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Appraisal of Free Radical Scavenging Activities and Inhibitory Effect on Lipid Peroxidation Related to Phenolic Content of Seed Extracts from Lychee (*Litchi chinensis* Sonn.)

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Abstract: **Five cultivars of Lychee (*Litchi chinensis* Sonn.) seed cultivated in northern Thailand namely: O-Hia, Kwangchoa, Chakrapad, Hong-Huay and Kim Cheng, were collected, dried and ground into powder and then extracted by maceration with 85% ethanol and sequentially partitioned with *n*-hexane, ethyl acetate and water. Each fraction of the extracts was determined their antioxidant activities in three different assays and total phenolic content. The results revealed that the ethanolic extract (EE) and ethyl acetate (EA) partitioned extract from all the Lychee cultivars exhibited various level of free radical scavenging activities against α, α- diphenyl–ß-picrylhydrazyl (DPPH assay), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS assay) and inhibitory effect on lipid peroxidation (TBARS assay). Interestingly, we found that all the ethyl acetate (EA) partitioned extract of Lychee seeds possessed potent antioxidant activities, especially that from Kim Cheng presented the highest activity in the three test models which is comparable to vitamin E and gallic acid, with IC_{50} of 0.09 ± 0.00 mg mL^{-1} (DPPH), TEAC value of 1.03 ± 0.01 mg Trolox mg $sample^{-1}$ (ABTS) and IC_{50} of 0.31 ± 0.04 mg mL^{-1} (TBARS). This bioactive fraction consists of high total phenolic content (GAE of 0.44 ± 0.00 mg gallic mg $sample^{-1}$). The chromatographic fingerprint of lychee seed extracts by HPLC at wavelength 280 nm found that the EE and EA extracts presented the same pattern with six major peaks, but different in peak height. The total phenolic content assay and HPLC analysis presented the corresponding data to the antioxidant activities. In the conclusion, Lychee seed extracts can be used as natural source of potent antioxidant that may substitute for synthetic antioxidant in the future. These results will be further investigated for the development into anti-aging cosmetic products.**

Keywords: **Lychee Seed Extracts, Free Radical Scavenging Activities, Lipid Peroxidation Inhibition, Total Phenolic Content, Chromatographic Fingerprint.**

I. INTRODUCTION

Oxidation reactions can produce free radicals, which start chain reactions that damage cells.[1] An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules.[2] Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols.[3] Antioxidants can be either endogenous compound, produced by the organism as part of its ROS defense, or can be exogenous compounds acquired from the diet. The endogenous system includes both enzymes and nonenzymatic antioxidants, and dietary antioxidants are small molecules.[4] Phenolic compounds are capable of protecting them from oxidative damage and defending them against yeast, fungi, virus and bacteria that might inhibit their germination. Considering the developing nutraceutical industry and escalating demand for natural functional food additives and cosmetic products. Thus, Lychee seeds could be further assessed and utilized in view of their phenolics content which have been claimed to impart health benefit effects. Many studies have demonstrated the radical scavenging properties of plant phenolic compounds. Rice-Evans *et al.* (1997) measured the radical scavenging activity of flavonoids and phenolic acids based on their ability to scavenge a preformed radical cation chromophore of 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+\bullet}$) at pH 7.4. Among flavonoids, the highest scavenging activities were found for the flavonol quercetin, the anthocyanidins cyanidin and delphinidin, and the green tea flavan-3-ols epicatechin gallate and epigallocatechin gallate.[2]

Lychee (*Litchi chinensis* Sonn.), a tropical to subtropical fruit in the family Sapindaceae, is originating from China, Vietnam, Indonesia and the Philippine, and now also grown elsewhere in the world especially in the north of Thailand. Lychee ranks the eleventh in value of fruit production in Thailand. The important cultivars were O-Hia, Kwangchao, Chakapad, Hong-Huay and Kim Cheng.[5] Lychee is one of an important fruit in canned food industry with lots of lychee seed waste. Nagendra Prasad, K., *et al* (2009) reported that fruit seed extracts of Lychee gave potent antioxidant properties and antityrosinase activity in the fraction consisted of polyphenols and flavonoids.[6] However, there is no information reported on Lychee seed cultivated in northern Thailand, which are growing in various cultivars. In the present study, the potential of seed extracts from various cultivars of Lychee cultivated in northern Thailand being used as a natural antioxidant for cosmetic application was investigated.

II. MATERIAL AND METHOD

Plant material: Five cultivars (O-Hia, Kwangchao, Chakapad, Hong-Huay and Kim Cheng) of lychee (*Litchi chinensis* Sonn.) seed that cultivated in northern Thailand were used in this study. The lychee seeds were washed and dried for 24 hrs by using hot air oven at 60°C and grounded to reduce their size for further use.

Chemica and Reagent: 1,1-diphenyl-2-picryl hydrazyl (DPPH), Gallic acid, Quercetin, t-octylphenoxy polyethoxyethanol) Triton X-100), Trolox and 2-Thiobabituric acid 98% (TBA) were purchased from Sigma chemical Co. (St. Louis, Mo, USA). 2,2'-azino-bis)3-ethylbenzthia zoline-6-sulphonic acid((ABTS) were purchased from Wako Pure Chemical Industries, Japan**.** Phosphatidylcholine) Epikulon 200) was purchased from Degussa, Germany.

Extraction: The lychee seed powder was macerated in 85% ethanol at room temperature for 2 hours 3 times. The extracts were filtered and evaporated to dryness with rotary evaporator (EYELA®, Japan) to obtain ethanol extract (EE). A part of the crude ethanol extract (EE) was re-dissolved in 95% ethanol and added purified water (to obtain 85% ethanol) then partitioned with *n*-hexane, ethyl acetate and water, sequentially. Each fraction was concentrated with rotary evaporator at 40°C to obtain hexane extract (HE), ethyl acetate extract (EA) and aqueous extract (AE), respectively.

DPPH radical scavenging assay: The experimental procedure was adapted from Brem, Seger et al. (2004).[7] In this assay, the stable free radical 2, 2-diphenyl-1-picrylhydrazyl which has a strong absorption at 540 nm, reacts with antioxidants and produces colorless 2, 2-diphenyl-1-picryl hydrazine independently of enzymatic activities. Dilution series of test compounds, dissolved in EtOH, were performed in sterile disposable microplates, using freshly prepared 167 µM $DPPH^{\bullet}$ in ethanol, 180 µL.Ellagic acid and Gallic acid served as known antioxidants. The samples were tested in dilutions ranging from 0.1-0.5 mg/mL with a final volume of 200 µL for all of the assays. Results were determined after 30 min of reaction time in order to analyse antiradical activities. The disappearance of the free radical $DPPH^{\bullet}$ was measured spectrophotometrically at 540 nm with a microplatereader (Backman coulter®, DTX 880 multimode detector, Austria). All measurements were performed in triplicate. The percentage inhibition was calculated by the following equation**:** % Inhibition = $(A_{control} - A_{sample}/ A_{control})* 100$ where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of test compound. Extract concentration providing 50% inhibition was also calculated from the graph plotted percentage inhibition against extract concentration as IC_{50} value (mg mL^{-1}).

ABTS cation radical scavenging assay: ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h, before use[8]. The solution was diluted with ethanol to obtain the absorbance of 0.9±0.1 units at 734 nm. The extracts solution of 70 µl of ethanolic extracts test solution of each sample was added to 630 µl of ABTS free radical cation solution. The absorbance, monitored for 50 min, was measured spectrophotometrically at 734 nm by using a spectophotometer (Shimadzu UV-Vis 2450, Japan). All measurements were performed in triplicate. Trolox served as known antioxidant. The free radical-scavenging activity of each sample was expressed as trolox equivalent antioxidant capacity(TEAC, mg Trolox mg $sample^{-1}$).

Inhibitory effect on lipid peroxidation with Thiobarbituric Acid Reaction Species (TBARS) assay: A modified thiobarbituric acid-reactive substances (TBARS) assay was used to measure the antioxidant activity of the lychee seed extracts in term of inhibition on lipid peroxidation[9,10]. Liposome suspension, consisting of cholesterol, phosphatidylcholine and 0.2 M potassium phosphate buffer (pH 7.2), was prepared in a sonicator for 20 min. The lychee seed extract in ethanol was mixed with a mixture of the sonicated solution, and AAPH. The resulting mixture was incubated for 24 hr at 50° C. After incubation, the solution was mixed with 0.2% BHT, 3%Triton-X , 20% acetic acid and 0.6% TBA. Then heated at 90°C for 30 min and cooled to room temperature. The absorbance of the mixture was measured spectrophotometrically at 540 nm with a microplatereader. The percentage inhibition and IC_{50} value (mg $mL^{-1)}$ were also calculated as mentioned above in DPPH assay. Gallic acid and Quercetin served as known antioxidants.

Determination of Total Phenolic content in lychee seed extracts: The total phenolic content of all extracts were determined using the Folin-Ciocalteu reagent.[11,12] Each filtrated of sample was transferred into an eppendorf tube that contained distilled water and then mixed thoroughly with of Folin- Ciocalteu reagent. After mixing for 3 min, 7.5% (w/v) sodium carbonate was added. The mixtures were agitated with a vortex mixer, then allowed to stand for a further 30 min in the dark. The absorbance of extracts and blanks were measured at 765 nm using a spectrophotometer (Shimadzu UV-Vis 2450, Japan). The concentration of total phenolic compounds in each extract was expressed as gallic acid equivalent (GAE, mg gallic mg $sample^{-1}$)

Determination of chromatographic fingerprint of lychee seed extract by HPLC: The chromatographic fingerprint of each lychee seed extract was determined by reversed phase high performance liquid chromatography. The separation of phenolic compounds was performed on a HP HPLC series 1100 (Hewlett Packard, Waldbronn, Germany) equipped with CHEM STATION software, a degasser G1322A, a binary gradient pump G1311A, a thermoautosampler G1313A, a column oven G1316A and UV-Visible detection system G1314A. The column was an Alltech Altima 5 mm C (25034.6 mm I.D.) and a C18 guard column (Phenomenex 4 x 3.0 mm.). The column was operated at a temperature of 40°C. The mobile phase consisted of 3% acetic acid in water (eluent A) and methanol (eluent B) [13,14]. The gradient program was as

follows: 10% B to 55% B (50 min), 55% B to 100% B (10 min), 100% B to 10% B (5 min). The injection volume for all samples was 20 µl. Simultaneous monitoring was performed at 280 nm at a flow-rate of 1 ml min^{-1}.

III. Results

From our preliminary study, using various extraction methods of lychee seed (macerated in acetone, macerated in methanol: 1.5 N HCl at ratio 85:15 and macerated in 85% ethanol) found that maceration in 85% ethanol gave the highest antioxidant activity by DPPH assay (data not shown). Therefore, five cultivars of lychee seed cultivated in northern Thailand, O-hia, Kwangchoa, Chakrapad, Hong-Huay and Kim Cheng, were macerated with 85% ethanol to gave the ethanolic extract (EE) and then semi-purified by liquid-liquid extraction, a part of EE was partitioned with *n*-hexane, ethyl acetate and water to obtain HE, EA and AE extracts, respectively. Each fraction was evaluated their antioxidant capacity by DPPH assay. The EE and EA extracts showed strong free radical scavenging activity (IC_{50} of 0.15 ± 0.01 and 0.09 ± 0.00 mg mL^{-1}) while hexane extract (HE) and aqueous extract (AE) showed weak free radical scavenging activity (IC_{50} of 0.38 ± 0.04 and 0.63 ± 0.16 mg mL^{-1}) by DPPH assay. Therefore, the EE and EA were chosen for further investigation. The percentage yield and the physical appearance of EE and EA from each cultivar of lychee seed extracts are given in Table 1. The EE from Kim Cheng possessed the highest yield of extraction (5.96 ± 0.01%), whereas the EA from Chakrapad showed the lowest (0.24 ± 0.01%). In the previous study on the other plant seed such as *Tamarindus indica* by Siddhuraju, P.,in 2007, they separated only the seed coat for the extraction and found that the seed coat extract shows strong antioxidant activity due to the high content in polyphenolic compounds while the seed kernel consists of oil, starch and protein[15]. In our studies, we used the whole seeds of Lychee, the waste from canned lychee industry which will reduce the cost. Although, may be the separation of seed coat before extraction give no different in percentage yield in five cultivars but the cost of industrial extraction procedure will be increased. From the previous report, it is still lacking the supportive scientific information on using of whole seed extract of lychee.

Table 1: Percentage yield (w/w) and physical appearance of the extracts from lychee seeds

Cultivar of Lychee	Physical Appearance		% yield (w/w), mean±SD	
	EE	EA	EE	EA
O-Hia (A)	dark brown powder	Light brown powder	4.47 ±0.01	0.89 ± 0.01
Kwangchao (B)	dark brown powder	Light brown powder	2.78 ±0.01	0.39 ± 0.01
Chakrapad (C)	dark brown powder	Light brown sticky semisolid	3.93 ±0.01	0.24 ± 0.01
Hong-Huay (D)	Dark brown very sticky semisolid	Dark brown very sticky semisolid	4.11 ±0.01	0.44 ±0.01
Kim Cheng (E)	Light brown powder	Light brown powder	5.96 ±0.01	0.39 ±0.01

Note: EE is ethanol fraction, EA is ethyl acetate fraction

DPPH radical scavenging assay: The DPPH radical scavenging activity of all lychee seed extracts were shown in Table 2 and Figure 1. Kim Cheng showed the highest scavenging activity on DPPH radical in both EE and EA with IC_{50} of 0.13 ± 0.01 mg mL^{-1} and 0.09 ± 0.01 mg mL^{-1}. Whereas the EE from Hong Huay showed the lowest antioxidant activity with IC_{50} of 0.27 ± 0.01 mg mL^{-1}. In the part of EA, Kwangchao showed the lowest antioxidant activity but not significantly different between Kwangchao, Chakrapad and Hong Huay were found (IC_{50} of 0.15 ± 0.02, 0.14 ± 0.02 and 0.14 ± 0.01 mg mL^{-1} respectively). It was noticed that EA from all cultivars exhibited higher scavenging activity than EE due to the higher consisting phenolic compounds in the partitioned ethyl acetate extract.(Table2)

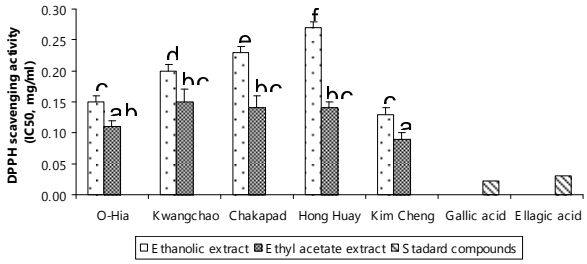

Figure 1. DPPH radical scavenging activity of the ethanolic fraction and ethyl acetate fraction from five cultivars of lychee seed extracts. For each treatment, means within the all bar followed by different letters are significantly different at *P< 0.05.*

ABTS cation radical scavenging assay: The results from ABTS cation radical scavenging assay of all lychee seed extracts were shown in Table 2 and Figure 2. The ethyl acetate fraction (EA) revealed higher scavenging activity than ethanolic fraction (EE). Among them, both of EE and EA from Kim Cheng exhibited the highest activity with TEAC value of 0.77 ± 0.01 and 1.03 ± 0.01 mg Trolox mg $sample^{-1}$ and the Chakapat showed the lowest activity in both EE and EA with TEAC value of 0.39 ± 0.05 and 0.64 ± 0.05 mg Trolox mg $sample^{-1}$. The ABTS cation radical scavenging capacity of the ethyl acetate fraction from Kim Cheng and O-Hia were comparable to trolox which is a vitamin E analog (Table 2).

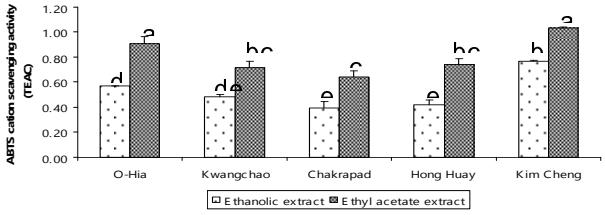

Figure 2. ABTS cation scavenging activity of ethanolic fraction and ethyl acetate fraction from five cultivars of lychee seed extract. For each treatment, means within the all bar followed by different letters are significantly different at *P< 0.05.*

There was the accordance between DPPH and ABTS assay because these two methods show the same mechanism of action to scavenge the free radicals. However the different mechanism of action was necessary to confirm the good antioxidant property.

Inhibitory effect on lipid peroxidation with TBARS assay: Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cell and tissues. Thiobarbituric acid reactive substances species (TBARS) assay measured the pink pigment produced through reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) (an indicator of lipid peroxidation). The antioxidant activity of each part of lychee seed extracts was as shown in Table 2 and Figure 3. For the ethanolic fraction, O-Hia exhibited the highest antioxidant activity with IC_{50} of 0.56 ± 0.08 mg mL^{-1} whereas Hong Huay showed the lowest antioxidant activity with IC_{50} of 1.74 ± 0.03 mg mL^{-1}. Whereas in the ethyl acetate fraction, Kim Cheng possessed the highest antioxidant activity with IC_{50} of 0.31 ± 0.04 mg mL^{-1} and Chakapad showed the lowest antioxidant activity with IC_{50} of 0.86 ± 0.10 mg mL^{-1}. In this assay, the results also strongly indicated that the ethyl acetate fraction of lychee seed extracts possessed the higher inhibitory effect on lipid peroxidation than the ethanolic fraction. Interestingly, the IC_{50} of EA from Kim Cheng was comparable to gallic acid, a known antioxidative substance and it contained the highest amount of total phenolic content.

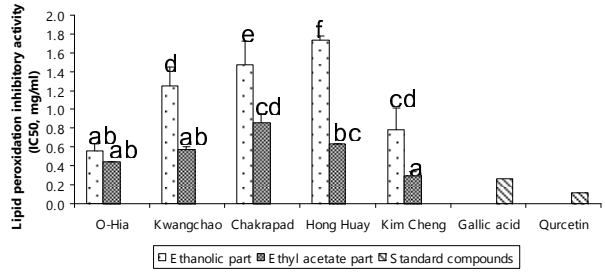

Figure 3. Lipid peroxidation inhibitory activity of ethanolic fraction and ethyl acetate fraction from five cultivars of lychee seed extract.

For each treatment, means within the all bar followed by different letters are significantly different at *P< 0.05.*

Although in TBARS assay, which showed the different mechanism of action as lipid peroxidation inhibitory effect which might have been due to chain termination of peroxyl radical,[6] but it presented the same pattern of the results as in DPPH and ABTS assay.

The three different methods, DPPH, ABTS and TBARS assay, were used to determine the antioxidant activities of lychee seed extracts for confirming their antioxidant capacity and searching out for which lychee cultivars that possess the highest antioxidant activity. As previously described, the used of different methods and concentration are necessary in antioxidant activity assessment [16,17].

The present study shows that no single assay is sufficient to estimate the antioxidant activity of a studies compound. These method represented different mechanism of antioxidant action. A sample possessed DPPH or ABTS free radical scavenging property indicated that its mechanism of action was hydrogen donor and termination the oxidation process by converting free radicals to more stable product. TBARS assay presented that its mechanism was the ability to inhibit the lipid peroxidation reaction of sample by chain termination of peroxyl radicals. Therefore, our results can be strongly confirmed that the lychee seed extracts play a promising role as natural antioxidant by both free radical scavenging ability as well as inhibition effect on lipid peroxidation and are valuable for further applications especially in cosmeceutical use that preventing skin aging.

Total Phenolic content of lychee seed extracts: The Folin-Ciocaltue assay is widely used procedure for quantification of total phenolic in plant materials. Folin-Ciocalteu reagent is not specific and detects all phenolic groups found in extracts including those found in the extractable proteins. The concentrations of total phenolic compounds in all extracts were expressed as gallic acid equivalent (GAE). The total phenolic content of each fraction of lychee seed extract had shown in Table 2 and Figure 4. The EE and EA from Kim Cheng exhibited the highest phenolic content with GAE of 0.30 ± 0.00 and 0.44 ± 0.00 mg gallic mg $sample^{-1}$. Chakapat shown the lowest phenolic content in both EE and EA with GAE of 0.17 ± 0.00 and 0.30 ± 0.00 mg gallic mg $sample^{-1}$, respectively.

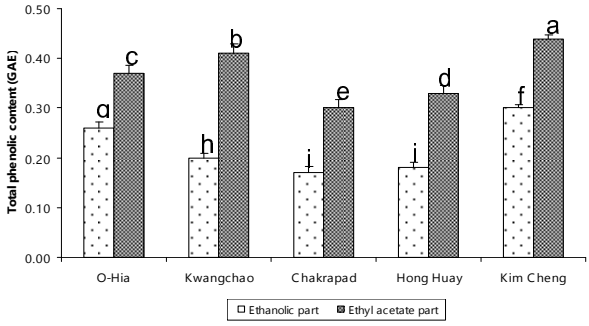

Figure 4. Total phenolic content of ethanolic fraction and ethyl acetate fraction of five cultivars of lychee seed extract.For each treatment, means within the all bar followed by different letters are significantly different at P< 0.05.

Table 2: Total phenolic content and antioxidant activities of all the extracts from 5 cultivars of Lychee seed.

Lychee Cultivar	Total phenolic content GAE [a] (mg gallic mg $sample^{-1}$)	DPPH IC_{50} [a] (mg mL^{-1})	ABTS TEAC [a] (mg Trolox mg $sample^{-1}$)	TBARS IC_{50} [a] (mg mL^{-1})
EE				
O-Hia	0.26 ± 0.00	0.15 ± 0.01	0.57 ±0.00	0.56 ± 0.08
Kwangchao	0.20 ± 0.00	0.20 ± 0.01	0.48 ± 0.02	1.25 ± 0.20

Chakapat	0.17 ± 0.00	0.23 ± 0.01	0.39 ± 0.05	1.47 ± 0.26
Hong Huay	0.18 ± 0.00	0.27 ± 0.01	0.42 ± 0.04	1.74 ± 0.03
Kim Cheng	0.30 ± 0.00	0.13 ± 0.01	0.77 ± 0.01	0.78 ± 0.23
Gallic acid	-	0.02 ± 0.00	-	0.26 ± 0.02
Ellagic acid	-	0.03 ± 0.00	-	-
Quercetin	-	-	-	0.12 ± 0.02
EA				
O-Hia	0.37 ± 0.00	0.11 ± 0.01	0.90 ± 0.06	0.44 ± 0.00
Kwangchao	0.41 ± 0.00	0.15 ± 0.02	0.72 ± 0.05	0.57 ± 0.03
Chakapat	0.30 ± 0.00	0.14 ± 0.02	0.64 ± 0.05	0.86 ± 0.10
Hong Huay	0.33 ± 0.00	0.14 ± 0.01	0.74 ± 0.05	0.63 ± 0.00
Kim Cheng	0.44 ± 0.00	0.09 ± 0.01	1.03 ± 0.01	0.31 ± 0.04
Gallic acid	-	0.02 ± 0.00	-	0.26 ± 0.02
Ellagic acid	-	0.03 ± 0.00	-	-
Quercetin	-	-	-	0.12 ± 0.02

[a]Mean±SD
Note: EE is ethanol fraction, EA is ethyl acetate fraction

From the antioxidant and total phenolic content determination in each fraction of the extracts from five cultivars of lychee seed, the results found that the antioxidant activities were related to their total phenolic content. The ethyl acetate fraction of all cultivars of lychee seed extracts exhibited higher antioxidant activity than the ethanolic fraction in all assay methods (DPPH, ABTS and TBARS assay), which also revealed the higher total phenolic content. Kim Cheng cultivar possessed the highest antioxidant activity and the highest total phenolic content (Table 2). So, the phenolic compounds contained in the extracts played an important roles for their antioxidant activites. The previous study in China, lychee was extracted by 50% ethanol and five compounds were identified, namely gallic acid, procyanidine B2, (-)-gallocatechin, (-)-epicatechin, and (-)-epicatechin-3-gallate.[6] In this study, the lychee seed extracts cultivated in northern Thailand might be contained gallic acid derivatives or others and its derivatives as main constituent that need for further investigated.

Determination of chromatographic fingerprint of lychee seed extracts by High Performance Liquid Chromatography (HPLC): High Performance Liquid Chromatography (HPLC) is used almost exclusively for the qualitative and quantitative analysis. Retention times were utilized as primary criterion for peak identification. The chromatographic fingerprint of EE and EA lychee seed extracts were shown in Figure 5. The same chromatographic pattern with six major peaks were found, but in different peak height. At the same concentration, the EA exhibited the higher peak than EE extract. The results of this study might correspond to the antioxidant analysis that the EA had higher activity than EE extract. According to the retention time presented in HPLC chromatogram, the major peaks are not gallic acid or ellagic acid as the previous data reported in lychee seed coat (data not shown). The bioactive compound in whole seed of lychee should be further investigated.

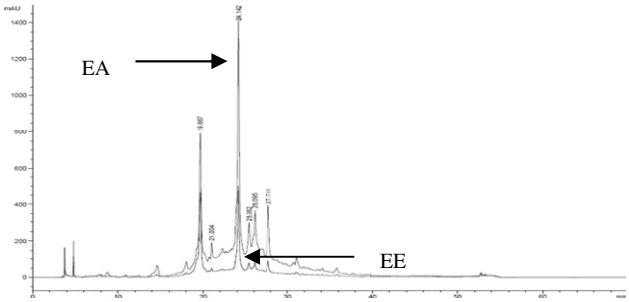

Figure 5. HPLC Chromatogram of EE and EA from Lychee seed.

IV. Conclusion

Although the presence of Lychee cultivated in China had been suggested in their antioxidant activities[6] but the Lychee cultivated in Thailand and their antioxidant capacity in various test models has not been reported up to date. As the results, we found that the ethyl acetate fraction of Lychee seed possessed potent antioxidant activities, especially the extract of Kim Cheng presented the highest activity in the three test models which is comparable to vitamin E and gallic acid. The bioactive fraction contained high total phenolic content. The HPLC fingerprint of the EE and EA extract showed the same pattern with six major peaks, but different in peak height. The total phenolic content assay and HPLC analysis presented the corresponding data to the antioxidant activities. Thus, Lychee seed extracts can be used as natural source of potent antioxidant to substitute for synthetic antioxidants in the future. The identification of bioactive compounds and investigation for the development into anti-aging cosmetic products should be further study.

V. Acknowledgements

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