การสกัดสารออกฤทธิ์ทางชีวภาพจากเมล็ดหมาก (*Areca catechu* L.) ด้วยวิธีการสกัดของแข็งด้วยของเหลวโดยไมโครเวฟ

Microwave-assisted Solid-liquid Extraction of Biological Compounds from *Areca catechu* L. Seed

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สำนักวิชา วิทยาศาสตร์เครื่องสำอาง มหาวิทยาลัยแม่ฟ้าหลวง
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บทคัดย่อ

งานวิจัยนี้ศึกษาการสกัดสารออกฤทธิ์ทางชีวภาพจากผลหมาก (*Areca catechu* L.) โดยใช้ไมโครเวฟช่วยในการสกัดด้วยตัวละลายที่มีขั้วต่างกัน 6 ชนิดคือ เฮกเซน เอทิลอะซิเตท อะซีโตน เอทานอล 95% เอทานอล 50% และน้ำ นำสารสกัดที่ได้มาวิเคราะห์ด้วย HPLC และปริมาณสารประกอบฟีนอลิก (EPC) และฟลาโวนอยด์ (EFC) และวิเคราะห์ความสามารถการกวาดอนุมูลอิสระด้วยวิธี DPPH และความสามารถในการให้อิเล็กตรอนด้วยวิธี FRAP พบว่าสารสกัดอะซีโตนให้ปริมาณสารออกฤทธิ์ทางชีวภาพสูงสุด โดย EPC มีปริมาณ 733.11 มก. สมมูลของกรดแกลลิกต่อกรัมของสารสกัดแห้ง (mg GAE/g extract) และ EFC ปริมาณ 113.42 มก. สมมูลของเคอเซตินต่อกรัมของสารสกัดแห้ง (mg QE/g extract) สารสกัดจากอะซีโตนยังให้ฤทธิ์ในการต้านอนุมูลอิสระสูงสุดเช่นกัน โดยพบว่ามีความสามารถเทียบเท่ากับ 184.36 และ 126.15 มก. ทรอลอกซ์ต่อกรัมของสารสกัดแห้ง (mg TEAC/g extract) จากการทดสอบด้วยวิธี DPPH และ FRAP ตามลำดับ สารสกัดเฮกเซนที่ได้จากขั้นตอนแรกและ สารสกัดน้ำจากขั้นตอนสุดท้ายให้ปริมาณสารและฤทธิ์ทางชีวภาพต่ำสุด โครมาโตแกรมจาก HPLC แสดงให้เห็นว่าสารประกอบที่มีคาเทชิน เป็นส่วนประกอบหลักในสารสกัดหมาก การศึกษานี้แสดงให้เห็นว่าอะซีโตนเป็นสารละลายที่เหมาะสมในการสกัดสารออกฤทธิ์ทางชีวภาพจากผลหมาก

คำสำคัญ : สารต้านอนุมูลอิสระ ผลหมาก การสกัดเป็นลำดับขั้น HPLC การสกัดของแข็งด้วยของเหลว

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Abstract

This study was purposed to extract biological compounds from *Areca catechu* L. (betel nut) seed using microwave-assisted solid-liquid extraction with various solvents. Sequential extraction by 6 different polarity of organic solvents, including hexane, ethyl acetate, acetone, 95% ethanol, 50% ethanol, and water were employed. Each fractionated extract was investigated for its elution profile by HPLC. The colorimetric assays of extractable phenolic (EPC) and flavonoids (EFC) contents, DPPH radical scavenging capacity (DPPH) and ferric reducing antioxidant power (FRAP) of the extract were determined. Amongst all fractions, acetone extract possessed the highest EPC and EFC of 733.11 mg gallic acid equivalent per gram extract (mg GAE/g extract) and 113.42 mg quercetin equivalent per gram extract (mg QE/g extract), respectively. The most powerful antioxidant capacity was also obtained from the acetone fraction exhibiting 184.36 and 126.15 mg trolox equivalent antioxidant capacity per g extract (mg TEAC/g extract) when assayed by DPPH and FRAP method, respectively. The first fraction of hexane and the last fraction of water exhibited lowest bioactive compounds and activities. The HPLC chromatogram showed the major components of raw betel nut seed were catechin-like compounds with the remarkable highest peak area in acetone fraction. This study shows the similar characteristics of raw betel nut seed in the solubility and polarity range of acetone. The suitable solvent was revealed for bioactive compounds and antioxidant activities extraction from raw betel nut seed.

Keywords : Antioxidant, *Areca catechu* L., Fractionation, HPLC, Solid-liquid extraction

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Introduction

A. catechu L. or betel nut is widely distributed in South East Asia, including Thailand. This palm family plant was reported its potential biological activity obtaining from many of its aerial parts. The nut was the most potential part. Its seed was used in traditional medicine as it had been reported the anti-inflammatory, anti-hyaluronidase [Lee & Choi, 1999; Bhandare *et al*., 2010], anti-tyrosinase, anti-elastase [Moon *et al*., 2010], anti-fungal activities [Yenjit *et al*., 2010], and enzymatic antioxidant [Lee *et al*., 2003]. The biological activities are attributed to the secondary metabolites of polyphenols in *A. catechu* L. Polyphenols which can be found in plants are divided into various groups. Flavonoids are included in the phenolics group that contain C6-C3-C6 backbone and the OH groups which possess potential antioxidant activities [Rice-Evan *et al*., 1996].

Antioxidants are employed in wide range of applications such as food process, packaging development, and also in cosmetics which also well-known as anti-aging agent [Lee & Choi, 1999]. However, the previous reports used only alcohol as a solvent for extraction without employing the lower or higher polarity solvents. Recently, there are many interesting methods for the development of bioactive compound extraction. The solid-liquid extraction is a common method in plant extraction using solvent as an extractant. The microwave-assisted method is widely used in the plant secondary metabolite extraction because the cell disruption process via microwave is powerful without thermal effect [Proestos & Komatis, 2008]. The multi residual extraction or re-extraction process complementary with proper solvent polarity is commonly used as it can achieve even the small amounts of the target compounds [Sobhanzadeh *et al*., 2012].

This study was aimed to compare the phenolic antioxidant from betel nut seed using stepwise microwave extraction of six different polarity solvents. The phenolic and flavonoid content, antioxidant capacity, reducing power and HPLC profile of the extracts were evaluated to compare the efficiency of each solvent extraction.

Materials and Methods

A. catechu L. seed extract preparation

Raw material: Raw betel nut seed was obtained from Nakorn Sri Thammarat, Thailand. The washed seed was dried at 50°C in a hot air oven. The dried sample was ground by hammer mill and sieved into 500 µm. The sample powder was kept at -20°C until used.

Extraction: The sample (10 g) was used in stepwise extraction of bioactive compounds with 100 ml of hexane, ethyl acetate, acetone, 95% ethanol, 50% ethanol, and water, respectively. The microwave power of 900 watts for 30 min was employed to assist the extraction efficacy. After extraction, the mixture was filtered and then filtrates were evaporated using rotary evaporator. The 50% ethanol and water fractions were further completely dried by lyophilizer. The obtaining dried crude extracts were kept in -20°C freezer until used.

Determination of bioactive compounds

The *in vitro* assays of extractable phenolics and flavonoids contents were carried out with some modifications from the previous report [Kumar *et al*., 2008].

Extractable phenolics content (EPC): The Folin-Ciocalteu's method was used in the determination of EPC. The reactions were colorimetric measured at 765 nm by microplate reader. The result was expressed in mg gallic acid equivalent per g extract (mg GAE/g extract)

Extractable flavonoids content (EFC): The EFC of each extract was determined by aluminum chloride colorimetric method. The reactions were colorimetric measured at 510 nm by microplate reader. The result was expressed in mg quercetin equivalent per g extract (mg QE/g extract).

Antioxidant activities determination

The *in vitro* assays of antioxidant activities by mean of DPPH and FRAP [Thaipong *et al*., 2006] were determined with some modifications.

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DPPH radical scavenging activity (DPPH): The stable radical 2,2-Diphenyl-1-picrylhydrazyl was used to test radical scavenging of the extracts. The reactions were colorimetrically measured at 517 nm by microplate reader. The result was expressed as mg trolox equivalent antioxidant capacity per g extract (mg TEAC/g extract).

Ferric reducing antioxidant power (FRAP): The reducing power of the extracts determined by the Fe(II)-TPTZ complex. The reactions were colorimetric measured at 593 nm by microplate reader. The result was expressed as mg trolox equivalent antioxidant capacity per g extract (mg TEAC/g extract).

HPLC analysis

The Waters Alliance HPLC system (Waters, Milford, MA, U.S.A.) of 2695 separation module equipped with 2996 photodiode array detector was used in this study. The stationary phase was 5 µm Altima C18 column (250 × 4.6 mm). The linear gradient of 100% solvent A (5% acetic acid in DI water) to 100% solvent B (methanol) was carried out through 75 min with the flow rate of 0.8 ml/min [Wang & Lee, 1996]. The resulting elution profiles were detected at 280 nm and compared with the standards.

Results and Discussion

A. catechu L. seed extracts

Appearances of dried crude extracts were shown in Figure 1. The first extraction step of hexane gave the pale-yellow semi-solid crude. The light-brown extract was obtained from extraction with ethyl acetate and 50% ethanol and the reddish-brown extract was from acetone and 95% ethanol extraction. The last fraction of water was almost white. The yielding weight was shown in Table 1.

Bioactive compounds in the *Areca catechu* L. seed extracts

Extractable phenolics content (EPC)

The EPC values of the extracts were found statistically different. As shown in Table 1, the acetone extract exhibited the highest content of 733.11 mg GAE/g extract, followed by 95% EtOH and EtOAc extract. This may imply that phenolic compounds are highly soluble after elimination of fats by the lowest polarity solvents of hexane. There have been reported the use of acetone in phenolic extraction from areca nut [Wang & Lee, 1996; Chavan & Singhal, 2013] employing 80% acetone at pH 4.0 combined with ultrasonic assistance provide maximum total phenol of 362.59 mg GAE/g from areca nut [Chavan & Singhal, 2013]. The 301 mg GAE/g from the whole fresh unripe areca fruit by using 80% acetone was also reported [Wang & Lee, 1996]. The lowest phenolics content was found in the last extraction with water. This may be due to the least residue bioactive compounds available after 5 steps of extraction

Extractable flavonoids content (EFC)

The highest EFC of 113.04 mg QE/g extract was found in acetone extract. However, it was not significantly different from that of ethyl acetate extract. The 95% EtOH and 50% EtOH extracts exhibited the EFC of 75.22 and 82.17 mg QE/g extract, respectively. These were comparable with the report of Zhang *et al*. (2009) in which the 77.36 mg/g was obtained from 70% EtOH reflux extraction. Similar to the EPC, the lowest EFC was found in the last step of water extract. 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, 1996]. The similar results also obtained in the partition extraction of phenolics antioxidant from *Lespedeza cuneata* [Kim & Kim, 2010]. The study showed that the last fraction of water extract exhibited the lowest EPC and EFC. The first fraction of hexane possessed EPC of 39.62 mg GAE/g sample which lower than methanol extract (46.33 mg GAE), but higher in EFC, 67.97 (hexane) and 32.73 mg QE/g sample (methanol). The results from this experiment also showed the higher correlation of the reducing power to EPC than EFC.

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Table 1 The bioactive compounds and biological activities of *A. catechu* L. seed extracts.

Extract	**Yield** (%)	**EPC** (mg GAE)	**EFC** (mg QE)	**DPPH** (mg TEAC)	**FRAP** (mg TEAC)
Hexane	8.45±0.85^{b**}	7.02±0.52^{f}	11.33±0.76^{d}	0.36±0.09^{e}	0.27±0.00^{d}
EtOAc	1.60±0.20^{e}	446.96±5.07^{c}	112.17±0.44^{a}	105.51±0.47^{c}	99.05±2.38^{b}
Acetone	11.25±1.85^{a}	733.11±5.57^{a}	113.04±3.35^{a}	184.36±7.75^{a}	126.15±13.44^{a}
95%EtOH	5.70±0.10^{c}	479.05±10.44^{b}	75.22±0.73^{c}	126.83±11.13^{b}	92.16±5.93^{b}
50%EtOH	4.95±0.05^{cd}	240.88±13.63^{d}	82.17±0.64^{b}	84.90±4.44^{d}	21.55±2.91^{c}
Water	3.80±0.00^{d}	41.47±0.43^{e}	6.73±0.20^{e}	0.61±0.07^{e}	5.44±0.55^{d}

* The results were presented in mean±SD (n=3) standard equivalent per gram extract

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

Antioxidant activities of *A. catechu* L. seed extracts

DPPH radical scavenging activity (DPPH)

The radical scavenging activity of the extracts was investigated by means of the decolorizing of stable DPPH radical. The highest activity was obtained from acetone extract of 184.36 mg TEAC/g extract followed by the 95% ethanol and ethyl acetate extracts, respectively. The first and last extraction steps of hexane and water gave the lowest activity. Comparison of antioxidant capacity in the seed, flower and husk of areca nut showing the seed extract possessed the higher DPPH and hydroxyl radical scavenging activities and reducing power than other two extract [Zhang *et al*., 2009].

Ferric reducing antioxidant power (FRAP)

The reducing power of the extracts was determined by observing its ability to reduce Fe(III) to Fe(II) with the complex with TPTZ. The activity was similar to the DPPH, and the highest reducing capacity was also found in the acetone extract. The extracts were found to have significant difference with each others. Similarly, the lowest value of water and hexane extracts was not statistically different.

The antioxidant activities were found to be related with the EPC rather than the EFC. High EPC and antioxidant were observed in the acetone extract. This result indicated that the phenolic antioxidant in the betel nut probably exhibit polarity similar to acetone. The similar results was reported in the liquid-liquid extraction of antioxidant from *Callistemon lanceolatus* stem [Kim *et al*., 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 of Zhang *et al*. (2010) showed that the partitioning of areca nut seed extract by light petroleum, ethyl acetate, and n-butanol possessed the consistent results. The highest DPPH radical scavenging activity exhibited from ethyl acetate fraction with the IC_{50} of 27.6 µg/ ml. The lower activities were found in n-butanol (85.1 µg/ ml) and light petroleum fraction (>100 µg/ ml).

HPLC profiles of *A. catechu* L. seed extracts

The extract (5.0 mg/ml) were investigated their elution profiles by HPLC. The obtained profiles in Figure 1 show the presence of the valuable compounds in the sample. After the elimination of the fatty compounds by hexane, the higher extraction potential was obtained. The interested peaks of all extracts, except for hexane extract, had similar retention time range of 12- 25 min. which is also in the range of catechin-like compounds in the standard (Figure 1). The literatures also reported that the major polyphenols in the betel nut are catechin,

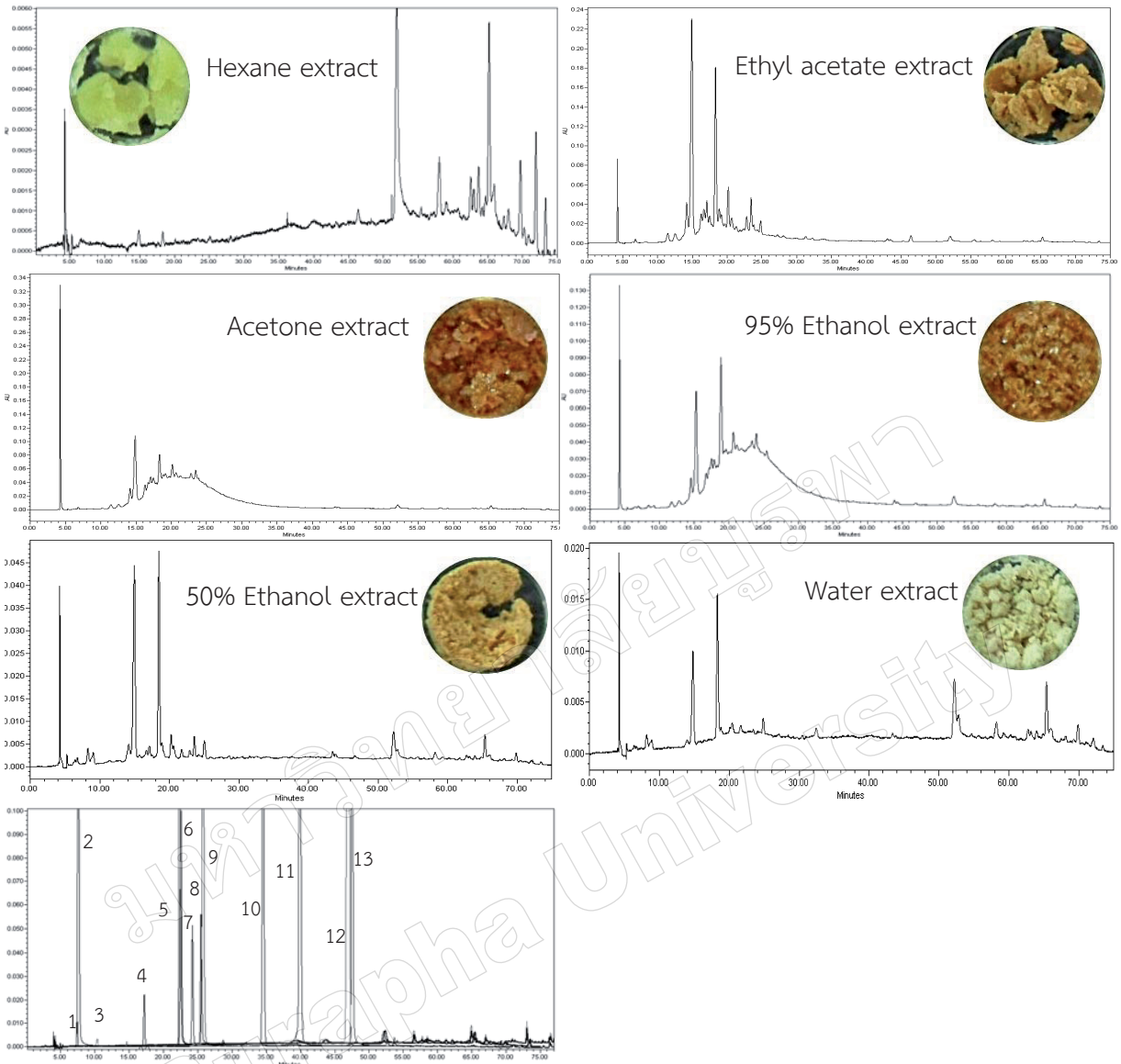

Figure 1 HPLC elution profile and the appearance of *Areca catechu* L. seed extracts and phenolics standards; (1) kojic acid, (2) gallic acid, (3) gallocatechin, (4) catechin, (5) epigallocatechingallate, (6) chlorogenic acid, (7) epicatechin, (8) gallocatechingallate, (9) caffeic acid, (10) ferulic acid, (11) ellagic acid, (12) cinnamic acid, (13) quercetin

epicatechin, gallic acid ellagic acid and anthocyanins [Wang & Lee, 1996; Huang *et al*., 2010]. The higher peak areas were obtained from the acetone and 95% ethanol with the elution profile in the catechin-like region. In addition, unknown peak in all extract was found in the early retention time of approximately 4 min which may be related to others bioactive compound. After the broad peak were extracted out, the sharper peaks, but lower in the peak area, were obtained from 50% ethanol and water extract at 14, 15, and 19 min, approximately.

Conclusion

The *A. catechu* L. seed extracted from different polarity solvents showed alter phenolic content,

antioxidant capacity, reducing power and characters in HPLC elution profiles. Acetone gave the highest potential for multi-residual solid-liquid microwave-assisted extraction of bioactive compounds from *A. catechu* L. seed. The interesting peaks which were suspected to be the catechin-like groups required further investigation to elucidate the other bioactive compounds in *A. catechu* L. seed.

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