

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).

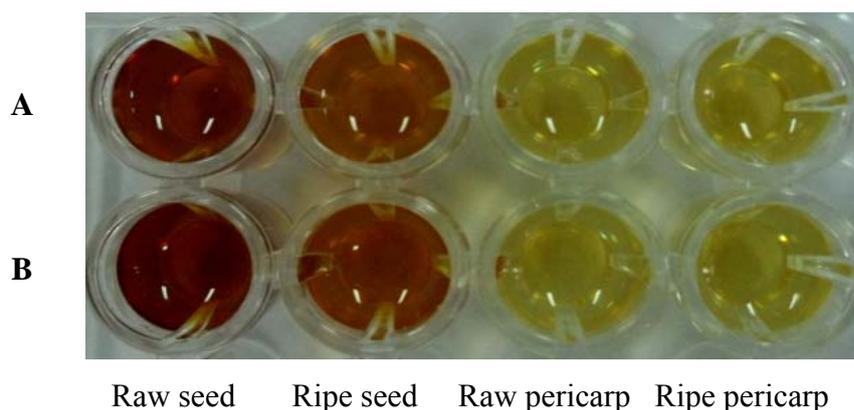


Figure 4.1 Appearance of Betel Nut Crude Extract Prepared by Shaking (row A) and Microwave-Assisted Extraction Methods (row B)

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) *
<i>Shaking</i>			
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
<i>Microwave assistance</i>			
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 IC₅₀ values of the raw betel nut seed extracts. The most potential scavenging activity was possessed by the raw seed extract with the lowest IC₅₀ 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 IC₅₀ 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 IC₅₀ 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 IC₅₀ 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 IC₅₀ 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 Fe³⁺-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 IC₅₀ 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 IC₅₀ 0.19 and 0.18 mg/mL for ShE and MAE, respectively while the raw pericarp extract showed the highest IC₅₀ 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 IC₅₀ values.

Table 4.3 Cosmetic Bioactivities in Term of IC₅₀ 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)
<i>Shaking</i>					
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
<i>Microwave assistance</i>					
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
<i>Standard</i>					
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)
<i>Shaking</i>					
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
<i>Microwave assistance</i>					
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 $\mu\text{g/mL}$ for ShE and MAE, respectively. The lower activity was found in the ripe seed extract of 11.30 and 9.33 $\mu\text{g/mL}$ 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) $\mu\text{g/mL}$. The lowest tyrosinase inhibitory activity was found in the raw pericarp extract which the IC_{50} of 233.94 and 199.63 $\mu\text{g/mL}$ 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 than 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) $\mu\text{g/mL}$. The lower activity was governed by the ripe seed extracts of 140.70 and 146.17 $\mu\text{g/mL}$ for ShE and MAE, respectively. Both raw and ripe seed extract possessed the higher anti-elastase potential than the epigallocatechin gallate (EGCG) (147.07 $\mu\text{g/mL}$). 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) $\mu\text{g/mL}$, whereas the ripe pericarp extracts possessed the lowest activity of 1,627.85 and 2,006.40 $\mu\text{g/mL}$ 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 $\mu\text{g/mL}$, respectively). The moderate activity was found in 25 $\mu\text{g/mL}$ 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.

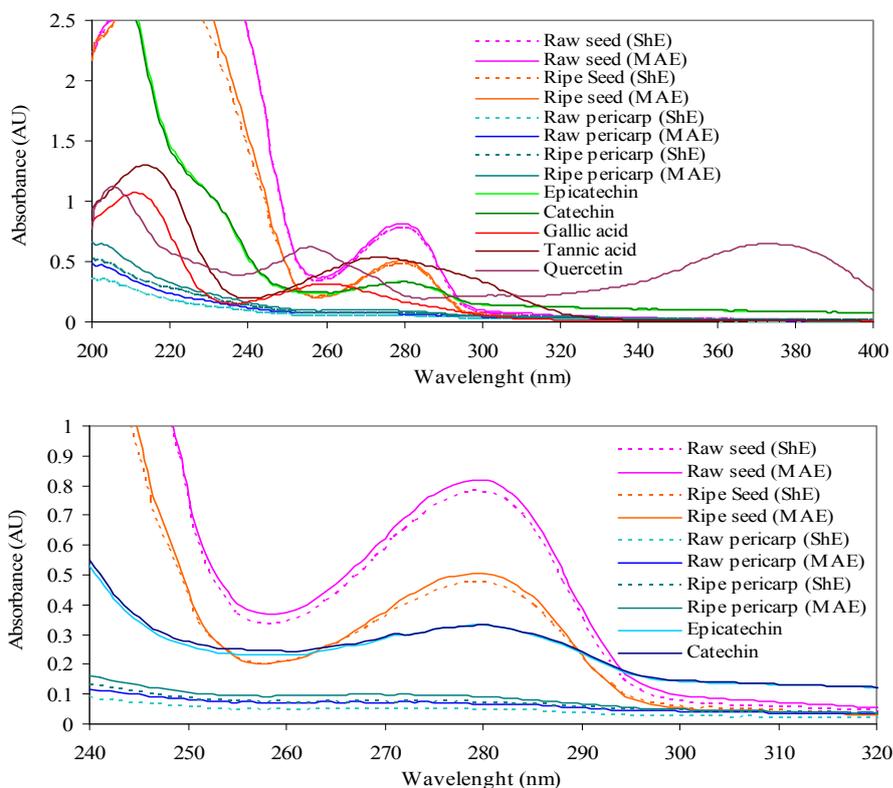
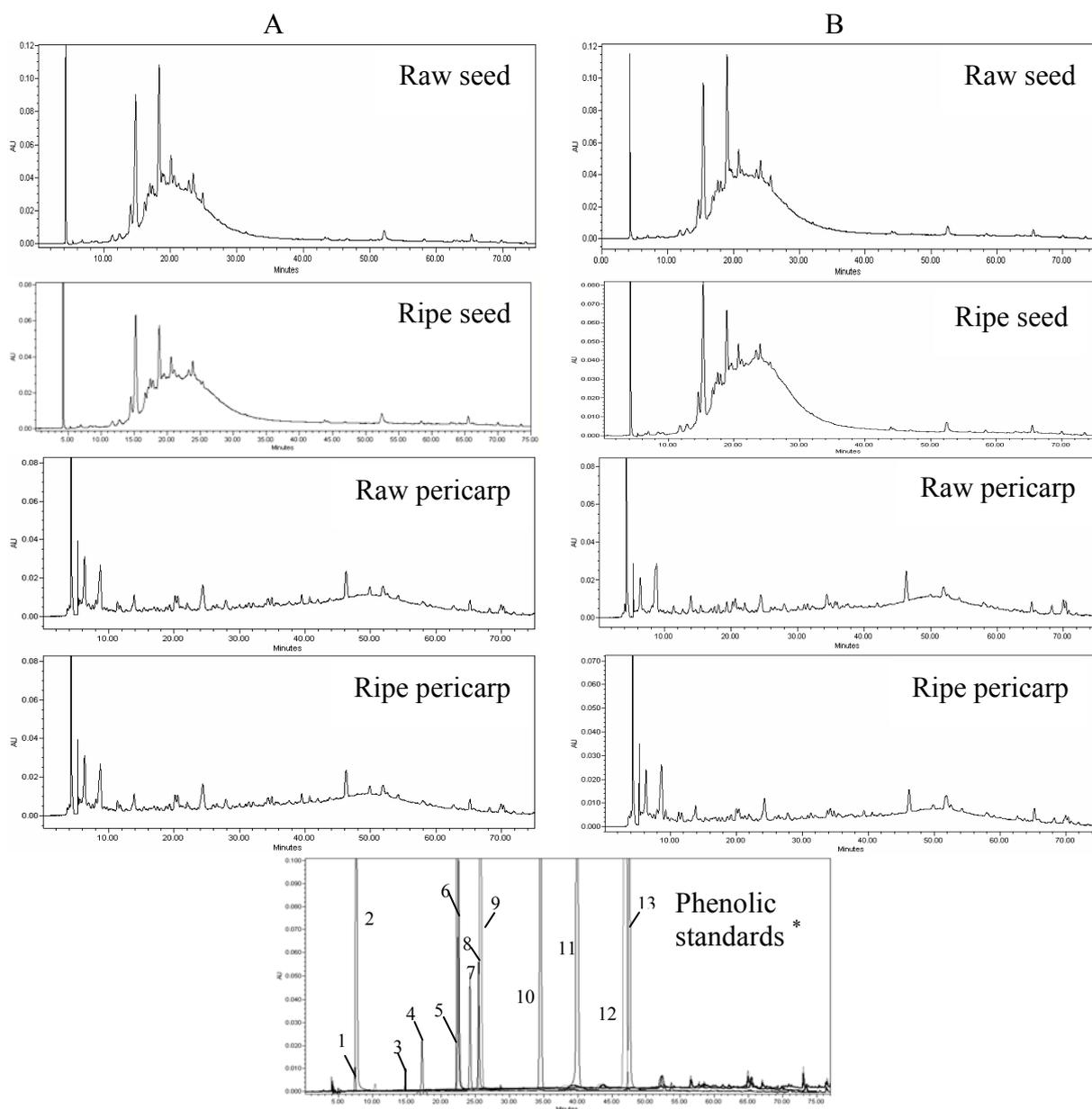


Figure 4.2 UV-scanning of Betel Nut Extracts

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) galocatechin, (4) epigallocatechin, (5) epigallocatechingallate, (6) chlorogenic acid, (7) epicatechin, (8) galocatechingallate, (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*
<i>E. coli</i>	15.00±0.00	-	-	-	-	-
<i>S. aureus</i>	15.00±0.00	-	13.67±1.15	-	12.00±1.00	-
<i>S. epidermidis</i>	19.67±0.58	-	11.00±0.00	-	11.67±0.58	-
<i>Ps. aeruginosa</i>	15.00±0.00	-	-	-	-	-
<i>S. typhimurium</i>	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 IC₅₀ 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 ($\mu\text{g/mL}$)	% Survival*	SD	IC ₅₀ ($\mu\text{g/mL}$)
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 \leq 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 ($\mu\text{g/mL}$)	% Survival*	SD	IC ₅₀ ($\mu\text{g/mL}$)
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 \leq 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 well 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-assisted 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 $\mu\text{g/mL}$, 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 (IC₅₀) from Extraction between Shaking (ShE) and Microwave-Assisted (MAE) Methods

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

Note: * Values were presented in mean±SD

MD: mean difference of the results between shaking and microwave-assisted 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-assisted 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 $\mu\text{g/mL}$ 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 ultrasonic 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.

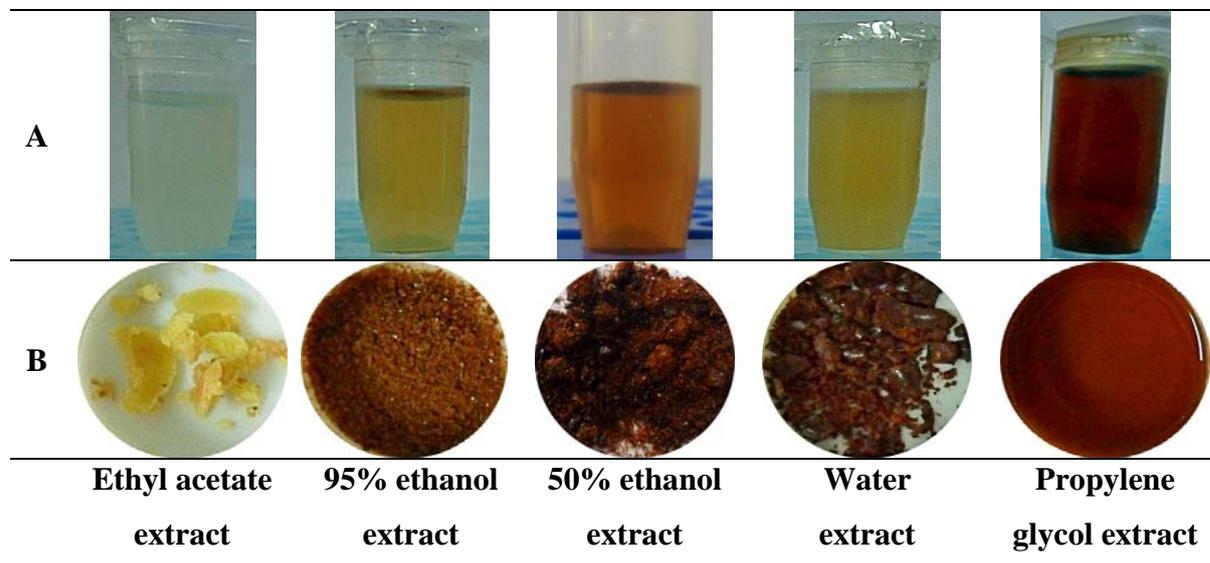


Figure 4.4 Appearance of Betel Nut Seed Extract, Crude Extract (row A) and Dried Crude Extract (B)

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 ($60,750 \times 10^{-4}$) than water ($122,931 \times 10^{-4}$). 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 (*Capsicum 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 IC₅₀ 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 IC₅₀ 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 IC₅₀ Values of Betel Nut Seed Extracts

Extract*	DPPH ($\mu\text{g/mL}\pm\text{SD}$)	ABTS ($\mu\text{g/mL}\pm\text{SD}$)	Anti-LOOH ($\text{mg/mL}\pm\text{SD}$)	Anti-Tyr ($\mu\text{g/mL}\pm\text{SD}$)	Anti-Ela ($\mu\text{g/mL}\pm\text{SD}$)
EtOAc	115.14 \pm 1.02 ^{c**}	43.34 \pm 0.51 ^c	5.83 \pm 0.12 ^d	41.49 \pm 0.15 ^c	1,154.42 \pm 21.11 ^d
95%EtOH	30.57 \pm 0.12 ^b	1.43 \pm 0.13 ^a	0.16 \pm 0.01 ^b	9.64 \pm 0.04 ^a	57.63 \pm 0.10 ^a
50%EtOH	19.17 \pm 0.09 ^a	1.06 \pm 0.00 ^a	0.18 \pm 0.01 ^a	6.24 \pm 0.07 ^a	111.50 \pm 0.44 ^a
Water	31.69 \pm 0.11 ^b	1.30 \pm 0.06 ^a	0.34 \pm 0.01 ^b	10.79 \pm 0.03 ^a	120.70 \pm 0.23 ^b
PG	557.22 \pm 8.24 ^d	301.46 \pm 4.30 ^d	2.59 \pm 0.03 ^c	159.36 \pm 0.47 ^d	969.67 \pm 10.47 ^c
Standard					
Trolox	4.11 \pm 0.03 ^a	4.34 \pm 0.03 ^b	-	-	-
BHT	-	-	4.19 \pm 0.04 ^a	-	-
Kojic acid	-	-	-	15.65 \pm 0.05 ^b	-
EGCG	-	-	-	-	47.07 \pm 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 IC₅₀ value (1.30 $\mu\text{g/mL}$). However, the ABTS cation radical scavenging capacity of ethanol and water extract (1.43 $\mu\text{g/mL}$) 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 $\mu\text{g/mL}$). 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 ethanolic 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 IC₅₀ 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 glycol with water on myrobalan fruit extraction were reported (Chulasiri, M., Wanasawas, P., sriam, D., Nakamat, S., Wongkrajang, Y., Kongsaktragoon, 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 $\mu\text{g}/\text{mL}$. However, the IC_{50} of ethanol extract was not statistical significantly different with water extract (10.79 $\mu\text{g}/\text{mL}$). The ethanol and water extract also found having more potential that the kojic acid standard (15.65 $\mu\text{g}/\text{mL}$). The lower activity was found in ethyl acetate extract of 41.49 $\mu\text{g}/\text{mL}$, and the lowest was found in propylene glycol extract (159.36 $\mu\text{g}/\text{mL}$). 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 $\mu\text{g}/\text{mL}$). The activity of ethanol extract was not statistical significantly lower than epigallocatechin gallate (EGCG) of 47.07 $\mu\text{g}/\text{mL}$. The significantly lower anti-elastase activity was found in water extract (120.70 $\mu\text{g}/\text{mL}$) and the lowest was of ethyl acetate extract (1,154.42 $\mu\text{g}/\text{mL}$). 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.

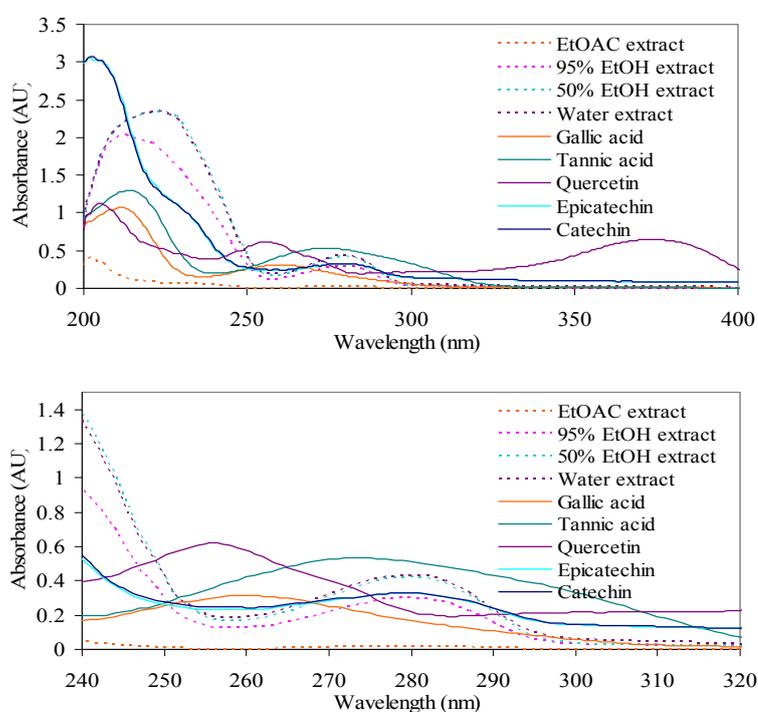
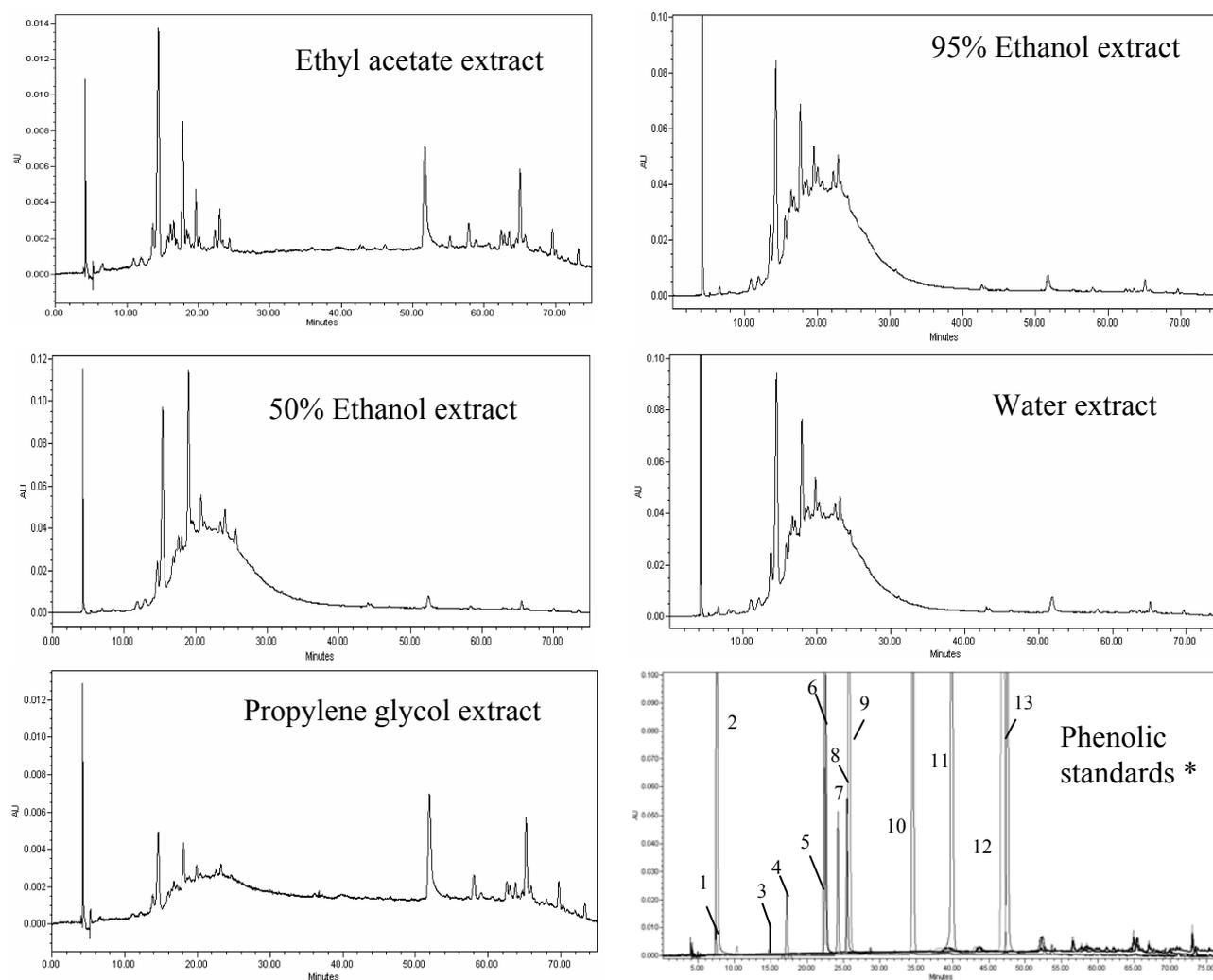


Figure 4.5 UV-Scanning of Betel Nut Extracts

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) galocatechin, (4) epigallocatechin, (5) epigallocatechingallate, (6) chlorogenic acid, (7) epicatechin, (8) galocatechingallate, (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*
<i>E. coli</i>	15.00±0.00	-**	-	-	-	-
<i>S. aureus</i>	15.00±0.00	-	-	12.00±0.00	13.67±1.15	13.67±0.58
<i>S. epidermidis</i>	18.33±0.58	-	-	10.67±0.58	11.00±0.00	12.67±0.58
<i>Ps. aeruginosa</i>	15.00±0.00	-	-	-	-	-
<i>S. typhimurium</i>	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 IC₅₀ 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)
<i>Human dermal fibroblast</i>			
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	
<i>B16-F10 melanoma cell line</i>			
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 ^c	0.95±0.07 ^d	0.09±0.01 ^c	0.03±0.01 ^c	0.02±0.00 ^c
Ethyl acetate	1.60±0.20 ^c	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 IC₅₀ 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.

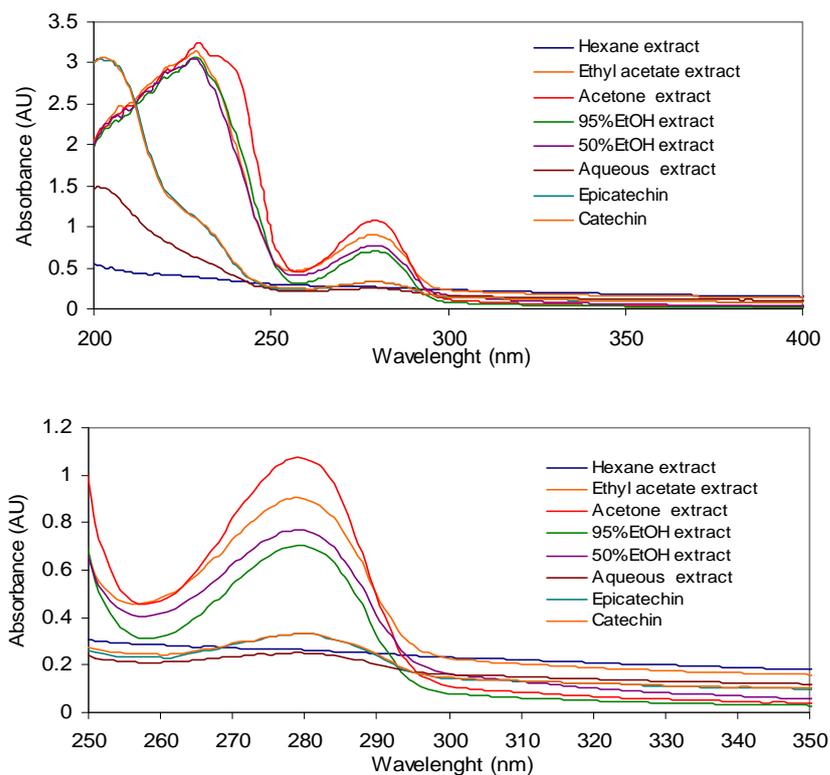


Figure 4.7 UV-Scanning of Betel Nut Seed Extracts and Phenolic Standards; 200-400 and 250-350 nm

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.

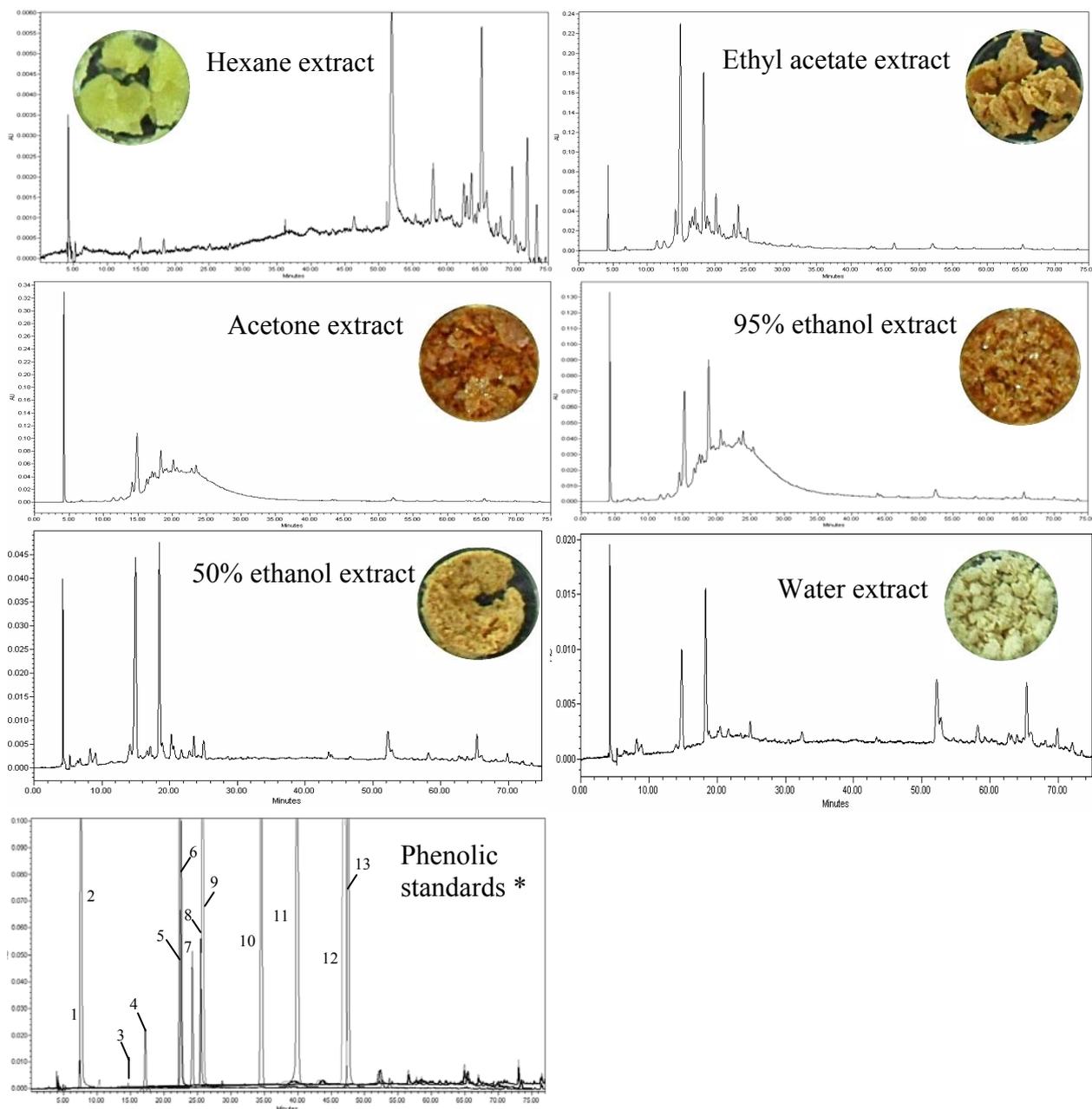
The obtained profiles in Figure 4.8 displayed the manner of the valuable compounds in the sample. The hexane extract mostly comprised of low polar compound which were eluted after 50 min. The interested peaks of all extracts, except for hexane extract, were similar in the retention time range of 12- 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 epicatechin 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) galocatechin, (4) catechin, (5) epigallocatechingallate, (6) chlorogenic acid, (7) epicatechin, (8) galocatechingallate, (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 leucocyanidins 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. The 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.

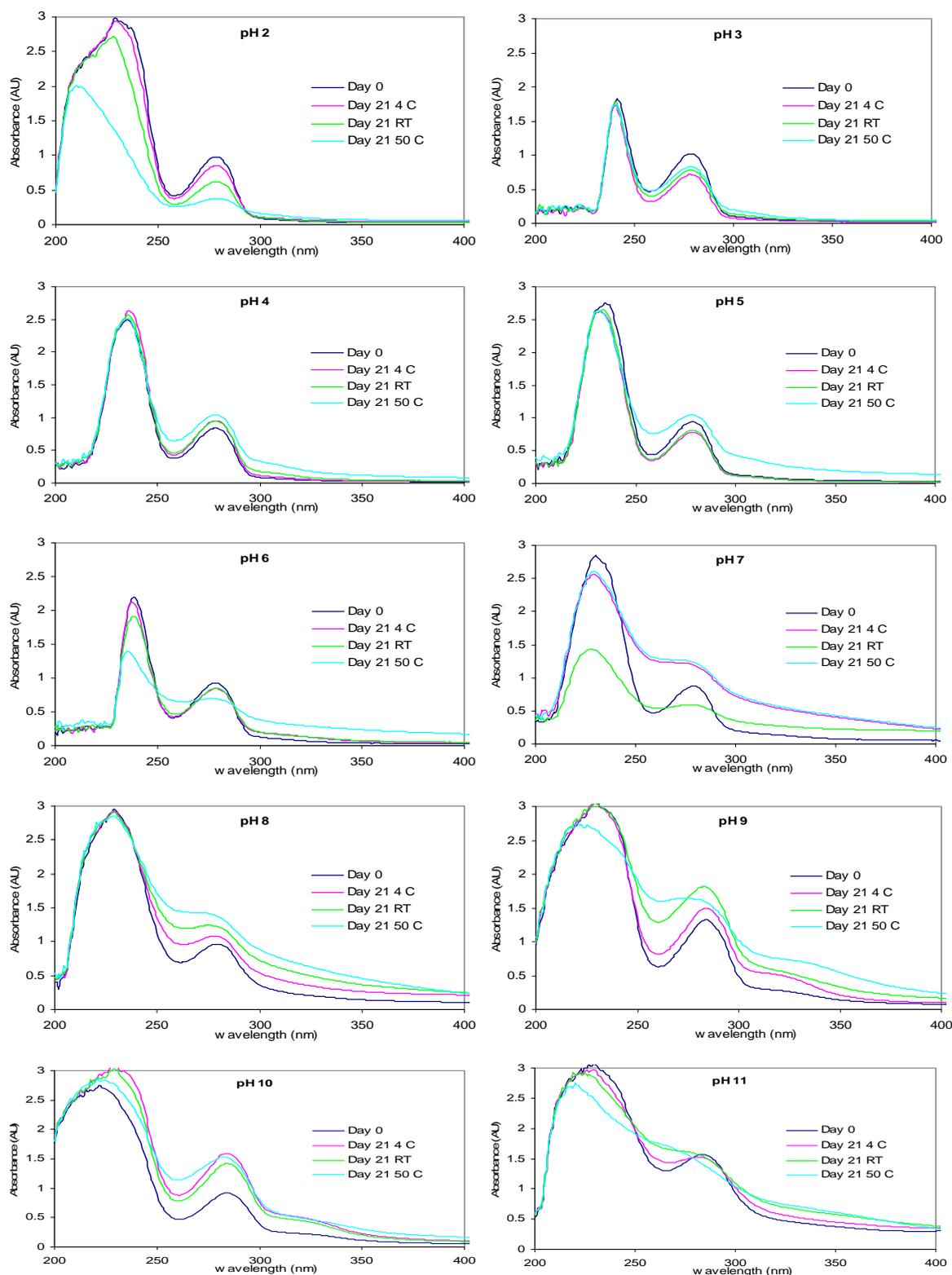
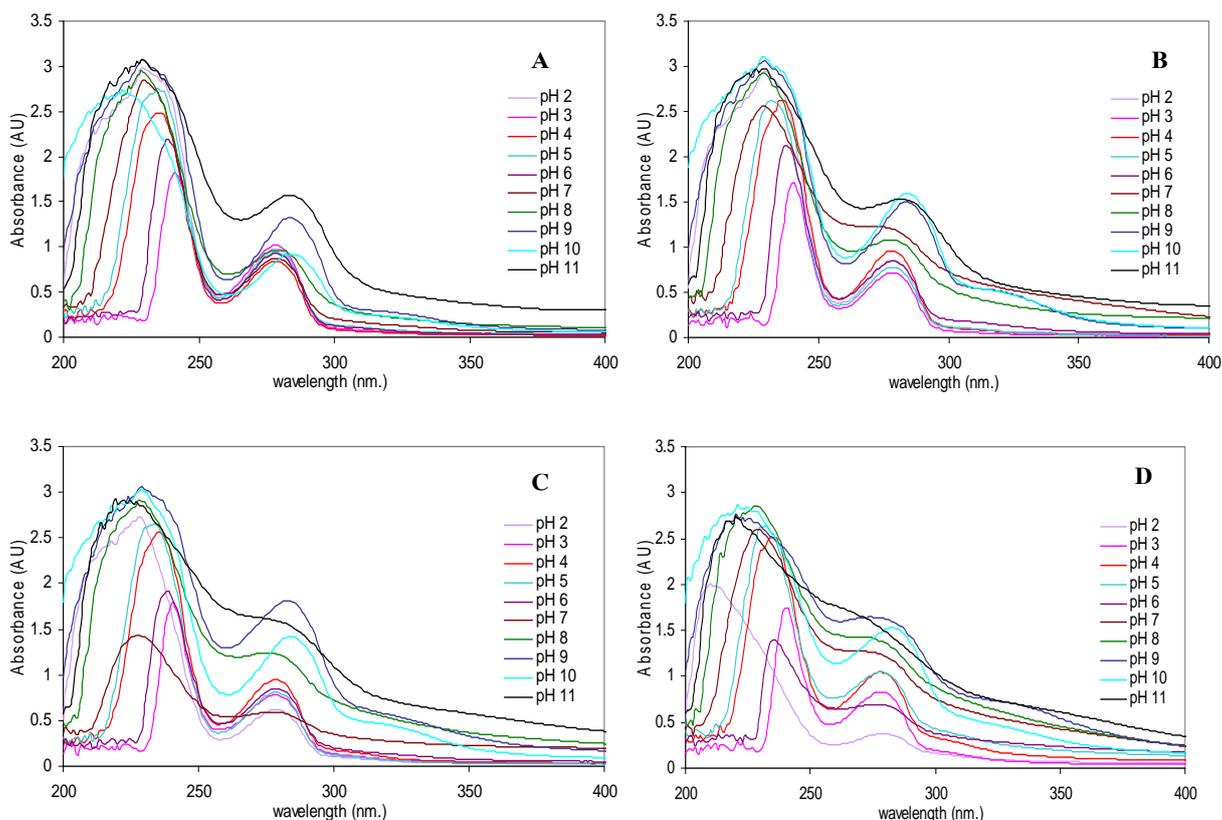


Figure 4.9 Absorption Stability of Raw Betel Nut Seed Extract Solution for 21 Days



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 consisted 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 weak 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 hydroxycinnamic, 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

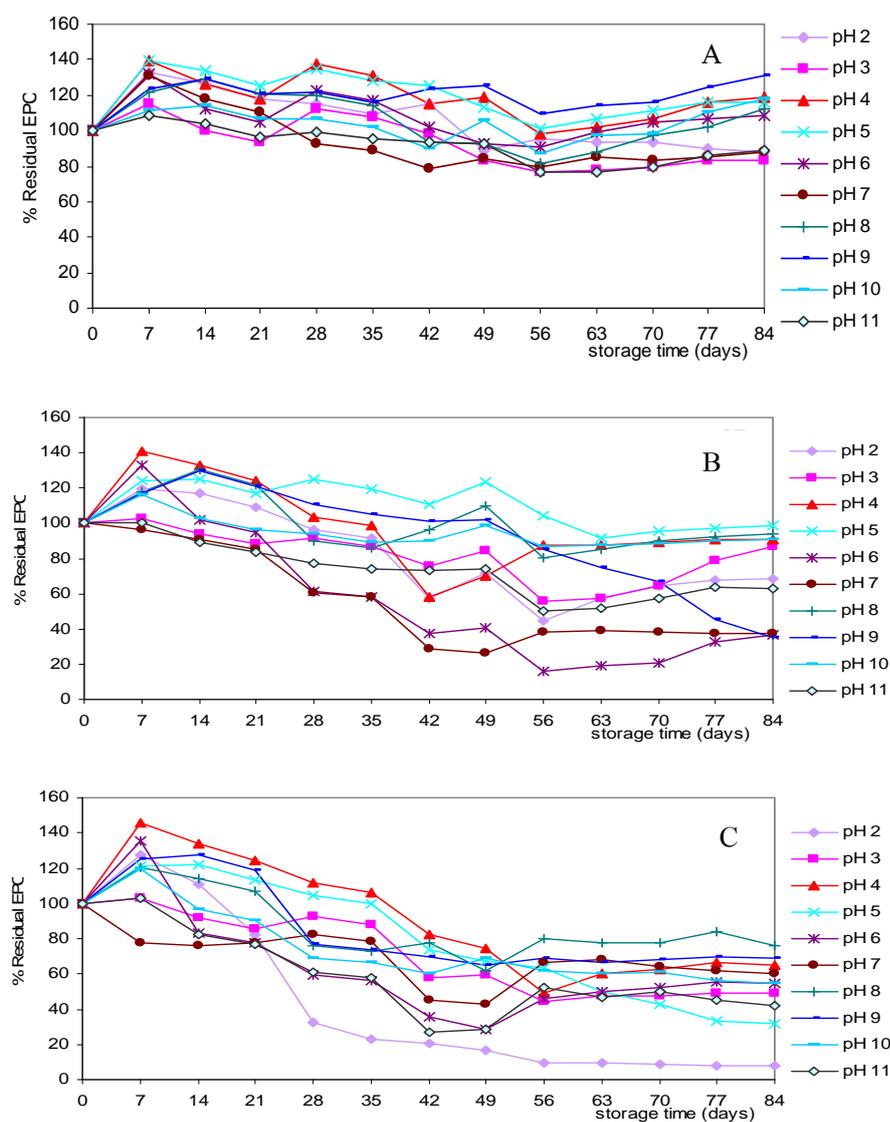
The EPC in the extract was weekly evaluated and calculated into gallic acid equivalent then expressed as percentage of residual EPC (Figure 4.11). At the first 7 days storage, increment of the EPC in every condition was remarked. The raising in EPC in the beginning of storage was explained by the releasing 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

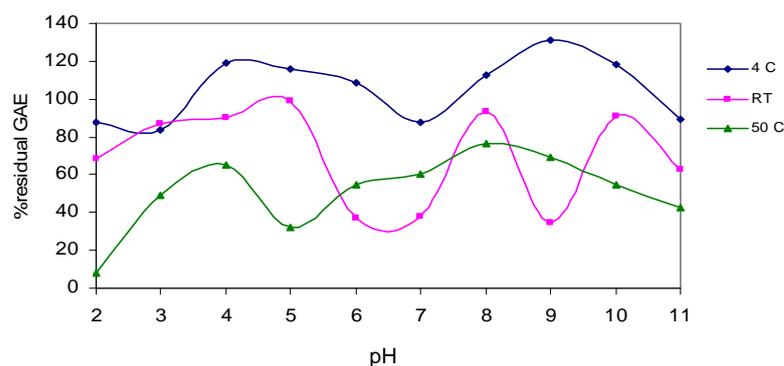


Figure 4.12 Percentage of Residual Extractable Phenolics Content of Raw Betel Seed Nut Extract Solutions after 84 Days Storage

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.

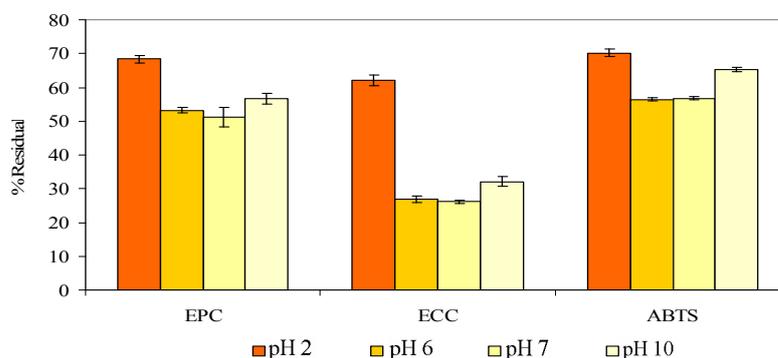


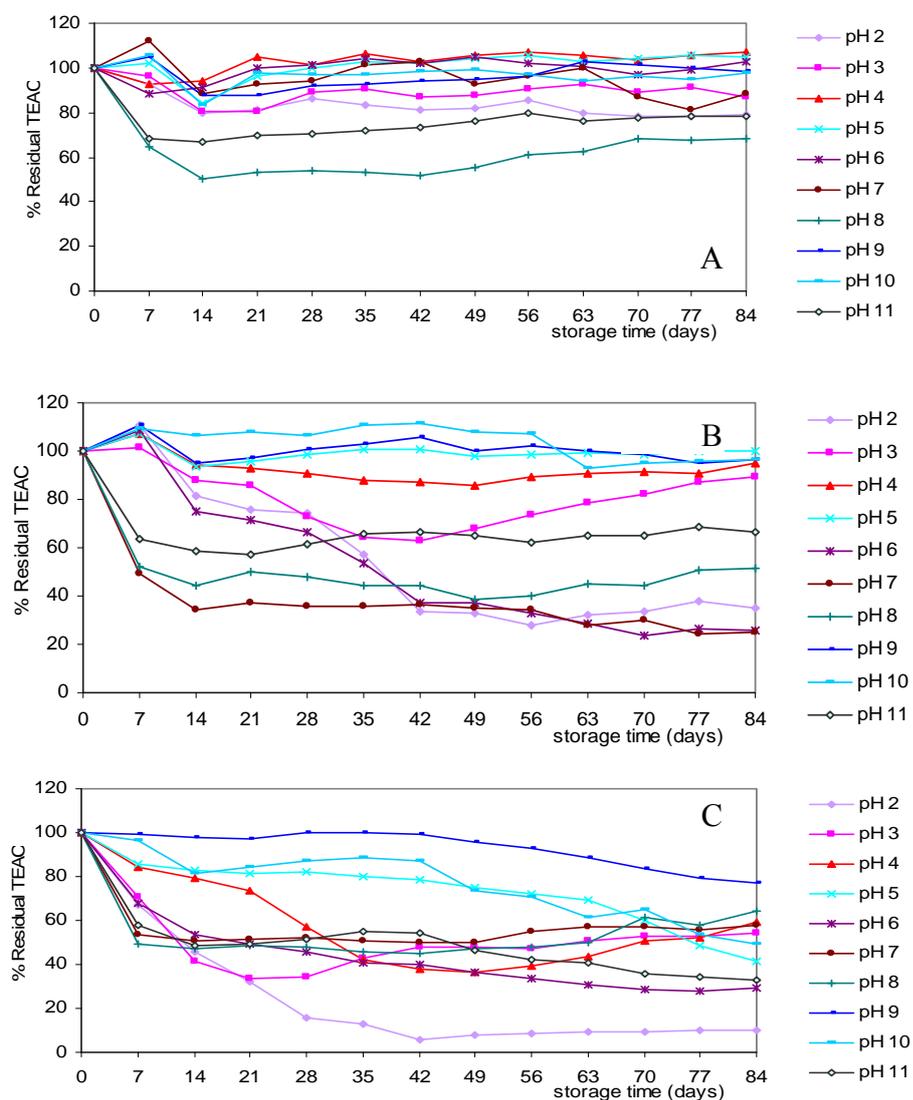
Figure 4.13 Residual Extractable Phenolic Content (EPC), Extractable Catechin Content (ECC), and ABTS Radical Scavenging Activity (ABTS) of the Betel Nut Seed Extract after Heating/Cooling Cycle

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

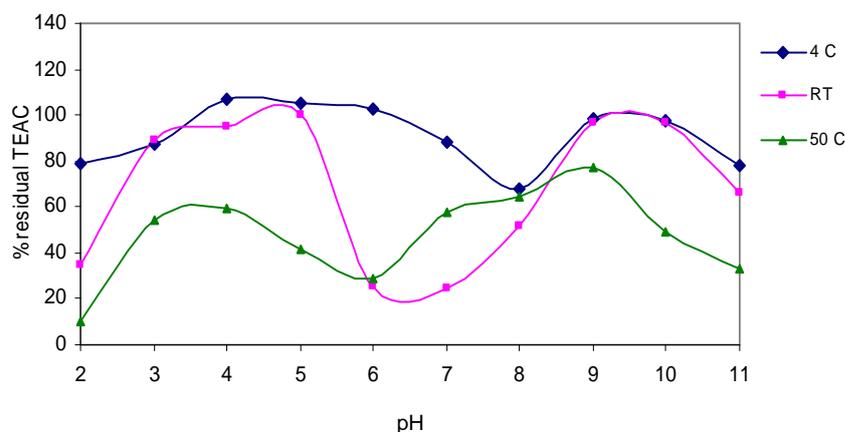


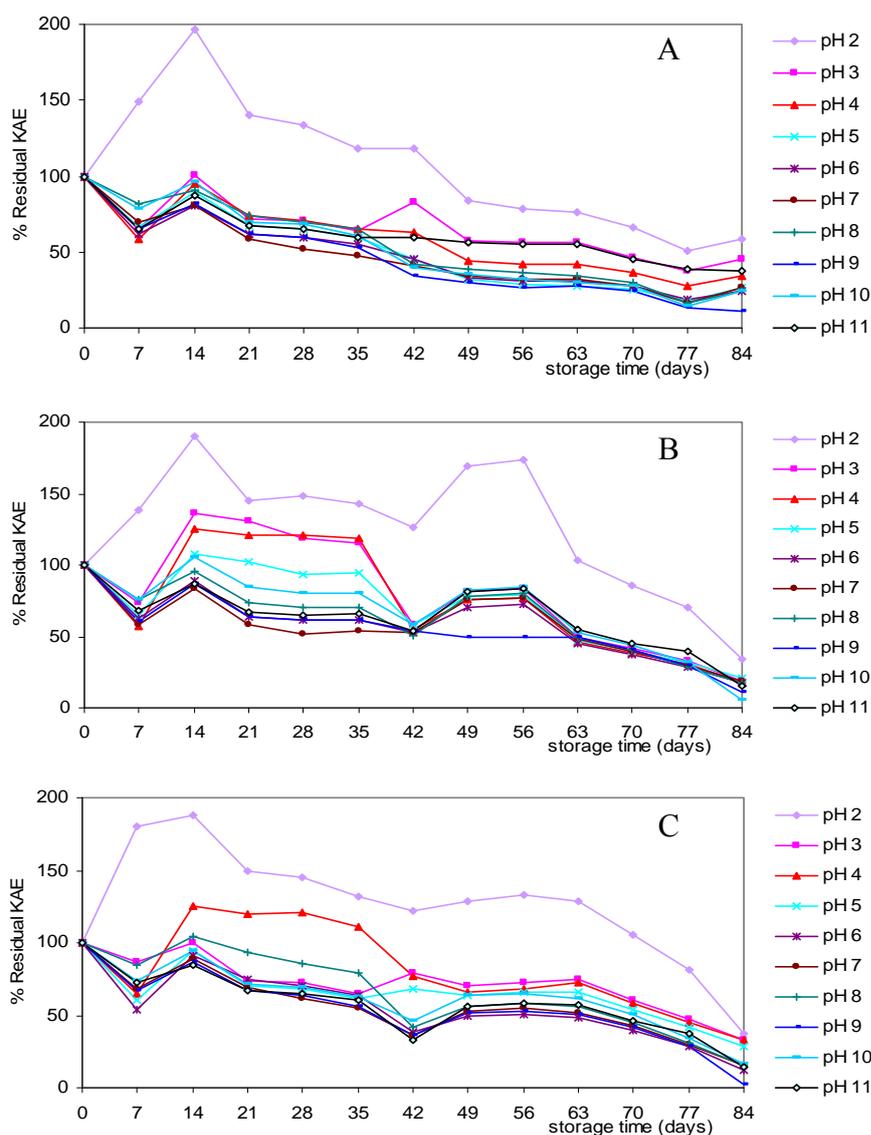
Figure 4.15 Percentage of Residual ABTS Radical Scavenging Capacity of Raw Betel Seed Nut Extract Solutions after 84 Days Storage

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.

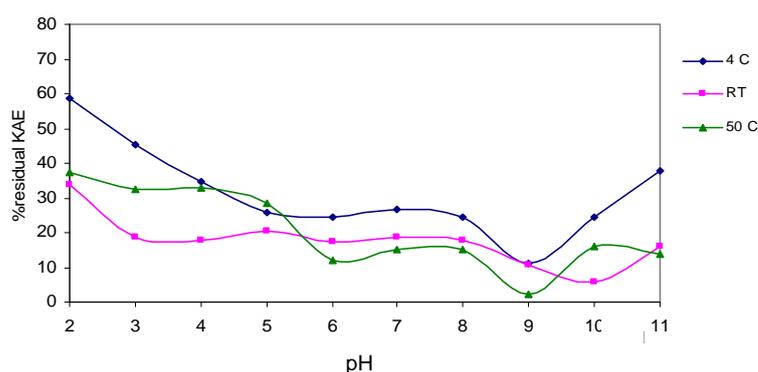


Figure 4.17 Percentage of Residual Tyrosinase Inhibition Activity of Raw Betel Nut Seed Extract Solutions after 84 Days Storage

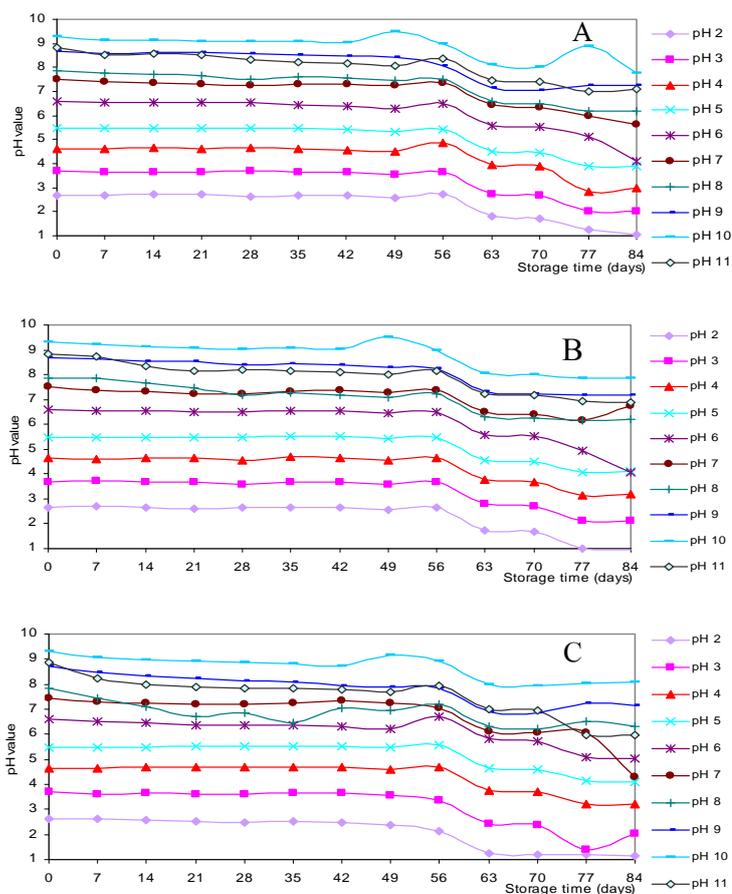
4.6.5 Physio-Chemical Evaluation

In addition to the extract, the finished product containing extract was also in considered to its 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

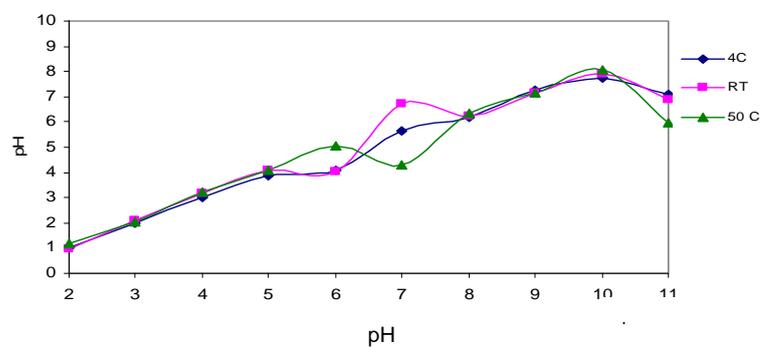
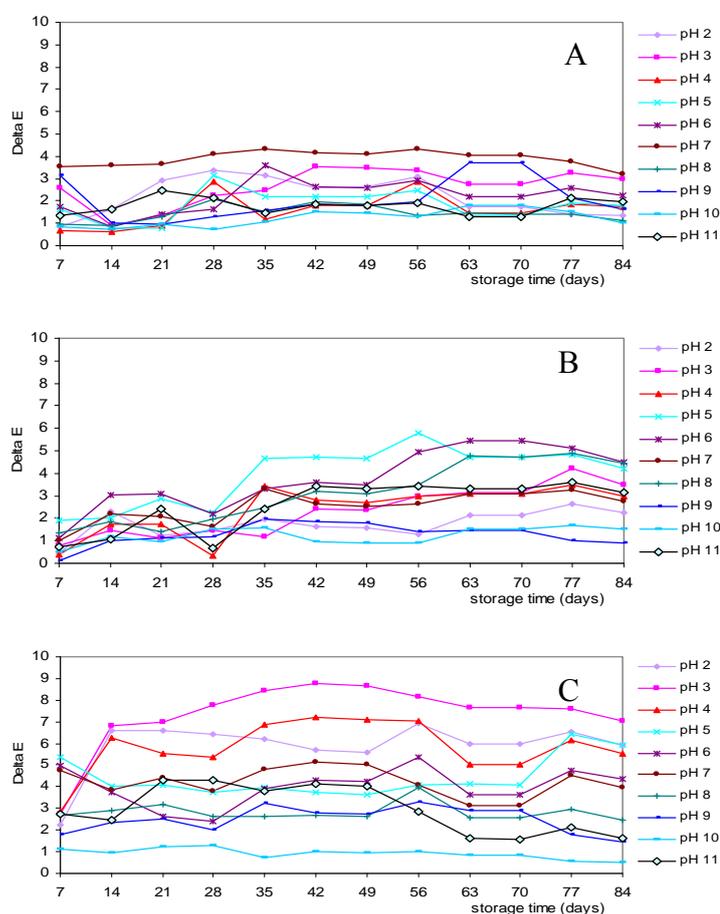


Figure 4.19 pH Changes of Raw Betel Nut Seed Extract Solutions After 84 Days Storage

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.

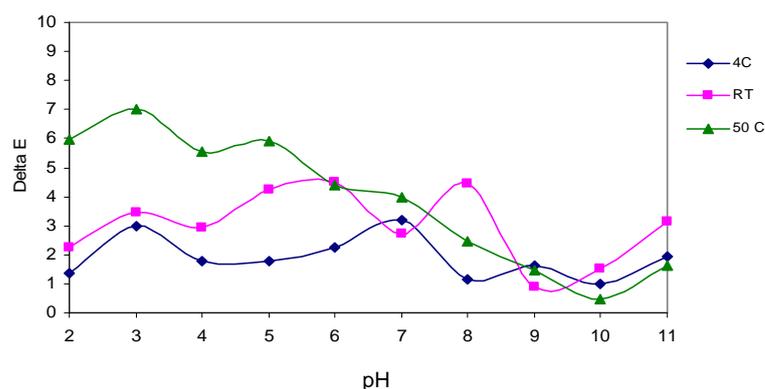


Figure 4.21 Color Changes (ΔE^*) of Raw Betel Seed Nut Extract Solutions after 84 Days Storage

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.

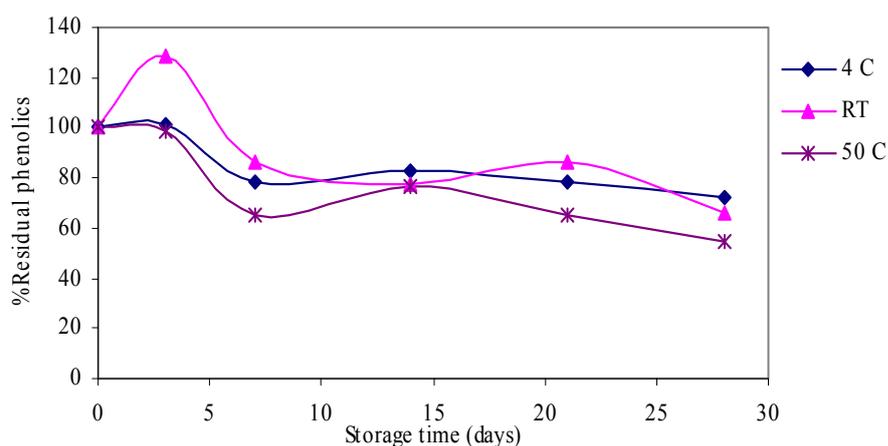


Figure 4.22 Percentage of Residual Phenolics Content of the Formula Containing 0.5% Raw Betel Nut Seed Extract

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
<i>Base formula</i>					
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
<i>Formula containing 0.5% raw betel nut extract</i>					
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).

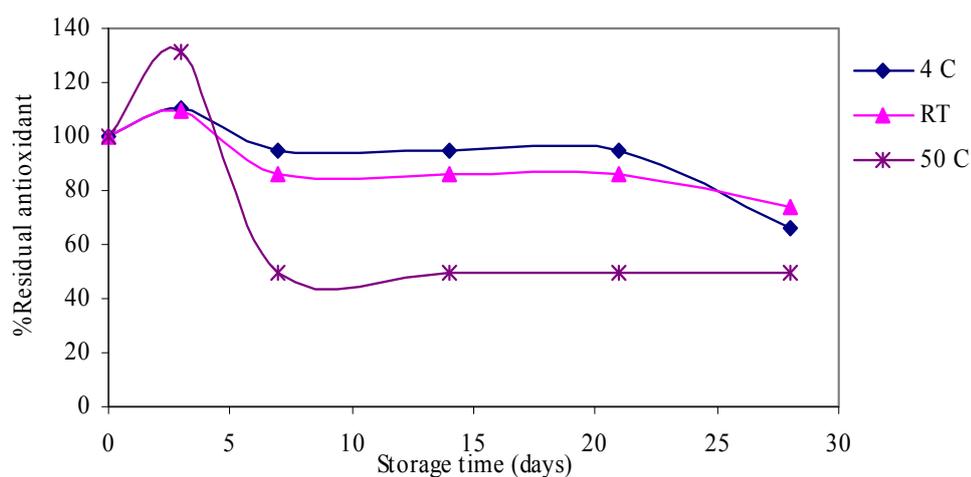


Figure 4.23 Percentage of Residual Antioxidant Activity of the Formula Containing 0.5% (w/w) Raw Betel Nut Seed Extract

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.

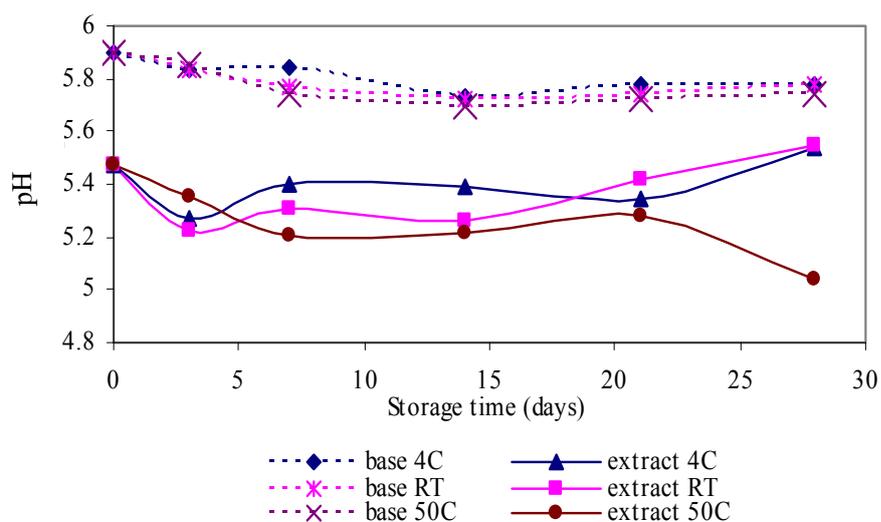


Figure 4.24 Changes of pH of the Base Formula and Formula Containing 0.5% (w/w) Raw Betel Nut Seed Extract

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).

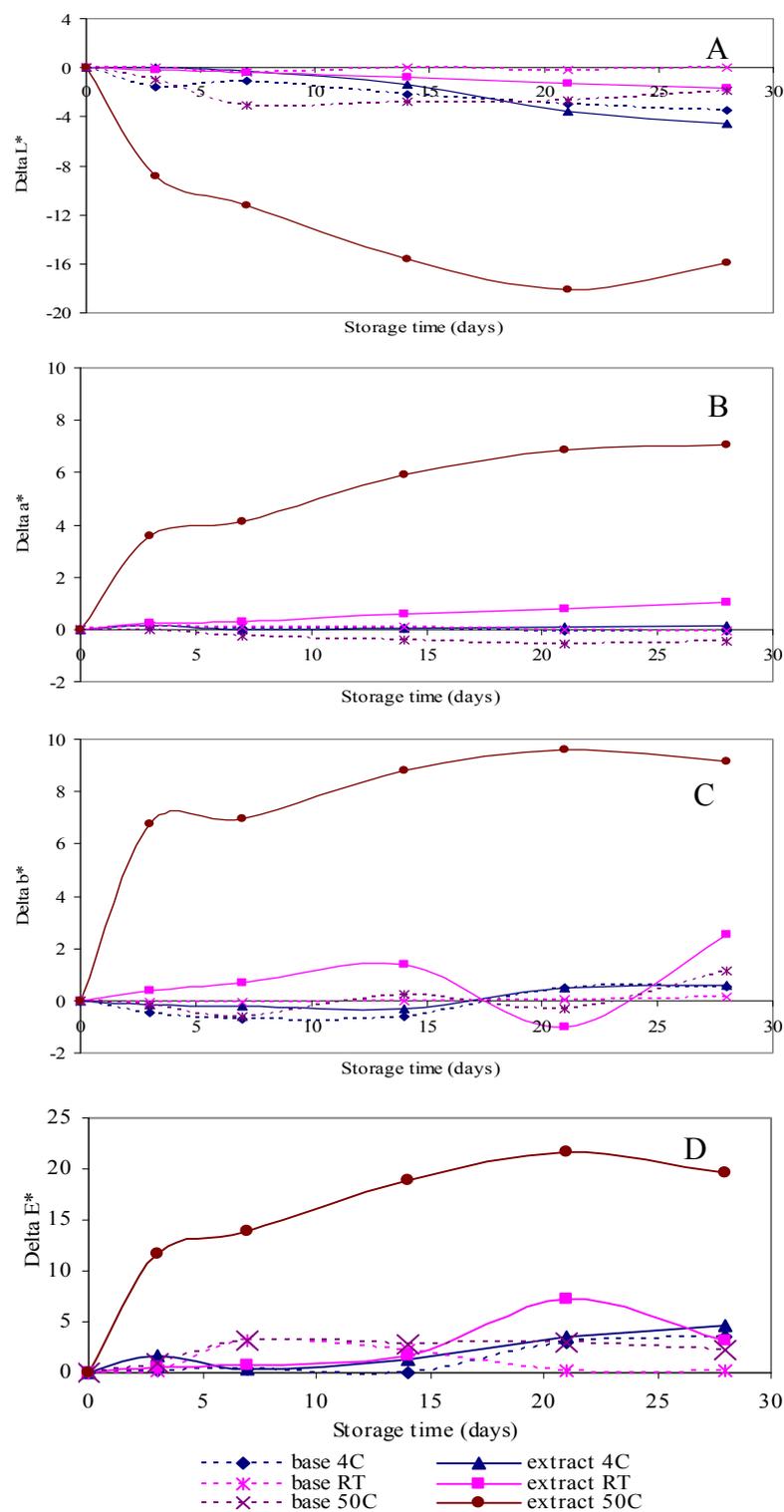


Figure 4.25 Changes of Color of the Base Formula and Formula Containing 0.5% (w/w) Raw Betel Nut Seed Extract

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 summarize the human eyes detection of the color differences of the formulas at the final day compared to the initial day and their base formula.

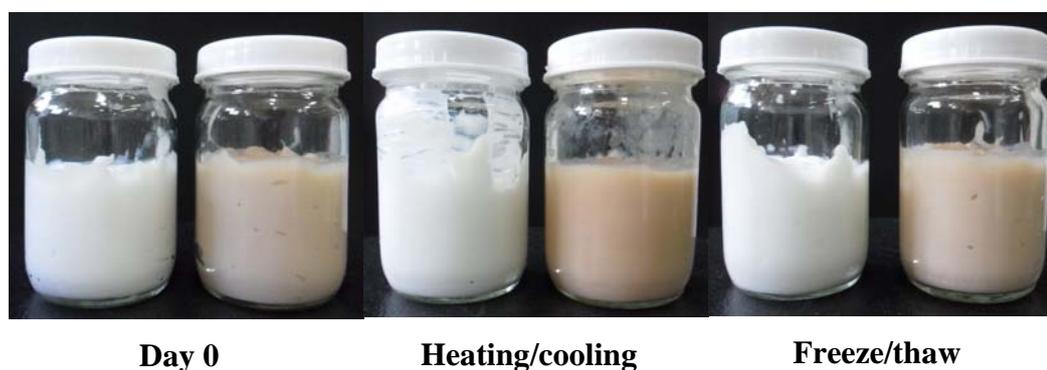


Figure 4.26 Appearance of the base formula (left) and the formula containing 0.5% (w/w) raw betel nut seed extract (right)

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.

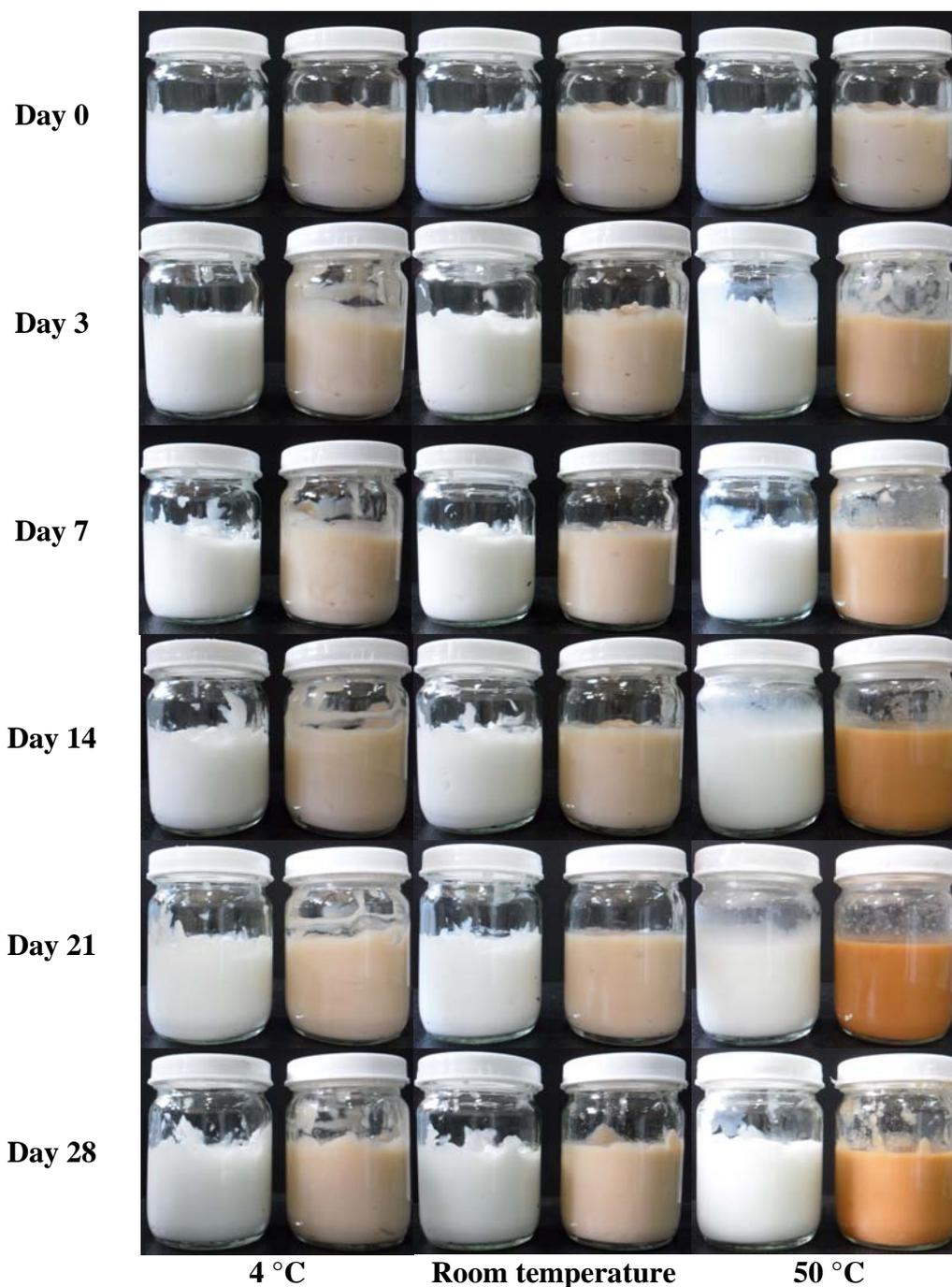


Figure 4.27 Appearance of the Base Formula (left) and the Formula Containing 0.5% (w/w) Raw Betel Nut Seed Extract (right)

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.

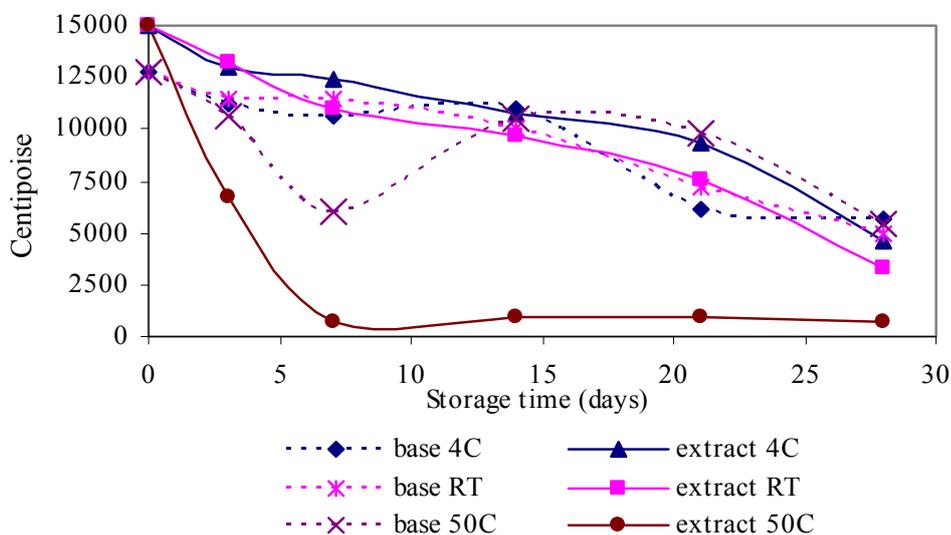


Figure 4.28 Changes of Viscosity of the Base Formula and Formula Containing 0.5% (w/w) Raw Betel Nut Seed Extract

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 lag 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 obtain maximum *in vivo* efficacy in the further study.