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Policosanol contents, volatile profile and toxicity test of granulated cane sugar enriched with rice bran materials

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Abstract

Sugarcane and rice bran are the most important sources of commercial policosanol (PC) wax which exhibits a cholesterol lowering bioactivity. Both defatted rice bran (DRB) as agricultural waste and rice bran oil (RBO) retain a varying but significant amount of PC wax. Non-centrifugal cane sugar (NCS) has been consumed worldwide, and possesses various health benefits. It is mostly produced in hardened block form, which is not convenient for use compared with granular form. We aimed to increase PC contents of the granular sugar by adding wax extracted from DRB and RBO and to investigate the toxicity of the products. The results showed that the total PC contents including long chain aldehyde of products were increased to the maximum level of 147.97 mg/100 g. DRB is promising source of policosanol (6,044.7 mg/100 g). The main volatile components of developed sugar product was aldehyde and alcohol compounds. The 28 day toxicity evaluations of the developed sugar revealed no adverse effects.

Introduction

Nearly 24 million tons of rice *(Oryza sativa)* is produced annually in Thailand with nearly 2.28 million tons of rice bran (RB) produced as a by-product of the rice milling process. RB is normally used to produce value added rice bran oil which can be used for cooking or industrial applications. Thailand is the third largest rice bran oil (RBO) production (60,000 ton) in the world after Japan and India *(http://www.business-standard.com)*. In recent years, the interest in cold pressed extraction has been increasing as the oil obtained from cold-pressing contains better nutritive properties than oil from a chemical extraction method (Thanonkaew *et al.*, 2012). The extracted oil is further processed only through membrane-based filtration techniques, resulting in RBO with better nutritional value and preservation of bioactive substances and compounds and is rich in functional properties. However, the cold pressed process results in low oil recovery of only 16-22% compared with the solvent extraction method (90-95% oil recovery) (Reddi *et al*., 1948). The cold-pressed method lead to 84-78% of the defatted rice bran (DRB) being a waste product which has very low value and is used for animal feed or is discarded as agricultural waste.

RB contains a high content of waxes which consist of very long-chain alcohol compounds collectively known as policosanols (PC) (Cravotto *et al*., 2004). Consequently, both RBO and DRB retain a varying but significant amount of wax depending on various extraction conditions. PC commonly refer to a mixture of aliphatic primary alcohols and aldehyde (C20 to C36) containing mainly docosanol (C22), tetracosanol (C24), hexacosanol (C26), octacosanol (C28) and triacontanol (C30) (Irmak *et al*., 2006; Harrabi *et al*., 2009) which are produced from a diversity of natural sources such as beeswax, sugar cane, rice bran, and wheat germ (Irmak *et al.*, 2006). However, the most important sources of commercial PC wax are sugarcane and rice bran. Many clinical studies have shown the potential serum lipid-lowering properties of PC and have indicated that they are interesting cholesterol lowering nutraceuticals (Janikula, 2002; Chen *et al*., 2008; Francini-Pesenti *et al*., 2008). The researches on acute and chronic toxicity, carcinogenicity and mutagenicity have shown no treatment-related toxicity and also have been shown to be safe when used in long term clinical

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studies (Aleman *et al.*, 1994; Gamez *et al.*, 2001). Its bioavailability has been reported to be between 5% and 12%, with absorption after oral administration ranging from 10 – 35% (Kabir and Kimura, 1993). On a total lipid basis, RBO with 3 – 4% wax has been reported (Sayre and Saunders, 1990).

Non-centrifugal sugar (NCS), that is, whole cane sugar, unrefined brown or black sugar is a solid product obtained by evaporating sugarcane juice and is consumed in many countries under different names such as Jaggery, Panela, Rapadura, Muscovado, Kokuto, Nam Taan Oi (Jaffe, 2012). Scientific researchers have reported significant positive health effects of NCS including immunological, anti-toxicity, cytoprotective, anticariogenic, and anti-hypertension effects (Jaffe, 2012). As the traditional process of NCS is based on evaporating sugar cane juice using open pans without molasses removal, the sugar product has its own characteristic taste and aroma and is rich in nutrients. In most countries the traditional NCS is produced in solid form with different shapes such as blocks or cones (Jaffe, 2012) since it is difficult to control the final product in granulated form due mainly to the impurity of cane juice from the dirt from the whole stalks. Compared with the granular form, the blocks or cones form is not convenient for use either domestically or industrially.

Our preliminary experiments found that peeling the stalks simply generates the novel granulated cane sugar product in the final process regardless of cane cultivars. On the other hand, we found that the PC contents of this granulated sugar also were reduced compared with the traditional process since PC wax is mainly in the outer layer rinds (Asikin *et al.*, 2012). The purpose of the present study, therefore, is to develop the granulated NCS enriched with PC from wax extracted from DRB and RBO based on only the evaporation process. The long term toxicity test in animal and volatile profile were also investigated.

Materials and Methods

Chemicals

The policosanol standards docosanol (C22-OH), tetracosanol (C24-OH), hexacosanol (C26-OH), octacosanol (C28-OH), and triacontanol (C30-OH) were purchased from Sigma Chemical Company (St. Louis, MO, USA). The derivatization reagent N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was obtained from GL Science (Tokyo, Japan). Pyridinium chlorochromate (Sigma Chemical Company) was used in the synthesis of long-chain aldehyde standards. Cold-pressed rice bran oil was obtained from the mill community enterprise Makhamraeng Nakhonsawan Province, Thailand.

Wax extraction from defatted rice bran

Dried defatted rice bran was mixed with hot hexane and isopropanaol to separate the crude wax through wax crystallization process. The crude wax was dried at 60°C for 12 h. The dried wax was heated with hexane and was saponified by refluxing with potassium hydroxide in 95% ethanol for 2 h to obtain lipid alcohol. To separate long chain alcohol from soap, hot water, toluene and ethanol were applied by refluxing. The toluene layer which contains the long chain alcohol was removed and refluxed using hot water and ethanol. The separated toluene layer was washed with hot water 3 times following removing toluene by rotary evaporator and oven drying.

Granulated cane brown sugar preparation

The granulated cane sugar product was prepared from Suphanburi 50 cultivar collected at stage of maturity (cultivated for 12 months old). Harvested canes were immediately brought back to the laboratory. The outer layer rinds were separated from the sugarcane stalks by hand peeling following the juice extraction by a two-roller power crusher. In order to make traditional granulated brown sugar, the cane juice was then filtered using a layer muslin cloth and was transferred to a hold house open pan with temperature at 110 - 120ºC to concentrate the sugarcane juice by boiling. Wax from DRB (0.7% and 1.3%) and rice bran oil (8% and 10%) were added during process of evaporation. The times were monitored during the boiling-concentration process till the granulated brown sugar was formed. All sugar samples were ground using a dry blender (Iwatani Corporation, Tokyo, Japan), sieved through a 30 mesh size and kept dry at -20°C until further analysis.

Policosanol and long-chain aldehyde determination

Sample extraction

The Soxhlet extraction method was followed Asikin *et al*. (2008). Briefly, 9 g of cane sugar products were placed in a thimble filter (Advantec No. 84, Tokyo, Japan) and extracted using a Soxhlet apparatus with approximately 150 mL of a mixture of hexane and methanol (20:1 v/v) with extraction time of 10 h both for policosanol and long-chain aldehyde analysis. The solvent solution was removed using a rotary-evaporator under a vacuum at 40°C following dilution of dried residue extract with toluene or chloroform to obtain 1mL sample volumes for analysis.

Standard and sample preparation

For quantification of policosanol compounds by GC-FID, a mixture of the policosanol standards was prepared in toluene. The policosanol standards or samples were also prepared in chloroform and derivatized with MSTFA (2:1 v/v) at 50°C for 15 min for mass spectrum identification. The mixed derivatization solution including 0.5 mL of sample in chloroform and 250 µL of MSTFA, was heated at 50°C for 15 min, followed by the addition of chloroform to obtain a 1mL sample for analysis. The aldehyde standards were synthesized from their alcohol forms by oxidation with pyridinium chlorochromate as described by Pérez-Camino *et al.* (2003). Briefly, the corresponding 1 mM alcohol standards (17.73 mg tetracosanol, 19.14 mg hexacosanol, 20.54 mg octacosanol, and 21.94 mg triacontanol) and 9 mM pyridinium chlorochromate (97.5 mg) were stirred in 50 mL of dichloromethane for 1.5 h at room temperature. The reaction mixture was eluted with dichloromethane through a short column (6× 2 cm i.d.) packed with silica gel-60. The reaction products were then dried with N_2 and diluted in toluene. The synthesized long-chain aldehyde standards were then subjected to gas chromatography (GC) analysis and their mass fragments were identified using GC-MS.

GC-FID analysis

A Shimadzu GC-2010 equipped with a fused capillary column (DB 5, 0.25 mm i.d. × 30 m; J&W Scientific, Folsom, CA, USA) and a flame ionization detector were used for quantitative analysis of policosanols and long-chain aldehydes. The GC injector and the flame ionized detector were both set at 350°C. Samples (1 µL) were injected with a split ratio of 1:10 under helium atmosphere. The oven temperature was initially set to 150°C, increased to 320°C at 4°C/min, and then maintained at 320°C for 15 min. The relationship between concentration and peak area was calibrated by injecting mixture standards of policosanols and aldehydes of different concentrations over the concentration levels of the extract samples. Policosanol and long-chain aldehyde content were expressed as mg/100 g sample on a wet basis.

GC-MS analysis

Trimethylsilyl derivatives of alcohols were analyzed by a Shimadzu GC-MS QP-2010 plus equipped with a fused capillary column DB-5 MS (0.25 mm i.d. × 30 m, J and W Scientific) under the same GC conditions described above. However, the mass spectra of aldehydes were analyzed without silylation. Samples (0.3 µL) were injected with a split ratio of 1:10. For MS detection, the electron impact (EI) ion source and transfer line temperatures were set to 200 and 280oC, respectively, and the ionization energy was set to 70 eV. The mass acquisition scan range and rate were 30 - 500 amu and 2 scans/s, respectively.

Volatile compound analysis

Three grams of samples were placed in a 20-mL glass GC vial and sealed with aluminum crimp cap. Agilent G1888 headspace autosampler was used. The GC-FID analysis was performed using an Agilent 7890A GC system equipped with a fused silica capillary (column: DB-Wax column (60 m × 0.25 mm i.d., 0.25 µm film thickness, Agilent J and W) and a flame ionization detector (FID). The GC injector and FID were both set at 250°C, and the oven was initially programed at 40°C (held for 5 min), then increased to 200°C at a rate of 5°C/min and maintained for 3 min. Helium was used as carrier gas at a flow rate of 23 cm/s. Samples were injected using a split ratio of 1:10.The peak area response of the volatile compounds was monitored in order to evaluate the relative amounts (%) of the volatile components in cane brown sugar. The mass spectra of volatile aroma compounds were analyzed using an Agilent 7890A GC coupled with an Agilent 5975C mass spectrometer. The column and oven programs for GC-MS analysis were as described above. For MS detection, the ion source and interface were both programmed at 230°C, the electron impact ionization at 70 eV, and the acquisition range (m/z) at 29 – 300 amu. The aroma components were identified by comparison of the linear retention indices (RIs), the mass spectra fragmentation patterns with the MS data of the corresponding compounds obtained from the National Institute of Standards and Technology (NIST) MS Library, Version 2008, and the peak enrichment upon co-injection with authentic volatile standards. Linear RIs of the volatile components were determined relative to the retention times of a series of n-alkanes (C5–C20). All analyses were carried out in triplicate (Asikin *et al*., 2014).

Toxicity test

Four-week-old male ICR mice were purchased from the BioLASCO Experimental Animal Center (Taiwan Co., Ltd, Taipei, Taiwan). After 1 wk of acclimation, animals were randomly distributed into five groups. All animals were housed in a controlled atmosphere (25 ± 1°C at 50% relative humidity) and with a 12-h light/12-h dark cycle. Animals had free access to food and water at all times. Food cups were replenished with fresh diet every day. All experimental

animal care and treatment followed the guidelines set up by Institutional Animal Care and Use Committee of National Kaohsiung Marine University (IACUC, NKMU). All groups of mice were fed a controlled diet daily. The mice were orally administered original brown sugar (BS), brown sugar enriched with 10% RBO (OBS30), brown sugar enriched with 0.7% wax extract (WBS2), and brown sugar enriched with 1.3% wax extract (WBS4) at 100 mg/ kg consecutive for 4 weeks, while distilled water served as the control group. The animals were sacrificed under CO_2 asphyxiation after the study period of four weeks. Blood was collected from the mice in each group in the mice model through heart puncture when sacrificed. After coagulation, the activities of serum glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), triglyceride (TG) and total cholesterol (T-cho) were analyzed. The liver, kidneys, and spleen were removed and weighed.

Blood samples

Blood samples were collected from the left ventricle under anesthesia. The samples were mixed in 10 µL of heparin sodium and centrifuged at 3,500 rpm and 4°C for 10 min. The plasma was then stored at - 80°C until use. The total cholesterol, triglyceride (TG), glutamic-oxaloacetic transaminase (GOT), and glutamic-pyruvic transaminase (GPT) levels were analyzed by enzyme methods using Fuji DRI-CHEM4000 (Fujifilm, Hsinchu, Taiwan) and Fuji DRI-CHEM slides (TCHO, TG,GOT/AST, and GPT/ALT-P3, Fujifilm).

Necropsy

Following the 28 days of experimental period, all mice were sacrificed. The vital organs of mice in both the control and the treatment groups, such as liver, heart, spleen, lung and kidney, were isolated and examined for any lesions. All of the individual organs were weighed and observed macroscopically to compare between both the treated and the control groups.

Statistical analysis

All the data were expressed as means ± standard deviation (SD) of three replications, and one factor ANOVA followed by the Tukey test was used for the statistical analysis. Trends were considered significant when the means of compared sets differed at $p < 0.05$.

Results and Discussion

Several reports and many clinical trials have shown that policosanol (PC) have significant health-promoting effect such as the reduction of lipid levels (Kato *et al.*, 1995; Gouni-Berthold *et al*., 2002; Reiner *et al.*, 2005), platelet aggregation reduction (Arruzazabala *et al*., 1996), antiviral activity (Pope *et al*., 1998), reduction of prostate enlargement, and relief of intermittent claudication (Castano *et al.*, 2004). Sugarcane and rice bran (RB) are the most important sources of commercial PC wax globally. Defatted rice bran (DRB) is a waste product discarded as agricultural waste from rice bran oil extraction process, although it contains a valuable PC wax and could be used as a source of PC for food manufacture. Both rice bran oil (RBO) and DRB retain a varying but significant amount of PC wax. With the traditional process method for non-centrifugal sugar (NCS) or cane brown sugar, controlling the final sugar product in granulated form is difficult, partially due to the dirt of cane juice from the rind. Consequently, the NCS is mostly produced in hardened block form, which is not a convenient form for use compared to the granular form. The granulated brown sugar could be developed by additional processing of peeling the rind to improve the purity of the juice. However, PC wax of sugarcane is contained mainly in the rind of the cane (Asikin *et al*., 2012). The purpose of this study was to increase the policosanol contents of granulated cane brown sugar products (NCS) by enriching with RBO and wax extracted from DRB, and to evaluate volatile profile. Also toxicity test in mice were conducted. SP50 cane cultivar was applied to develop PC- enriched sugar product.

Policosanol and long chain aldehyde contents of rice bran oil and wax extracted from defatted rice bran

Table 1 indicated that there were substantial amount of policosanol and long chain aldehyde of wax extracted from DRB (6,005.28 and 39.49 mg/100 g, respectively), and RBO (72.71 and 24.22 mg/100 g, respectively). The major policosanol of wax was triacontanol (C30) (51.6%), followed by hexacosanol (C26) (18.5%) and octacosanol (C28) (15.9%) while those of RBO was octacosanol (33.6%) and tetracosanol (C24) (32.2%). Vali *et al.* (2005) indicated that predominant fatty alcohol of rice bran wax was tetracosanol (C24) and triacontanol (C30), while Ishaka et al. (2014) reported octacosanol (C28) and hexacosanol (C26) were the main policosanol of rice bran wax. Kim et al. (2014) studied on PC content in rice bran and reported that the octacosanol (C28) was the predominant component (46.4%), followed by triacontanol (C30) (31.6%), and tetracosanol (C24) (24.3%), which was a similar result reported by Cravotto et al. (2004). Our result was similar, with

Table 1. Policosanol and long-chain aldehyde contents of rice bran oil and wax extracted from defatted rice bran, obtained using GC-FID

Sources	Policosanol content[1] (mg/100 g sample)					Total
	C22	C24	C26	C28	C30	
Wax	92.24±14.55	739.95±34.13	1,114.34±57.91	959.55± 8.92	3,099.20±122.33	6,005.28±305.75
RBO	6.11±0.20	23.42±7.00	13.53±0.38	24.42± 0.90	5.22±0.78	72.71± 8.13
	Long-chain aldehyde contents (mg/100 g sample)					Total
	C22	C24	C26	C28	C30	
Wax	ND	ND	39.49±1.18	ND	ND	39.49±1.18
RBO	ND	2.88±0.02	11.15±0.56	10.20±2.61	ND	24.22±2.95

[1]Values are expressed as means ± standard deviation (n = 3); RBO=rice bran oil
ND: not detected

Table 2. Policosanol contents of granulated sugarcane products enriched with policosanol from rice bran oil and wax extracted from defatted rice bran, obtained using GC-FID

cane cultivars	policosanol contents[1] (mg/100 g)					Total
	C22	C24	C26	C28	C30	
BS	0.45±0.07^{a}	0.39±0.01^{d}	0.14±0.00^{d}	0.16±0.01^{c}	0.86±0.12^{c}	2.00± 0.20^{d}
WBS2	1.97±1.18^{a}	12.78±0.42^{b}	20.46±0.44^{b}	2.30±0.52^{c}	18.46±1.45^{b}	55.97± 0.59^{b}
WBS4	1.06±0.03^{a}	23.31±0.08^{a}	36.55±0.25^{a}	46.04±1.26^{a}	34.52± 0.35^{a}	141.48± 1.89^{a}
OBS24	0.78± 0.12^{a}	3.14± 0.18^{c}	2.03± 0.02^{c}	4.62± 0.19^{b}	1.00± 0.07^{c}	11.58± 0.30^{c}
OBS30	1.10± 0.24^{a}	3.12± 0.70^{c}	1.86± 0.05^{c}	4.20± 0.43^{b}	0.78± 0.13^{c}	11.06± 032^{c}

[1]Values are expressed as means ± standard deviation (n = 3) and values with different superscript letters within the same columns are significantly different ($p < 0.05$).
BS: brown sugar by SP50 cultivar, WBS2: brown sugar with 0.7% wax extracted from DRB, WBS4: brown sugar with 1.3% wax extracted DRB, OBS24: brown sugar with 8% RBO, OBS30: brown sugar with 10% RBO.
Not detected is ND

small differences to those of these previous studies and the variation probably being due to different varieties of rice cultivars, area of origin, and the chemical extraction method of wax and policosanol. Our results are, however, consistent with the report of Kaewkool and Krisnangkura (2010) showing that C30 is the highest component among long chain alcohol from rice bran wax. The reason may be due to the same area of rice origin, Thailand. In our study, even hexacosanol (C26) (1,114.34 mg/ 100 g) and octacosanol (C28) (959.55 mg/100 g) of wax from DRB were found at much higher levels than those of rice bran wax reported by Ishakal *et al*. (2014) (283.1 mg/100 g rice bran wax) and Kim *et al.* (2014) (26.6 mg/100 g rice bran wax).

We could not find long chain aldehyde docosanal (C22) and triacontanal (C30) in either RBO or in wax extracted from DRB. Hexacosanal (C26) was the only long chain aldehyde found in wax from DRB whereas octacosanal (42.1%) was the major long chain aldehyde found in RBO followed by hexacosanal (C26) and tetracosanal (C24). In the literature, no reports on policosanol and long chain aldehyde in DRB and RBO have been found, indicating that our study is the first to report this result.

Policosanol and long chain aldehyde contents of cane sugar products enriched with PC from rice bran material

Based on international unit of color for sugars, we found that the brown color of granulated sugar products with wax from DRB and RBO addition was lighter (IU 937.5) than the control product (without rice bran materials) (IU 2,641.7) (data not shown). The developed sugar products with 1.3% wax addition had higher water activities (0.58) and moisture content (3.11%) compared with the control (0.20% and 0.98%, respectively). The granulated sugar products with rice bran material addition slightly reduced total soluble solid (16.4) compared with the control sugar (17.3), while they had no impact on pH of products (5.75) (data not shown). The PC contents of the granulated sugar products enriched with rice bran materials are shown in Table 2. The retention times of the four synthesized aldehyde compounds, tetracosanal (C24), hexacosanal (C26), octacosanal

Table 3. Long-chain aldehyde content of granulated sugarcane products enriched with policosanol from rice bran oil and wax extracted from defatted rice bran, obtained using GC-FID

cane	long-chain aldehyde contents1 (mg 100 g-1 sample)				Total
cultivars	C24	C26	C28	C30	
BS	0.23±0.12^{b}	0.19±0.04^{e}	0.21±0.02^{c}	ND	0.63 ± 0.18^{e}
WBS2	0.49±0.02^{a}	2.91±0.23^{b}	0.63±0.04^{c}	ND	4.03±0.26^{d}
WBS4	0.54± 0.03^{a}	5.05±0.16^{a}	0.91±0.09^{c}	ND	6.49±0.08^{c}
OBS24	0.63±0.02^{a}	1.75±0.04^{c}	22.34±1.16^{b}	ND	24.72±1.21^{b}
OBS30	0.63±0.02^{a}	0.79±0.06^{d}	27.66±0.38^{a}	ND	29.08± 0.33^{a}

[1]Values are expressed as means ± standard deviation (n = 3) and values with different superscript letters within the same columns are significantly different ($p < 0.05$).
BS: brown sugar by SP50 cultivar, WBS2: brown sugar with 0.7% wax extracted from DRB, WBS4: brown sugar with 1.3% wax extracted from DRB, OBS24: brown sugar with 8% RBO, OBS30: brown sugar with 10% RBO.
Not detected is ND.

(C28) and triacontanal (C30), were 1 min less than their corresponding alcohol compounds.

The GC analysis of PCs revealed that the control brown sugar made from SP50 cane cultivar (BS) contained 2 mg/100 g and the most abundant of PC was mainly triacontanol (C30) (43%), followed by docosanol (C22) (22.5%). The result differs from previous studies of sugarcane policosanol that showed that octacosanol (C28) was the main component in Kokuto (Japanese brown cane sugar) and cane wax (Menendez *et al*., 2005; Irmak *et al*., 2006; Marrison *et al*., 2006; Asikin *et al.*, 2008). The original policosanol contents of sugar product here (2.0 mg/100 g) is much lower than that of Kokuto, which ranges from 6.96 – 85.7 mg/100g (Asikin *et al*., 2008). This might be due to the difference of cane cultivars, hand peeling process application, and the duration of policosanol Soxhlet extraction which was 10 h comparing to 24 h.

There was a significantly increase ($p < 0.05$) of each and total PC amount in the alcohol form after RBO and wax from DRB were added (Table 2). Increasing amount of wax addition caused the huge increasing level of total policosanol in sugar products. The developed sugar product with 0.7% (WBS2) and 1.3% (WBS4) wax contained the total PC contents at 55.97 and 141.48 mg/100 g, respectively (Table 2). The PC components of sugar products with 1.3% wax (WBS4) was higher than that of Kokuto (86 mg/100 g) (Asikin *et al*., 2008). The substantial increase of octacosanol (C28), hexacosanol (C26) and triacontanol (C30) of sugar products with wax from DRB addition was attributed to the most abundant amount of these policosanols in the extracted wax (Table 1).

PC distribution profile of sugar products with RBO addition at 8% (OBS24) and 10% (OBS30), was no significant difference ($p \geq 0.05$). Their major PC was octacosanol (C28) (39.9% and 38.0%, respectively) following tetracosanol (C24) (27.1% and 28.8%, respectively) since these two PC are the main policosanol in rice bran oil (Table 1). There was no significantly change ($p \geq 0.05$) of docosanol (C22) contents either RBO or wax from DRB addition.

With the increasing the level of rice bran material addition, the total long chain aldehyde (policosanal) was significantly increased ($p < 0.05$) from the original product (BS) (Table 3). The total long chain aldehyde amount in developed products with RBO addition was also higher than that found in Kokuto (8.7 mg/100 g) by Asikin *et al.* (2008). The major long chain aldehyde of sugar product made from SP50 cultivar was tetracosanal (C24) (36.5%) but that of Kokuto was octacosanal (C28). Adding wax from DRB and RBO caused the major change in long chain aldehyde profile and contents from tetracosanal (C24) (36.51%) to hexacosanal (C26) (72.2% and 77.8%) and to octacosanal (C28) (90.4% and 95.1%), respectively. These results were due to the fact that hexacosanal (C26) was the only long chain aldehyde in wax whereas octacosanal (C28) was than major long chain aldehyde in rice bran oil (Table1).

Gouni-Berthold and Berthold (2002) revealed that policosanol at doses of 10 mg to 20 mg per day lowers total cholesterol by 17% to 21% and low-density lipoprotein (LDL) cholesterol by 21% to 29%, and raises high-density lipoprotein cholesterol by 8% to 15%, and at dosages of policosanol at the amount of up to 20 mg per day is safe and well tolerated. In this finding, the total amount PC (alcohol together with aldehyde forms) of sugar products enriched with 0.7% wax (WBS2) and 1.3% wax (WBS4) were 60 and 147.97 mg/100 g , respectively, while that with 8% (OBS24) and 10% (OBS30) of RBO were 36.3 and 40.14 mg/100 g, respectively. The guideline for the sugar intake to maintain health recommended 4-8

Table 4. Volatile components (relative concentration (%) and mg/100 g fresh weight) of granulated cane sugarcane products enriched with PC from rice bran wax and oil

No	RI[b]	Volatile compounds	Contents (%)					Identification[c]
			BS	OBS24	OBS30	WBS2	WBS4	
1	697	Acetaldehyde	3.94±0.08[c]	12.25±0.34[b]	11.06±0.94[b]	14.47±0.16[a]	3.43±0.74[c]	RI, MS, Std
2	781	Propanal	ND	2.28±0.17	ND	ND	ND	RI, MS, Std
3	807	2-Methyl propanal	ND	10.43±1.96[a]	1.76±0.28[b]	14.71±2.48[a]	1.91±0.93[b]	RI, MS, Std
4	907	2-Methyl butanal	0.56±0.19[d]	6.57±0.31[a]	4.70±0.43[bc]	4.71±0.09[b]	1.35±0.32[cd]	RI, MS, Std
5	911	3-Methyl butanal	0.72±0.32[e]	17.77±0.66[a]	9.89±0.52[bc]	9.40±0.38[c]	2.66±0.51[d]	RI, MS, Std
6	973	Pentanal	2.11±0.66[b]	2.37±0.28[b]	6.34±0.75[a]	2.48±0.67[b]	1.17±0.26[b]	RI, MS, Std
7	1077	Hexanal	0.96±0.11[b]	10.34±1.06[a]	11.67±1.03[a]	2.78±0.16[b]	1.36±0.19[b]	RI, MS, Std
		Aldehyde						
8	738	Dimethyl sulfide	14.96±2.42[a]	19.48±1.59[a]	17.25±1.11[a]	15.05±0.55[a]	2.60±0.70[b]	RI, MS, Std
		Sulfur						
9	894	Methanol	3.74±0.39[b]	4.80±0.08[b]	8.72±0.75[a]	9.67±1.39[a]	2.29±0.37[b]	RI, MS, Std
10	925	Isopropyl alcohol	34.70±4.74[a]	ND	ND	ND	0.66±0.02[b]	RI, MS, Std
11	932	Ethanol	22.57±5.61[b]	2.80±0.59[c]	13.83±0.21[b]	15.88±1.58[b]	79.28±3.60[a]	RI, MS, Std
12	1301	1-Hydroxy-2-propanone	ND	ND	ND	0.85±0.11	ND	RI, MS, Std
		Alcohol						
13	998	Decane	3.41±0.94[a]	2.05±0.20[ab]	3.20±0.54[a]	2.62±0.73[ab]	0.68±0.17[b]	RI, MS, Std
14	1091	Undecane	1.66±0.10[a]	2.07±0.27[a]	2.54±0.48[a]	ND	0.46±0.09[b]	RI, MS, Std
15	1192	Dodecane	2.36±0.23[a]	ND	ND	1.09±0.48[b]	0.41±0.04[b]	RI, MS, Std
16	1293	Tridecane	1.66±0.10	ND	ND	ND	ND	RI, MS, Std
		Hydrocarbon						
17	1456	Acetic acid	1.13±0.42[a]	ND	ND	0.89±0.25[a]	ND	RI, MS, Std
18	1635	Butanoic acid	1.03±0.41	ND	ND	ND	ND	RI, MS, Std
19	1658	2-Propenoic acid	0.47±0.10	ND	ND	ND	ND	RI, MS
		Acid						
Total relative percentage			96.03±0.27	93.25±0.61	90.96±0.29	94.61±0.22	98.24±0.24	
Total identified (peak area 1.E + 06)			3.25±0.39	2.44±0.20	1.59±0.13	2.65±0.04	8.30±0.99	

[a] Each value is expressed as the mean ± standard deviation (n = 3) and values with different superscript letters within the same rows are significantly different ($p < 0.05$); ND: not detected (< 0.01%).
[b] Retention indices relative to n-alkanes on a polar DB-Wax column.
[c] RI: identification based on retention index; MS: identification based on the NIST MS library; Std: identification based on authentic standards analyzed by mass spectrometry.
Each value is expressed as the mean ± standard deviation (n=3).

teaspoons (16-32 g) daily depending on the energy needed (1,600-2,400 kcal a day). Consequently, the recommended daily maximum amount of sugar is 32 g. WBS2 and WBS4 contains approximately 21.6 and 53.3 mg of total PC (alcohol together with aldehyde forms) per 32 g (8 teaspoons), respectively while OBS24 and OBS30 contains optimum daily level of total PC, 13.1 and 14.5 mg per 32 g sugar, respectively, compared to sugar products with wax addition.

RB are the important sources of large groups of phytochemicals including phenolic acid, flavonoids, phytosterols, polyphenols (γ-oryzanol), and tocopherols which were reported by several studied on their bioactivities and their health promotion effect (Liang *et al*., 2014; Sharif *et al*., 2014). Our GC analysis also revealed several chromatograms related to these functional compounds, therefore further study to characterize and quantify the compounds are needed. This is the first report of granulated cane brown sugar product with policosanol enrichment. The developed products are produced using the concept of food to food enrichment. Defatted rice bran is promising sources of policosanols as natural functional ingredients. These developed sugar products have a potential to be used in functional food and functional beverage manufactures.

Volatile components

A total of 19 volatile components were identified in products, including, 7 aldehydes, 1 sulfur compounds, 4 alcohols, 4 hydrocarbons, and 3 acid compounds (Table 4). The total intensity of the identified volatile compounds of sugar products made by SP50 cultivar (control), represented by the total peak area, was 3.25.E+0.6. After RBO was added at 8% (OBS24) and 10% (OBS30), PC-enriched cane brown sugar product was observed to reduce aroma intensity to 2.44.E+0.6 and 1.59.E+0.6, respectively. Regarding its relative percentage to the total peak area of volatile compounds, more than half of the volatile components in initial cane brown sugar were determined to be ethanol compounds (63.5%), followed by sulfur compound, dimethylsulfide (15.6%). Isopropyl alcohol was found to be the predominant acid alcohol compound, followed by ethanol. The volatile pattern found differed from the study of Asikin *et al.* (2008) on Kokuto which acid compound (58.70%), followed by MRPs and alcohols (26.52% and 11.96%, respectively) were the major volatile component. Butanoic acid was

Table 5. Biochemical parameters for rats after 28 days treatment with PC-enriched brown sugar products (BS, OBS30, WBS2 and WBS4).

Activity	GOT (u/L)	GPT (u/L)	TG (mg/dL)	T-cho (mg/dL)
Control	86.0 ± 18.8	27.4 ± 9.0	328.2 ± 52.8	164.6 ± 20.9
BS	155.2 ±79.7	33.3 ± 12.0	323.7 ± 34.4	165.2 ± 20.8
OBS30	127.0 ± 49.4	35.5 ± 10.9	379.5 ± 35.4	177.7 ± 24.2
WBS2	109.7 ± 62.3	39.5 ± 15.1	329.7 ± 54.2	154.0 ± 50.7
WBS4	314.8 ± 103.5**	56.0 ± 42.1	373.8 ± 46.7	180.8 ± 29.9

Blood was collected from mice in each group in the mice model through heart puncture when sacrificed. After coagulation, the activities of serum GOT, GPT, TG and T-cho were analyzed. Data are presented as the mean ± SE (n = 6 per group), and statistical analysis was done by Student's t test. * $p < 0.05$ and ** $p < 0.01$, statistically significant differences from control group. Control is a control diet group. BS: brown sugar by SP50 cultivar, OBS30: brown sugar with 10% rice bran oil, WBS2: brown sugar with 0.7% wax extract, WBS4: brown sugar with 1.3% wax extract.

found to be the predominant acid constituent while pyrazine, pyranone, pyrrole, furanone, were the main MRP group components. In this study, we could not detect MRP compound from Maillard reaction in all samples due to the short time of evaporation process (20 min) used.

Regarding its relative percentage to the total peak area of volatile compounds, aldehyde compounds and dimethyl sulfide of the brown sugar products with RBO addition at both levels were found at 66.5% and 50%, respectively. Adding RBO significantly increases ($p < 0.05$) the level of aldehydes and dimethylsulfide. The major of aldehyde was acetaldehyde, 3-methylbutanal, and hexanal. It seemed that hydrocarbon and acid compounds were reduced when rice bran oil was added into the sugar products. The total intensity of the identified volatile compounds of brown sugar product with wax addition at 0.7% represented by the total peak area, was slightly reduced to be 2.65.E+0.6, but increasing wax addition to 1.3% caused substantially increase of the total intensity of the identified volatile compounds (8.30.E+0.6). Based on relative percentage to the total peak area, the major volatile components of products added 0.7% wax was aldehyde compounds (51.3%), followed by ethanol compound (27.9%), and dimethyl sulfide (16.54%). Increasing the wax level to 1.3% resulted in major change on the main volatile in the product. The main volatile compound was alcohol components (83.7%) followed by aldehydel compounds (12.1%) while dimethyl sulfide was reduced to 2.6%. Like the same effect of rice bran oil adding, wax addition to sugar products caused the reduction of the hydrocarbon and acid compounds.

Toxicity test

We evaluated the toxicity of PC-enriched brown sugar products using a 28-day oral feeding study in mice and therefore, this is the first report on the evaluation of the toxicity of food products enriched with wax extracted from DRB and RBO. The granulated sugar products enriched with high doses of RBO (10%) and 2 levels of wax extract addition (0.7 and 1.3%) were examined for the toxicity tests. The histology of organs change and serum biochemical indicators were investigated. Inspection of the physical appearance of the mice revealed that the mice were healthy throughout the period of study and no mortality was recorded in any of the experimental groups. Daily oral administration of PC- enriched sugar products at all tested for a period of 28 days did not induce any symptoms of toxicity, morbidity or mortality in mice. No significant difference was observed in body weight between the control and treated groups ($p \geq 0.05$).The gross appearance of livers demonstrated that they showed normal appearance in mice treated with these compounds (data not shown). Therefore, the results indicated no toxicity in OBS30, WBS2, and WBS4 treated mice and longtime safety of these dietary PC-enriched granulated brown sugar products.

Therefore we further evaluated the biochemical profiles in the plasma (Table 5). GOT (glutamate oxaloacetate transaminase) and GPT (glutamic pyruvic transaminase), which are serum biochemical indicators for liver inflammation. These enzymes are released into the bloodstream when the liver is injured. No significant difference was observed for the serum total triglycerides (TG) and cholesterol (T-cho) concentration. As showed in Table 5, the level of GOT was elevated significantly ($p < 0.01$) in the BSW4 treated groups only, when compared with the control group. These results also indicated toxicity in WBS4 treated mice, but at the low level. Since toluene was applied in the wax extraction, the toxicity found here for sugar product enriched with 1.3% wax (BSW4) might be derived from either the impurity of wax extract or amount of PC itself.

For a nutraceutical wax application, these quality characteristics were required further purification of extract. This study demonstrated the consumption of sugar product enriched with 10% RBO (OBS30) and 0.7% wax (WBS2) was safe.

Conclusion

This is the first report of granulated cane brown sugar product with policosanol enrichment. The developed products are produced using the concept of food to food enrichment. Wax extracted from defatted rice bran, which is the waste of rice bran oil process, are promising sources of policosanols as natural functional ingredients. The enrichment results in brown sugar products which contain higher amount of policosanol than sugar products produced using traditional processes. The volatile profile of developed sugar products were changed when either rice bran oil or wax extract was added into the products. The toxicity test showed no evidence of systemic toxicity attributable to sugar products enriched with 10% rice bran oil and 0.7% wax extracted from defatted rice bran. Additional research on the cholesterol lowering effect, mutagenic and carcinogenic potential of policosanol enriched brown sugar products would valuable be included in future studies to further support the health benefit and safety of its consumption.

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