Research article

Enhancing the Antioxidant, Anti-tyrosinase and Anti-elastase Activity of Pigmented Rice Extracts by Sonication, Heating, Enzyme Digestion and Fermentation

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Abstract

Keywords

anti-tyrosinase;
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pigmented rice

In the modern cosmetics industry, there exists a major trend for replacement of synthetic chemicals with plant-based ingredients in skincare formulation. This research aimed to assess the efficiency of different methods to extract bioactive compounds (as measured by total phenolic content; TPC and total flavonoid content; TFC) responsible for three important cosmeceutical properties (antioxidation, anti-tyrosinase, and anti-elastase) from selected rice grains. The grains of two varieties of purplish-black rice (Neaw Leum Pua; NLP) and black rice (Hom Nil; HN) were extracted with phosphate buffered saline (PBS) and then supplementary treated with sonication, heating, and proteolytic enzyme digestion or fermented with *Aspergillus niger*. The results showed that sonication slightly enhanced TPC and TFC, but significantly increased antioxidant capacity and anti-tyrosinase activity. The high temperature of 60°C and 100°C significantly increased antioxidant capacity based on FRAP assay but reduced that based on DPPH radical scavenging assay. Anti-tyrosinase activity was enhanced by high temperature but that of anti-elastase was inhibited. The addition of papain to the seed extracts slightly enhanced anti-elastase and anti-tyrosinase activity. Notably, fermented rice grains exhibited much greater yields of TPC and TFC, and higher biochemical activities than other methods. The fermented rice exhibited the highest TFC after 3 days; the highest TPC, antioxidant capacity, and tyrosinase inhibition after 6 days; and the highest elastase

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inhibition activity after 9 days. Therefore, fermentation was the most promising technique to enhance all three desirable properties for cosmetic ingredients when pigmented rice was used as the raw material.

1. Introduction

Growing awareness of the need for environmental sustainability and the potential hazards of chemically synthesized compounds in the cosmetics industry have led to a continuous expansion in research and development in the use of plants as cosmetic ingredients [1]. A wide range of plant species, including rice, have been explored as the sources of cosmetic ingredients that have multiple functions in skin care [2]. In addition to being consumed as an important daily source of carbohydrate, rice (*Oryza sativa* L.) is also processed into a wide range of foods, beverages, dietary supplements, and cosmetic products [3]. Rice has been used for cosmetic purposes for centuries, and its values as cosmetics ingredients have recently been demonstrated for antiaging, anti-inflammatory, whitening, photoprotective, and moisturizing properties [4]. Although white rice is consumed worldwide, the folks in some Asian countries also consume pigmented rice varieties that are known to contain higher levels of phytochemicals, which are mainly found in the outer aleurone layer [5]. The extracts of pigmented rice varieties exhibit excellent properties for cosmetic applications including antioxidant capacity, whitening activity (due to inhibitory effects on tyrosinase), and antiaging activity (through an inhibition of elastase and collagenase) [6].

Phenolic compounds or polyphenols are one of the main groups of phytochemicals known to possess crucial properties for human nutrition and skincare products [7, 8]. The major groups of phenolic compounds present in pigmented rice are phenolic acids and flavonoids [9]. The antioxidation properties of polyphenolic compounds can be resulted from various mechanisms of action including direct reaction with reactive oxygen species (ROS), enhancement of dismutation of free radicals to compounds with much lower reactivity, chelating prooxidative metal ions, increase of expression of antioxidant enzymes such as catalase and superoxide dismutase, and chelation of prooxidative metal ions such as iron or copper [8].

Several phenolic acids present in red and black rice bran that possess strong antioxidant capacities were identified [9]. Phenolic acids such as coumaric, sinapic and ferulic acids were reported to be positively correlated with the antioxidant capacity and tyrosinase inhibitory activity of fermented rice extracts [10]. A previous study identified protocatechuic methyl ester and protocatechuic acid in black rice bran extracts as being responsible for tyrosinase inhibition [11]. Flavonoids also act as strong antioxidants by donating electrons or hydrogen atoms to free radicals, delocalizing the unpaired electrons of the free radicals, and chelating metal ions [8]. The flavonoid compounds that contributed to the antioxidant capacity of pigmented rice included catechin, quercetin, epicatechin, naringin, anthocyanin, and proanthocyanidin [12].

Conventional methods of extraction of plant bioactive compounds involve the use of toxic organic solvents, laborious processes, and high energy cost. Scientists are focusing on exploring more eco-friendly, efficient, and cost-effective techniques such as sonication, heat treatments (e.g., microwave, hot air, parboiling, autoclaving, pressurized hot water extraction), supercritical fluid extraction, pulse electric field, and enzyme-assisted among others [13]. Most of the bioactive compounds, particularly phenolic compounds, that are found in rice grains are located in the bran (the outer layer of grains composing the pericarp, seed coat, nucellus, and aleurone layer), and are mostly present in insoluble bound forms covalently linked to cell wall structural components (e.g., lignin, cellulose, proteins, and sugars), and are therefore difficult to be released and extracted [14]. The mechanisms of sonication or ultrasonic treatment that can enhance the efficiency of bioactive compound extraction involves the breakdown of plant cells, better solvent penetration, capillary

effect, and higher mass transfer [15]. The sonication method was reported to significantly increase total phenolic content (TPC) and antioxidant capacity of pigmented rice bran extracts when compared to conventional extraction methods [16]. The mechanisms of heat-assisted extraction of bound phenolic compounds are based on breaking the bonds between lignin and phenolic acids, and the release of phenolics due to the degradation of lignin. However, too high temperature can lead to thermal degradation of polyphenols [17]. Increased temperature up to 80°C resulted in significant elevation of TPC and TFC in aqueous [18] and ethanolic [19] extracts of rice.

In addition to secondary metabolite compounds, proteins, low molecular weight proteins and peptides resulting from the activity of proteolytic enzymes also contribute to cosmetic properties including hydrating effects, film-forming properties, exfoliating effect, improving skin healing properties, and inhibiting oxidative damage in skin cells [20, 21]. Rice protein hydrolysates were previously reported to exhibit TPC, TFC, antioxidant capacity as well as anti-tyrosinase and anti-hyaluronidase activities [22]. Proteolytic enzymes such as papain and bromelain have been directly incorporated in skincare formulations to improve the efficacy of moisturizers, reduce skin scaling and dryness, facilitate peeling, and improve penetration of active substances [23]. Recently, research in fermentation with fungi such as *Aspergillus* spp. and *Rhizopus* spp., has grown enormously as an alternative technology to increase yield of secondary metabolites and antioxidant capacity from various types of plant materials [24]. A few authors have reported enhanced cosmetic properties of the fermentation products of rice after an optimum period of fungal fermentation [10, 25, 26].

This research aimed to increase the TPC and TFC yields from pigmented rice grains and to enhance the crucial properties for potential cosmetic ingredients (i.e., antioxidant capacity, anti-tyrosinase, and anti-elastase activity) using sonication, heating, proteolytic enzyme digestion, and fermentation. The multi-functional cosmeceutical properties obtained from plant extracts or fermentation products have great potential in replacing or supplementing synthetic chemical components in conventional skincare products. The use of rice-based cosmetic ingredients is likely to be beneficial not only for reducing exposure of skin to potentially toxic chemicals but also promoting utilization of pigmented rice, and thus raising the income of poor farmers in many low-middle income Asian countries.

2. Materials and Methods

2.1 Plant and fungus materials

Two varieties of pigmented rice were used in this study. Neaw Leum Pua (NLP) is a commercial purplish-black glutinous rice obtained from farmers in Phetchaboon Province, Thailand. Hom Nil (HN) is a commercial black rice obtained from a local supermarket. The stock of *Aspergillus niger* van Tieghem was obtained from the culture collection belonging to Department of Microbiology, Faculty of Science, Khon Kaen University, Thailand.

2.2 Methods for extraction of bioactive compounds

2.2.1 Sonication

The dehulled pigmented rice grains were finely ground and sieved through a 200-mesh stainless steel wire screen. The ground rice samples were then used for each extraction method with three replications. For basic extraction (PBS), each ground rice sample (3 g) was soaked in 30 mL of phosphate buffered saline (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 130 mM NaCl, 2.7 mM KCl, pH 7.4) for 1 h. For sonication-assisted extraction (PBSS), each sample was soaked in 30 mL PBS (pH

7.4) for 1 h followed by sonication for 1 h at 28 kHz in an ultrasonic cleaner (Model JT-113T, Jato, Shenzhen, China). The PBS and PBSS samples were then centrifuged at 15,000 rpm, and the supernatants were aliquoted, and kept in -20°C until further analysis.

2.2.2 Heating

The PBSS samples were incubated for 30 min in a water bath (Model WNB14, Memmert Gmbh, Schwabach, Germany) at 40°C (PBSS40), 60°C (PBSS60), or 100°C (PBSS100). The heated samples were centrifuged at 15,000 rpm, the supernatants were aliquoted, and kept in -20°C until further analysis.

2.2.3 Proteolytic enzyme digestion

The PBSS40 samples (which were extracted with PBS (pH 7.5), sonication and heating at 40°C) were used for enzymatic treatment using Pronase E and papain. The PBSS40 samples were centrifuged at 15,000 rpm, and the supernatant was collected. For enzymatic digestion, 20 µL of each supernatant was mixed with Pronase E enzyme at the concentration of 4U/µL and kept in a freezer at -20°C to serve as control (PE0). For digestion, 20 µL of each PBSS40 supernatant was incubated at 40°C in a dry bath for 4 h. After completion of digestion, each sample was stored at -20°C until further analysis (PE4) or heated at 95°C for 10 min to inactivate the Pronase E enzyme (PEX) before storage. For treatment with papain, the PBSS40 samples were processed in the same manner as those for Pronase E except that PBS at pH 6.0 was used for extraction. The control samples were mixed with 4U/µL papain and stored at -20°C (PP0). For digestion, the samples were incubated with 4U/µl papain for 4 h at 40°C (PP4) and then stored at -20°C until further analysis. For PPX, the samples were digested with papain for 4 h at 40°C followed by heating at 95°C for 10 min to inactivate papain before storing at -20°C for further analysis.

2.2.4 Fermentation with *Aspergillus niger*

The dehulled rice grains of both varieties were finely ground, sieved through a 200 mesh and then sterilized by autoclaving at 121°C for 15 min. Ground rice (40 g) was used as substrate for each fermentation reaction. The experiment was set up with three replications. *Aspergillus niger* was subcultured and grown on potato dextrose agar (PDA) at 30°C. After 5 days of incubation, the spores of *A. niger* were collected and transferred to 10 mL of sterile distilled water that had been added with 0.1% Tween 80 to disperse spores. The concentration of spore suspension was adjusted to 10^6 spores/mL with a haemacytometer. The suspension of *A. niger* spores (4 mL) was thoroughly mixed with a 40 g sample of the sterilized ground rice seeds in a 250-mL flask. Sterile water (10 mL) was then added to obtain 50% moisture content. The contents were mixed well and incubated in an incubator at 30°C. The rice grains (3 g) were sampled on day 0 (prior to incubation), and 3, 6, 9 and 12 days after incubation. Each fermented rice seed sample (3 g) was added into a 50-mL centrifuge tube, and 30 mL of PBS pH 7.4 was then added to the tube before sonication for 30 min. The mixture was then centrifuged at 15,000 rpm for 20 min. The supernatant was sterilized by filtering through a 0.45 µm filter and stored in 1-mL aliquots at -20°C until analysis.

2.3 Determination of total phenolic content (TPC), total flavonoid content (TFC) and proteins

The total phenolic content (TPC) was determined according to Razak *et al.* [26] in 96-well microplates 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 the TPC was expressed as mg of gallic acid equivalent (GAE) per g of sample (mg GAE/g sample). For total flavonoid content (TFC), the aluminum chloride colorimetric method modified from that of Razak *et al*. [26] was performed 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 TFC was determined in triplicate and expressed as quercetin equivalents per g of sample (mg QE/g sample). The protein content in the extract sample was determined using Bradford reagent [27]. 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. (SRL). 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.

2.4 Determination of antioxidation capacity

Ferric reducing antioxidant power (FRAP) assay was performed according to Benzie and Strain [28] with some modifications. Three stock solutions for the assay included Solution 1: 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution 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 a dark bottle. An extract sample (20 µL) was aliquoted into a 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 mmol Fe(II) equivalents per gram of sample. The DPPH radical scavenging assay was performed according to Razak *et al*. [26] with some modifications. A 40 µL extract 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 (A_s). A 40 µL of rice extract was mixed with 100 µL methanol and the absorbance was measured and recorded as A_c. For the blank, the absorbance of 40 µL PBS buffer mixed with 100 µL of 0.25 mM DPPH in methanol was recorded as A_b. The DPPH radical scavenging activity was determined in triplicate and expressed as DPPH radical scavenging activity (%) according to the following equation.

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

2.5 Determination of tyrosinase and elastase inhibitory activity

Tyrosinase inhibitory assay, which is based on the reduction of dopachrome synthesis from L-3,4-dihydroxyphenylalanine (L-DOPA), was carried out in 96-well microplates. Each reaction contained 40 µL of the extract sample or PBS as blank, 40 µL of air-saturated 0.1 M phosphate buffer pH 6.8, and 40 µL of mushroom tyrosinase (32 U/mL). The reaction was 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 the dopachrome product at 490 nm from the sample and blank (A_{sample} and A_{blank}, respectively) [26]. The A_{blank} was subtracted by the sample background containing extract sample of 40 µL and 80 µL air-saturated 0.1 M phosphate buffer pH 6.8 ($A_{background}$). Tyrosinase inhibitory activity was carried out in triplicate and expressed as % tyrosinase inhibitory activity.

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

The elastase inhibitory activity was carried out in the light-protected condition in 96-well microplates following the method of Thring *et al*. [29]. A 20 µL aliquot of the sample (or distilled water as blank) was mixed with 100 µL of 0.2 M Tris-HCl buffer (pH 8.0) and 20 µL elastase enzyme solution (Porcine pancreatic elastase 0.01 mg/mL in 0.2 mM Tris-HCL buffer pH 8.0). The mixture was incubated for 10 min at room temperature. After that, 20 µL of elastase substrate N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (NS3APN) solution (0.8 mM NS3APN in 0.2 M Tris HCl pH 8.0) was added. The reaction mixture was incubated for 10 min and the absorbance of the product (*p*-nitroanilide) was determined at 410 nm in a microplate reader. The absorbance for the background ($A_{background}$) was obtained by mixing 20 µL of the sample with 120 µL Tris-HCl buffer and 20 µL of elastase substrate. Elastase inhibitory activity (%) was calculated by the equation below:

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

2.6 Statistical analysis

Data were presented as mean±SE, and the test of significance differences among data was performed using one-way ANOVA in IBM SPSS@ Statistics version 28.0.1(142).

3. Results and Discussion

3.1 Effects of sonication and heating on TPC, TFC and antioxidation capacity

The effects of sonication and heating on the bioactive compounds and antioxidation activities of seed extracts of NLP (the black glutinous rice) and HN (the black rice) are displayed in Figure 1. Most researchers used organic solvents such as methanol and acetone to extract plant phenolic compounds [30] while PBS was used in this study as the basic solvent because both phenolics and proteins can be recovered [31]. The amounts of TPC extracted by PBS in this study were comparable to earlier reports [32, 33] which also used ground pigmented rice as starting materials but methanol was used as extraction solvent. The strong relationship between high phenolic content of pigmented rice and the antioxidant capacity for cosmeceutical applications is well-documented [25, 26]. Phenolic compounds act as antioxidants by reacting with a variety of free radicals by hydrogen atom transfer (assessed by DPPH radical scavenging assay), single electron transfer, and transition metal chelation (assessed by FRAP assay) [34]. The prominent phenolic compounds in HN which are responsible for antioxidation were *p*- coumaric acid and ferulic acid [35] whereas those in NLP were protocatechuic acid, *p*-coumaric acid, and vanillic acid [36]. The dominant flavonoid responsible for antioxidation capacity in both HN and NLP was cyanidin-3-glucoside [35, 36]. Sonication (PBSS) significantly increased the antioxidant capacity of NLP based on both FRAP and DPPH radical scavenging activity (Figure 1E, G) but enhanced only DPPH radical scavenging activity of HN (Figure 1H). The mechanism of ultrasonic assisted extraction is based on mechanical, cavitation and thermal phenomena which cause cell wall rupture and enhance solvent penetration thereby increase the efficiency of extraction of bioactive substances from plants [14, 37, 38]. In this study, the PBSS extraction did not significantly raise the amount of TPC and TFC extracted. The much lower efficiency of sonication-assisted extraction in this study in comparison to previous reports could be due to several factors including the starting materials (whole grains vs bran), solvent type

(PBS vs ethanol), temperature (room vs warmer temperature), and ultrasonic frequency and processing time [39].

Heating at 40℃ and 60℃ (PBSS40 and PBSS60) tended to increase TPC content in seed extracts of NLP and HN compared with extraction with PBSS without heating (Figure 1A,B), although the values were not significantly different. Generally, the TPC contents in seed extracts of NLP were slightly higher than those of HN. The amounts of TPC and TFC obtained were in the same range as earlier reported for Thai pigmented rice [32, 33]. The TPC in NLP seed extracted by varying methods were in the range of 4.36 (PBS) to 5.56 (PBSS+40℃) mg GAE/g while that of HN seeds were between 2.97 (PBS) to 4.08 (PBSS+60℃) mg GAE/g (Figure 1A, B). Interestingly, TFC in the seed extracts of NLP (8.15 to 11.05 mg QE/g) were severalfold higher than that of HN (0.82 to 3.47 mg QE/g) (Figure 1C, D). Similarly, Teeranachaideekul *et al*. [40] also recorded three times higher TFC in NLP than HN. A wide variation in TFC was noted among ten varieties of purplish-black rice showing as high as 4.9 times difference between the lowest and the highest variety [33]. Heating significantly increased TFC only in NLP when applied at 40℃ while none of the heating treatments significantly enhanced TFC in HN. During heating, plant tissues are softened, thereby weakening phenol–protein and phenol–polysaccharide interactions. Such weakened interactions may enhance the diffusion of secondary metabolites such as phenolics and flavonoids into the extraction media [19]. In KDML105 white rice bran, the amounts of TPC and TFC extracted in water showed 31% and 15% increase, respectively, with increase of temperature from 50℃ to 80°C [18]. It was reported that the yields of TPC and TFC from white rice grains were 1.5 times higher at 50℃ than 25°C [41]. Heat-assisted increased amounts of extracted phenolic compounds were also reported for red and brown rice bran [19]. Therefore, high temperature tended to increase the amount of TPC and TFC extracted with PBS from rice seeds, but the most effective temperatures varied with rice genotype, seed color, type of starting material (whole grains, brans, or ground grains), and solvent type.

With respect to antioxidation potential, the highest FRAP activity for NLP was obtained with extraction at boiling (PBSS100) temperature which was 20% higher than that in PBSS without heating (Figure 1E). However, the maximum FRAP activity for HN was obtained with the extraction at 60°C. Previous studies also reported an increase in antioxidant capacity based on the FRAP assay of rice grains when extraction took place at higher temperatures between 50°C to 80°C [18, 41]. Conversely, heating at 40℃, 60℃ and 100°C caused dramatic reductions in DPPH scavenging activity (Figure 1G, H) and HN tended to be more sensitive to temperature increase than NLP. This could be explained by the thermolability of specific bioactive metabolites in HN and NLP which are responsible for DPPH radical scavenging activity. Similarly, heating reduced the DPPH scavenging activity of pigmented rice by 10% [42], and the reduction was attributed to breakdown of specific antioxidant molecules, chemical reactions, and leaching of water-soluble antioxidant components [43]. As analyzed by Wanyo *et al*. [18], high temperature during extractions exerted different effects on the contents of various types of phenolic compounds. For example, temperatures up to 80°C caused an increase in protocatechuic acid and ferulic acid, but a reduction in vanillic acid. When the temperature exceeded the optimum, oxidation and decomposition of secondary metabolites results in a reduction of bioactive compounds in the extracts [44]. Therefore, heating at 40℃ in a water bath was a simple treatment suitable for enhancing the extraction of phenolic and flavonoid compounds from NLP and HN rice seeds. Heating at 40-100°C tended to enhance antioxidation activity based on the FRAP assay but was detrimental to DPPH scavenging activity.

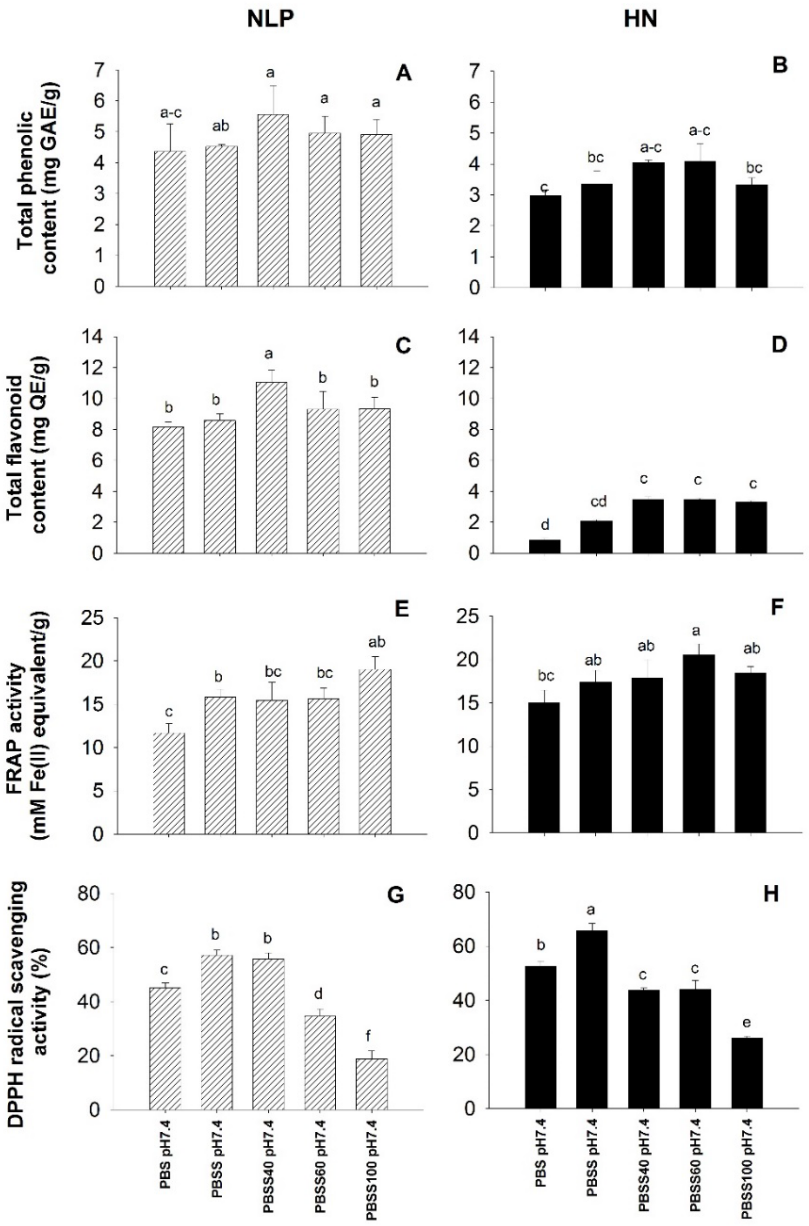

Figure 1. Effects of sonication and heating at 40°C, 60°C, and 100°C on total phenolic content (A, B), total flavonoid content (C, D), FRAP activity (E, F), and DPPH radical scavenging activity (G, H) of seed extracts of rice varieties Neaw Leum Pua (NPL: A, C, E, G) and Hom Nil (HN: B, D, F, H). PBS = ground seeds were soaked in PBS; PBSS = ground seeds were soaked in PBS followed by sonication; PBSS40, PBSS60 and PBSS100 = ground seeds were soaked in PBS, sonicated, and heated at 40°C, 60°C and 100°C, respectively. For each parameter, means with different lowercase letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.

3.2 Effects of sonication and heating on total protein, anti-tyrosinase and anti-elastase activity

The effects of sonication and heating of the seed extracts of NLP and HN on total protein content, and inhibitory activity on enzymes relating to aging are displayed in Figure 2. Heating did not have any positive effects on extraction of proteins from the seed extracts of both NLP and HN. All five extraction treatments resulted in similar protein contents ranging from 9.99 to 11.47 mg/g in NLP, and 12.06 to 13.77 mg/g in HN (Figure 2A, B). The tyrosinase inhibitory activity in seed extracts of HN subjected to heating tended to increase compared to that in the PBSS extract, with the highest activity of 68.64% inhibition obtained when the PBSS extract was heated at 100℃ (Figure 2D). For NLP, sonication significantly increased tyrosinase inhibitory activity to 64.49% compared to 45.78% in the extract using PBS alone (Figure 2C). Heating at 40℃, 60℃, and 100℃ raised tyrosinase inhibitory activity to 64.83, 67.98 and 71.27%, respectively. In contrast, the high temperatures at 60 and 100°C destroyed some bioactive compounds, resulting in significant reduction in % inhibition of elastase in the seed extracts of both NLP and HN (Figures 2E, 2F). Extractions using PBS, PBSS, and PBSS40 resulted in similar levels of elastase inhibitory activity for both NLP (44.72-45.80%) and HN (41.82-42.40%). In rice bran, it was found that anti-elastase activity was associated with ferulic acid and syringic acid while anti-tyrosinase was related to the synergistic effects of ferulic acid, syringic acid, gallic acid and hydroxybenzoic acid [45]. A recent report in rice found that oven heating at 60 and 80°C for 24 h and heating by pasteurization at 80℃ and 90°C for 10 min significantly increased tyrosinase inhibition activity but only oven heating boosted the anti-elastase activity [46]. This indicated that different methods of heating had differential effects on bioactive compounds involved in the inhibition of tyrosinase and elastase. Therefore, if both anti-pigmentation and anti-wrinkle properties are required from black rice, more research needs to be performed to obtain the best thermal process and optimum temperature. Therefore, heating at 40℃ to 100℃ tended to enhance tyrosinase inhibitory activity but reduce elastase inhibitory activity in both NLP and HN.

3.3 Effects of proteolytic enzyme digestion on anti-tyrosinase and anti-elastase activity

In this study, the PBSS40 extracts from HN and NLP were further treated for 4 h with either Pronase E (PE4) or papain (PP4) to yield protein hydrolysates mixed with other extracted compounds. The mixture of low molecular weight proteins, small peptides, and other bioactive compounds in the PE4 and PP4 fractions of HN gave significantly higher tyrosinase and elastase inhibition activities than those of the controls (PE0 and PP0) (Figure 3B, D). On the other hand, only the PP4 fraction of NLP showed significantly higher elastase inhibition activity than PP0, but not that of tyrosinase (Figure 3A, C). From Figures 2A and B, the proximate protein content of HN was higher (12.56%) than that of NLP (10.63%), which implied more variable peptides, digestion products, from HN. However, when the digestion mixtures were heated at 95°C before being assayed (PEX and PPX), the tyrosinase and elastase inhibitory activity dramatically decreased, suggesting that the increased inhibitory activity (in PE4 and PP4 compared with PE0 and PP0) was presumably associated with the direct proteolytic action of Pronase E and papain on tyrosinase and elastase molecules. Heating also damaged bioactive compounds in the extracts because the anti-tyrosinase and anti-elastase activities in the PEX and PPX treatments were lower than those originally present in PE0 and PP0. An interesting exception was found in the HN extracts digested with papain followed by heat inactivation (PPX), which showed higher tyrosinase inhibitory activity than the control (PP0). This was similar to the increased tyrosinase inhibitory activity with boiling earlier described (Figure 2D). This confirmed that heating enhanced extractability and induced some chemical reactions such as the Maillard reaction leading to synthesis of specific bioactive compounds [47] with anti-tyrosinase

activity. Papain is incorporated into many skincare products for its hydrolyzing, hydrating, and exfoliating properties [23]. In this study, papain also inhibited tyrosinase, suggesting its potential use for skin whitening.

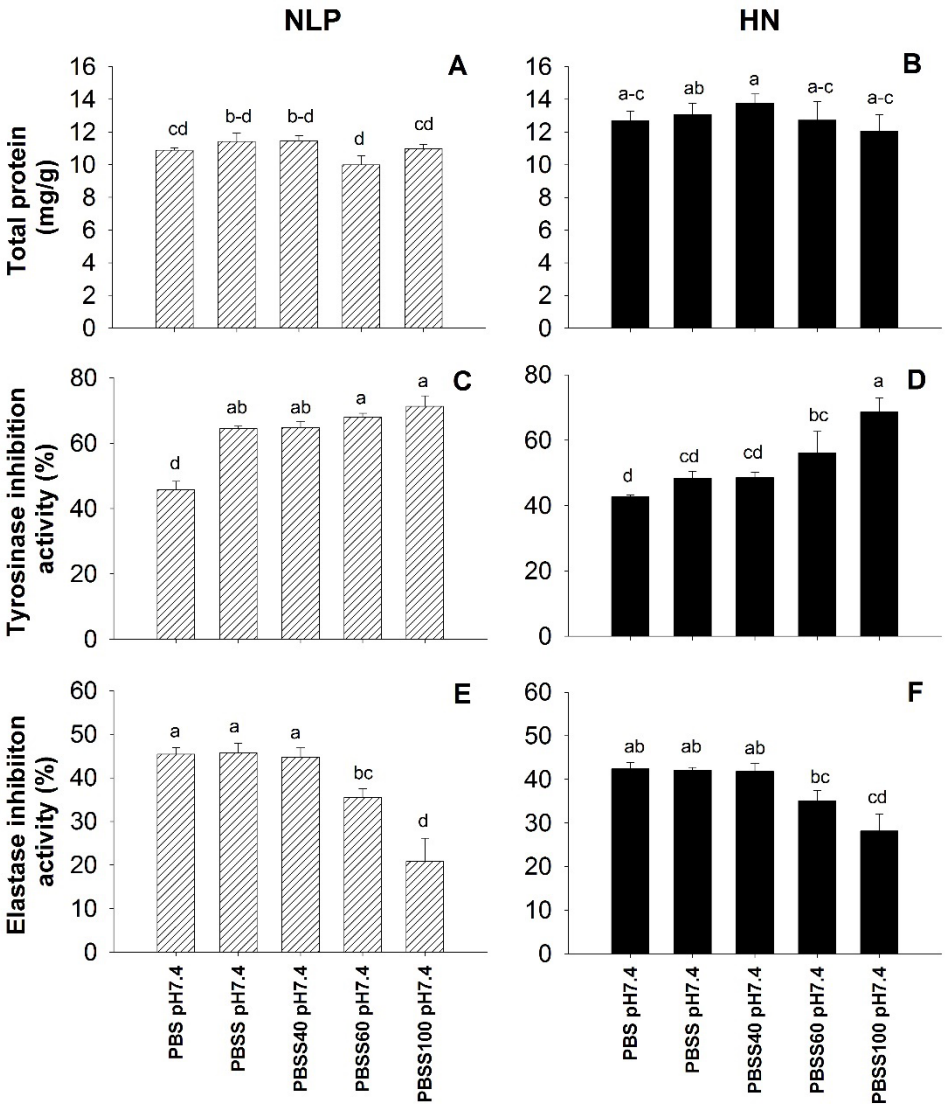

Figure 2. Effects of sonication and heating at 40°C, 60°C, and 100°C on total protein (A, B), tyrosinase inhibitory activity (C, D), and elastase inhibitory activity (E, F) in seed extracts of rice varieties Neaw Leum Pua (NPL: A, C, E) and Hom Nil (HN: B, D, F). PBS = ground seeds were soaked in PBS; PBSS = ground seeds were soaked in PBS followed by sonication; PBSS40, PBSS60 and PBSS100 = ground seeds were soaked in PBS, sonicated, and heated at 40°C, 60°C and 100°C, respectively. For each parameter, means with different lowercase letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.

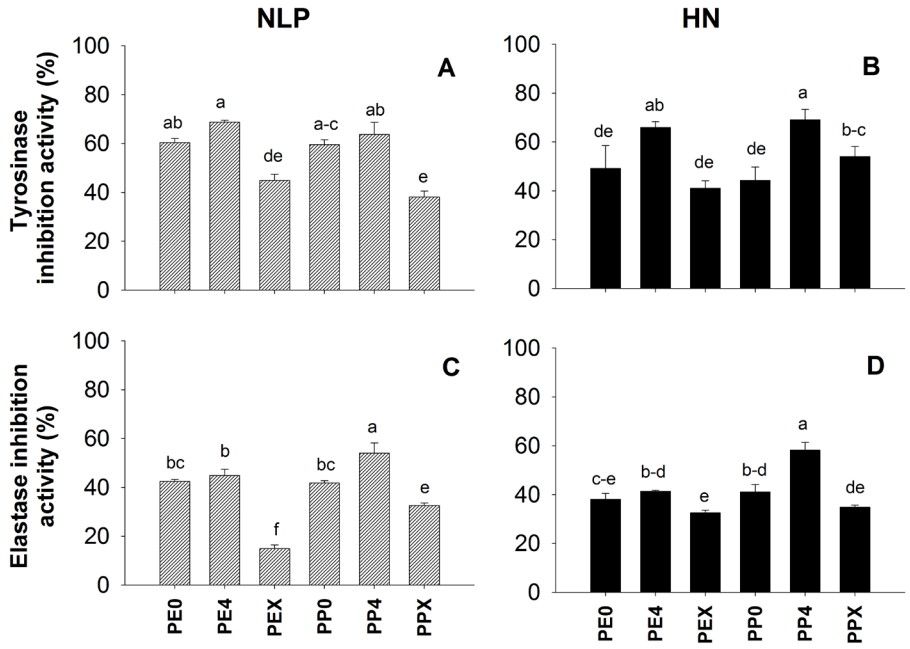

Figure 3. Effects of Pronase E and papain digestion on tyrosinase inhibition activity (A, B) and elastase inhibition activity (C, D) in seed extracts of rice varieties Neaw Leum Pua (NPL: A, C) and Hom Nil (HN: B, D). PE0 = seed extracts mixed with Pronase E then stored frozen to serve as control; PE4 = seed extracts digested with Pronase E for 4 h then stored frozen; PEX = seed extracts digested with Pronase E for 4 h then heated at 95°C to inactivate the enzyme then stored frozen; PP0 = seed extracts mixed with papain then stored frozen to serve as control; PP4 = seed extracts digested with papain for 4 h then stored frozen, PPX = seed extracts digested with papain for 4 h then heated at 95°C to inactivate the enzyme and stored frozen. For each parameter, means with different lowercase letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.

3.4 Bioactive compounds and cosmeceutical properties in fermented rice

3.4.1 Total phenolic and total flavonoid compounds in fermented rice

The data in Figure 4A clearly showed that fermentation of the ground rice seeds of both NLP and HN with *A. niger* significantly enhanced TPC, producing the maximum content after 6 days of fermentation, which was 2.8 times (for NLP) and 4.2 times (for HN) that of the unfermented seeds (day 0). However, the TPC slightly decreased after 9 days of fermentation, and after 12 days the content was still 1.8 times (for NLP) and 3.2 times (for HN) that of the unfermented seeds. The TPC in the extracts from fermented seeds of NLP and HN were comparable throughout the 12-day fermentation period. Similarly, TPC in non-fermented broken rice (0.03 mg GAE/g) increased several times to 2.79 and 3.87 mg GAE/g after 18 days of fermentation by *A. oryzae* and *Rhizopus oligosporus*, respectively [48]. After 14 days of fermentation of rice bran with three species of

Aspergillus spp., TPC increased 2.0 to 3.4 times [10]. Analysis of phenolic acid composition during fermentation revealed dramatic increases in coumaric acid, ferulic acid, and sinapic acid [10]. Coumaric acid was reported to be an antioxidant that inhibited skin aging [49] while ferulic acid exhibited tyrosinase inhibition activity [50].

In this study, total TFC of NLP and HN rapidly increased by 211% and 70%, respectively, after 3 days of fermentation compared with the content on day 0 (Figure 4B) and then progressively declined. When broken rice was fermented with *A. sojae*, Ritthibut *et al*. [10] reported that TFC increased to a maximum on the sixth day of fermentation and then slowly declined. During fermentation, hydrolytic enzymes (e.g., cellulase, esterase, xylanase, and β-glucosidase) released by fungi break down the components of plant cell walls leading to efficient release of phenolic and flavonoid compounds [51]. A metabolomic analysis of corn stalk before and after fermentation with *A. niger* revealed significant increase in 20 bioactive compounds including sugar and glycosides, organic acids, benzene and derivatives, amino acids, phenolic compounds, and flavonoids [24].

3.4.2 FRAP activity and DPPH radical scavenging activity in fermented rice

The patterns of change in antioxidation capacity, in both NLP and HN, based on FRAP and DPPH scavenging activity were similar, i.e., the activity increased to a maximum on day 6 of fermentation, and then continuously declined to the levels similar to the unfermented seeds after 12 days of fermentation. The maximum FRAP activities on day 6 were 1.9 and 2.18-fold that of the unfermented seeds (day 0) for NLP and HN, respectively (Figure 5A). The FRAP activities in the unfermented HN seeds, as well as fermented seeds on days 3 and 6, were significantly lower than that of NLP seeds, although the activities on days 9 and 12 were similar for both varieties. The levels of DPPH radical scavenging activity, on the other hand, were similar for NLP and HN throughout the fermentation period (Figure 5B). The maximum DPPH activities in the fermented seeds on day6 were 2.4 and 2.03-fold those of the unfermented seeds (day 0) for NLP and HN, respectively. It was noted that the pattern of changes in DPPH and FRAP activity with fermentation time closely followed the changes in TPC, indicating that the phenolic compounds could be responsible for the radical scavenging activity [9, 10]. A previous study found that DPPH radical scavenging activity increased by eight times in broken rice fermented with *A. niger* for 18 days when compared with

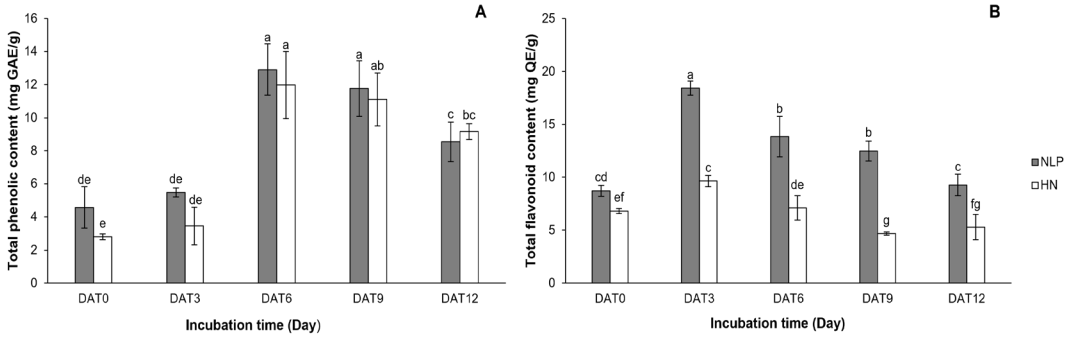

Figure 4. Total phenolic content (A), and total flavonoid content (B) in the extracts of unfermented rice seeds (DAT0), and fermented seeds after fermenting with *Aspergillus niger* for 3, 6, 9 and 12 days. (NLP, Neaw Leum Pua; HN, Hom Nil). Each value is the mean±SE, means with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

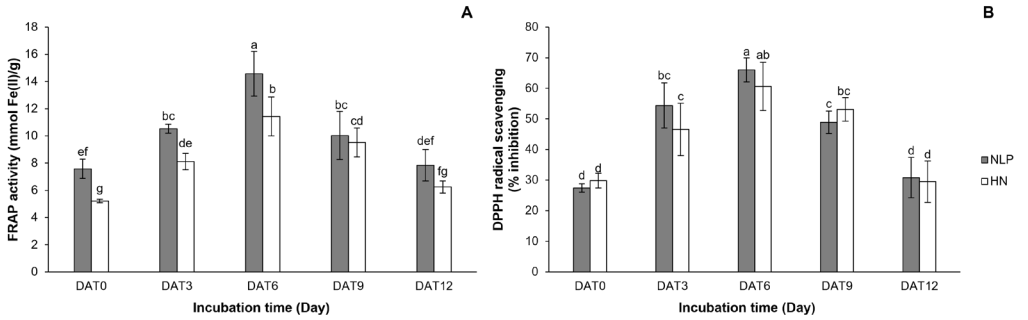

Figure 5. Antioxidation capacity based on FRAP assay (A) and DPPH radical scavenging activity (B) in the extracts of unfermented rice seeds (DAT0), and fermented seeds after fermentation with *Aspergillus niger* for 3, 6, 9 and 12 days. (NLP, Neaw Leum Pua; HN, Hom Nil). Each value is the mean±SE, means with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

that in the unfermented rice [26]. Rice bran fermented with *A. brasiliensis* exhibited an increase in the DPPH radical scavenging activity to 33.8% after 8 days, which approximately doubled the activity seen in the unfermented rice bran (15.7%) [10]. In the same experiment [10], *A. sojae* was found to be the most effective fungus for increasing the DPPH scavenging activity in rice bran, producing a maximum activity of 63.9% on day 10. Several fold increases in antioxidant capacity based on FRAP assay in rice bran fermented with *A. oryzae*, *R. oryzae*, and the mixed culture were also reported [52].

3.4.3 Total protein in fermented rice

The total protein contents of the unfermented NLP seeds were 10.24 mg/g, and remained stable until day 6 of fermentation, after which it significantly declined to 6.52 and 6.84 mg/g on days 9 and 12 (Figure 6). For HN, the total protein in unfermented seeds was 9.56 mg/g, after which it continuously declined during fermentation to reach the level of 6.77 mg/g on day 12. Seed proteins were metabolized and used as a substrate for fermentation of *A. niger* as an energy source for growth, metabolism, and production of biomolecules with cosmeceutical properties [53]. A rise in amino acids such as serine, alanine and glycine after fermentation was found to contribute to moisturizing effects for human skin [54].

3.4.4 Tyrosinase and elastase inhibitory activity in fermented rice

Fermentation of NLP and HN rice grains with *A. niger* enhanced anti-tyrosinase and anti-elastase activity. The anti-tyrosinase activity of NLP increased from 66.48% on day 0 to a maximum of 78 and 79% on day 3 and day 6 of fermentation, which was approximately a 19% increase, while 81% increase was recorded for HN on day 6 (increase from 43 to 78%) (Figure 7A). A previous work [29] using broken rice fermented with *A. niger* also displayed maximum anti-tyrosinase activity (approximately 35%) on day 6 and the activity remained relatively stable until day 20. However, in our study, the anti-tyrosinase activity started to decline significantly on day 9. This could be due to the difference in the ratio of the rice substrate to the number of inoculated spores, the metabolic activity of different *A. niger* strains, and the nature of substrates. Rice bran fermented with *A. sojae*, also showed a maximum anti-tyrosinase activity of 72.68% on day 6 (approximately 4.6 times

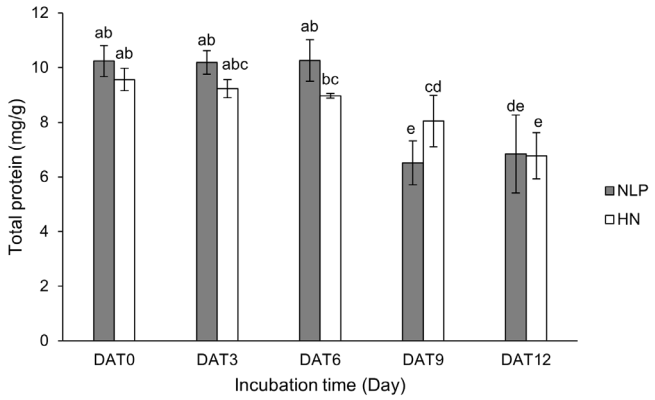

Figure 6. Total protein in the extracts of unfermented rice seeds (DAT0), and fermented seeds after fermentation with *Aspergillus niger* for 3, 6, 9 and 12 days. (NLP, Neaw Leum Pua; HN, Hom Nil). Each value is the mean±SE, means with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

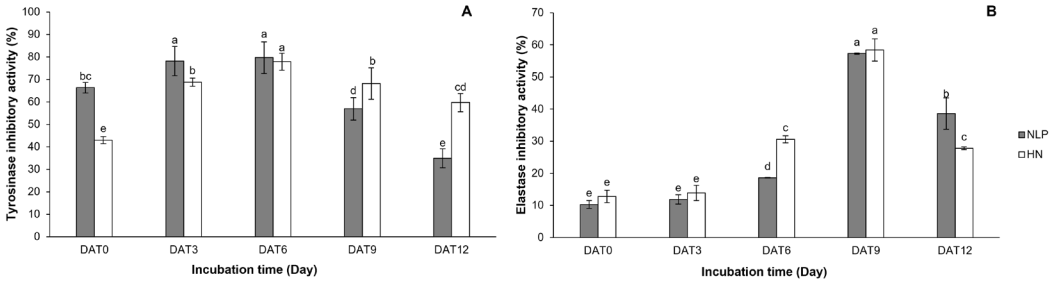

Figure 7. Tyrosinase inhibitory activity (A) and elastase inhibitory activity (B) in the extracts of unfermented rice seeds (DAT0), and fermented seeds after fermentation with *Aspergillus niger* for 3, 6, 9 and 12 days. (NLP, Neaw Leum Pua; HN, Hom Nil). Each value is the mean ± SE, means with different letters are significantly different at $P < 0.05$.

higher than that on day 0) [10]. Moreover, the changes in the amount of kojic acid (a potent inhibitor of tyrosinase) during the time course of fermentation was closely related to the anti-tyrosinase activity. It was well documented that the ability of *A. niger* to inhibit tyrosinase was due to the synthesis of kojic acid [55].

The pattern of change in the elastase inhibitory activity of fermented rice during fermentation with *A. niger* (Figure 7B) was different from that of tyrosinase inhibitory activity. The starting elastase inhibitory activities in the unfermented seeds were 10.32 and 12.82% in NLP and HN, respectively. The elastase inhibitory activity slowly increased to reach its maximum on day 9 exhibiting the activity of 57.26% for NLP and 58.44% for HN, and then significantly decreased on day 12 (38.60% for NLP and 27.74% for HN). So, the enhancement of anti-elastase activity occurred later than anti-tyrosinase, which peaked on day 3 or day 6. A similar observation was reported that anti-elastase activity began to appear 8 days after fermenting broken rice with *A. niger* and the activity continued to increase until day 20 reaching a maximum inhibition value of 50% [26]. Therefore, the bioactive compounds responsible for inhibition of tyrosinase and elastase are likely

to be different. The elevation of anti-elastase activity during fermentation of rice bran with *A. oryzae* coincided with the increased production of ferulic acid and syringic acid [52], which have long been shown to have anti-aging properties [56]. It was suggested that, in addition to phenolic compounds, elastase inhibitory activity may also take place owing to the presence of other functional compounds, possibly organic acids as well as peptides and/or lipophilic components [10].

The number of research work on the production of bioactive molecules for cosmetic ingredients based on solid state fermentation of rice that is similar to this study is quite limited [10, 25, 26, 46, 48] compared to the fermented rice filtrate, particularly, *Galactomyces* ferment filtrate (Pitera™), which has been widely commercialized for decades [57]. Previous research employed white rice bran as the starting material for solid state fermentation and the resultant extracts were assessed for *in vitro* cosmeceutical characteristics and identification of bioactive compounds [10, 25, 26, 46, 48]. Therefore, future research is needed in several aspects including a search for more effective microorganisms and suitable pigmented rice varieties for multifunctional ingredients. In addition, there should be a search for an optimization of culture conditions, effective extraction methods with minimal loss of bioactive compound and efficient elimination of microorganism, *in vitro* and cellular testing of desirable cosmeceutical properties, safety and toxicology assessment, product formulation, *in vivo* human skin test and cost-effective manufacturing consideration [53]. Nevertheless, the results of this study suggest that whole grains of pigmented rice may serve as potential sources of bioactive raw materials for cosmeceuticals produced by solid state fermentation using *A. niger* based on the *in vitro* antioxidation, anti-tyrosinase and anti-elastase activity.

4. Conclusions

The last decade has seen growing interest in the extraction of bioactive compounds from plants for use in cosmetic formulations with an aim to reduce the use of toxic chemicals. Bioactive compounds were extracted from two varieties of pigmented rice that had purplish black pericarps using non-toxic PBS supplemented with sonication, heating, enzyme digestion, and fermentation to enhance yield of bioactive compounds and functional properties relating to cosmetic application. Sonication increased TPC, TFC, antioxidant capacity, anti-tyrosinase and anti-elastase activity. Heating was beneficial for TFC, anti-tyrosinase, and FRAP activity but destroyed DPPH scavenging and anti-elastase activity. Digestion with papain promoted anti-tyrosinase and anti-elastase activity. Fermentation of ground rice with *Aspergillus niger* for 3 to 9 days was the most promising method, producing the best yields of TPC, TFC, and the highest levels of all three functional properties: antioxidation, anti-pigmentation (due to anti-tyrosinase activity), and anti-wrinkle (due to anti-elastase activity). Therefore, compounds produced via fermentation exhibited great potential to replace several synthetic chemicals in conventional skincare products. The desirable compounds and properties can be further improved by screening for more effective strains of *A. niger*, *Aspergillus* spp., or other genera of fungi. One limitation of this fermentation process lies in the extra cost of getting rid of residual fungal mycelium and spores from the fermented rice without reducing the contents of desirable products before further use as cosmetic ingredients.

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