Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.5327/fst.129722

Analysis of polyphenols in millet and processing effects on antioxidant activity

Liangyu LI[1,2,3] Yang LING[4], Tianfeng HE[4], Chaoyang LI[1,2,3], Rong-an CAO[4]*

Abstract

In this study, millet is used as the raw material to study the composition of millet polyphenols and the effects of different processing interventions on the antioxidant activity of millet polyphenols. The composition of millet polyphenols is determined by liquid analysis, and the effects of different processing interventions on their antioxidant activity are studied through five kinds of processing interventions – boiling, baking, microwave treatment, high-pressure boiling, and extrusion expansion – and the changes are discussed. The millet polyphenols mainly contained three substances: 7-O-β-D-glucopyranosyl-6-C-β-D-glucopyranosylluteolin ($C_{27}H_{30}O_{16}$), p-coumaric acid ($C_9H_8O_3$), and isoscoparin-7-O-β-D-glucoside ($C_{28}H_{32}O_{16}$). The antioxidant activity of millet polyphenols obtained from treatment in five kinds of processing interventions was in the following order: boiling > high-pressure boiling > baking > microwave > extrusion. Boiling was the best processing method to retain antioxidant activity of polyphenols, while extrusion was the least favorable.

Keywords: millet; polyphenols; LC-MS; processing intervention; antioxidant activity.

Practical Application: This study will contribute to the development of functional foods based on millet, ensuring the antioxidant activity of millet polyphenols during processing.

1 Introduction

Millet(*Setaria italica (L.) Beauv. var. germanica (Mill.) Schrad*), is one of the cereal grains commonly eaten in China. It has advantages of short cultivation period, strong vitality, and easy storage (Li et al., 2021b). It is a high-quality dual-purpose material for medicine and food, which is rich in nutrients and contains less-active ingredients such as polyphenols. Nambiar et al. (2012) summarized the types of polyphenols in millet of various cultivated varieties, and found that the main phenolic acids were ferulic, p-coumaric, and cinnamic acids. It is generally believed that the main way to intake polyphenols is to eat fruits and vegetables, but studies in recent years have shown that cereals are also rich in polyphenols (Ryan et al., 2011).

In many countries, millet is mostly deep processed, while processing in China is still relatively simple, mainly focusing on producing foods such as millet porridge and millet noodles (Pang et al., 2015). The deep processing of millet has not been fully carried out. Deep processing technology and industrial development can improve the added value of millet (Du, 2016). Meanwhile, attention should be paid to the changes of processing characteristics and functional characteristics during millet processing. Filonova et al. (2022) studied the influence of the japanese millet flour and the method of dough preparation on the formation of the aroma of bakery products, by the smell analyzer. Li et al. (2020a) characterized the starch physicochemical properties of 95 accessions of proso millet (Panicum miliaceum L.). Senevirathne et al. (2021) studied the Antiamylase, Antiglucosidase, and Antiglycation Properties of Millets and Sorghum from Sri Lanka. In China, millet research has mainly focused on analysis of its chemical composition and nutritional components, while there are few studies on the antioxidant properties of millet polyphenols. Li et al. (2020c) studied neural protective effects of millet and millet polyphenols on high-fat diet-induced oxidative stress in the brain. Wang et al. (2022) studied the effects of different processing methods on the millet polyphenols and their anti-diabetic potential.

Polyphenols have a strong antioxidant effect, which can benefit people's health with anti-aging and anti-tumor functions (Nazzaro et al., 2020), the research mainly focused on the extraction of polyphenols (León-Roque et al., 2023; Chavez-Santiago et al., 2022; Safdar et al., 2022a, b; Liu et al., 2022; Tran et al., 2022).Millet polyphenols in the body can resist aging directly as antioxidants (Masisi et al., 2016), and they retain bioactive properties after digestion (Gangopadhyay et al., 2015). Different processing methods will affect the antioxidant property of phenols, but there are few relevant studies. In this study, millet was processed by five methods: high-pressure boiling, extruding, baking, steam boiling, and microwaving. The effects of processing methods on the antioxidant properties of millet polyphenols are discussed to determine the best processing methods and conditions for maintaining the antioxidant properties of millet polyphenols.

Received 30 Nov., 2022
Accepted 19 Jan., 2023
[1] *National Coarse Cereal Engineering Technology Research Center, Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang Province, China*
[2] *Engineering Research Center of Processing and Utilization of Grain By-products, Ministry of Education, Daqing, Heilongjiang Province, China*
[3] *Technology Innovation Center of Separation of natural products by simulated moving bed chromatography of Heilongjiang Province, Daqing, Heilongjiang Province, China*
[4] *College of Food Science, Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang Province, China*
*Corresponding author: racao@163.com

2 Materials and methods

2.1 Materials

Materials and reagents

Millet was obtained from Gulong Town, Zhaoyuan County, Heilongjiang Province, China. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were supplied by Sigma-Aldrich USA.

Instruments and equipment

Equipment: autoclave MLS-3781L (Panasonic, Japan), pneumatic extruder XSS-QPC (Xinshishang Machinery, Wuhan China), high performance liquid chromatograph AcquityTM ultra (Waters, USA), TOF mass spectrometer Triple TOF 5600+ (ABSCIEX, USA), and Eppendorf MiniSpin centrifuge (Eppendorf, Germany).

2.2 Methods

Extraction method of millet polyphenols

Impurities were removed from millet and it was dried at constant temperature until the water content was below 8%. Twice the volume of n-hexane was added and stirred at 45 °C for 2 h, centrifuged at 3550 × g for 10 min, and the supernatant removed. The operation of extracting polyphenols follows: extraction solution 70% ethanol, liquid:material ratio 1:15, and extraction at 45 °C for 3 h.

Effects of processing interventions on antioxidant activity of millet polyphenols

(1) High-pressure boiling

Impurities were removed from millet and it was high-pressure cooked at 120 °C for 3, 9, and 15 min, polyphenols were extracted and their content determined, and then their antioxidant activity was determined.

(2) Extrusion

Impurities were removed from millet and it was extruded, polyphenols were extracted, and the content and antioxidant activity of millet polyphenols were determined.

(3) Baking

Impurities were removed from millet and it was baked at 100, 120, and 140 °C for 40, 60, and 80 min, respectively. Then polyphenols were extracted, and the content and antioxidant activity of millet polyphenols were determined.

(4) Boiling

Impurities were removed from millet and it was cooked at 80, 90, and 100 °C for 10, 30, and 50 min, respectively. Then millet polyphenols were extracted, and their content and antioxidant activity were determined.

(5) Microwave

Impurities were removed from millet and it was microwaved at 200, 400, and 600 W for 3, 9, and 15 min, respectively. Then millet polyphenols were extracted, and their content and antioxidant activity were determined.

(6) Blank

Impurities were removed from millet, the polyphenols were extracted, and their content and antioxidant activity were determined.

Liquid chromatography-mass spectrometry (LC-MS) methods for components of millet polyphenols

(1) Sample pretreatment

The sample was freeze-dried and 2 mL of 50% acetonitrile was added. It was then ultrasonicated for 30 min, centrifuged at 3550 × g for 30 min, and the supernatant taken for testing.

(2) LC-MS condition

This used the method of Li et al. (2021a) with slight modification. Wavelength of the UV detector was set at 280 nm.

Chromatographic conditions: injection volume was 5 μL and column temperature was 30 °C. Mobile phase A consisted of a water solution containing 0.1% (v/v) formic acid, while mobile phase B was acetonitrile solution containing 0.1% (v/v) formic acid. A gradient program at a flow rate of 0.8 mL/min was as follows: 0–25 min, 5% B; 25–35 min, 25%B; and 35–38 min, 95% B.

Mass spectrometry conditions: equipped with electrospray ionization source and positive ion mode monitoring, the atomizing gas was 50 psi, air curtain gas was 35 psi, ion source temperature was 600 °C (positive), ion source voltage was 5500 V (positive), and m/z setting range was 100–1500. First level scan: cluster voltage 100 V and focus voltage 10 V. Secondary scanning: mass spectrometry data were collected by TOF MS-Product Ion-IDA mode. The CID energy was 20, 40, and 60 V.

Determining millet polyphenol content

Millet polyphenols were determined by Folin phenol method (Fu et al., 2011; Wei et al., 2019) with slight modification. To 1 mL of polyphenol sample, 0.5 mL of 0.5 mol/L Folin monophenol and 2.5 mL of 75 g/L Na_2CO_3 were added, mixed evenly, kept in darkness for 2 h at 25 °C, and then absorbance of the reaction solution was measured at 760 nm wavelength. The polyphenol determination standard curve was $Y = 0.2061x + 0.1052$, $R^2 = 0.9987$, obtained by pre-experiment.

Determination of DPPH radical scavenging activity

The DPPH free-radical scavenging activity was determined by the method of Li et al. (2020b) with slight modification. To 2 mL of samples at different concentrations (0–50 μg/mL), 2 mL of 0.4 mM DPPH radicals was added. The mixture was shaken

vigorously and left to stand for 30 min in darkness before absorbance was measured at 517 nm against a methanol blank.

Ascorbic acid (Vc) was used as a positive control. The percent inhibition of DPPH was calculated according to the following Equation 1:

$$DPPH\ scavenging\ activity\ (\%) = \left[(A - B)/A\right] \times 100 \quad (1)$$

where A is absorbance of DPPH radical + methanol and B is absorbance of DPPH + test samples. All determinations were performed in triplicate.

Determination of ABTS+· radical scavenging activity

The ABTS+· free-radical scavenging activity was determined by the method of Li et al. (2020b) with slight modification. The ABTS solution (7 mmol/L, 5 mL) and potassium persulfate solution (2.45 mmol/L, 5 mL) were mixed in darkness for 12 h, to produce ABTS+·. The ABTS+· solution was diluted with water, and the absorbance was 0.70 ± 0.02 at 734 nm. The percentage inhibition of ABTS was calculated according to the following Equation 2:

$$ABTS\ scavenging\ activity\ (\%) = (A_0 - A_1)/A_0 \times 100 \quad (2)$$

where A_0 is absorbance of absolute ethanol and ABTS solution and A_1 is absorbance of the sample and ABTS solution.

Determination of reducing power

The reducing power activity was determined by the method of Ji et al. (2019) with slight modification. To 0.5 mL of polyphenol sample, 2.5 mL of PBS and 2.5 mL of potassium ferricyanide were added, the mixture was shaken vigorously and left to stand for 30 min at 50 °C, cooled quickly, 2.5 mL of trichloroacetic acid was added, and then centrifuged at 3550 × g for 10 min.

To 5 mL of supernatant, 5 mL of distilled water was added, then 1 mL of ferric chloride was added, and it was fully mixed and reacted for 10 min. Then absorbance was measured at 700 nm and recorded as A_1. The absorbance of 0.5 mL of absolute ethanol was measured and noted as A_0. Calculation Formula 3:

$$reducing\ power = A_1 - A_0 \quad (3)$$

Statistical analysis

Statistical analyses were done using the SAS statistical program, and the significance of each group was verified by one-way ANOVA followed by Duncan's test at $P < 0.05$.

3 Results and discussion

3.1 LC-MS results of millet polyphenols

The total ion flow diagram showed that millet polyphenols (Figure 1a) included three substances, with peak times of 10.89, 11.05, and 12.46 min, respectively. The fitted molecular formula for component 1 was $C_{27}H_{30}O_{16}$ (Figure 1b), with peak time at 10.89 min. The second-order mass spectrometry indicated that there were carboglycosides and oxyglycosides in the structure of component 1. According to database search and speculation, the compound was 7-O-β-D-glucopyranosyl-6-C-β-D-glucopyranosylluteolin. The fitted molecular formula

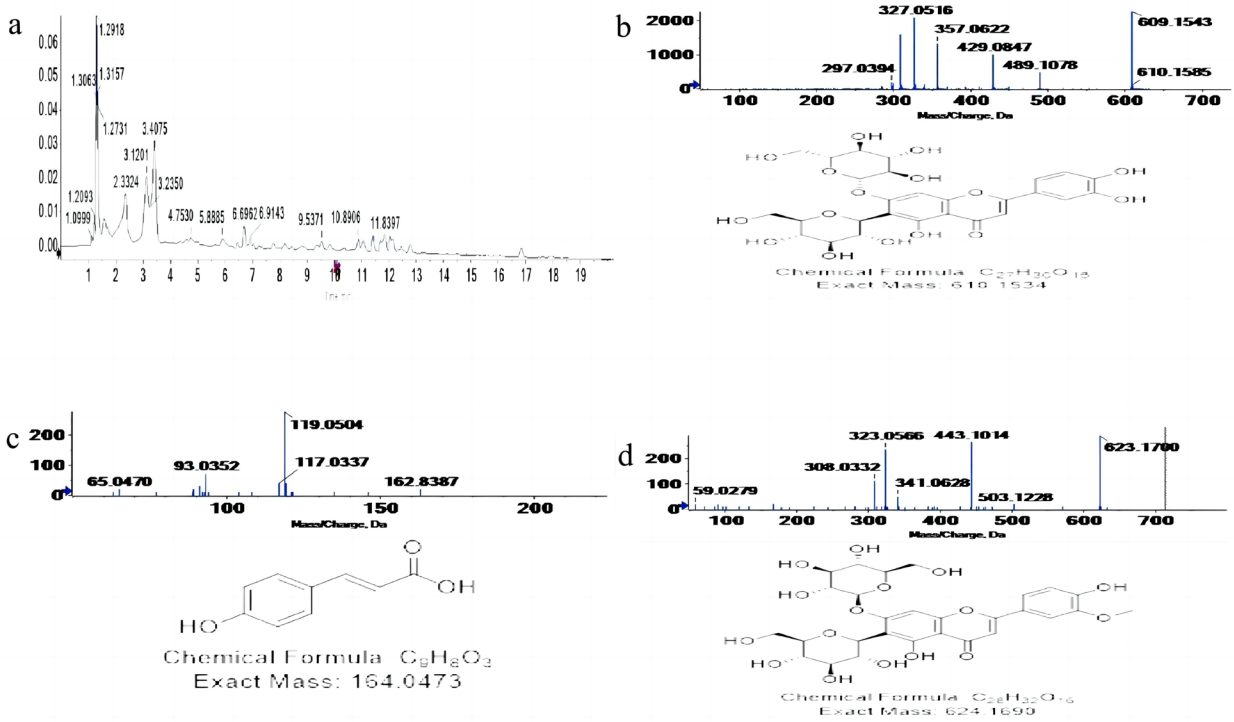

Figure 1. The results of LC-MS of polyphenols in millet. Fig. 1a is the total ion chromatogram of polyphenols in millet; Fig. 1b is mass spectrometry and possible structural formula of component 1; Fig. 1c is mass spectrometry and possible structural formula of component 2; Fig. 1d is mass spectrometry and possible structural formula of component 3.

for component 2 was $C_9H_8O_3$ (Figure 1c), with peak time at 11.05 min. The second-order mass spectrometry indicated that there were carboxy and ethyl in the structure of component 2. According to database search and speculation, the compound was p-coumaric acid. The fitted molecular formula for component 3 was $C_{28}H_{32}O_{16}$ (Figure 1d), with peak time at 12.46 min. The second-order mass spectrometry indicated that there were carboglycoside, oxyglycoside, and methoxy in the structure of component 3. According to database search and speculation, the compound was isoscoparin-7-O-β-D-glucoside.

3.2 Effects of processing interventions on antioxidant properties of millet polyphenols

High-pressure boiling and antioxidant properties

The different high-pressure boiling interventions resulted in significant differences in millet polyphenols' ability for scavenging DPPH and ABTS free-radicals, as well as their reducing capacity ($P < 0.05$; Figure 2). The rate of scavenging DPPH free-radicals initially increased with time and then decreased. The rate of scavenging ABTS free-radicals decreased with time, while the reducing capacity decreased. There was no significant difference in the reducing capacity between the 10-min and 30-min intervention groups ($P > 0.05$), but there was a significant difference between the 10-min and 50-min groups ($P < 0.05$). There was a significant difference in antioxidant properties between the high-pressure boiling and the non-intervention groups ($P < 0.05$), with the DPPH and ABTS scavenging ability of the high-pressure boiling group significantly higher than that of the non-intervention group, while reducing capacity was lower than that of the non-intervention group. There was a significant difference in the antioxidant property between the high-pressure boiling and the Vc control group ($P < 0.05$), and the antioxidant property was lower than that of the Vc group. The reason is that the total phenol content in millet decreased significantly following high-pressure boiling, which may lead to structural change in phenols, reducing their antioxidant property (Cardoso et al., 2015).

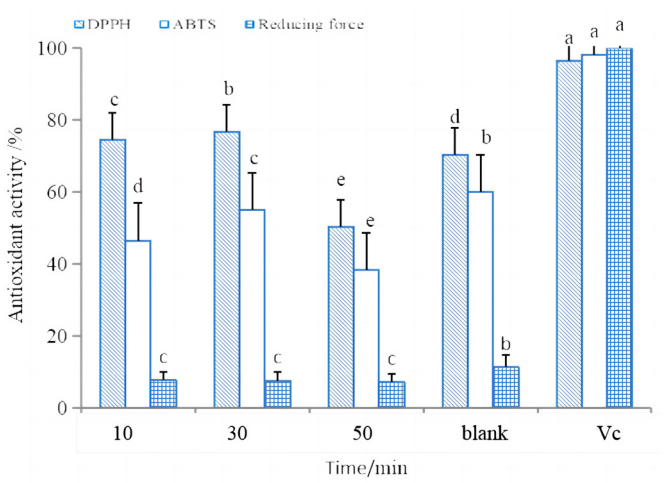

Figure 2. Effect of high-pressure boiling on antioxidant activity.

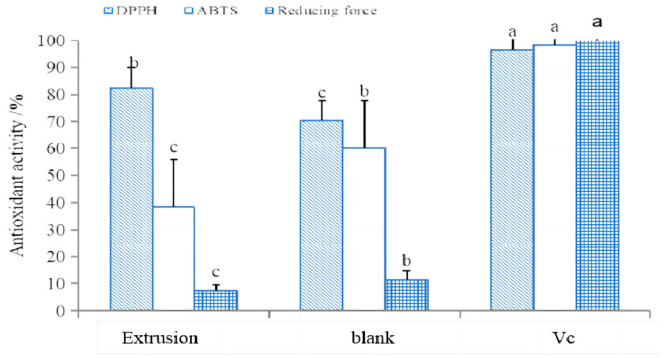

Figure 3. Effect of extrusion on antioxidant activity.

Extrusion and antioxidant properties

There was a significant difference in antioxidant properties of polyphenols between the extruding intervention and non-intervention groups ($P < 0.05$, Figure 3). The DPPH scavenging capacity was higher than that of the non-intervention group, but the ABTS scavenging and reducing capacity were lower. The antioxidant activity of millet polyphenols changed significantly after extruding. The reason may be that extruding helps to destroy the cell wall of millet, promoting the release of antioxidant substances and enhancing overall antioxidant properties (Qi et al., 2016). Meanwhile, due to the destructive effect of extrusion on millet polyphenols, the antioxidant activity was lower than for the non-intervention group (Li, 2017).

Baking and antioxidant properties

The different baking interventions resulted in significant differences in ability of millet polyphenols to scavenge DPPH and ABTS free-radicals, as well as in their reducing capacity ($P < 0.05$; Figure 4). The rate of scavenging DPPH free-radicals and reducing ability initially increased with time and then decreased; however, the rate of scavenging ABTS free-radicals increased with time (Figure 4a). The rate of scavenging DPPH free-radicals increased with time, while the rate of scavenging ABTS radicals decreased with time, and the reducing ability initially increased and then decreased with time (Figure 4b). The antioxidant properties of polyphenols were significantly lower in the baking intervention than the non-intervention group ($P < 0.05$), and also significantly lower than that in the Vc group ($P < 0.05$). The reason is that during baking, the sugars and amino acids in millet may undergo a Maillard reaction in dry heat. The reaction products may increase the content of total phenols, and also produce some substances with antioxidant properties, which could somewhat improve antioxidant activity. In addition, with the increase of baking intensity, some polyphenols are sensitive to heat, altering their structure and reducing antioxidant activity (Xia & Cao, 2017).

Steam boiling and antioxidant properties

The different steam boiling interventions resulted in significant differences in ability of millet polyphenols to scavenge DPPH and ABTS free-radicals, as well as in their reducing capacity ($P < 0.05$; Figure 5). The rates of scavenging DPPH and ABTS free-radicals and reducing capacity initially increased and then decreased with time (Figure 5a). The rates of scavenging DPPH

and ABTS free-radicals initially increased and then decreased with the increase of steam boiling temperature, but reducing capacity initially decreased and then increased (Figure 5b). The antioxidant activities significantly differed between the steam boiling and non-intervention groups ($P < 0.05$), and there was also a significant difference between the boiling intervention and Vc control groups. The antioxidant activity was lower than that of the Vc group. The reason is that heating millet can lead to the release of corresponding phenolic acids from bound phenols and increase the content of free phenols (Zhang et al., 2017). Steam boiling can greatly affect the content of phenols, which will destroy the structure of polyphenols and reduce the antioxidant activity over time. In addition, steam boiling may destroy the cell wall structure and release some stable bound phenols. However, when temperature is excessively high, the stable polyphenol structure will be destroyed, resulting in a decline in antioxidant properties (Yan et al., 2018).

Microwaving and antioxidant properties

The different microwaving interventions resulted in significant differences in ability of millet polyphenols to scavenge DPPH and ABTS free-radicals, as well as in their reducing capacity ($P < 0.05$; Figure 6). The scavenging rates of DPPH and ABTS free-radicals and reducing capacity initially increased and then decreased with time (Figure 6a). The rate of scavenging DPPH free-radicals and reducing ability initially increased and then decreased with the increase of microwave power, while the rate of scavenging ABTS free-radicals increased (Figure 6b). The antioxidant capacity was significantly greater for the microwaving intervention compared to the non-intervention group ($P < 0.05$). There was significantly lower antioxidant activity for the microwaving intervention compared to the Vc control group ($P < 0.05$). The reason is that when microwave intensity is low, microwaving helps to extract polyphenols (Zhang et al., 2013); however, when microwave power exceeded 440 W, the

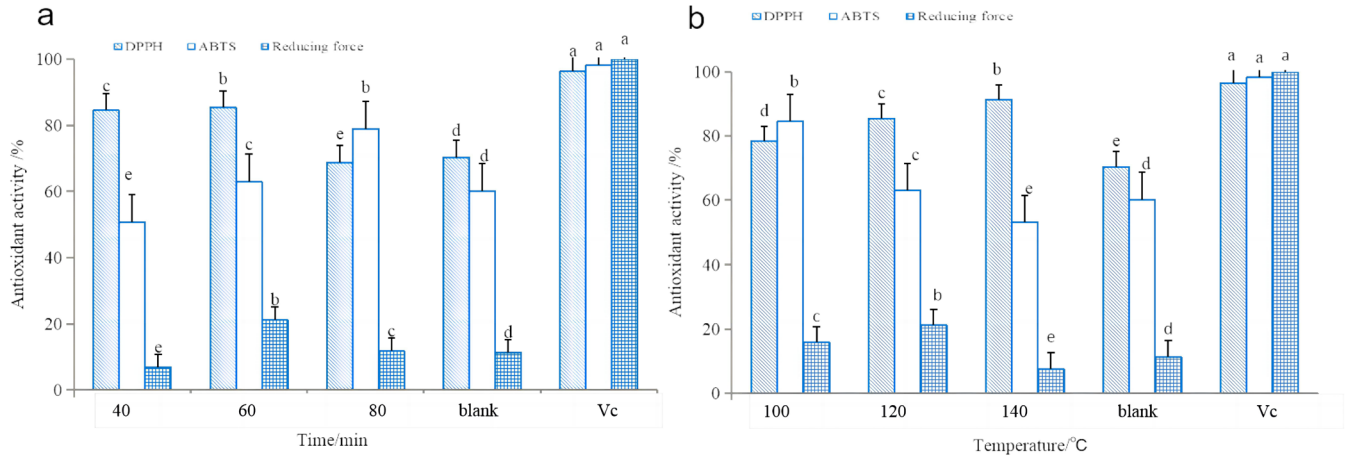

Figure 4. Effect of baking on antioxidant activity. Fig 4a is effect of baking time on antioxidant activity, Fig. 4b is effect of baking temperature on antioxidant activity.

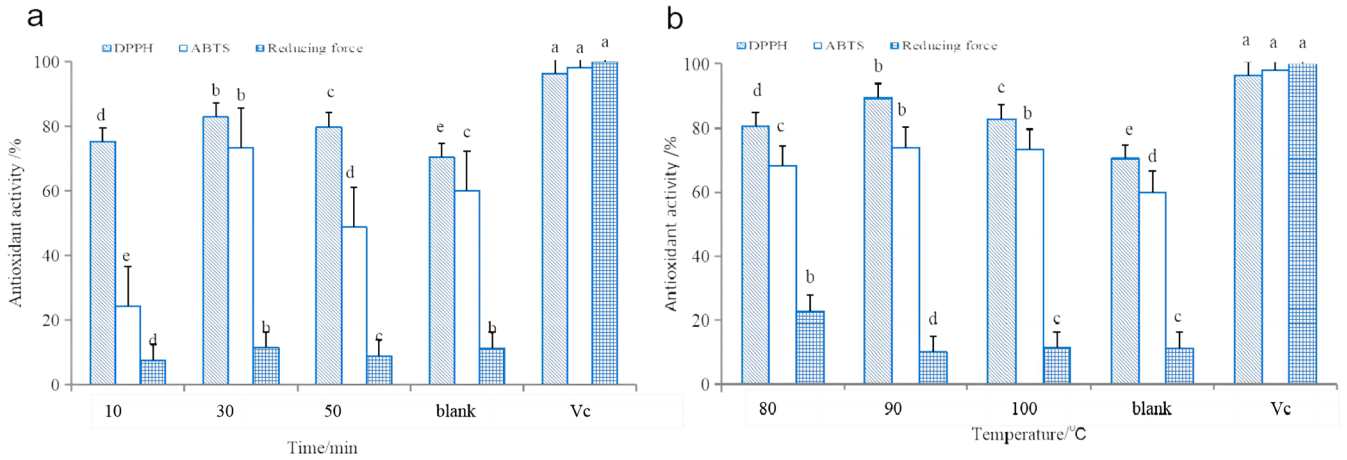

Figure 5. Effect of boiling on antioxidant activity. Fig. 5a is effect of boiling time on antioxidant activity, Fig. 5b is effect of boiling temperature on antioxidant activity.

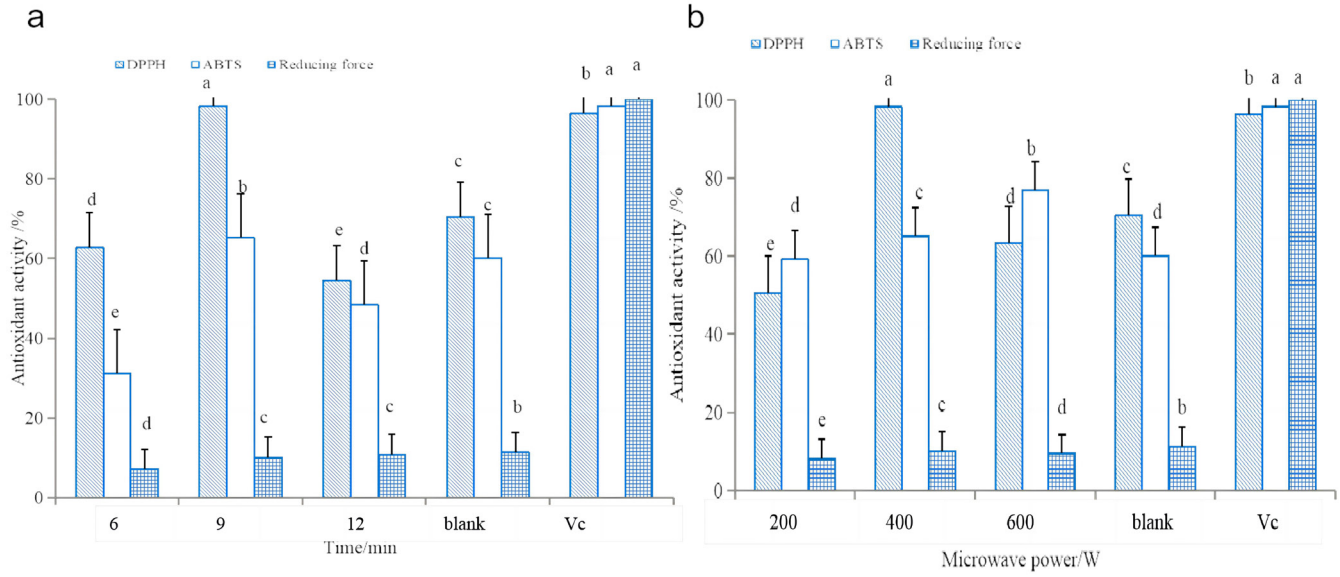

Figure 6. Effect of microwave treatment time antioxidant activity. Fig. 6a is effect of microwave time on antioxidant activity, Fig. 6b is effect of microwave power on antioxidant activity.

polyphenol activity decreased. In addition, excessive power also leads to a large amount of internal heat accumulation, destroying the structure of polyphenols and reducing their antioxidant activity (Wan, 2018).

4 Discussion

There were significant differences in the antioxidant properties of millet polyphenols among the five processing intervention groups ($P < 0.05$; Figure 7). After comprehensive consideration gave the order of the effects of the five intervention methods on antioxidant activity of millet polyphenols: steam boiling > high-pressure boiling > baking > microwaving > extruding. The five processing interventions involved heat treatment, and polyphenols are sensitive to heat. There are three stages of changes in antioxidant activity during heat treatment. The relationship between polyphenols and heating can be compared with the relationship between oil oxidation and water activity, while the antioxidant activity and temperature of millet also showed a changing trend of initially increasing and then decreasing. The first is the decreasing stage, in which the heating of millet degrades polyphenols, resulting in the overall decline of antioxidant activity. The second stage is increasing, in which the cell structure is damaged due to heating of millet, and some bound polyphenols are released, resulting in overall improvement of antioxidant activity. The third stage is decreasing, in which the newly released polyphenols are degraded by heat, resulting in a decline in antioxidant activity. Min et al. (2014) heated rice by steam boiling, and showed a significant increase in polyphenol content. Compared with baking, steam boiling is a wet and hot processing method. Due to the presence of water, the heating conditions are milder than other interventions and cause less damage to polyphenols, thus the antioxidant activity is the highest. The experimental data showed that steam boiling maintained the highest antioxidant activity of millet polyphenols,

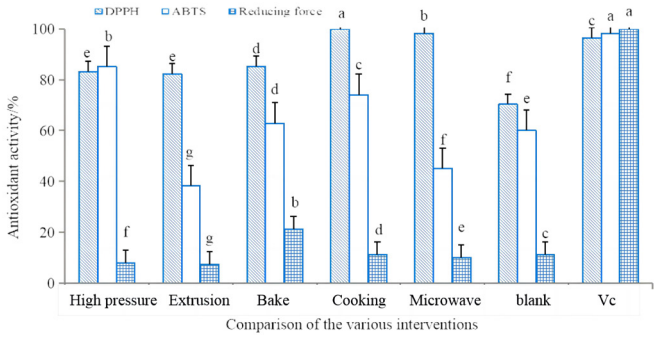

Figure 7. Comparison of the various interventions.

consistent with the results of Zhang et al. (2103). Cardoso et al. (2015) processed millet by high-pressure boiling, and found that the content of polyphenols increased. Compared with normal-pressure boiling, high-pressure boiling may lead to changes in the structure of phenols, resulting in lower antioxidant activity. Cardoso et al. (2015) conducted dry heating of sorghum and found improved antioxidant capacity of polyphenols. Muyonga et al. (2014) dry heat treated amaranth seeds and showed improved antioxidant capacity of polyphenols. The possible reason is that in the process of baking, the millet is affected by the dry and hot environment, and the Maillard reaction occurs (Pierre & Carolem, 2010); this increases the content of total polyphenols, which improve the antioxidant activity of polyphenols to a certain extent. Tian (2011) microwave treated millet and found that when power exceeded a certain value, the polyphenol yield decreased. Microwaving produces heat through collision and friction among molecules. When a large amount of heat accumulates, the structure of polyphenols may be destroyed, lowering the antioxidant activity. According to Zheng (2016), extruding can improve the taste and quality of food. Wang (2018) showed that extruding effectively improved the content

of soluble solids in rice bran. Extruding integrates the effects of high temperature, high-pressure, and high shear to achieve the purpose of puffing. However, excessive temperature, pressure, and shear can destroy the structure of polyphenols and reduce the antioxidant activity.

Finally, the antioxidant activity changed after processing by various methods, due to changed of the millet polyphenols contents. Now, many studies to date have only focused only on the changes in the content and antioxidant activity of polyphenols before and after processing. Few comparative studies have been done on how the changes in polyphenols composition and structure before and after processing, HPLC, GC-MS, and other techniques can be adopted to quantify the contents of certain phenolics, but it's not convincing, further improvements. The reduction of antioxidant activity during processing by various methods could affect effects on other functions. In addition, though polyphenols like catechins and ferulic acid are known for their antioxidant, hypoglycemic, and anti-inflammatory effects, other trace polyphenols and their derivatives in millets also need to be studied to understand their potential benefits for human health. At present, there is a lack of research on the interaction between polyphenols and other functional components during processing and this area needs further exploration.

5 Conclusion

The millet polyphenols mainly contained three substances: 7-O-β-D-glucopyranosyl-6-C-β-D-glucopyranosylluteolin ($C_{27}H_{30}O_{16}$), p-coumaric acid ($C_9H_8O_3$), and isoscoparin-7-O-β-D-glucoside ($C_{28}H_{32}O_{16}$). The order of antioxidant activities of millet polyphenols obtained from five kinds of processing treatments follow: boiling > high-pressure boiling > baking > microwave > extrusion. Boiling was the best processing method to retain antioxidant activity of polyphenols, while extrusion was the least favorable.

Conflict of interest

The authors declare no conflict of interests.

Funding

The present study was supported by the Scientific Research Initiation fund for Completion and Introduction of Talents (XDB202213) of Heilongjiang BaYi Agricultural University.

Author contributions

LL and RC were responsible for the design, overall management of the entire study, and editing. YL, TH, and CL provided the validation and formal analysis. LL and RC: writing review and editing. YL, TH, and CL analyzed the data. RC: supervision, writing-review and editing, and funding acquisition. All authors have read and agreed to the publishing of the current version of the manuscript.

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