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Original Article

Effect of extraction methods on biological activities of Thai rice bran extracts

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Abstract

Rice bran is an essential source of natural bioactive compounds with widely applications. This study aimed to determine the effects of extraction methods on the bioactivities of the extracts. Jasmine and Riceberry rice bran were extracted using 50% ethanolic maceration, cold pressed and subcritical dimethyl ether (DME) extraction. The extracts were analyzed for antioxidant, pro-collagen type I synthesis and 5α-reductase inhibition activities. The results showed that Riceberry rice bran extract exhibited superior antioxidant activity than Jasmine rice bran extract. The highest pro-collagen type I synthesis of Jasmine and Riceberry rice resulted from subcritical DME extraction (50.91%) and maceration (46.99%) respectively. It is a newly found that the subcritical DME extract of Jasmine rice bran displayed the strongest 5α-reductase inhibition activity (73.14 %inhibition) resulting from the biological markers linoleic acid (22.39±0.61 %w/w) and oleic acid (39.48±1.07 %w/w). Therefore, subcritical DME extraction is suitable for preparing rice bran extract for 5α-reductase inhibitor application.

Keywords: 5α-reductase, antioxidant, *Oryza sativa*, pro-collagen, rice bran, subcritical fluid extraction

1. Introduction

Rice is the main cereal crop in the world. Jasmine rice is a famous rice variety in Thailand and South Asia with its signature long white grain and a floral aroma. It has significant nutritional benefits including carbohydrates and vitamin B (Bureau of Rice Research and Development, 2010). Riceberry rice, a deep purple Thai rice provides optimum nutritional benefits has been developed by cross-breeding strains from the Chao Hom Nin variety which contains high antioxidant properties, and the Khao Dawk Mali 105 rice variety,

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known as fragrant rice (Rice Gene Discovery Unit and Rice Science Center, 2014; Thamnarathip *et al.*, 2016).

Rice bran is a by-product from rice milling process. It composes beneficial components such as sterols, vitamin B, γ-oryzanol, tocopherols, fatty acids and phenolic compounds (American college of toxicity, 2006; Chotimarkorn, Benjakul, & Silalai, 2008). In addition, the biological activities of rice bran include antioxidant, anti-inflammatory, tyrosinase inhibition, 5α-reductase (type 1) inhibition, anticancer, and reduction of blood cholesterol levels (Manosroi *et al.*, 2010; Ruksiri wanich, Manosroi, Abe, Manosroi, & Manosroi, 2011).

5α-reductase is an enzyme that converts testosterone to dihydrotestosterone (DHT). Excessive DHT results in androgen-dependent disorders which can cause pathogenetic conditions such as prostate cancer (Pham & Ziboh, 2002), benign prostatic hyperplasia, alopecia, hirsutism, and acne (Kettyle & Arky, 1998; Norman & Litwack, 1997). In contrast, linoleic acid and γ-oryzanol, which are the major biological compounds of rice bran, demonstrate inhibition of 5α-reductase (Choi *et al.*, 2014).

Several extraction methods are compatible with the *green* extraction concept including subcritical fluid extraction. Subcritical fluid extraction uses extractants at elevated temperatures while applying high pressure to maintain their liquid state (Tangkhavanich, Kobayashi, & Adachi, 2014). Dimethyl ether (DME) is used as an alternative solvent, due to its low subcritical temperature (-24.8 ˚C to 126.9 ˚C), low toxicity, weak hydrogen bond, slight polarity, and its ease of separation from final products. As the boiling point of DME is -24.8 ˚C, it has saturated vapor pressure of 0.51 MPa at 20 ˚C. Furthermore, DME has also been reported for use in extraction of non-polar compounds such as lipids and antioxidative compounds (Boonnoun, Tunyasitikun, Clowutimon, & Shotipruk, 2017; Kanda, Li, Goto, & Makino, 2015).

Therefore, this research aimed to determine the effects of extraction methods including maceration, cold pressed method, and subcritical fluid extraction on the biological activity as well as the bioactive compounds in Jasmine and Riceberry rice brans. Finally, processes demonstrated and proven in this research can support the food and cosmetic industries, based on extracted rice bran products, which will in turn add value to Thai rice.

2. Materials and Methods

2.1 Samples and chemicals

Rice brans of Jasmine and Riceberry obtained during milling process in 2016 from Thai Smart Life Company Ltd. (Thailand). Acetonitrile, isopropanol and methanol were HPLC grade as well as ethanol (AR grade) which purchased from ACI Labscan (Bangkok, Thailand). The standard linoleic acid, finasteride, 1,1, -Diphenyl-2-picrylhydrazyl, and dimethylsulfoxide were from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), Fetal bovine serum, Gluta max, Phosphate buffer, Trypsin, and Thiazolyl blue were purchased from Life Technologies (California, USA). Sodium bicarbonate obtained from VWR Life Science Amresco (Canada, USA). L-ascorbic acid purchased from Chem-Supply (USA). The standard γ-oryzanol and oleic acid purchased from Tokyo Chemical Industry (Japan). Dimethyl ether (Spray-work air can 420D) was from Siam Tamiya Co., Ld. (Thailand). Hydroxylamine hydrochloride was supplied from Carlo Erba Reagent (Milan, Italy). Dihydronicotinamide-adenine dinucleotide phosphate (NADPH) was purchased from OYC (Tokyo, Japan).

2.2 Extraction of rice bran

2.2.1 Plant preparation

Rice bran powder (Jasmine rice, Riceberry rice) has obtained from a local grinding mill. It was sieved pass through 177–297 µm sieves to separate grain from rice bran. The obtained powder was heated by hot air oven at 100 ˚C for 15 min to inactivate endogenous lipase.

2.2.2 Maceration

The rice bran 10 g was extracted by 100 ml of 50 %v/v ethanol in water with an agitation rate of 160 rpm at room temperature (approximately 25˚C) for 12 h. The extract was filtered through Whatman No.1 filter paper. The remained rice bran powder was re-extracted twice with the fresh solvent. The filtrates were combined, and the solvent was removed. The residual crude of rice bran extract was weighted and then stored at -20 ˚C until use.

2.2.3 Cold pressed method

The cold pressed Jasmine and Riceberry rice bran oils were gifts from Thai Smart Life Co., Ltd. The rice bran samples were placed in the receiver compartment of mechanical pressing machine (Lopburi vegetable cold press, Thailand). The screw was pressed in the high pressure at the temperature range of 40-60 ˚C. The crude oil was collected and stored at -20 ˚C until use.

2.2.4 Subcritical DME fluid extraction

Rice bran powder 10 g was contained in a cellulose extraction thimble along with a magnetic bar. Then, the thimble was placed in an extractor. The extraction apparatus was set as presented by Boonnoun *et al.* (2017). The 60 g DME was filled to the pre-weight extractor. The process was controlled at 35 ˚C, 0.69 MPa with the agitation rate of 400 rpm. After 30 min, the extracts flowed out through a stainless-steel filter. The DME solvent was defeated by evaporation at room temperature. Extracts were collected and stored at -20 ˚C until use.

2.3 Bioactive marker determination

2.3.1 γ-oryzanol

The γ-oryzanol was analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) according to the method reported by Sakunpak, Suksaeree, Pathompak, & Sermkaew, (2014). Briefly, 50 mg rice bran extract was dissolved in 5 ml isopropanol before filtrated through 0.45 µm PTFE. RP-HPLC was performed including an autosampler and a column oven equipped with a VertiSep™ pHendure C18 column (4.0×250 mm, 5 µm) and a variable wavelength UV–vis detector at 325 nm. The mobile phase, acetonitrile: metha-

nol (60:40, v/v) was flowed at 1.5 ml/min with an injection volume of 20 µl. The γ-oryzanol content was calculated from the peak area of standard γ-oryzanol.

2.3.2 Linoleic acid and oleic acid

The fatty acid content in the rice bran extract was determined by RP-HPLC following the method reported by Manosroi *et al.*, (2010) with some modification. In brief, 50 mg rice bran extract was dissolved in 5 ml isopropanol before filtration through 0.45 µm PTFE. The RP-HPLC was performed including auto sampler and a column oven equipped with a VertiSep™ pHendure C18 column (4.0×250 mm, 5 µm) and a UV–vis detector at 205 nm. The mobile phase, acetonitrile and 0.1% (v/v) glacial acetic acid (95:5) was flowed at 1 ml/min with an injection volume of 20 µl. The contents of linoleic acid and oleic acid were calculated from the peak area of standard linoleic acid and oleic acid.

2.4 Biological activity study

2.4.1 Cell culture preparation

The human dermal fibroblast cells (HDF) and Androgen-dependent LNCaP cells were cultured as following; The HDF cells were cultured in complete high glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum, 2%(v/v) Gluta max and 2%(v/v) of 10 µg/ml penicillin/streptomycin at 37 ˚C with 5% CO_2 in a humidified atmosphere. The LNCaP cells were cultured in RPMI-1640 containing 10% Fetal Bovine Serum and 100 U/ml penicillin, 100 µg/ml streptomycin at 37˚C with 5% CO_2 in humidified atmosphere. Cells were sub cultured at ≥80% cell confluence.

2.4.2 Cytotoxicity

The toxicity of the rice bran extracts was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The extract solutions (31.25 -500 µg/ml) were prepared in PBS containing 2%v/v dimethylsulfoxide (DMSO). Firstly, the cells were seeded into 96 well plates at a density of 1×10^4 cells/well and left at 37 ˚C in 5% CO_2 with a humidified atmosphere for 24 h. Then, the cells were exposed to 100 µl of the extracts and blank control before incubating for 24 h. Next, 50 µl of culture medium from each well were replaced by 50 µl of MTT solution. After three hours, the formazan products were dissolved in DMSO, and measured at 490 nm. The amount of formazan is directly proportional to the number of living cells according to the following equation:

$$\text{Cell viability (\%)} = (Abs_{sample}/Abs_{blank})\times100$$

Where; Abs_{sample} is absorbance of the sample
Abs_{blank} is absorbance of the blank control

2.4.3 Analysis of pro-collagen type I synthesis

Pro-collagen synthesis activity was examined by sandwich ELISA using Human Pro-collagen alpha 1 matched antibody pair kit (ab216064, Abcam). The extract solution, 31.25 µg/ml and a positive control, L-ascorbic acid were prepared in PBS containing 2%v/v DMSO. In brief, the HDF cells were seeded into 96 well plates at a density of 1×10^4 cells/well and left overnight at 37 ˚C in 5% CO_2 with a humidified atmosphere. After that, 100 µl of the medium in each well were replaced by 100 µl of the extract solution or the positive control prior to incubating for 24 h. Then, the culture medium was collected and the quantity of pro-collagen was determined according to a description of the assay kit. The result was expressed in term of %increase of the pro-collagen content in comparing to the control.

2.4.4 Antioxidant test by DPPH radical scavenging assay

The antioxidant activity was measured by DPPH radical scavenging assay. The serial concentration (0.2 µg/ml-10 mg/ml) of the extract was prepared in methanol. Then, 100 µl of the extracts or a positive control, L-ascorbic acid were mixed with 100 µl DPPH (0.2 mM of DPPH in methanol). Reactions were kept in room temperature under a dark condition for 30 min. The absorbance of the resulting solution was measured at 517 nm. Inhibition of free radical DPPH in percent was calculated as the following equation:

$$\text{Free radical DPPH inhibition (\%)} = [(Abs_{blank}-Abs_{sample})/Abs_{blank}] \times 100$$

Where Abs_{sample} is absorbance of the sample, and Abs_{blank} is absorbance of the blank.

2.4.5 5α-reductase inhibition assay

2.4.5.1 5α-reductase steroid enzyme preparation

The 5α-reductase enzyme in this study obtained from the LNCaP cells. For preparation, the cells with more than 80% cell confluence were suspended in lysis buffer containing 10 mM Tris HCl buffer pH 7.4, 50 mM KCl, 1 mM EDTA and 0.5 mM phenylmethanesulfonyl fluoride. The cell suspension with 9×10^7 cells/ml was homogenized on ice using sonication probe with 10 s pulse on and 10 s pulse off for 1 min with 40% amplitude. The free cell supernatant inferred as the crude enzyme.

2.4.5.2 Enzymatic inhibitory assay

The assay was determined by measuring the potential of the extract on interrupting 5α-reductase to convert testosterone to DHT according to the assay reported by Srivilai *et al.*, (2016). Finasteride was used as a positive control. The reaction contained 100 µl of the extract (100 µg/ml in DMSO), 20 µl of 34.7 µM testosterone, 40 µl of Tris buffer pH7.4, 50 µl of 1mM NADPH in Tris buffer and 80 µl enzyme (5α-reductase). The reaction was maintained at 37˚C for 60 min prior to stopping the reaction by adding 300 µl hydroxylamine and keeping at 60˚C for 60 min. The supernatant was collected, and DHT was estimated by LC-MS. In the control group (the reagent blank and inhibitor blank), the reaction mixture only added DMSO and no inhibitor. For the reagent blank (C_0), the reaction mixture was quenched at time 0. While, the inhibitor blank (C_{60}), the reaction mixture was quenched at 60 min after incubation with uninhibited DHT

production. Percentage of 5α-reductase inhibition calculated using peak area from LC-MS chromatogram and as the following equation:

$$\text{Inhibition of } 5\alpha\text{-reductase } (\%) = 100 - [(\text{Sample} - C_0)/(C_{60} - C_0) \times 100]$$

Where sample is DHT peak area in the sample treatment, C_0 is DHT peak area of the reagent blank, and C_{60} is DHT peak area of the inhibitor blank.

3. Results and Discussion

3.1 Extraction yields

The extraction yields of both kind of rice depended on the extraction method (Table 1). Extraction by maceration offered the maximum yields of 14.20±0.67 and 27.18± 0.19%w/w for Jasmine (J) and Riceberry (R) rice brans respectively. While, the lowest extracted yields found in subcritical DME extract with 4.93±0.08 and 4.90±0.19%w/w for Jasmine (JD) and Riceberry (RD) rice brans respectively. The difference in yield among these processes dues to the difference in their principles as well as the solvent polarity. The cold pressed extraction relies on the mechanical force to squeeze oil from raw materials. The advantages of this extraction are low cost and rapid. Moreover, the compounds in the crude oil are generally hydrophobic such as fatty acid despite the yield is slightly small. The 50% ethanol maceration exhibited the highest yield because of the process bases on the principle "like dissolve like" between the extraction solvents and the compounds available in the raw materials. However, the obtained compounds are mostly hydrophilic due to the high polarity of ethanol and water. Whilst, in subcritical DME fluid extraction, dimethyl ether is lower in polarity than that of ethanol. The effects of solvent polarity were reported by Lai, Li, Lu, & Chen, (2009) who showed that the extraction yield was increased with increasing polarity value. The dielectric constants of the solvents were compared to demonstrate their polarities. Ethanol (dielectric constant = 24.5) showed a higher polarity than DME (dielectric constant = 3.01-5.34) that related with the lower in yield of subcritical DME fluid extract comparing to maceration extract. In contrary, the advantages of subcritical DME fluid extraction are that it is less time consuming, provides easy separation of the DME from the extract.

3.2 Bioactivity marker determination

3.2.1 Determination of γ-oryzanol content

The γ-oryzanol contents in rice bran extracts were presented as the total γ-oryzanol in Table 1 which was the summation of cycloartenylferulate (A), 24-methylenecycloartanyl ferulate (B), campesterylferulate (C) and β-sitosterylferulate (D) as depicted in the HPLC chromatogram (Figure 1A). The results obviously showed that γ-oryzanol content from different rice varieties and extraction processes were significantly different ($p<0.05$). For Jasmine rice, cold pressed extract (JO) provided the yield of 4.67±0.01%w/w, which was higher than the 2.47±0.01%w/w yield found in subcritical DME extract (JD). On the other hand, the best γ-oryzanol yield of 6.01±0.03%w/w in Riceberry rice found in the subcritical DME extract (RD), which was significantly higher than the 5.43±0.04%w/w obtained from the cold pressed extract (RO). The higher γ-oryzanol content in the RO over JO was not in agreement with the previous report of Mingyai (Mingyai *et al.*, 2018). The highest γ-oryzanol content of 6.01±0.03%w/w in RD suggested its efficiency for commercial applications. However, γ-oryzanol was not detected in 50% ethanolic maceration extracts from both rice varieties. Ethanol/water blending formed a high polarity solvent and therefore it was difficult to retrieve low polar compounds, especially γ-oryzanol, which is composed mainly of aromatic ring and cycloalkyl groups. A general extraction solvent for γ-oryzanol in rice bran was shown to be hexane and propanol (50:50) (Xu & Godber, 2000).

3.2.2 Determination of linoleic acid and oleic acid contents

The linoleic acid and oleic acid contents were quantified from peak area of the standard compounds at the retention time of 6 and 8 min respectively (Figure 1B-C). Regard to these fatty acid contents (Table 1), subcritical DME provided the highest linoleic acid and oleic acid contents with, 22.39±0.61%w/w and 39.48±1.07%w/w for Jasmine rice bran and 19.96±0.54%w/w and 33.48±0.39 %w/w for Riceberry rice bran. These were significantly higher ($p<0.05$) than those from the maceration and cold pressed methods. These results were in agreement with other reports, where DME was suitable for extraction of lipids and functional compounds (Goto, Kanda, Wahyudiono, & Machmudah, 2015). For Jas-

Table 1. Percentage yield (%w/w), γ-oryzanol, linoleic acid and oleic acid contents of Jasmine and Riceberry rice bran extracts of different extraction methods.

Rice bran variety	Extraction method	Extract yield (%w/w)	γ-oryzanol (%w/w)	Linoleic acid (%w/w)	Oleic acid (%w/w)
Jasmine	Maceration (J)	$14.20\pm0.67^{A,a}$	ND	$7.18\pm0.35^{A,a}$	$7.52\pm1.00^{A,a}$
	Cold pressed (JO)	$7.00\pm0.00^{B,b}$	$4.67\pm0.01^{A,a}$	$5.1\pm0.21^{B,b}$	$7.36\pm0.44^{A,a}$
	Subcritical DME (JD)	$4.93\pm0.08^{C,c}$	$2.47\pm0.01^{B,b}$	$22.39\pm0.61^{C,c}$	$39.48\pm1.07^{B,b}$
Riceberry	Maceration (R)	$27.18\pm0.19^{D,d}$	ND	$2.18\pm0.18^{D,d}$	$1.76\pm0.51^{C,d}$
	Cold pressed (RO)	$5.00\pm0.00^{C,e}$	$5.43\pm0.04^{C,d}$	$13.90\pm0.25^{E,e}$	$25.04\pm3.75^{D,e}$
	Subcritical DME (RD)	$4.90\pm0.19^{C,e}$	$6.01\pm0.03^{D,e}$	$19.96\pm0.54^{C,f}$	$33.48\pm0.39^{E,f}$

ND = Non detect; One way ANOVA; Post hoc multiple comparisons; Equal variances assumed by Tukey; $p<0.05$; A, B, C, D and E: Mean values with different letters within the column are significantly different for all extracts from the different rice bran varieties and the different extraction methods; a, b and c: Mean values with different letters within the column are significantly different for Jasmine rice bran extract from the different extraction methods (J, JO, JD). d, e and f: Mean values with different letters within the column are significantly different for Riceberry rice bran extract from the different extraction methods (R, RO, RD).

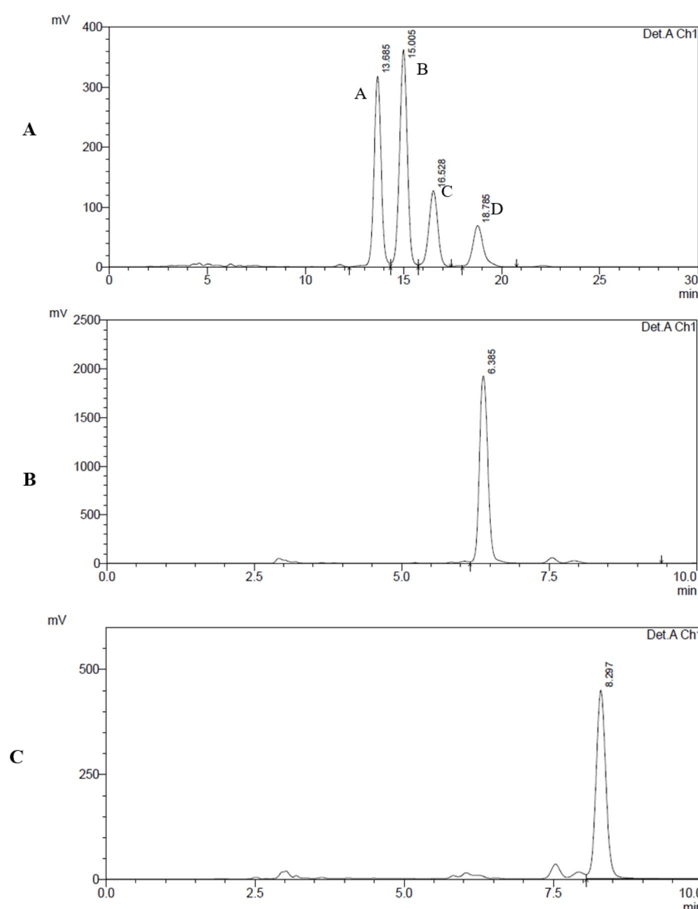

Figure 1. HPLC chromatogram of the standard compounds (A) γ-oryzanol, (B) linoleic acid and (C) oleic acid

mine rice, the levels of linoleic and oleic acid were practically similar in the range of 5.10±0.21-7.52±1.00%w/w from maceration and cold pressed extraction. Meanwhile, these were evidently difference in Riceberry rice since the amount of linoleic and oleic acid from cold pressed were around 6.3 and 14.2 folds higher than those obtained from maceration. The fatty acid yield results from different methods were different may due to their different in extraction conditions and the physically property of the desired products. This can be explained by when the solvent temperature being above the normal boiling point at elevating pressure in subcritical fluid extraction, DME are in liquid phase with gas-like diffusion rate due to hydrogen weakening and polarity decreasing. Therefore, the dissolving power is greater than in the gas phase at the same temperature or pressure. All of which play a role in the permittivity of the solution with more elution power between solvent and bioactive compounds (Haghighi & Khajenoori, 2013; Kanda, Li, Goto, & Makino, 2015; Zakaria & Kamal, 2016).

On the other hands, the maceration method using 50% ethanol as a high polarity solvent was poorly to snatch lipophilic compounds like fatty acids from the raw materials. Linoleic acid and oleic acid were confirmed as a marker for 5α-reductase inhibition activity by correlation coefficient study (Choi *et al.*, 2014; Ruksiriwanich *et al.*, 2011).

3.3 Biological activity study

3.3.1 Cytotoxicity on human dermal fibroblast cells

The cytotoxicity on human dermal fibroblast cells (HDF) was studied to observe the safe concentration of each extract for further biological activity studies. The chosen concentration should ensure cell viability of more than 90% comparing to the control group. At these concentrations, cell necrosis and morphology change should not occur. The cell viability effect of the Jasmine rice bran maceration extract (J), Riceberry rice bran maceration extract (R) and Jasmine rice bran cold press extract (JO) were greater than 90% at concentration range 31.25-500 µg/ml, but the cell morphology change was observed when treated with R and JO at 500 µg/ml. Moreover, the cell morphology changes were also observed when treated at lower concentration (250 µg/ml) of Riceberry rice barn cold press extract (RO) and the percentage of cell viability was also decreased significantly when treated with 500 µg/ml RO. With subcritical DME extraction, the JD and RD caused HDF morphology changes at 62.5 µg/ml, with percentages of cell viability significantly reduced at a concentration range between 250 and 500 µg/ml. Therefore, the selected concentration of all extracts was 31.25 µg/ml.

3.3.2 Analysis of Pro-collagen type I synthesis

For Jasmine rice, the high pro-collagen synthesis was found in the cells treated with JD and JO (50.91±7.05% and 44.49±8.75% respectively) while the lowest pro-collagen synthesis of 20.49±5.11% was found in the cells treated with J as showed Table 2. Comparing to Riceberry rice, the highest pro-collagen synthesis obtained from the cells treated with R (46.99±17.04%). This was around 2.4 and 2.3 times higher than that observed in RO and RD with 19.52±5.21% and 20.49±1.70 respectively. The pro-collagen type I synthesis activity of Jasmine rice bran extract and Riceberry rice bran extract on HDF cells has not yet been characterized and reported. Our finding is merely in agreement with the activity of Black glutinous rice that was previously reported (Han *et al.*, 2018; Phetpornpaisan, Tippayawat, Jay, & Sutthanut, 2014). However, the highest pro-collagen synthesis in the cells treated with JD and R were around 1.4 and 1.5 times lower than that found with positive control, L-ascorbic acid (125 mM) with 73.50±5.11%. The effect of extraction methods showed different trend of pro-collagen type I synthesis. This result might be because of the different in biological compounds existing in the rice bran varieties. The result also supports the previous report that bioactive compounds extracted from rice bran demonstrated anti-aging activity (Manosroi, Chutoprapat, Abe, Manosroi, & Manosroi, 2012).

3.3.3 Antioxidant test by DPPH radical scavenging assay

The results showed the EC_{50} of DPPH scavenging activity of rice bran extracts (Table 2). The highest activity values in both kinds of rice clearly found in the maceration extract. In Jasmine rice, the highest activity obtained from the maceration extract (J) with the EC_{50} of 1.31±0.01 mg/ml that was approximately 2.2 and 6.1 times higher than that observed in JO and JD respectively. The highest activity in Riceberry rice was from R with 0.2±0.05 mg/ml calculating as around 11.7 and 18.2 times higher than that found in RO and RD respectively. The maximum activity from both samples was however lower than the positive control (Ascorbic acid) that exhibited the EC_{50} of 0.004±0.00 mg/ml. The result suggested that high polarity solvents should be applied to achieve the extracts containing high antioxidant activity. In general, phenolic compounds were the primary functional compound in antioxidant properties of cereal or even rice bran. Previously, it has been reported that an increase in solvent polarity and solvent viscosity increased the extraction yield of phenolic compounds and antioxidant properties (Lai, Li, Lu, & Chen, 2009). Comparing to the highest activity of two kinds of rice in the maceration, the EC_{50} found in Riceberry rice was 6.5 times higher than that found in Jasmine rice. Riceberry rice appeared in deep purple and it composed abundantly with anthocyanins, the potent antioxidant compound that widely reported as the component in pigmented rice (Deng *et al.*, 2013). This finding is also in agreement with Nam *et al.*, (2006) who reported that the rice bran extracts from pigmented rice had higher antioxidant compounds, such as anthocyanins and ferricyanide, and demonstrated higher levels of activity than the non-pigmented varieties.

3.3.4 5α-reductase inhibition assay

5α-reductase is an enzyme that converts testosterone to DHT. In our study, the 5α-reductase enzyme was taken from the LNCaP cells. The amount of DHT in the reaction was detected by LC-MS which represented in the Extracted-Ion Chromatograms (EIC) at the *m/z* of 306.24. Figure 2 showed the EIC chromatograms of DHT derivatives in the reaction. The DHT peak did not detect in the reagent blank reaction (Figure 2A) while it was detected in the inhibitor bank reaction that allowed 5α-reductase to convert testosterone to DHT (Figure 2B). However, the reaction treated with Jasmine rice bran extract and finasteride resulted in the reduction of DHT peak (Figure 2C-D). The 5α-reductase inhibition activity of the rice bran extract (100 µg/ml in DMSO) was presented in Table 2. The superior activities were found in the extracts from subcritical DME extraction with 73.14± 4.06% and 70.49±3.11% inhibition for JD and RD respectively. This was the remarkable result with high activity of the

Table 2. Biological activities of Jasmine and Riceberry rice bran extracts from different extraction methods.

Sample	Extraction method	Pro-collagen synthesis (% increase)	DPPH assay (EC_{50}, mg/ml)	%5αR Inhibition
Jasmine		*(31.25 ug/ml)*		*(100 µg/ml)*
	Maceration (J)	$20.49±5.11^{A,a}$	$1.31±0.01^{A,a}$	$7.72±2.76^{A,a}$
	Cold pressed (JO)	$44.49±8.75^{B,b}$	$2.95±0.23^{B,b}$	$19.89±2.87^{B,b}$
	Subcritical DME (JD)	$50.91±7.05^{B,b}$	$8.12±1.50^{C,c}$	$73.14±4.06^{C,D,c}$
Riceberry		*(31.25 ug/ml)*		*(100 µg/ml)*
	Maceration (R)	$46.99±17.04^{B,d}$	$0.20±0.05^{D,d}$	ND
	Cold pressed (RO)	$19.52±5.21^{A,e}$	$2.34±0.12^{E,e}$	$65.85±3.13^{C,d}$
	Subcritical DME (RD)	$20.49±1.70^{A,e}$	$3.64±1.21^{F,f}$	$70.49±3.11^{C,D,d}$
L-ascorbic acid* (22.02 µg/ml)	-	$73.50±5.11^{C}$	$0.004±0.00^{D}$	-
Finasteride* (1.5 µg/ml)	-	-	-	$97.86±0.07^{E}$

ND = Non detectable, * = Positive control; One way ANOVA; Post hoc multiple comparisons; Equal variances assumed by Tukey; $p<0.05$; A, B, C, D, E and F: Mean values with different letters within the column are significantly different for all extracts from the different rice bran varieties and the different extraction methods; a, b and c: Mean values with different letters within the column are significantly different for Jasmine rice bran extract from the different extraction methods (J, JO, JD); d, e and f: Mean values with different letters within the column are significantly different for Riceberry rice bran extract from the different extraction methods (R, RO, RD).

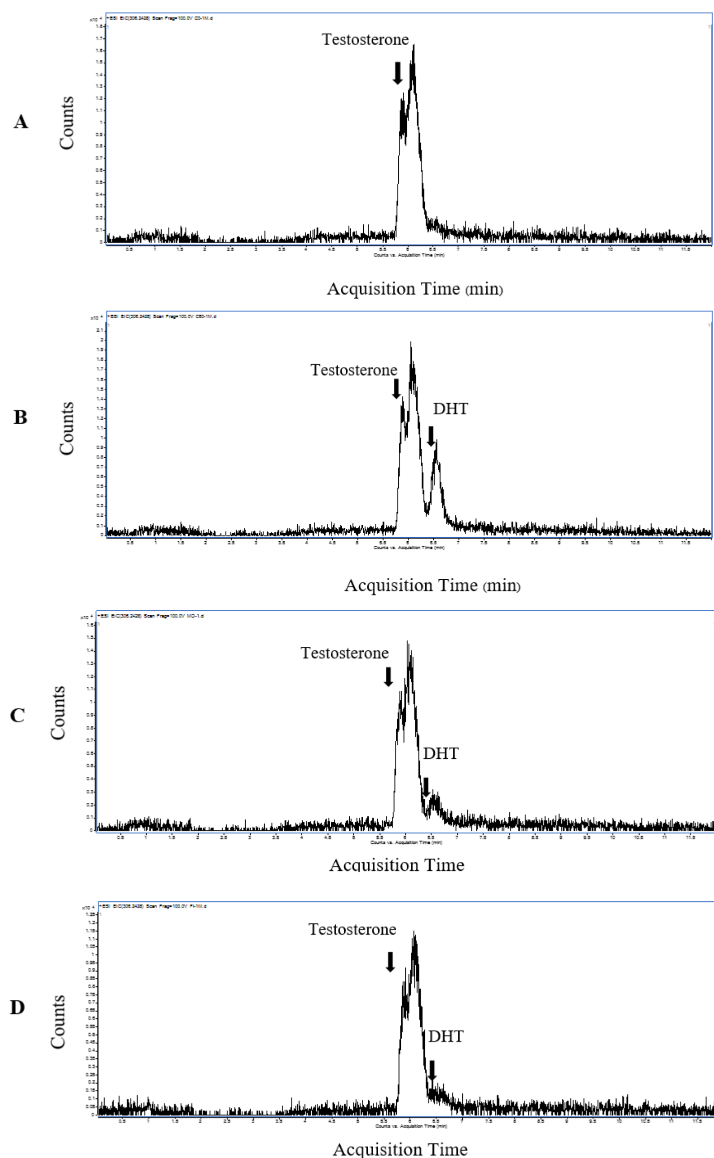

Figure 2. EIC chromatogram of DHT at *m/z* 306.24 in the reaction of (A) the reagent blank (C_0), (B) the inhibitor blank C_{60}, (C) the Jasmine rice bran extract (JD) and (D) finasteride (positive control).

unpurified crude extract, although this activity was still lower than that found in the positive control, finasteride (1.5 µg/ml) which exhibited of 97.86±0.07% inhibition. In case of Jasmine rice, the activity observed in JD was significantly higher ($p<0.05$) than that found in the maceration (J) and cold press method (JO) for 9.4 and 3.6 times respectively. Unlike Jasmine rice, the activities found in Riceberry rice were not significantly different between RD (70.49±3.11%) and RO (65.85±3.13%) while the activity from the maceration (R) was not found. This result might be due to the bioactive compounds existing in the extracts. Regard to the fatty acids found in rice bran, linoleic acid was previously determined as the significant 5α-reductase inhibition compound while oleic acid and γ-linolenic acid less affected on this inhibition (Ruksiri wanich *et al.*, 2011).

In contrast, subcritical DME extraction gave the highest linoleic acid content among three extraction processes with both Jasmine and Riceberry rice which were 22.39±0.61 and 19.96±0.54% w/w for JD and RD respectively (Table 1). This result corresponded with the highest 5α-reductase inhibition activity found in subcritical DME extraction (JD, RD). The positive relation of linoleic acid amount and 5α-reductase inhibition activity was clearly found in Riceberry rice extract. As the highest linoleic acid content in RD (19.96±0.54%w/w) that also expressed the highest 5α-reductase inhibition activity of 70.49±3.11%, the second linoleic acid content in RO (13.90±0.25) exhibited the activity of 65.85±3.13% while the lowest linoleic acid content in J (2.18±0.18%w/w) did not show the inhibition activity. This evidence also revealed the chance to approach the desired activity of the 5α-reductase

inhibition from Riceberry rice with subcritical DME extraction and cold press method. The result of Jasmine rice was slightly different. Although the highest activity found in JD can be anticipated, but the activity seen in J and JO did not correlate with linoleic content since the 5α-reductase inhibition activity found in JO (19.89±2.87%) was around 2.5 times higher than the activity seen in J (7.72±2.76%) despite, the linoleic content observed in JO and J were 5.1±0.21 and 7.18±0.35 %w/w respectively. Considering the other bioactive compounds in rice bran, γ-oryzanol has been reported to involve in the inhibition of 5α-reductase (Ruksiriwanich *et al.*, 2011). By using cold pressed extraction, the γ-oryzanol content of 4.67±0.01%w/w was observed in JO while it was not found in J and this might be the reason for low 5α-reductase inhibition activity in J in comparing to JO. In our study, three bioactive compounds, γ-oryzanol, linoleic acid and oleic acid were analyzed and the result clearly revealed the relationship between linoleic acid and 5α reductases inhibition activity in pigmented rice bran extract. In addition, it is possible that other compounds also involved in the activity found in the pigmented rice since some compounds such as ferulic acid, vanillic acid, γ -oryzanol and phytic acid were reported to be synergistic in 5α reductases inhibition activity (Das, Chaud huri, Kaimal, & Bhalerao, 1998; Ruksiriwanich, Manosroi, Abe, Manosroi, & Manosroi, 2011). The result certainly provides superior benefits on acquiring the 5α-reductase inhibition activity from rice bran extract and this was, therefore, possible to operate in a commercial scale. In our study, the correlation between 5α-reductase inhibition activity and the fatty acids was estimated as r^2=0.8925 and r^2=0.9342 for linoleic acid and oleic acid respectively.

4. Conclusions

The extraction processes affected the extract yield, bioactive compounds and biological activities of rice bran extracts. The maceration provided the highest yield with remarkable antioxidant activity in both rice while in Riceberry rice, this extract also exhibited the high activity of procollagen type I synthesis. Cold press method gave the extracts with high γ-oryzanol content. Significantly, the Jasmine rice bran extract from subcritical DME extraction provided the highest linoleic acid and oleic acid content and the extracts gave the highest activity of 5α-reductase inhibitor. This suggested the relationship between the extraction method and the bioactive compounds related to their biological activity, and it can be applied in a commercial scale. Remarkably, the high 5α-reductase inhibitor activity in the rice bran extract from subcritical DME extraction can be used as a natural androgenic dependent disorder treatment and cosmetics.

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