Antioxidant capacity and amino acid profile of millet bran wine and the synergistic interaction between major polyphenols

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Abstract Millet bran, the by-product of millet processing industry, contains an abundance of phytochemicals, especially polyphenols. The main objective of this study was brewing antioxidant wine from millet bran, as well as the nutritional evaluation. The total polyphenol content of wine samples was determined by Folin–Ciocalteu colorimetric method, and the antioxidant capacity was evaluated by DPPH radical-scavenging capacity, Trolox equivalent antioxidant capacity (TEAC), and ferric reducing antioxidant power (FRAP). Results showed that millet bran wine (MBW) contained as much as six times of total polyphenols compared with millet wine (MW), and performed considerably stronger antioxidant activity in DPPH, TEAC and FRAP assays. More than sixfold of total amino acids (AA) were found in MBW than in MW. Moreover, the indispensable AA and functional AA were also abundant in MBW. The major polyphenol compounds in MBW were identified using HPLC, including vanillic acid, syringic

acid (SA), *p*-coumaric acid (CA) and ferulic acid (FA). They exhibited synergism in the antioxidant assays, especially the combinations of SA and CA, SA and FA. This study not only provides evidence for MBW as a nutraceutical with antioxidant activity, but also opens new avenues in the area of making comprehensive utilization of agricultural by-products.

Keywords Foxtail millet bran · Wine · Antioxidant · Polyphenol compound · Synergism

Introduction

Foxtail millet (*Setaria italica*) is one of the oldest cultivated crops which originated in China, and is now cultivated throughout the world. It was reported that foxtail millet has many nutritious and medical functions including antioxidant, antimicrobial, and anticancer activities (Saleh et al. 2013). Foxtail millet grains are dehulled and milled to improve the sensory and edible quality before they reach the market. However, the millet bran is rich in dietary fiber, unsaturated fatty acid, protein, phytosterol, γ -oryzanol, and especially polyphenol compounds. These multiple phytochemicals and proteins endow millet bran with a variety of biological activities including antioxidative (Chandrasekara and Shahidi 2011), anti-tumor (Shan et al. 2014), and immunomodulating activities (HOSODA et al. 2012). Researchers have also demonstrated that millet bran has higher bioactivity than millet grain, especially in antioxidant activity (Chandrasekara and Shahidi 2011; Saleh et al. 2013). In this regard, millet bran may serve as a natural source of antioxidants in food applications and as a nutraceutical in promoting health and reducing disease risks. However, millet bran is extensively used as animal

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feed in China, which leads to a great waste of valuable resources. Thus, making comprehensive utilization of millet bran is an urgent task in China.

The present research aims at providing new insights into the utilization of agricultural by-products. The defatted millet bran used in the present study contains starch 57.6%, protein 19.5%, and fibre 13% of the dry weight. The high starch content makes it suitable for brewing wines. On the other hand, removing the starch from millet bran facilitates the development of other nutrients like protein and dietary fibre from the residue. As far as we know, almost no research has brewed wine from the bran of grain. There is only one previous research that detected aroma compounds in the wine made from red rice bran (Ueki et al. 1991). Although aroma and taste are important qualities of rice wine, the nutritious value should also be considered, as it directly influences human health. The millet bran wine (MBW) in the present study was supposed to be rich in polyphenol compounds. Therefore it was assessed for its antioxidant capacity by comparing with millet wine (MW), aiming at providing alternative drug from nature sources for treating oxidative stress-related diseases. High performance liquid chromatography (HPLC) was performed to elucidate the main polyphenols in MBW that are responsible for the antioxidant activity. The interactions of the major polyphenols were further identified. Moreover, as the precursors for aroma compounds and nutrient components of the wine, the amino acid profiles of MBW and MW were identified in this study (Fig. 3).

Materials and methods

Chemicals

Folin–Ciocalteu's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-*s*-triazine (TPTZ), 2,2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), Trolox, and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA); all the standard polyphenol compounds (purity, 98%) for HPLC analysis were purchased from Mansite Biotechnology Co. Ltd. (Chengdu, Sichuan, China); HPLC-grade methanol was obtained from Fisher Scientific (Waltham, MA, USA). Dry yeast for winemaking (*Saccharomyces cerevisiae*) was purchased from Angel Yeast Co. Ltd. (Yichang, Hubei, China).

Wine samples

Foxtail millet and millet bran were obtained from Nanyang Town Millet Processing Factory (Shijiazhuang, Hebei, China) and stored in 4 °C cold room before wine making. The brewing procedure was modified from the traditional

brewing method for Chinese rice wine (Que et al. 2006a). The powdered millet and millet bran were defatted using petroleum ether at 1:4 (w/v) by ultrasonication for three times. The defatted materials were subjected to the starch saccharification and fermentation procedures. Briefly, liquefaction was carried out at a material–water ratio of 1:2 (w/v) using 24 U g^{−1} thermostable α -amylase at 95 °C for 2 h, and then the pH of the mixture was adjusted to 4.0–4.5 and 500 U g^{−1} glucoamylase was added. Then the mixture was kept at 60 °C for 1 h before filtered with gauze. The supernatant was subjected to fermentation process which was started by adding 0.15% (yeast weight/material weight) dry yeast, and the primary fermentation was carried out at 25 °C for 7 days, post-fermentation was at 14 °C for 15 days. In the end of fermentation process, both MW and MBW reached alcoholic strength of approximately 12.5%. Commercially available millet wine (CAMW) was provided by Hanlin Winery (Nanyang, Henan, China), with an alcoholic percentage of 16%.

Sensory evaluation

Sensory evaluation of the wine samples was carried out using the method of quantitative descriptive analysis (QDA) by 20 trained sensory panels. The following parameters were analyzed: sweet/sour/bitter/astringent tastes, colour, millet (bran) odor, as well as alcohol level. The intensity of taste and odor was indicated on the scale 1–5, where 1 means extremely weak and 5 means extremely strong. Overall acceptability was also evaluated using the mean score from 1 (poor) to 5 (outstanding). The means were obtained and plotted as a polygonal graph.

Total polyphenol content (TPC)

The TPC were determined using the Folin–Ciocalteu colorimetric method (Slinkard and Singleton 1977). In brief, 0.5 mL of diluted wine samples was mixed with 2.5 mL of Folin–Ciocalteu reagent (diluted tenfold) and 2 mL of 7.5% (v/v) Na₂CO₃ solution before being kept at 30 °C for 90 min. The absorbance of the mixture was recorded at 765 nm using a microplate reader (SpectraMax M2e, USA). The total phenolic content was expressed as gallic acid equivalents (mg gallic acid equivalents per mL).

Trolox equivalent antioxidant capacity (TEAC)

TEAC assay used in this study was slightly modified on the basis of previous report (Re et al. 1999). In brief, a stock solution of 7 mM ABTS⁺ was prepared in 100 mM PBS (pH 7.4) with 2.45 mM potassium persulfate in the dark at ambient temperature for 16 h. Before use, the stock solution was diluted to an absorbance of 0.70 ± 0.05 at

734 nm with PBS. 20 μL of the samples was added to 980 μL of ABTS⁺ working solution, then the mixture was left to stand in the dark at room temperature for 6 min. The absorbance at 734 nm was recorded to calculate the TEAC values. The TEAC results were expressed as Trolox equivalents (mg Trolox equivalents per mL).

Ferric reducing antioxidant power (FRAP) assay

The reducing power of wine samples was assessed by FRAP assay (Pulido et al. 2000). The FRAP reagent was freshly prepared as a mixture of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ at a ratio of 10:1:1. Then a mixture of 1.8 mL of FRAP reagent and 0.2 mL of diluted wine samples was left in the dark at 37 °C for 8 min before recording the absorbance of the reaction mixture at 595 nm. The calibration curve was constructed with aqueous solutions of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0–2.0 mmol L^{-1}), and the FRAP value was expressed as $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents (mmol $\text{Fe}^{2+} \text{L}^{-1}$).

DPPH radical-scavenging capacity

The DPPH radical-scavenging capacity was determined by a microplate method adapted from Brand-Williams et al. (1995). In brief, 20 μL of samples was added to 0.18 mL of DPPH methanolic solution (0.1 mmol L^{-1}) and mixed immediately. The mixture was allowed to stand in the dark at room temperature for 15 min. The absorbance at 540 nm was measured, and the result was expressed as remaining percentage of DPPH.

Amino acid (AA)

For measurement of total AA, wine samples were hydrolyzed with equal volume of 6 M HCl at 110 °C for 22 h under nitrogen. The dried hydrolysate was dissolved in 0.02 M HCl and then analyzed with an automatic AA analyzer (L-8900; Hitachi, Tokyo, Japan). For measurement of total Cys, wine samples were oxidized with performic acid at 0 °C for 16 h. The dried products were dissolved in distilled water and then lyophilized prior to the standard acid hydrolysis. For measurement of protein-associated or total Trp, wine samples were hydrolyzed with 4.2 M NaOH by heating at 110 °C for 26 h. The hydrolysate was acidified to pH 6–7 using 6 M HCl after cooled to 0 °C. The mixture was then centrifuged at $13,000 \times g$ for 5 min, and the resulting supernatant was subjected to HPLC on a Agilent ZORBAX SB-C18 column (4.6×250 mm, particle size 5 mm) (Agilent, Palo Alto, CA, USA). An isocratic elution of 10% methanol in water

was performed, and the absorbance at 280 nm was detected with a photodiode array detector (SPD-M10Avp; Shimadzu, Kyoto, Japan).

Polyphenol compounds by HPLC

The wine samples were pretreated as previously described with minor modifications (Paixão et al. 2008). Briefly, wine samples/mixed standards were extracted three times with equal volume of diethyl ether. The organic layer was concentrated to dryness and redissolved in 50% methanol before HPLC analysis.

The HPLC apparatus is the same as that in AA analysis. For HPLC analysis, 20 μL sample was injected into the column with a constant flow rate of 0.8 mL min^{-1} , and the column temperature was set at 30 °C. The mobile phase was composed of methanol, acetic acid and water at a ratio of 10:2:88 for mobile phase A, and 90:2:8 for mobile phase B. The elution conditions were: 0–18 min, 0–30% B; 18–24 min, 30% B; 24–36 min, 30–50% B; 36–56 min, 50–90% B; 56–66 min, 90–0% B; 66–70 min, 0% B. The identification and peak assignment of phenolic components in wine was determined by comparing their retention times and spectral data with those of authentic standards, and the identified phenolic components were further quantified using calibration curves of authentic standards.

Synergistic interaction

The evaluation of synergistic interaction is based on the previous reports (Li et al. 2012; Wang et al. 2014). The interaction of the main phenolic compounds was investigated in binary pairs at the actual ratio in MBW. In the ABTS and DPPH radical-scavenging assays, the interaction types were evaluated using the combination index (CI), which was expressed as CI₃₀, CI₅₀ and CI₇₀ when the fraction affected by the dose was 0.30, 0.50, 0.70, respectively. CI was calculated as follows:

$$\text{CI} = (\text{D})_1 / (\text{Dx})_1 + (\text{D})_2 / (\text{Dx})_2$$

where $(\text{Dx})_1$ and $(\text{Dx})_2$ indicate the individual dose of the two components required to scavenge a given level of free radical, and $(\text{D})_1$ and $(\text{D})_2$ are the doses of each component necessary to produce the same effect in combination, respectively. The interaction types can be indicated as follows: $\text{CI} \leq 0.90$, synergism; $0.90 < \text{CI} < 1.10$, additive effect; $\text{CI} \geq 1.10$ antagonism.

In the FRAP assay, the synergistic effect is determined by comparing between the theoretical and experimental values. In brief, the theoretical value was calculated as the sum of the two components' individual values multiplying their proportion in the combination. If the experimental value of the combination was significantly larger than their

theoretical value ($p < 0.05$), then the interaction is termed as synergism.

Statistical analysis

Statistical significance was analyzed by one-way ANOVA using Origin version 8.5, followed by Tukey's tests. A p value less than 0.05 was considered to be statistically significant. The results were expressed as the mean $\pm$ standard deviation (SD) of at least three individual experiments.

Results and discussion

Sensory analysis of wine samples

As shown in Fig. 1, the sour taste was relatively strong in MBW and CAMW, while CAMW showed the strongest bitter taste. MW and MBW reserved an abundance of millet/millet bran odor. And MBW had the darkest colour. The overall acceptability showed that MW had the highest acceptability, scored between very good and outstanding. MBW ranked second in terms of acceptability, with a score between good and very good. The CAMW had the lowest score, which could be potentially explained by its bitter taste and high acidity.

Comparison of TPC and antioxidant capacities of wine samples

Post-fermentation is a period characterized by receding alcohol fermentation activity and the senescence of yeast. At the same time, aroma components, organic acids, higher alcohol, aldehydes and esters are continuously generating.

Fig. 1 The quantitative descriptive analysis (QDA) for odors and tastes of MBW, MW, and CAMW. *MBW* millet bran wine, *MW* millet wine, *CAMW* commercially available millet wine

Many researchers focused on the changes in amino acid profiles and aroma components during post-fermentation period (Simpson 1978; Valero et al. 2003). However, the changes in TPC and antioxidant activity have not been reported. As can be seen in Fig. 2, TPC rarely changed in both MW and MBW during post-fermentation, which could be explained by the weakened yeast activity ($p > 0.05$). With regard to antioxidant activity, the post-fermentation exerted a positive effect on the antioxidant activity of MBW. The TEAC, FRAP values and DPPH-scavenging rate of MBW significantly increased during the 15-day's post-fermentation ($p < 0.05$). Although polyphenol compounds contribute extensively to the antioxidant activity, other components like AA, which mainly came from the autolysis of yeast during post-fermentation, and the aroma compounds might also contribute to the antioxidant activity of MBW (Junior et al. 2009). In comparison, the antioxidant capacity, except for DPPH-scavenging activity, has not significantly changed in MW during post-fermentation period ($p > 0.05$).

Considerably higher amounts of polyphenol compounds were found in MBW compared with MW and CAMW, which reached up to 1.35 mg mL^{-1} and was 6 times that of MW and 2.6 times that of CAMW ($p < 0.05$, Fig. 2). With respect to antioxidant activity, MBW showed remarkable antioxidant capacity with TEAC value of $4.29 \text{ mg Trolox equivalents per mL}$ after 15 days of post-fermentation, which was almost 10 times that of MW and 2.5 times that of CAMW. Moreover, the FRAP value of MBW was nearly four times that of MW. MBW with 15 days of post-fermentation (MBW-15d), when diluted four times, could scavenge 70% of the DPPH-radical, compared with 20 and 28% for MW-15d and CAMW, respectively. Our results provided evidence that the waste fractions of milled millet grain may serve as a potential source of natural antioxidants. Also, the wine brewed from millet bran could be regarded as a nutraceutical with strong antioxidant capacity, which can help promoting health and reducing disease risks.

Over the last decade, considerable experiments demonstrated the relation between oxidative stress and many chronic diseases. Increasing evidence indicated that diets high in selected natural antioxidants can reduce the incidence of chronic diseases. However, synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole, and propyl gallate showed potential health hazards (Lee et al. 2008). A great number of efforts have been made to find naturally occurring bioactive substances that possess antioxidant activities. Chinese rice wine, which is frequently used in Chinese traditional medicine, has been claimed to possess antioxidant activity and show beneficial effects on the prevention of cancer and cardiovascular diseases (Que et al. 2006a; Xie et al. 2004). As is well

Fig. 2 TPC and antioxidant capacities of wine samples. **a** TPC, **b** TEAC, **c** FRAP and **d** DPPH-scavenging rate. The data represent the mean $\pm$ SD of three determinations. Mean values with different letters are significantly different ($p < 0.05$). The wine samples for

DPPH assay were diluted four times. MBW millet bran wine, MW millet wine, CAMW commercially available millet wine, TPC total polyphenol content, TEAC Trolox equivalent antioxidant capacity, FRAP ferric reducing antioxidant power

known, polyphenols contribute largely to the antioxidant activity. However, only less than 0.2 mg mL^{-1} polyphenols was detected in traditional Chinese rice wine (Que et al. 2006b), compared with 1.35 mg mL^{-1} in MBW, which is almost sevenfold that of Chinese rice wine. Red wine is a well-known strong antioxidant beverage with health-promoting activity on reducing risks of cardiovascular disease and certain cancers. The polyphenol contents in red wines range from 1.6 to 3.73 mg mL^{-1} (Di Majo et al. 2008; Minussi et al. 2003), and in this regard, MBW is comparable to red wine and shows potential to serve as a new kind of antioxidant beverage.

AA profiles of wine samples

The AA profiles of the three wine samples are summarized in Table 1. MBW contained the most abundant AA content, reaching up to 6857 mg L^{-1} , which is six times more than that in MW, and was nearly two times that of CAMW. A previous study investigated the AA profiles of 98 Chinese rice wine samples, indicating that the AA content in

Chinese rice wine ranged from 2527.5 to 5290.4 mg L^{-1} , with an average concentration of 3749.2 mg L^{-1} (Shen et al. 2010). Greek white wines were reported to possess an average AA content of 417 mg L^{-1} (Soufleros et al. 2003). Another research revealed that the AA contents of red wines and white wines were 1917 and 1187 mg L^{-1} on average (Gómez-Alonso et al. 2007). As we can see, MBW significantly surpasses them in AA contents.

As can be seen in Table 1, Glu was found to be the most abundant AA in MBW with the concentration of 1249 mg L^{-1} . Other AA like Gly, Arg, Asp and Ala were also abundant in MBW, with concentrations higher than 600 mg L^{-1} . The top five AA represented more than 56% of the total AA in MBW, and exerted various bioactivities. For example, Glu is the precursor of γ -aminobutyric acid, which is an important excitatory neurotransmitter in brain (Perry et al. 1973). Gly and Ala could protect kidney cells from stress injury (Nissim et al. 1992), and Arg is able to enhance wound healing and immune responses (Barbul et al. 1990). The predominant AA in MW and CAMW was also Glu (Table 2). Apart from Glu, MW was also rich in

Fig. 3 HPLC chromatograms of mixed standards (a–d) and MBW (e–h) recorded at 270, 307, 320 and 360 nm. MBW millet bran wine. Peak identification: (2) protocatechuic acid, (4) vanillic acid, (5)

syringic acid, (6) vanillin, (8) *p*-coumaric acid, (9) ferulic acid, (10) ellagic acid, (14) hesperetin, (16) apigenin

Gly and Arg, and for CAMW, Ala and Pro were abundant. In regards to the indispensable AA (IAA), MBW contained far more IAA than MW, about 11-fold of Met, sixfold of Thr, Lys and Trp, and fivefold of Val and Leu were found in MBW compared to MW. Although total AA content in MBW was only twice as much as in CAMW, certain kinds of IAA were extremely abundant in MBW compared to

CAMW, such as Lys (tenfold), Trp (tenfold), Thr (eightfold) and Met (threefold).

AAs are precursors for aroma compounds in wine. They undergo a series of biotransformations, yield higher alcohols, aldehydes, esters and ketonic acids, and have an impact on the organoleptic properties of wine (Huang and Ough 1989). On the other hand, AAs are also nutrient

Table 1 Amino acid profiles of wine samples^a (mg L⁻¹)

Amino acid	MBW	MW	CAMW
Asp	620	92	101
Thr	424	70	55
Ser	320	64	68
Glu	1249	124	835
Gly	702	100	271
Ala	610	84	594
Val	273	54	259
Met	68	6	24
Ile	123	40	103
Leu	258	56	329
Tyr	181	44	49
Phe	91	22	134
Lys	403	70	39
His	279	40	30
Arg	669	98	21
Pro	274	54	637
Trp	60	10	6
Cys	78	30	74
Total	6857	1058	3629

MBW millet bran wine, MW millet wine, CAMW commercially available millet wine

^aData are expressed as means of three independent determinations

components of the wine. There is a category of AAs called functional AA (FAA), including Arg, Cys, Glu, Leu, Pro, and Try (Wu 2009). Growing evidence shows that besides

their role as building blocks of proteins and polypeptides, they are also important in regulating key metabolic pathways that are necessary for maintenance, growth, reproduction, and immunity in organisms (Wu et al. 2007). Supplementing diet with one or a mixture of these AAs may be beneficial for health. Comparing among the three wine samples, 100 mL of MBW provides 258.8 mg FAA, ranking the highest of the three wine samples. As for MW and CAMW, they provide 37.2 and 190.2 mg FAA per 100 mL, respectively. Thus, MBW surpasses MW and CAMW not only in total of AA contents, but also in the contents of IAA and FAA as well.

HPLC analysis of polyphenols in wine samples

Polyphenols are the principal compounds responsible for the health-beneficial effect of wines due to their antioxidant and free radical scavenging properties. Thus, the present study tentatively identified and quantified the polyphenol compounds in MBW using HPLC. Sixteen standard compounds could be separated by HPLC, among which nine polyphenols were detected in MBW based on their retention times and UV–Vis spectra. Table 2 presents the characteristic spectra, retention times, and contents of individual polyphenols found in MBW sample. Syringic acid (SA) was found to be the most abundant polyphenol in MBW, with concentration of 236.18 mg L⁻¹. And the content of vanillic acid (VA) was just below SA as 226.34 mg L⁻¹. Aside from SA and VA, *p*-coumaric acid (CA) and ferulic acid (FA) were also abundant in MBW,

Table 2 Identification of polyphenols in MBW by HPLC method

Peak no.	Compound	UV (nm)	RT	Content ^a (mg L ⁻¹)
1	Gallic acid	270	5.82	ND
2	Protocatechuic acid	260	10.62	26.12 ± 1.22
3	Gentisic acid	320	15.73	ND
4	Vanillic acid	260	19.20	226.34 ± 9.08
5	Syringic acid	270	20.96	236.18 ± 11.26
6	Vanillin	280	22.65	40.72 ± 0.97
7	Syringaldehyde	307	23.98	ND
8	<i>p</i> -Coumaric acid	307	25.30	159.16 ± 3.78
9	Ferulic acid	320	27.27	133.88 ± 3.25
10	Ellagic acid	270	34.72	2.22 ± 0.23
11	<i>o</i> -Coumaric acid	360	37.58	ND
12	Quercetin	360	44.19	ND
13	Luteolin	360	46.28	ND
14	Hesperetin	280	46.76	13.34 ± 0.59
15	Kaempferol	360	48.71	ND
16	Apigenin	320	49.83	4.06 ± 0.34

RT retention time, ND not detected, MBW millet bran wine

^aResults represent mean values ± SD of three determinations

Table 3 DPPH radical-scavenging ability and synergistic effect for binary combinations of major polyphenols in MBW

Compounds and combinations	Ratio (w/w)	IC30 ^a (mg mL ⁻¹)	IC50 ^a (mg mL ⁻¹)	IC70 ^a (mg mL ⁻¹)	CI30	CI50	CI70
VA	–	1.28	2.19	3.09	–	–	–
SA	–	0.03	0.05	0.08	–	–	–
CA	–	7.02	12.05	17.07	–	–	–
FA	–	0.08	0.14	0.20	–	–	–
VA + SA	19:20	0.06	0.11	0.16	1.12 AN	1.08 AD	1.07 AD
VA + CA	7:5	2.09	3.58	5.07	1.08 AD	1.08 AD	1.08 AD
VA + FA	17:10	0.22	0.37	0.53	1.09 AD	1.07 AD	1.06 AD
SA + CA	3:2	0.05	0.08	0.12	0.90 SY	0.89 SY	0.88 SY
SA + FA	7:4	0.03	0.06	0.09	0.85 SY	0.86 SY	0.86 SY
CA + FA	6:5	0.18	0.30	0.43	1.00 AD	0.97 AD	0.96 AD

MBW millet bran wine, VA vanillic acid, SA syringic acid, CA *p*-coumaric acid, FA ferulic acid, AN antagonistic interaction, AD additive interaction, SY synergistic interaction

^aData are expressed as means of three independent determinations

Table 4 ABTS radical-scavenging ability and synergistic effect for binary combinations of major polyphenols in MBW

Compounds and combinations	Ratio (w/w)	IC30 ^a (mg mL ⁻¹)	IC50 ^a (mg mL ⁻¹)	IC70 ^a (mg mL ⁻¹)	CI30	CI50	CI70
VA	–	0.27	0.44	0.61	–	–	–
SA	–	0.44	0.73	1.01	–	–	–
CA	–	0.16	0.26	0.37	–	–	–
FA	–	0.25	0.43	0.60	–	–	–
VA + SA	19:20	0.29	0.49	0.68	0.87 SY	0.88 SY	0.89 SY
VA + CA	7:5	0.21	0.35	0.49	1.02 AD	1.02 AD	1.02 AD
VA + FA	17:10	0.23	0.39	0.54	0.88 SY	0.89 SY	0.89 SY
SA + CA	3:2	0.21	0.36	0.50	0.84 SY	0.84 SY	0.84 SY
SA + FA	7:4	0.24	0.42	0.59	0.69 SY	0.72 SY	0.73 SY
CA + FA	6:5	0.18	0.31	0.44	0.95 AD	0.97 AD	0.97 AD

MBW millet bran wine, VA vanillic acid, SA syringic acid, CA *p*-coumaric acid, FA ferulic acid, SY synergistic interaction, AD additive interaction

^aData are expressed as means of three independent determinations

with concentrations of 159.16 and 133.88 mg L⁻¹, respectively. Other identified phenolics were protocatechuic acid, vanillin, ellagic acid, hesperetin and apigenin. It is worthy to note that SA, VA, CA and FA accounted for almost 90% of the identified polyphenols, indicating their significant role in the antioxidant activity of MBW. The total amount of the nine identified polyphenols was only over half of the TPC determined by the Folin–Ciocalteu colorimetric method (Fig. 2), which could be explained by the fact that some phenolics are not identified in the present study, as well as by the fact that some components like proanthocyanidins, various polymeric/oligomeric phenolics, and tannins are difficult to identify individually by HPLC (De Beer et al. 2004).

Synergistic interactions between major polyphenol compounds in MBW

As the major polyphenols in MBW, VA, SA, CA and FA were evaluated individually for their antioxidant activities, as they may contribute mostly to the antioxidant property of MBW. Surprisingly, as shown in Table 4, VA and CA possessed extremely poor DPPH-scavenging activity, with IC50 of 2.19 and 12.05 mg mL⁻¹, respectively. This finding is consistent with other researches (Ellnain-Wojtaszek et al. 2003; HiRoTA et al. 2000). However, in terms of TPC, the IC50 of MBW in DPPH assay was only 0.186 mg L⁻¹, which is considerably lower than that of VA and CA. We speculated that synergistic interaction might exist between the major polyphenols in MBW. Thus

Table 5 FRAP assay and synergistic effect for binary combinations of major polyphenols in MBW

Compounds and combinations	Ratio (w/w)	Experimental value ^a (mmol Fe ²⁺ g ⁻¹)	Theoretical value ^a (mmol Fe ²⁺ g ⁻¹)	Interaction
VA	–	0.10 ± 0.00	–	–
SA	–	10.02 ± 0.36	–	–
CA	–	0.02 ± 0.00	–	–
FA	–	6.89 ± 0.02	–	–
VA + SA	19:20	5.15 ± 0.06	5.20 ± 0.19	AD
VA + CA	7:5	0.08 ± 0.00	0.08 ± 0.00	AD
VA + FA	17:10	3.87 ± 0.08*	3.60 ± 0.01	SY
SA + CA	3:2	7.39 ± 0.08*	6.58 ± 0.24	SY
SA + FA	7:4	11.43 ± 0.16*	10.10 ± 0.2	SY
CA + FA	6:5	3.44 ± 0.07	3.55 ± 0.01	AD

FRAP ferric reducing antioxidant power, MBW millet bran wine, VA vanillic acid, SA syringic acid, CA *p*-coumaric acid, FA ferulic acid, AD additive interaction, SY synergistic interaction

* $p < 0.05$

^aResults represent mean values ± standard deviation (SD) of three independent measurements

DPPH, ABTS-radical scavenging and FRAP assays were employed to investigate the synergistic action between binary combinations of the four compounds. Previous studies demonstrated that combinations of compounds with different ratio might exhibit different interaction types (Popovich et al. 2010). Therefore, in the present study we employed the same ratio as in MBW to mimic the actual situation in MBW.

Table 3 represents the DPPH-scavenging activity of individual and combinations of VA, SA, PA and FA. Results show that although CA possessed poor DPPH-scavenging activity, it showed antioxidant synergism when used in combination with SA, with IC₅₀ of 0.08 mg L⁻¹, compared with 12.05 mg L⁻¹ when CA acted alone. Therefore, when a weaker radical-scavenger is combined with a stronger one, it might regenerate the antioxidant activity of the latter one, exhibiting the increased antioxidant capacity of the combination (Peyrat-Maillard et al. 2003). However, VA, which is also a weak DPPH-scavenger, could not enhance the antioxidant activity of other compounds, and exhibited additive effect in DPPH assay. Synergistic interaction was also observed in the combination of SA and FA. Other combinations were all additive except for VA and SA, the CI 30 of which exhibited a slight antagonistic interaction. This indicated that the combination of VA and SA at a low dose might counteract with each other on antioxidant activity, while the antagonism was weakened and became additive at higher doses. Table 4 shows the results in ABTS-scavenging assay. Synergistic interactions were observed in four combinations, including VA and SA, VA and FA, SA and CA, and SA and FA. Among these, the combination of SA and FA showed the best synergistic interaction with CI from 0.69

to 0.73. Ferric reducing antioxidant capacities of these combinations were also measured in order to evaluate their synergistic effects (Table 5). Among the six combinations, synergistic interactions were observed between VA and FA, SA and CA, and SA and FA, among which SA and FA exhibited the biggest difference between experimental and theoretical values, indicating the strongest synergistic action. It is worthy to note that the combinations of SA and CA, SA and FA exhibited synergism in all three assays, indicating their significant roles in the antioxidant activity of MBW. The synergistic antioxidant activity among the major polyphenols may be due to the hydroxyl groups in them. The number of free hydroxyl groups might potentially increase after being combined, which could further promote access of the free radical scavengers to the radical center (Cai et al. 2006), and exhibit a synergistic effect. Three antioxidant methods were employed in the present study to explore the interaction types between polyphenol compounds. The results indicated that the interaction types are not in full accordance with the three methods, showing the difference in various antioxidant systems. Thus, rather than a single evaluation method, more methods should be applied to give a comprehensive prediction of antioxidant synergism.

Conclusion

The present study provided a practical method concerning the utilization of millet bran by brewing wines. The wine brewed from millet bran performs remarkable antioxidant activities compared with millet wine, and showed more potential for being a nutraceutical. Four major phenolics

were identified including vanillic acid, syringic acid, *p*-coumaric acid, and ferulic acid. They exhibited synergism in the in vitro antioxidant assays, especially the combinations of syringic acid and *p*-coumaric acid, syringic acid and ferulic acid, indicating their potential role in the design of functional foods, dietary supplements or medicinal drugs. Moreover, millet bran wine is a rich source of indispensable amino acids and functional amino acids. Apart from its nutritional value, millet bran wine is also well-accepted as a wine beverage based on the sensory analysis. The present study not only provides theoretical basis for the purported antioxidant activity of the MBW, but also opens new avenues in the area of making comprehensive utilization of agricultural by-products.

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