical-radical interaction to render stable molecules, via radical disproportionate, although these secondary reactions are greatly hindered. It is important to notice that when studying plant extracts there is the existence of several possible radical scavenger species in the extracts. The capacity of biological reagents to scavenge the DPPH radical can be expressed as its magnitude of antioxidation ability. The DPPH alcohol solution is deep purple in color with an absorption peak at 517 nm, which disappears with the presence of the radical scavenger in the reactive system and when the odd electrons of the nitrogen in the DPPH are paired. The reactive rate and the ability of the radical scavenger depend on the rate and the peak value of disappearance of the DPPH (Li, Y.J., Chern, J., Li, Y., Li, Q., Zheng, Y.F., Fu, Y. & Li, P., 2011). The results of the DPPH assay have been presented in many ways. The majority of studies express the results as the IC_{50} value, defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%. This value is calculated by plotting inhibition percentage against extract concentration.

2.6.4 Ferric Reducing Antioxidant Power (FRAP)

Total antioxidant activity is measured by ferric reducing antioxidant power (FRAP) assay (Benzie, I.F.F. & Strain, J.J., 1999). FRAP assay uses antioxidants as a reductant in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. At low pH (3.6), the reduction of ferric-tripyridyl triazine (Fe^{3+}-TPTZ) complex has an intense color and can be monitored by measuring the change in absorption at 593 nm. The reaction is non specific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric ferrous half reaction, will drive the ferrous ($Fe^{3+}$to Fe^{2+}) ion formation. The change in absorbance is therefore, directly related to the combined or total reducing power of the electron donating antioxidants present in the reaction mixture (Benzie & Strain, 1996).

2.6.5 ABTS Radical Scavenging Activity

The 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) was pre-oxidized with potassium persulphate to generate $ABTS^{\bullet+}$. The mixture was incubated for 12-16 h to reach to complete reaction. The $ABTS^{\bullet+}$ stock was diluted with buffer for using in hydrophilic and with ethanol for lipophilic assay. The decolorizing reaction was monitored at 743 nm for radical scavenging activity of the antioxidant (Arnoa, M.B., Cano, A. & Acosta, M., 2001).

2.6.6 Lipid Peroxidation Inhibitory Activity

Lipid peroxidation is highly determined as its mechanism toxic to the oil, fatty acid and also lipid bilayer membrane, the basis structure of many cellular membranes. The presence of polyunsaturated fatty acids (PUFAs) in the lipid bilayer's phospholipids critically impacted to the membrane fluidity. Lipid peroxidations affect to the membrane's phase properties, electrical resistance, membrane protein moiety as well as lysosomes's latency becoming fragile and leaky. Peroxidation also well-known to decrease membrane associated enzymes and membrane pump homeostasis maintenances. The major toxicological product been interested is 4-hydroxynonenal (4-HNE), various 2-alkenals, and malonaldehyde (MDA). In the perspective of food and dietary, rancidity of oil is the commonly lipid peroxidation event which is the oxygen-dependent deterioration of lipid. The major product LOOHs, the non radical intermediates derived from unsaturated fatty acids, phospholipids, glycolipids, esters and cholesterol are formed from the reaction to the reactive oxygen species (ROS). The alkoxyl radical are formed as the result of the fission of cyclic endoperoxides (O-O bonds). The transition of metal complex catalyzes the forming of reduced iron compounds and react with LOOH, which is fairy stable molecules at physiological temperature. The interesting malonaldehyde (MDA) can be measured as an adduct formation with thiobarbituric acid (TBA). The resulted thiobarbituric reactive substance (TBARS) was spectrophotometrically monitored the pink color at 532 nm (Devasagayam, T.P.A., Boloor, K.K. & Rasmasarma., 2003).

2.6.7 Tyrosinase Inhibitory Activity

Recently the destruction of ozone layer leads to the more harmfulness of ultraviolet (UV) radiation. The excessive exposure to UV radiation causes post-inflammatory pigmentation. Pigmentary disorders are caused by many factors such as inflammatory, hormonal imbalance, and excessive exposure to UV (Lee & Choi, 1999a). The integrated mechanism for the formation and delivery of melanin within melanosomes from melanocyte to the keratinocytes (Moon, J.Y., Yim, E.Y., Sng, G., Lee, N.H. & Hyun, C.G., 2010) is to be considered. Melanin plays a critical role in UV absorption and free radical affects. The regular of cellular pigmentation can be controlled at many different stages of melanogenesis. The enzymatic producing process of melanin was involved with tyrosinase enzyme (Lee & Choi, 1999a). The process was depicted in Figure 2.8, Initial melanin synthesis is catalyzed by tyrosinase and is then divided into eumelanogenesis or pheomelanogenesis. The other melanogenic enzymes are involved in eumelanogenesis that is, l-3,4-dihydroxyphenylalanine (DOPA) chrome tautomerase (DCT) and tyrosinase-related protein 1 (TYRP1 and no specific enzymes have been found that are involved in pheomelanogenesis (Ando, H., Kondoh, H., Ichihashi, M. & Hearing, V.J., 2007). The spectrophotometry assays determined the inhibition of dopachrome melanin production via the inhibition of tyrosinase activity.

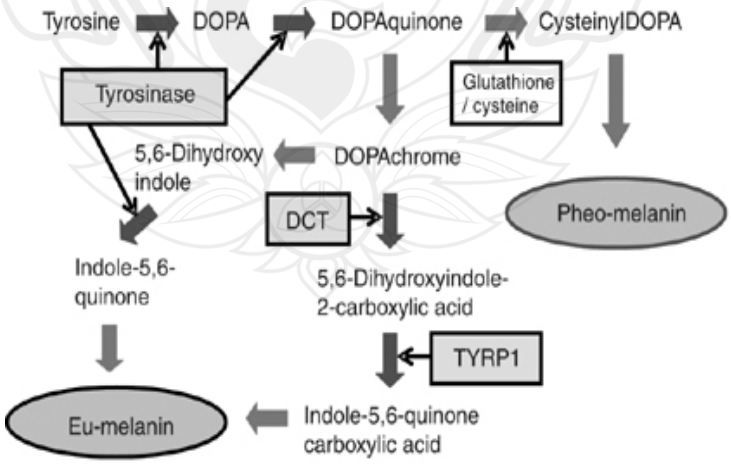


Figure 2.8 Melanin Synthetic Pathway and the Involvement of Melanogenic Enzymes

2.6.8 Elastase Inhibitory Activity

The skin elasticity significantly decreased as the age increased, especially significance at the age above 40 years old resulted in sagging. Biologically, elastase activity significantly increased with age (Lee & Choi, 1999a). Elastase is the only enzyme owning the elastin fiber breaking down property. The destruction of elastin leads to the destruction of skin demonstrates the skin-aging signs including sagging, wrinkles, and other fine lines (Moon et al., 2010). The elastase (EC 3.4.21.36) inhibition was assessing and N-succinyl-ala-ala-ala-*p*-nitroanilide was used as the substrate which specific to elastase. The hydrolysis by elastase prefers the alanine cleavage releasing the primary aromatic amine of *p*-nitroaniline. The colorimetric determination monitors the decrement of cleaved product by adding the inhibitor by the decrement of the absorbance at 510 nm.

2.6.9 Antibacterial Activity

The antibacterial activities of the plant extracts were widely study as the meaningful application as natural agent. The recent interesting strains to be inhibited are wide range depend on the application. The use in cosmetic product, antibacterial agents were purposed in both being the active ingredient and self preservative. The hygienic and sanitary cosmetics as well as anti-acne and cleansing products were inquired to be tested against the normal occurred strains. There were indicated bacteria which considered being avoided in cosmetic product. Since being the water containing formula, cosmetics contamination ability has been known. Microorganisms may cause spoilage or chemical changes in cosmetic products and injury to the user (Dashen, M.M., Chollom, P.F., Okechalu, J.N. & Ma-aji, J.A., 2011). The generally concerned bacterial are *Staphylococcus aureus*, *Staphylococcus epidermidis* (gram-positive bacteria) and *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*. In addition to the mentioned bacteria, the occasionally occurrences of *Candida albican*, yeast and the recently regulation of *Clostridium* spp. contaminant in natural cosmetic products.

There were many testing method commonly employed in antimicrobial activity. The widely applied dilution method or agar disc and paper disc diffusion method were employed. The diffusion method was published being the most suitable

for using in antimicrobial therapeutic test by the manufacturers. This well known method was nick named as the critical development by Bauer and Kirby (Bauer, A.W., Kirby, W.M.M., Sheris, J.C. & Turck, M., 1966). The inhibitory activity is observed by eyes and clear zone diameter measurement. The ability of the tested agents is compared to the synthetic antibiotic or drugs.

2.7 Extraction Method

The extraction process is one of the most concerned factors of developing natural extract. There are many factors including in the process of extraction. The target compound to be extracted and their nature and preferences was the firstly concern. The factor of extraction including, extraction method, extraction solvent, time, and temperature were in account of the extraction efficacy. The target compound has its own preferences. The volatile oil may extracted more in the high temperature accompany with the closed extraction apparatus like soxhlet or distillation clevenger. While the thermolabile substance like secondary metabolites are considered to extract with the less thermal involved method like conventional shaking or ultrasonication at room temperature to avoid the decomposition of the target compounds.

The extraction solvents are generally chosen by the solubility properties and the polarity index (Table 2.3) matching to the target compounds. The low polarity oil or fatty acid may be chosen to be extracted with low polar organic solvent such as hexane and petroleum ether while the medium polarity compounds appreciate dichloromethane chloroform or ethyl acetate. The higher polarity compound of polyphenols will be extracted more in higher polar organic solvent of acetone, ethanol, methanol or water (Kumoro, A.C., Hasan, M. & Singh, H., 2009). Many studies also literate the synergistic effect of the combination of two or more solvent in the system.

The alternative extraction method microwave assistance (MAE) was employed in the recent study. MAE was currently regarded as a robust alternative to traditional extraction techniques, especially in the case of the sample preparation for analytical

purpose (Zhang, H.F., Yang, X.H. & Wang, Y., 2011). The advantages of MAE are, for example, suitable for the recovery of vast array of compounds, versatile and efficient for secondary metabolites extraction. Compared to the classical reflux and soxhlet extraction MAE shows the shorter extraction time, higher extraction yield, selectivity, and better quality target compounds (Mandal, V., Mohan, Y. & Hemalatha, S., 2007: Chen, Y., Xie, M.Y. & Gong, X.F., 2007). The cost-effective is one of the most factor concerning in the extraction development. MAE possessed lower cost compared to the supercritical fluid extraction, lower solvent consumption than accelerated solvent extraction and lower extraction time than ultrasonic-assisted extraction and suit to the thermolabile constituents (Zhang et al., 2011; Mandal et al., 2007). However, there were the demerit reports of MAE with the cooperated with high pressure (Zhang et al., 2011). Flavonoids extraction was found having higher degradation rate of rutin, hyperin, quercetin, and quercitin when the pressure in the system increased (Liu, Z.Y., Hu, X.L., Bu, F.Q., Ding, L. & Zhang, H.Q., 2007). Moreover, extraction efficacy of MAE may be poor when either extraction solvent or target compounds are non-polar, or when the viscosity of solvent is extremely high (Wang, L. & Weller, C.L., 2006).

Numbers of literatures, more than 20 reports stated the application of MAE combined with various additional functions such as focused oven, closed vessel, ultrasound, dry-diffusion system, and the simple domestic microwave oven as well. The potential extraction efficacy of microwave is the disruption of plant tissues and/or cells (even organelles) (Zhang et al., 2011). The plant material particle size impacted to the extraction efficacy as the smaller size allow more specific surface area of the sample to be extracted by solvent via microwave assistance (Gao, M., Huang, W., Moytri, R.C. & Lui, C., 2007). The same research also reported the different structure of plant material extracted by MAE and heat-reflux extraction, MAE possessed higher cell disruption observed by light micrographs (Gao et al., 2007).

Table 2.3 Some of the Solvent Polarity Index and Dielectric Constant

Solvent	Polarity index	Dielectric constant
Hexane	0.1	1.89
Ethyl acetate	4.4	6.08
Isopropanol	3.9	19.9
Acetone	5.1	20.7
Methanol	5.1	32.6
Ethanol	5.2	24.3
Acetronitrile	5.8	37.5
Water	9.0	78.3

Moreover, the extraction solvent employed in MAE is to be considered, in addition to the polarity, of its dissipation factor or the ability of the solvent to absorb microwave energy and pass it on as heat to the surrounding molecule (Mandal et al., 2007). The factors in calculated, dielectric loss and dielectric constant (Table 2.3) are also important to the extraction manner of the solvent. The former indicates the efficacy of converting microwave energy into heat and, more in considered in this study, the later is the ability of the solvent to absorb microwave energy.

2.8 Cosmetic and Multifunctional Agent

The world wide cosmetic market has been raised gradually. Thai Ministry of Commerce recently reported the 2012 cosmetics export value of Thailand was 82,747.2 million baht which expanded about 10% from 2011 (Thai Ministry of Commerce, 2012). Thai cosmetic product market segmentation of the skin care was of 41% for facial product. The lightening product was 48%, 43% for anti-aging and moisturizing, and another 10% for sun-protection and others (Marketing Oops, 2008). Skin care product in Asian countries also has a decidedly more dominant market share

of 40% in both Japan and China market (Global Insight, 2007). Moreover, the consumer demands interested in the development of the product contains natural ingredients and the environmental friendly products. The Green Marketing in Europe, America, and Asia was the main export market of Thailand. Thai cosmetic product, approximately 70%, was recognized to be natural products. The worldwide natural product was investigated having the market value over 3 trillion baht (Kasikorn Research, 2010).

The main market share was of skin care product. The major attractiveness of consuming Thai cosmetic product was the lower cost with the high unique concept. The important export market was Asian countries of more than 40% of total share because of the similar perspective of natural and organic products. The second market share was of Japan (24.85%) who also interesting in natural concept. European countries (5.68%) and U.S.A. (0.68%) also interested in Thai cosmetic products (Thai Ministry of Commerce, 2012). The major expectation of the consumer from Thai cosmetic is the natural and safety cosmetic. The export value of natural extracts in 2012 was also reported by Thai Ministry of Commerce of 164.2 million baht raised more than 18% from 2011. Asian countries still being the main market of 58.52% and the lower was U.S.A. (14.24%). Europe and Japan also being where Thai natural extracts taken the export value (3.05% and 3.82%, respectively) (Thai Ministry of Commerce, 2012). The raising in export value indicated the gradually interesting in natural perspective. Cosmetics were one of the main products developed to be the natural concept. Including the natural active ingredients is one of the best claims. Thailand native plants and herbals were widely researched and developed. Natural extracts widely stated of their various cosmetic activity including, antioxidant, anti-melanogenesis, antibacterial, and moisturizing effects. The safety in use of natural was competitive with the synthetic one.

In addition, the cosmetic import value also critically raised to over 25% which will be considerable of wasting more than 20,927 million baht to the Europe (34.08%), U.S.A. (18.39%), Asian (14.07%), and Japan (13.18%) (Thai Ministry of Commerce, 2012). The Thai cosmetic research and development with adding the unique Thai concept serves the sustainable agriculture as well as the consuming of Thai cosmetic.

The multifunctional cosmetic also widely claimed as the simultaneous effects which suitable to the recent life style of the consumers. The effects can be, for example, moisturizing, anti-acne, anti-wrinkle, whitening, anti-bacteria, sunscreen, and also as the colorant properties. These can be obtained from the ingredients both as the basic, active or additive ingredients in the formulation. These inquiries lead the research and development of the natural multi-functional agent which achieve more than those synthetic chemical agents in the safety perspective.

CHAPTER 3

METHODOLOGY

Table 3.1 Chemicals

Chemical	**Chemical formula**	**Molecular weight (g/ mol)**	**Company**
2,2'-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid) (ABTS)	$C_{18}H_{18}N_4O_6S_4$	514.62	Sigma
2,4,6-Tripyridyl-s-Triazine (TPTZ)	$C_{18}H_{12}N_6$	312.33	Sigma
6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (Trolox)	$C_{14}H_{18}O_4$	250.29	Sigma
Absolute ethanol	C_2H_6O	46.07 (d= 0.789)	Merck
Acetic acid	$C_2H_4O_2$	60.50 (d= 1.049)	Fisher Sci.
Agar	-	-	Himedia
Aluminium chloride	$AlCl_3 \cdot 5H_2O$	241.43	Ajax Finechem
Ascorbic acid	$C_6H_8O_6$	176.12	Fisher Sci.
Boric acid	H_3BO_3	61.83	Ajax Finechem
Caffeic acid	$C_9H_8O_4$	180.16	Sigma

Table 3.1 (continued)

Chemical	Chemical formula	Molecular weight (g/ mol)	Company
Catechin	$C_{15}H_{14}O_6$	290.27	Sigma
Cetyl alcohol	$C_{16}H_{34}O$	242.44	Dow Corning
Chloramphenicol	$C_{11}H_{12}Cl_2N_2O_5$	323.13	Merck
Chlorogenic acid	$C_{16}H_{18}O_9$	354.31	Sigma
Citric acid	$C_6H_8O_7 \cdot H_2O$	210.14	Ajax Finechem
Cross-linked poly acrylate polymer (Carbopol®940)	-	-	Dow Corning
DC 9045 (silicone elastomer blend)	-	-	Dow corning
3,4 dihydroxyphenyl alanine (L-Dopa)	$C_9H_{11}NO_4$	197.19	Sigma
Dimethyl sulfoxide (DMSO)	C_2H_6OS	78.13 (d= 1.100)	Fisher Chem.
Dipotassium phosphate	K_2HPO_4	174.2	Ajax Finechem
Disodium EDTA	$C_{10}H_{16}N_2O_8 \cdot 2Na$	292.24	Dow Corning
DPPH (2,2'-diphenyl-1-picrylhydrazyl) (DPPH)	$C_{18}H_{12}N_5O$	394.32	Sigma
Epicatechin	$C_{15}H_{14}O_6$	290.27	Sigma
Epigallocatechin	$C_{15}H_{14}O_7$	306.27	Sigma
Epigallocatechin gallate	$C_{22}H_{18}O_{11}$	458.37	Sigma
Ethanol (95%)	C_2H_6O	46.07 (d= 0.789)	Merck
Ethyl acetate	$C_4H_8O_2$	88.11 (d= 0.897)	Merck
Ferric chloride	$FeCl_3 . H_2O$	270.30	Ajax Finechem

Table 3.1 (continued)

Chemical	Chemical formula	Molecular weight (g/ mol)	Company
Ferrous sulfate	$FeSO_4 \cdot 7H_2O$	278.05	Ajax Finechem
Ferulic acid	$C_{10}H_{10}O_4$	194.18	Sigma
Follin-Ciocalteu reagent	$C_6\ H_6\ O$	94.11	Carlo Erba
Gallic acid	$C_7H_6O_5$	170.12	Fluka
Gallotannin (tannic acid)	$C_{76}H_{52}O_{46}$	1701.2	Himedia
Gentamicin	$C_{21}H_{43}N_5O_7$	477.60	Merck
Glycerine	$C_3H_5(OH)_3$	92.09	Dow Corning
Glyceryl monostearate	$C_{21}H_{42}O_4$	358.57	Dow Corning
Hydrochloric acid (12M)	HCl	36.5	Fisher Chem.
4-Hydroxy-3-methoxy benzaldehyde (vanillin)	$(CH_3O)(OH)C_6H_3CHO$	152.14	Merck
Kojic acid	$C_6H_6O_4$	142.11	Sigma
Methanol	CH_4O	32.04 (d= 0.79)	Fisher Chem.
Methyl paraben	$C_8H_8O_3$	152.15	Dow Corning
Mineral oil	-	-	Dow Corning
Mushroom tyrosinase	-	-	Sigma
N-Succinyl $(Ala)_3$ *p*-nitro aniline	$C_{19}H_{25}N_5O_8$	451.43	Sigma
Nutrient broth	-	-	Himedia
Brij 58 (Polyoxyethylene 20 cetyl ether)	$C_{16}H_{33}(OCH_2CH_2)_{20}OH$	1,122.00	Dow Corning
Brij 72 (Polyoxyethylene-2-stearyl ether)	$C_{18}H_{37}(OCH_2CH_2)_2OH$	358.60	Dow Corning
Porcine pancreas elastase	-	-	Sigma
Potassium dihydrogen phosphate	KH_2PO_4	136.09	Ajax Finechem

Table 3.1 (continued)

Chemical	Chemical formula	Molecular weight (g/ mol)	Company
Potassium persulfate	$K_2S_2O_8$	270.322	Ajax Finechem
Propyl paraben	$C_{10}H_{12}O_3$	180.20	Himedia
Propylene glycol	$C_3H_8O_2$	76.09	BDH
Quercetin	$C_{15}H_{10}O_7$	302.236	Sigma
Sodium acetate	$C_2H_3NaO_2$	82.03	Ajax Finechem
Sodium borate	$Na_2B_4O_7 \cdot 10H_2O$	381.37	Ajax Finechem
Sodium carbonate	Na_2CO_3	105.99	Fisher Chem.
Sodium citrate	$C_6H_7NaO_7$	214.11	Ajax Finechem
Sodium dihydrogen phosphate	NaH_2PO_4	119.98	Ajax Finechem
Sodium hydroxide	NaOH	40.00	Ajax Finechem
Sodium nitrite	$NaNO_2$	69.00	Ajax Finechem
Stearic acid	$C_{18}H_{36}O_2$	284.48	Dow Corning
Stearyl alcohol	$C_{18}H_{38}O$	270.49	Dow Corning
Sulfuric acid	H_2SO_4	98.08 (d=1.84)	Merck
Triethanolamine (99%)	$C_6H_{15}NO_3$	149.19	Dow Corning
Tris Hydrochloride (Tris-HCl)	$NH_2C(CH_2OH)_3 \cdot HCl$	157.6	Calbiochem
Tryptic soy agar	-	-	Himedia
Tryptic soy broth	-	-	Himedia

Table 3.2 Instruments

Instrument	Model	Company
2-Digit digital balance	ARC 120	Adventurer,USA
4-Digit digital balance	TB-214	Denver, Germany
Adjustable micropipette	-	Eppendrof, Germany
Aspirator	A-1000	Eyela, Japan
Blender	-	Sharp
Centrifuge	Spectrafuge/16M	LABNET
Chromameter	TA/CR-400	KONICA MINOLTA
Freeze dryer	FreeZone	Labconco
Freezer	SF C697(GYN)	Sanyo
Hammer mill	CMC-20	Thailand
Homogenizer	KA/T25D digital	ULTRA-TURRAX
Hot air oven	UM500, UFE600	Memmert, Germany
Hot plate	HS-115	HL Instrument
Microplate reader	UVM 340	Biochrom
Microwave	SEVERIN	ART MW7853
pH meter	CyberScan pH 1100	EUTECH
Refrigerator	SJ-D24N-SLG	SHARP
Rotary evaporator	CCA-1110	Eyela, Japan
Sieving machine	AS 200 digit	Retsch
SPF analyzer	LLC/SPF-290S	Optometics, USA
UV-VIS spectrophotometer	Libra S22	Biochrom, UK
Vertical shaker	KS 4000i Control	IKA
Viscometer	RVDV-II +P	Brookfield, Germany
Vortex mixer	KMC-1300V	VISION, Korea

3.1 Sample Preparation

The betel nut samples were obtained from Thasala, Nakorn Sri Thammarat, Thailand in the ages of 3-6 months (raw) and 7-9 months (ripe). The samples were tap water washed and air dried then separated into 2 parts of pericarp and seed. Four samples; raw seed, ripe seed, raw pericarp, and ripe pericarp were obtained to the study (Figure 3.1.1). The samples were dried by using 50°C hot air oven to obtain the consistent weight. The dried samples were ground by a hammer mill and sieved into 500 µm size. The obtaining samples were kept at -20°C for further extractions.

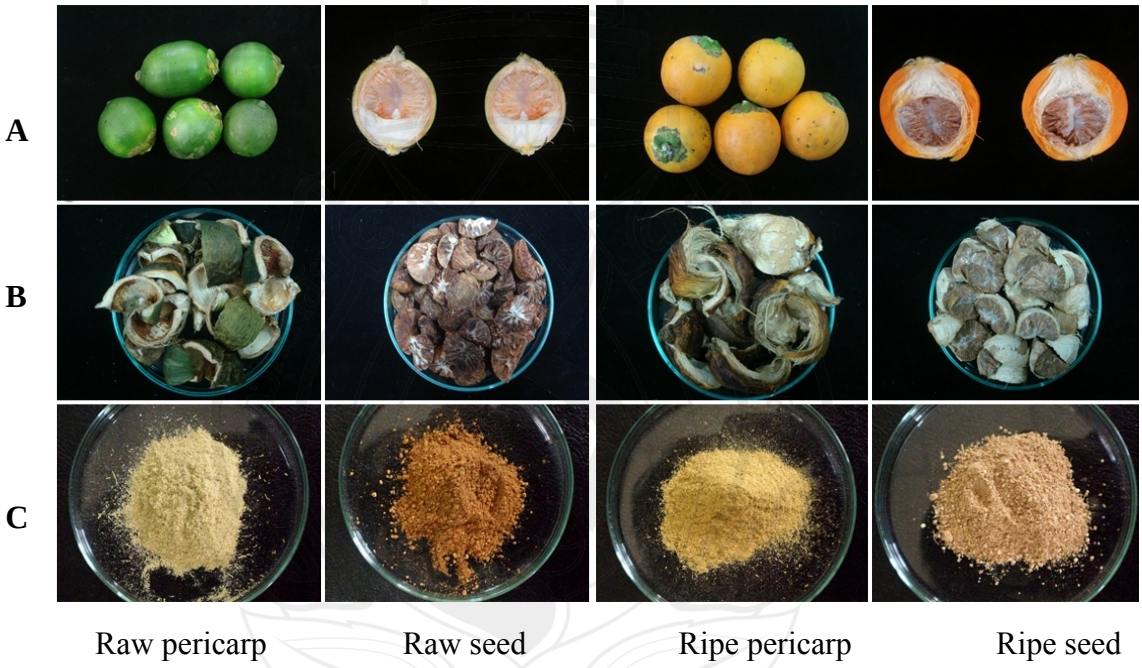


Figure 3.1.1 Appearance of the Betel Nut Samples Before Drying (A), After Drying (B), and After Milling (C)

3.2 Extraction of Bioactive Compounds from *A. catechu* Linn.

3.2.1 Effect of Sample and Extraction Method on Bioactive Compound Extraction

The sample (5 g) was mixed with 50% ethanol (50 mL) immediately before the extraction. The mixtures of sample and solvent were extracted for 30 min for microwave-assisted extraction (MAE) method with the power of 900 watts. The conventional shaking extraction (ShE) method was carried out using vertical shaker at 125 rpm for 3 hrs at room temperature. The extracted matters were immediately filtered through No.1 filter paper using vacuum suction flask. The filtrates were completely dried by using vacuum rotary evaporator. The extractions were done in triplicate. The dried crude extracts were kept at -20°C for further determinations of cosmetic bioactivities.

3.2.2 Effect of Solvent on Bioactive Compound Extraction from Betel Nut

Because the raw seed exhibited the highest cosmetic activity, it was subsequently selected for this experiment. The sample (5 g) was mixed with 50 mL of either ethyl acetate, 95% ethanol, 50% ethanol, water, or propylene glycol. The extraction was carried out by microwave-assistance with 900 watts power for 30 min at room temperature. The extraction was done in triplicate. The filtrates of ethyl acetate, 95% ethanol, and 50% ethanol extracts were completely dried by using vacuum rotary evaporator. The filtrate of water extract was lyophilized by using freeze-dryer. The excepted propylene glycol extract was not dried. The obtaining ethyl acetate, 95% ethanol, water, and propylene glycol were kept at -20°C for further determination.

3.3 Solid-liquid Fractionation Extraction

The partial separation was carried out in order to determine the polarity group molecules in the raw betel nut seed. The raw betel nut seed (5 g) was step-wisely

extracted as shown in Figure 3.2 by 50 mL of hexane, ethyl acetate, acetone, 95% ethanol, 50% ethanol, and water, respectively. The extraction was carried out by microwave-assistance with 900 watts power for 30 min at room temperature. The residue of each step was re-extracted orderly. After that, the extracted solution was immediately filtered through No.1 filter paper. The extraction was done in duplicate. The filtrates were completely dried by using vacuum rotary evaporator. The dried crude extracts were kept at -20°C for further determinations.

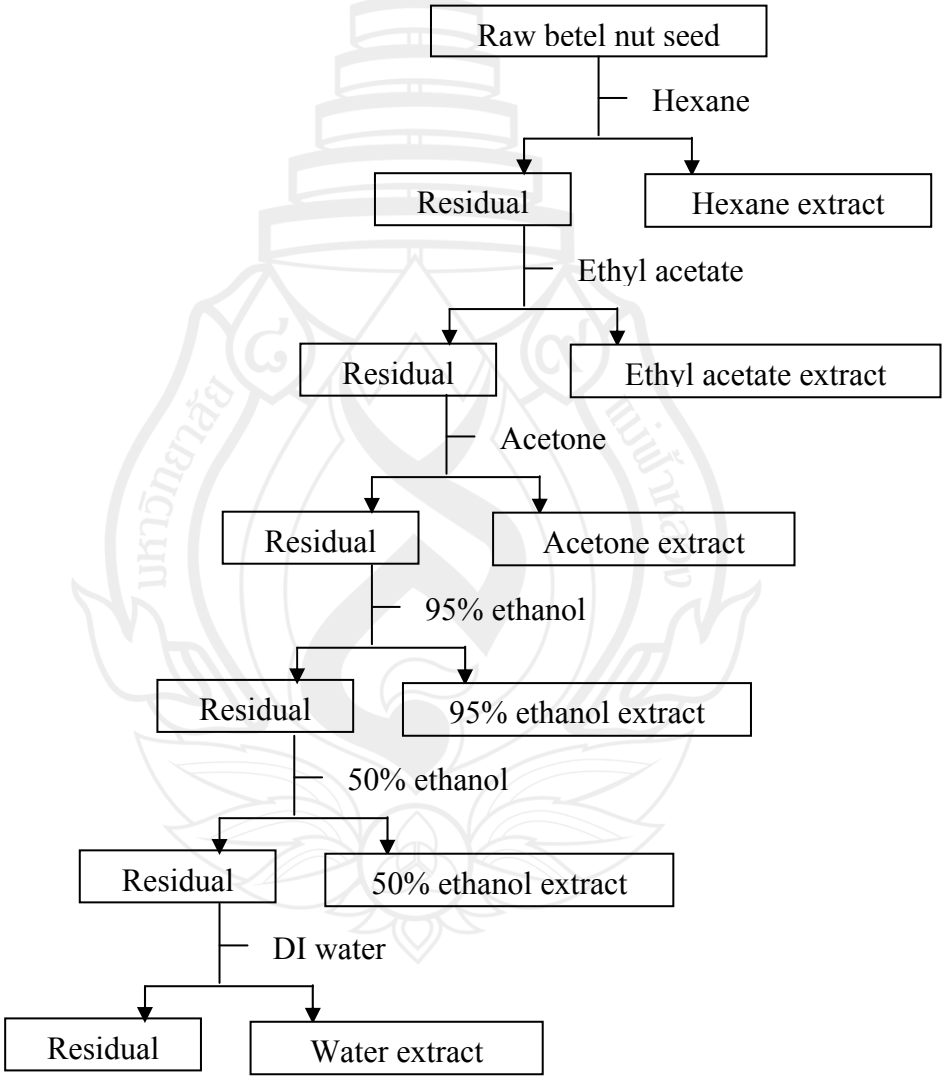


Figure 3.2 Scheme of Solid-liquid Fractionation Extractions

3.4 Determination of Polyphenolics Compound Content

3.4.1 Determination of Extractable Phenolics Content (EPC)

Extractable phenolics content of betel nut extract was determined according to the Folin-Ciocalteu method (Kumar, S., Kumar, D. & Prakash, O., 2008) with some modifications. The standard phenolic compound used in this study was gallic acid.

Reagents preparation

1. Gallic acid (0.10 mg/m:): Gallic acid (2.5 mg) was completely dissolved in deionized water. Then the final volume of 25 mL was adjusted with the deionized water.

2. Sodium carbonate (7.5%, w/v): Sodium carbonate (Na_2CO_3) (7.5 g) was completely dissolved in deionized water. Then the final volume of 100 mL was adjusted with the deionized water.

The proper volume of diluted sample was added with the adjusting of DI H_2O into 1.25 mL. Then 0.25 mL of Folin-Ciocalteu reagent was added. The alkaline condition of the reaction was done by the adding of 1.50 mL of 7.5% Na_2CO_3. The mixture was vortexed and incubated at ambient for 30 min. The experiment was done in triplicate. Then the absorbance of each sample was measured at 765 nm by spectrophotometer. Extractable phenolics contents (EPC) of all extracts were expressed as milligram gallic acid equivalents per gram of dried sample (mg GAE/g sample) which calculated from gallic acid standard curve.

3.4.2 The Extractable Flavonoids Content (EFC)

Extractable flavonoids content of betel nut extract was determined according to the aluminium colorimetric assay (Kumar et al., 2008) with some modifications. The standard flavonoid compound used in this study was quercetin.

Reagents preparation

1. Quercetin (0.25 mg/mL): Quercetin (6.25 mg) was totally dissolved and adjusted to 25 mL with ethanol. The standard was freshly prepared and kept in amber bottle.

2. Sodium nitrite (5% w/v): Sodium nitrite ($NaNO_2$) was weighed in 5.0 g and totally dissolved in 100 mL the deionized water.

3. Aluminium chloride (10% w/v): Aluminium chloride ($AlCl_3$) was weighed in 10.0 g and totally dissolved in 100 mL the deionized water.

4. Sodium hydroxide (4% w/v): Sodium hydroxide (NaOH) was weighed in 4.0 g and totally dissolved in 100 mL the deionized water.

Aluminium colorimetric assay was employed for flavonoids content determination. The proper volume of diluted sample was added with the adjusting of DI H_2O into 3.7 mL. Then 0.15 mL of 5% $NaNO_2$ was added followed by the same volume of 10% $AlCl_3$. The mixture was vortexed and incubated at ambient for 5 min then 1.0 mL of 4% NaOH was added. After the incubation at ambient for 5 min., the reaction was measured the absorbance at 510 nm against the blank with the absence of sample. The result was expressed as milligram quercetin equivalent per gram of dried sample (mg QE/g sample).

3.4.3 The Extractable Catechin Content (ECC)

The vanillin assay (Gunaratne, A., Wu, K., Li, D., Bentota, A., Corke, H. & Cai, Y.Z., 2013; Sun, B., Ricardo-da-Silva, J. M., & Spranger, I., 1998) with some modifications was used for proanthocyanidins content determination. The standard proanthocyanidins compound used in this study was catechin.

Reagents preparation

1. Catechin (0.1 mg/mL): Catechin (1.00 mg) was totally dissolved and adjusted to 10 mL with methanol. The standard was freshly prepared and kept in amber bottle.

2. Sulfuric acid (25% v/v): Sulfuric acid (H_2SO_4) (25 mL) was mixed with methanol and adjusted into 100 mL.

3. Vanillin solution (1% w/v): Vanillin was weighed in 1.0 g and totally dissolved in 100 mL methanol.

The proper volume of diluted sample was added with the adjusting of methanol into 0.4 mL. Then 1.0 mL of 25% H_2SO_4 was added followed by the same volume of 1% vanillin solution. The mixture was vortexed and incubated at 30◦C.

After 15 min incubation, the reaction was measured the absorbance at 500 nm against the blank with the adding of methanol instead of vanillin solution for correcting the absorbance by nonvanillin reactive compounds to eliminate the influence of the interference (e.g., anthocyanins). The result was expressed as milligram catechin equivalent per gram of dried sample (mg CE/g sample).

3.5 Determinations of Cosmetic Bioactivity

3.5.1 DPPH Radical Scavenging Activity Assay

The decolorizing reaction of 2,2-diphenyl-1-picrylhydrazyl stable radical scavenging activity was employed (Thaipong, K., Boonprakob, U., Crosby, K., Cineros-Zevallos, L. & Byrne, D.H., 2006).

Reagent preparation

1. Trolox (0.125 mg/mL): 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) was weighed (1.25 mg) and totally dissolved with 10 mL ethanol. The standard was freshly prepared and kept in amber bottle.

2. DPPH reagent (0.1 mM): 2,2-diphenyl-1-picrylhydrazyl (0.0198 g) was totally dissolved with 500 mL ethanol. The reagent was freshly prepared and kept in amber bottle.

The reagent was freshly prepared by 0.1 mM DPPH in ethanol and was kept in amber bottle. The proper volume of diluted sample was added with the adjusting of ethanol into 1.0 mL. Two milliliters of DPPH reagent was added then the mixture was vortexed and incubated at dark ambient condition for 30 minute. The decolorized reaction was measured at 517 nm and the decrement of absorbance was calculated against the control of stable radical of DPPH reagent into percentage of inhibition as the equation below.

$$\% \text{ Radical scavenging activity} = \left(\frac{A_{517} \text{ control} - A_{517} \text{ sample}}{A_{517} \text{ control}} \right) \times 100$$

Where, A_{517} control = the absorbance of the control (reaction without antioxidant)

A_{517} sample= the absorbance of the tested sample extract or trolox standard

The trolox standard curve is prepared by plotting the percentage of inhibition versus trolox concentration. The result was expressed as milligram trolox equivalent antioxidant capacity per gram of dried sample (mg TEAC/g sample) and the concentration giving 50% inhibitory activity (IC_{50}, µg/mL).

3.5.2 ABTS Cation Radical Scavenging Capacity

The decolorizing of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) cation radical scavenging capacity of the extract was determined (Thaipong et al., 2006).

Reagent preparation

1. Trolox (0.125 mg/mL): Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was weighed (1.25 mg) and totally dissolved and adjusted to 10 mL with ethanol. The standard was freshly prepared and kept in amber bottle.

2. Potassium phosphate buffer (PB) (50 mM) pH 7.4: The mixture of dipotassium hydrogen phosphate (4.35 g) and potassium dihydrogen phosphate (3.40 g) were dissolved with DI H_2O. The buffer solution was adjusted into pH 7.4 with either monobasic or dibasic phosphate. The solution with desired pH value was then adjusted to 500 mL.

3. $ABTS^{\bullet+}$ reagent: $ABTS^{\bullet}$ (7.4 mM) was prepared by totally dissolved 0.0406 g $ABTS^{\bullet}$ in 10 mL DI H_2O. Dipotassium persulfate (2.45 mM) was prepared by totally dissolved 0.0066 g $K_2S_2O_8$ in 10 mL DI H_2O. Then $ABTS^{\bullet}$ (7.4 mM) was pre-incubated with 2.45 mM $K_2S_2O_8$ to generate the $ABTS^{\bullet+}$ at dark ambient condition for 12-16 hrs prior use. The stock $ABTS^{\bullet+}$ was then mixed with 50 mM PB pH 7.4 at the ratio of 1:20 (v/v) and used within 4 hrs.

The proper volume of diluted sample was added with the adjusting of 50 mM PB pH 7.4 into 1.0 mL. Two milliliters of $ABTS^{\bullet+}$ reagent was added then the mixture

was vortexed and incubated at dark ambient condition for 30 min. The blue decolorized reaction was measured at 734 nm and the decrement of absorbance was calculated against the control of stable cation radical of ABTS as the equation below.

$$\% \text{ Radical scavenging activity} = \left(\frac{A_{734} \text{ control} - A_{734} \text{ sample}}{A_{734} \text{ control}} \right) \times 100$$

Where, A_{734} control = the absorbance of the control (reaction without antioxidant)

A_{734} sample= the absorbance of the tested sample extract or trolox standard

The trolox standard curve is prepared by plotting percentage of inhibition versus trolox concentration. The result was expressed as milligram trolox equivalent antioxidant capacity per gram of dried sample (mg TEAC/g sample) and the concentration giving 50% inhibitory activity (IC_{50}, µg/mL).

3.5.3 Ferric Reducing Antioxidant Power

The reducing power of the betel nut extract was determined (Thaipong et al., 2006; Benzie & Strain, 1999). The complex of reduced form of ferrous, Fe(II) with 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) was measured.

Reagent preparation

1. Trolox (0.125 mg/mL): 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) was weighed (1.25 mg) and totally dissolved and adjusted to 10 mL with ethanol. The standard was freshly prepared and kept in amber bottle.

2. Acetate buffer (0.3 M) (AcB) pH 3.6: The mixture of acetic acid (8.59 mL) and sodium acetate (12.30 g) were dissolved with DI H_2O. The buffer solution was adjusted into pH 3.6 with either acetic acid or sodium acetate. The solution with desired pH value was then adjusted to 500 mL.

3. FRAP reagent: Ferric chloride (20 mM) was prepared by totally dissolved 0.1622 g $FeCl_3$ in 50 mL DI H_2O. TPTZ (10 mM) was prepared by totally dissolved 0.1562 g TPTZ in 50 mL 40 mM HCL. The FRAP reagent was prepared by mixing the 10 mM TPTZ, 20 mM $FeCl_3$ and 0.3 M AcB pH 3.6 at the ratio of 1:1:10 by volume and kept in amber bottle.

The ferric reducing antioxidant power (FRAP) was determined. The proper volume of diluted sample was added with the adjusting of AcB pH 3.6 into 1.0 mL. Then 2.0 mL of FRAP reagent was added, vortexed and incubated at 37°C. After 30 min incubation, the reaction was measured the absorbance at 593 nm against the blank with the absence of sample. The result was expressed as milligram trolox equivalent antioxidant capacity per gram of dried sample (mg TEAC/g sample).

3.5.4 Lipid Peroxidation Inhibitory Activity

The measuring of thiobarbituric acid reactive substances (TBARS) formed by the complex of thiobarbituric acid (TBA) and malonaldehyde (MDA) product of the lipid peroxidation was determined (Choi, C.W., Kim, S.C., Hwang, S.S., Choi, B.K., Ahn, H.J., Lee, M.Y., Park, S.H. & Kim, S.K., 2002).

Reagent preparation

1. Butylated hydroxy toluene (5 mg/mL): BHT (50 mg) was totally dissolved and adjusted to 10 mL with ethanol.

2. Ferrous sulfate (4 mM): Fe_2SO_4 (11.1 mg) was totally dissolved and adjusted to 10 mL with DI H_2O.

3. TBA-TCA solution: Thiobarbituric acid (1%) was prepared by totally dissolve 0.5 g of TBA with 50 mL 0.1 N NaOH. Trichloroacetic acid (5.5%) was prepared by totally dissolve TCA (2.75 g) with 50 mL DI H_2O. The TBA and TCA solution was mixed in the ratio of 3:7 (v/v) and kept in 4°C.

4. Ascorbic acid (20 mM): Ascorbic acid (3.5 mg) was totally dissolved in 50 mL DI H_2O

5. Tris-hydrochloride buffer (0.1 M) pH 7.5: Tris-HCl (7.88 g) was totally dissolved in 400 mL DI H_2O. The solution was adjusted to pH 7.5 with 0.1 M NaOH. The solution with desired pH value was then adjusted the volume to 500 mL.

6. Linoleic acid emulsion: linoleic acid (20 mM) was weighed in 0.3505 g and mixed with 100 mL 0.1 M Tris-HCl buffer pH 7.5. Then, 0.3505 g Tween 20 was added. The emulsion was homogenously mixed with the adjustment to 125 mL with 0.1 M Tris-HCl buffer pH 7.5.

The linoleic acid emulsion (0.5 mL) and Tris-HCl buffer was added in the screw cap test tube. The reaction was initiated by adding 0.1 mL of 4 mM Fe_2SO_4. The proper volume of diluted sample was added with the adjusting of Tris-HCl buffer into 1.1 mL. The 0.1 mL of 20 mM ascorbic acid was added to activate the reaction. The mixture was pre-incubated at 37°C. After 10 min, 1.0 mL of TBA-TCA solution was added. The reaction was carried out in 95 °C water bath for 5 min. The reaction was then stopped in the ice bath for another 5 min. The emulsion reaction was centrifuged at 7,000 rpm for 5 min, the supernatant was measured the absorbance of TBARS at 532 nm. The blank was set without the adding the initiator, 4 mM Fe_2SO_4 and the control was absence of sample. The TBARS production inhibition was calculated into percentage of inhibition as the equation below.

$$\% \text{ Lipid peroxidation inhibitory activity} = \left(\frac{A_{532} \text{ control} - A_{532} \text{ sample}}{A_{532} \text{ control}} \right) \times 100$$

Where, A_{332} control = the absorbance of the control (reaction without antioxidant)

A_{532} sample= the absorbance of the tested sample extract or BHT standard

The BHT standard curve is prepared by plotting percentage of inhibition versus BHT concentration. The result was expressed as milligram BHT equivalent per gram of dried sample (mg BHTE/g sample) and the concentration giving 50% inhibitory activity (IC_{50}, µg/mL).

3.5.5 Tyrosinase Inhibitory Activity

The mushroom tyrosinase (EC 1.14.18.1) inhibitory activity of the extract was determined using L-Dopa as the monophenolase substrate (Onar, H.C., Yusufoglu, A., Turey, G. & Yanardag, R., 2012).

Reagent preparation

1. Kojic acid (0.125 mg/mL): Kojic acid (1.25 mg) was weighed and totally dissolved and adjusted to 10 mL with DI H_2O. The standard was freshly prepared and kept in amber bottle.

2. Potassium phosphate buffer (50 mM) (PB) pH 6.8: The mixture of dipotassium hydrogen phosphate (K_2HPO_4) (4.35 g) and potassium dihydrogen phosphate (KH_2PO_4) (3.40 g) were dissolved with DI H_2O. The buffer solution was adjusted into pH 6.8 with either monobasic or dibasic phosphate. The solution with desired pH value was then adjusted to 500 mL.

3. L-Dopa (25 mM): L-Dopa (122.5 mg) was totally dissolved with PB pH 6.8 and adjusted to 25 mL.

The mushroom tyrosinase (EC 1.14.18.1) was prepared into 1,000 unit/mL and was used in 0.04 mL. Then the proper volume of diluted sample was added with the adjusting of 50 mM PB buffer pH 6.8 into 1.76 mL. The mixture was pre-incubated at 37°C for 5 min prior adding 0.2 mL of 20 mM L-DOPA substrate. The reaction was carried out at 37°C for exactly 10 min. The measuring of dopachrome product was conducted at 475 nm. The blank was set without the adding the tyrosinase enzyme and the control was absence of sample. The resulting dopachrome production inhibition was calculated into percentage of inhibition as the equation below.

$$\% \text{ Tyrosinase inhibitory activity} = \left(\frac{A_{475} \text{ control} - A_{475} \text{ sample}}{A_{475} \text{ control}} \right) \times 100$$

Where, A_{475} control = the absorbance of the control (reaction without kojic acid)

A_{475} sample= the absorbance of the tested sample extract or kojic acid

The kojic acid standard curve is prepared by plotting percentage of inhibition versus kojic acid concentration. The result was expressed as milligram kojic acid equivalent per gram of dried ample (mg KAE/g sample) and the concentration giving 50% inhibitory activity (IC_{50}, µg/mL).

3.5.6 Elastase Inhibitory Activity

The inhibitory activity of the betel nut extract to the porcine pancreas elastase (PPE) (EC 3.4.21.36) against the substrate of N-Succinyl-$(Ala)_3$-*p*-nitroanilide (STANA) was determined (Onar et al., 2012). Epigallocatechin gallate (EGCG) was used as standard.

Reagent preparation

1. Epigallocatechin gallate (0.2 mg/mL): EGCG (1.0 mg) was dissolved and adjusted to 10 mL with DI H_2O.

2. Tris-hydrochloride buffer (0.2 M) pH 7.5: Tris-HCl (7.88 g) was totally dissolved in 200 mL DI H_2O. The solution was adjusted to pH 7.5 with 0.1 M NaOH. The solution with desired pH value was then adjusted to 250 mL

3. N-succinyl-$(Ala)_3$-*p*-nitroanilide (5 mM): STANA (22.6 mg) was totally dissolved in Tris-HCl buffer pH 7.5 and adjusted to 10 mL.

The porcine pancreas elastase (PPE) was prepared in 10 unit/mL and was used in 2.5 µL. Then the proper volume of diluted sample was added with the adjusting of 0.2 M Tris-HCl buffer pH 8.0 into 157.5 µL. The mixture was pre-incubated at 37°C for 10 min prior 2.5 µL of STANA (5 mM) was added. The reaction was carried out at 37°C for exactly 20 min. The measuring of *p*-nitroaniline product was conducted at 410 nm. The blank was set without the adding the elastase enzyme and the control was absence of sample. The result of the *p*-nitroaniline production inhibitory activity was calculated into percentage of inhibition as the equation below.

$$\% \text{ Elastase inhibitory activity} = \left(\frac{A_{410} \text{ control} - A_{410} \text{ sample}}{A_{410} \text{ control}} \right) \times 100$$

Where, A_{410} control = the absorbance of the control (reaction without EGCG)

A_{410} sample= the absorbance of the tested sample extract or EGCG standard

The EGCG standard curve is prepared by plotting percentage of inhibition versus EGCG concentration. The result was expressed as the concentration giving 50% inhibitory activity (IC_{50}, µg/mL) compared with epigallocatechin gallate.

3.5.7 Anti-Microbial Activity

The paper disk diffusion method (Bauer et al., 1996) was achieved against 5 microorganisms. The bacteria were gram-negative of *E. coli, Ps. aeruginosa,* and *S. typhimurium* and gram- positive *of S. aureus* and *S. epidermidis*. The inhibitory activity was observed by means of its clear zone.

Reagent preparation

The culture media was prepared as nutrient broth and agar for *E. coli, Ps. aeruginosa, S. aureus* and *S. epidermidis*. Tryptic soy broth and agar were used in *S. typhiurium* cultured.

1. Nutrient broth: The nutrient broth powder was weighed in 8 g and totally dissolved in 1 L DI H_2O. The mixture was kept in glass media bottle and auto-claved for 20 min.

2. Nutrient agar: The nutrient broth powder was weighed in 8 g with the addition of agar (15 g) and totally dissolved in 1 L DI H_2O. The mixture was kept in glass media bottle and auto-claved for 20 min.

3. Tryptic soy broth: The tryptic soy broth powder was weighed in 30 g and totally dissolved in 1 L DI H_2O. The mixture was kept in glass media bottle and auto-claved for 20 min.

4. Tryptic soy agar: The tryptic soy agar powder was weighed in 30 g with the addition of agar (15 g) and totally dissolved in 1 L DI H_2O. The mixture was kept in glass media bottle and auto-claved for 20 min.

5. Tested sample: The dried samples or positive antibiotic were dissolved into the required concentration with DMSO.

6. Tested paper disc: No. 1 filter paper was punctured in the size of 6 mm and auto-claved for using as the paper disc. The paper disc was filled with the tested sample.

The obtained bacterium was streaked in three planes and incubated for 24 hr. The few colonies were picked with the wire loop and were introduced to the test tube containing 4 mL broth. The overnight inoculum stock was diluted with the broth to obtain the 0.5 McFarland standards equal to 1.5×10^8 CFU/m: (colony-forming unit per milliliter). The working bacterial broth was prepared by the turbidity measurement at 625 nm using spectrophotometer in the range of 0.08-0.10 AU.

The 15 mm glass plates (5-6 mm depth) were poured with approximately 12 mL culture medium. The swabbing cotton was totally put into the working inoculum and the excess suspension was removed by rotated against the side of the tube. Then the working bacterial cotton swap was evenly streaked in 3 planes onto the medium

surface and reaches the edge of the plate. The inoculum plate was leaved to dry. Then, within 3-5 min, the test sample paper disc was place onto the medium surface with the flamed forceps and gently pressed down to ensure contact. The plates were inverted incubated in the 37°C incubator within 30 min. After overnight incubation, the clear zone diameters (including the 6 mm paper disc) were measured with the ruler on the undersurface of the petri dish.

3.6 Characterization of Betel Nut Extracts

3.6.1 UV Absorption Scanning

The extracts were scanned their absorption in the UV region, wavelength of 200-400 nm with the frequency of 1 nm by UV-visible spectrophotometer. The subtraction of the absorption was computed with the dissolved solvent (50% ethanol). The absorbability of the extracts was then compared to the standard substances of gallic acid, tannic acid, quercetin, catechin, and epicatechin.

3.6.2 HPLC Analysis

High performance liquid chromatography (HPLC) was employed to determine the chromatogram pattern of the extracts. The linear gradient of 5% acetic acid and methanol was used for 75 min analysis. The sample was automatically injected for 10 μL with the constant flow rate of 0.8 mL/ min through reverse phase column (C18, Altima) (Wang, C.K. & Lee, W.H., 1996). The chromatograms pattern were detected at 280 nm and were compared to the standard substances of kojic acid, gallic acid, catechin, gallocatechin, epigallocatechin, epigallocatechingallate, chlorogenic acid, epicatechin, caffeic acid, gallocatechingallate, ferulic acid, ellagic acid, cinnamic acid, and quercetin.

3.7 Cytotoxicity Test

The cytotoxicity of samples were investigated by MTT assays with the normal cell line (human skin fibroblast) and cancer cell line (B16-F10, mouse skin melanoma) by BIOTEC, Thailand. The results were obtained with the reference testing protocol as described below.

3.7.1 Sample Preparation

Each sample was dissolved in dimethylsulfoxide (DMSO) to make a stock concentration of 20 mg/mL. The samples were then serial diluted in the culture medium of cells giving 8 concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 µg/mL.

3.7.2 Cell Culture

The target cells were B16-F10 (mouse skin melanoma; ATCC Cat. No. CRL-6475) and human skin dermal fibroblast cell lines. The B16-F10 cells were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin. The human dermal fibroblast cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/m: penicillin and 100 µg/mL streptomycin. The cells were incubated at 37°C in a fully humidified, 5% CO_2: air atmosphere.

3.7.3 MTT Cytotoxicity Test

The brief summary of method was a modified version of conventional direct and indirect contact tests conformed to the published standard methods (BS-EN30993-5 and ISO10993-5). The MTT assay is a tetrazolium-dye based colorimetric micro-titration assay. Metabolism competent cells are able to metabolize the tetrazolium (yellow) to formazan (blue). The color change was measured spectrophotometrically with a plate reader. It is assumed that cells are metabolically deficient will not survive, thus the MTT assay is also an indirect measurement of cell

viability. The cells were seeded in a 96-well plate at a density of 2,000 cells/ well, and incubated for 48 hours. The sample at various concentrations were added to the cells and incubated for 24 hours. The test samples were removed from the cell cultures and the cells were re-incubated for a further 24 hrs in fresh medium and then tested with MTT assay.

Briefly, 50 μL of MTT in PBS at 5 mg/mL was added to the medium in each well and the cells were incubated for 4 hours. Medium and MTT were then aspirated from the wells, and formazan solubilized with 200 μL of DMSO and 25 μL of Sorensen's Glycine buffer, pH 10.5. The absorbance was read with a microplate reader (Molecular Devices) at a wavelength of 570 nm. The average of 4 wells was used to determine the mean of each point. The experiments were done in triplicate to get the values and standard deviation. The data were analyzed with the SoftMax Program (Molecular Devices) to determine the IC_{50} for each toxin sample. A dose-response curve was derived from 8 concentrations in the test range using 4 wells per concentration. Results of toxic compounds are expressed as the concentration of sample required to kill 50% (IC_{50}) of the cells compared to controls.

3.8 Stability Test of the Extract

The artificial condition of food and cosmetic application was conducted in the stability test. The different storage factors of pH and temperature were tested. Fifty millimolar of buffer solutions were prepared from sodium phosphate (pH 2, 7, and 11), sodium citrate (pH 3, 6), sodium acetate (pH 4, 5), Tris-HCl (pH 8), and sodium borate (pH 9, 10) (Chaiwut, P., Nitsawang, S., Shank, L. & Kanasawud, P., 2007).

The 50% ethanol raw betel nut seed extract were dissolved in the concentration of 1% (w/v) with each pH buffer for physical stability tests of color and pH changes. The extract at the concentration of 2.0 mg/mL was used in biological activities stability determination; phenolic content, ABTS cation radical scavenging, and tyrosinase inhibitory activities. The sample solutions were kept at 4°C, room temperature, and 50°C. The determination was carried out weekly through 12 weeks.

3.9 Development of Cream Containing Betel Nut Seed Extract

3.9.1 Preparation of Cream Base Formula

The cream base formula in Table 3.3 was developed from the master formula as described in Ernest, W.F. (2001). The orders of mixing were performed under the regular mixing method of oil in water emulsion type. The ingredients of each phase were weighed separately. The oil phase (phase A) was completely melted by a hot plate. Then, phase A was slowly added into hot water phase (phase B) and vigorously stirred. Then the emulsion was subjected to the homogenizer for 10 minutes. The additive of silicone (phase C) and 50% ethanol raw betel nut seed extract in the concentration of 0.5% (w/w) (phase D) were added after the emulsion was cooled down. Then the emulsion was homogeneously mixed.

Table 3.3 The Cream Base Master Formula

Part	Ingredient	% w/w
A	Oil	20.0
	Brij 72	3.5
	Brij 721S	1.5
	Propyl paraben	0.2
B	Water	73.6
	Methyl paraben	0.2
	Xanthan	0.5
C	Silicone	-
D	Active substance	1.0

3.9.2 Conditions of Stability Test

3.9.2.1 Centrifugation: The test formula was weighed of 1.5 g and added into a 1.5 mL size of centrifuge tube and centrifuged at acceleration of 6,000 rpm for 30 min. Then, the phase separation of the tested formulations was observed.

3.9.2.2 Freeze/thaw cycles: A glass bottle with a plastic screw cover was filled with the test sample. The sample was vertically stored in a freezer at -20 ◦C for 12 h and then stored at room temperature for 12 h. The sample was repeated in three cycles. Separation of emulsion phase or any change in product property was observed.

3.9.2.3 Heating/cooling cycles: A glass bottle with a plastic screw cover was filled with the test sample. The sample was stored in hot air oven at 55◦C for 12 h and then stored in the refrigerator at 4◦C for 12 h. The sample was repeated in three cycles. Separation of emulsion phase or any change in product property was observed.

3.9.3 Physio-chemical evaluation: The pH, viscosity and color of developed base formula were determined before and after stability tests according to evaluate their stability. The experiment was performed in triplicate of cycle 0 and 3.

3.9.3.1 pH measurement: The pH value of 5% (w/v) of formula in deionized water was measured by using a CyberScan pH 1100 pH meter.

3.9.3.2 Viscosity measurement: Viscosity of the formula was measured by using of Brookfield RVDV-II+P at room temperature. The measurement was performed in triplicate.

3.9.3.3 Color measurement: Color of the formula was measured by using of Chromameter KONICA MINOLTA CR-400 at room temperature. The data value L* (luminance), a* (green-red spectrum), and b* (blue-yellow spectrum) were recorded. The measurement was performed in triplicate.

3.10 Data and Statistical Analysis

All experiments were performed in triplicate (n=3). The recorded results were subjected to statistical analysis with 95 percentage of confidential level.

CHAPTER 4

RESULTS AND DISCUSSIONS

The results of this study were categorized into 6 main parts. The first part was studied the raw and ripe seed and raw and ripe pericarp on the bioactive compound contents. Simultaneously, the comparison of extraction method between conventional shaking (ShE) and microwave-assistance (MAE) was also investigated and resulted in the second part. The different extraction potential from 5 solvents of ethyl acetate, 95% ethanol, 50% ethanol, water, and propylene glycol were investigated in the third part. The solid-liquid fractionated extraction was studied in the fourth part in order to partial purify and determine the polarity group compounds in the betel nut extract. The fifth part of the extract stability test was performed in the condition of pH 2-11 with the storage at 4°C, room temperature, and 50°C. The final part included the application of betel nut extract in oil in water emulsion. The stability of the formula containing 0.5% extract was studied under the accelerated conditions of freeze/thaw and heating/cooling as well as storage at 4°C, room temperature, and 50°C. The pH, viscosity, color, residual phenolic content and residual radical scavenging activity were employed to evaluate the emulsion stability.

4.1 Effect of Shaking Speed on Bioactive Compound Extraction from Betel Nut Seed

The conventional shaking method in this study was preliminary tested for its optimal shaking speed for the extraction of bioactive compound from betel nut seed. Three speed levels of 75, 125, and 175 rpm were used. Table 4.1 showed the comparative result of the cosmetic bioactivities. The speed of 125 rpm provided significantly higher EPC even compared to 175 rpm extraction. For antioxidant capacity, 125 rpm also provided higher activity compared to 75 and 175 rpm when determined using FRAP assay. There was no significantly higher DPPH radical scavenging activity when using high speed of 175 rpm compared to 125 rpm speed. Therefore, the shaking speed of 125 rpm was chosen to use in the extraction of cosmetic active compound from the betel nut compared to the microwave-assisted method which the maximum power of 900 watt was used.

Table 4.1 Preliminary Test of the Shaking Speed for Using in Shaking Method

Shaking speed (rpm)	EPC (mg GAE)*	DPPH (mg TEAC)*	FRAP (mg TEAC)*
75	132.50	299.43	294.85
125	172.61	413.30	351.61
175	158.24	417.19	332.21

Note: * The result presented in mg standard equivalent per gram dried weight sample

4.2 Effect of Sample Part and Ripening Stage on Bioactive Compound Extraction from Betel Nut

The raw and ripe seed as well as raw and ripe pericarp were studied for their bioactive content. The 50% ethanol was used as the extraction solvent. The betel nut extract from each sample are shown in Figure 4.1. The obvious differences in the color of extracts were noted reddish brown from the seed and yellow from the pericarp. The most reddish brown were found in raw betel nut seed extract than that of ripe seed. The pericarp extracts were almost similar. The different extraction method displayed alternation of the color. Lighter color shade was obtained from shaking (Figure 4.1A) than those of microwave-assisted method (Figure 4.1B).

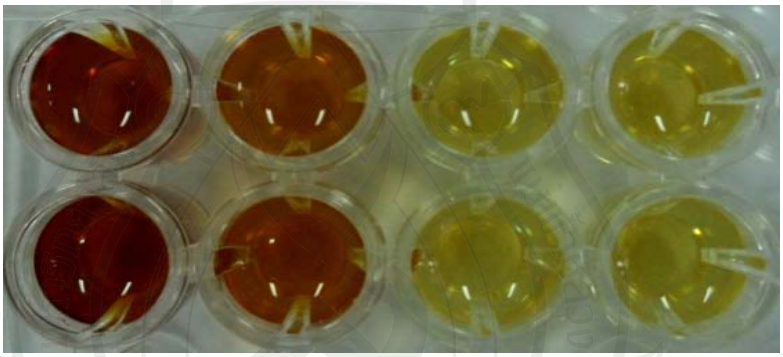


Figure 4.1 Appearance of Betel Nut Crude Extract Prepared by Shaking (row A) and Microwave-Assisted Extraction Methods (row B)

4.2.1 Determination of Polyphenolics Compound Content

4.2.1.1 Determination of extractable phenolics content (EPC)

The EPC of betel nut extracts are shown in Table 4.2. The value were determined by the Folin-Ciocalteu reagent containing molybdenum on superior oxidation state (Mo^{+6}) with a yellow color. Polyphenols compounds which contain at least one hydroxyl group (-OH) was determined its reducing activity of Mo^{6+} to

inferior states of oxidation (Mo^{4+} or Mo^{5+}) which have a blue color and can be monitored spectrophotometry at 765 nm (Kumar et al., 2008).

In comparison of the sample, raw betel nut seed extract exhibited the highest EPC in both extraction method of shaking (ShE) (172.61 mg GAE/g sample) and microwave-assistance (MAE) (226.58 mg GAE/g sample). According to the ripening stage, the raw seed provided the higher EPC, approximately 1.04 - 1.68 fold than those ripe seeds. It is notice that extract from betel nut seed, both raw and ripe, showed the higher EPC than the pericarp extracts. However, the ripe pericarp showed the higher EPC than that of the raw pericarp approximately 3.47-3.59 folds. The EPC of ripe pericarp was 4.55 and 5.97 mg GAE/g sample from ShE and MAE, respectively. The lowest was obtained from raw pericarp of 1.31 mg GAE/g sample (ShE) and 1.66 (MAE) mg GAE/g sample.

4.2.1.2 The extractable flavonoids content (EFC)

The EFC was determined by aluminium colorimetric assay (Kumar et al., 2008). Aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminium chloride forms acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids (Chang et al., 2002).

The EFC results in Table 4.3 showed that highest EFC was obtained from the raw seed extracts which the ShE and MAE provide the values of 132.48 and 140.79 mg QE/g sample, respectively. The lower values were obtained from the ripe seed exhibiting 99.76 and 111.78 mg QE/g sample from extraction by ShE and MAE, respectively. The raw seed extracts possessed the EPC approximately 1.26-1.33 fold higher than the ripe seed. Similarly to the EPC trend, the pericarp exhibits the lower EFC than the seed part. The EFC of the ripe pericarp were 4.23 and 3.87 mg QE/g sample, respectively by ShE and MAE showing the higher EFC approximately 2.01-2.27 fold than raw pericarp.

4.2.1.3 The extractable catechin content (ECC)

Vanillin assay was employed in the determination of flavanols. The assay was widely used as its simplicity and specificity to a narrow range of flavanols (monomers and polymers) and dihydrochalcones (Sun et al., 1998).

Table 4.2 showed the highest ECC from raw betel nut seed extracts which are 56.04 and 67.23 mg CE/g sample from ShE and MAE, respectively. The ripe seed possessed the lower value of 23.35 (ShE) and 36.17 mg CE/g sample (MAE). The lowest ECC was obtained from raw pericarp, 0.61 (ShE) and 0.68 mg CE/g sample (MAE).

Table 4.2 Extractable Phenolic (EPC) and Extractable Flavonoid (EFC) of Betel Nut Extracts Prepared by Shaking (ShE) and Microwave-Assisted (MAE) Extractions

Extraction method	**EPC (mg GAE)** *	**EFC (mg QE)** *	**ECC (mg CE)** *
Shaking			
Raw seed	172.61±1.21[a**]	132.48±0.33 [a]	56.04±0.67 [a]
Ripe seed	102.51±1.68 [b]	99.76±0.10 [b]	23.35±0.70 [b]
Raw pericarp	1.31±0.09 [d]	1.86±0.33 [d]	0.61±0.02 [d]
Ripe pericarp	4.55±0.10 [c]	4.23±0.17 [c]	2.48±0.02 [c]
Microwave assistance			
Raw seed	226.58±1.58 [a]	140.79±0.85 [a]	67.23±0.60 [a]
Ripe seed	134.57±2.21 [b]	111.78±0.55 [b]	36.17±0.14 [b]
Raw pericarp	1.66±0.09 [d]	1.93±0.15 [d]	0.68±0.03 [d]
Ripe pericarp	5.97±0.13 [c]	3.87±0.44 [c]	2.40±0.02 [c]

Note: * Values were presented in mean±SD per gram of dried weight sample
** Small letter subscribed the statistical significantly difference among the samples in each test ($p<0.05$)

The polyphenol contents in the seed and pericarp were consistent with previous reports. The seed portion of *A. catechu* L. showed the highest phenolics content than the other parts of leaf, spike, vein, and tender shoot, respectively (Wang et al., 1997). It was also reported to contain the phenolic compound higher than husk

and flower, respectively (Wetwitayaklung et al., 2006). The comparison of the ripening stage in each seed and pericarp showed the opposite trend. The raw seed contained higher polyphenols contents than the ripe seed. In contrast with the pericarp, raw pericarp exhibited lower polyphenols contents than ripe. These results described the effect of ripening stage on the distribution of phenolics compound in the process of maturity of betel nut. Study of the change in tannin content along 1-8 month seed reported the increasing line from 1-6 month then gradually deducted at 6-8 month (Wang et al., 1997). Some of the study reported the contrast result which the polyphenols content decreased substantially along the maturity (Wetwitayaklung et al., 2006). On the other hands, it was reported that the bioactive compound, tannin, decreased as the ripening stage of betel nut increased (Bhandare, A.M., Kshirsagar, A.D., Vyawahare, N.S., Hadambar, A.A. & Throve, V.S., 2011).

4.2.2 Determinations of Cosmetic Bioactivity

4.2.2.1 DPPH radical scavenging activity assay

The anti-oxidant properties of the betel nut extracts by means of the stable DPPH radical scavenging activity was determined. The stable purple DPPH radical was hydrogen donated by the bioactive compounds giving the yellow reduced form.

Table 4.3 shows the IC_{50} values of the raw betel nut seed extracts. The most potential scavenging activity was possessed by the raw seed extract with the lowest IC_{50} of 21.03 and 19.17 µg/mL by ShE and MAE, respectively. The extracts were about 5 times less potent than trolox which is the derivative of tocopherol (4.11 µg/mL). The lower potential was obtained from ripe seed of 29.07 (ShE) and 27.30 µg/mL (MAE). The result of DPPH scavenging activity was similar with the phenolic content as the pericarp extracts were less potent than those of seed extracts. The ripe pericarp possessed higher activity, 119.35 and 114.65 µg/mL, obtained by ShE and MAE, respectively. The lowest activity was found in the raw pericarp extracts of 153.33 (ShE) and 138.33 (MAE) µg/mL.

The antioxidant activity was also calculated to be the milligram trolox equivalent antioxidant capacity per gram of dried weight sample (mg TEAC/g sample). The results are shown in Table 4.4. The similar results were obtained as the highest activity was raw betel nut seed extract (413.30 (ShE) and 495.51 (MAE) mg

TEAC/g sample). The lower was derived from ripe seed extracts of 293.82 (ShE) and 379.75 (MAE) mg TEAC/g sample. In comparison between the parts of betel nut fruit, the seed has the significantly higher activity than the pericarp. The lowest TEAC from the ripe pericarp extracts were 7.48 (ShE) and 8.95 (MAE) mg TEAC/g sample.

4.2.2.2 ABTS cation radical scavenging capacity

The ABTS cation radical scavenging activity of the betel nut extract can be seen in Table 4.4. The significantly highest potential was obtained from raw seed extract with the lowest IC_{50} of 1.26 and 1.06 μg/mL obtained by ShE and MAE, respectively. The ripe stage of betel nut seed exhibited the lower potential with IC_{50} of 4.66 (ShE) and 4.39 (MAE) μg/mL. The lowest ABTS cation radical scavenging activity was found in the raw pericarp of 30.31 (ShE) and 27.78 (MAE) μg/mL. In comparison with the IC_{50} of trolox standard (4.34 μg/mL), the raw seed extracts were even possessed the higher potential of approximately 4 folds over the standard. Moreover, the extraction methods were found having some affected on ABTS activity.

The IC_{50} values in Table 4.3 were positively correlated with the standard equivalent values in Table 4.4. The raw betel nut seed obviously showed the greatest antioxidant capacity, followed by the ripe seed, ripe pericarp, and raw pericarp, respectively.

4.2.2.3 Ferric reducing antioxidant power (FRAP)

The ferric reducing power interprets the antioxidant activity by the electron donating via reduction reaction cause the intense color of Fe^{3+}-TPTZ complex. The results in Table 4.4 show the activity of betel nut extracts which expressed as trolox equivalent antioxidant capacity (TEAC). The reducing capacity was also same as the previous determination. The highest potential raw seed extracts were 413.30 and 495.51 mg TEAC/g sample by ShE and MAE, respectively. The lower was obtained from ripe seed of 293.82 (ShE) and 379.75 (MAE) mg TEAC/g sample. The reducing activity of the pericarp was consisted to the previous determination that it was lower than the seed part. The ripe pericarp possessed the higher reducing activity of 7.48 and 8.95 mg TEAC/g sample by ShE and MAE, respectively.

The study of antioxidant and phenolic compounds of 112 traditional Chinese medicinal plants found that *A. catechu* was at the top rank (Cai, Y., Luo, B., Sun, M. & Corke, H., 2004). The 70 Korean medicinal plants were study for their free radical scavenging effect (Ahn, B.Y., 2009). The *A. catechu* seed exhibited the highest activity which was close to BHA activity and higher than obtained from BHT and L-ascorbic acid.

4.2.2.4 Lipid peroxidation inhibitory activity

The inhibitory activity against the lipid peroxidation process was one of the most focused determinations in the anti-oxidant capacity of the natural extract. The interruption of unsaturated fatty acid oxidation of the betel nut extracts was investigated. The results in Table 4.3 shows the IC_{50} values of the betel nut extract. The seed part has the higher activity than the pericarp as same as the previous results. The raw seed extract displayed the IC_{50} 0.19 and 0.18 mg/mL for ShE and MAE, respectively while the raw pericarp extract showed the highest IC_{50} in the range of 1.80-1.93 mg/mL. It is worth to notice that the betel nut extracts, even the low potential pericarp extracts, exhibited significantly higher potential lipid peroxidation inhibitory activity than the standard of BHT (4.19 mg/mL). The BHT is the commonly synthesis antioxidant used to prevent the lipid oxidation process. Therefore, the betel nut extract could be substantially substituted the use of BHT.

The lipid peroxidation inhibitory activity of the extract was also interpreted as BHT equivalent. The results are shown in Table 4.4 as the highest activity was also obtained in the raw seed extract (58.76 and 60.51 mg BHTE/g sample for ShE and MAE, respectively). The other results were obviously similar to the sequence of IC_{50} values.

Table 4.3 Cosmetic Bioactivities in Term of IC_{50} of Betel Nut Extracts Prepared by Shaking (ShE) and Microwave-Assisted (MAE) Extraction Methods

Extract	DPPH (µg/mL)	ABTS (µg/mL)	Anti-LOOH^{-} (mg/mL)	Anti-Tyr (µg/mL)	Anti-Ela (µg/mL)
Shaking					
Raw seed	21.03±0.11 [a]	1.26±0.00 [a]	0.19±0.01 [a]	7.15±0.05 [a]	125.14±0.45 [a]
Ripe seed	29.07±0.11 [b]	4.66±0.12 [b]	0.43±0.02 [b]	11.30±0.08 [b]	140.70±0.31 [b]
Raw pericarp	158.33±1.25 [d]	30.31±0.14 [d]	1.93±0.02 [d]	233.94±7.64 [d]	1,627.85±11.25 [d]
Ripe pericarp	119.35±1.13 [c]	28.24±0.40 [c]	1.42±0.02 [c]	196.45±3.13 [c]	1,440.25±9.49 [c]
Microwave assistance					
Raw seed	19.17±0.09 [a]	1.06±0.00 [a]	0.18±0.01 [a]	6.24±0.07 [a]	111.50±0.44 [a]
Ripe seed	27.30±0.12 [b]	4.39±0.00 [b]	0.38±0.01 [b]	9.33±0.04 [b]	146.71±0.57 [b]
Raw pericarp	138.93±0.99 [d]	27.78±0.18 [c]	1.80±0.02 [d]	199.63±3.83 [d]	2,006.40±11.96 [d]
Ripe pericarp	114.65±0.63 [c]	26.04±0.40 [c]	1.40±0.01 [c]	144.00±5.73 [c]	1,610.10±13.46 [c]
Standard					
Trolox	4.11±0.03	4.34±0.03	-	-	-
BHT	-	-	4.19±0.04	-	-
Kojic acid	-	-	-	15.65±0.05	-
EGCG	-	-	-	-	47.07±0.17

Note: [*] Values were presented in mean±SD
[**] Small letter subscribed the statistical significantly difference among the samples in each test ($p<0.05$)

Table 4.4 Cosmetic Bioactivities in Term of Standard Equivalent of Betel Nut Extracts Prepared By Shaking (ShE) and Microwave-Assisted (MAE) Extraction Methods

Extract	DPPH (mg TEAC/g sample)	ABTS (mg TEAC/g sample)	FRAP (mg TEAC/g sample)	Anti-LOOH^{-} (mg BHTE/g sample)	Anti-Tyr (mg KAE/g sample)
Shaking					
Raw seed	413.30±0.90 [a]	719.05±1.14 [a]	351.16±2.99 [a]	58.76±0.38 [a]	334.63±3.83 [a]
Ripe seed	293.82±0.38 [b]	511.46±1.59 [b]	219.10±1.90 [b]	23.87±0.19 [b]	179.09±1.1 [b]
Raw pericarp	6.36±0.26 [d]	15.82±0.28 [d]	23.37±0.35 [d]	7.90±0.06 [d]	18.99±1.68 [d]
Ripe pericarp	8.95±0.39 [c]	16.32±0.32 [c]	43.18±0.87 [c]	13.85±0.07 [c]	23.69±1.33 [c]
Microwave assistance					
Raw seed	495.51±0.69 [a]	908.39±2.70 [a]	383.17±2.25 [a]	60.51±0.44 [a]	348.81±9.12 [a]
Ripe seed	379.75±0.91 [b]	629.49±3.83 [b]	297.93±1.18 [b]	24.56±0.16 [b]	189.78±3.99 [b]
Raw pericarp	6.69±0.31 [d]	15.12±0.21 [d]	37.07±0.85 [d]	8.16±0.09 [d]	29.68±0.65 [d]
Ripe pericarp	7.48±0.18 [c]	36.59±0.61 [c]	70.29±0.66 [c]	14.12±0.10 [c]	34.35±1.09 [c]

Note: [*] Values were presented in mean±SD
[**] Small letter subscribed the statistical significantly difference among the samples in each test ($p<0.05$)

4.2.2.5 Tyrosinase inhibitory activity

Tyrosinase is a key enzyme for melanin synthesis. Substance that can inhibit this enzyme can be considered as whitening agent. The most effective tyrosinase inhibitory activity expressed as the lowest IC_{50} values. As in Table 4.3, the raw seed extract gave the lowest IC_{50} of 7.51 and 6.24 µg/mL for ShE and MAE, respectively. The lower activity was found in the ripe seed extract of 11.30 and 9.33 µg/mL for ShE and MAE, respectively. The pericarp extract also as same as the previous determinations. The ripe pericarp extract exhibited the activity of 196.45 (ShE) and 144.00 (MAE) µg/mL. The lowest tyrosinase inhibitory activity was found in the raw pericarp extract which the IC_{50} of 233.94 and 199.63 µg/mL were observed for ShE and MAE, respectively.

The tyrosinase inhibitory activity of betel nut extracts were also expressed as the kojic acid equivalence per gram of dried sample (mg KAE/g sample) (Table 4.4). Similarly, the highest inhibition activity was obtained from raw seed extracts (189.78 and 348.81 mg KAE/g sample for ShE and MAE, respectively). The ripe pericarp extract also exhibited greater activity for tyrosinase inhibition that the raw sample.

4.2.2.6 Elastase inhibitory activity

Elastase is one of the metallo proteinase (MMP) enzymes which degrade the elastin in the dermis layer. Natural extract that can inhibit the elastase activity may considered as anti-aging substance. The elastase inhibitory activity of the betel nut extracts were also in the same order as the previous bioactivity determinations. In comparison of the part of betel nut, the seed was significantly higher than the pericarp. The seed exhibited the higher activity in the raw stage than the ripe seed (Table 4.3). The raw seed extracts showed the lowest IC_{50} of 125.14 (ShE) and 111.50 (MAE) µg/mL. The lower activity was governed by the ripe seed extracts of 140.70 and 146.17 µg/mL for ShE and MAE, respectively. Both raw and ripe seed extract possessed the higher anti-elastase potential than the epigallocatechin gallate (EGCG) (147.07 µg/mL). The lower elastase inhibitory activity was also obtained from the pericarp extracts. The higher activity was obtained from ripe pericarp of 1,440.25 (ShE) and 1,610.10 (MAE) µg/mL, whereas the ripe pericarp extracts possessed the lowest activity of 1,627.85 and 2,006.40 µg/mL by ShE and MAE, respectively. The

previous study (Thring, T.S.A., Hili, P. & Naughton, D.P., 2009) studied the anti-elastase activity of 21 plants and used EGCG as the standard. The very high inhibition potential (89%) was found in white tea and was equally active with EGCG (25 and 114 μg/mL, respectively). The moderate activity was found in 25 μg/mL green tea and pomegranate (10 and 14%, respectively). This study also reported very high correlation of elastase inhibitory activity and phenolics content (p=0.001). The isolated catechin and EGCG from green tea extract was reported the mix-type inhibitory activity (Kim, Y., Uyama, H. & Kobayashi, S., 2004).

The result implies that the antioxidant capacity, tyrosinase and elastase inhibitory activity were probably from polyphenols contents. Similar to the previous study of antioxidant, elastase and hyaluronidase inhibitory activities, the results were correlated to the concentration of phenolic-rich betel nut seed extract (Onar et al., 2012; Anthikat, R.R. & Michel, A., 2012). It has been documented that superoxide scavenging activity and inflammation delayed activity via nitric oxide synthase of betel nut seed extract were attributed to polyphenolics such as simple phenolics, tannins and proanthocyanins contents in the extract (Lee, S.E., Hwang, H.J., Ha, J.S., Jeong, H.S. & Kim, J.H., 2003). There was the report of the different part of *A. catechu* on polyphenols contents, antioxidant, and glutathione-S-Transferase (GST) inhibitory activity (Hamsar, M.N., Ismail, S., Mordi, M.N., Ramanathan, S. & Mansor, S.M., 2011). The unripe seed possessed the highest phenolic content among ripe seed and root (Hamsar *et* al., 2011). Flavonoids and phenolic acids are classified as mixed antioxidants because of the ability to donate protons to free radicals, and their capable of preventing the formation of reactive oxygen species (ROS) either by the metal traces chelating possess the inhibition of enzymes involved in the process of ROS production (Silva, M. C. A. & Paiva, S. R., 2012).

4.2.3 UV Absorption Ability

The scanning profiles of betel nut extracts in Figure 4.2 showed distinguish pattern of molecule content in the seed and pericarp parts. The interesting absorbability was observed at approximately 258-295 nm with the maximum absorbance at 279.8 nm was found in the seed extracts.

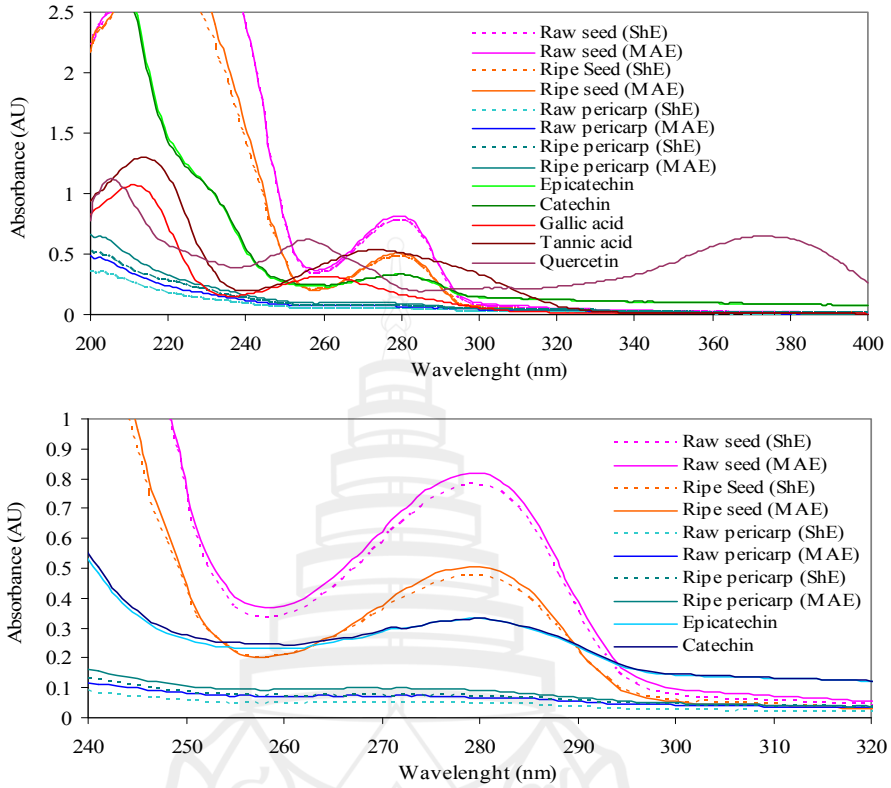
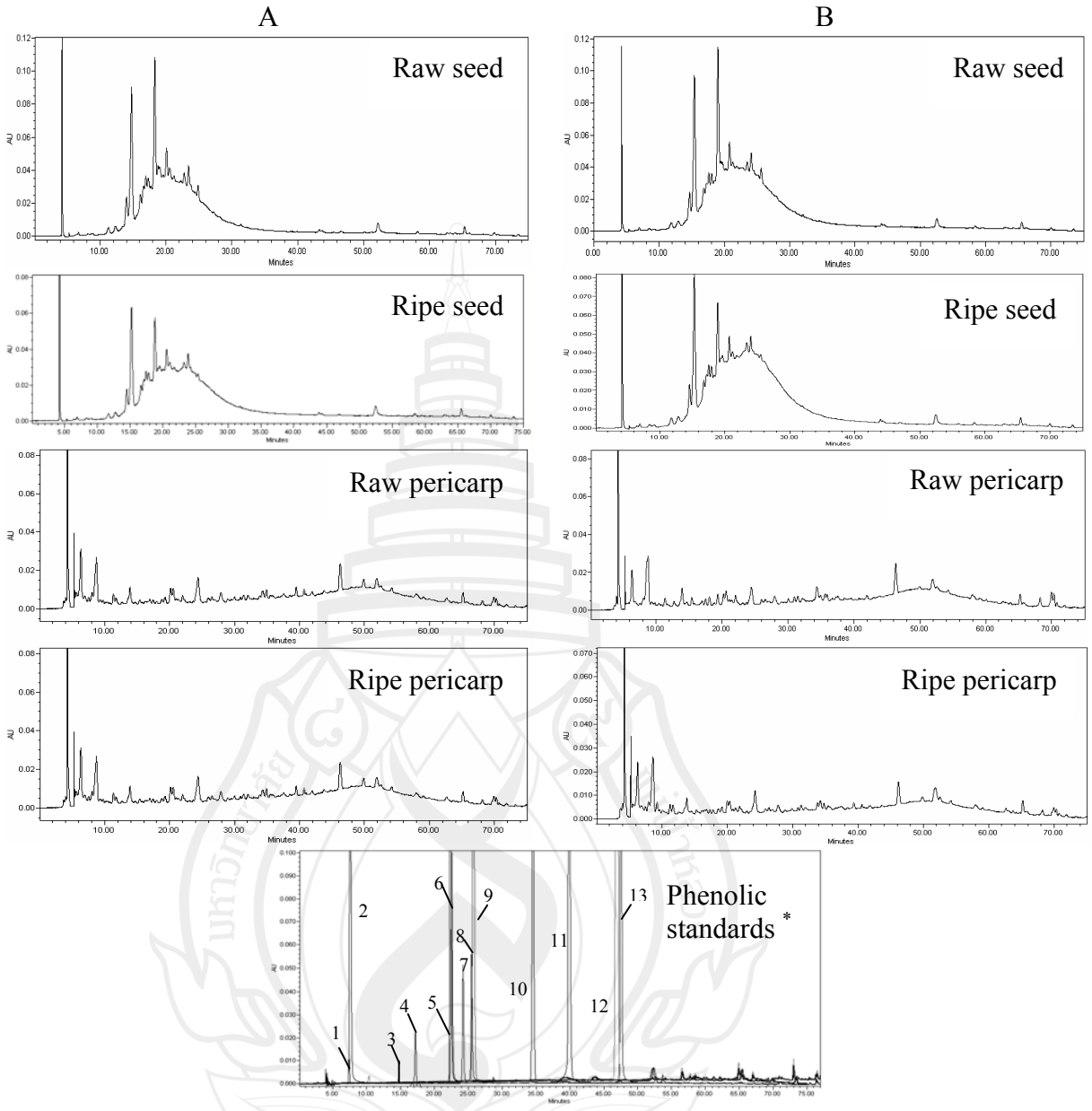


Figure 4.2 UV-scanning of Betel Nut Extracts

When compared with phenolics standards, the maximum absorbance at approximately 280 nm was observed to be the pattern of catechin and epicatechin. There was no interesting peak from betel nut pericarp extracts along the UV-region. The absorbability of the raw betel nut seed was higher than the ripe which related to previous the colorimetric determination of biological contents and activities.

4.2.4 HPLC Analysis

The elution profiles of the betel nut extracts in Figure 4.3 showed the comparison of extraction methods efficiency and the chemical compositions in each sample part. None significantly differences in peak area were found in the pericarp extracts. The raw and ripe seed extracts contained the molecules mostly eluted at 15-25 min, while the pericarp comprised higher polar molecules predominantly obtained at 5-10 min of elution.



Note: * (1) kojic acid, (2) gallic acid, (3) gallocatechin, (4) epigallocatechin, (5) epigallocatechingallate, (6) chlorogenic acid, (7) epicatechin, (8) gallocatechingallate, (9) caffeic acid, (10) ferulic acid, (11) ellagic acid, (12) cinnamic acid, (13) quercetin

Figure 4.3 HPLC Separations of Betel Nut Extracts Prepared by Shaking (column A) and Microwave-Assisted Method (column B) Compared with Standard Phenolic Substances

The fingerprints of betel nut extracts were compared with the mixture of 13 phenolics standard substances (Figure 4.3). There is some similar retention time of the compounds in the extract with the region of catechin-like family. Spectra scanning of the sample peak (Appendix B) confirmed that the main peaks in the betel nut seed belongs to catechin group (Appendix A). It was also found that catechin and epicatechin are the main phenolic compounds in betel nut seed (Wang & Lee, 1996). The seed was reported to be the main container of secondary metabolite of betel nut and the pericarp is major composed of fibrous (Wang et al., 1997). There have been found that the bioactive constituents in the betel nut were simple phenolics, catechin, caffeic acid, ferulic acid, non-tannin flavans, hydrolysable and condensed tannins, alkaloids and flavonoids (Wang & Lee, 1996; Bhandare et al., 2010).

4.2.5 Anti-Microbial Activity

The bacterial inhibitory activity of the betel nut extracts are depicted in Table 4.5. The positive result was found only from the seed extract which showed the activity against gram-positive microbes (*S. aureus* and *S. epidermidis)*. The seed extracts (3.2 mg) possessed inhibitory activities close to the positive of Gentamicin (10 μg). There were no statistical significantly differences between raw and ripe seed extracts. The low bioactive compound pericarp extracts were not effective to inhibit any microbes tested implying that the anti-microbial activity was attributed to the phenolics compounds of the extracts.

The bacterial growth inhibitory was considered to be taken in response by the polyphenols compounds. The catechin group, which promised to be the major constituents in the betel nut, was reported its high potential on microorganism growth inhibitory activity (Taylor, W.P., Hamilton-Miller, M.T.J. & Stapleton, D.P., 2009). Strong presence of tannins in all extracts may explain its potent bioactivities as tannins are known to possess potent antimicrobial activities (Kaur, G.J. & Arora, D.S., 2009). The possible reason of only gram-positive anti-bacterial activity was due to cell wall structure of bacteria. The cell wall of the gram-positive bacteria is only the peptidoglycan layer which might be easier to break down by the betel nut seed extract. In contrast for the gram-negative bacteria, the outermost cell wall contains additional lipopolysaccharide making it more resistant.

Table 4.5 Anti-Bacterial Activity of Betel Nut Extracts

	Clear zone (mm±SD)					
	Gentamicin (positive)	DMSO (negative)	Raw seed*	Raw pericarp*	Ripe seed*	Ripe pericarp*
E. coli	15.00±0.00	-	-	-	-	-
S. aureus	15.00±0.00	-	13.67±1.15	-	12.00±1.00	-
S. epidermidis	19.67±0.58	-	11.00±0.00	-	11.67±0.58	-
Ps. aeruginosa	15.00±0.00	-	-	-	-	-
S. typhimurium	17.00±0.21	-	-	-	-	-

Note: * Extracts used were 3.2 mg versus 10 µg of positive Gentamicin

- : no inhibitory zone was detected

4.2.6 Cytotoxicity Test

The extracts from microwave-assisted extraction were subjected to the cytotoxicity test against normal cell line of human skin fibroblast. Table 4.6 showed the negative results with more than 50 percent viable cells when treated with the betel nut extracts (>200 µg/mL). There were no significant cell damaging by the extracts those interpret the safety of using the betel nut extract onto the skin.

The cytotoxicity against B16-F10 melanoma cell line (Table 4.7) was interpret the anti-tumor or said to be anti-cancer property of the extracts. The cancer cell line was tested for the non-viable cell after cultured with the various concentrations of the betel nut extracts. Although most of the extracts were not able to inhibit the melanoma cell line, the raw betel nut seed extract at 200 µg/mL could damage almost 50% of the cells. The results were utilized the safe of use of raw betel nut seed extract in cosmetic products. The previous DPPH and ABTS radical scavenging capacity and lipid peroxidation, tyrosinase, elastase inhibitory activities determination, the raw betel nut seed extract possessed the IC_{50} values lower than 200 µg/mL.

Table 4.6 The % Survival of The Human Dermal Fibroblast Cell Line Cultured with Betel Nut Extracts at Various Concentrations

Samples	Concentration (µg/mL)	% Survival*	SD	IC_{50} (µg/mL)
Raw seed	200.00	81	3	>200
	100.00	116	12	
	50.00	106	2	
	25.00	102	5	
	12.5	113	6	
	6.25	107	9	
	3.13	114	14	
	1.56	102	8	
Ripe seed	200.00	91	6	>200
	100.00	105	7	
	50.00	115	7	
	25.00	105	4	
	12.5	93	7	
	6.25	96	3	
	3.13	93	5	
	1.56	98	3	
Raw pericarp	200.00	88	3	>200
	100.00	90	1	
	50.00	91	7	
	25.00	97	4	
	12.5	100	3	
	6.25	92	1	
	3.13	100	1	
	1.56	99	1	
Ripe pericarp	200.00	89	4	>200
	100.00	90	2	
	50.00	98	2	
	25.00	93	1	
	12.5	101	5	
	6.25	105	3	
	3.13	105	3	
	1.56	103	6	

Note: * % Survival>50% indicated no cytotoxicity; % survival≤50% indicated cytotoxicity

Table 4.7 The % Survival Of The B16-F10 Melanoma Cell Line Cultured With Betel Nut Extracts At Various Concentrations

Samples	Concentration (µg/mL)	% Survival*	SD	IC_{50} (µg/mL)
Raw seed	200.00	51	1	>200
	100.00	69	4	
	50.00	98	1	
	25.00	101	7	
	12.5	100	2	
	6.25	100	1	
	3.13	99	8	
	1.56	100	3	
Ripe seed	200.00	99	2	>200
	100.00	101	2	
	50.00	100	7	
	25.00	101	7	
	12.5	99	9	
	6.25	100	2	
	3.13	97	2	
	1.56	100	1	
Raw pericarp	200.00	100	1	>200
	100.00	101	2	
	50.00	100	5	
	25.00	100	4	
	12.5	99	5	
	6.25	99	6	
	3.13	100	5	
	1.56	100	1	
Ripe pericarp	200.00	101	7	>200
	100.00	102	2	
	50.00	100	6	
	25.00	100	5	
	12.5	100	1	
	6.25	100	2	
	3.13	99	3	
	1.56	99	3	

Note: * % Survival>50% indicated no cytotoxicity; % survival≤50% indicated cytotoxicity

4.3 Comparison of Shaking and Microwave-Assisted Methods on Bioactive Compound Extraction from Betel Nut

This study compared the efficiency of bioactive compound extraction by 2 methods of conventional shaking and microwave-assisted extraction. The EPC, EFC, ECC, DPPH and ABTS radical scavenging capacity, lipid peroxidation inhibitory activity, reducing capacity as wall as tyrosinase and elastase enzyme inhibition activities were used for evaluation the efficiency. Statistical analysis of T-test was employed to investigate the comparison.

4.3.1 Determination of Polyphenolic Compounds Content

4.3.1.1 Determination of extractable phenolics content (EPC)

The polyphenols extractions were highly effected by extraction method as showed in Table 4.8. In comparison of microwave-assisted (MAE) and conventional shaking (ShE) extraction method, MAE possessed significantly higher in phenolic extraction potential. The highest EPC obtained from raw seed exhibited the significantly mean difference of 48.86 mg GAE/g sample which can be calculated as 1.26 folds higher than ShE. The higher EPC of ripe seed, raw, and ripe pericarp also obtained from MAE with the mean differences of 11.54, 0.46, and 3.69 mg GAE/g sample, respectively. The higher MAE extraction potential was calculated as 1.09, 1.27, and 2.62 folds over those obtained from ShE in ripe seed, raw, and ripe pericarp extraction, respectively.

4.3.1.2 The extractable flavonoids content (EFC)

The flavonoids content in all samples were higher from MAE than ShE. The EFC as well as their mean difference in sample were showed in Table 4.8. In comparison of microwave-assisted (MAE) and conventional shaking (ShE) extraction method, MAE possessed significantly higher in EFC extraction potential. The highest EFC was same as EPC, raw seed exhibited the significantly mean difference of 8.31 mg QE/g sample which can be calculated as 1.06 folds higher than ShE. The significantly higher EFC of ripe seed and pericarp also obtained from MAE with the mean differences of 12.02 and 0.36 mg QE/g sample, or 1.12 and 1.09 folds over

those from ShE. The raw pericarp extracts were not statistical significantly affected by the extraction method of MAE and ShE with the mean difference of only 0.07 mg QE/g sample (1.03 folds).

4.3.1.3 The extractable catechin content (ECC)

Table 4.8 showed the mean differences of ECC between ShE and MAE. The extraction of raw seed showed the highest ECC which higher by MAE which the statistical significantly extraction potential was calculated being about 1.2 folds greater than ShE. The similar higher extraction potential of MAE was also found in ripe seed extraction (1.55 folds). The pericarp samples showed not significantly extraction potential of MAE over those from ShE.

Table 4.8 Extractable Bioactive Compounds of Betel Nut Extracts and the Mean Differences between Shaking (ShE) and Microwave-Assisted (MAE) Extraction Methods

Sample	Extraction method	EPC		EFC		ECC	
		mg GAE*	MD	mg QE*	MD	mg CE*	MD
Raw seed	ShE	179.72 ±0.52	46.86s	132.48±0.50	8.31s	56.04±0.67	11.19s
	MAE	226.58±1.41		140.79±0.30		67.23±0.60	
Ripe seed	ShE	123.03±0.32	11.54s	99.76±0.56	12.02s	23.35±0.70	12.82s
	MAE	134.57±1.96		111.78±0.97		36.17±0.14	
Raw pericarp	ShE	1.72±0.11	0.46s	1.86±0.29	0.07	0.61±0.02	0.07
	MAE	2.18±0.12		1.92±0.13		0.68±0.03	
Ripe pericarp	ShE	2.28±0.28	3.69s	3.87±0.39	0.36s	2.48±0.02	0.08s
	MAE	5.97±0.12		4.23±0.16		2.40±0.02	

Note: * Values were presented in mean±SD per gram of dried weight sample

MD: mean difference of the results between shaking and microwave-assisited extraction method

s: Significant difference at $p \geq 0.05$

4.3.2 Determinations of Cosmetic Bioactivity

4.3.2.1 DPPH radical scavenging activity assay

Similar results can be observed in Table 4.9. The antioxidant properties of the betel nut extracts was determined by DPPH assay as in 4.2. The similar results with the polyphenols contents were obtained as the highest activity was raw betel nut seed extract, 413.30 (ShE) and 495.51 (MAE) mg TEAC/g sample. The lower activity was obtained from ripe seed extracts with 293.82 (ShE) and 379.75 (MAE) mg TEAC/g sample. In comparison between the parts of betel nut fruit, the seed has the significantly higher activity than the pericarp. The ripe pericarp extract exhibited the higher TEAC of 7.48 and 8.95 mg TEAC/g sample by ShE and MAE, respectively. The lowest TEAC from the ripe pericarp extracts were 7.48 (ShE) and 8.95 (MAE) mg TEAC/g sample.

4.3.2.2 ABTS cation radical scavenging capacity

The ABTS cation radical scavenging activities were shown in Table 4.9. The same activity as those of DPPH assay. The raw seed extract antioxidant capacity were 719.05 (ShE) and 908.39 (MAE) mg TEAC/g sample. Lower antioxidant capacities were belonged to the ripe seed, ripe pericarp, and raw pericarp, respectively. The ripe seed exhibited the activity of 511.46 (ShE) and 629.49 (MAE) mg TEAC/g sample. The pericarp extract was in the same previous trend, the ripe possessed the higher activity than raw pericarp. The ripe pericarp showed 16.32 and 36.59 mg TEAC/g sample by ShE and MAE, respectively. The raw pericarp which is the lowest activity extracts exhibited 15.82 (ShE) and 15.12 (MAE) mg TEAC/g sample.

4.3.2.3 Ferric reducing antioxidant power

The reducing capacity results in Table 4.9 were also same as the previous determination. The highest potential raw seed extracts were 413.30 and 495.51 mg TEAC/g sample by ShE and MAE, respectively. The lower was obtained from ripe seed of 293.82 (ShE) and 379.75 (MAE) mg TEAC/g sample. The reducing activity of the pericarp was consisted to the previous determination that was lower than the seed part. The ripe pericarp possessed the higher reducing activity of 7.48 and 8.95 mg TEAC/g sample by ShE and MAE, respectively. The lowest activity of raw pericarp was 6.36 (ShE) and 6.69 (MAE) mg TEAC/g sample.

4.3.2.4 Lipid peroxidation inhibitory activity

The extract concentration give 50% lipid peroxidation inhibition were showed in Table 4.9 with the mean difference of each sample. The IC_{50} was not statistical significantly different between MAE and ShE in any sample. The lipid peroxidation inhibitory activity in Table 4.10 showed that the raw seed extract possessed the highest activity of 58.76 and 60.51 mg BHTE/g sample by ShE and MAE, respectively. The ripe seed gave lower activity of 23.87 (ShE) and 24.56 (MAE) mg BHTE/g sample. The raw pericarp possessed the lower activity than the ripe one. The ripe pericarp extract exhibited 13.85 and 14.12 mg BHTE/g sample by ShE and MAE, respectively. The lowest were found in the raw pericarp of 13.85 (ShE) and 14.12 (MAE) mg BHTE/g sample. Table 4.9 portray the mean different of BHT equivalent was not significant in any sample.

4.3.2.5 Tyrosinase inhibitory activity

The IC_{50} value of the extracts was showed in Table 4.10. The higher activity with lower IC_{50} was obtained from MAE in all samples with the significantly mean differences. Raw betel nut possessed the lowest IC_{50} with the statistical mean difference of 0.91 μg/mL, or about 1.15 folds higher from MAE than ShE. The MAE possessed higher extraction potential than ShE of 1.21, 1.17, and 1.36 folds in ripe seed, raw and ripe pericarp, respectively. The tyrosinase inhibitions in the kojic acid equivalent were showed in Table 4.10. The highest equivalences were obtained from raw seed extracts of 189.78 and 348.81 mg KAE/g sample by ShE and MAE, respectively. The highest activity was followed by the ripe seed of 179.09 (ShE) and 189.78 (MAE) mg KAE/g sample. The lower tyrosinase inhibitory activities were found in the pericarp extracts. The ripe pericarp has the higher activity of 29.68 (ShE) and 34.35 (MAE) mg KAE/g sample than the 18.99 (ShE) and 23.69 (MAE) mg KAE/g sample of raw pericarp extracts. The mean differences of each sample were showed in Table 4.9. The method effected to the tyrosinase inhibitory activity extraction in all sample.

Table 4.9 Comparison on the Mean Differences of the Betel Nut Cosmetic Bioactivities (IC_{50}) from Extraction between Shaking (ShE) and Microwave-Assisted (MAE) Methods

Extract	Extraction method	DPPH		ABTS		Anti-LOOH$^{\bullet}$		Anti-tyrosinase		Anti-elastase	
		µg/mL*	MD	µg/mL*	MD	mg/mL*	MD	µg/mL*	MD	µg/mL*	MD
Raw seed	ShE	1.03±0.11	1.86s	1.26±0.00	0.20	0.19±0.01	0.01	7.15±0.05	0.91s	125.14±0.45	13.64s
	MAE	19.17±0.09		1.06±0.00		0.18±0.01		6.24±0.07		111.50±0.44	
Ripe seed	ShE	29.07±0.11	1.77s	4.66±0.12	0.27	0.43±0.02	0.05	11.30±0.08	1.97s	146.71±0.57	6.01s
	MAE	27.30±0.12		4.39±0.00		0.38±0.01		9.33±0.04		140.70±0.31	
Raw pericarp	ShE	158.33±1.25	19.40s	30.31±0.14	2.53s	1.93±0.02	0.13	233.94±7.64	34.31s	2,006.40±11.96	372.55s
	MAE	138.93±0.99		27.78±0.18		1.80±0.02		199.63±3.83		1,627.85±11.25	
Ripe pericarp	ShE	119.35±1.13	4.70s	28.24±0.40	2.20	1.42±0.02	0.02	196.45±3.13	52.45s	1,610.10±13.46	169.85s
	MAE	114.65±0.63		26.04±0.40		1.40±0.01		144.00±5.73		1,440.25±9.49	

Note: * Values were presented in mean±SD

MD: mean difference of the results between shaking and microwave-assisited extraction method

s: Significant difference at $p \geq 0.05$

Table 4.10 Comparison of Means Difference on Cosmetic Bioactivities (Standard Equivalent) from Extraction by Shaking (ShE) and Microwave-Assisted (MAE) Methods

Sample	Extraction method	DPPH		ABTS		FRAP		Anti-LOOH-		Anti-TYR	
		mg TEAC*	MD	mg TEAC*	MD	mg TEAC*	MD	mg BHTE*	MD	mg KAE*	MD
Raw seed	ShE	413.30±0.72	82.20s	719.05±1.33	189.33s	351.16±2.63	32.01s	58.76±0.38	1.75	334.60±7.77	159.03s
	MAE	495.51±1.35		908.39±1.43		383.17±2.09		60.51±0.44		348.81±14.28	
Ripe seed	ShE	293.82±3.41	85.93s	511.46±4.76	118.03s	219.09±1.80	18.83s	23.87±0.19	0.69	179.09±3.90	155.54s
	MAE	379.75±2.08		629.49±3.69		297.93±1.52		24.56±0.16		189.78±9.81	
Raw pericarp	ShE	6.36±0.23	0.33	15.12±0. 19	0.70s	37.07±0.09	6.11s	7.90±0.06	0.26	18.99±1.56	5.99s
	MAE	6.69±0.29		15.82±0.28		43.18±0.76		8.16±0.09		23.69±1.56	
Ripe pericarp	ShE	7.48±0.18	1.47	16.32±0.30	20.28s	7.03±0.30	16.34s	13.85±0.07	0.27	29.68±1.04	15.35s
	MAE	8.95±0.35		36.59±0.53		23.37±0.31		14.12±0.10		34.35±1.04	

Note: * The values were presented in mean±SD of mg standard equivalent per gram of dried weight sample

MD: mean difference of the results between shaking and microwave-assisited extraction method

s: Significant difference at $p \geq 0.05$

4.3.2.6 Elastase inhibitory activity

Table 4.9 showed the higher activity with the lower IC_{50} was obtained from MAE in all samples with the significantly mean differences. Raw betel nut which possessed the lowest IC_{50} with the statistical mean difference of 13.64 µg/mL or about 1.12 folds higher from MAE than ShE. MAE possessed higher extraction potential than ShE of 1.04, 1.23, and 1.12 folds in ripe seed, raw and ripe pericarp, respectively. The mean differences of each sample were showed in Table 4.9. The method effected to the tyrosinase inhibitory activity extraction in all sample.

The MAE method used only 30 min at 900 watt when comparing to 6 h at 125 rpm of shaking. This work clearly showed that MAE was the potential method for extraction the bioactive compound from the betel nut. The result was agreement with previous report that the extraction of phenolic content from *Rosmarinus officinalis, Origanum dictamnus, Origanum majorana, Teucrium polium*, *Vitex agnus–cactus, Styrax officinalis* by MAE were superior to that using conventional reflux extraction (Proestos, C. & Komaitis, M., 2008). The comparison of MAE with heat reflux and ultrasonicate extraction found that MAE found having higher precision and significantly higher extraction yield with significant reduction in extraction time (Zhu, X., Su, Q., Cai, J. & Yang, J., 2006: Flora, et al., 2009). However, there was the reported of other significant effects of solvents, power, and duration on the MAE potential (Li & Lin, 2010).

There were many focused on microwave-assisted technique in these few years on its promising environmental friendly process and potential extraction results. When compared with classical reflux extraction and soxhlet extraction, microwave assisted extraction generally shows evident advantages with shorter extraction time, higher extraction yield, higher selectivity and better quality of the target extracts such as polyphenols as well as essential oils (Zhu et al., 2006). MAE is relatively cost-effective when compared to accelerate solvent extraction (Mandal et al., 2008: Zhang et al., 2011: Proestos & Komaitis, 2008: Zhu et al., 2006) and surpasses supercritical fluid extraction owing to its operation simplicity and low cost. Moreover the main advantage of MAE over ultrasonic-assisted extraction is its reduced extraction time consumption (Zhang et al., 2011).

The simple principle of microwave assisted extraction consists of microwave energy which encourages the extractant in contact with solid sample. The process involves disruption of hydrogen bonds, as a result of microwave-induced dipole rotation of molecules, and migration of ions, which enhance the penetration of solvent into matrix. Cell wall disruption by microwave also allows the releasing of the intracellular products of plant to be extracted (Mandal et al., 2008: Zhang et al., 2011: Proestos & Komaitis, 2008: Zhu et al., 2006).

4.4 Effect of Solvent on Bioactive Compound Extraction from Raw Betel Nut Seed

The betel nut extracts from each solvent were showed in Figure 4.4. The filtrates showed the notable color differences (Figure 4.4A). The clear pale yellow extract was obtained from ethyl acetate extract. The similar brownish were obtained from 95% ethanol, 50% ethanol, and water extract. The propylene glycol extract possessed the most brownish extract. After completely dried (Figure 4.4B), the ethanol extract possessed waxy yellow crude. The ethanol crude was powdery brown, while crude of water extract was flaky. The noticeably different was found in propylene glycol extract. The propylene glycol is the solvent with the high melting point (approximately 121°C) then it can not be eliminated. The extraction yield of each extract was showed in Table 4.11.

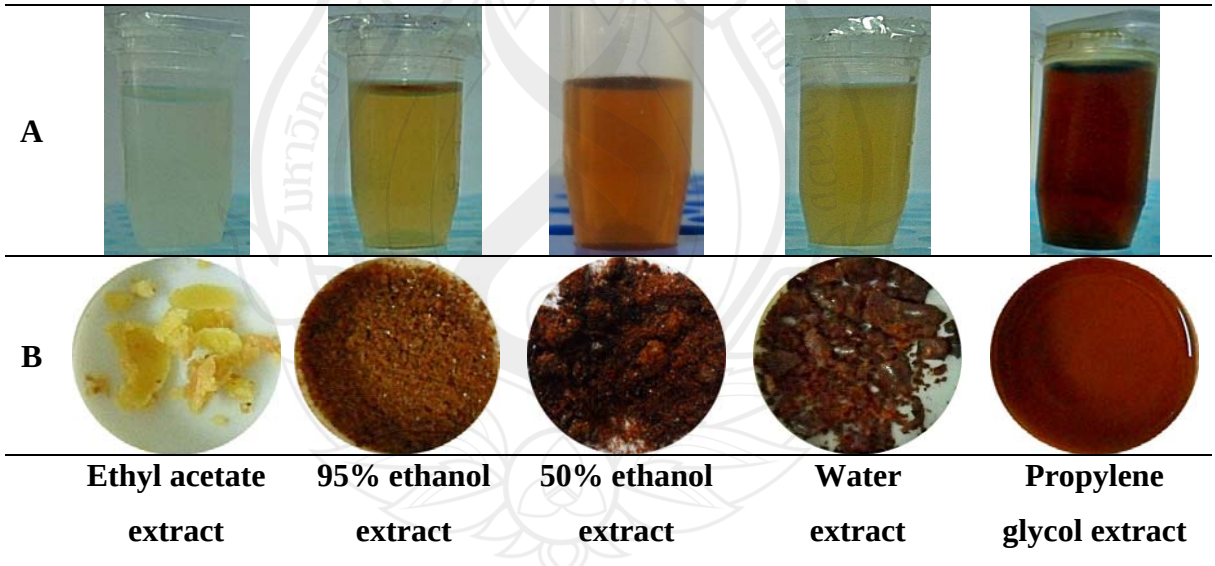


Figure 4.4 Appearance of Betel Nut Seed Extract, Crude Extract (row A) and Dried Crude Extract (B)

4.4.1 Determination of Polyphenolics Compound Content

4.4.1.1 Determination of extractable phenolics content (EPC)

Extractable phenolic content of betel nut extracts are shown in Table 4.11. In comparison of the solvent, 50% ethanol extract exhibited the highest EPC of 226.58 mg GAE/g sample. The lower EPC were obtained from 95% ethanol, propylene glycol, and water extract of 117.73, 109.51, and 70.06 mg GAE/g sample, respectively. The significantly lowest EPC of 13.48 mg GAE/g sample was possessed by ethyl acetate extract. The raw betel nut extracts of 50% ethanol extract exhibited about 1.92 folds than higher than 95% ethanol extract and 1.68 and 1.07 folds over than water and propylene glycol extract. The highest EPC of ethanol extract was also found having significantly higher extraction potential than the from ethyl acetate extract from low polarity ethyl acetate (8.73 folds).

4.4.1.2 The extractable flavonoids content (EFC)

The EFC results in Table 4.11 show that highest EFC was obtained from 50% ethanol extract which was not statistical significantly different with 95% ethanol extract of 140.79 and 132.19 mg QE/g sample, respectively. The lower EFC values obtained from propylene glycol (109.51 mg QE/g sample) and water extract (70.06 mg QE/g sample), respectively. The ethyl acetate extract possessed the significantly lowest EFC of 5.06 mg QE/g sample. Ease perspective of the comparing in folds depicted that the ethanol extract possessed 1.06, 1.1.8, and 1.50 folds higher EFC than 95% ethanol, propylene glycol, and water extract, respectively. The ethyl acetate extract exhibited approximately 26 folds lower than ethanol extract.

4.4.1.3 The extractable catechin content (ECC)

Table 4.11 showed the ECC results of each sample. The significantly highest value was obtained from propylene glycol (128.91 mg CE/g sample). The lower was obtained from 50% ethanol, water, and 95% ethanol, respectively. The lowest ECC was similar to the previous results of EPC and EFC. Ethyl acetate possessed the lowest extractant potential (3.50 mg CE/g sample).

The ethyl and methyl alcohol were considered as the potential extractant in many of extraction methods (Sultana, B., Anwar, F. & Ashraf, M., 2009). As the like-dissolve-like theory, the lower polar solvents such as hexane and ethyl acetate were not suitable for phenolics compound extraction. The phenolics compounds containing

the –OH group makes it being polar. The previous study of Patel, A., Patel, A., Patel, A. & Patel, N.M. (2010) reported the higher total phenolics, flavonoids; quercetin and rutin contents in ethanolic *Tephrosia purpurea* Linn. extract (18.44%, 1.56% and 2.54%) than those of water extract (9.44%, 0.91%, 1.85%). The study of *A. catechu* seed extraction (Li & Lin, 2010) also reported higher extraction potential of methanol than water. The higher total phenolic contents was obtained in methanol than water extract (466.70 and 278.25 μg gallic acid equivalent) as same as flavonoids content (933.33 and 286.36 μg rutin equivalent) (Li & Lin, 2010).

Table 4.11 Extractable Phenolic (EPC), Extractable Flavonoid (EFC) Contents of Betel Nut Seed Extracts

Extract*	**% Yield (w/w)**	**EPC (mg GAE)****	**EFC (mg QE)****	**ECC (mg CE)****
EtOAc	8.70±0.60 [d***]	13.48±0.28 [d]	5.06±0.09 [d]	3.5±0.76 [e]
95% EtOH	26.90±0.80 [b]	117.73±1.66 [a]	132.19±0.04 [a]	36.47±0.75 [d]
50% EtOH	21.00±0.72 [c]	226.58±1.58 [a]	140.79±0.85 [a]	67.23±0.60 [b]
Water	20.30±0.30 [c]	70.06±0.87 [c]	88.38±0.49 [c]	56.33±0.69 [c]
PG	-	109.51±0.94 [b]	112.31±0.38 [b]	128.91±2.07 [a]

Note: * EtOAc: ethyl acetate, 95% EtOH: 95% ethanol, 50% EtOH: 50% ethanol and PG: propylene glycol

** The values were presented in mean±SD of mg standard equivalent per gram of dried weight sample

*** Small letter subscribed the statistical significantly difference among the samples in each test ($p<0.05$)

In addition of the solvent polarity, the dissipation factor influences the extraction efficacy in microwave-assisted method. The significant lower extraction efficacy low polar solvent was described as the lower dielectric constant or the ability to absorb microwave energy, i.e. 20.7 (acetone) and 1.89 (hexane) while the higher

ability were found in water (78.3) and ethanol (24.3) (Mandal et al., 2007). Furthermore, the dissipation factor or the ability of the solvent to absorb microwave energy and pass it on as heat (Zhang et al., 2011) which can be calculated from the dielectric constant and dielectric loss values (Mandal et al., 2007) was greatly influence to the extraction yield. The constant interpret the extractable manner of the target compounds. The reason of higher polyphenols extraction yield of ethanol was attributed to the lower dissipation factor of ethanol ($60{,}750\times10^{-4}$) than water ($122{,}931\times10^{-4}$). The polyphenols which thermal instability was less degraded after extraction by ethanol. The use of small amount of water also reported to increase the solvent penetration to plant cell wall (Mandal et al., 2007). These influences of solvent using in MAE were also stated (Kiss, G.A.C., Forgacs, E., Cserhati, T., Mota, T., Morais, H. & Ramos, A., 2000) the optimization of the microwave-assisted extraction of pigments from paprika (*Capsisum annum* L.) found the linear relationships between extraction potential and extraction solvent. The dielectric constant of the solvent mixtures exerted a significant influence both on the strength and selectivity of extraction (Kiss et al., 2000).

4.4.2 Determinations of Cosmetic Bioactivity

4.4.2.1 DPPH radical scavenging activity assay

The results in Table 4.12 showed the similar results to the bioactive compounds content. The ethanol extract showed the highest antioxidant capacities with the lowest IC_{50} values of DPPH assay (30.57 µg/mL). However, the results of ethanol extract was not statistical significantly different from water extract of 31.69 µg/mL. A number of researches showed the higher DPPH inhibition of methanol extracts when compared to those reached with water (Li & Lin, 2010). The lower potential extract with the higher IC_{50} values were ethyl acetate extract (115.14 µg/mL) and propylene glycol extract (557.22 µg/mL), respectively. The antioxidant activity was also calculated to be the milligram of trolox equivalent antioxidant capacity per gram of dried weight sample (mg TEAC/g sample). The results were shown in Table 4.13. The similar results were obtained, the highest activity was found in ethanol extract (407.50 mg TEAC/g sample). The lower was obtained from propylene glycol and water extract with no significantly difference of 361.65 and 351.36 mg TEAC/g

sample, respectively. The lowest antioxidant activity from ethyl acetate extract (5.61 mg TEAC/g sample) was significantly lowest similar to those of polyphenolics content results.

Table 4.12 Cosmetic Bioactivities in Term of IC_{50} Values of Betel Nut Seed Extracts

Extract*	DPPH (µg/mL±SD)	ABTS (µg/mL±SD)	Anti-LOOH (mg/mL±SD)	Anti-Tyr (µg/mL±SD)	Anti-Ela (µg/mL±SD)
EtOAC	115.14±1.02 c**	43.34±0.51 c	5.83±0.12 d	41.49±0.15 c	1,154.42±21.11 d
95%EtOH	30.57±0.12 b	1.43±0.13 a	0.16±0.01 b	9.64±0.04 a	57.63±0.10 a
50%EtOH	19.17±0.09 a	1.06±0.00 a	0.18±0.01 a	6.24±0.07 a	111.50±0.44 a
Water	31.69±0.11 b	1.30±0.06 a	0.34±0.01 b	10.79±0.03 a	120.70±0.23 b
PG	557.22±8.24 d	301.46±4.30 d	2.59±0.03 c	159.36±0.47 d	969.67±10.47 c
Standard					
Trolox	4.11±0.03 a	4.34±0.03 b	-	-	-
BHT	-	-	4.19±0.04 a	-	-
Kojic acid	-	-	-	15.65±0.05 b	-
EGCG	-	-	-	-	47.07±0.17 a

Note: * EtOAc: ethyl acetate, 95% EtOH: 95% ethanol, 50% EtOH: 50% ethanol and PG: propylene glycol

** Small letter subscribed the statistical significantly difference among the samples in each test ($p<0.05$)

4.4.2.2 ABTS cation radical scavenging capacity

The results in Table 4.12 showed the similar results to the previous determination toward DPPH radical. The water extract showed the highest antioxidant capacities with the lowest IC_{50} value (1.30 µg/mL). However, the ABTS cation radical scavenging capacity of ethanol and water extract (1.43 µg/mL) were not significantly different. This result showed the higher potential of ethanol and water extracts in the scavenging of ABTS radical than the trolox standard. The results of

ethanol and water extract, the raw seed extracts were even possessed the higher potential of approximately 4 folds over trolox standard (4.34 μg/mL). The results of trolox standard equivalent antioxidant capacity (Table 4.13) also showed the related results of ABTS with the previous DPPH assay. The highest antioxidant capacity was possessed by ethanol extract of 407.50 mg TEAC/g sample. The lower capacity with no statistical significantly difference of water and propylene glycol extract were obtained (351.36 and 361.65 mg TEAC/g sample, respectively). The ethyl acetate extract exhibited the lowest activity of 5.61 mg TEAC/g sample.

4.4.2.3 Ferric reducing antioxidant power (FRAP)

The results in Table 4.13 show the activity of betel nut extracts which expressed as trolox equivalent antioxidant capacity (TEAC). The ferric reducing power interprets the antioxidant activity by the electron donating via reduction reaction cause the intense color of Fe^{3+}-TPTZ complex. The reducing capacity was also same as the previous determination. The significantly highest reducing potential was found in ethanol extract of 252.94 mg TEAC/g sample. The lower was obtained from water and propylene glycol extract with no statistically different of 48.69 and 49.89 mg TEAC/g sample, respectively. The reducing activity of the pericarp was consisted to the previous determination that the lowest was found in ethyl acetate extract (6.71 mg TEAC/g sample).

4.4.2.4 Lipid peroxidation inhibitory activity

The results in Table 4.12 showed the IC_{50} values of the betel nut extracts. The lowest IC_{50} of ethanol extract was not statistical significantly different with the water extract of 0.16 and 0.34 mg/mL, respectively, followed by propylene glycol extract of 2.59 mg/mL. The mentioned extracts were more effective than the standard of butylated hydroxytoluene (BHT), the commonly synthesis antioxidant used to prevent the lipid oxidation process of 4.19 mg/mL. The lowest activity was obtained from ethyl acetate extract of 5.83 mg/mL. The similar results of the equivalence to BHT standard were showed in Table 4.13. The highest activity was obtained in water extract (46.28 mg BHTE/g sample) which not statistical significantly difference with ethanol extract (46.28 mg BHTE/g sample). The lowest lipid peroxidation inhibitory activity was found in propylene glycol extract (1.22 mg BHTE/g sample).

Table 4.13 Cosmetic Bioactivities in Term of Standard Equivalent of Betel Nut Seed Extracts

Extract*	DPPH (mg TEAC)**	ABTS (mg TEAC)**	FRAP (mg TEAC)**	Anti-LOOH• (mg BHTE)**	Anti-Tyr (mg KAE)**
EtOAc	5.61±0.03	15.63±0.42	6.71±0.06	2.08±0.04	9.19±0.73
95% EtOH	407.50±0.72	494.99±0.25	252.94±3.69	45.61±3.19	301.53±4.00
50% EtOH	495.51±0.69	908.39±2.70	383.17±2.25	60.51±0.44	348.81±9.12
Water	351.36±1.96	307.73±0.49	49.69±1.18	46.28±1.63	105.18±4.23
PG	361.65±0.44	330.89±2.37	49.89±0.32	1.23±0.01	193.52±4.30

Note: * EtOAc: ethyl acetate, 95% EtOH: 95% ethanol, 50% EtOH: 50% ethanol and PG: propylene glycol

** The values were presented in mean±SD of mg standard equivalent per gram of dried weight sample

The previous study reported the higher total phenolics, flavonoids contents in ethanolic *Tephrosia purpurea* Linn. extract than those of water extract as mentioned above (Patel et al., 2010). The results of antioxidant activities by means of FRAP was higher in ethaolic than water extract (76.56 and 64.94 mg GAE). The ethanolic extract also possessed higher inhibitory of nitric oxide, hydrogen peroxide, and DPPH-RSA with the IC_{50} at 805.85, 341.55, 561.18 µg/ml than water extract (938.92, 653.28, and 831.36 µg/ml) (Patel et al., 2010). The comparison of different solvents on horseradish roots also stated the synergistic effect of 80%ethanol (Tomsone, L., Kruma, Z. & Galoburda, R., 2012). Total phenolic content and DPPH radical scavenging activity was highest in 80%ethanol extract than 95% ethanol and water, respectively. The mixture ratio (30 to 100%) of either ethanol or propylene glvcol with water on myrobalan fruit extraction were reported (Chulasiri, M., Wanasawas, P., sriaum, D., Nakamat, S., Wongkrajang, Y., Kongsaktrakoon, B., Phronchirasilp, S., Songchitsomboon, S. & Leelarungrayub, D., 2011). The total phenolic content and DPPH radical scavenging activity were found highest at the ratio

of 70% in both ethanolic and hydroglycolic extract. The previous study of the effect of solvent on *A. catechu* seed extraction also reported the similar results. The high extraction potential of the 50% methanol than water extract of 461.17 and 278.25 µg GAE/mL extract, respectively (Li & Lin, 2010). Flavonoids and phenolic acids are classified as mixed antioxidants because they are able to donate protons to free radicals, and are still capable of preventing the formation of reactive oxygen species (ROS) either by the inhibition of enzymes involved in the scavenging activity was obtained from water extract (Li & Lin, 2010).

4.4.2.5 Tyrosinase inhibitory activity

Tyrosinase is a key enzyme for melanin synthesis. Substance that can inhibit this enzyme can be considered as whitening agent. The most effective tyrosinase inhibitory activity expressed IC_{50} values were showed in Table 4.12. In consistent with the previous determinations, the highest activity was found in ethanol extract of 9.64 µg/mL. However, the IC_{50} of ethanol extract was not statistical significantly different with water extract (10.79 µg/mL). The ethanol and water extract also found having more potential that the kojic acid standard (15.65 µg/mL). The lower activity was found in ethyl acetate extract of 41.49 µg/mL, and the lowest was found in propylene glycol extract (159.36 µg/mL). The tyrosinase inhibitory activity of betel nut extracts were also expressed in the kojic acid equivalence per gram of dried sample (mg KAE/g sample) (Table 4.13). The highest equivalence was obtained from ethanol extract (301.53 mg KAE/g sample). The lower were obtained in propylene glycol and water extract of 193.52 and 105.18 mg KAE/g sample, respectively. The ethyl acetate extract exhibited the lowest tyrosinase inhibitory activity of 9.19 mg KAE/g sample which consistent to the previous assays.

4.4.2.6 Elastase inhibitory activity

The elastase inhibitory activity was also in the same order as the previous determinations. The IC_{50} value of the extracts was showed in Table 4.12. The highest activity with the lowest IC_{50} was also found significant in ethanol extract (57.63 µg/mL). The activity of ethanol extract was not statistical significantly lower than epigallocatechin gallate (EGCG) of 47.07 µg/mL. The significantly lower anti-elastase activity was found in water extract (120.70 µg/mL) and the lowest was of ethyl acetate extract (1,154.42 µg/mL). The same trend of phenolic content and the

activity implied that the antioxidants, tyrosinase and elastase inhibitory activities were probably from polyphenols contents.

Similar to the previous study of antioxidant, elastase and hyaluronidase inhibitory activities, the results were correlated to the concentration of phenolic-rich betel nut seed extract (Lee & Choi, 1999a,b). It has been documented that superoxide scavenging activity and inflammation delayed activity via nitric oxide synthase of betel nut seed extract were attributed to polyphenolics such as simple phenolics, tannins and proanthocyanins contents in the extract (Onar et al., 2012; Anthikat & Michel, 2012).

4.4.3 UV Absorption Ability

The scanning profiles of betel nut extracts in Figure 4.5 showed the distinguish pattern of the seed and pericarp part. The interesting absorbability was observed at approximately 258-295 nm with the maximum absorbance at 279.8 nm was found in the all extract but not significant in ethyl acetate extract.

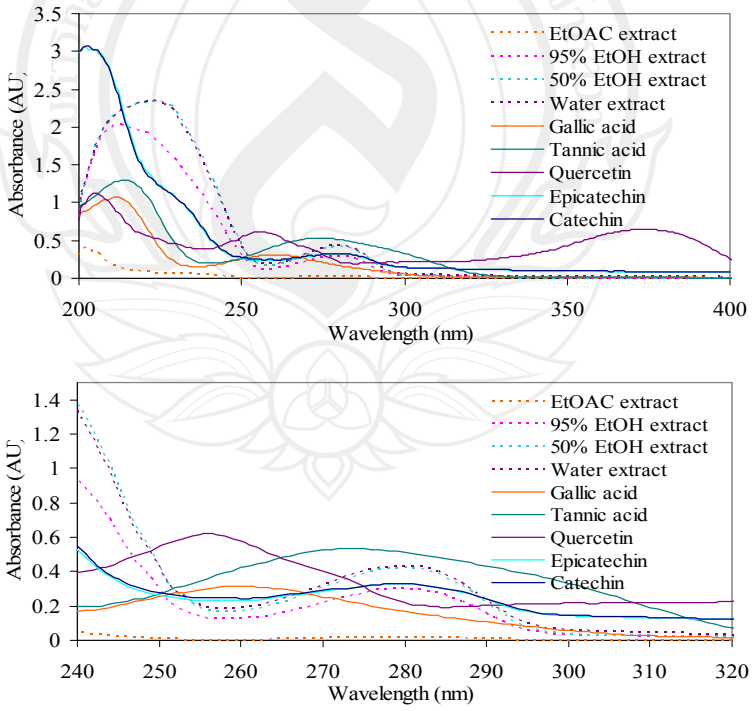
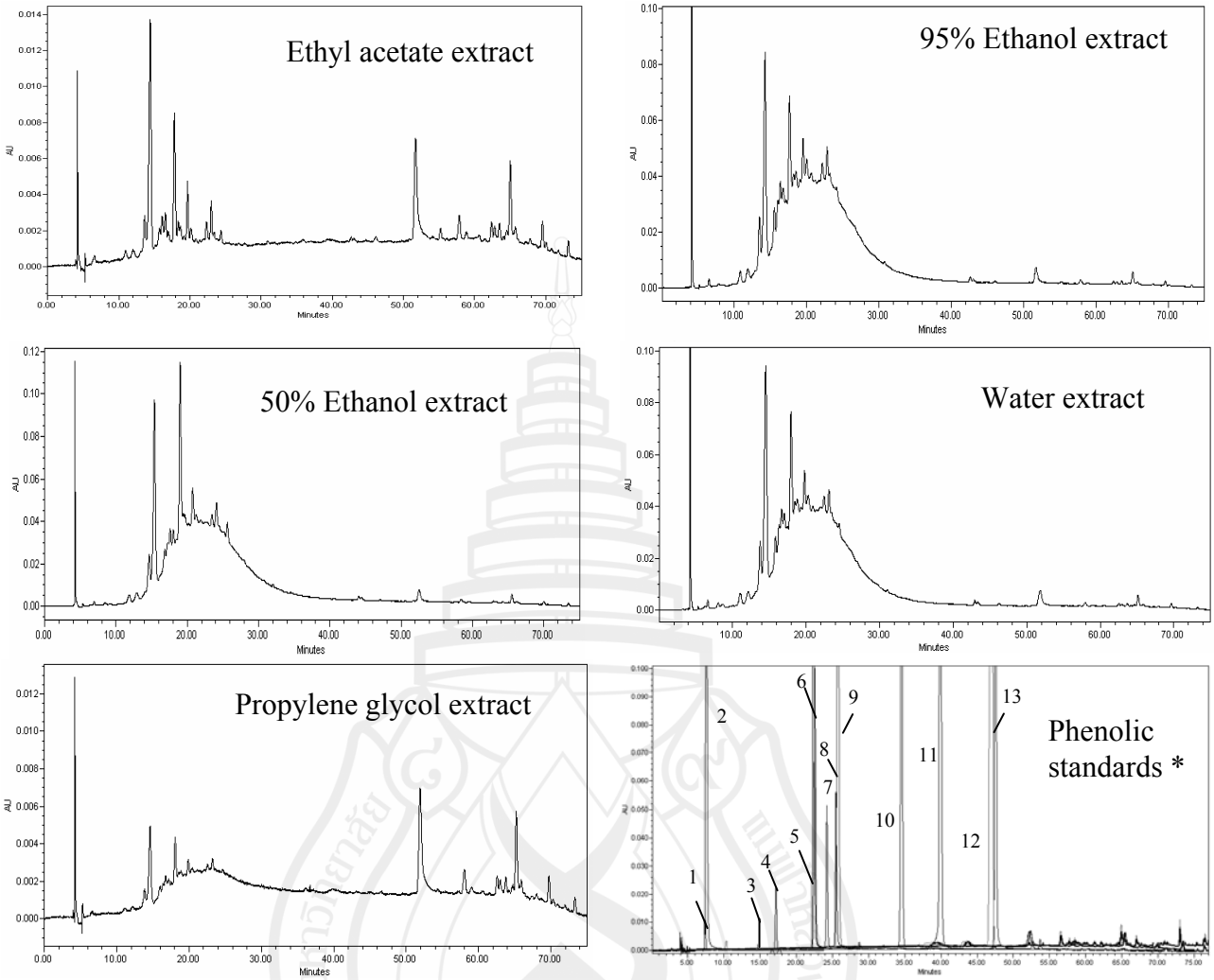


Figure 4.5 UV-Scanning of Betel Nut Extracts

There was no interesting peak from ethyl acetate extract along the UV-region. When compared with phenolics standards, the maximum absorbance at approximately 280 nm was also found to be the pattern of catechin and epicatechin. The absorbability of the raw betel nut seed was higher than the ripe which related to previous the colorimetric determination of biological contents and activities.

4.4.4 HPLC Analysis

The fingerprints of betel nut extracts were compared with the mixture of 13 phenolic standard substances (Figure 4.6). There is some similar retention time of the compounds in the extract with the region of catechin-like family. Spectra scanning of the sample peak confirmed that the main peaks in the betel nut seed belongs to catechin group. The previous study also found that catechin and epicatechin are the main phenolic compounds of betel nut seed (Wang & Lee, 1996). The seed was reported to be the main container of secondary metabolite of betel nut and the pericarp is major composed of fibrous (Wang & Lee, 1996; Bhandare et al., 2010; Wang et al., 1997; Wetwitayaklung et al., 2006). There have been found that the bioactive constituents in the betel nut were simple phenolics, catechin, caffeic acid, ferulic acid, non-tannin flavans, hydrolysable and condensed tannins, alkaloids and flavonoids (Wang & Lee, 1996: Bhandare et al., 2010: Wang et al., 1997). Furthermore, there was the study reported the betel nut seed contained catechin based on procyanidins which range from dimmer to decamers and polymers (Huang et al., 2010).



Note: * (1) kojic acid, (2) gallic acid, (3) gallocatechin, (4) epigallocatechin, (5) epigallocatechingallate, (6) chlorogenic acid, (7) epicatechin, (8) gallocatechingallate, (9) caffeic acid, (10) ferulic acid, (11) ellagic acid, (12) cinnamic acid, (13) quercetin

Figure 4.6 HPLC Separations of Betel Nut Extracts Compared with Standard Phenolic Substances

4.4.5 Anti-Bacterial Activity

The bacterial inhibitory activity of the betel nut extracts in Table 4.14 showed the positive result against gram-positive microbes (*S. aureus* and *S. epidermidis)*. The water and ethanolic raw seed extracts (3.2 mg) possessed inhibitory activities close to the positive of gentamicin (10 µg). There were no statistical significantly differences between ethanol and water extract. The low bioactive compound ethyl acetate extract was not effective to inhibit any microbes implying that the anti-microbial activity was attributed to the phenolics compounds of the extracts. The catechin group, which promised to be the major constituents in the betel nut, was reported its high potential on microorganism growth inhibitory activity (Anthikat & Michel, 2009).

The possible reason of only gram-positive anti-bacterial activity was the stronger cell wall structure of negative bacteria. The cell wall of bacteria is the peptidoglycan layer. In addition of the gram-negative bacteria, the outermost cell wall contains lipopolysaccharide and protein makes it more resistant to the growth inhibitors. The minimum concentration which has been used in the test (1 mg) was also observed to inhibit the gram-positive bacteria. The results at 1 mg extract possessed either equal or higher inhibition zone when compared to 1 µg positive gentamicin.

Table 4.14 Anti-Bacterial Activity of Betel Nut Extracts

	Clear zone (mm)					
	Gentamicin (positive)	**DMSO (negative)**	**EtOAc***	**95%EtOH***	**50%EtOH***	**Water***
E. coli	15.00±0.00	-**	-	-	-	-
S. aureus	15.00±0.00	-	-	12.00±0.00	13.67±1.15	13.67±0.58
S.epidermidis	18.33±0.58	-	-	10.67±0.58	11.00±0.00	12.67±0.58
Ps. aeruginosa	15.00±0.00	-	-	-	-	-
S. typhimurium	17.00±0.21	-	-	-	-	-

Note: * Extracts used were 3.2 mg versus 10 µg of positive Gentamicin
** - : no inhibitory zone was detected

4.3.6 Cytotoxicity test

The cytotoxicity results in Table 4.15 against normal and melanoma cell lines showed the negative results with more than 50 viable cells when treated with the betel nut extracts (>200 µg/mL). There were no significant cell damaging by the extracts those interpret the safety of using the betel nut extract onto the skin. The results were utilized the use of raw betel nut seed extract in cosmetic products. The previous DPPH and ABTS radical scavenging capacity and lipid peroxidation, tyrosinase, elastase inhibitory activities determination, the ethanolic raw betel nut seed extract possessed the IC_{50} values lower than 200 µg/mL**.**

Table 4.15 The % Survival of the Human Dermal Fibroblast Cell Line and B16-F10 Melanoma Cell Line Cultured with Samples at Various Concentrations

Concentration (µg/mL)	% Survival	SD	IC_{50} (µg/mL)
Human dermal fibroblast			
200.00	75	7	>200
100.00	72	4	
50.00	93	6	
25.00	89	5	
12.5	100	2	
6.25	87	4	
3.125	96	3	
1.56	99	1	
B16-F10 melanoma cell line			
200.00	98	5	>200
100.00	99	2	
50.00	100	5	
25.00	102	2	
12.5	101	3	
6.25	100	4	
3.125	102	2	
1.56	99	4	

Note: * % Survival > 50% indicated no cytotoxicity; % survival ≤ 50% indicated cytotoxicity

4.5 The Solid-liquid Fractionation Extraction

In order to investigate polarity group of the compounds presenting in the raw betel nut seed, solid-liquid sequential extraction with various solvents was employed. Microwave was still used in this study. The fractionation was done by the re-extraction of the residual sample stepwisely.

The extraction yield was found to be significantly highest in acetone extract of 11.25% (w/w), followed by those from hexane, 95% ethanol, 50% ethanol, and water extract, respectively. The lowest yield was presented in ethyl acetate extract of 1.60% (w/w). The appearances of the extracts were showed in Figure 4.8.

4.5.1 Phenolic Compounds in Betel Nut Seed Extracts

4.5.1.1 Extractable phenolics content (EPC)

The EPC values of the extracts were found to be statistically different. As shown in Table 4.16, the acetone extract exhibited the significantly highest EPC of 82.39 mg GAE/g sample and followed by 95% ethanol extract of 27.30 mg GAE/g sample. The 50% ethanol extract showed non significant difference to ethyl acetate extract which obtained 11.92 and 8.05 mg GAE/g sample, respectively. The first hexane extract possessed the lowest EPC of 0.59 mg GAE/g sample. The last extraction step of water extract was found to have low EPC of 1.58 mg GAE/g sample. This may be due to the least residue bioactive compounds available after 5 steps of extraction. There have been reported the use of acetone in phenolic extraction from areca nut (Chavan, Y. & Singhal, R.S., 2013a; Wang & Lee, 1996) employing 80% acetone at pH 4.0 combined with ultrasonic assistance provide maximum total phenol of 362.59 mg GAE/g sample from areca nut (Chavan, Y. & Singhal, R.S., 2013b). The 301.00 mg GAE/g sample from the whole fresh unripe areca fruit by using 80% acetone was also reported (Wang & Lee, 1996).

4.5.1.2 Extractable flavonoids content (EFC)

The significantly highest EFC was obtained from acetone extract of 12.71 mg QE/g sample (Table 4.16). Similarly to the EPC, the 95% ethanol showed the lower EFC of 4.29 mg QE/g sample. The ethyl acetate and 50% ethanol has no

significant effect on EFC value (p<0.05). The lowest EFC was found in the last water extract of 0.26 mg QE/g sample which did not significantly differ from the hexane extract of 0.95 mg QE/g sample. The EFC from this experiment was interpreted in term of quercetin equivalent per gram of dried betel nut.

4.5.1.3 Extractable proanthocyanidins content (ECC)

Table 4.16 showed the statistical significantly highest ECC in acetone fraction of 1.51 mg CE/g sample. The lower was obtained from 95% ethanol fraction, 0.36 mg CE/g sample. The rest fractions exhibited low ECC with no statistical significantly differences to each others. By the way the lowest values were similar to EPC and EFC which obtained from the first and last fraction of hexane and water, respectively.

There was also report by compared to other standard. There was the report of using of 70%ethanol for extraction the flavonoid from betel nut by reflux method obtaining 77.36 mg catechin equivalent/g crude extract (Zhang et al., 2009). The solid-liquid extraction used in this study depends on the solubility of the solute (target compounds) and the solvent. The simple phenolics prefer the higher polarity solvent. In contrast, the large compounds of flavonoids prefer the lower polarity solvent (Rice-Evans, C., Miller, N. & Paganga, G., 1996). The similar results also obtained in the partition extraction of phenolics antioxidant from *Lespedeza cuneata* (Kim, J.S.J. & Kim, M.J., 2010). The study showed the last fraction of water extract exhibiting the lowest EPC and EFC. The first fraction of hexane possessed the EPC of 39.62 mg GAE/g sample which lower than methanol extract (46.33 mg GAE/ g sample). The results from this experiment also showed the higher correlation of the reducing power to EPC than EFC.

Table 4.16 The Bioactive Compounds and Biological Activities of Betel Nut Seed Extracts

Extract	%Yield (w/w)	EPC (mg GAE)	EFC (mg QE)	ECC (mg CE)	DPPH (mg TEAC)	FRAP (mg TEAC)
Hexane	8.45±0.85 ^{b**}	0.59±0.03 ^{e}	0.95±0.07 ^{d}	0.09±0.01 ^{c}	0.03±0.01 ^{e}	0.02±0.00 ^{e}
Ethyl acetate	1.60±0.20 ^{e}	8.05±0.09 ^{c}	2.03±0.01 ^{c}	0.06±0.01 ^{c}	1.90±0.01 ^{d}	1.78±0.04 ^{c}
Acetone	11.25±1.85 ^{a}	82.39±15.05 ^{a}	12.71±2.07 ^{a}	1.51±0.28 ^{a}	20.76±4.14 ^{e}	14.31±3.78 ^{a}
95%EtOH	5.70±0.10 ^{c}	27.30±0.35 ^{b}	4.29±0.08 ^{b}	0.36±0.02 ^{b}	7.23±0.71 ^{b}	5.25±0.38 ^{b}
50%EtOH	4.95±0.05 ^{cd}	11.92±0.54 ^{c}	2.02±0.02 ^{c}	0.11±0.00 ^{c}	2.11±0.13 ^{c}	0.53±0.09 ^{d}
Water	3.80±0.00 ^{d}	1.58±0.02 ^{d}	0.26±0.01 ^{e}	0.01±0.00 ^{c}	0.02±0.00 ^{e}	0.21±0.02 ^{de}

Note: * The results were presented in mean±SD (n=3) of standard equivalent per g sample

** The different small letter in the same column described the statistical significantly difference ($p \geq 0.05$)

4.5.2 Antioxidant Activities of the Betel Nut Seed Extracts

The radical scavenging activity of the extracts was investigated by means of the decolorizing of stable DPPH radical. As shown in Table 4.16, the highest antioxidant activity was obtained from acetone extract of 20.76 mg TEAC/g sample. The lower activities were found in the 95% ethanol, 50% ethanol, and ethyl acetate extracts of 7.23, 2.11, and 1.90 mg TEAC/g sample, respectively. The first and last extraction step of hexane and water gave the lowest activity of 0.03 and 0.02 mg TEAC/g sample, respectively. The reducing power of the extracts were determined as its ability to reduce Fe(III) to Fe(II) with the complex with TPTZ. The results in Table 4.16 were similar to the DPPH radical scavenging activity, the highest was obtained from acetone extract of 14.31 mg TEAC/g sample. The lower reducing powers were found in the extracts from acetone, ethyl acetate, and 50% ethanol (5.25, 1.78, and

0.53 mg TEAC/g sample, respectively). The lowest reducing power was of the first fraction of hexane extract (0.02 mg TEAC/g sample).

The similar results of antioxidant activity was reported in the liquid-liquid extraction of antioxidant from *Callistemon lanceolatus* stem (Kim, J.H., Byun, J.C., Bandi, A.K.R., Hyun, C.G. & Lee, N.H., 2009) using the partitioning in the order of hexane, ethyl acetate, butanol, and water. The lowest DPPH radical scavenging activity was obtained from the first fraction of hexane. The fraction of ethyl acetate, water, and butanol extracts possessed higher activity, respectively.

The previous study (Zhang, X., Wu, J., Han, Z. Mei, W.L. & Dai, H.F., 2010) showed that of the partitioning of areca nut seed constituents by light petroleum, ethyl acetate, and *n*-butanol possessed similar trend with the result of this experiment in which the first and last fraction showing the lower activities. The highest DPPH radical scavenging activity exhibited from ethyl acetate fraction with the IC_{50} of 27.6 µg/mL. The lower activities were found in *n*-butanol (85.1 µg/mL) and light petroleum fraction (>100 µg/mL). It was reported that the better lipid peroxidation inhibitor was derived from condensed tannin fraction than in non-condensed tannin fraction (Wang & Lee, 1996). However, the crude extract which is the mixture of both non-condensed and condensed tannin possessed the better lipid peroxidation inhibitory activity (Wang & Lee, 1996).

4.5.3 UV Absorption Scanning

The absorption profiles of the betel nut extracts were scanned through the UV region of 200-400 nm compared with the catechin and epicatechin standards. The maximum absorption at approximately 230-235 nm and 270-290 nm were observed. The early absorption region at 230-235 nm responses to the UVC (200-280 nm) protection efficacy of the sample extracts. In addition of the UVB (280-320 nm) protection, the absorption at the range of 270-290 nm showed the significant similarity of the maximum profile of catechin and epicatechin compounds at 279.8 nm. This result implied the composition of catechin group in the extracts. The absence of these peaks in hexane and water extracts related to the lowest in EPC, EFC and also those of the antioxidant activities obtained in the first and last fractions of raw betel nut seed extraction.

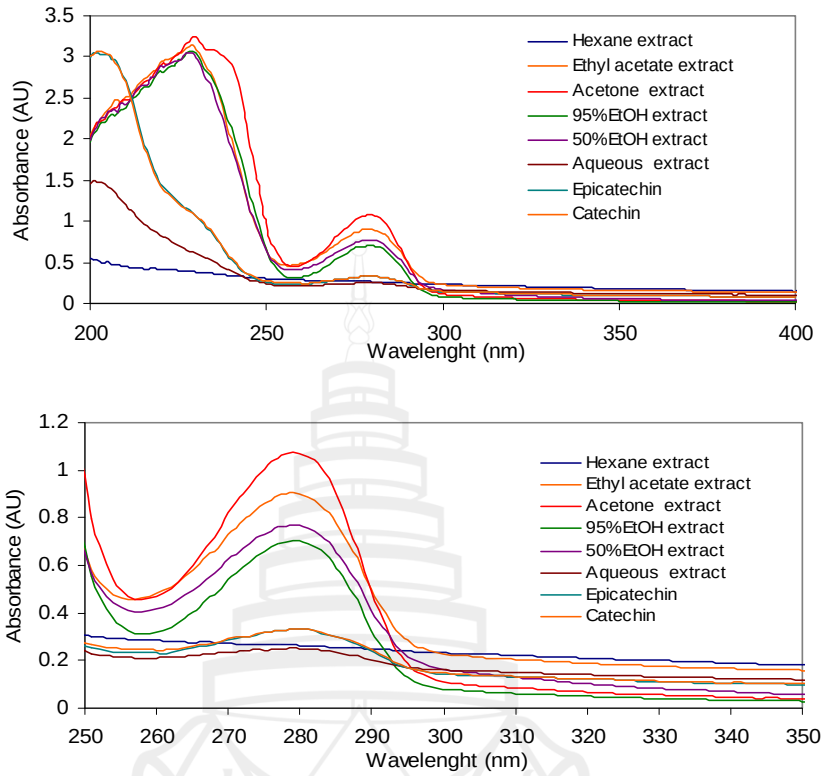


Figure 4.7 UV-Scanning of Betel Nut Seed Extracts and Phenolic Standards; 200-400 and 250-350 nm

4.5.4 HPLC Analysis

HPLC elution profile of the betel nut seed extracts was performed by using the same extract concentration of 5.0 mg/mL. The appearances of dried crude extracts of each fraction were also shown in Figure 4.8. The first extraction step of hexane gave the pale-yellow semi-solid crude. The light-brown color was obtained from ethyl acetate and 50% ethanol extracts and the reddish-brown was observed in acetone and 95% ethanol extracts. The last fraction of water was almost white.

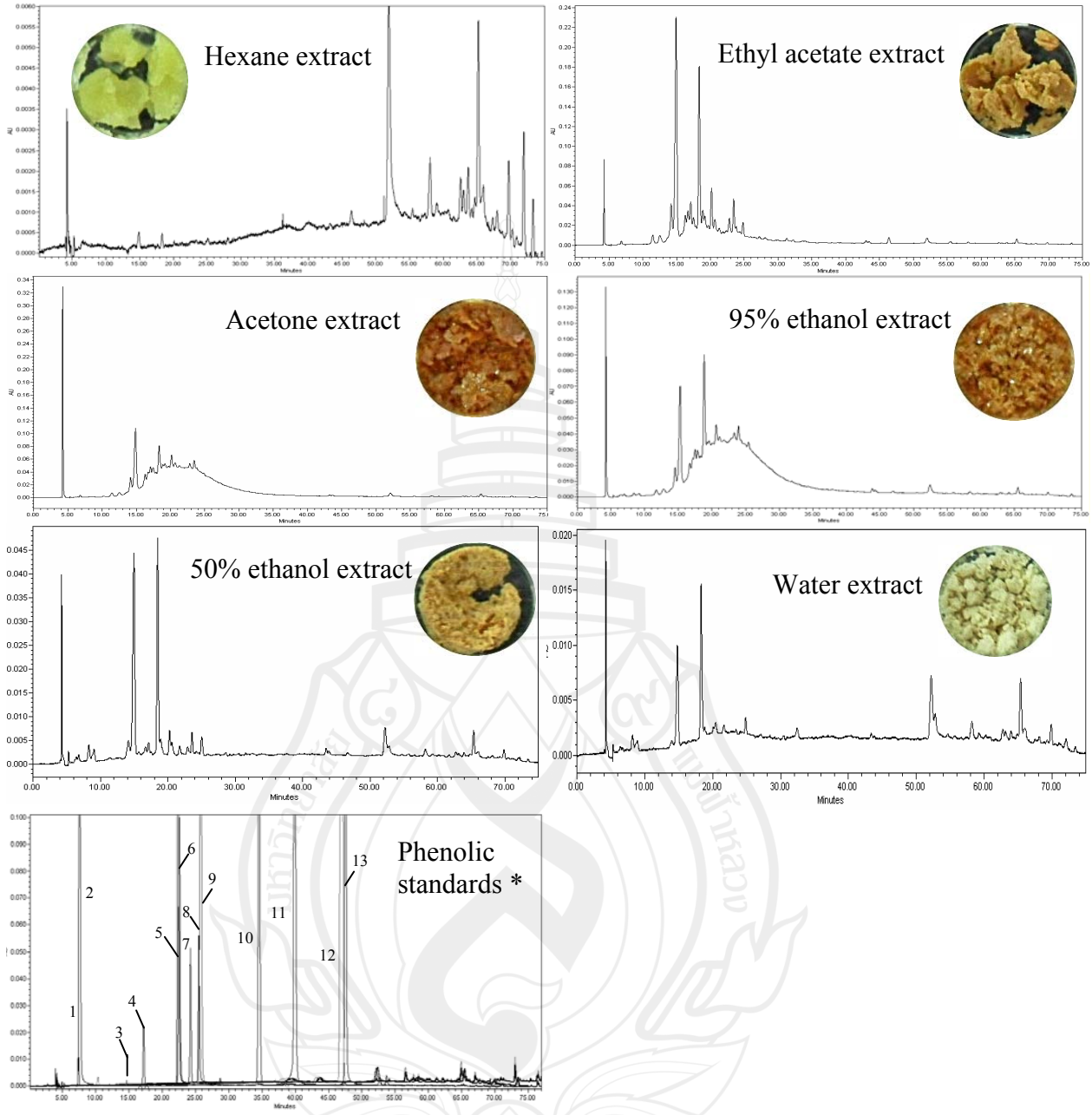
The obtained profiles in Figure 4.8 displayed the manner of the valuable compounds in the sample. The hexane extract mostly comprised of low polar compound which were eluted after 50 min. The interested peaks of all extracts, except for hexane extract, were similar in the retention time range of 12- 25 min. which are also in the range of catechin-like compounds in the standard (Figure 4.8). The higher

peak areas were obtained from the acetone and 95% ethanol with the shoulders in the catechin-like region.

There were unknown interesting peak in all extract in the early retention time of approximately 4 min which may also in considered being the bioactive compound. Elution profile of acetone and 95% ethanol fractions displayed the group of compound with shoulder peak at around 15-25 min of retention time. This result was consistent with the phenolic content and antioxidant capacity which were obviously detected in these 2 fractions. After the shoulders were extracted out, the sharper peaks were clearly seen obtained from the 50% ethanol and water extract at approximately 14, 15, and 19 min.

The resulted chromatograms were comparable to previous work (Wang & Lee, 1996). The crude areca fruit extract was subjected to Sephadex LH-20 with exhaustive washing by 95% ethanol to obtain non-condensed tannin fraction. The residue was successive washed by 50%acetone to obtain the condensed tannin fraction. The HPLC of three samples were analyzed. The crude extract of 80%acetone showed several peaks. The sharp peaks of catechin and epicatehin were observed from the condensed tannin fraction. The residue large molecules of condensed tannin were found having board peaks. The existences of catechin group in each fraction were confirmedly shown as their spectrum (Appendix D).

The literatures also shows the major polyphenols in the betel nut were flavonoids of catechin and epicatechin with minor of others flavanols compounds (Zhang et al., 2009). HPLC and liquid chromatography-mass spectrometry results of Chavan & Singhal (2013a) confirmed the presence of catechin and epicatechin in the extract. Proanthocyanidins (PACs) in areca nut were analyzed using HPLC combined with electrospray-ionization mass spectrometry (ESI-MS) and compared to grape seed extract (Wu, Q., Yang, Y. & Simon, J.E., 2007). The PACs distribution in areca nut was found to be very similar to that in grape seed, but lacking any gallate conjugates. The PAC monomers (+)-catechin and (-)-epicatechin were successfully quantified by reversed phase HPLC with ESI-MS in the selected-ion-monitoring (SIM) mode.



Note: * (1) kojic acid, (2) gallic acid, (3) gallocatechin, (4) catechin, (5) epigallocatechingallate, (6) chlorogenic acid, (7) epicatechin, (8) gallocatechingallate, (9) caffeic acid, (10) ferulic acid, (11) ellagic acid, (12) cinnamic acid, (13) quercetin

Figure 4.8 HPLC Chromatogram and the Appearance of Betel Nut Seed Extracts and Phenolics Standards

The phenolic compounds in raw areca fruit were analyzed and categorized into 4 groups (Wang & Lee, 1997) analyzed. The main group of non-tannin flavans of monomeric anthocyanins, catechin, and leucocyanindins were accounted for 41.05%. The lower percentage of 34.20% was analyzed to be condensed tannins such as polymers or copolymers of catechin and leucocyanins. The hydrolysable tannins of sugar polyester or related polyhydric alcohols and a phenolic carboxylic, gallic and ellagic acid were accounted for 22.19%. The simple phenolics which are hydroxybenzoic and hydroxycinnamic acids were reported taken the lowest account of 2.56%. Wang & Lee (1996) also elucidated areca crude extract by Sephadex LH-20 into 2 fraction; A and B. The polymerization degrees were reported. The lower degree of polymerization (70%) revealed the lower molecules in fraction A. The non-condensed tannin, catechin and epicatechin were then explored by further HPLC. The higher degree of polymerization was found in fraction B (174%) and suspected to be the condensed tannin (Wang & Lee, 1996). In order to get the sharp peak of catechin and epicatechin, the mentioned authors had to used 23 min for the crude extraction, 240 min to elute fraction A and another 360 min for fraction B, more than 500 min was used to obtain the pattern of extract. The recent study of solid-liquid microwave-assistance extraction used only 30 min per extraction step.

4.6 Stability of Raw Betel Nut Seed Extract

Because of the 50% ethanolic extract of raw betel nut seed showed the highest phenolic content and cosmetic bioactivities, it was subsequently selected for stability investigation in various pH and temperature conditions.

4.6.1 Effect of pH and Temperature on Absorption Stability

When the molecules lost their stability during storage, change in absorption spectrum of the substance can be detected. Te betel nut seed extract was stored at various pH and temperature for 21 days. The extracts were scanned through 200-400 nm at the initial day (Day 0) and after 21 days storage (Day 21). The absorption profiles of each pH were depicted in Figure 4.9. The changes of the absorption at this range were different in each pH condition. The notable absorbability at the early of 200-250 nm of the extract was differentiated in its possible derivative occurrences in the acid-basic conditions. The interested compound which absorbed in the range of 270-290 nm as previously said to be catechin group was also observed. It should be noted that the raw betel nut extract solutions were stable the most at pH 3-5 in every storage temperatures. This pH covered natural pH of the betel nut seed extract which appeared as pH 4.5.

The spectra were also categorized in the storage temperature as Figure 4.10. The absorption of the extracts were recorded before storage, Day 0 (Figure 4.10A). The least change in absorption patterns were found at 4°C (Figure 4.10B). The most alteration patterns were observed when stored at 50°C (Figure 4.10D). The storage temperature effects to the extract absorption stability. The heat condition of 50°C was not only effect to the absorbability decrement but also found to increase the absorption of some extract solution at the range of 270-290 nm. The thermal also affects to the pattern of the extract solution along 200-400 nm. The changes in absorption shape revealed the decomposition of the compounds or the occurrence of their derivatives.

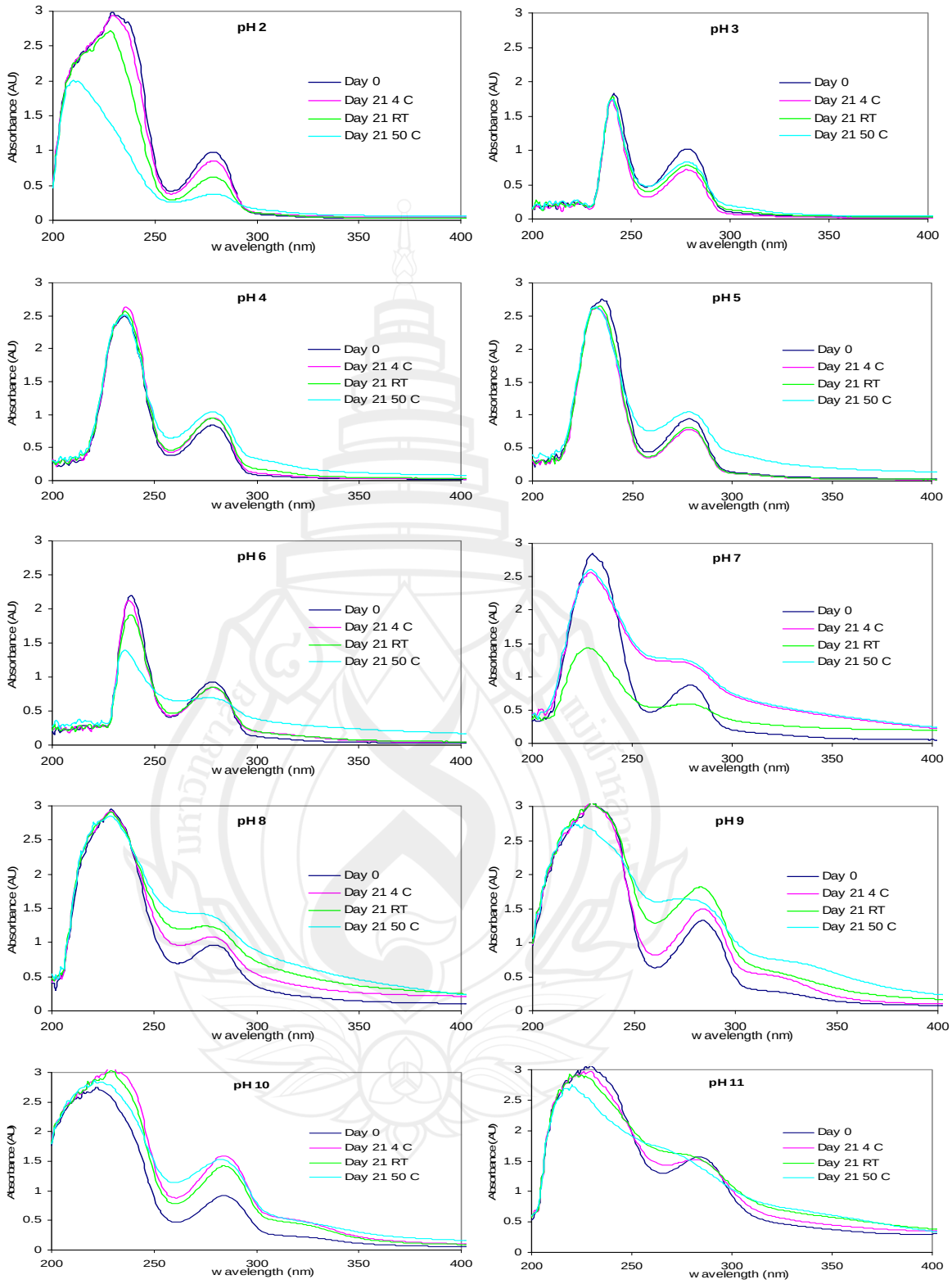
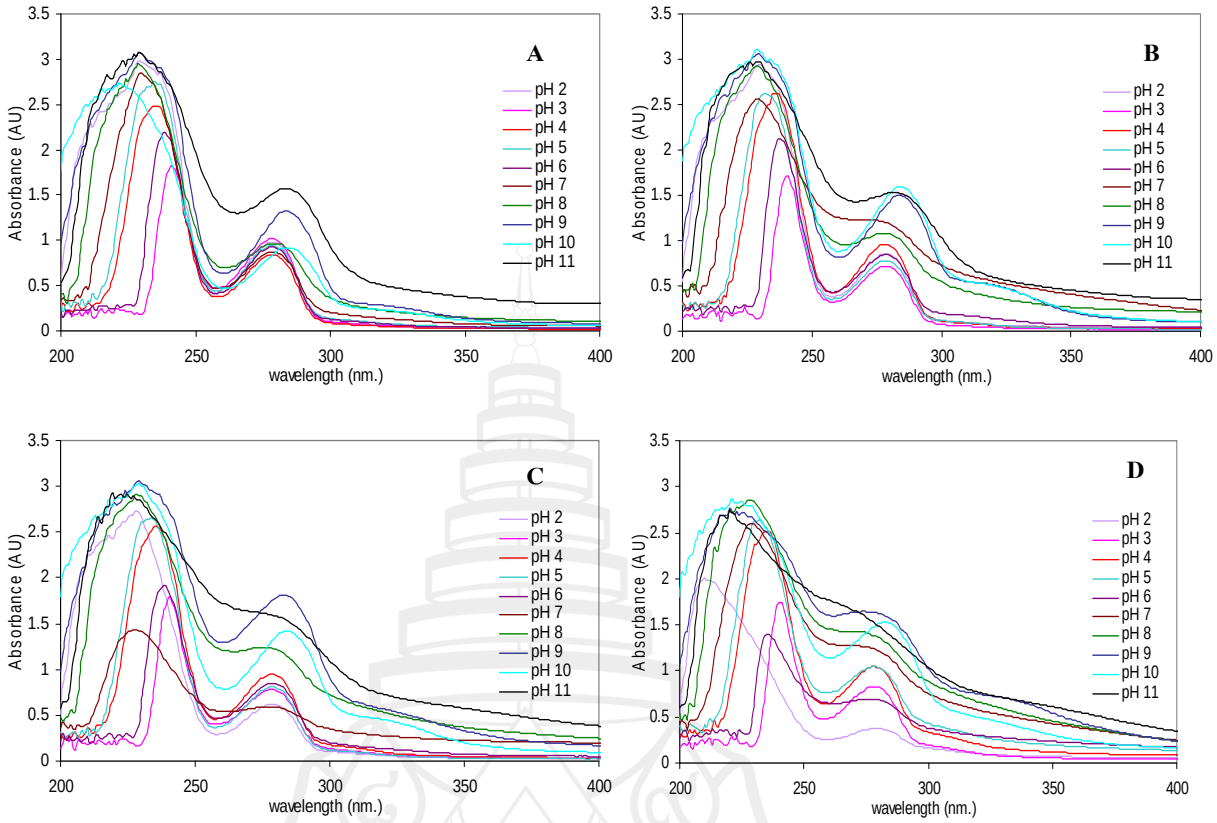


Figure 4.9 Absorption Stability of Raw Betel Nut Seed Extract Solution for 21 Days



Note: A: initial day and at day 21 of B: storage at 4°C, C: room temperature, D: 50°C

Figure 4.10 Absorption Stability of Raw Betel Nut Seed Extract

The effect of pH 3-11 on phenolic compounds stability by UV-spectroscopy was studied (Friedman, M. & Jurgens, H.S., 2000). The meaningful changes of phenolic spectrums were reported. The study also comsisted to the primary well-known that treating phenolic compounds with alkaline condition causes the side reaction including their destructions (Friedman & Jurgens, 2000).

The character of phenols could be characterized by spectra (Dearden, J.C. & Forbes, W.F., 1959). The phenols group was described having two bands in the range of 200-360 nm. The shorter wave called B-band and longer was C-band of phenols. The absorption spectrums of the compound were influenced by the solvent, electron-

withdrawing and electron-donating substitutions in benzene ring(s), intra and inter molecular hydrogen-bonding, and the pH dependent formation of resonance forms with altered conjugation compared to the parent compounds (Dearden & Forbes, 1959). The degree of polymerization of a complex mixture of monomeric and oligomeric tannins of ellagitannins was studied under acid and base condition (Vrhovsek, U., Palchetti, A., Reniero, F., Guillou, C., Masuero, D. & Mattivi, F., 2006). The study showed the hydrolysis of ellagitannin ester bonds under acid condition resulting in the releasing of ellagic acid in various degrees (Vrhovsek et al., 2006). The recent spectrum showed the consistent result to the apple juice spectrum stabilities which appeared to be the highest at pH 3-4 condition (Friedman & Jurgens, 2000). The mentioned study also stated that the phenolic OH groups may be primarily responsible for the observed spectral changes. The 8 tested compounds were found irreversible unstable in caffeic acid (2 OH), its ester chlorogenic acid and 3 OH owner, gallic acid. The non-phenols compound, *trans*-cinnamic acid and single OH, ferulic acid was reversible to the original after were neutral from pH 11 back into pH 7 (Friedman & Jurgens, 2000). The multi-ring aromatic structure of catechin, epigallocatechin, and rutin were found more stable via pH induced conditions. The explanation was structurally resistance of the compounds. Catechin, epigallocatechin, and rutin have more resistant ionized and resonance forms to the degradation by pH than those of monocyclic phenolic compounds (Friedman & Jurgens, 2000). The major phenolics in betel nut seed was reported to be catechin and epicatechin as mentioned before (Wang & Lee, 1996). Then the recent results of the most stable spectrum in pH 3 and 4 can be concurred the well-known week acid stable of catechin. There is the study (Sani, I.M., Iqbal, S., Chan, K.W. & Ismail, M., 2012) showed the disappeared of some phenolics in the alkaline medium (vanillic and ferulic acid). The acid medium possessed more disappearing of hydroxylcinnamic, caffeic, syringic, *p*-coumaric, and protocatechuic acid. The phenolics, including betel nut phenolics, protocatechuic acid, were majored effected by acid conditions (Sani et al., 2012).

4.6.2 Effect of pH and Temperature on EPC Stability

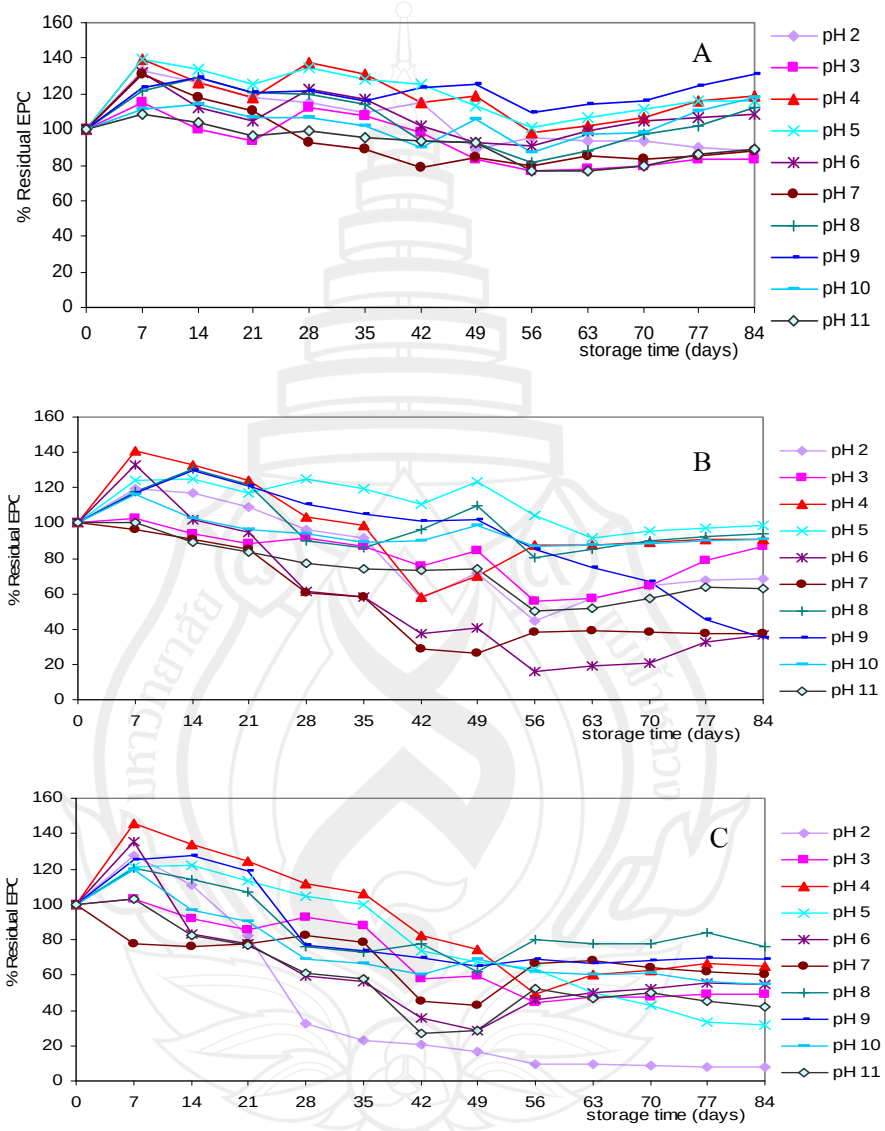
The EPC in the extract was weekly evaluated and calculated into gallic acid equivalent then expressed as percentage of residual EPC (Figure 4.11). At the first 7 days storage, increment of the EPC in every condition was remarked. The raising in EPC in the beginning of storage was explained by the releasing of free phenolics which were hydrolyzed by both acid and alkaline conditions, but not in the neutral pH 7. The EPC was the most stable at 4°C and its stability decrease when the temperature raising. After 7 days, the EPC in all pH storage were gradually decreased.

There are mainly two groups of phenolic acids, benzoic and cinnamic acid. Furthermore, most phenolic acids in plants primary occur in the bound form as conjugated with sugars, fatty acids, or proteins. The pH influences the extractable yield and profile of phenolic acids. The study showed that both alkaline and acid conditions cause the releasing of free phenolics and higher in the former condition (Kim, K.H., Tsoa, R., Yang, R. & Cui, S.W., 2006). The product of hydrolysis, free phenolic acids were then being easier degraded as their simple structure.

After 84 days storage, Figure 4.11 showed that the most stable EPC at 4°C in pH 4-6 and 8-10. Moreover, the phenolic residual was not less than 80% in all pH solution. At room temperature, neutral pH of 6, 7, and 9 tend to possess the high degradation rate, while the rest solution retained the phenolic content more than 60%. High temperature of 50°C results in the decrement of phenolic content. At the most acidic condition of pH 2, phenolic content was dramatically degraded into lower than 20%. This result verified the observation from Figure 4.12 that the higher temperature increased the oxidation and degradation of the phenolic compounds in the extract.

As discussed before that the phenolics were structurally changes under pH conditions, the EPC residue was discussed again as the point of thermal effect. The cleaved phenolics were existed in the simple form which reacted easily with the accelerated condition of 50°C. The previous study also stated the degradation of cinnamic acids derivatives, *p*-coumaric, caffeic and ferulic acids under hot acidic conditions (Robbins, R.J., 2003). The heat treatment effect also stated (Friedman & Jurgens, 2000), nonenzymatic browning reactions with other components in the extract were present to form Maillard browning products (Friedman, M., 1997) leads to the instability of bioactive compounds. The different results were obtained from the

effect of acid and base catalyzed hydrolysis on the yield of phenolics from germinated brown rice (Sani et al., 2012). The total phenolics as well as flavonoids contents were higher in acidic condition (Sani et al., 2012).



Note: A: storage at 4°C, B: room temperature, C: 50°C

Figure 4.11 Residual of Extractable Phenolics Content of Raw Betel Nut Seed Extract

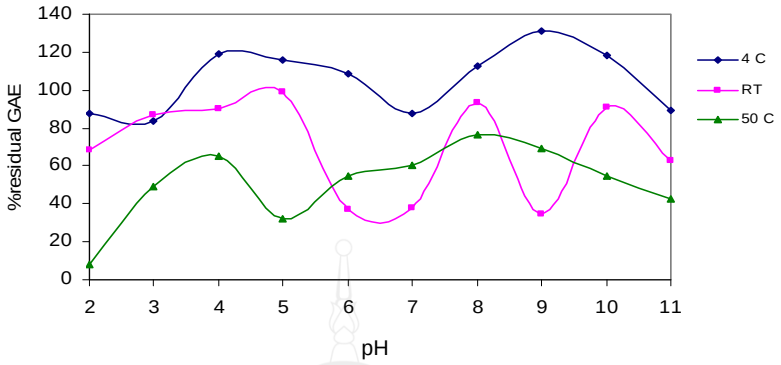


Figure 4.12 Percentage of Residual Extractable Phenolics Content of Raw Betel Seed Nut Extract Solutions after 84 Days Storage

Due to phenolic stability and absorbability of the extract at pH 6 and 7 were less than other pH condition storage, even these pH are neutral, the stability of the extract in buffer solution pH 6 and 7 were rechecked under accelerated condition of heating/cooling for 3 cycles. The storage pH 2 and 10 which provided the highest EPC stability were also employed to compared the result. The results in Figure 4.13 showed the consistent results to the previous long term stability that the storage of extract at pH 6 and 7 could retained the least polyphenols contents and antioxidant activity.

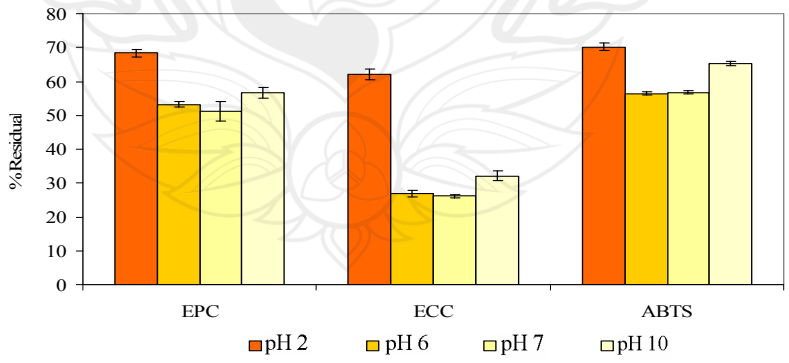


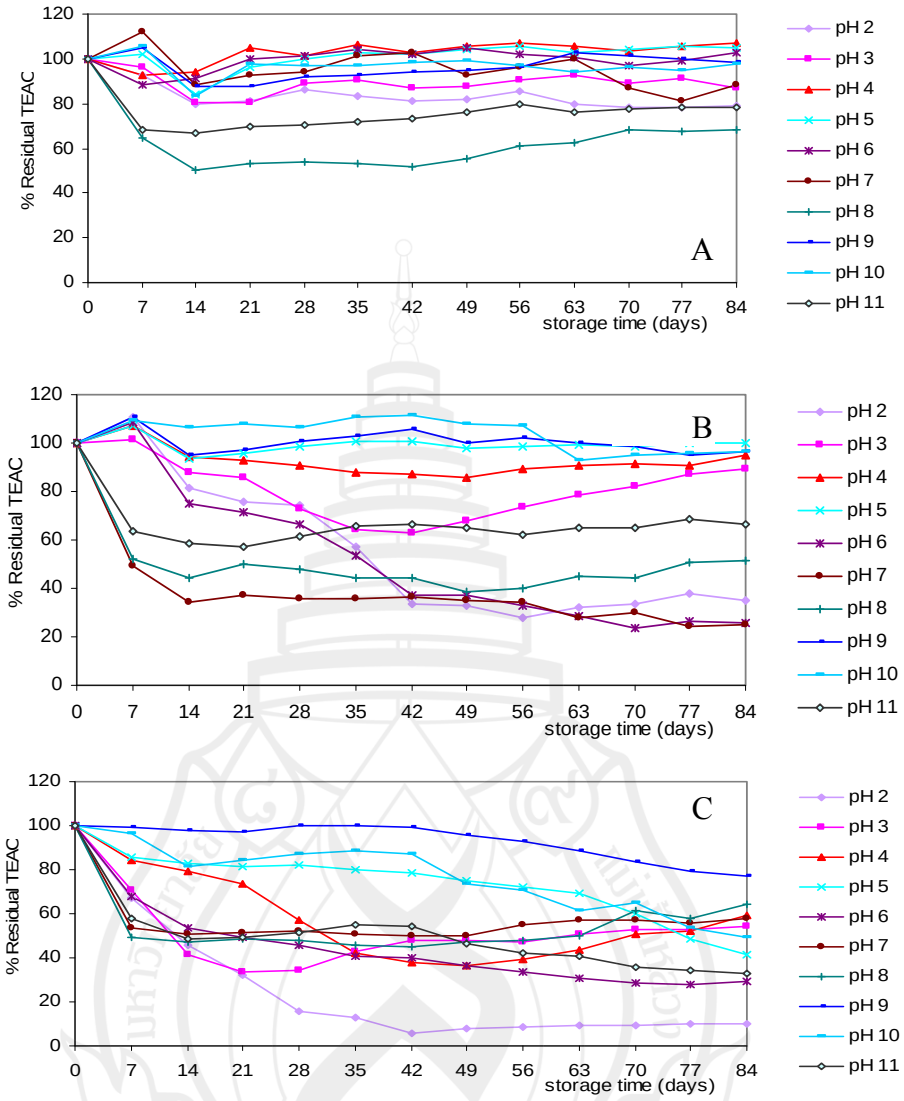
Figure 4.13 Residual Extractable Phenolic Content (EPC), Extractable Catechin Content (ECC), and ABTS Radical Scavenging Activity (ABTS) of the Betel Nut Seed Extract after Heating/Cooling Cycle

4.6.3 Effect of pH and Temperature on Antioxidant Stability

The residual ABTS radical scavenging activities of extract at the tested conditions were showed in Figure 4.14. The residual antioxidant activity of 4°C storage was showed in Figure 4.14A. The decrements of the activity were found higher than 80% compared to the initial day, except at high basic condition of pH 8 and 11. The antioxidant stability of the extracts at room temperature was showed in Figure 4.14B. At pH 3-5 and 9-10, the residual activities were found more than 80% after 84 days storage. However, at pH 7, 8, and 11, the activity was decreased dramatically after 1 week storage then at 2 weeks pH 2 and 6 were also observed deducted. The antioxidant activity was lower stable as the temperature increased. The high temperature condition at 50°C, the antioxidant activities were lowered to below 80% after 6 weeks storage in all pH.

After 84 days storage, the antioxidant activities of the extract solutions were retained more than 50% except at pH 2, 5, and 7. After 84 days storage, the residual antioxidant activity of each extract pH is illustrated in Figure 4.15. It is mentioned again that higher temperature caused more stability lost of the antioxidant. However, it is obviously seen that the pH 4 and 9 could provide the stable radical scavenging activity at all temperature. The low temperature of 4°C was able to prolong the activity more than 80% at all pH.

According to the recheck of pH 6 and 7 stability, residual antioxidant results in Table 4.13 also in the same trend as residual polyphenols contents. Under accelerated condition of heating/cooling for 3 cycles, neutral condition of pH 6 and 7 also possessed the lower stability when compared to pH 2 and 9.



Note: A: storage at 4°C, B: room temperature, C: 50°C

Figure 4.14 Residual of ABTS Radical Scavenging Capacity of Raw Betel Nut Seed Extract

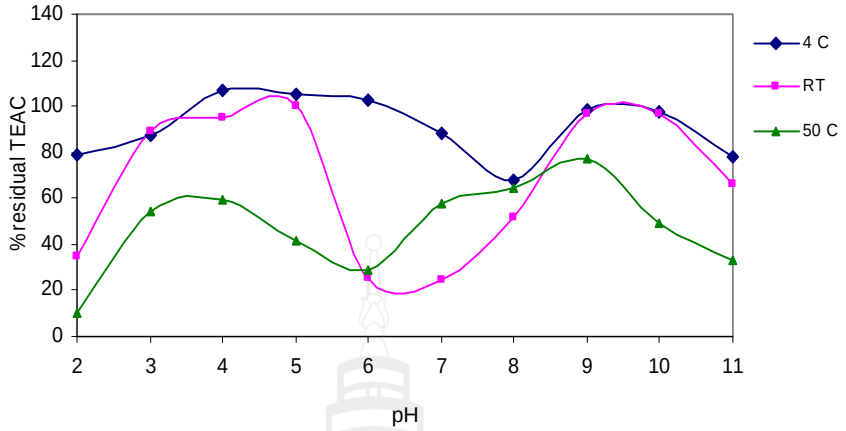


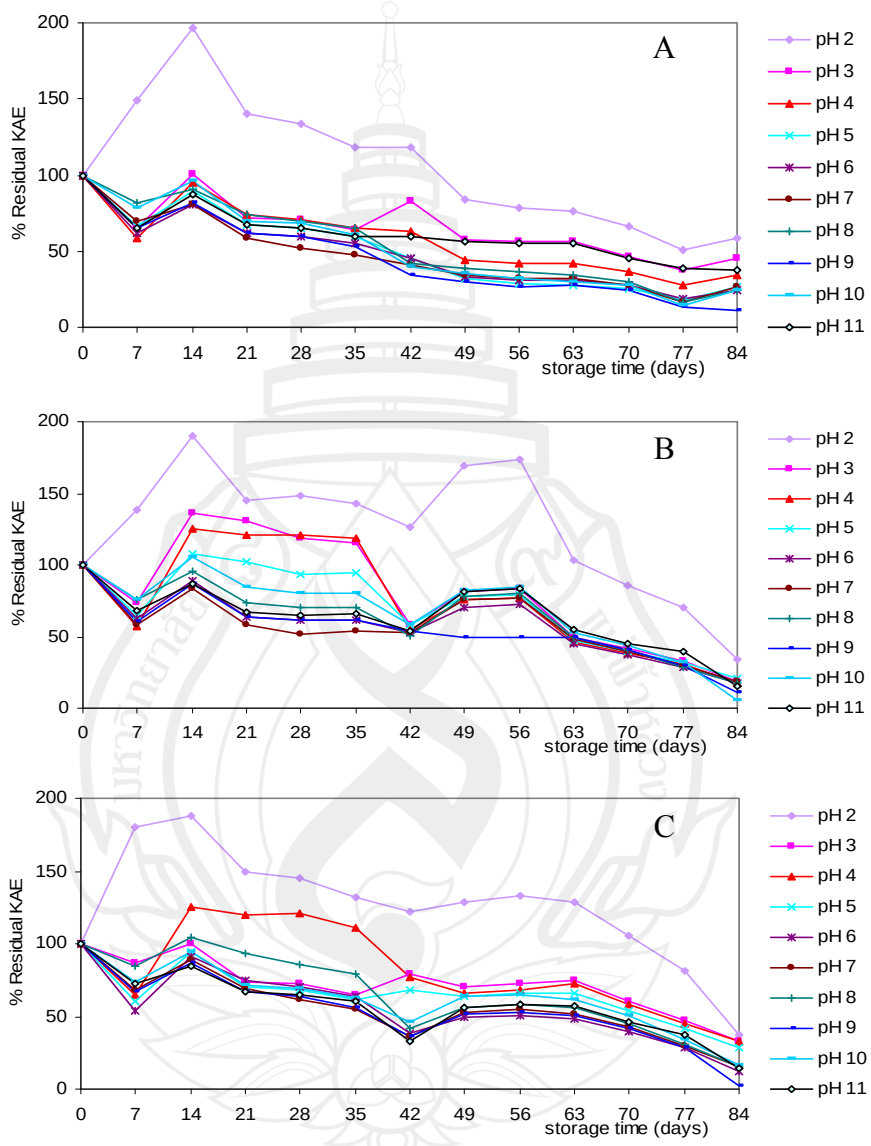
Figure 4.15 Percentage of Residual ABTS Radical Scavenging Capacity of Raw Betel Seed Nut Extract Solutions after 84 Days Storage

Beside of the recent results of the relation of EPC and antioxidant activity, it was supported by the previous study (Kim et al., 2006). The antioxidant activity of wheat bran extract was found higher in alkaline than acidic hydrolysis (Kim et al., 2006). The antioxidant; ABTS radical scavenging and ferric reducing power of germinated brown rice extract also higher in the basic than acid condition, while DPPH radical scavenging was higher in the later condition (Sani et al., 2012). The study was discussed its unrelated result of phenolic content and antioxidant as the higher relation of flavonoids were observed to related with antioxidant activities than the total phenolic content (Sani et al., 2012).

4.6.4 Effect of pH and Temperature on Tyrosinase Inhibitor Stability

The tyrosinase inhibitory stability results were showed in Figure 4.16 in categorized as the storage temperatures, the decreasing of the residual activity was critically observed. The activity in all condition was retained 50% activity until 6 weeks of storage. The manner of activity changes almost the same in all pH except for pH 2. The tyrosinase inhibitory activity was about 1 fold increasing after 2 weeks at 4°C and room temperature and just for 1 week at 50°C storage. The low temperature condition in Figure 4.16A showed almost similar trend lines of pH 3, 4, and 11 and of

pH 5-10. The gradually decrement of the activity was observed. Only pH 2 extract solution was found having more than 50% activity. The rest pH solutions could retain more than 20% activity except the lowest residual activity in pH 9.



Note: A; storage at 4°C, B; room temperature, C; 50°C

Figure 4.16 Residual of Tyrosinase Inhibition Activity of Raw Betel Nut Seed Extract

The stability at the ambient temperature depicted in Figure 4.16B, the pH 2 extract solution was noticed again as its different residual activity changing profile from others pH. The tyrosinase inhibitory activity was left over than 50% and lower after 10 weeks storage. However, at the final day the activity could not hold more than 25% compare to the initial day in all pH condition except 30% of pH 2.

The thermal accelerated at 50°C storage in Figure 4.16C also showed the lowered trend lines in all pH solution. The acidic conditions of pH 2-5 showed the higher stabilized activity than the higher pH of 6-11. The activity of the neutral to basic condition were lower than 50% after 9 weeks storage, while 2 more weeks were possessed in the acidic condition. The noted pH 2-4 solutions were retained slightly lower than 40% residual after 84 days storage. The pHs above 6 were considered as low stabilizing activity as they were lower than 10% residual.

The summary of the effect of pH and temperature on tyrosinase inhibitory stability of the raw betel nut seed extract was depicted in Figure 4.17. The results were obviously exhibited the low relation to the previous determinations on EPC and ABTS cation radical scavenging activity. This imply the attribution of the different bioactive compound on tyrosinase inhibitory and ABTS radical scavenging activity which credited by the phenolics by EPC.

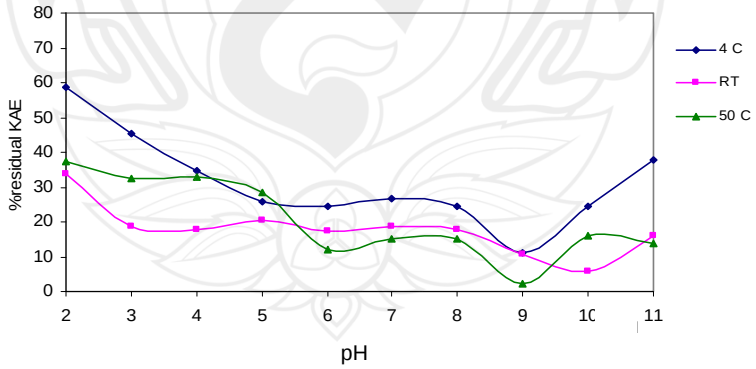


Figure 4.17 Percentage of Residual Tyrosinase Inhibition Activity of Raw Betel Nut Seed Extract Solutions after 84 Days Storage

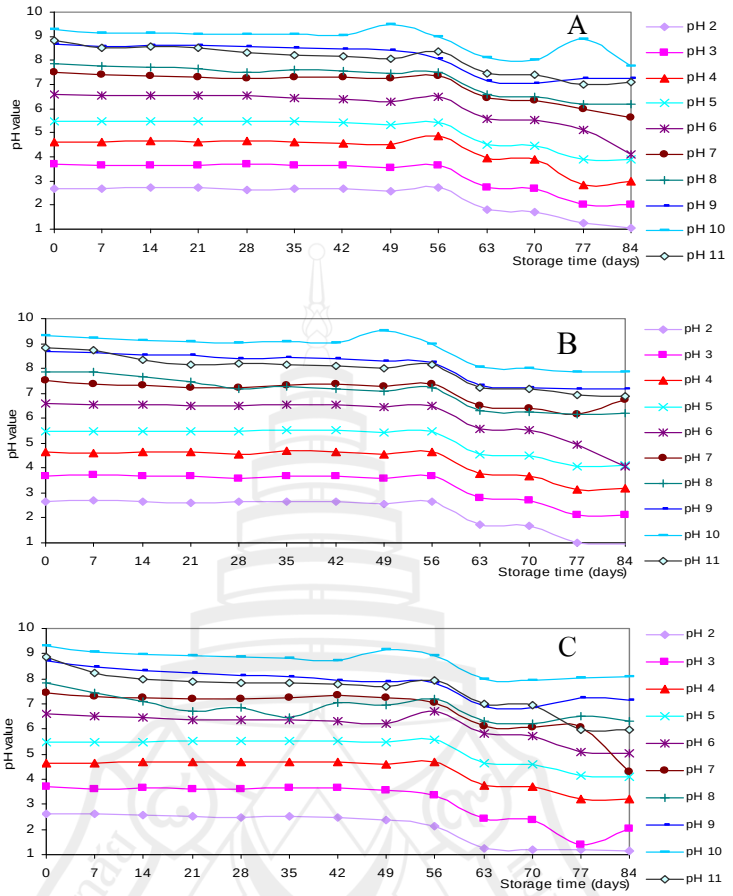
4.6.5 Physio-Chemical Evaluation

In addition to the extract, the finished product containing extract was also in considered to tits instability. The oxidative degradation of botanical phenolics can be initiated by light, sonication, oxygen, basic pH conditions, heat, redox-active solvents, and formulation additives (Baugh, S. & Ignelzi, S., 2000).

4.6.5.1 pH measurement during storage

The pH stability of raw betel nut seed extracts in Figure 4.18 shows high pH stability of the extract in all pH solution during 56 days storages. The gradually decreased was found in the last 4 weeks of the study. Noticeably, the pH values of the extract solutions were not exactly the same pH with the buffer since the initial day. The extract solutions under pH 8 have pH value over the buffers, while pH 9-11 solutions have been decreased. These were according to the extract itself was a weak acidic (about pH 4 in DI water). The storage temperatures seem to be not affected the pH stability.

The pH profile of all extract solution after 84 days storage is depicted in Figure 4.19. Most of the raw betel nut extract solution shows no critically changes in pH value at any storage buffer and temperature. Some change in pH was observed when storage in the buffer pH between 6-7 and 11. The extract solution at pH 7 only showed the significant effect of the storage temperature at 4°C and 50°C.



Note: A: storage at 4°C, B: room temperature, C: 50°C

Figure 4.18 pH Stability of Raw Betel Nut Seed Extract

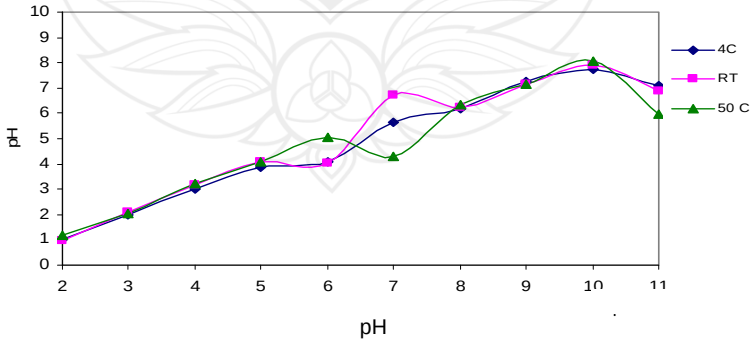
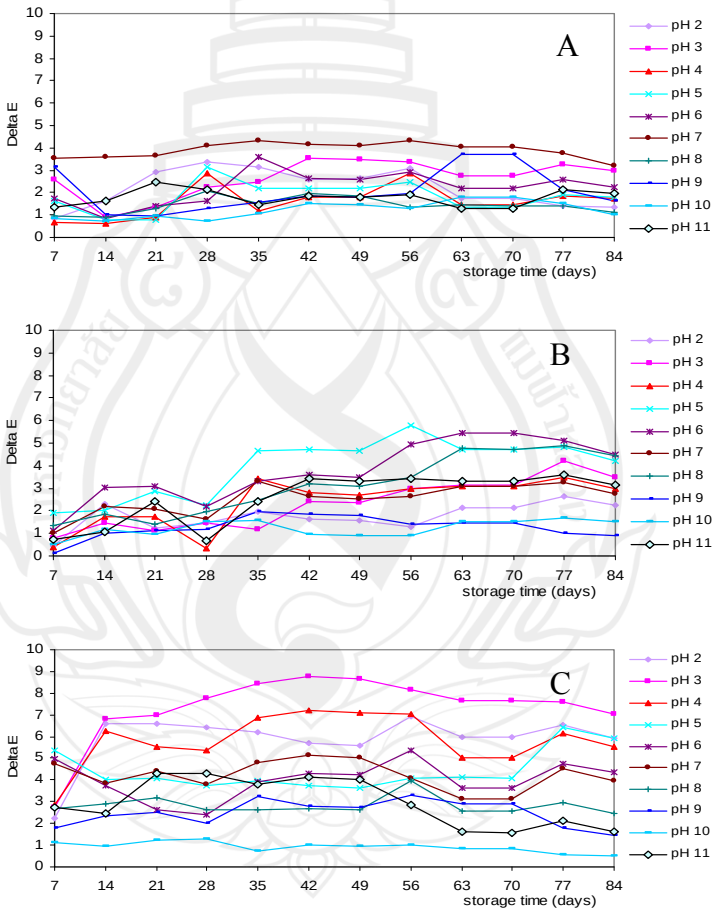


Figure 4.19 pH Changes of Raw Betel Nut Seed Extract Solutions After 84 Days Storage

4.6.5.2 Color measurement during storage

The color of extracts was expressed as ΔE* which is the color change detected by human eyes. The more value of ΔE*, the more color changes. For the stability of extract color in Figure 4.20, thermal is rather considered as the significant factor than pH condition. At 4°C and room temperature showed in Figure 4.20A and B, the color seems to be stable at every pH. In contrast at the high temperature (Figure 4.20C), the color of extracts, especially at pH 2-4, was obviously changed due to the precipitation of the extract under accelerated condition of 50°C.



Note: A; storage at 4°C, B; room temperature, C; 50°C

Figure 4.20 Stability of Color Changes (ΔE*) of Raw Betel Nut Seed Extract

For color stability at day 84, as showed in Figure 4.21, high temperature storage was greatly effected to the color changing, expressed as ΔE* value. When temperature rose, more change in extraction color was observed in acidic condition. The storage conditions of various pH and temperatures influence the maintenance of color, phenolics and biological activities of the extract. Betel nut seed extract contained high amount of phenolic compounds (Uchino, K., Matsuo, T. & Iwamoto, M., 1988). High temperature accelerated oxidation of these substances and subsequently darker color was observed. There has been reported that the synthetic phenolics stability found the basic-induced degradation with the irreversible structural deformation by heat (Friedman & Jurgens, 2000). In contrast with the color, temperature was not influenced to the extract pH. Therefore, the pH of extract was stabilized even the temperature increase.

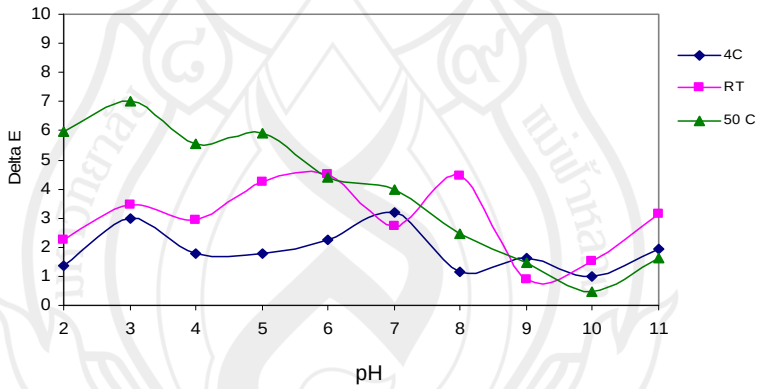


Figure 4.21 Color Changes (ΔE*) of Raw Betel Seed Nut Extract Solutions after 84 Days Storage

For the phenolic stability test, increment of the EPC in every condition was remarked at the first 7 days storage. This might be due to liberation of small phenolic compounds from complex phenolic substances in the betel nut seed extract. However, gradual decrease of the EPC after 7 days can be explained that high temperature enhanced oxidation and degradation of the phenolic compounds in the extract (Friedman & Jurgens, 2000). The different in rate of antioxidant activity

degradation depended on the thermal and pH tolerance of phenolic compounds. The phenolics in the betel nut extract mainly exhibited the biological activity. Loss of the phenolics at high temperature resulted as decrease of the antioxidant and tyrosinase inhibition activities. The degradation of phenolic compounds such gallic acid and its ester in plant extract under high pH was reported to be irreversible by the possible formation of unstable quinone (Friedman & Jurgens, 2000).

4.7 Stability of Cream Containing Raw Betel Nut Seed Extract

4.7.1 Phenolic Content

The residual phenolic contents in the formulation during stability test are shown in Table 4.17. The storage under heating/cooling cycle possessed higher residual EFC (96.27%) than freeze/thaw condition (93.50%). The slight difference may be caused by the thermal acceleration of heating (50°C) causes the releasing of the bound polyphenolics. However, both condition exhibited the excellent residual activity of the formulas after 3 cycles. The stilling condition of 3 different temperatures of 4°C, room temperature, and 50°C, the formulas retained different residual EPC (Figure 4.22). After 7 days storage, the extract contained in the formula could retain the most in the room temperature condition (86.07%). The condition of 4°C and 50°C showed the lower residual of 78.67 and 65.47%, respectively. The EPC gradually decreased till day 28, the residual EPC was 71.87% (4°C), 66.00% (room temperature), and 54.80% (50°C) (Table 4.17). The results could interpret the thermal instability of the extract.

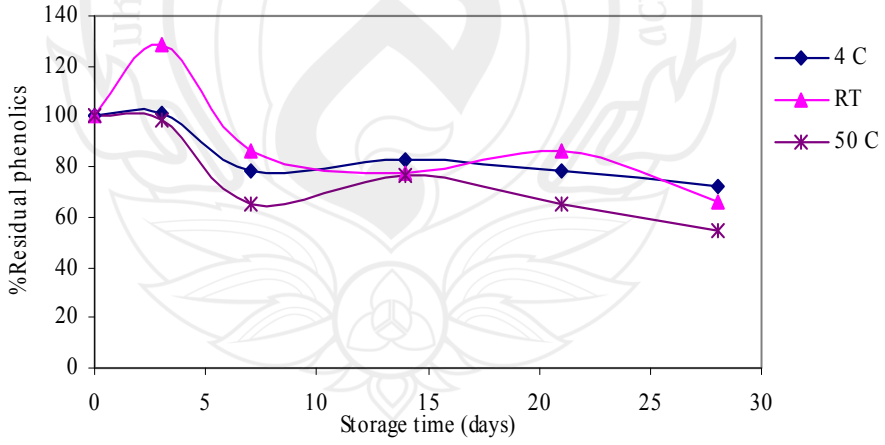


Figure 4.22 Percentage of Residual Phenolics Content of the Formula Containing 0.5% Raw Betel Nut Seed Extract

Table 4.17 Stability of the Cosmetic Formulation Containing Raw Betel Nut Seed Extract after 3 Accelerated Cycles of Freeze/Thaw and Heating/Cooling and after 28 Days Storage at 4°C, Room Temperature, and 50°C

Evaluation parameters	Acceleration (3 cycle)		Storage for 28 days		
	Freeze/thaw	Heating/cooling	4°C	Room temperature	50°C
Base formula					
ΔpH	-0.075	-0.128	-0.123	-0.115	-0.152
ΔL*	-0.440	-0.880	-3.517	0.020	-1.920
Δa*	0.090	0.187	-0.043	-0.037	-0.440
Δb*	-0.163	-0.153	0.520	0.153	1.120
ΔE*	0.900	0.504	3.555	0.169	2.266
ΔcP	-400	-1,400	-7,100	-10,430	-7,820
Formula containing 0.5% raw betel nut extract					
ΔpH	-0.736	-0.738	0.069	0.074	-0.432
ΔL*	-4.210	-0.397	-4.523	-1.643	-15.943
Δa*	1.34	0.24	0.117	-0.037	-0.440
Δb*	2.06	0.217	0.610	2.553	9.137
ΔE*	4.880	0.504	4.566	3.212	19.684
ΔcP	-8,100	-10,000	-10,430	-11,660	-14,241
%Residual EPC	93.50	96.27	71.87	66.00	54.80
%Residual antioxidant	90.78	102.35	66.27	73.54	49.35

4.7.2 Antioxidant Capacity Stability

The residual DPPH radical scavenging activity of the formulas was showed in Table 4.17. The accelerated conditions of heating-cooling and freeze-thaw showed the notable difference. Under heating/cooling cycle possessed higher residual activity of 102.35%. The freeze/thaw condition possessed the lower residual activity of 90.78%.

The difference in residual scavenging activity was related to the residual EPC. The thermal condition of heating/cooling cycle may effect to the releasing of the bound polyphenolics and also other effective compounds cause more radical scavenging efficacy.

The storage condition of 3 different temperatures of 4°C, room temperature, and 50°C, the formulas retained different residual antioxidant activity (Table 4.17). At day 3, the notable increasing of antioxidant activity was found in 50°C storage (131.45%). This result implied the effected of thermal in the acute polyphenols decomposition but the derivative(s) were not longer stable after day 3. At day 7 of storage the residual antioxidant activity of all storage conditions were decreased especially critical at 50°C. The most activity decrement was found in high temperature storage (50°C) of 49.48% and remained stable until day 28 (49.35%). The results could interpret the highly thermal instability of the extract after derivatives may occur at the early day and critically decomposed. At 4°C and room temperature storage, the antioxidant activity reach plateau and was slightly decreased after day 21. At the final tested day, the raw betel nut extract in the formula remained 66.27% (4°C) and 73.54% (room temperature).

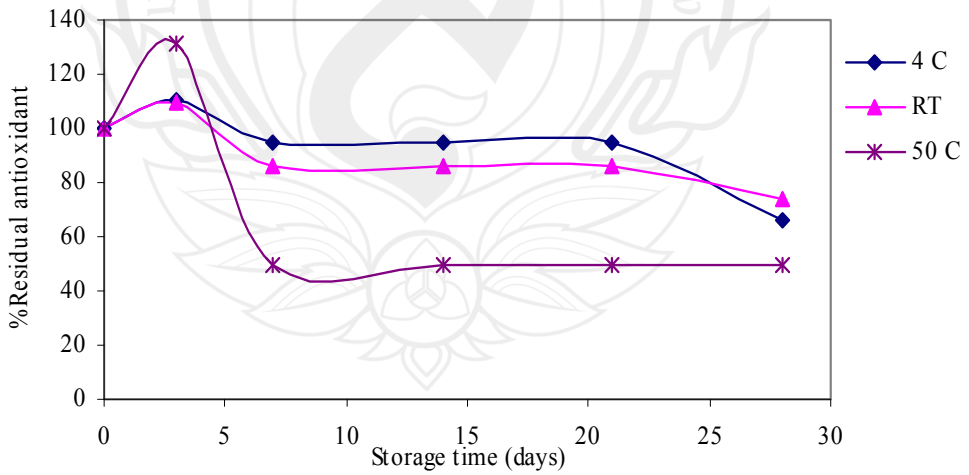


Figure 4.23 Percentage of Residual Antioxidant Activity of the Formula Containing 0.5% (w/w) Raw Betel Nut Seed Extract

The changing in physio-chemical properties of pH, color, and viscosity of the formulation containing 0.5% (w/w) raw betel nut extract were determined comparing to those of the base formulas.

4.7.3 pH Stability

The pH values were decreased into more acidic state in every condition tested. During storage, the base formulas at all tested conditions were quite stable which the pH decreased less than 0.15. The differences of pH changing of the formula containing extract compared to its base formula were showed in Table 4.17. The accelerated conditions of freeze/thaw and heating/cooling, the formula contained raw betel nut seed extract possessed similar acidic trends, -0.66 and -0.61, respectively.

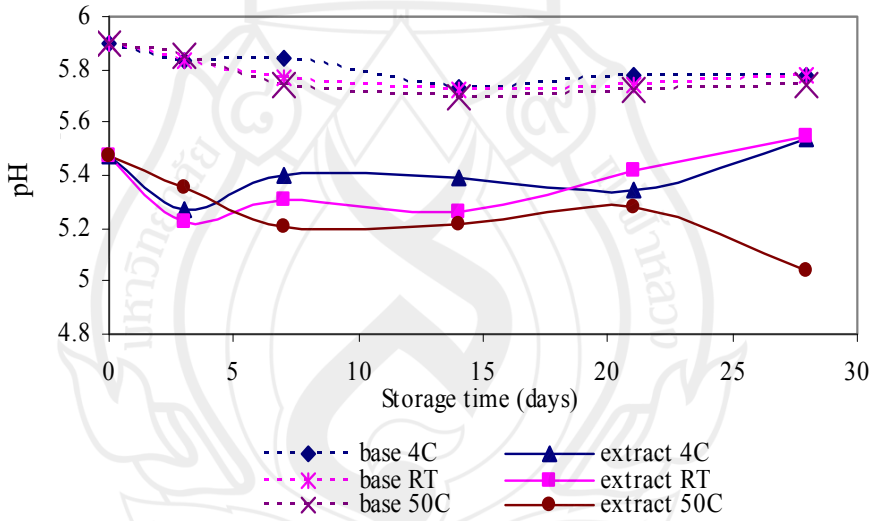


Figure 4.24 Changes of pH of the Base Formula and Formula Containing 0.5% (w/w) Raw Betel Nut Seed Extract

Under different storage temperature of 4°C, room temperature, and 50°C, the base formulas were slightly different in pH changes, 0.06, 0.13, and 0.15, respectively. The pH profiles of the formula containing raw betel nut seed extract were showed in Figure 4.24. The different storage temperatures exhibited the

significant in the pH stability. At the final day 28, the storage at 4°C and room temperature exhibited similar profile of 0.23 and 0.24 reductions, respectively. The high temperature condition, 50°C, exhibited the highest pH lowered effect of 0.71 at day 28. The high acidic effect under high temperature implied the releasing of free phenolics acids leads some changes in pH detection.

4.7.4 Color Stability

The delta values (Δ) in Table 4.17 shows the changes of the color parameters (CIELab) at the final day compared to the initial day. The color changing profiles of the formulas containing 0.5% raw betel nut seed extract were showed in Table 4.17. At the accelerated conditions, the L* of the formula containing 0.5% extract was lowered the most in heating/cooling cycle, -3.33, the minus value of L* indicated the lower luminance of the formula. The freeze/thaw cycle showed no effect to the luminance of the formula compare to the base (0.04). At the still storage of 4°C, room temperature and 50°C, L* were variety lowered (Figure 4.25A).

The heat condition of 50°C obviously affected the most (-19.06) and at 4°C and room temperature the formula also changed (-14.63). The green-red hue changing was indicated as a* parameter. Comparing between the accelerated conditions, heating cooling possessed the higher effect to the red color of the formula (1.25) while freeze/thaw cycles possessed not significantly higher a* of 0.05 (Table 4.17). The minus Δa* value interpret the greener and the positive Δa* shows the redder compared to the base formula. All formulas possessed the positive Δa*. Figure 4.25B showed Δa* profiles of the formula stored at 4°C, room temperature, and 50°C. The storage at 4°C possessed slightly lower Δa* (4.11) than at room temperature (4.89). The storage at 50°C exhibited almost 3 folds changed (11.63) compared to 4°C and room temperature.

The blue-yellow color was indicated by the parameter of b* (Figure 4.25C). The base formulas were slightly lower b* in all storage condition. The accelerated cycles of heating/cooling exhibited significantly higher Δa* than freeze/thaw cycles of 2.22 and 0.37, respectively (Table 4.17).

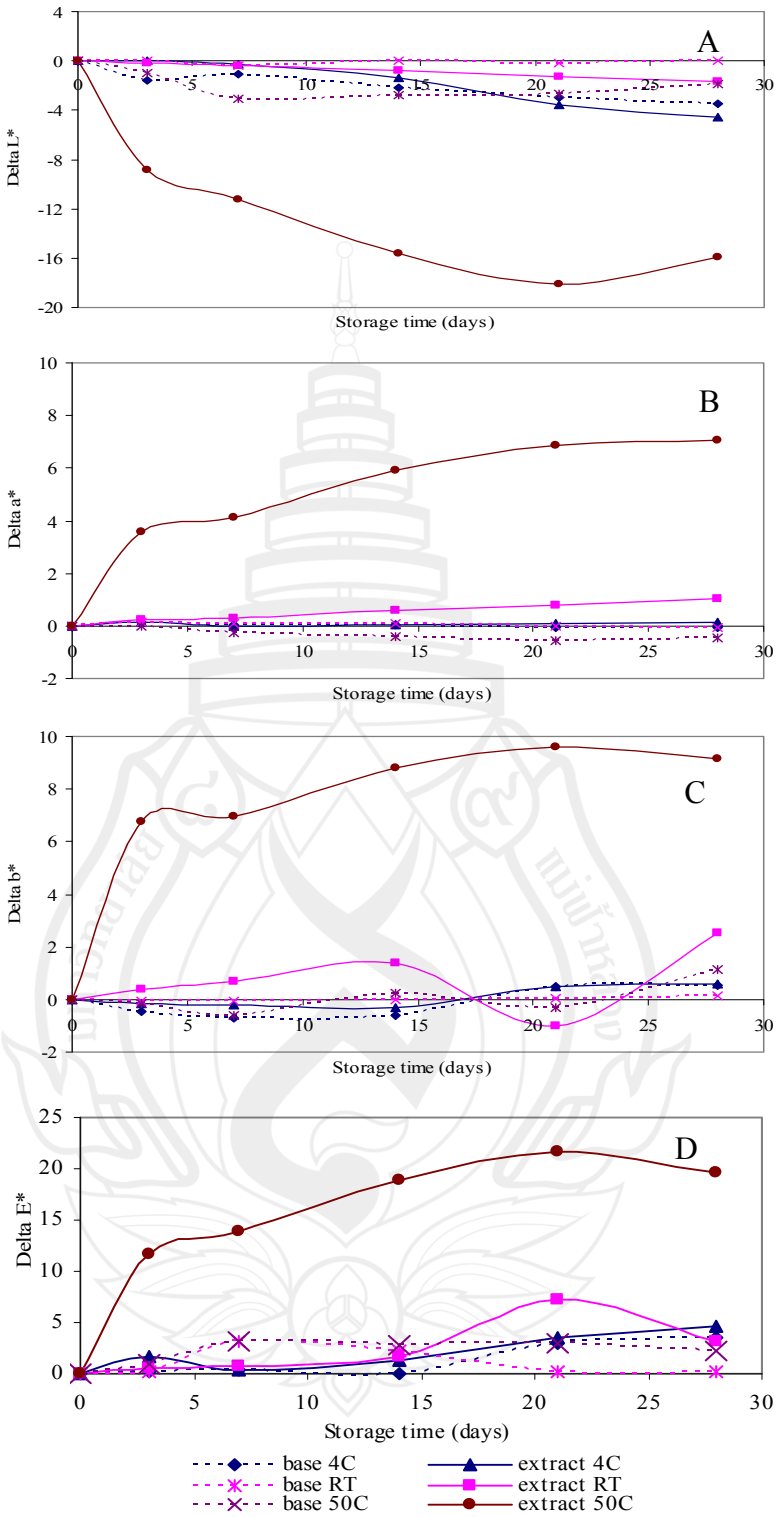


Figure 4.25 Changes of Color of the Base Formula and Formula Containing 0.5% (w/w) Raw Betel Nut Seed Extract

The storage at 4°C possessed low positive change in b* of 8.15, this may be the result of higher color conservation at low temperature. At room temperature, the higher yellowish was observed (10.38) and the highest yellowish was induced at 50°C storage (16.25). The total color differences were calculated as ΔE*. The ΔE* would summarized the human eyes detection of the color differences of the formulas at the final day compared to the initial day and their base formula.

Day 0 **Heating/cooling** **Freeze/thaw**

Figure 4.26 Appearance of the base formula (left) and the formula containing 0.5% (w/w) raw betel nut seed extract (right)

The accelerated heating/cooling cycles possessed higher ΔE* of 3.98 compared to 0.05 from freeze/thaw condition after 3 cycles (Table 4.17). The appearances of the tested formulas under accelerated conditions were showed in Figure 4.26. The ΔE* of the base formulas were not observed excepted for the storages at 4°C and 50°C, the slightly ΔE* were influenced by the decreasing in luminance of the formula. The ΔE* profiles of the formula containing 0.5% raw betel nut seed extract were showed in Figure 4.25D. The storage at 4°C exhibited slightly higher ΔE* of 1.01 and 3.04 than at room temperature at day 28 of storage, respectively. At heat condition of 50°C storage, the total color difference was found dramatically of 17.42 (Table 4.17). The high ΔE* value of the formulation containing 0.5% raw betel nut seed extract at 50°C storage was affected by all color parameters. The critically lower luminance, higher red as well as yellow hue makes the formula

begin turned to brownish after 3 days storage. The appearances of the tested formulas at 4°C, room temperature, and 50°C were showed in Figure 4.27.

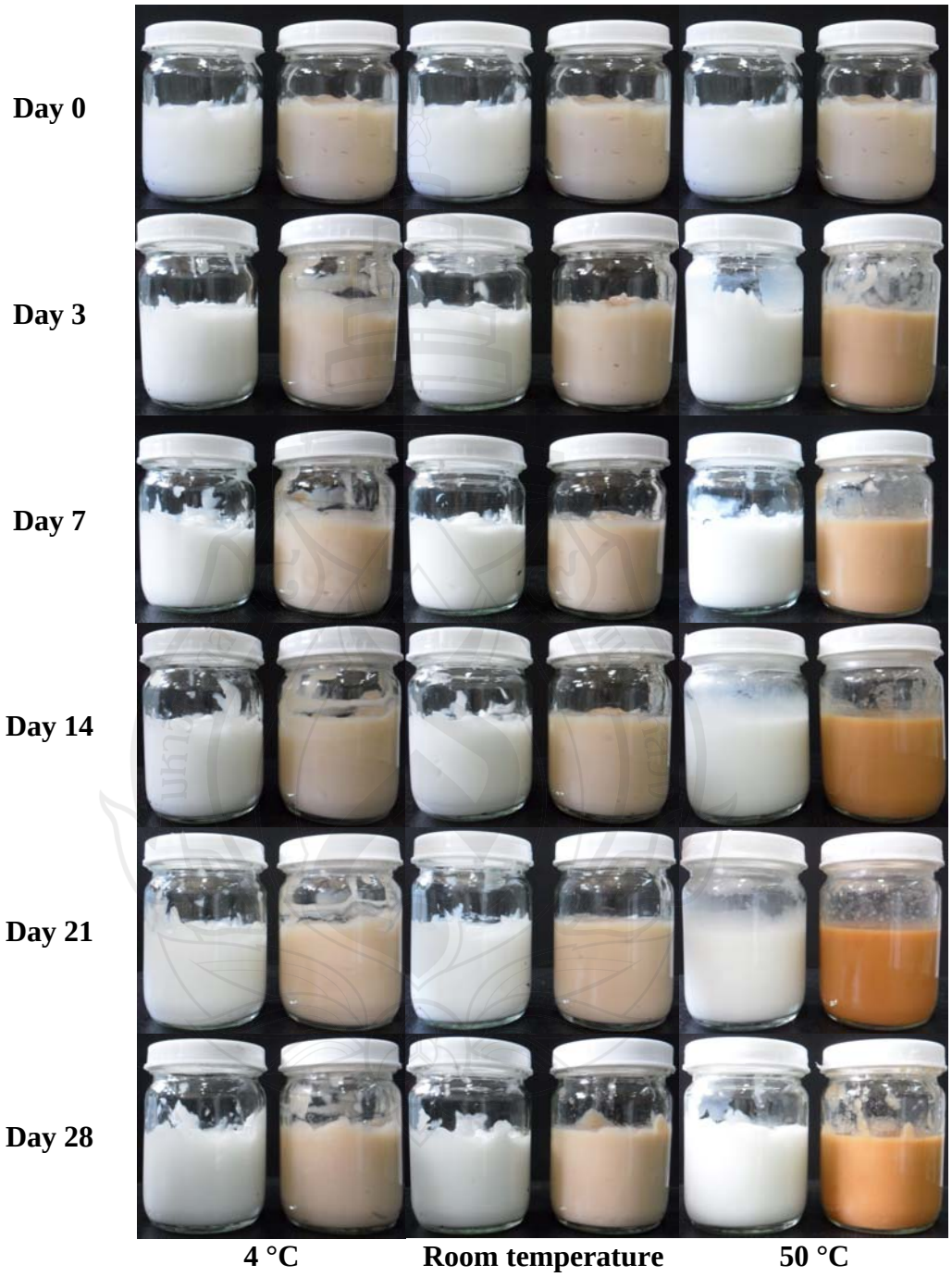


Figure 4.27 Appearance of the Base Formula (left) and the Formula Containing 0.5% (w/w) Raw Betel Nut Seed Extract (right)

4.7.5 Viscosity Stability

The emulsion stability was determined the viscosity by Brookfield viscometer and reported in Table 4.17. The formulas exhibited the lower in viscosity interpreted by the lower centipoises values (cP). After 3 cycles of heating/cooling the viscosity of base formula was slightly decreased (400 cP), while the viscosity of the formula containing 0.5% extract was significantly decreased (1,400 cP). The formula containing extract was similar to the base, heating/cooling possessed higher viscosity decrement (6,400 cP). The higher viscosity stability was found in freeze/thaw cycles (5,500 cP) (Table 4.17).

The storage at 4°C and room temperature were found having similar lowered viscosity of 1,130 and 1,640 cP, respectively. The heat condition at 50°C showed the critical viscosity decreasing (4,731 cP) (Table 4.17). The viscosity profiles of the formula containing raw betel nut seed extract stored at 4°C, room temperature, and 50°C showed in Figure 4.28.

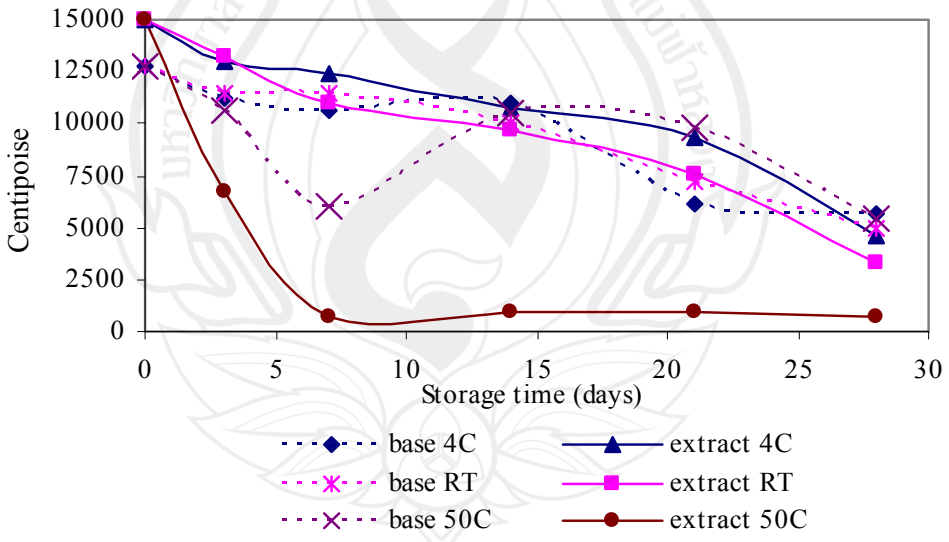


Figure 4.28 Changes of Viscosity of the Base Formula and Formula Containing 0.5% (w/w) Raw Betel Nut Seed Extract

The change in viscosity of the emulsion was obviously effected by heat. Both accelerated heating/cooling cycles and 28 days storage at 50°C possessed the lowering in viscosity. The formulas also possessed the creaming effected which is reversible to the homogenous form by tender shaking. This creaming effect also influences the viscosity of emulsion. The chemical reaction of the particles may change the physical properties as well.

4.8 Suggestion

The author would express recommendations to complete this study. First, the substrate for tyrosinase activity testing could be mono-, di-, or tri-hydroxyphenols. Tyrosinase activity was reported most specific to dihydroxyphenols (catechol, L-dopa, D-dopa, catechin, chlorogenic acid). This study used L-dopa, dihydroxyphenol as substrate because of its less log period of diphenolase when compared to monophenolase activity using monohydroxyphenols (*p*-cresol or tyrosine) as substrate (Seo, S.Y., Sharma, V.K. & Sharma, N., 2003).

By the way, the specific oxidation reaction inhibitory activity was suggested to achieve in the further study. Both monohydroxyphenol and dihydroxyphenol could be tested for specific inhibition stage of the extract to tyrosine.

In this study, the cytotoxicity result of the extract utilized the primary safety in use as the active ingredient in topical formulation. In order to complete the consumer safety, the irritation test should be carried out. Furthermore, the concentration of the extract in the formula (0.5%, w/w) was based on the *in vitro* assay with two folds over the results in order to obtained maximum *in vivo* efficacy in the further study.

CHAPTER 5

CONCLUSION

The 50% ethanolic raw betel nut seed extract from microwave assistance was found having the highest EPC, EFC, and ECC with values of 226.58 mg GAE/g sample, 140.79 mg QE/g sample, and 67.23 mg CE/g sample, respectively. This extract also exhibited the highest antioxidant capacities of 495.51, 908.39 and 383.17 mg TEAC/g sample when determined by DPPH, ABTS and FRAP assays, respectively. The lipid peroxidation and tyrosinase inhibitory activity of 60.51 mg BHTE/g sample and 348.81 mg KAE/g sample were obtained. The lower bioactivities were obtained from ripe seed extract. The pericarp possessed lower activity than seed part. In contrast, ripe stage of pericarp exhibited higher bioactivities than the raw pericarp. The microwave-assisted extraction method exhibited the significantly higher extraction potential.

Amongst the fractions from solid-liquid extraction, acetone extract possessed the highest EPC, EFC, and ECC of 82.39 mg GAE/g sample, 12.71 mg QE/g sample, and 1.51 mg CE/g sample, respectively. The most powerful antioxidant capacity was also obtained from the acetone fraction exhibiting 20.76 and 14.31 mg TEAC/g sample when assayed by DPPH and FRAP method, respectively. The first fraction of hexane and the last fraction of water exhibited lowest bioactive compounds and activities. The HPLC chromatogram showed the major components of raw betel nut seed were catechin-like compounds.

The stability test of the extract found that when the pH and temperature storages increased, the color of extract solution more intense. The raw betel nut seed extract retained residual EPC and radical scavenging activity at pH 4-6 and 8-10, especially when storing at low temperature.

The formula containing 0.5% raw betel nut seed extract retained more than 90% residual EPC after 3 accelerated cycles. The residual antioxidant activity of 90.78 and 102.35% were investigated. The EPC and antioxidant were stable more at the storage at 4°C and room temperature than at 50°C storage. The study revealed the potentially use of *A. catechu* Linn. fruit as the antioxidant, whitening, and anti-bacterial agent in cosmetic product. The using of betel nut is also present the sustainable development and value-adding to Thai agricultural product.

REFERENCES

Ahn, B.Y. (2009). Free radical scavenging effect of ethanol extract from *Areca catechu*. **J. Applied Biol. Chem., 52**, 92-95.

Ando, H., Kondoh, H., Ichihashi, M. & Hearing, V. J. (2007). Approaches to identify inhibitors of melanin biosynthesis via the quality control of tyrosinase. **J. Invest. Dermatol., 127**, 751-761.

Anthikat, R. R. N. & Michel, A. (2009). Study on the areca nut for its antimicrobial properties. **J. Young Pharmacists., 1**. 42-45.

Anthikat, R. R. N. & Michel, A. (2012). Anti-inflammatory and antioxidant effect of *Areca catechu*. **Inter. J. Phamaceutic. Res. Scholars., 3**, 2031-2037.

Arnao, M. B., Cano, A. & Acosta, M. (2001). The hydrophilic and lipophilic contribution to total antioxidant activity. **Food Chem., 73**, 239-244.

Ashawat, M. S., Shailendra, S. & Swarnlata, S. (2007). *In vitro* antioxidant activity of ethanolic extracts of *Centella asiatica*, *Punica granatum*, *Glycyrrhiza glabra* and *Areca catechu*. **Res. J. Med. Plant., 1**, 13-16.

Balasundram, N., Sundram, K. & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. **Food Chem., 99**, 191-203.

Bauer, A. W., Kirby, W. M. M., Sheris, J. C. & Turck, M. (1996). Antibiotic susceptibility testing by a standardized single disk method. **Am. J. Clin. Pathol., 45**, 493-496.

Baugh, S. and Ignelzi, S. (2000). Hydrolysis and redox factors affecting analysis of common phenolic marker compounds in botanical extracts and finished products. **J. AOAC Inter., 83**(5), 1135-1140.

Benzie, I. F. F. & Strain, J. J. (1999). Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. **Meth. Enzymol., 299**, 15-27.

Bhandare, A. M., Kshirsagar, A. D., Vyawahare, N. S., Hadambar, A. A. & Thorve, V. S. (2010). Potential analgesic, anti-inflammatory and antioxidant activities of hydroalcoholic extract of *Areca catechu* L. nut. **Food Chem. Toxicol., 48**, 3412-3417.

Cai, Y., Luo, B., Sun, M. & Corke, H. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. **Life Sciences, 74**, 2157-2184.

Chang, C. C., Yang, M. H., Wem, H. M. & Chern, J. C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. **J. Food Drug Anal., 10**(3), 178-182.

Chaiwut, P., Nitsawang, S., Shank, L. & Kanasawud, P. (2007). A Comparative study on properties and proteolytic components of papaya peel and latex proteases. **Chiang Mai J. Sci., 34**(1), 109-118.

Chavan, Y. & Singhal, R. S. (2013a). Seperation of polyphenols and arecoline from areca nut (*Areca catechu* L.) by solvent extraction, its antioxidant activity, and identification of polyphenols. **J. Sci. Food Agric.** DOI: 10.1002/jsfa.6081

Chavan, Y. & Singhal, R. S. (2013b). Ultrasound-assisted extraction (UAE) of bioactives from arecanut (*Areca catechu*L.) and optimization study using response surface methodology. **Innov. Food Sci. Emerg., 17**, 106-113

Chen ,Y., Xie, M. Y. & Gong, X. F. (2007). Microwave-assisted extraction used for the isolation of total terpenoid saponins from *Ganoderma artrum*. **J. Food Engineer., 81**, 162-170.

Cheng, H. Y., Lin, T. C., Yu, K. H., Yang, C. M. & Lin, C. C. (2003). Antioxidant and free radical scavenging activities of *Terminalia chebula*. **Biol. Pharm. Bull., 26**, 1331-1335.

Choi, C. W., Kim, S. C., Hwang, S. S., Choi, B. K., Ahn, H. J., Lee, M.Y., Park, S.H. & Kim, S. K. (2002). Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. **Plant Science, 163**, 1161-1168.

Chulasiri, M., Wanasawas, P., Sriaum, D., Nakamat, S., Wongkrajang, Y., Kongsaktrakoon, B., Phornchirasilp, S., Songchitsomboon, S. & Leelarungrayub, D. (2011). Utilizing hydoglycolic extract fro myrobalan fruits to counteract reactive oxygen species. **Inter. J. Cosmet. Sci., 33**, 371-376.

Dashen, M. M., Chollom, P. F., Okechalu, J. N. & Ma-aji, J. A. (2011). Microbiological quality assessment of some brands of cosmetics powders sold within Jos Metropolis, Plateau State. **J. Microbiol. Biotech. Res., 1**(2), 101-106.

Dearden, J. C. & Forbes, W. F. (1959). Light absorption studies, Part XIV: The Ultraviolet absorption spectra of phenols. **Can. J. Chem., 37**, 1294-1305.

Devasagayam, T. P. A., Boloor, K. K. & Ramasarma. (2003). Methods for estimating lipid peroxidation: An analysis of merits and demerits. **Indian J. Biochem. Biophys., 40**, 300-308.

Ernest, W. F. (2001). **Cosmetic and toiletry formulations** (2nd ed., vol 8). New York: Noyes Publication and William Andrew Publishing.

Farah, A. & Donangelo, C. M. (2006). Phenolic compounds in coffee. **Brazil. J. Plant Physiol., 18**(1), 23-36.

Flora, S. J. S. (2009). Structural, chemical and biological aspects of antioxidants for strategies against metal and metalloid exposure. **Oxid. Med. Cell Longev., 2**(4), 191-206.

Friedman, M. (1997). Chemistry, biochemistry, and dietary role of potato polyphenols. A review. **J. Agric. Food Chem., 45**, 1523-1540.

Friedman, M. & Jurgens, H. S. (2000) Effect of pH on the stability of plant phenolic compounds. **J. Agric. Food Chem., 48**, 2101-2110.

Gao, M., Huang, W., Moytri, R. C. & Lui, C. (2007). Microwave-assisted extraction of scutellarin from *Erigeron breviscapus* Hand-Mazz and the determination by high performance liquid chromatography. **Anal. Chem. Acta., 591**, 161-166.

Global Insight. (2007). **A study of the European cosmetics industry: final report.** Retrieved March 17, 2013, from http://s3.amazonaws.com/zanran_storage/

Gunaratne, A., Wu, K., Li, D., Bentota, A., Corke, H. & Cai, Y.Z. (2013). Antioxidant activity and nutritional quality of traditional red-grained rice varieties containing proanthocyanidins. **Food Chem., 138**,1153–1161.

Halliwell, B. (2005). Free radicals and other reactive species in disease. **eLS.**

Hamsar, M.N., Ismail, S., Mordi, M.N., Ramanathan, S. & Mansor, S.M. (2011). Antioxidant activity and the effect of different parts of areca catechu extracts on Glutathione-S-Transferase activity *in vitro*. **Free Rad. Antiox., 1**(1), 28-31.

Huang, D., Ou, B. & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. **J. Agric. Food Chem., 53**(6), 1841-1856.

Huang, P. L., Chi, C. W. & Liu, T. Y. (2010). Effect of *Areca catechu* L. containing procyanidins on cyclooxygenase-2 expression *in vitro* and *in vivo*. **Food Chem. Toxicol., 48**, 306-313.

Kasikorn Research. (2010). **เครื่องสำอางนำเข้าขยายตัวอย่างต่อเนื่อง ผู้ประกอบการไทยเร่งปรับตัว**. Retrieved August 14, 2011, from http://www.kasikornreserch.com/th

Kaur, G. J. & Arora, D. S. (2009). Antibacterial and phytochemical screening of *Anethum graveolens, Foeniculum vulgare and Trachyspermum ammi*. **BMC Compl. Altern. Med., 9**, 30.

Kim, B. J., Kim, J. H., Kim, H. P. & Heo, M. Y. (1997). Biological screening of 100 plant extracts for cosmetic use (II): anti-oxidative activity and free radical scavenging activity. **Inter. J. Cosmet. Sci., 19**, 299–307.

Kim, J.H., Byun, J.C., Bandi, A.K.R., Hyun, C.G. & Lee, N.H. (2009). Compounds with elastase inhibition and free radical scavenging activities from *Callistemon lanceolatus*. **J. Med. Plant. Res., 3**(11), 914-920.

Kim, J. S. J. & Kim, M. J. (2010). *In vitro* antioxidant activity of *Lespedeza cuneata* methanolic extracts. **J. Med. Plants Res., 4**, 674-679.

Kim, K. H., Tsao, R., Yang, R. & Cui, S. W. (2006). Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. **Food Chem., 95**, 466-473.

Kim, Y., Uyama, H. & Kobayashi, S. (2004). Inhibition effects of (+)-catechin-aldehyde polycondensates on protienases causing proteolytic degradation of extracellular matrix. **Biochem. Biophys. Res. Commun., 320**, 26-261.

Kiss, G.A.C., Forgacs, E., Cserhati, T., Mota, T., Morais, H. & Ramos, A. (2000). Optimization of the microwave-assisted extraction of pigments from paprika (*Capsicum annuum* L.) powders. **J. Chromatogr. A., 889**, 41-49.

Kumar, S., Kumar, D. & Prakash, O. (2008). Evaluation of antioxidant potential phenolic and flavonoid content of *Hibiscus tiliacius* flowers. **Elec. J. Environ. Agric. Food. Chem., 7**, 2863-2871.

Kumoro, A. C., Hasan, M. & Singh, H. (2009). Effects of solvent properties on the soxhlet extraction of diterpenoid lactones from *Andrographis paniculata* leaves. **Sci. Asia., 35**. 306-309.

Lee, K. K., Cho, J. J., Park, E. J. & Cho, J. D. (2001). Anti-elastase and anti-hyaluronidase of phenolic Substance from *Areca catechu* as a new anti-ageing agent. **Inter. J. Cosmet. Sci., 23**, 341-346.

Lee, K. K. & Choi, J. D. (1999a). The effects of *Areca catechu* L. extract in anti-inflammation and anti-melanogenesis. **Int. J. Cosmet. Sci., 21**, 275-284.

Lee, K. K. & Choi, J. D. (1999b). The effects of *Areca catechu* L. extract on anti-aging. **Int. J. Cosmet. Sci., 21**, 285-295.

Lee, S. E., Hwang, H. J., Ha, J. S., Jeong, H. S. & Kim, J. H. (2003). Screening of medicinal plant extracts for antioxidant activity. **Life Sciences, 73**, 167-179.

Li, C. C. & Lin, E. S. (2010). Antiradical capacity and reducing power of different extraction method of *Areca catechu* seed. **Afr. J. Biotechnol., 9**, 7831-7836.

Li, Y. J., Chen, J., Li, Y., Li, Q., Zheng, Y. F., Fu, Y. & Li, P. (2011). The reactive rate and the ability of the radical scavenger depend on the rate and the peak value of disappearance of the DPPH. **J. Chromatogr., A. 1218**, 8181-8191.

Liu, Z. Y., Hu, X. L., Bu, F. Q., Ding, L. & Zhang, H. Q. (2007). Studies on the chemical change in the process of microwave-assisted extraction of flavonoids from *Acanthpanax senticosus* harms. **Chem. J. Chinese Univ., 28**, 431-435.

Mandal, V., Mohan, Y. & Hemalatha, S. (2007). Microwave assisted extraction-An innovative and promising extraction tool for medicinal plant research. **J. Phamaceut. Biomed. Anal., 46**,322-327.

Marketing Oops. (2008). **ส่วนแบ่งตลาดผลิตภัณฑ์ดูแลเส้นผม ยาสีฟัน และโลชั่นบำรุงผิว.** Retrieved March 17, 2013, from www. marketingoops.com/ reports/ matrix/market-share-2/

Moon, J. Y., Yim, E. Y., Song, G., Lee, N. H. & Hyun, C. G. (2010). Screening of elastase and tyrosinase inhibitory activity from Jeju Island plants. **EurAsia. J. BioSci., 4**, 41-53.

Nelson, B. S. & Heischober, B. (1999). Betel nut: a common drug used by naturalized citizens from India, far east Asia, and the south Pacific islands. **Ann. Emerg. Med., 34**(2), 238-243.

Norton, S. C. (1998). Betel: Consumption and consequences. **J. Am. Acad. Dermatol., 38**(1), 81-88.

Onar, H. C., Yusufoglu, A., Turker, G. & Yanardag, R. (2012). Elastase, tyrosinase and lipoxygenase inhibition and antioxidant activity of an aqueous extract from *Epilobium angustifolium* L. leaves. **J. Med. Plants Res., 6**, 716-726.

Oxenham, M. F., Locher, C., Cuong, N. L. & Thuy, N. K. (2002). Identification of *Areca catechu* (betel nut) residues on the dentitions of bronze age inhabitants of Nui Nap, Northern Vietnam. **J. Archaeol. Sci., 29**, 909–915.

Patel, A., Patel, A., Patel, A. & Patel, N. M. (2010). Estimation of flavonoid, polyphenolics content and *in vitro* antioxidant capacity of leaves of *Tepharosia purpurea* Linn. (Leguminosae). **Int. J. Pharma Sci. Res., 1**(1), 66-77.

Proestos, C. & Komaitis, M. (2008). Application of microwave-assisted extraction to the fast extraction of plant phenolic compounds. **LWT, 41**, 652–659.

Rice-Evans, C., Miller, N. & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. **Free Radic. Biol. Med., 20**(7), 933-956.

Robbins, R. J. (2003). Phenolic acids in foods: An overview of analytical methodology. **J. Agric. Food Chem., 51**, 2866-2887.

Sani, I. M., Iqbal, S., Chan, K. W. & Ismail, M. (2012). Effect of acid and base catalyzed hydrolysis on the yield of phenolics and antioxidant activity of extracts from germinated brown rice (GBR). **Molecules., 17**, 7584-7594.

Seo, S.Y., Sharma, V.K. & Sharma, N. (2003). Mushroom Tyrosinase: recent prospects. **J. Agric. Food Chem., 51**, 2837-2853.

Shalini, S. (2012). Natural antioxidants- a review. **Inter. J. Phytotherapy., 2**, 7-15.

Silva, M. C. A. & Paiva, S. R. (2012). Antioxidant activity and flavonoid content of *Clusia fluminensis* Planch. and Triana. **An. Acad. Bras. Cienc., 84**(3), 609-616.

Singleton V. L., Orthofer R. & Lamune-Raventos R. M. (1999). Analysis of total phenols and other oxidation substance and antioxidants by means of Folin-Ciocalteu reagent. **Meth. Enzymol., 299**,152-17

Staples, G. W. & Bevacqua, R. F. (2006). ***Areca catehu* (Betel nut palm).** Retrieved August 1, 2011, from http://www.agroforestry.net/tti/Areca-catechu-betel-nut.pdf

Sultana, B., Anwar, F. & Ashraf, M. (2009). Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. **Molecules., 14**, 2167-2180.

Sun, B., Ricardo-da-Silva, J. M., & Spranger, I. (1998). Critical factors of vanillin assay for catechins and proanthocyanidins. **J. Agric. Food Chem., 46**, 4267-4274.

Taylor, W. P., Hamilton-Miller, M. T. J. & Stapleton, D. P. (2009). Antimicrobial properties of green tea catechins. **Food Sci. Technol. Bull., 2**, 71-81.

Thai Bureau of Cosmetics and Hazardous Substance. (2004). **นวัตกรรมผลิตภัณฑ์เครื่องสำอาง ศูนย์ข้อมูลเครื่องสำอาง.** Retrieved August 14, 2011, from http://web.dmsc.moph.go.th

Thai Department of Agriculture Extension. (2002). **อนาคตหมากไทยจะเป็นอย่างไร.** Retrieved August 10, 2011, from http://www.doae.go.th/library/html/detail/

Thaipong, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L. & Byrne, D. H. (2006). Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. **J. Food. Compos. Anal., 19**, 669-675.

Thai Ministry of Commerce. (2012). **มูลค่าสินค้าออก สินค้าเข้า และดุลการค้าของไทย.** Retrieved March 10, 2013, from www2.ops3.moc.go.th

Thring, T. S. A., Hili, P. & Naughton, D. P. (2009). Anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants. **BMC Complem. Altern. M., 9**(27), 1-11.

Tomsone, L., Kruma, Z. & Galoburda, R. (2012). Comparison of different solvents and extraction methods for isolation of phenolic compounds from horseradish roots (*Armoracia rusticana*). **World Acad. Sci., 64**, 903-908.

Uchino, K., Matsuo, T. & Iwamoto, M. (1988) New 5'-nucleotidase inhibitors, NPF-86IA, NPF-86IB, NPF-86IIA, and NPF-86IIB from *Areca catechu*. II. Anti-tumor effects. **Planta Med., 54**(5), 419-422.

Vrhovsek, U., Palchetti, A., Reniero, F., Guillou, C., Masuero, D. & Mattivi, F. (2006). Concentration and mean degree of polymerization of *Rubus* ellagitannins evaluated by optimized acid methanolysis. **J. Agric. Food Chem., 54**, 4469-4475.

Wang, C. K. & Lee, W. H. (1996). Separation, characteristics, and biological activities of phenolics in Areca fruit. **J. Agric. Food Chem., 44**, 2014-2019.

Wang, C. K., Lee, W. H. & Peng, C. H. (1997). Contents of phenolics and alkaloids in *Areca catechu* Linn. during maturation. **J. Agric. Food Chem., 45**, 1185-1188.

Wang, L. & Weller, C. L. (2006). Recent advances in extraction of neutraceuticals from plants. **Trends in Food Sci. Technol., 17**, 300-312.

Wells, P. G., Bhuller, Y., Chen, C. S., Jeng, W., Kasapinovic, S., Kennedy, J. C., Kim, P. M., Laposa, R. R., McCallum, G. P., Nicol, C. J., Parman, T., Wiley, M. J. & Wong, A. W. (2005). Molecular and biochemical mechanisms in teratogenesis involving reactive oxygen species. **Toxicol. Applied Phaemacol., 207**(2), 354-366.

Wetwitayaklung, P., Phaechamud, T., Limmatvapirat, C. & Keokitichai, S. (2006). The study of antioxidant capacity in various parts of *Areca catechu* L. **Naresuan Univ. J., 14**, 1-14.

Wu, Q., Yang, Y. & Simon, J. E. (2007). Qualitative and quantitative HPLC/MS determination of proanthocyanidins in Areca nut (*Areca catechu*). **Chem. Biodiverse., 4**(12), 2817-2826.

Yenjit, P., Issarakraisila, M., Intana, W. & Chantrapromma, K. (2010). Fungicidal activity of compounds extracted from the pericarp of *Areca catechu* against *Colletotrichum gloeosporioides in vitro* and in mango fruit. **Postharvest Biol. Technol., 55**, 129–132.

Zhang, H. F., Yang, X. H. & Wang, Y. (2011). Microwave assisted extraction of secondary metabolites from plants: Current status and future direction. **Trends Food Sci. Technol., 21**, 672-688.

Zhang, W. M., Li, B., Han, L. & Zhang, H. D. (2009). Antioxidant activities of extract from Areca (*Areca catechu* L.) flower, husk, and seed. **Elec. J. Environ. Agric. Food. Chem., 8**, 740-748.

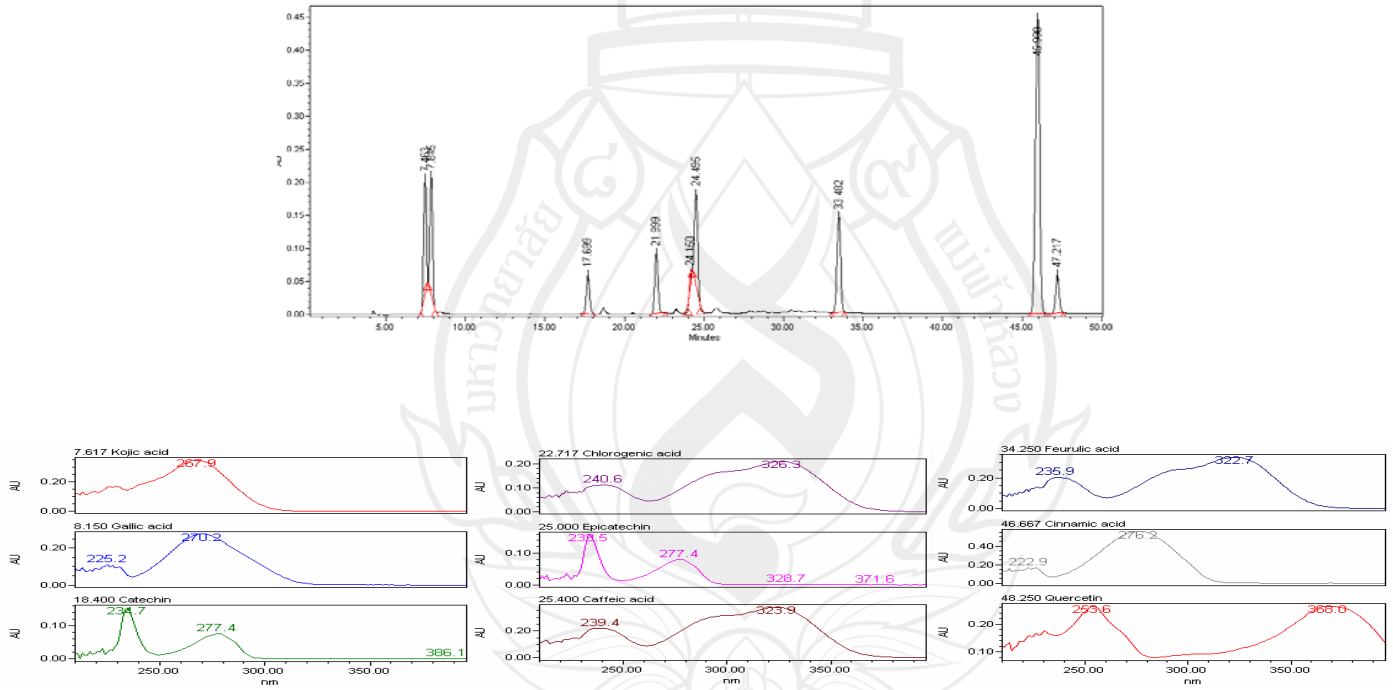
Zhang, X., Wu, J., Han, Z., Mei, W. L. & Dai, H. F. (2010). Antioxidant and cytotoxic phenolic compounds of areca nut (*Areca catechu*). **Chem. Res. Chinese Univer., 26**, 161-164.

Zhu, X., Su, Q., Cai, J. & Yang, J. (2006). Optimization of microwave-assisted solvent extraction for volatile organic acids in tobacco and its comparison with conventional extraction methods. **Analytica Chemica Acta., 579**, 88-94.

APPENDICIES

APPENDIX A

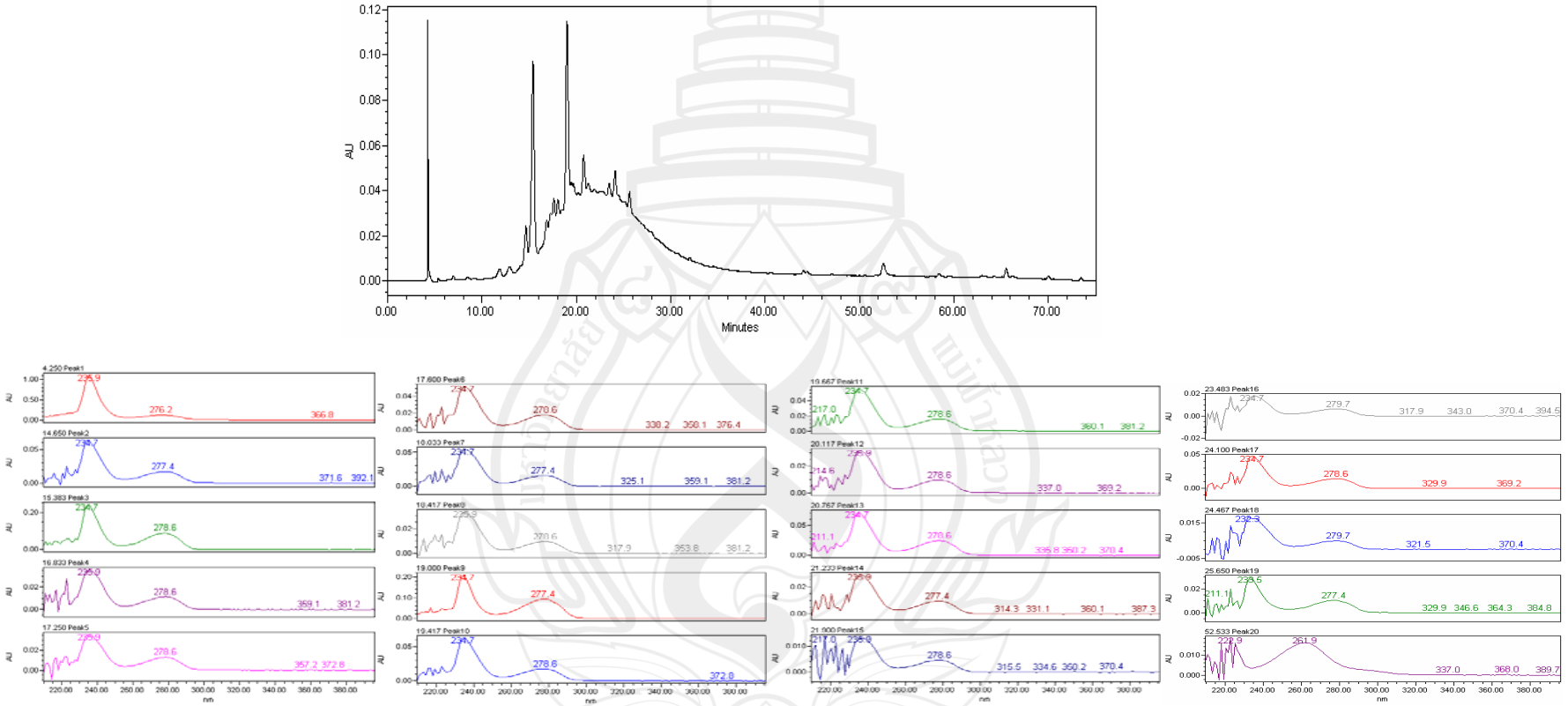
Spectrum of phenolic standards



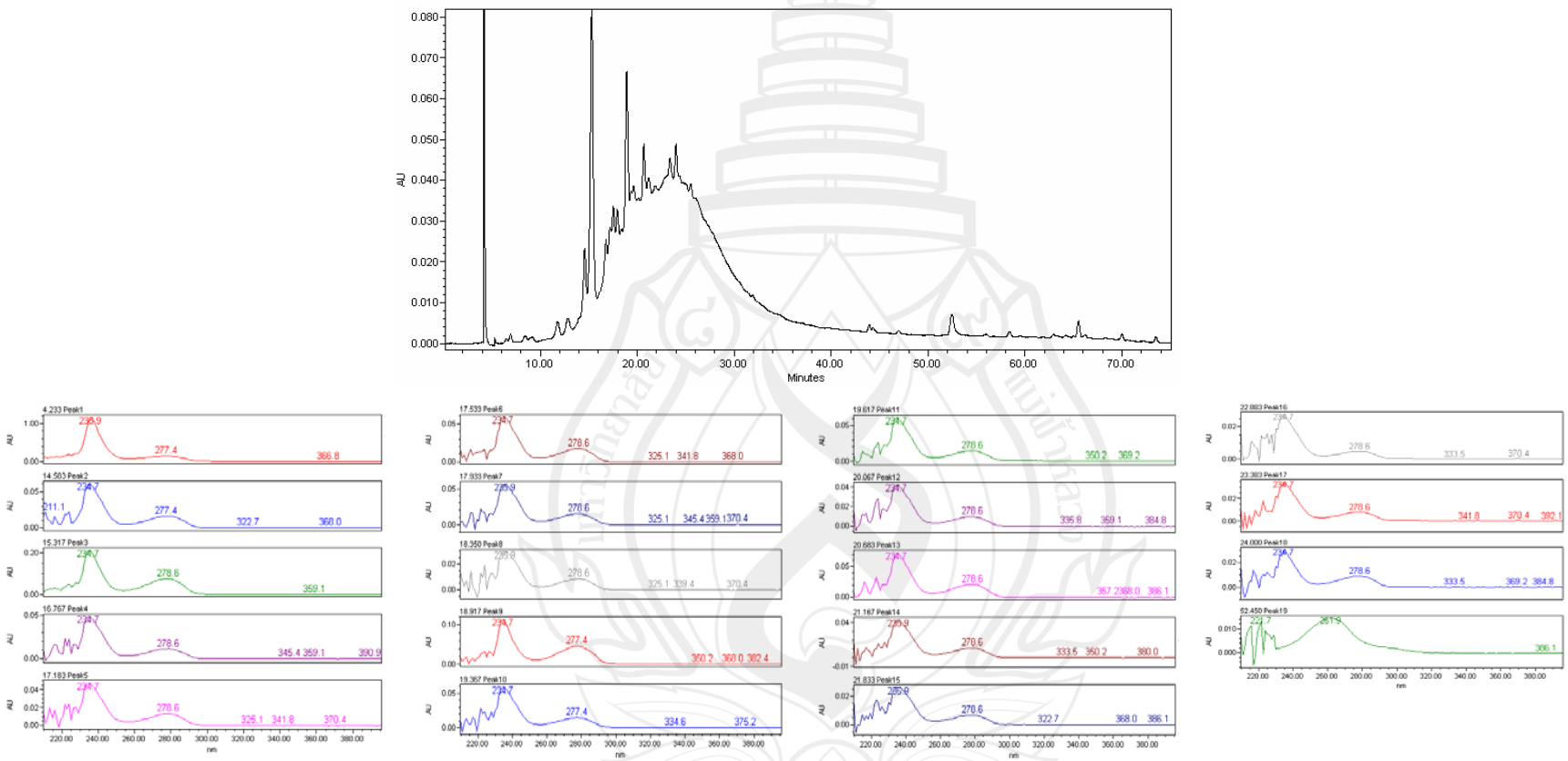
APPENDIX B

Spectrum of betel nut extracts from the different sample

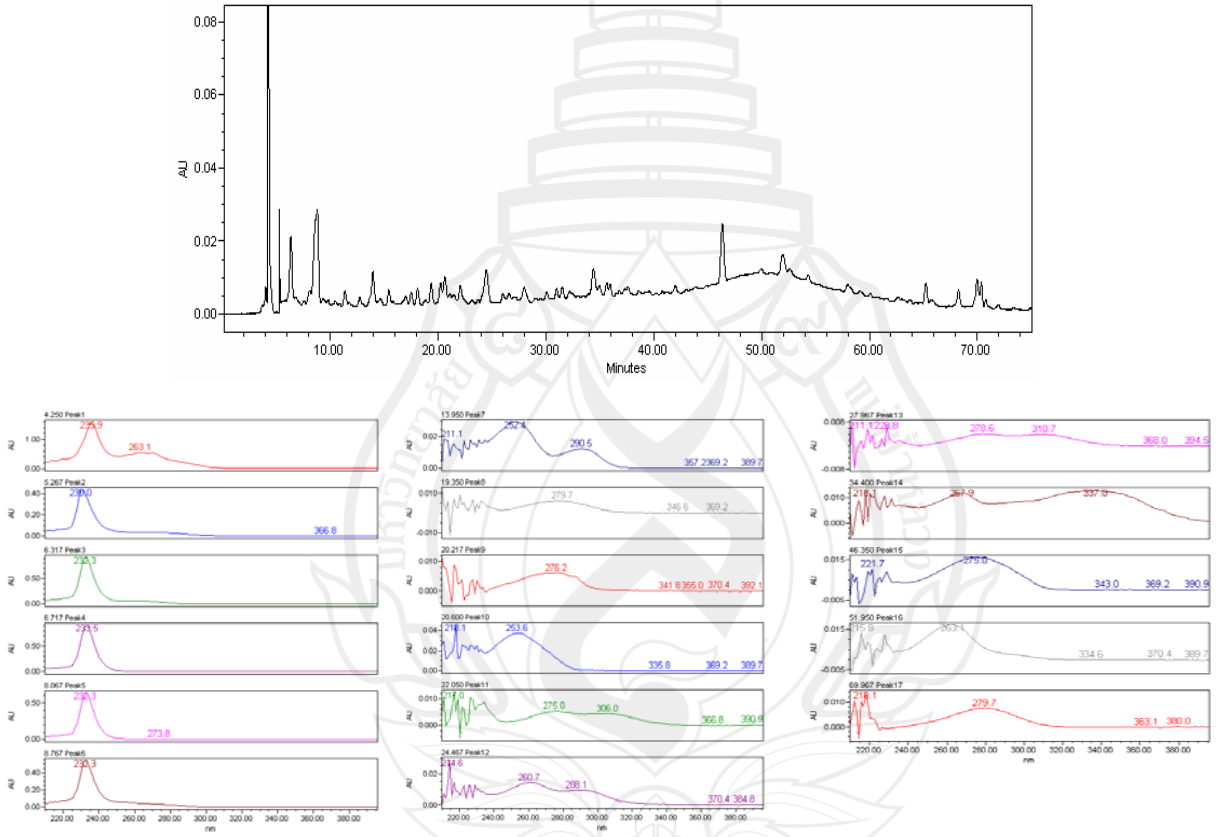
Spectrum of raw betel nut seed extract prepared by microwave-assistance



Spectrum of ripe betel nut seed extract prepared by microwave-assistance



Spectrum of raw betel nut pericarp extract prepared by microwave assistance



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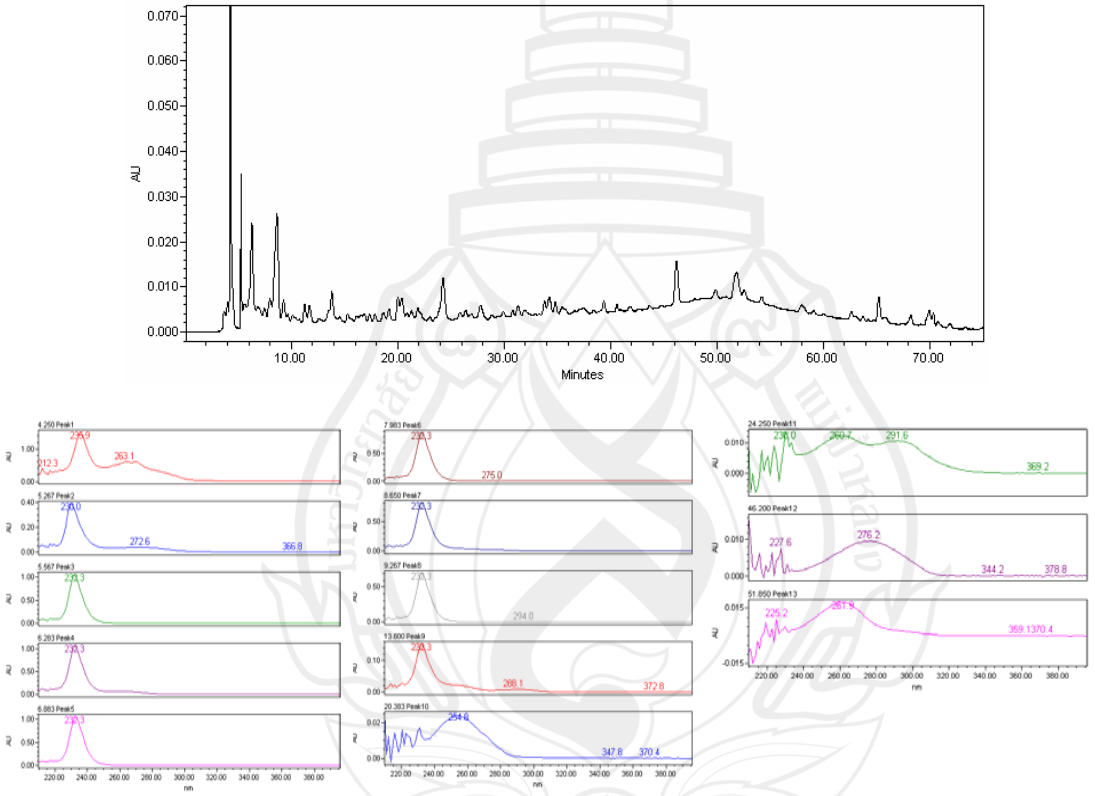
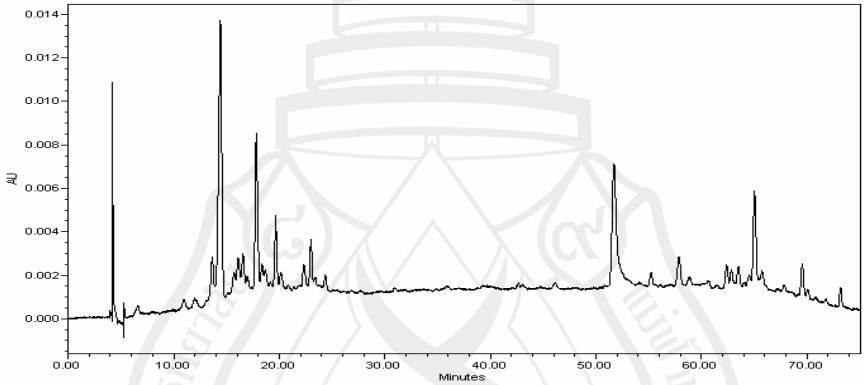
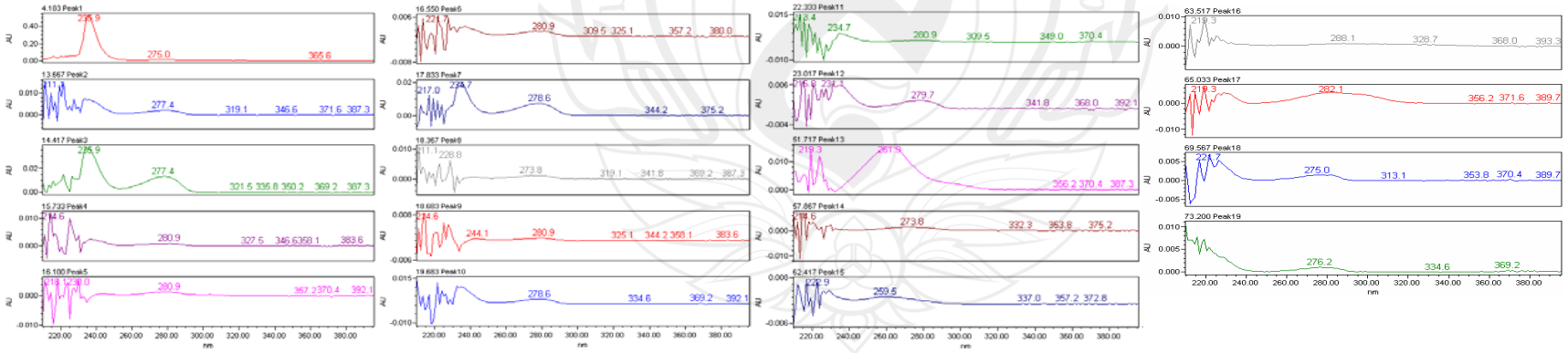


APPENDIX C

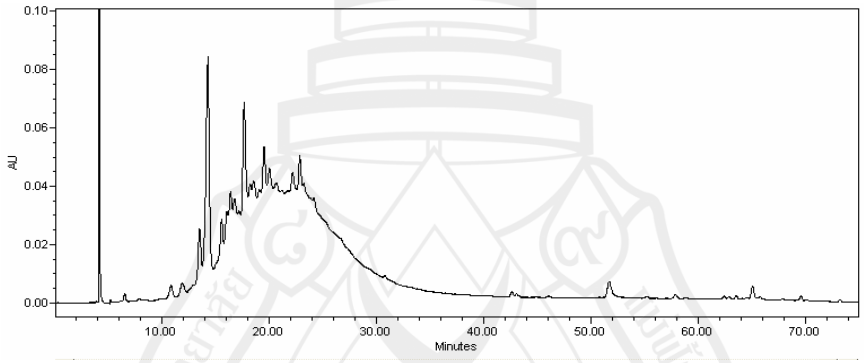
Spectrum of betel nut extracts from the different solvent

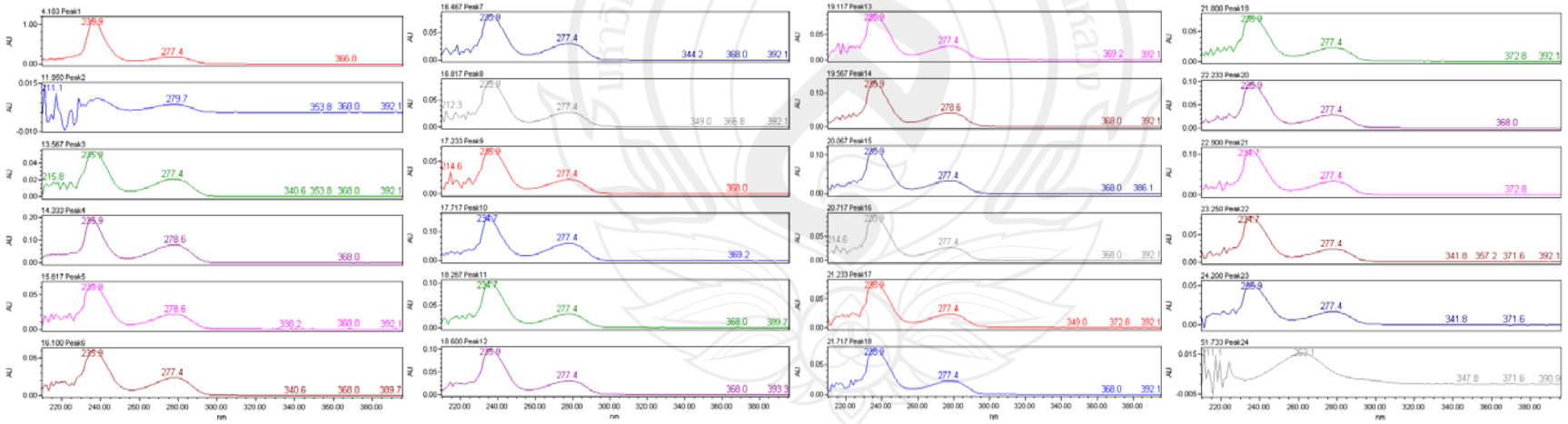
Spectrum of raw betel nut seed ethyl acetate extract



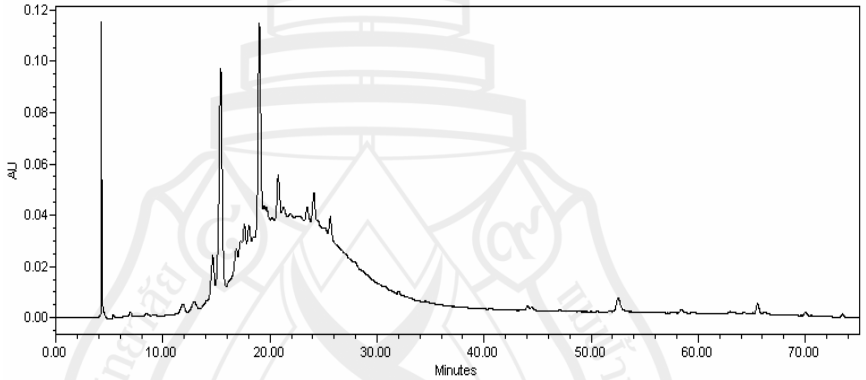


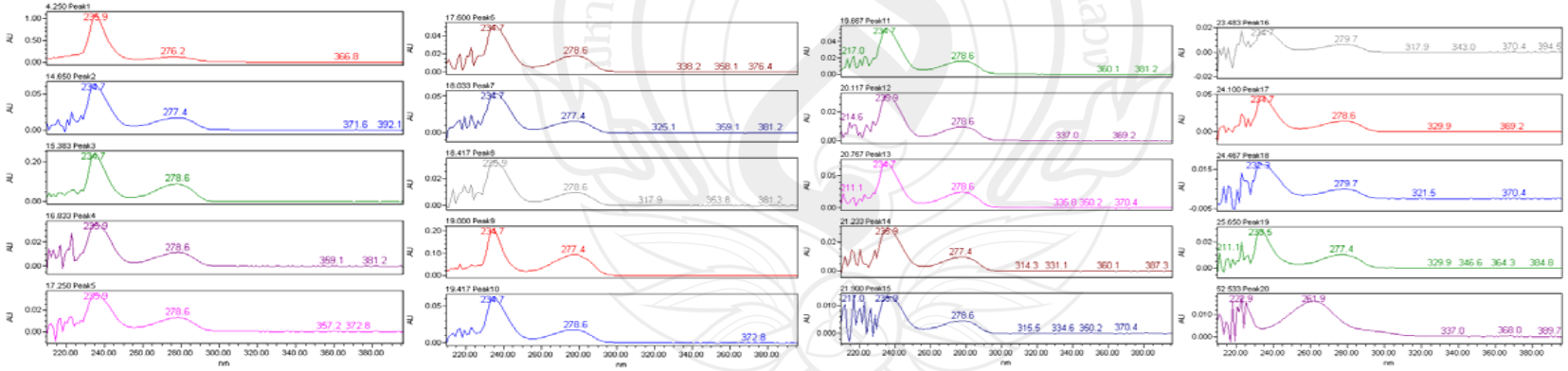
Spectrum of raw betel nut seed 95%ethanol extract



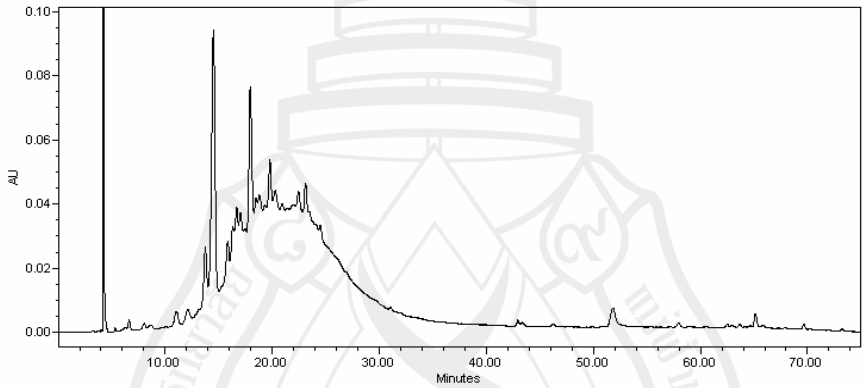


Spectrum of raw betel nut seed 50%ethanol extract

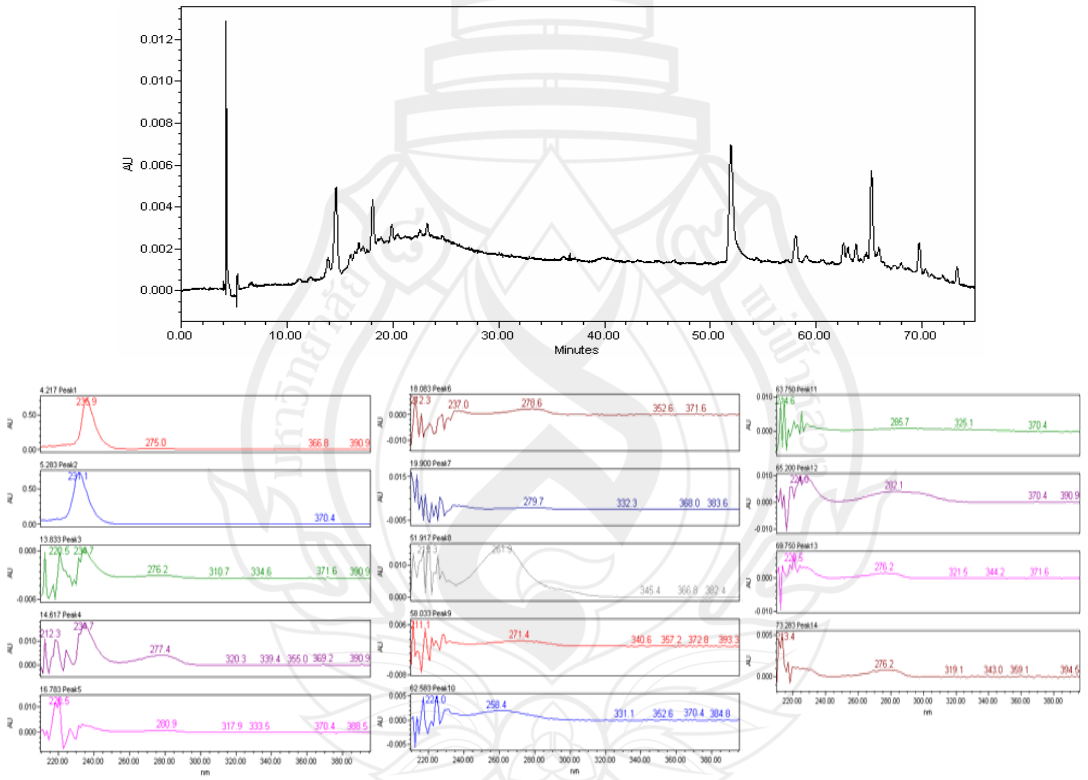




Spectrum of raw betel nut seed water extract



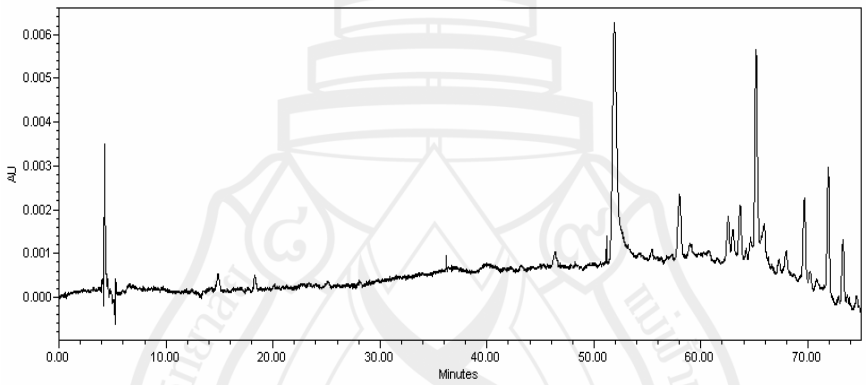
Spectrum of raw betel nut seed propylene glycol extract

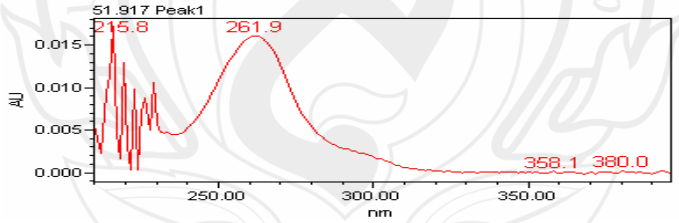


APPENDIX D

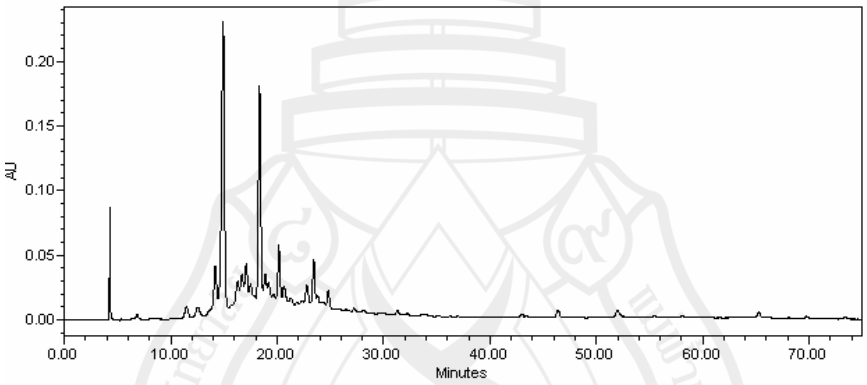
Spectrum of betel nut extracts from the solid-liquid fractionated extraction

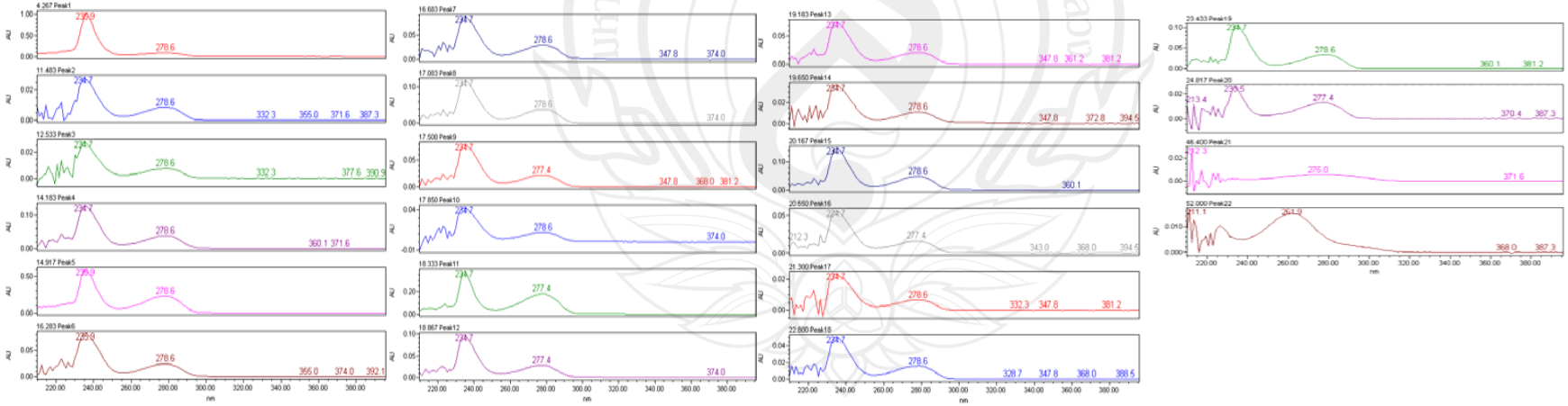
Spectrum of raw betel nut seed hexane fraction



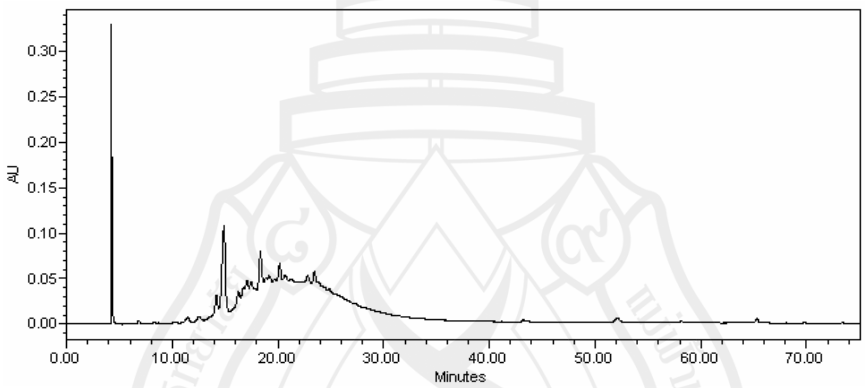


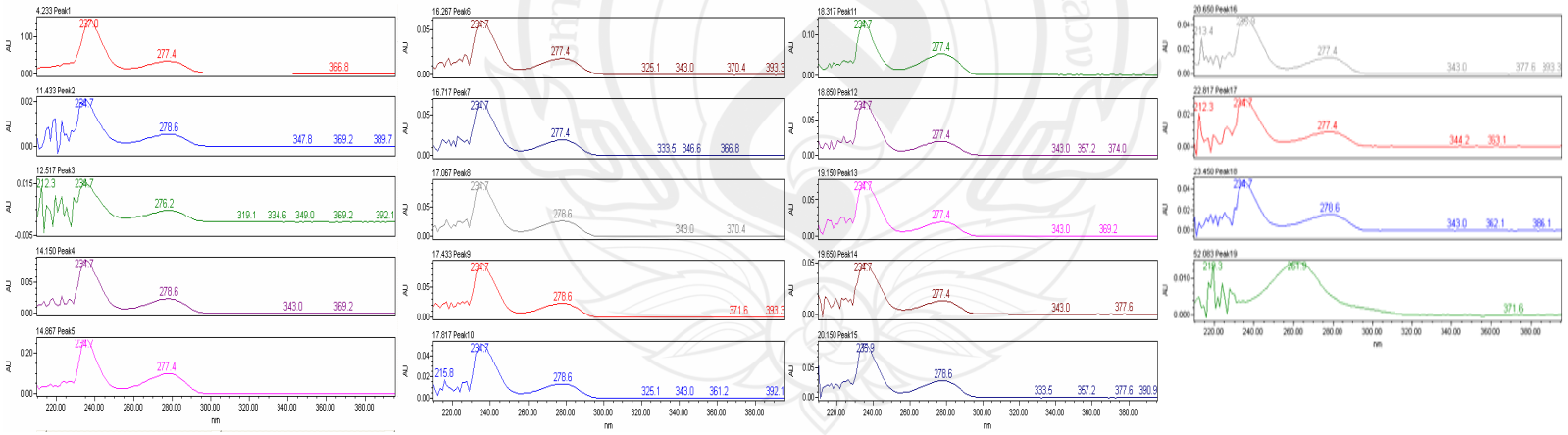
Spectrum of raw betel nut seed ethyl acetate fraction



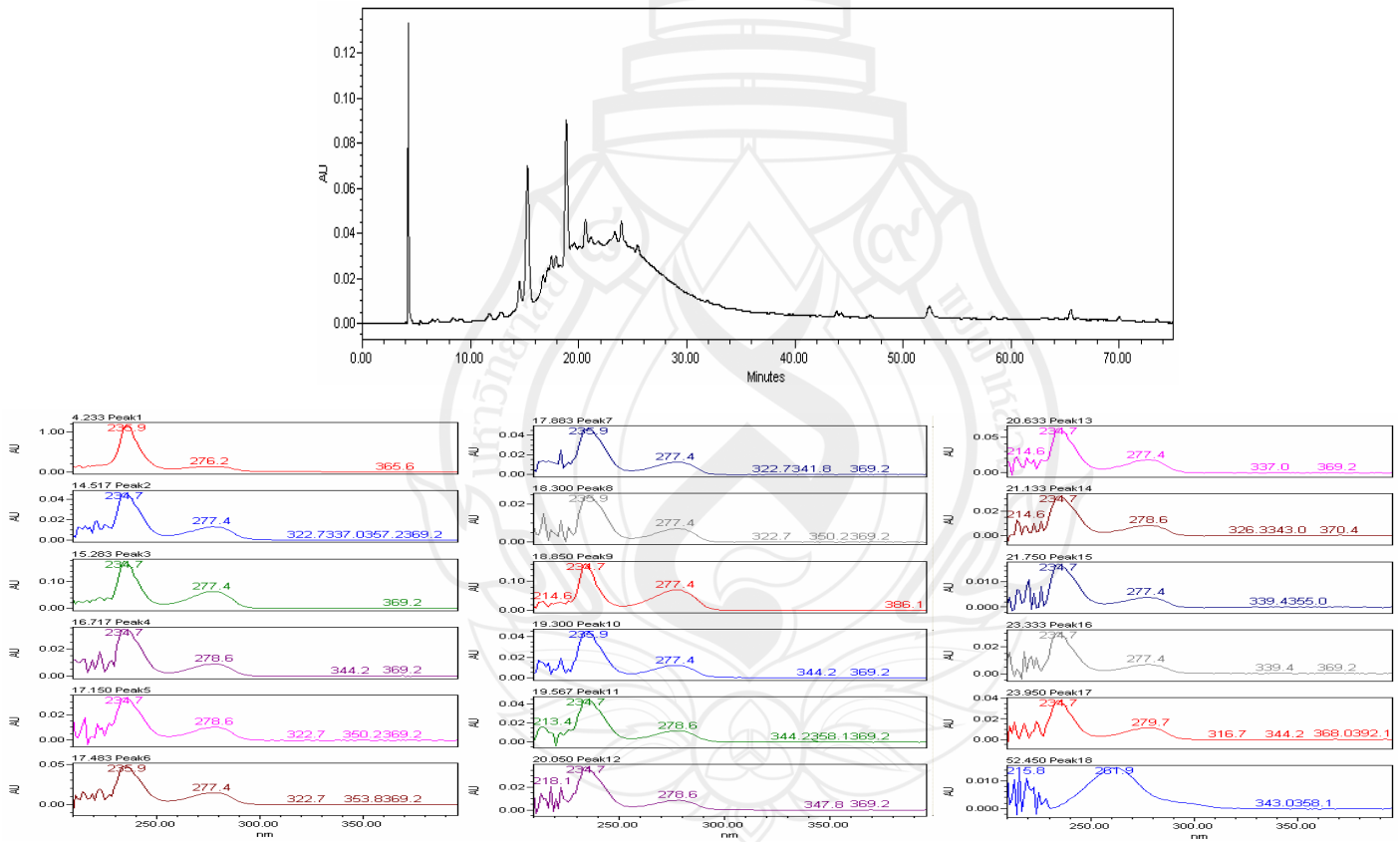


Spectrum of raw betel nut seed acetone fraction

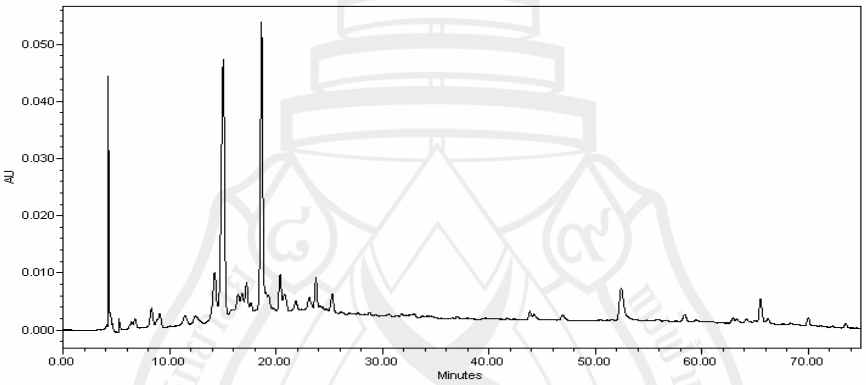


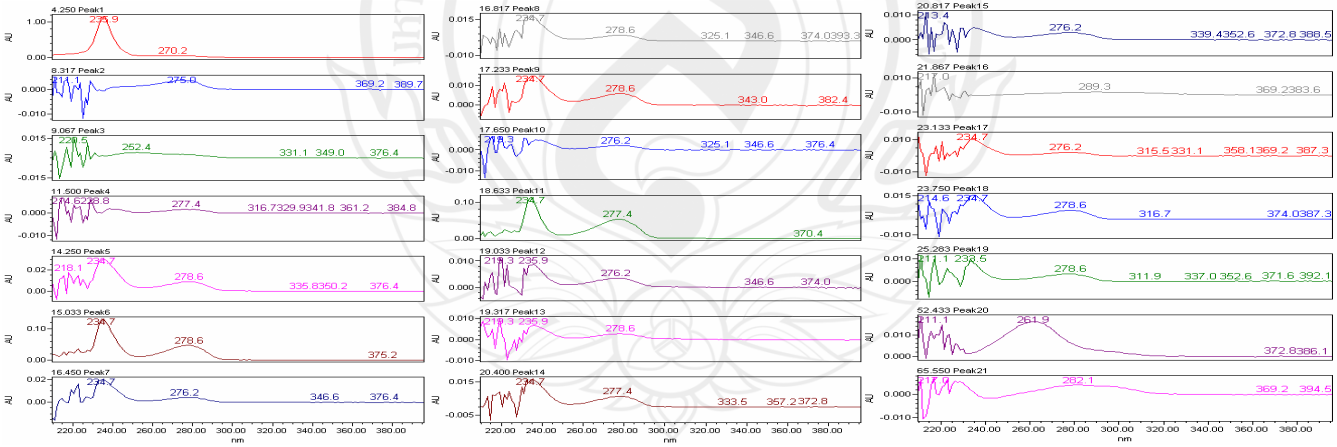


Spectrum of raw betel nut seed 95%ethanol fraction

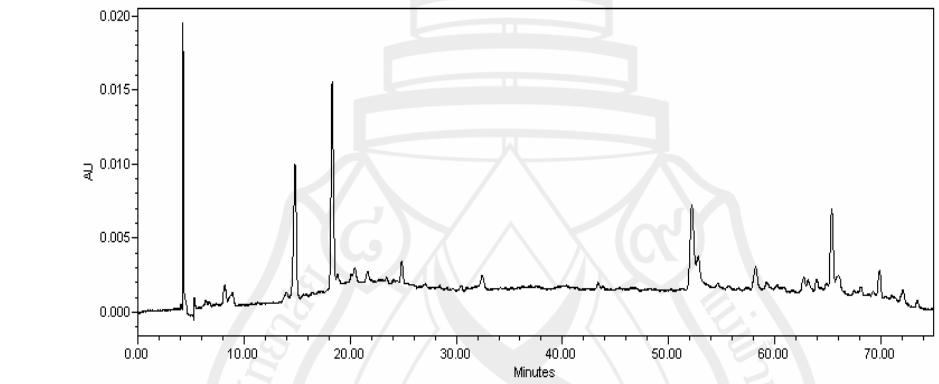


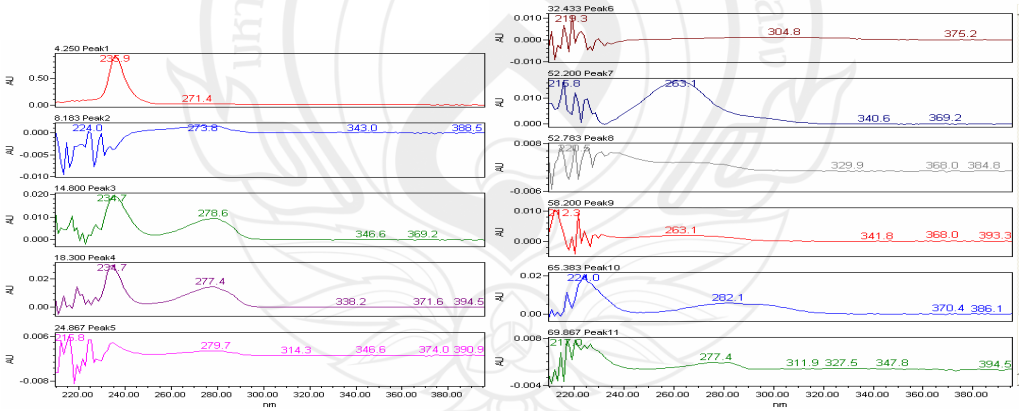
Spectrum of raw betel nut seed 50%ethanol fraction





Spectrum of raw betel nut seed water fraction



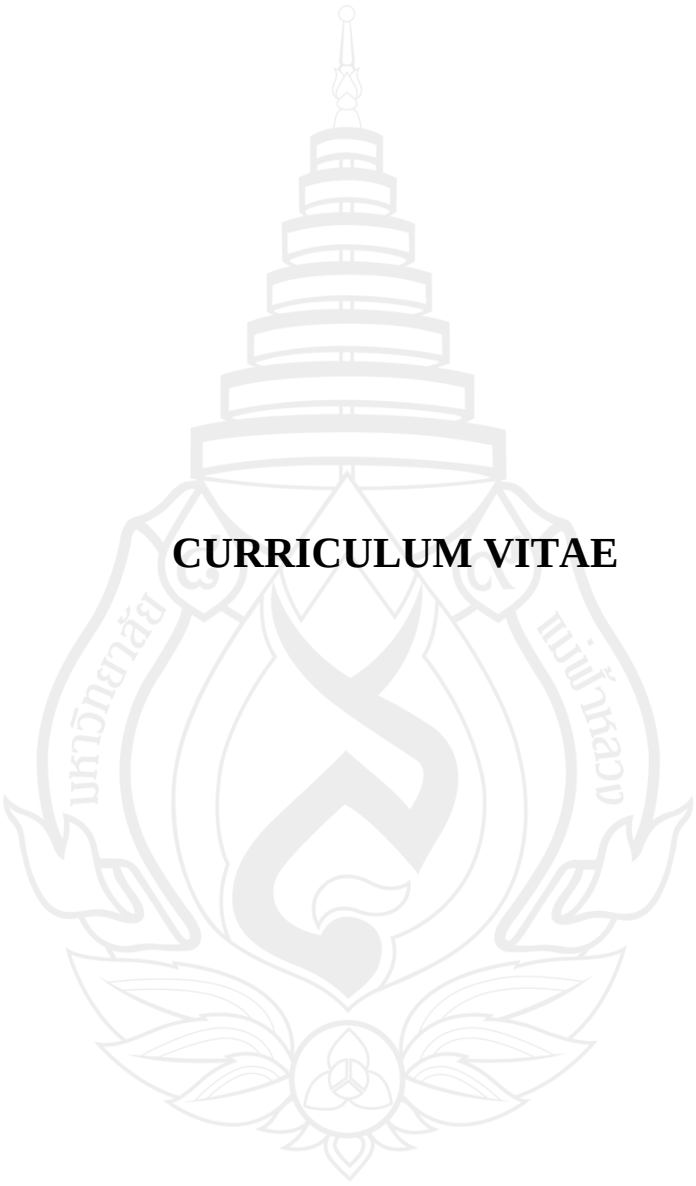


APPENDIX E

Stability of the cosmetic formulation containing raw betel nut seed extract

		Base formula					Formula containing 0.5% (w/w) raw betel nut extract				
		L*	a*	b*	Viscosity (cP)	pH	L*	a*	b*	Viscosity (cP)	pH
Accelerated condition											
Cycle 0	H/C	69.74	-1.82	-0.09	12,800	5.90	68.86	-1.73	-0.25	12,800	5.77
	F/T	68.04	-1.85	0.07	12,800	5.90	67.60	-1.66	-0.08	12,800	5.82
Cycle 3	H/C	56.50	2.33	8.68	12,400	5.77	52.29	3.67	10.74	4,000	4.74
	F/T	56.58	2.03	7.96	13,400	5.82	56.19	2.27	8.18	6,900	4.73
Storage temperature											
Day 0	4 ◦C	64.93	-1.83	0.07	12,800	5.90	61.41	-1.87	0.59	15,000	5.78
	RT	67.74	-1.81	-0.11	12,800	5.90	67.76	-1.85	0.05	15,000	5.78
	50 ◦C	64.27	-1.91	0.05	12,800	5.90	62.35	-2.35	1.17	15,000	5.75
Day 28	4 ◦C	56.83	2.12	8.13	11,172	5.78	52.31	2.24	8.74	12,980	5.54
	RT	54.77	1.99	7.88	11,480	5.78	53.13	3.04	10.43	13,240	5.55
	50 ◦C	59.23	2.22	8.29	10,660	5.75	43.29	9.28	17.43	6,695	5.04

Note: H/C: and heating/cooling, F/T: freeze/thaw, RT: room temperature



CURRICULUM VITAE

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