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Binary ethanol-water solvents affect betalain contents and health-promoting properties of red *Celosia argentea* inflorescence extracts

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Abstract

The present work aimed to compare the betalain profiles and contents in the red inflorescence of *Celosia argentea* extracts obtained using different ethanol-water ratios. The impact of betalain content on the health-promoting properties was also evaluated. Freeze-dried inflorescence powder was extracted three times with aqueous-ethanol (0, 20, 40, 60, and 80% (v/v) ethanol). The highest total betacyanin content was found in the 40 and 60% aqueous-ethanolic extracts. Total phenolic content and antioxidant activities of the extracts increased with increasing ethanol concentration. The antioxidant activities measured by oxygen radical absorbance capacity (ORAC) assay of the 60 and 80% aqueous-ethanolic extracts were significantly higher than that of ascorbic acid. The IC_{50} of α-glucosidase inhibitory activities of all extracts were comparable to that of acarbose. Phenolics were the major responsible compounds for antioxidant ($r = 0.975$, $p < 0.01$ by ORAC assay), α-amylase ($r = -0.725$, $p < 0.01$), and lipase ($r = 0.607$, $p < 0.05$) inhibitory activities, whilst betacyanins corresponded to α-glucosidase inhibitory activities ($r = 0.627$, $p < 0.05$). The 60% aqueous-ethanolic extract was superior to the others in terms of colour, phytochemical contents, and health-promoting activities. These extracts can be utilised as natural food colorant, functional ingredients, and nutraceuticals.

Introduction

Consumers are more likely to choose delightful, appealing, and appetising food products, and increasingly seeking safer, more nutritious, and healthier food products. Colour is one of the most important qualities and attributes which plays a major role in consumer acceptance and buying behaviour (Martins *et al.,* 2016). Recently, natural colorants have drawn considerable attention among the consumers and food industry, because they improve organoleptic properties, contribute functional properties, and provide health benefits to the products, and are safer (Martins *et al.,* 2016; Rodriguez-Amaya, 2016). Natural pigments, particularly those from fruits and vegetables, commonly used in foods include anthocyanins, carotenoids, chlorophylls, and betalains (Rodriguez-Amaya, 2016).

Betalains are water-soluble nitrogenous pigments which can be categorised into two groups: red-violet (betacyanins, BC) and yellow-orange (betaxanthins, BX) pigments (Slimen *et al.,* 2017). Betalains have attracted significant attention since their colour intensity is three times that of anthocyanins (Moreno *et al.,* 2008), and they also have biological properties such as anti-cancer, anti-diabetic, anti-lipidemic, anti-inflammatory, neuroprotective, and cardioprotective properties (Gengatharan *et al.,* 2015; Rahimi *et al.,* 2019). Besides, the antioxidant activities of betalains were higher than that of well-known antioxidants such as ascorbic acid and certain phenolic compounds (Cai *et al.,* 2003). The major betalain sources are plants in the Amaranthaceae family such as beet, prickle pear, and amaranth. Yet betalain extracted from red beet is the only commercially available food additive in this group (Delgado-Vargas *et al.,* 2000). The study for exploring new potential sources of betalains is ongoing.

Celosia argentea (CA) has been suggested as an alternative betalain source for food applications. CA is known as ornamental and edible plant widely spread around the world including sub-tropical Africa, North America, and Asia (Schliemann *et al.,* 2001). Betalains generally accumulate in all plant parts, but more abundantly in the inflorescence. We were interested in CA inflorescences due to the

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colour and the biological activities linked to its use in treating dysentery, haemoptysis, haemorrhoids, leucorrhoea, profuse uterine bleeding, eczema, and menstruation problems (Pingale *et al.,* 2012). Besides, betalains in CA including amranthin, isoamranthin (C-15 isomer), dopamine-BX, 3-methoxytyramine-BX, and (*S*)-tryptophan-BX (Schliemann *et al.,* 2001) have been less studied than betanin and indicaxanthin derived from beet and prickle pear (Tesoriere *et al.,* 2004; Sanchez-Gonzalez *et al.,* 2013).

Extraction is the first significant step to obtain large amounts of target compounds. A single solvent is less effective for extraction; thus, a mixture of water and organic solvent has been applied in many studies. Water extracts the polar compounds while the organic solvent extracts less polar components (Waszkowiak and Gliszczyńska-Świgło, 2016; Lim *et al.,* 2019). Solvents for betalain extraction include water, alcohol (ethanol and methanol), and their mixture (0 - 80%) (Cai *et al.,* 2001; Roy *et al.,* 2004; Sanchez-Gonzalez *et al.,* 2013; Miguel, 2018; Righi Pessoa da Silva *et al.,* 2018). Water is usually preferable to extract pigments to be used in the food systems (Cai and Corke, 1999). Nevertheless, the optimum ratio between water and alcohol to obtain rich betalain extracts vary in different raw materials used (Cai *et al.,* 1998; Sanchez-Gonzalez *et al.,* 2013; Miguel, 2018; Lavanya *et al.,* 2019).

Few studies have explored betalain extraction from CA inflorescences, but mostly used water or methanol-water mixture to obtain a high compound content or purity (Cai *et al.,* 2001; Schliemann *et al.,* 2001; Sanchez-Gonzalez *et al.,* 2013; Lavanya *et al.,* 2019). However, methanol is unsafe for human consumption. The present work therefore aimed to extract betalains from red CA inflorescence using a water-ethanol system which is more suitable for food applications (Waszkowiak and Gliszczyńska-Świgło, 2016). The profile and content of betalain from red CA inflorescence extracted with different ratios of water and ethanol (ranged from 0 to 80%) were investigated. Phenolic compounds, which are the abundant secondary metabolites in plants, and widely used in food products (Mark *et al.,* 2019) were also determined. The different ratios of the solvents not only affect the component profiles, but also their biological properties (Yin *et al.,* 2014; Waszkowiak and Gliszczyńska-Świgło, 2016). The health-promoting properties, including antioxidant and inhibitory activities of digestive enzymes (α-amylase, α-glucosidase, and lipase) were also evaluated in the present work. The correlations between the constituents and health-promoting properties were also investigated. We searched for the best ethanol concentration to obtain a promising CA inflorescence extract with a vivid colour and good health-promoting properties which could be safely applied in food products.

Materials and methods

Chemicals and reagents

Gallic acid, lipase from porcine pancreas (Type II, 100 - 400 unit/mg protein in olive oil), and α-amylase from porcine pancreas (Type VI-B, 9 U/mg solid) were purchased from Sigma Chemical Co. Ltd. (St. Louis, Mo., U.S.A.). α-Glucosidase from yeast (100 U/1.4 mg) was purchased from Oriental Yeast Co. Ltd. (Itabashiku, Tokyo, Japan). Ascorbic acid, ferric chloride, and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acarbose, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 4-nitrophenyl-α-glucopyranoside (PNPG), and orlistat were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Folin-Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany). Lipase Kit S was purchased from DS pharma biomedical Co. Ltd. (Osaka, Japan). All reagents and solvents were of analytical or HPLC grade.

Sample preparation and extraction

Fresh red CA inflorescences were harvested from Tokyo, Japan. The plants were washed, cleaned, and then freeze-dried. The dry samples were ground into powder, and kept at -20°C for further extraction. Dry powder was extracted with pre-cooled 0, 20, 40, 60, and 80% aqueous-ethanol at a ratio of 1:100 (sample to extraction solvent). The mixture was vortexed, sonicated, shaken, and centrifuged at 2,600 *g* for 10 min at 4°C. The supernatant was filtered, and the residue was re-extracted two more times. The supernatant was pooled, evaporated at 30°C, and freeze-dried. The extracts were re-dissolved with deionised water for further analysis. The extraction yield (%) was calculated using Eq. 1.

$$\text{Extraction yield (\%)} = \frac{\text{Extract weight}}{\text{Sample weight}} \times 100 \qquad \text{(Eq. 1)}$$

Colour measurement

The extracts at a concentration of 0.2 mg/mL were measured for their L* (lightness; 0 – 100), a* (negative and positive values indicate green and red colour, respectively), and b* (negative and positive values indicate blue and yellow colour, respectively) using a CR-13 Colour Reader (Konica Minolta, Osaka, Japan).

Total betacyanin contents (TBC)

The extracts were diluted with water at the appropriate dilution, and absorbance was measured at 536 nm using a UV-Visible spectrophotometer (UV-1600 PC, Shimadzu, Japan). The TBC was calculated by Lambert Beer's law using Eq. 2 (Cai *et al.,* 1998):

$$\text{Total betacyanin content (mg/g extract)} = \frac{AV(DF)MW}{\varepsilon LW} \quad \text{(Eq. 2)}$$

where, A = absorbance at 536 nm for the maximum absorption of amaranthine, V = total extract volume (mL), DF = dilution factor, MW and ε = molecular weight and molar extinction coefficient of amaranthin, respectively (MW = 726; $\varepsilon = 5.66 \times 10^4$ M^{-1} cm^{-1}), L = path length of the cuvette (1 cm), and W = weight of the extract (g).

Total phenolic contents (TPC)

The TPC of the extracts was determined by the Folin-Ciocalteu method according to Judprasong *et al.* (2013) with slight modification. Appropriate dilution of extracts (25 µL) was reacted with 10% Folin-Ciocalteu reagent (50 µL) for 5 min at ambient temperature. Then, 200 µL of 7.5% sodium carbonate was added, and the mixture incubated for 2 h at ambient temperature in the dark. Absorbance at 650 nm were read using a microplate reader (THERMO max, Molecular Devices, Sunnyvale, California, U.S.A.). The results were expressed as gallic acid equivalent (GAE).

HPLC-DAD-MS analysis of betalains

Betalain analysis was performed on LCMS Shimadzu-2010 consisted of degasser, binary pump, autosampler, column oven, diode array detector (DAD), and LCMS solution software version 3.41.324 (Shimadzu, Kyoto, Japan). The Devolosil ODS-HG5 column (2.0 × 250 mm, 5 µm; Nomura Chemical Co., Ltd., Japan) was used at 30°C at a flow rate of 0.1 mL/min. Mobile phase A was 1% formic acid in water, and mobile phase B was methanol. Betalains were separated under gradient elution starting from 0 - 12 min, 5 - 25% B; 12 - 16 min, 25 - 70% B; and 16 - 20 min, 70 - 5% B. The injection volume was 10 µL. The BC and BX were detected at 535 and 462 nm, respectively. The HPLC-DAD was equipped with electrospray ionisation (ESI) source operating in the positive ionisation mode. The sample were scanned from *m/z* 50 - 2000. Nitrogen was used as a nebuliser gas at a flow rate of 1.5 L/min. The CDL and heat block temperature were 250 and 300°C, respectively. The detector voltage was 1.2 kV.

Antioxidant activity

The antioxidant activities of the extracts were evaluated using the ferric reducing antioxidant power (FRAP), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and oxygen radical absorbance capacity (ORAC) assays slightly modified from Judprasong *et al.* (2013). The activities of the extracts were compared with ascorbic acid.

FRAP assay: The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl solution, and 20 mM ferric chloride at a proportion of 10:1:1 (v/v/v), respectively. Next, 40 µL of the extracts at the appropriate concentration, ascorbic acid, and trolox standard (0 - 100 µM) were a reacted with 160 µL of FRAP reagent, and incubated for 8 min at 37°C in the dark. The absorbance was read at 590 nm. The FRAP values were presented as µmol trolox equivalent (TE)/g extract.

DPPH assay: The 150 µM DPPH in 50% ethanol (210 µL) was mixed with 30 µL of extracts or ascorbic acid at various concentrations. The mixture was incubated at ambient temperature in the dark for 30 min. Absorbance was read at 490 nm. DPPH radical scavenging activity (%) was calculated using Eq. 3:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad \text{(Eq. 3)}$$

where, $A_{control}$ = absorbance of DPPH solution without the extract, and A_{sample} = absorbance of the extract reacted with the DPPH solution. The results were reported as the concentration of the extract required to decrease DPPH radical by 50% (IC_{50}, mg/mL).

ORAC assay: The extracts, ascorbic acid, and trolox standard (0 - 100 µM) were diluted with ORAC buffer (75 mM potassium phosphate buffer (pH 7.2)). Then, 150 µL of fluorescein solution (81.7 nM in ORAC buffer) was added in the extracts, and incubated for 30 min at 37°C in the dark. After

adding 25 µL of freshly prepared AAPH solution(153 mM in ORAC buffer) into the mixture, the fluorescence intensity was read using an automate plate reader with a 96-well plate (SPECTRAFluor Plus, TECAN, Salzburg, Austria). The excitation and emission wavelengths were 485 and 535 nm, respectively. The ORAC values were reported as µmol TE/g extract.

Inhibitory activity of digestive enzymes

The α-amylase inhibitory activity was performed based on starch-iodine colour assay according to Xiao *et al.* (2006) with slight modification. The extracts or acarbose (an anti-diabetic drug) were diluted with 20 mM sodium phosphate buffer (pH 6.9) containing 6 mM NaCl (PBS) to obtain different concentrations. The diluted extracts (50 µL) were mixed with 50 µL of PBS buffer, and then 50 µL of α-amylase (0.225 U/mL dissolved in PBS buffer) was added. After incubating the mixture for 10 min at 37°C in the dark, soluble starch solution (0.12%, 50 µL) was added. The reaction occurred for 10 min at 37°C in the dark. The enzyme reaction was stopped by adding 100 µL of iodine-hydrochloric reagent (0.5 mM I_2, 0.5 mM KI, and 0.1 M HCl). Absorbance was read at 590 nm. The percent inhibition of α-amylase was calculated using Eq. 4:

$$\% \text{ inhibition of } \alpha\text{-amylase} = \left[1-\frac{A_{Sbl}-A_{SRx}}{A_{bl}-A_{Rx}}\right] \times 100 \quad \text{(Eq. 4)}$$

where, A_{Sbl} = absorbance of sample without the enzyme, A_{SRx} = absorbance of the sample after reacting with the enzyme, A_{bl} = absorbance of PBS buffer without the enzyme, and A_{Rx} = absorbance of PBS buffer with the enzyme.

The inhibitory activities of α-glucosidase were evaluated according to Zhu *et al.* (2014) with slight modification. The substrate, PNPG, was prepared at a concentration of 1 mM in 0.1 M potassium phosphate buffer (pH 7) (KPB). The diluted extracts or acarbose (50 µL) were mixed with α-glucosidase (100 µL, 0.5 U/mL in KPB), then incubated for 10 min at 37°C in the dark. After adding of substrate (50 µL) and incubation for 10 min at 37°C in the dark, absorbance was measured at 405 nm. The percent inhibition of α-glucosidase was calculated using Eq. 5:

$$\% \text{ inhibition of } \alpha\text{-glucosidase} = \left[1-\frac{A_{SRx}-A_{Sbl}}{A_{Rx}-A_{bl}}\right] \times 100 \quad \text{(Eq. 5)}$$

where, A_{SRx} = absorbance of the sample after reacting with the enzyme, A_{Sbl} = absorbance of the sample without the enzyme, A_{Rx} = absorbance of KPB with the enzyme, and A_{bl} = absorbance of KPB without the enzyme.

The lipase inhibitory activities of the extracts were compared with orlistat, a drug designed to treat obesity. The assay was followed with Lipase Kit S. Briefly, diluted extracts or orlistat with water (50 µL) were mixed with BAL-tributyrate (a substrate, 10 µL) and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DNTB, a colour substance, 30 µL). Lipase solution was prepared by mixing lipase (5 mg) with sodium dodecyl sulphate (a surfactant, 1 mg) in 1 mL of water, then diluted 100 times. After incubating the former mixture for 5 min at 37°C in the dark, 10 µL of lipase solution was added. The mixture was incubated for 20 min at 37°C in the dark. The absorbance at 405 nm was read after adding the stop solution. The percent inhibition of lipase was calculated using Eq. 6:

$$\% \text{ inhibition of lipase} = \left[1-\frac{A_{SRx}-A_{Sbl}}{A_{Rx}-A_{bl}}\right] \times 100 \quad \text{(Eq. 6)}$$

where, A_{SRx} = absorbance of the sample after reacting with the enzyme, A_{Sbl} = absorbance of the sample without the enzyme, A_{Rx} = absorbance of water with the enzyme, and A_{bl} = absorbance of water without the enzyme.

Statistical analysis

All experiments were analysed in triplicate (n = 3), and presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used to compare the mean differences among all parameters. Differences were considered significant at $p < 0.05$. Pearson's correlation was performed to assess the relationship between parameters. The interpretation of Pearson's correlation coefficients (r) is as follows: 1 = perfect; 0.8 - 0.9 = strong; 0.6 - 0.7 = moderate; 0.3 - 0.5 = fair; 0.1 - 0.2 = poor; and 0 = none (Akoglu, 2018). Statistical analysis was performed using the IBM® SPSS® Statistic version 19 (IBM SPSS, Chicago, IL, USA).

Results and discussion

The CA extract properties

The CA inflorescences were extracted with binary ethanol-water solvent at different concentrations (0 - 80% ethanol). The extraction

yield of the CA extracts ranged from 25.03 ± 1.80 to 32.71 ± 1.55%. The lowest yield was obtained in the 80% aqueous-ethanolic extract which resulted from the low amount of water-soluble compounds such as protein and carbohydrate. It was noted that the purplish-red colour of all samples could be completely extracted within three extractions, except for the 80% aqueous-ethanol solution. The 80% aqueous-ethanol solution was less able to extract polar compounds including the major BC in CA due to low polarity. To complete extraction, all purplish-red pigments with 80% aqueous-ethanol should be extracted six to seven times.

The colour characteristics are shown in Table 1. The 40% aqueous-ethanolic extract had the most intense colour. Meanwhile, the 80% aqueous-ethanolic extract had the least intense colour as it was brighter (high L*), less red (less positive a*), and less blue (less negative b*) than the others.

Total betacyanin and total phenolic contents in CA extracts

The TBC of all the CA extracts (Table 2) were not different due to the varied materials. Nevertheless, the 80% aqueous-ethanolic extract had the lowest content since amaranthin and isoamaranthin are water-soluble compounds. It was noted that 100% ethanol could not extract BC from red CA inflorescence, as reported by Righi Pessoa da Silva *et al.* (2018). Moreover, our samples were in a dry form, but BC are hydrophilic compounds, meaning that BC extraction requires some water in the extraction solvent. The optimum extraction solvent ratio to obtain the highest amount of betalains varied between different plants depending upon the plant matrices, types, and amount of betalains. Water was the best solvent for betalains extraction in *Bougainvillea spectabilis, Celosia argentea* inflorescence, and *Amaranthus gangeticus*, whereas ethanol extract of *Amaranthus blitum* showed the highest of total betalain content (Chong *et al.,* 2014; Lavanya *et al.,* 2019). Additionally, the highest total betalain content was obtained from 20% aqueous-ethanolic extract in *Opuntia joconostle* (Sanchez-Gonzalez *et al.,* 2013).

We also investigated the TPC because the compounds are one of the most abundant secondary

Table 1. The colour characteristics of CA extracts (at 0.2 mg/mL) at different ethanol concentrations.

Ethanol (%)	Extraction yield (%)	L*	a*	b*
0	32.18 ± 1.97^{a}	32.1 ± 0.5^{b}	11.4 ± 0.8^{a}	-1.5 ± 0.3^{ab}
20	31.04 ± 2.16^{a}	31.8 ± 1.0^{b}	13.1 ± 0.5^{a}	-2.6 ± 1.2^{bc}
40	31.89 ± 2.89^{a}	31.6 ± 1.8^{b}	12.7 ± 2.2^{a}	-2.8 ± 0.4^{c}
60	32.71 ± 1.55^{a}	31.7 ± 0.3^{b}	13.1 ± 0.3^{a}	-2.4 ± 0.5^{bc}
80	25.03 ± 1.80^{b}	35.4 ± 0.8^{a}	9.2 ± 1.2^{b}	-0.4 ± 0.2^{a}

Values are mean ± S.D of three replicates (n = 3). Means in a column with different lowercase superscripts indicate significant difference ($p < 0.05$).

Table 2. Total betacyanin content (TBC), total phenolic content (TPC), and TPC/TBC of CA extracts at different ethanol concentrations.

Ethanol (%)	Total betacyanin content (mg/g extract)	Total phenolic content (mg GAE/g extract)	TPC/TBC
0	12.27 ± 4.25	81.75 ± 12.06^{d}	7.06 ± 1.76^{b}
20	14.52 ± 3.80	102.02 ± 4.71^{d}	7.29 ± 1.53^{b}
40	14.91 ± 3.38	133.47 ± 14.06^{c}	9.13 ± 1.25^{b}
60	13.99 ± 1.67	154.59 ± 6.67^{b}	11.21 ± 1.94^{b}
80	9.06 ± 2.04	186.86 ± 15.08^{a}	21.47 ± 5.89^{a}

Values are mean ± S.D of three replicates (n = 3). Means in a column with different lowercase superscripts indicate significant difference ($p < 0.05$). GAE, gallic acid equivalent.

metabolites in plants, and they exhibit various health-promoting properties (Williamson *et al.,* 2018). Phenolics have lower polar than BC. Thus, TPC increased significantly in an ethanol concentration-dependent manner (Table 2). Similar to betalain extraction, the optimum solvent extraction ratio to yield the highest TPC should be determined in each plant. In the present work, 80% ethanol was the optimum ratio to extract phenolics in red inflorescence of CA. In accordance with Nour *et al.* (2013), the TPC of blackcurrant increased with increasing ethanol concentration up to 96%. In contrast, the decrease in TPC was observed when ethanol concentration increased from 60 - 90% in flaxseed (Waszkowiak and Gliszczyńska-Świgło, 2016). Pistachio hull had the greatest level of TPC when extracted with 40% aqueous-ethanol (Özbek *et al.,* 2020).

Ethanol concentration further affected TPC/TBC. A drastic and significant increase in the ratio was observed in the 80% aqueous-ethanol extract (Table 2) since this extract resulted in an incomplete extraction of BC. Notwithstanding the evidence, the TPC/TBC appeared to increase with increasing ethanol concentration.

The betalain profiles of CA extracts by HPLC-DAD-MS

Betalain profiles were identified by HPLC-DAD-MS. BC and BX were detected at their maximum wavelength, 535 and 462 nm, respectively. The betalain profiles of CA extracts are illustrated in Figure 1. The major BC in all the extracts was amaranthin followed by isoamaranthin. Dopamine-BX and 3-methoxytyramine-BX were the main BX. The results are in accordance with a previous report which found 373.6, 10.2, 1.8, and 0.2 mol/g of isoamaranthin, dopamine-BX, 3-methoxy-tyramine-BX, and (*S*)-tryptophan-BX, respectively, in fresh inflorescence of red CA var *cristata* (L.) Kuntze (Schliemann *et al.,* 2001). However, we could not detect (*S*)-tryptophan-BX in the present work due to the different of variety of the CA, and lower content of this compound in red varieties as compared to the yellow varieties (Schliemann *et al.,* 2001). In the present work, trace amounts of other betalains were also detected in the extracts by means of their spectrum. The effect of ethanol concentration on betalain content was reflected via the peak area (Figure 1). Overall, 40 and 60% ethanol were found to be the most efficient concentrations to yield maximum isoamaranthin. The lowest peak areas of isoamaranthin were observed in the 80% aqueous-ethanolic extract which corresponded to TBC. Two major BX in CA extracts were less polar than BC, so the peak areas of BX increased with higher ethanol concentrations. Nevertheless, dopamine-BX was not detected in the 0 and 20% aqueous-ethanolic extracts as the compound was less polar. Thus, the compound could not be extracted by the polar extraction solvent, or the amount of compound was below the detection limit.

Health-promoting properties of CA extracts and their relationships with phytochemical contents

The health-promoting properties of CA extracts in terms of antioxidant properties and inhibitory activities on digestive enzymes were investigated (Figure 2). The relationship between the health-promoting properties and phytochemical contents showing the estimation of major contributor of the activities is tabulated in Table 3.

Antioxidant properties

The antioxidant activities of the extracts were measured by three assays namely FRAP, DPPH, and ORAC assays since the true total antioxidant capacity could not be measured using only a single method (Prior *et al.,* 2005).

All the assays showed a similar tendency of antioxidant activities in an ethanol concentration-dependent manner. The ORAC values of the 60 and 80% aqueous-ethanolic extracts were greater than that of ascorbic acid, a well-known antioxidant. However, the FRAP values and IC_{50} evaluated by DPPH assay of ascorbic acid were stronger than that of the CA extracts. This indicated that the antioxidants in the CA extracts are likely to be chain-breaking antioxidants that can scavenge peroxyl radical via the hydrogen atom transfer mechanism rather than the single electron transfer mechanism due to the basic principle of ORAC assay reported previously (Prior *et al.,* 2005). Peroxyl radical produced from the lipid peroxidation process is one of the most abundant radicals in the human biological system. However, there are many radicals generated in the biological system. The imbalance of free radicals and antioxidants causes non-communicable diseases (Lobo *et al.,* 2010). The results show the potential of the CA extracts to scavenge the radicals. Consequently, further research regarding *in vivo* experiment is necessary to confirm these findings. Lavanya *et al.* (2019) also reported promising antioxidant activities from water extract of CA inflorescence measured by ABTS, DPPH, FRAP, cupric ion reducing antioxidant capacity, and chelating potential assays. However,

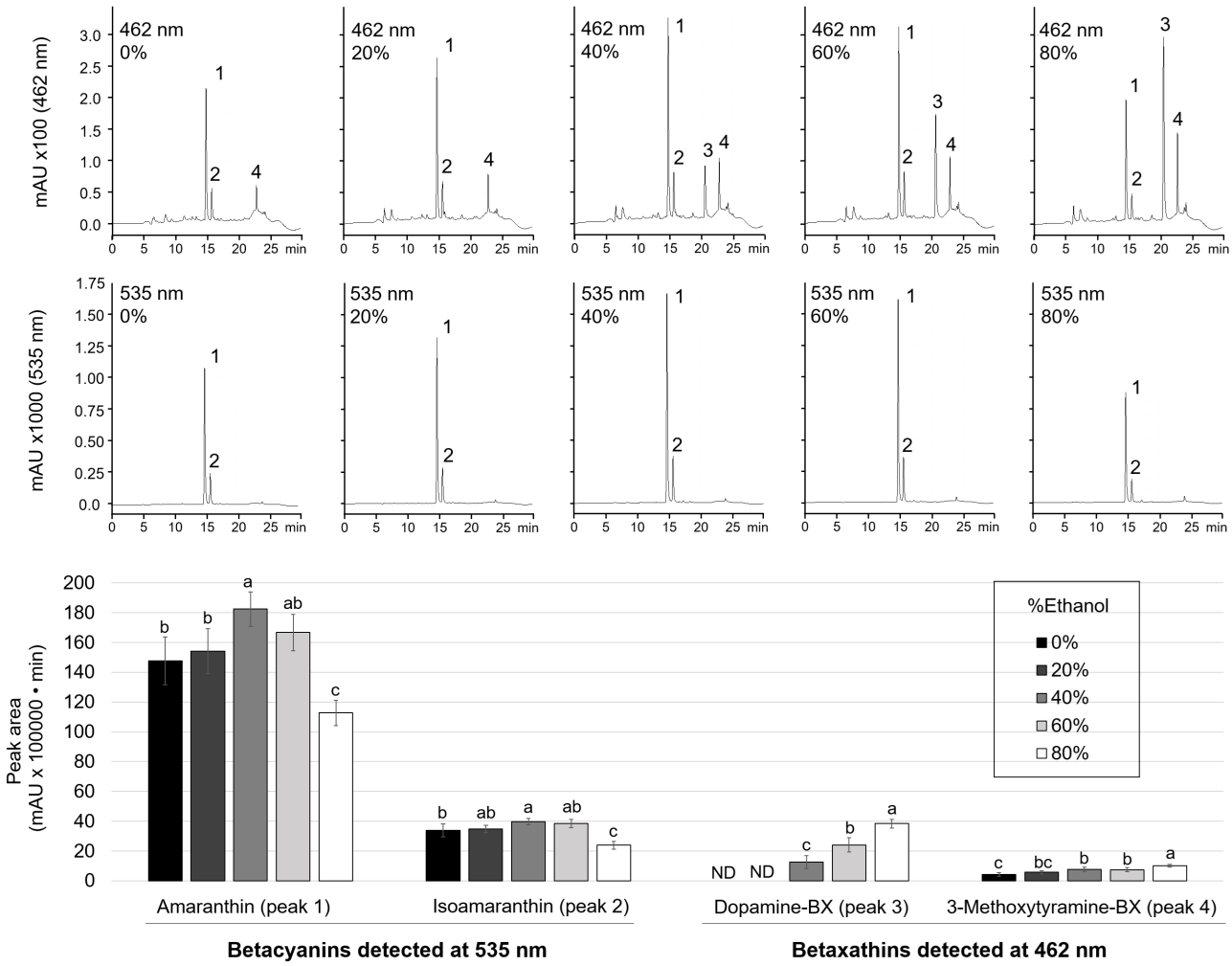

Figure 1. Chromatograms and relative quantitation of betalains in CA extracts at different ethanol concentrations: 0, 20, 40, 60, and 80% at 462 and 536 nm. Peak areas were calculated from each chromatogram: at 535 nm for betacyanins: amaranthin (1) and isoamaranthin (2); and at 462 nm for betaxanthins: dopamine-BX (3) and 3-methoxytyramine-BX (4). Values are mean ± S.D of three replicates (n = 3). Means with different lowercase letters indicate significant differences ($p < 0.05$) among the extracts at different ethanol concentrations within each compound. ND: not detected.

the properties were less than that of ascorbic acid.

Phenolic compounds were the major contributors to antioxidant activities (r = 0.963, -0.887, and 0.975 ($p < 0.01$) for FRAP, DPPH, and ORAC assay, respectively). In addition, moderate to strong correlations were observed between BX and antioxidant activities (FRAP assay: r = 0.885 and 0.917; DPPH assay: r = -0.673 and -0.729; ORAC assay r = 0.926 and 0.920 ($p < 0.01$ or 0.05) for dopamine-BX and 3-methoxytyramine-BX, respectively). A strong correlation was observed both between antioxidant activities and TPC, in addition to TBC (Nour *et al.,* 2013; Waszkowiak and Gliszczyńska-Świgło, 2016; Righi Pessoa da Silva *et al.,* 2018; Özbek *et al.,* 2020). Phenolics and betalains consist of an aromatic ring in the structure bearing at least 1 –OH group. The H atom from phenolics is donated to the free radical, thereafter, the resonance of phenoxyl radicals is formed. The electron is delocalised and shared throughout the aromatic ring. In the case of betalains, the electronic resonance system also can be supported by the imino and the tetrahydropyridine groups (Craft *et al.,* 2012; Slimen *et al.,* 2017). Although betalains exhibited antioxidant activities, results of the present work showed that only BX had stronger activities than that of BC, in accordance with Cai *et al.* (2003).

Inhibitory activities on digestive enzymes

Promising alternatives for the reduction of metabolic risk factors lie in investigating compounds with the ability to suppress or inhibit digestive enzymes related to carbohydrate and fat metabolisms. In the present work, we investigated the inhibitory activities of CA extract on α-amylase, α-glucosidase, and lipase.

The α-amylase and α-glucosidase inhibitors have been previously studied by various researchers due to their ability to prevent or treat diabetic condition by reducing the glucose absorption

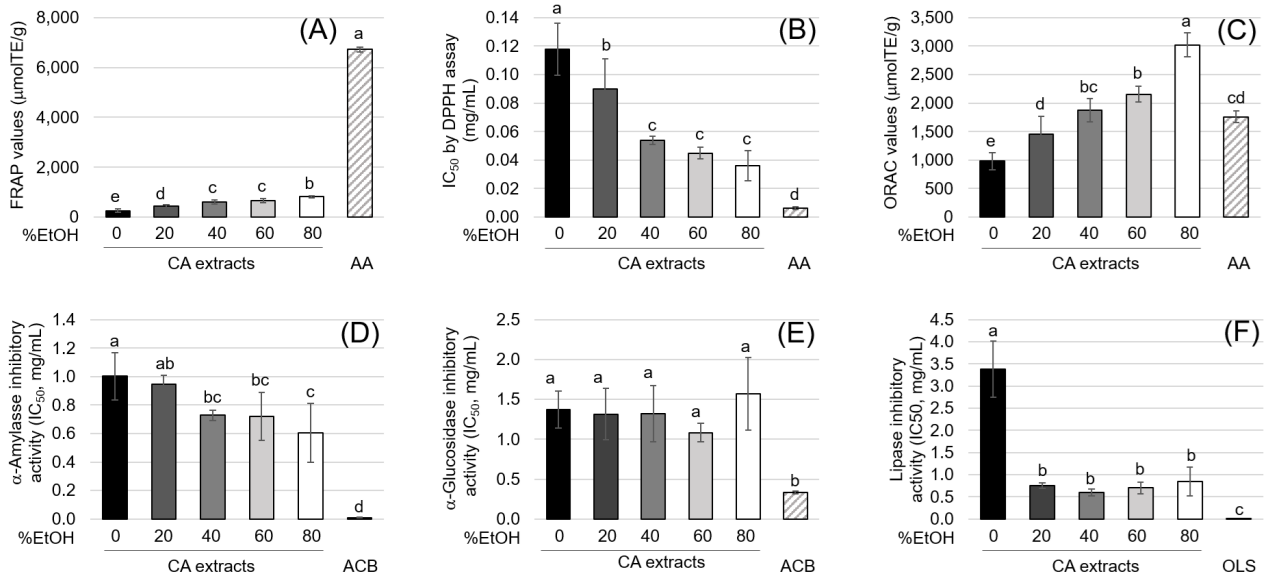

Figure 2. Antioxidant and inhibitory activities of CA extracts prepared with different ethanol concentrations. Antioxidant activities evaluated by FRAP (A), DPPH (B), and ORAC (C) assays. Inhibitory activities were evaluated with digestive enzymes including α-amylase (D), α-glucosidase (E), and lipase (F). Values are mean ± S.D of three replicates (n = 3). Means with different lowercase letters indicate significant difference ($p < 0.05$) among the extract at different ethanol concentrations within each graph. AA: ascorbic acid; ACB: acarbose; and OLS: orlistat.

Table 3. Pearson's correlation coefficient (*r*) of phytochemical contents and health-promoting properties.

	FRAP	DPPH	ORAC	Amy	Glc	Lip
TBC	-0.171	0.113	-0.229	0.227	-0.627*	-0.242
Am	-0.230	0.064	-0.449	0.165	-0.462	-0.188
IAm	-0.294	0.135	-0.499	0.234	-0.555*	-0.122
DBX	0.885**	-0.673*	0.926**	-0.538	0.257	0.366
MBX	0.917**	-0.729**	0.920**	-0.801**	-0.007	-0.620*
TPC	0.963**	-0.887**	0.975**	-0.725**	0.108	-0.607*

*Correlation is significant at the 0.05 level (two-tailed). **Correlation is significant at the 0.01 level (two-tailed). FRAP, ferric reducing power; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ORAC, oxygen radical absorbance capacity; Amy, α-amylase inhibitory activity; Glc, α-glucosidase inhibitory activity; TBC, total betacyanin contents; Am, amaranthin; IAm, isoamaranthin; DBX, dopamine-betaxanthin; MBX, 3-methoxytyramine-betaxanthin; and TPC, total phenolic contents.

(Rasouli *et al.,* 2017). The α-amylase inhibitory activities significantly increased with increasing ethanol concentration. In contrast, α-glucosidase inhibitory activities did not depend on the ethanol concentration. Acarbose had stronger α-amylase and α-glucosidase inhibitory activities than that of the CA extracts. It was noted that the inhibitory activity of acarbose against α-glucosidase was only three to five times higher than that of the CA extracts. 3-Methoxytyramine-BX and phenolics were responsible for the α-amylase inhibitory activity (r = -0.801 and -0.725, $p < 0.01$, respectively), while BC, particularly isoamaranthin, were the major contributor to α-glucosidase inhibitory activity (r = -0.627 and -0.555, $p < 0.05$, respectively). Telagari and Hullatti (2015) reported that hydrophilic substances from the whole plant CA extracts contained α-amylase and α-glucosidase inhibitors. It is widely known that phenolics are able to inhibit α-amylase and α-glucosidase depending on its

molecular size, the solubility of the compound in water, and the position of –OH group (Rasouli *et al.,* 2017). However, the inhibition of α-amylase and α-glucosidase by betalains was limited. Biswas *et al.* (2013) demonstrated that betalamic acid significantly inhibited α-amylase activity, but not amaranthin and BX in *Amaranthus tricolor* L. These results are different from the results of the present work which could be due to the different BX and other compounds in the raw materials. To the best of our knowledge, no previous studies have described the inhibition of betalains against α-glucosidase. Koss-Mikołajczyk *et al.* (2019) reported the inhibitory activities of α-amylase and α-glucosidase from prickly pears and beets; however, the responsible compounds were not from betalains; indicaxanthin and betanin in prickly pears, and vulgaxanthin I and betanin in beets. In contrast, we found that BC may have the potential to inhibit the α-glucosidase activity. We estimated that isoamaranthine has poor bioavailability similar to betanin, a major BC in red beetroot (Tesoriere *et al.,* 2004). Thus, they remain in the intestinal cavity for a long period during digestion. Hence, the ability of BC to inhibit α-glucosidase will effectively reduce the glucose absorption. Further research using *in vivo* studies could help to support this hypothesis.

The alternative obesity treatment approach is to inhibit lipase. At present, only orlistat has been clinically approved as a lipase inhibitor although it has gastrointestinal side effects (Buchholz and Melzig, 2015). Thus, there has been an increasing interest with regards to investigating this property from plant materials. The 20 - 80% aqueous-ethanolic CA extracts were found to have a similar degree of lipase inhibition which was higher than that of the water extract. Nevertheless, the CA extracts had significantly lower inhibitory activities than that of orlistat. *Celosia cristate* (L.) O. Kuntze extract was reported to have a weak lipase inhibitory activity when compared with other 75 medicinal plants (Sharma *et al.,* 2005). The most responsible compounds which acted as lipase inhibitors in CA extracts were 3-methoxytyramine-BX and phenolics, which showed a moderate correlation with this activity (r = -0.620 and -0.607, $p < 0.05$, respectively). Phenolics have been reported as lipase inhibitors (Buchholz and Melzig, 2015), whereas no reports have explored this property with betalains.

Conclusion

The present work demonstrated the effect of solvent ratios on the extraction of phytochemical profile and content related to health-promoting properties in red CA inflorescence extracts. The extracts are a valuable source of betalains and phenolic compounds. The ratio between water and ethanol determined the types and amounts of compounds extracted from the material. Increased ethanol concentration increased the phenolic and BX contents. Meanwhile, BC was the highest in 40 and 60% aqueous-ethanolic extracts. The antioxidant activities in 60 and 80% aqueous-ethanolic extracts were higher than that of ascorbic acid. Additionally, the α-glucosidase inhibitory activities of the extracts were comparable to that of acarbose. Phenolics and BX were the major contributors to antioxidant, α-amylase inhibitory, and lipase inhibitory activities. Further, BC, particularly isoamaranthin, were responsible for the α-glucosidase inhibitory activity. The best extraction solvent for a vivid purplish-red colour, high phytochemical contents, and great health-promoting activities was found to be 60% aqueous ethanol. Further study on the bioavailability and the health-promoting properties, particularly α-glucosidase inhibitory activity by *in vivo* assay, may shed light on the potential and utilisation of betalains from red CA inflorescence in the future.

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