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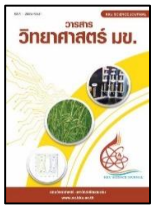

การใช้คลื่นเสียงความถี่สูงและไมโครเวฟช่วยในการสกัดสารออกฤทธิ์ทางชีวภาพจากเมล็ดข้าวสี

Sonication- and Microwave-Assisted Extraction of Bioactive Compounds from Pigmented Rice Varieties

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บทคัดย่อ

ข้าวสีของไทยจัดเป็นข้าวฟังก์ชันเพราะมีสารประกอบกลุ่มฟีนอลลิกและฟลาโวนอยด์ในปริมาณที่สูง ทำให้สามารถต้านออกซิเดชันได้ดี ซึ่งเป็นคุณสมบัติที่อุตสาหกรรมเครื่องสำอางต้องการ รวมทั้งมีสมบัติการยับยั้งเอนไซม์ที่เกี่ยวข้องกับความหมองคล้ำของผิว เช่น ไทโรซิเนส เป็นต้น งานวิจัยนี้ได้ศึกษาเปรียบเทียบวิธีการสกัดสารผสมที่มีสมบัติเป็นองค์ประกอบในเครื่องสำอางจากเมล็ดข้าวกล้องสี 3 วิธี คือ (1) การแช่ในสารละลายฟอสเฟตบัฟเฟอร์ซาไลน์ พีเอช 7.4 (Phosphate buffer saline: PBS, pH 7.4) (2) การแช่ใน PBS ร่วมกับการใช้คลื่นเสียงความถี่สูง (PBSS) เพื่อช่วยทำลายโครงสร้างของผนังเซลล์พืช และ (3) การแช่ใน PBS ร่วมกับการใช้คลื่นเสียงความถี่สูงและไมโครเวฟ (PBSSM) เพื่อช่วยเพิ่มอุณหภูมิในการสกัด การทดสอบด้วยข้าวสีของไทยจำนวน 8 พันธุ์ ประกอบด้วย ข้าวสีแดง 3 พันธุ์ คือ ข้าวเจ้ามะลิแดง (MD) ข้าวมะลิโกเมน (MK) และข้าวสังข์หยด (SY) ข้าวสีม่วงเข้ม 3 พันธุ์ คือ ข้าวเหนียวดำมั้ง (DM) ข้าวเหนียวดำลืมผัว (NLP) และ ข้าวไรซ์เบอร์รี่ (RB) ข้าวสีดำ 2 พันธุ์ คือ ข้าวหอมนิล (HN) และ ข้าวเมล็ดฝ้าย (MF) พบว่า PBSS และ PBSSM ให้ผลการสกัดสารประกอบกลุ่มฟีนอลลิก ฟลาโวนอยด์ โปรตีน กิจกรรมการต้านออกซิเดชันด้วย FRAP และกิจกรรมยับยั้งเอนไซม์ไทโรซิเนสสูงกว่าการแช่ใน PBS และเมื่อเทียบกับ PBSS การใช้ไมโครเวฟ (PBSSM) ให้ผลดีขึ้น ลดลง หรือเท่าเดิมขึ้นอยู่กับพันธุ์ข้าวและลักษณะที่ศึกษา ยกเว้นฤทธิ์การต้านออกซิเดชันด้วย DPPH ที่ลดลงอย่างมากในข้าวทุกพันธุ์เมื่อใช้ไมโครเวฟ ข้าวสีม่วงเข้ม

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หรือดำมีฟีนอลลิก ฟลาโวนอยด์ โปรตีน ฤทธิ์การต้านออกซิเดชัน และกิจกรรมยับยั้งไทโรซิเนสสูงกว่าข้าวสีแดง ดังนั้นข้าวเหนียวลืมผัว ข้าวหอมนิล ข้าวเหนียวดำมัง ข้าวเมล็ดฝ้าย และข้าวไรซ์เบอร์รี่มีศักยภาพที่จะนำมาใช้สกัดสารสำคัญเพื่อใช้เป็นส่วนประกอบในเครื่องสำอาง เนื่องจากมีสมบัติที่ดีในการต้านออกซิเดชันและต้านเอนไซม์ไทโรซิเนส นอกจากนี้ยังพบว่าสารละลายฟอสเฟตบัฟเฟอร์ซาไลน์สามารถสกัดสารสำคัญได้ดีไม่แพ้ตัวทำละลายอินทรีย์และมีประสิทธิภาพในการสกัดมากขึ้นเมื่อใช้ร่วมกับคลื่นเสียงความถี่สูง

ABSTRACT

Thai pigmented rice has been classified as functional rice because of high accumulation of phenolics and flavonoids, which are powerful antioxidants. This property attracts cosmeceutical industry in addition to the inhibitory effects on the skin aging enzymes such as tyrosinase. This work compared three extraction methods, to extract mixtures of compounds that possessed cosmeceutical ingredient properties from pigmented brown rice, including (1) soaking in phosphate buffer saline pH 7.4 (PBS), (2) soaking in PBS followed by incubation in an ultrasonic sonication bath (PBSS) to help destroy plant cell walls, and (3) soaking in PBS followed by sonication and microwave treatment (PBSSM) to increase extraction temperature. Eight varieties of Thai pigmented rice, comprising three varieties of red rice (Mali Dang: MD; Mali Ko Mane: MK; Sang Yod: SY), three deep-purple rice (Dam Mong (DM); Neaw Leum Pua (NLP); Riceberry (RB)) and two black rice (Hom Nil (HN); Maled Fai (MF)) were used. PBSS and PBSSM revealed higher phenolics, flavonoids, protein and better antioxidation activity based on Ferric reducing antioxidant power (FRAP) as well as tyrosinase inhibitory effect than that of PBS. In comparison with PBSS, the use of microwave (PBSSM) enhanced, reduced or did not alter the amounts of bioactive compounds or functional activities depending on rice varieties and the tested properties, except for antioxidant capacity based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity which was dramatically destroyed in all rice varieties with microwave treatment. Regardless of the extraction methods, the purple and black rice exhibited much higher bioactive compounds, antioxidant capacity, and anti-tyrosinase activity than red rice. Rice varieties with deep-purple or black pericarp like NLP, HN, DM, MF and RB, may therefore be employed as potential raw materials for application as cosmetic ingredients providing good antioxidant and anti-tyrosinase properties. Moreover, the amount of phytochemicals extracted by PBS was comparable to those reported using organic solvents, and sonication further enhanced the extraction efficiency.

คำสำคัญ: การยับยั้งเอนไซม์ไทโรซิเนส การต้านออกซิเดชัน การสกัดด้วยคลื่นเสียงความถี่สูง สารประกอบฟีนอลิก ข้าวสี

Keywords: Anti-tyrosinase, Antioxidation, Sonication-assisted Extraction, Phenolic Compounds, Pigmented Rice

INTRODUCTION

Rice is the most important staple food which feeds more than half of the world's population. In 2022, Thailand is ranked number two rice exporter after India, and exported 7.639 million tons of mostly polished white rice (Thai Rice Exporter Association, 2023). White rice is daily consumed worldwide whereas consumption of pigmented or colored rice is common in some countries in Asia like China, Japan, Korea, Thailand, India and Sri Lanka (Das *et al*., 2023). However, in recent years the interest in consuming pigmented rice, regarded as functional rice, has grown enormously due to its much greater health benefits associated with high contents of phytochemicals including phenolic compounds, proteins, vitamins, minerals, and dietary fiber which are present predominantly in the colored pericarp layer (Samyor *et al*., 2017; Priya *et al*., 2019). Phenolic compounds in pigmented rice were reported to exhibit high antioxidant capacity, anti-inflammatory, anti-glucosidase, anti-diabetic and cancer preventive effects (Boue *et al*., 2016; Gao *et al*., 2018; Sripanidkulchai *et al*., 2022). The major classes of phenolic compounds or polyphenols in pigmented rice are phenolic acids (such as p-phenoxybenzoic acid, protocatechuic acid, vanillic acid, p-coumaric acid, sinapic acid, and ferulic acid), flavonoids (including flavonols, anthocyanin and procyanidin), stilbene, coumarins and tannin (Zaupa *et al*., 2015). Both phenolic acids and flavonoid components were reported to be associated with antioxidant capacity in pigmented rice extracts (Chen *et al*., 2022).

Recently, high antioxidant capacity in pigmented rice has attracted interest among researchers to incorporate pigmented rice extracts in cosmetic formulations for skin anti-aging effects (Teeranachaideekul *et al*., 2018; Tikapunya *et al*., 2023). Skin aging is induced by both intrinsic and external factors resulting in dryness, wrinkles, loss of elasticity, rough textured appearance, and dark spots (Zhang and Duan, 2018). Oxidative stress in skin cells initiated by reactive oxygen species (ROS) was implicated as one of the most important causes of skin aging in relation to induction of melanogenesis by increasing the amount of tyrosinase, increasing wrinkles by accelerating collagen breakdown, altering skin texture due to oxidation of lipids and proteins, and induction of inflammatory lesions (Masaki, 2010; Zhang and Duan, 2018). It was well-documented that treatments with plant-derived polyphenols can enhance resistance to oxidative stress and prevent skin premature aging (Yadav *et al*., 2015). In a previous study, Teeranachaideekul *et al*. (2018) suggested that extracts from five pigmented rice varieties (with red, purple and black pericarp) were promising ingredients for cosmetics for anti-aging applications due to high antioxidant capacity, anti-collagenase, and anti-elastase activity. High total phenolic content (TPC), antioxidant capacity, and anti-tyrosinase activity were reported to be present in hot water extracts of deep purple rice grains variety Riceberry (Poomanee *et al*., 2021). Tikapunya *et al*., (2023) demonstrated the potential of Riceberry grain extracts as natural ingredients in body cream products due to their high TPC, high total flavonoid content (TFC), antioxidant capacity, and non-cytotoxicity. In addition to phytochemicals like polyphenols and flavonoids, rice proteins, protein hydrolysate and peptides have been reported to have functional properties relating to cosmetic applications. Rice protein hydrolysates were reported to exhibit high antioxidant capacity, anti-hyaluronidase and anti-tyrosinase activity (Chen *et al*., 2021).

Bioactive compounds from plants are conventionally extracted using large amount of toxic solvents (eg. ethanol, methanol, acetone and hexane), with laborious process like Soxhlet extraction and maceration (Ameer *et al*., 2017). Recently, more eco-friendly techniques such as sonication, microwave, pressurized hot water, parboiling, supercritical fluid extraction etc. have been suggested for more efficient extraction of bioactive compounds from rice (Andriani *et al*., 2022). Phenolic compounds in rice are present in free form or bound to structural components of the cell wall such as cellulose, proteins, sugars and lignin causing low extractability (Rocchetti *et al.*, 2022). Ultrasound-assisted extraction (UAE) has emerged as a simple and eco-friendly technology to increase extraction efficiency of phenolic compounds from plants (Tao *et al.*, 2014). Acoustic energy generated from ultrasound poses several actions that can increase the efficiency of extraction of bound phenolics such as breakdown of plant cell wall, capillary effects, more solvent penetration, higher mass transfer and higher diffusion (Mason *et al.*, 2011; Wang *et al.*, 2020). It was reported that UAE in a sonication bath for 22 min resulted in more than two folds increase in TPC yield from purple and black rice bran compared with the conventional extraction (Das *et al.*, 2017). Enhanced yields of bioactive compounds and antioxidant capacity from pigmented rice bran by sonication treatment were also reported by Tabaraki and Nateghi (2011), Irakli *et al.* (2018), and Surin *et al.* (2020). Microwave-assisted extraction (MAE) has recently attracted the attention of researchers as an alternative to conventional techniques to extract more bioactive compounds in shorter time using less volume of solvent (Ciulu *et al.*, 2018). Microwave treatment of rice bran at 440W for 2.5 min was reported to provide the best contents of TPC, TFC, and antioxidant activity (Pokkanta *et al.*, 2022). Jha *et al.* (2017) reported that the best yield of phenolic compounds in black rice husk was obtained from a combination of 10 min of sonication and 31 s of microwave treatment.

This study aimed to extract bioactive compounds (total phenolics, total flavonoids and total proteins) from pigmented rice grains using non-toxic aqueous solvent (Phosphate buffered saline: PBS) for future application as cosmetic ingredients, and to increase the efficiency of extraction of bioactive compounds, anti-oxidation capacity, and anti-tyrosinase activity using sonication- and microwave-assisted extraction.

MATERIALS AND METHODS

1. Plant Materials

Eight varieties of pigmented rice were used in this experiment. Four commercial varieties were purchased from local supermarkets including the black rice Hom Nil **(HN)**, the red rice Mali Dang **(MD)**, the deep-purple rice Riceberry **(RB)**, and the red rice Sang Yod **(SY)**. The fifth commercial variety Neaw Leum Pua **(NLP)**, a deep-purple glutinous rice, was obtained from farmers in Phetchaboon Province. The other three varieties were indigenous rice collected at and kindly provided by the Department of Agronomy, Faculty of Agriculture, Khon Kaen University. These varieties included the deep-purple glutinous rice Dam Mong **(DM)**, the black rice Maled Fai **(MF)**, and the red rice Mali Ko Mane **(MK)**.

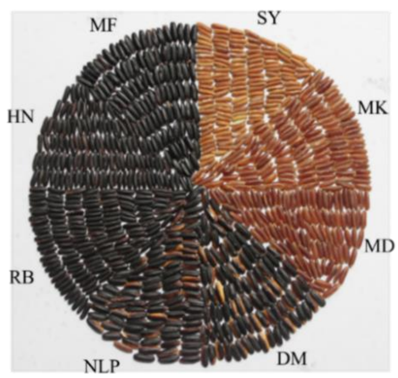

Figure 1 Dehulled grains of eight varieties of pigmented rice. (Sang Yod (SY); Mali Ko Mane (MK); Mali Dang (MD); Dam Mong (DM); Neaw Leum Pua (NLP); Riceberry (RB); Hom Nil (HN); Maled Fai (MF))

2. Methods of Extraction

The dehulled pigmented rice grains were finely ground and sieved through a 200-mesh stainless steel wire screen. Three grams of samples were then used for each extraction method with three replications. For Treatment 1 (Phosphate buffered saline **(PBS)** containing 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 130 mM NaCl, 2.7 mM KCl, pH 7.4), each sample was extracted with 30 ml of PBS (pH 7.4) for 1 h on a shaker. In Treatment 2 **(PBSS)** each sample was extracted with 30 ml PBS (pH 7.4) followed by 1 h in a sonication bath. For Treatment 3 **(PBSSM)**, each sample was extracted with 30 ml PBS (pH 7.4) followed by 1 h of sonication then by twice treatments in a microwave oven set at 800 w for 10 seconds each. All samples of seed extracts were centrifuged at 15,000 rpm, aliquoted, and kept in -20 °C freezer until use. This experiment started with NLP, HN and MD, which are representatives of deep-purple glutinous, black and red rice, respectively for the comparison of PBS, PBSS and PBSSM methods. After determination of the optimal conditions, the remaining 5 varieties of pigmented rice (DM, MF, RB, MK and SY) were then extracted following the optimal conditions.

3. Assays for bioactive compounds, antioxidant properties, proteins and tyrosinase inhibition activity

3.1 Total phenolic content (TPC)

The total phenolic content was determined according to Razak *et al*. (2019) in 96-well microplate using the Folin-Ciocalteu method with some modifications. The extract sample or standard gallic acid (50 µl) was added to 50 µl of 20% Folin-Ciocalteu reagent followed by 50 µl of 10% sodium carbonate. After 30 min the absorbance at 765 nm was measured in a microplate reader (SpectraMax M5, Molecular devices, USA). The reaction was carried out in triplicate and expressed as mg of gallic acid equivalent (GAE) per g of sample (mg GAE/g sample).

3.2 Total flavonoid content (TFC).

The aluminum chloride colorimetric method modified from that of Razak *et al*. (2019) was used for determination of the TFC in 96-well microplates. The extract sample or standard quercetin (50 µl) was added to 0.25M potassium acetate (40 µl) and left for 5 min at room temperature, then added with 2.5% $AlCl_3$ solution (40 µl). After 15 min the absorbance at 430 nm was measured in the microplate reader (SpectraMax M5, Molecular devices, USA). The total flavonoid content was determined in triplicate and expressed as quercetin equivalents per g of sample (mg QE/g sample).

3.3 Ferric reducing antioxidant power (FRAP) assay.

Ferric Reducing Antioxidant Power (FRAP) assay was performed according to Benzie and Strain (1996) and Razak *et al*. (2017) with some modifications. Three stock solutions for the assay included Solution 1: 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCL; Solution 2: 20 mM $FeCl_3$ $6H_2O$; and Solution 3: 20 mM sodium acetate buffer pH 3.6. The FRAP working solution was freshly prepared by mixing Solution 1, Solution 2 and Solution 3 at the ratio of 4:4:10 and kept in the dark bottle. The extract sample (20 µl) was aliquoted into 96-well microplate, then 180 µl of FRAP working solution was added and kept in the dark for 5 min. The absorbance was read at 595 nm in the microplate reader using 2 mM $FeSO_4.7H_2O$

as standard. FRAP activity was determined in triplicate and expressed as mM Fe(II) equivalent per gram of sample.

3.4 DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The DPPH assay was performed following that of Razak *et al.* (2019) with some modifications. Extract of 40 µl was mixed with 100 µl 0.25 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol and left in the dark for 5 min before measurement of absorbance at 490 nm (As). The As was subtracted by sample background of 40 µl sample in 100 µl methanol (A_{bg}) and control 40 µl PBS and 0.25 mM DPPH in 100 µl methanol (Ac). The DPPH radical scavenging activity was determined in triplicate and expressed as DPPH radical scavenging activity (%) according to the following equation.

$$\text{DPPH radical scavenging activity (\%)} = [\ A_s - (A_c + A_{bg}) / A_c] \times 100$$

3.5 Protein assay

The protein content in the extract sample was determined using Bradford reagent by performing in 96-well microplate based on the method described by Bradford (1976). The extract sample (20 µl) was added to a 96-well microplate followed by the addition of 100 µl Bradford reagent (Sisco Research Laboratories Pvt. Ltd.). The mixture was left at room temperature for 1 min and the absorbance was read at 595 nm (A_{595}). The protein assay was determined in triplicate and expressed as micrograms of bovine serum albumin (BSA) equivalent per gram of sample.

3.6 Tyrosinase inhibition activity

Tyrosinase inhibitory assay is based on the reduction of dopachrome synthesis from L-3,4-dihydroxyphenylalanine (L-DOPA) in 96-well microplates. Each reaction contains 40 µl of the extract sample or standard tyrosinase inhibitor (Kojic acid), 40 µl of 0.1 M phosphate buffer pH 6.8, 40 µl of mushroom tyrosinase (32 U/ml) and the reaction started by adding 40 µl of 10 mM L-DOPA, the reaction substrate. The reaction was left for 20 min at 25 °C in the dark before determining the absorbance of dopachrome product at 490 nm (A_{490}) (Razak *et al.*, 2019). Tyrosinase inhibition activity was carried out in triplicate and expressed as % inhibition activity (%) according to the equation below.

$$\text{Tyrosinase inhibition activity (\%)} = 100 - [\ (A_{sample} - A_{background}) \times 100 / A_{blank}]$$

4. Statistical analysis

The data were presented as mean ± SD, test of significance differences among data were performed using one-way ANOVA in IBM SPSS® Statistics version 28.0.1(142). Multivariate analysis including hierarchical cluster analysis was performed to differentiate rice samples based on the six investigated parameters including TPC, TFC, protein, FRAP and DPPH radical scavenging activity and anti-tyrosinase activity using OriginPro 2022 v.9.9.0.225 (OriginLab Corporation, Northampton, MA, USA).

RESULTS

1. Bioactive compounds in seed extracts of HN, MD and NLP using three extraction methods

The results on the preliminary evaluation of contents of bioactive compounds present in seed extracts of three varieties of pigmented rice (HN, MD and NLP) are shown in Figure 2. The PBSS and PBSSM method had neutral, positive, or negative effects, compared with the basic PBS, on phytochemical contents and bioactive properties depending on rice varieties. In comparison to extraction with PBS alone (PBS), sonication (PBSS) could enhance the efficiency of PBS extraction resulting in significantly increased total phenolic content (TPC) in seed extracts from HN and NLP (Figure 2A), total protein in NLP (Figure 2C), DPPH scavenging activity in MD (Figure 2E), and tyrosinase inhibition in NLP (Figure 2F). However, sonication did not promote the total flavonoid content (TFC) (Figure 2B) and FRAP activity in any of the three rice varieties (Figure 2D). Sonication combined with microwave (PBSSM) treatment significantly increased TPC in MD seed extracts compared with PBS and PBSS (Figure 2A). PBSSM significantly increased total protein contents in seed extracts from all three rice varieties (Figure 2C). With respect to antioxidant capacity, PBSSM was effective in increasing FRAP activity only in MD (Figure 2D). Interestingly, the highest tyrosinase inhibitory activity in all three rice varieties was observed in PBSSM extracts (Figure 2F). Surprisingly, microwave treatment dramatically reduced DPPH scavenging activity in all three rice varieties (Figure 2E).

Among the three rice varieties, MD (red rice) contained the lowest content of bioactive compounds (TPC, TFC and total protein), the lowest antioxidation capacity (FRAP and DPPH radical scavenging activity), and the lowest tyrosinase inhibition activity. However, MD tended to be more responsive to microwave treatment (PBSSM) showing significantly higher TPC, total protein, FRAP activity and tyrosinase inhibition activity compared with PBSS. The highest TPC was found in NLP (deep purple rice) treated with PBSS (3.57 mg GAE/g) followed by PBSSM (3.10 mg GAE/g). Similarly, the highest content of TFC was also present in NLP treated with PBSSM (9.05 mg QE/g) followed by PBSS (7.76 mg QE/g). Although microwave treatment was favorable for the extraction of TFC from NLP, it significantly destroyed TFC in HN (Figure 2B). The highest protein content was obtained from HN (black rice) extracted by PBSSM (12.46 mg/g) and the lowest in MD extracted with PBS (6.71 mg/g) (Figure 2C). The antioxidant capacity was comparable in HN and NLP which were almost two folds higher than that in MD. The highest FRAP activity was obtained in HN extracted by PBSS (15.99 mmol Fe(II) equivalent/g) and NLP extracted by PBSSM (15.16 mmol Fe(II) equivalent/g). Similarly, HN extracted by PBSS also showed the highest DPPH radical scavenging activity of 61.63%. The microwave heating considerably destroyed certain types of bioactive compounds leading to 3 to 4 folds reduction in DPPH activity in all rice varieties (Figure 2E). On the other hand, microwave significantly increased % tyrosinase inhibitory activity showing the highest activity in NLP extracted by PBSSM (64.10%) followed by HN extracted by PBSSM (61.04%). Therefore, PBSS and PBSSM were effective methods for increasing bioactive compounds and activity related to cosmetic applications and were further used for extraction of the second set of pigmented rice varieties including DM, MF, RB, MK and SY.

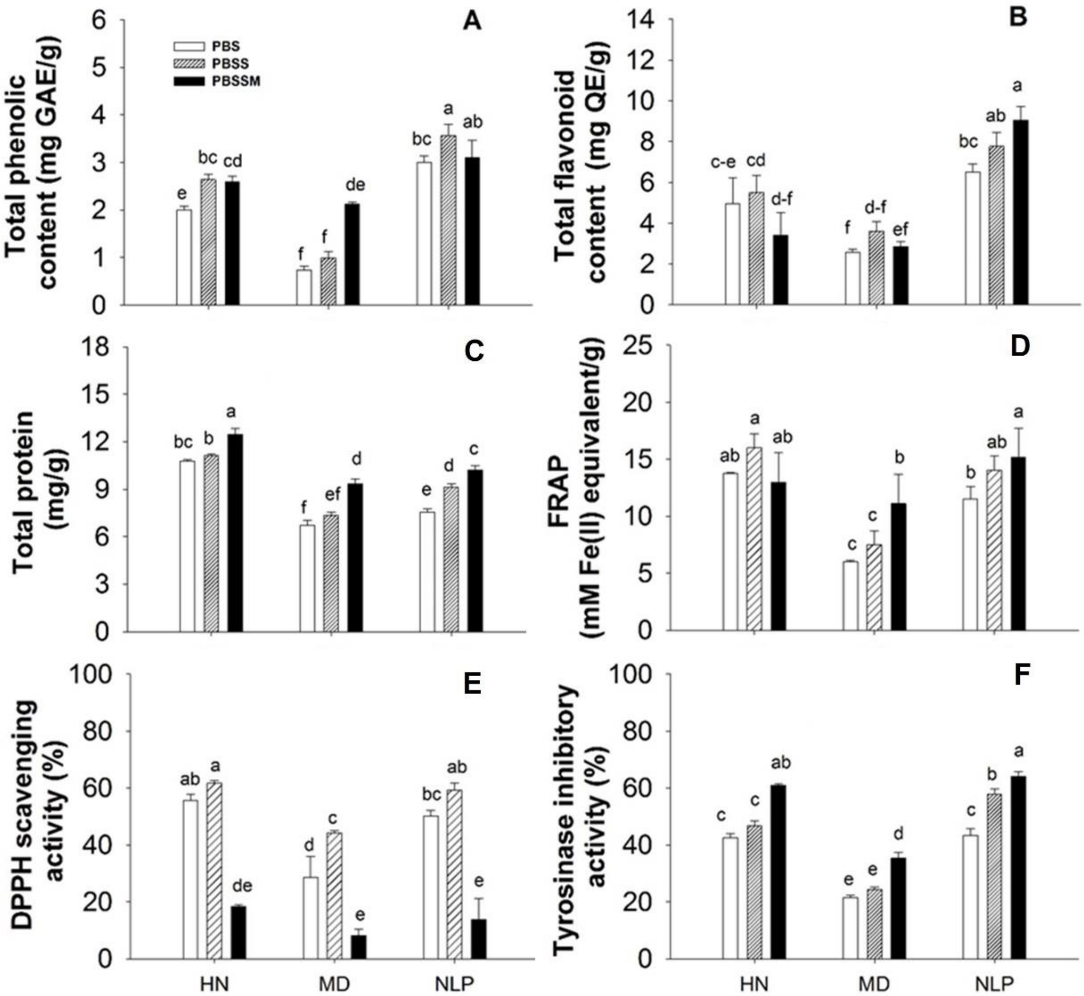

Figure 2 Contents of phytochemicals including total phenolic content (A), total flavonoid content (B) and total protein (C); antioxidant capacity including FRAP (D) and DPPH scavenging activity (E); and tyrosinase inhibitory activity (F) in seed extracts of rice varieties Hom Nil (HN), Mali Dang (MD) and Neaw Leum Pua (NLP) using three different extraction methods (PBS, Phosphate buffered saline; PBSS, PBS followed by sonication; PBSSM, PBSS followed by microwave heating). For each parameter, means ± SD with different lowercase letters are significantly different at $p < 0.05$ according to Duncan's multiple range test.

2. Bioactive compounds in seed extracts of five rice varieties using sonication- and microwave-assisted methods

The sonication-assisted (PBSS) and sonication + microwave-assisted (PBSSM) methods were performed to extract bioactive compounds from seeds of five rice varieties, and the results showing the TPC, TFC, total protein and % tyrosinase inhibitory activity are presented in Figure 3. Among the five varieties, MF (black rice) contained the highest TPC content followed by DM (deep-purple rice) (Figure 3A, B). The TPC in MF seeds extracted by PBSS and PBSSM were 7.8 and 7.34 mg GAE/g which were 2 to 6 folds more concentrated than those in RB (deep-purple rice), MK and SY (red rice). Microwave treatment significantly increased TPC in DM, MK and RB compared with those extracted by PBSS. Similarly, seeds of MF and DM contained the highest TFC which are 3 to 4 folds higher than that in MK, RB and SY (Figure 3C,D). When extracted by PBSS the TFC in seed extracts of DM and MF were almost equal (8.62 and 8.57 mg QE/g, respectively) (Figure 3C). However, microwave treatment was favorable for flavonoid extraction of DM leading to a significant increase in TFC to 11.25 mg QE/g (Figure 3D). When the extraction was assisted by sonication, seed extracts from all five varieties had comparable total proteins ranging from 9.49 mg/g in DM to 7.77 mg/g in SY (Figure 3E). Interestingly, microwave treatment (PBSSM) was able to extract significantly more proteins from seeds of all rice varieties compared with PBSS, especially DM and MF which showed 13.51 and 12.60 mg/g protein, respectively (Figure 3F). For tyrosinase inhibitory activity, DM and MF also showed significantly higher activity than MK, RB and SY (Figure 3G,H). Microwave treatment was able to significantly increase tyrosinase inhibitory activity in DM, MF and RB. The highest activity for tyrosinase inhibition was observed in extracts of DM (58.19%) and MF (57.79%) seeds by the PBSSM method. Although RB had much lower TPC and TFC than those of DM and MF (Figure 3A-D) its tyrosinase inhibitory activity was only slightly lower showing 41.60 and 54.95% by PBSS and PBSSM method, respectively.

The antioxidation capacity in seeds of the five rice varieties extracted by PBSS and PBSSM methods was displayed in Figure 4. The antioxidation capacity based on FRAP assay was 3 to 4 folds higher in DM and MF than that in MK, RB and SY (Figure 4A,B). Furthermore, microwave treatment (PBSSM) was favorable for DM, for extracting certain bioactive compounds which could reduce ferric to ferrous ions, leading to significantly higher FRAP activity compared with the PBSS extracts. The antioxidation capacity based on the levels of DPPH radical scavenging activity, on the other hand, was not much different among all five varieties ranging from 41.25 (in SY) to 53.63% (in DM) (Figure 4C). In contrast to the effects on FRAP activity, the microwave treatment (PBSSM) severely destroyed bioactive components relating to scavenging DPPH radicals in seed extracts of all five rice varieties compared to that in PBSS extracts, hence reducing DPPH assay values to the range of 25.47 (in RB) to 35.78% (in SY) (Figure 4D).

Therefore, the deep purple (DM) and black rice (MF) contained considerably higher contents of bioactive compounds (TPC, TFC and protein), higher antioxidation capacity (FRAP) and higher tyrosinase inhibitory activity than the red rice (MK and SY) in their extracts. Considering the cosmeceutical relating properties, the purple rice RB had a relatively high tyrosinase inhibitory activity comparable to that of DM and MF. The method combining microwave and sonication (PBSSM) enhanced the efficiency of extraction for all bioactive compounds and properties (except for the DPPH radical scavenging) compared with sonication alone.

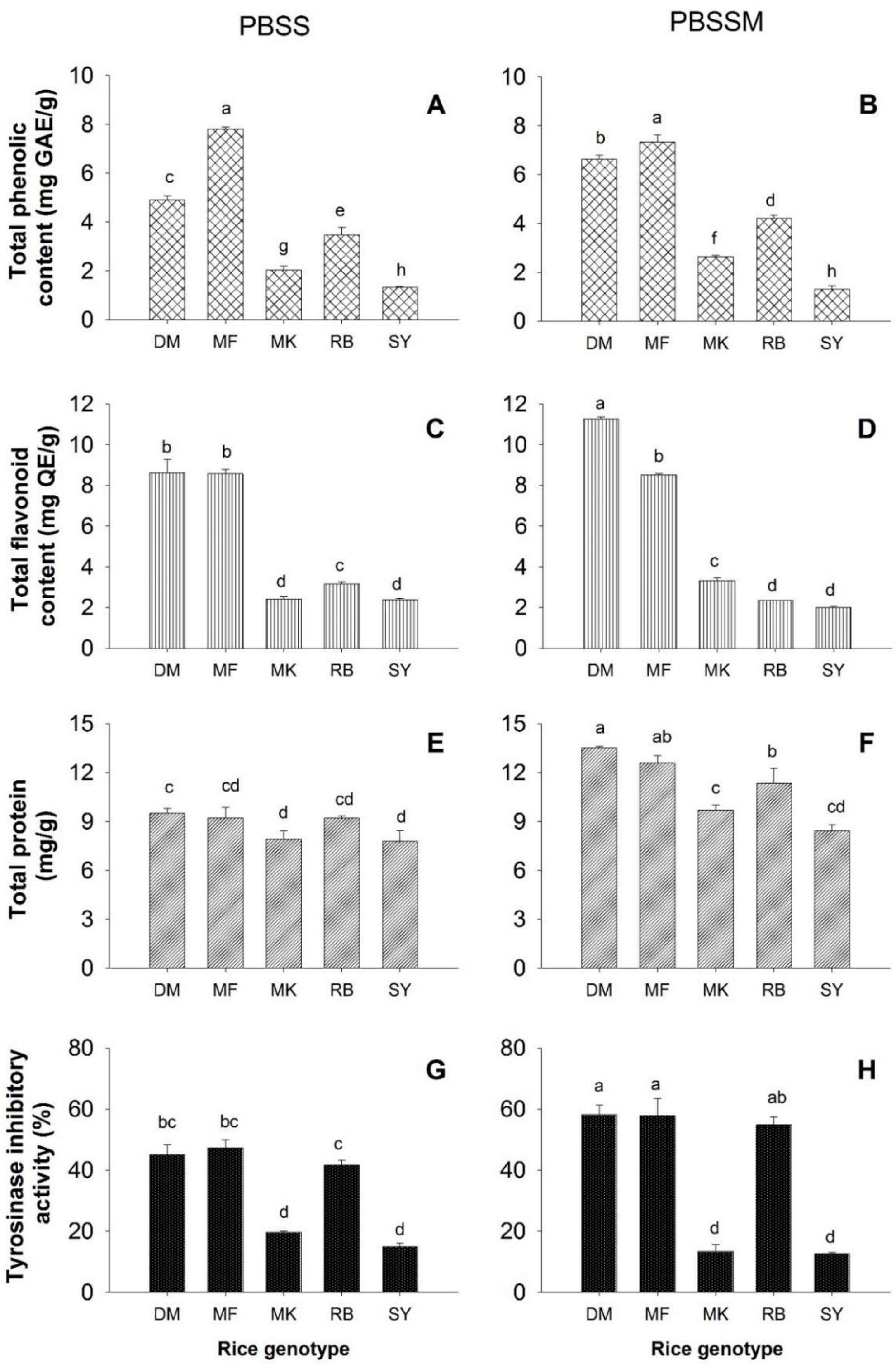

Figure 3 Contents of phytochemicals including total phenolic content (A,B), total flavonoid content (C,D), and total protein (E,F); and tyrosinase inhibitory activity (G,H) in seed extracts of five rice varieties using sonication-assisted (PBSS; A,C,E,G) and microwave-assisted (PBSSM; B,D,F,H) methods. Dam Mong (MD); Maled Fai (MF); Mali Ko Mane (MK); Riceberry (RB); Sang Yod (SY). For each parameter, means ± SD with different lowercase letters are significantly different at $p < 0.05$ according to Duncan's multiple range test.

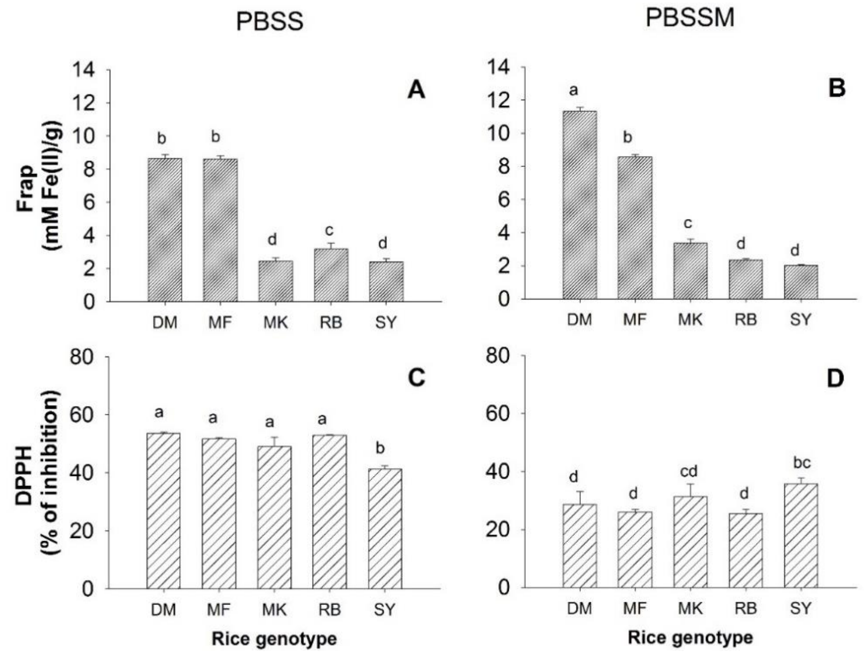

Figure 4 Antioxidation capacity as determined by FRAP activity (A,B) and DPPH radical scavenging activity (C,D) in seed extracts of five rice varieties by PBSS (A,C) and PBSSM (B,D) methods. Dam Mong (DM); Maled Fai (MF); Mali Ko Mane (MK,); Riceberry (RB,); Sang Yod (SY); PBSS, extraction using PBS buffer followed by sonication; PBSSM, extraction using PBS buffer followed by sonication and microwave treatment. For each parameter, means ± SD with different lowercase letters are significantly different at $p < 0.05$ according to Duncan's multiple range test.

3. Cluster analysis of rice varieties

In order to assess the relationship among all eight rice varieties, the data of TPC, TFC, proteins, FRAP, DPPH and anti-tyrosinase activity obtained from PBSS extracts were analyzed using hierarchical cluster analysis (HCA), and the dendrogram of eight rice varieties is presented in Figure 5. Three clusters of rice samples were well defined at the degree of similarity of 13. Cluster 1 was classified for HN and NLP, while MF, DM and RB are grouped in Cluster 2. The red rice (MD, MK and SY), which exhibited lower values for all six parameters than the purple and black varieties, were clearly separated from the purple and black rice and grouped in Cluster 3.

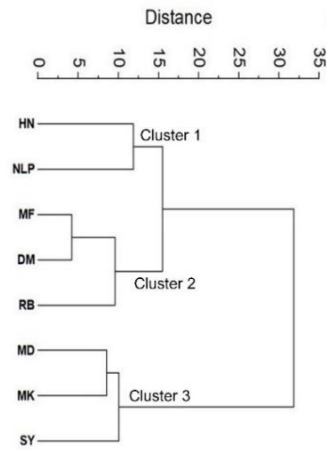

Figure 5 Dendrogram of hierarchical cluster analysis of eight rice varieties based on total phenolic content, total flavonoid content, total protein, FRAP and DPPH radical scavenging activity, and anti-tyrosinase activity of the seed extracts using the PBSS method.

DISCUSSIONS

The most prevalent methods for extraction of bioactive compounds from rice, particularly the phenolic compounds, employed organic solvents such as ethanol, methanol, and acetone while aqueous extractions were much less reported (Usman *et al.*, 2022). This experiment aimed to investigate the most effective methods to extract essential phenolic substances as well as proteins from pigmented rice for applications as cosmeceutical ingredients, therefore PBS was used for the extraction of ground brown rice. In this study, as shown in Figure 2, the TPC obtained from PBS extraction was 0.74 mg GAE/g for red rice (MD) and 2.0 – 3.0 mg GAE/g for black rice (HN and NLP). These values were well within the ranges reported by Sompong *et al.* (2011), Yodmanee *et al.* (2011), and Pramai and Jiamyangyuen (2016) that also used ground brown rice as starting materials, but methanol or ethanol were used as solvents. In addition, PBS is the most common solvent for extraction of proteins (Mansouritorghabeh *et al.*, 2017). Therefore, from this study PBS emerged as a suitable non-toxic aqueous solvent for effective extraction of bioactive compounds and proteins from pigmented rice grains.

In this study, the use of sonication- and microwave-assisted extraction were introduced to employ high frequency physical vibration in a sonication bath and short high heat in a microwave, respectively, to assist in releasing more bioactive compounds from cell wall matrix (Altemimi *et al.*, 2017). In comparison with PBS alone, sonication in an ultrasonic bath (PBSS) significantly increased TPC (for the black rice HN and NLP), total protein (in NLP), DPPH scavenging activity (in MD), and tyrosinase inhibition (in NLP) (Figure 2). Ultrasonic technology was earlier shown to improve the extraction efficiency of polyphenols and antioxidants from rice bran (Tabaraki and Nateghi, 2011). Recently, sonication-assisted extraction was effectively used to increase TPC yields and antioxidant capacity of pigmented rice bran (Das *et al.*, 2017; Irakli *et al.*, 2018; Surin *et al.*, 2020). In the bran of a black Indian rice variety, Das *et al.* (2017) found that sonication-assisted extraction yielded TPC of 19.78 mg GAE/g compared with 7.53 mg GAE/g (262% higher) obtained from the conventional method (heated in a water bath) at the same temperature (35.97 °C), timing (22.87 min), and solvent (23.78% ethanol). In this study, the PBSS extraction raised the amount of TPC in HN, MD, and NLP by 32, 34 and 19% compared with the basic PBS (Figure 2A). The much lower efficiency of sonication-assisted extraction in this study in comparison to that of Das *et al.* (2017) could be due to several factors including the starting materials (whole grains vs bran), solvent type (PBS vs ethanol), and temperature (room temperature vs 35.97 °C).

High heat from microwave was effective in increasing the amounts of extracted proteins (Figure 2C) and tyrosinase inhibition activity (Figure 2F) of HN, MD, and NLP but highly detrimental for antioxidant capacity based on DPPH radical scavenging (Figure 2E). The effects of microwave on extraction of phenolics, flavonoids and FRAP antioxidation activity were neutral, negative, or positive depending on rice varieties. Microwave-assisted extractions (MAE) have been shown to increase phenolics and antioxidant capacity from many types of plant materials such as tea residues (Tsubaki *et al.*, 2010) and capsicum fruits (Williams *et al.*, 2004). In rice, Pokkanta *et al.* (2022) reported that microwave treatment of KDML105 white rice bran at 440W for 2.5 min produced the highest TPC (39.62 μg GAE/g), TFC (11.61 μg CE/g), and DPPH scavenging activity (88%). In black rice husk extracted with ethanol, Jha *et al*. (2017) found that co-application of ultrasound (10.01 min sonication at 49 °C) and microwave (31.1 s microwave time) was the best condition to obtain the highest TPC and TFC. In our study, the benefit of co-application of ultrasound and microwave (PBSSM) compared with PBSS, on TPC, TFC, and FRAP activity varied with rice varieties. However, compared with PBSS, PBSSM was effective in increasing protein extracted from all eight rice varieties, except for SY (Figures 2C, 3E, 3F) and enhancing anti-tyrosinase of six varieties i.e., HN, MD, NLP (Figures 2F) and DM, MF, RB (Figure 3G, 3H).

Antioxidation capacity is by far the most outstanding nutritional and medicinal property of pigmented rice as compared with normal white rice. For applications in cosmetics, the antioxidant activity is highly correlated with the contents of phenolic compounds (Sompong *et al*., 2011; Pramai and Jiamyang-yuen, 2016). In this study, the PBSS extracts from red rice varieties tended to have low antioxidant capacity based on FRAP, that is MK and SY (Figure 4A) and MD (Figure 2D) showed FRAP activity of 2.44, 2.40 and 6.00 mmol Fe(II)/g, respectively. On the other hand, PBSS extracts of the deep-purple and black rice (with the exception of RB), had relatively high FRAP activity i.e., MF and DM (Figure 4A), HN and NLP (Figure 2D) showed FRAP activity of 8.62, 8.66, 10.77 and 14.05 mmol Fe(II)/g, respectively. Sompong *et al*. (2011) found that FRAP antioxidant activity of ten red rice varieties ranged from 0.85 to 8.08 mmol Fe(II)/100 g DW, and those of the three black rice varieties were 3.65 to 7.58 mmol Fe(II)/100 g DW. In addition, the DPPH radical scavenging activity among the ten rice varieties varied considerably from 12.99 (Thai red rice; Bahng Gawk variety) to 76.28% (red rice from Sri Lanka) while that of the three black rice varieties were 16 – 30% (Sompong *et al*., 2011). A similar range of DPPH radical scavenging activity was found in this study. In this study, the PBSS extracts of the red rice varied from 41.25 to 49.04% while those of the deep-purple and black rice were from 51.62 to 59.33%. The DPPH radical scavenging activity was highest in aqueous extracts of the black, followed by the red and lowest in the white rice (Saenkod *et al*., 2013).

The beneficial effect of proteins in cosmetics lies in its ability to bind water molecules to the outermost layer of epidermis, therefore creating suitable environment for healthy skin and hair (Secchi, 2008). In line with other bioactive compounds, darker color rice (deep purple to black) contained higher protein contents than red rice. The PBSS extracts of red rice MD (Figure 2C), SY and MK (Figure 3E) contained 7.36, 7.77 and 7.91% protein, respectively which are slightly higher than that of white rice (7%) (Wongsa, 2020). On the contrary, protein contents of PBSS extracts of 5 varieties of deep purple to black rice ranged from 9.12 (NLP) to 11.14% (HN) (Figure 2C). However, Sompong *et al*. (2011) found large variation in protein contents among ten red (7.16 – 10.36%), and three black (8.17 – 10.85%) rice varieties.

Tyrosinase is an enzyme responsible for the hydroxylation of tyrosine to O-diphenols and the oxidation of O-diphenols to O-quinones which are then converted to melanin pigments (Deniz *et al*., 2021). Excessive formation and accumulation of melanin causes skin color darkening, wrinkling, and ageing. Therefore, tyrosinase inhibitors such as kojic acid are often added in cosmetic products as active ingredients for skin whitening (Jiratchayamaethasakul *et al*., 2020). Several types of flavonoids and flavonoid derivatives were found to be active plant secondary metabolites that act as tyrosinase inhibitors (Chen *et al*., 2014;

Deniz *et al*., 2021). Phenolic acids such as protocatechuic methyl ester and protocatechuic acid from black rice bran extracts were also identified as being responsible for tyrosinase inhibition (Miyazawa *et al*., 2003). In this study, PBSS extracts of red rice including SY and MK (Figure 3G) and MD (Figure 2F) exhibited 15.02, 19.63 and 24.40% tyrosinase inhibition, respectively. While PBSS extracts of the deep purple or black rice (RB, DM, HN, MF and NLP) had much higher anti-tyrosinase activity ranging from 41.60% (for RB) to 57.77% (for NLP) (Figure 2F, 3G). In this study, TFC which was highly detected in PBSSM fractions of NLP (9.05 mg QE/g; Figure 2B), DM and MF (11.25 and 8.52 mg QE/g; Figure 3D) also revealed high tyrosinase inhibitory activity (64.10% for NLP; Figure 2F) and 58.19 and 57.79 % for MF and DM; Figure 3G). However, PBSSM fraction of RB (Figure 3D) contained relatively low TFC (2.33 mg QE/g sample, respectively), but expressed high tyrosinase inhibitory activity (54.95 %, Figure 3H). It was reported that not only did flavonoids played dominant role as tyrosinase inhibitor, but other groups of secondary compounds like phenolic acids, benzaldehyde, benzoate derivatives, long-chain fatty acids and steroids can act as tyrosinase inhibitor (Miyazawa *et al*., 2003; Chang, 2009).

Cluster analysis of eight rice varieties clearly separated the deep purple and black rice (HN, NLP, MF, DM, RB) from the red rice (MD, MK, SY) varieties. Similarly, Pramai and Jiamyangyuen *et al*. (2016) also reported that HN, NLP, and RB together with seven other black rice varieties were clearly separated from SY and three other red rice varieties based on darker pericarp color, more concentrated bioactive compounds (TPC, TFC, anthocyanin, **α**-tocopherol and **γ**-oryzanol) and greater antioxidant capacities (FRAP, DPPH and ABTS assay). It may be inferred that pigmented rice with darker color could be preferable to be employed as raw material for extraction of bioactive compounds with high antioxidation potential for cosmetic application.

Taken together, the results from this study suggested that aqueous-based solvent such as PBS can be used as an alternative to the commonly used organic solvents to extract comparable amounts of bioactive compounds and proteins from ground pigmented rice with high antioxidant capacity, and anti-tyrosinase activity. Moreover, the extraction efficiency can be enhanced by the assistance of sonication and microwave treatment. The promotion to use pigmented rice as a starting material for cosmetic ingredients will provide an additional income for poor farmers especially in the northeast Thailand.

CONCLUSION

The deep purple and black rice have significantly higher levels of phenolic compounds, flavonoids, proteins, antioxidant capacity, and tyrosinase inhibitory activity, as compared to red rice. They are then considered more suitable as raw materials for cosmetics ingredients. Aqueous extracts based on non-toxic PBS buffer provided reasonable amounts of phenolics, flavonoids, and proteins. Moreover, sonication-assisted extraction (PBSS) considerably increased the efficiency of PBS extraction of all investigated compound groups and functional activities. High temperature treatment using microwave was beneficial for protein extraction and anti-tyrosinase activity but significantly destroyed some bioactive compounds leading to a considerable reduction in DPPH radical scavenging activity and its use has to be further optimized.

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