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.

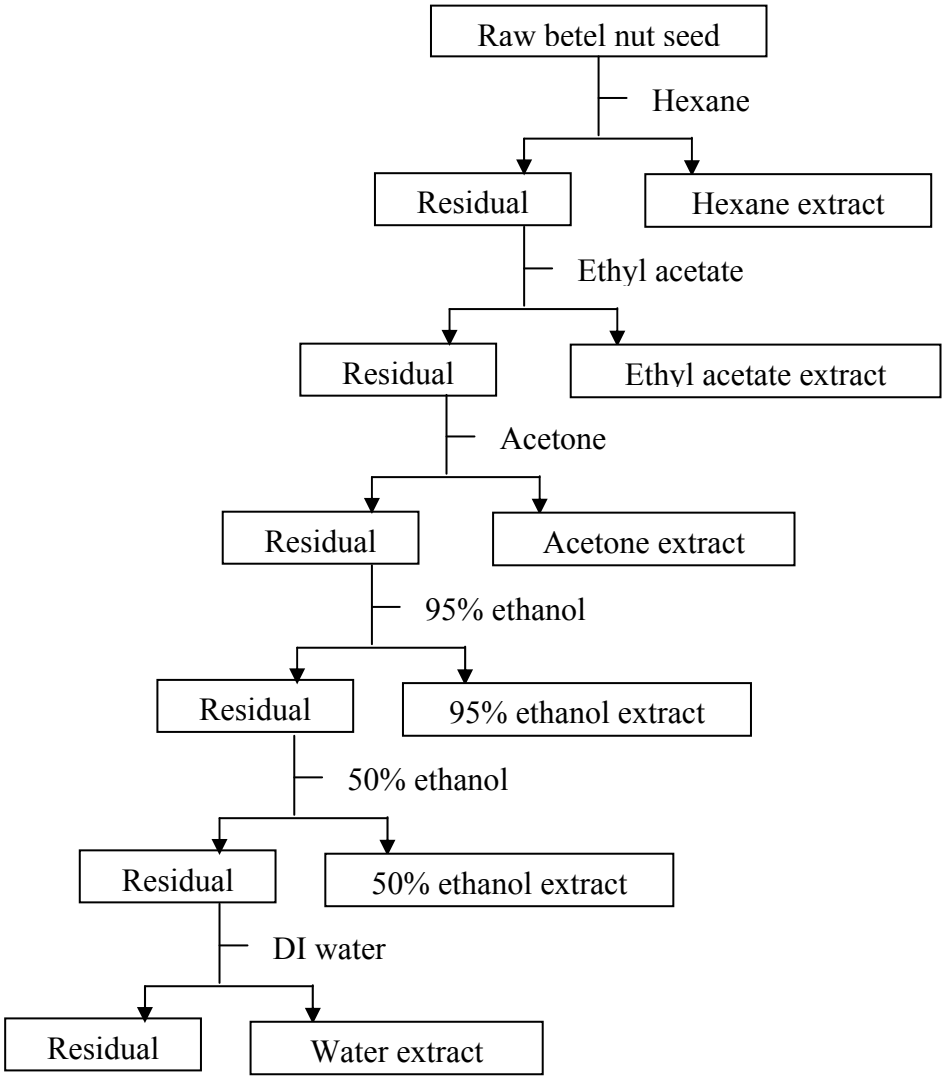

Figure 3.2 Scheme of Solid-liquid Fractionation Extractions

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.