Journal of Applied Biology & Biotechnology Vol. 12(1), pp. 111-116, Jan-Feb, 2024
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2024.149026

Increasing polyphenol antioxidant in *Orthosiphon stamineus* Benth leaves with fermentation extraction by *Saccharomyces cerevisiae* ATCC-9763

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ARTICLE INFO

Article history:
Received on: May 09, 2023
Accepted on: October 07, 2023
Available online: December 26, 2023

Key words:
Antioxidant activity,
Fermentation,
Flavonoid,
Orthosiphon stamineus,
Phenolic,
Saccharomyces cerevisiae.

ABSTRACT

This study aimed to elucidate the role of fermentation extraction by *Saccharomyces cerevisiae* in increasing polyphenol antioxidants in *Orthosiphon stamineus* Benth leaves. This study comprised five experimental extraction methods, i.e., fermentation, maceration, boiling, fermentation + boiling, and fermentation + maceration, with two types of samples (dry and wet) for each extraction method. The present study shows that fermentation extraction with dry samples has the highest polyphenol content. Interestingly, fermentation with *S. cerevisiae* on *O. stamineus* can increase the number of phenols and flavonoids, thereby increasing antioxidant activity. This study shows that dry leaf extract of *O. stamineus* leaves has been known as a good source of antioxidants, and the extraction method with fermentation is a better method to increase the antioxidant activity of *O. stamineus*. The result of the present study demonstrated that *S. cerevisiae* plays some important roles in increasing antioxidant polyphenol content in *O. stamineus* leaves.

1. INTRODUCTION

Orthosiphon stamineus Benth (Family *Lamiaceae*) is an important traditional folk medicine plant widely distributed in Southeast Asia, Australia, and South China [1]. *O. stamineus* contains secondary metabolites such as terpenoids, polyphenols, and sterols [2]. Polyphenols are the highest and dominant component in *O. stamineus.* Based on their structure, polyphenols function as antioxidant due to the benzene ring acting as a stabilizing antioxidant molecule. Furthermore, the polyphenol group is divided into five groups. The five groups are phenolic acids, flavonoids, stilbenes, coumarins, and tannins [3,4]. Phenolic acids have three hydroxyl groups, which act as antioxidants by binding free radicals with gallic acid, while flavonoids have five hydroxyl groups, so they have greater potential as antioxidants [5-7].

Antioxidants are compounds that can inhibit the oxidation of a molecule by ending the chain reaction of its initiation and deployment [8]. In case where there is an imbalance between free radicals and antioxidants, oxidative stress can occur, which can trigger diseases such as degenerative diseases, cancer, and aging [9]. The main role of antioxidant compounds is their ability to capture free radicals [10]. Many antioxidants have been found in herbal plants, such as *O. stamineus*. Herbal plants contain rosmarinic acid compounds, which are affluent of antioxidant activity.

Extraction was done to obtain polyphenolic compounds from the plant (*O. stamineus*). Extraction is the process of separating materials using suitable solvents. In the present study, the extraction method used is the maceration method. Maceration is one of the most frequently used methods of extraction [11]. Maceration is carried out by incorporating leaf simplicia and fresh leaves of the *O. stamineus* plant into the appropriate solvent, then tightly closed at room temperature for a predetermined time. The advantage of the maceration method is that it can avoid damage to thermolabile compounds because maceration does not use heat during extraction [12].

Indonesian people use boiled *O. stamineus* leaves as medicine. It is believed that boiled water from *O. stamineus* leaves can cure various diseases, one of which is diabetes mellitus [13,14]. On the other hand, in the aspect of biotechnology nowadays, extraction has been developed, which involves living things obtaining the antioxidant compounds present in *O. stamineus*, namely through fermentation. Fermentation is a process of chemical change in organic substrates caused by the activity of enzymes produced by microorganisms such as

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Saccharomyces cerevisiae [15]. Research by Ejuama *et al*. [16] shows that fermentation involving *S. cerevisiae* can increase antioxidant activity. Knowing the benefits of polyphenols, which have the potential to be antioxidant compounds. So, this is used as the basis for this study to critically review the optimal extraction method for increasing and producing better antioxidants in *O. stamineus*. Therefore, this aimed to elucidate the role of fermentation extraction by *S. cerevisiae* in increasing antioxidant polyphenol content in *O. stamineus* Leaves.

2. MATERIALS AND METHODS

2.1. Study Region

Fresh *O. stamineus* leaves were collected from the Biopharmaca Garden at IPB University, Bogor, West Java, Indonesia. Polyphenols were analyzed at the Tropical Biopharmaca Research Center and Research Laboratory Biochemistry, IPB University, Bogor, West Java.

2.2. Plant Material Preparation

This study used fresh *O. stamineus* leaves (a wet sample) and simplicia (a dry sample). Fresh young leaves were not far from the plant shoots picked directly from the Biopharmaca garden. A dry sample was made by drying *O. stamineus* leaves in a microwave at a temperature of 45°C for 48 h. The dried leaves were ground using a machine and filtered using a 100-mesh filter to obtain the simplicia.

2.3. Extracting *O. stamineus* Leaves by *S. cerevisiae* Fermented

Before being used as a fermenter, *S. cerevisiae* was rejuvenated with the modified Perwitasari *et al*. [17] method. *S. cerevisiae* used in this study came from the IPB Culture Collection with the ATCC-9763 strain. The stock culture of *S. cerevisiae* was taken as much as one OSE, then streaked into slants agar (Potato Dextrose Agar). The culture was incubated for 24 h at 37°C and was ready for use.

Fermentation of *O. stamineus* was according to the modification of Salar *et al*. [18]. *O. stamineus* leaves that have been sliced about 1 cm and simplicia were taken as much as 2 g and soaked in 20 mL of distilled water for 24 h. In an aseptic condition, the solution was inoculated with 5 mL of *S. cerevisiae* ($1–6 \times 10^7$ cells/mL). The suspension was incubated at 30°C for 72 h.

2.4. Extracting *O. stamineus* Leaves by Maceration

About 1 cm of Fresh leaves of *O. stamineus* or 2 g of simplicia were dissolved in 20 mL of 70% ethanol. Then, the solution was incubated for 48 h on a shaker at 120 rpm. Extract from *O. stamineus* was obtained by filtering the incubated solution using a 10-micron filter paper (Modification of Kawiji *et al*. [19]).

2.5. Extracting *O. stamineus* Leaves by Cooking Immediately

Fresh leaves of *O. stamineus* (50 g) were boiled with 500 mL of water for 15 min and then filtered using a filter. The boiled water from *O. stamineus* leaves was ready to analyze phenolic, flavonoid, and antioxidant activity levels.

2.6. Total Phenolic Compound Analysis

According to the Folin-Ciocalteu method, the total phenolic content (TPC) was obtained by mixing 100 μL of Folin-Ciocalteu (50%), and 20 μL of *O. stamineus* leaf extract was pipetted into 96-well microplate and incubated in a dark room for 5 min. The mixed solution was added to 80 μL of Na_2CO_3 (7.5%) and incubated for 2 h in a dark room. The absorbance that was incubated was measured at a wavelength of 750 nm using a spectrophotometer in triplicates, respectively. The TPC was expressed as milligrams of gallic acid equivalent per gram of extract (mg EAG/g). The standards used were gallic acid with concentrations of 150, 250, 300, 350, 400, 450, and 500 ppm (Modification of Calvindi *et al*. [20]).

2.7. Total Flavonoid Compound Analysis

About 10 μL of *O. stamineus* leaf extract was put into a *microplate* 96 *well* and was added with 60 μL methanol, 10 μL aluminum chloride (10%), 10 μL CH_3 COOK 1M, and 110 μL dissolved water; then the solution was incubated in the dark for about 30 min, and the absorbance was measured at a wavelength of 415 nm with triplicates, respectively. The total flavonoid content (TFC) was expressed as milligrams of quercetin equivalent per gram of extract (mg QE/g) (Modification of Calvindi *et al*. [20]).

2.8. Antioxidant Activity Analysis with the DPPH Method

Antioxidant activity analysis was carried out using the 2,2-diphenylpicrylhydrazyl, or DPPH, method. 100 μL of *O. stamineus* leaf extract was added to 100 μL of DPPH reagent (2.5 mg of DPPH powder in 50 mL of pro-analyzed ethanol), then the mixture was pipetted and transferred to a *96-well microplate*. The solution was incubated in the dark for about 30 min, and the absorbance was measured at a wavelength of 517 nm using a spectrophotometer with triplicates, respectively. The antioxidant activity of DPPH is expressed as micromol Trolox equivalent per gram of extract (μmol TE/g) (Modification of Nurcholis *et al*. [21]).

2.9. Antioxidant Activity Analysis with the ferric reducing antioxidant power (FRAP) Method

The other antioxidant analysis activities used the FRAP method. As much as 10 μL of *O. stamineus* leaf extract was added to 300 μL of FRAP reagent and transferred to a 96-well microplate. Microplates were incubated in the dark for 30 min. After incubation, the absorbance of the solution was measured at a wavelength of 593 nm using a spectrophotometer with triplicates, respectively (Modification of Benzie and Devaki [22]).

2.10. Statistical Analysis

The mean ± SD was established for three replicates. The ExpDes package in R was used to determine the ANOVA, followed by a Scott-Knott test (Ferreira *et al*. [23]). Values were considered significant at $P < 0.05$. Relationships between TPC, TFC, and antioxidant activities were evaluated using regression and correlation in the R Studio program.

3. RESULTS AND DISCUSSION

3.1. TPC

TPC was analyzed by the Folin-Ciocalteu method using gallic acid as a standard phenolic compound. The results of this study show that the TPC of *O. stamineus* leaves extracted has a different TPC depending on extraction methods and sample form. TPC from five different extraction methods and sample forms is shown in Table 1. Based on the method used for extraction in this study, the TPC in the wet samples ranged from 0.21 to 9.86 mg GAE/g, while in the dry samples, the TPC ranged from 3.88 to 25.28 mg GAE/g. The results of this study showed that the dry sample had the highest TPC compared to the wet sample

Table 1: Result of total phenolic content (as mg gallic acid equivalents (GAE)/g) in various extraction methods and samples of *Orthosiphon stamineus*.

Method	Total phenolic (mg GAE/g)	
	Wet sample	Dry sample
Fermentation method	9.86±0.25^{aB}	25.28±2.24^{aA}
Maceration method	6.07±0.54^{bB}	21.8±1.40^{bA}
Boiling method	5.56±0.01^{cB}	17.23±1.33^{cA}
Fermentation + Boiling method	0.21±0.01^{eB}	3.88±0.19^{eA}
Fermentation + Maceration method	1.06±0.00^{dB}	7.23±0.28^{dA}

Each value is presented as the mean of three replicates ± standard deviation; a-e means within a column with different letters are significantly different (Scott-Knott test, $P < 0.05$); A and B respectfully means within a row with different letters are significantly different (Scott-Knott test, $P < 0.05$), whereas a and A value is preferred

($P < 0.05$). This confirms previous studies showing that the dry sample has the highest TPC than the wet sample [24]. On the other hand, based on the extraction method used, this study showed that the fermentation method had the highest TPC ($P < 0.05$) in both wet and dry samples. The lowest phenolic content was in the combination method of fermentation and boiling. Basically, the leaves of *O. stamineus* have a TPC of 21.8 mg GAE/g. Fermentation treatment showed an increase in the TPC of *O. stamineus* leaves to 25.28 mg GAE/g. This shows an increase in the TPC through fermentation compared to the maceration method. These results indicated that the TPC could be increased by *S. cerevisiae*.

Research by Chrzanowski [25] shows yeast, or *S. cerevisiae,* is capable of producing phenolic compounds; as reported by Rodriguez *et al*. [26], yeast produces p-Coumaric acid (one of the phenolic compounds) up to 1.71 mg CAE/g wt. Jan *et al*. and Vlassa *et al.* [15,27] reported that fermentation with *S. cerevisiae* can increase the TPC in plants. Due to that, *S. cerevisiae* is capable of producing esterase enzymes during the fermentation process [28], as reported by previous studies demonstrating that the TPC can be increased by yeast fermentation due to the activity of esterase enzymes with β-glucosidase in glycosides that can hydrolyze phenolic compounds, which can free the phenolic that is bound to the glucose. The present study mostly refers to previous studies related to the increase by Zheng and Shetty [29].

3.2. TFC

TFC with quercetin as a standard of flavonoid compounds was analyzed using a colorimetric test. The results of the TFC test in this study are shown in Table 2. TFC obtained from wet samples ranged from 3.58 to 14.93 mg QE/g; furthermore, in dry samples, TFC ranged from 4.69 to 31.47 mg QE/g. The difference in TFC values in the two samples was significant; however, the combined extraction method showed that the TFC values in the wet and dry samples were not significantly different. The fermentation method shows the highest TFC ($P < 0.05$), with a TFC value of 14.93 mg QE/g in the wet sample and 31.47 mg QE/g in the dry sample. *S. cerevisiae* can produce metabolites such as quercetin with a productivity of 0.02038 mg QE/g weight [30]. The increase due to fermentation also occurs in the content of flavonoids; the leaves of *O. stamineus* have a phenolic content of 13.93 mg QE/g which increased to 31.47 mg QE/g after being fermented by *S. cerevisiae*. This confirms a previous study demonstrating that fermentation with *S. cerevisiae* can increase total flavonoids compared to the maceration method (as a control) [16]. The increase in total flavonoids in fermentation is caused by the yeast's release of enzymes that break down glucoside bonds, thereby increasing the recovery of flavonoids [31].

Table 2: Result of total flavonoid content (as mg quercetin equivalents (QE)/g) in various extraction methods and samples of *Orthosiphon stamineus.*

Method	Total flavonoid (mg QE/g)	
	Wet sample	Dry sample
Fermentation method	14.93±4.38^{aB}	31.47±0.61^{aA}
Maceration method	5.25±0.05^{bB}	13.93±0.65^{bA}
Boiling method	3.58±0.28^{bB}	10.67±1.18^{cA}
Fermentation + Boiling method	4.24±0.07^{bA}	4.69±0.27^{eA}
Fermentation + Maceration method	5.63±1.07^{bA}	7.48±0.92^{dA}

Each value is presented as the mean of three replicates ± standard deviation; a-e means within a column with different letters are significantly different (Scott-Knott test, $P < 0.05$); A and B respectfully means within a row with different letters are significantly different (Scott-Knott test, $P < 0.05$), whereas a and A value is preferred

The increase in total flavonoids in fermentation may be caused by the release of enzymes by yeast that can break down glucoside bonds, increasing the recovery of flavonoids [30]. The boiled method had the lowest TFC value in the wet sample, with 3.58 mg QE/g TFC, while in the dry sample, the lowest TFC value was shown in the fermentation + Boil combination method, which was 4.69 mg QE/g. Flavonoid compounds are known as compounds that play an important role as antioxidants in several medicinal plants [32]. This study's results indicate that fermentation extraction can increase the number of polyphenols—both phenolic and flavonoid compounds.

3.3. Antioxidant Activity

Antioxidant activity in this study was analyzed using the DPPH and FRAP methods. The antioxidant activity with the DPPH method can be seen in Table 3. The free radical DPPH, widely used to evaluate the ability of compounds to operate as free-radical scavengers and hydrogen suppliers, is a rapid, simple, and inexpensive method for testing antioxidant capabilities. The DPPH test relies on eliminating DPPH, a stabilized free radical. The free radical DPPH test with wet samples showed the highest value in the fermentation extraction method, 9.37 µmol TE/g. These results were significantly different from other free radical DPPH tests; the lowest DPPH activity was described in the boiled extraction method with a value of 0.10 µmol TE/g. The free radical DPPH test with dry samples showed significantly varied results; the highest value obtained in the fermentation method was 7.13 µmol TE/g, while the lowest was in the fermentation + boiling combination method at 0.13 µmol TE/g. The determination of antioxidant activity by fermentation in *O. stamineus* is still difficult. However, the results of this study confirm the previous research by Ejuama *et al*. [16], which stated that there was an increase in DPPH radical scavenging activity through the fermentation method.

The antioxidant activity measured by the FRAP method (Table 4) was higher than that of the DPPH method; it shows that the antioxidant in *O. stamineus* has a reducing role rather than binding or scavenging free radicals. The principle of the DPPH method is to bind unstable free radicals with an electron donor [33], while the FRAP method involves reducing Fe3+ compounds to Fe^{2+} for increased stability [34]. Boiling the wet sample showed the highest reducing activity of 89.11 µmol TE/g, which was not significantly different from the boiling method with a value of 87.11 µmol TE/g. In comparison, the lowest reducing activity of the wet sample was observed in the combined fermentation and boiling method at 2.09 µmol TE/g. The dried samples showed significantly higher antioxidant activity with significant variation, with the maceration method showing the highest reducing activity at 255.56 µmol TE/g

Table 3: DPPH antioxidant scavenging activity (as µmol Trolox/g) in various extraction methods and samples of *Orthosiphon stamineus.*

Method	Antioxidant activity with DPPH (µmol TE/g)	
	Wet sample	Dry sample
Fermentation method	9.37±0.42^{aA}	7.13±0.41^{aB}
Maceration method	0.23±0.05^{bB}	1.91±0.09^{cA}
Boiling method	0.10±0.04^{bB}	3.55±0.13^{bA}
Fermentation + Boiling method	0.25±0.02^{bA}	0.13±0.07^{dB}
Fermentation + Maceration method	0.36±0.01^{bA}	0.38±0.02^{dA}

Each value is presented as the mean of three replicates ± standard deviation; a-d means within a column with different letters are significantly different (Scott-Knott test, $P < 0.05$); A and B respectfully means within a row with different letters are significantly different (Scott-Knott test, $P < 0.05$), whereas a and A value is preferred

Table 4: FRAP antioxidant reducing activity (as µmol Trolox/g) in various extraction methods and samples of *Orthosiphon stamineus.*

Method	Antioxidant activity with FRAP (µmol TE/g)	
	Wet sample	Dry sample
Fermentation method	87.11±1.39^{aB}	212.49±3.57^{bA}
Maceration method	47.39±4.80^{bB}	255.56±23.7^{aA}
Boiling method	89.11±0.13^{aB}	198.00±6.35^{bA}
Fermentation + Boiling method	2.09±0.34^{dB}	75.44±8.85^{cA}
Fermentation + Maceration method	24.94±3.17^{cB}	47.93±3.11^{cA}

Each value is presented as the mean of three replicates ± standard deviation; a-d means within a column with different letters are significantly different (Scott-Knott test, $P < 0.05$); A and B respectfully means within a row with different letters are significantly different (Scott-Knott test, $P < 0.05$), whereas a and A value is preferred

and the combined fermentation and maceration method showing the lowest reducing activity at 47.93 µmol TE/g.

3.4. Correlation between Antioxidant Activity and Total Phenolics and Flavonoids

Pearson linear coefficient correlation was used to determine whether phenolic or flavonoid compounds contributed to the antioxidant activity of *O. stamineus*. The Pearson linear coefficient correlation analysis results can be seen in Figure 1. The antioxidant activity of DPPH has a significant positive correlation with total flavonoids ($r = 0.76$) and total phenolics ($r = 0.58$). A positive correlation between DPPH activity and total phenolics and flavonoids was also stated in a previous study [35,36]. Pearson linear coefficient correlation analysis on the antioxidant activity of FRAP also showed a significant positive correlation to total flavonoids ($r = 0.67$) and total phenolics ($r = 0.94$). These results follow a previous study conducted [37] showing that FRAP has a positive correlation with total phenolics and flavonoids.

The difference between the correlation results is due to other polyphenolic compounds besides phenolics and flavonoids, which can affect antioxidant activity [38]. According to Abdelwahab *et al.* [39], the *O. stamineus* plant has active compounds such as polyphenols, terpenoids, phenolic acids, and sterols. And on the other side, it may be that this study used the leaves as a sample to extract the bioactive compounds. Furthermore, most of the polyphenol components are found in the leaves. Moreover, apart from phenolics and flavonoids that act as antioxidants, polyphenol components are anthocyanins, lignans, and stilbenes [40].

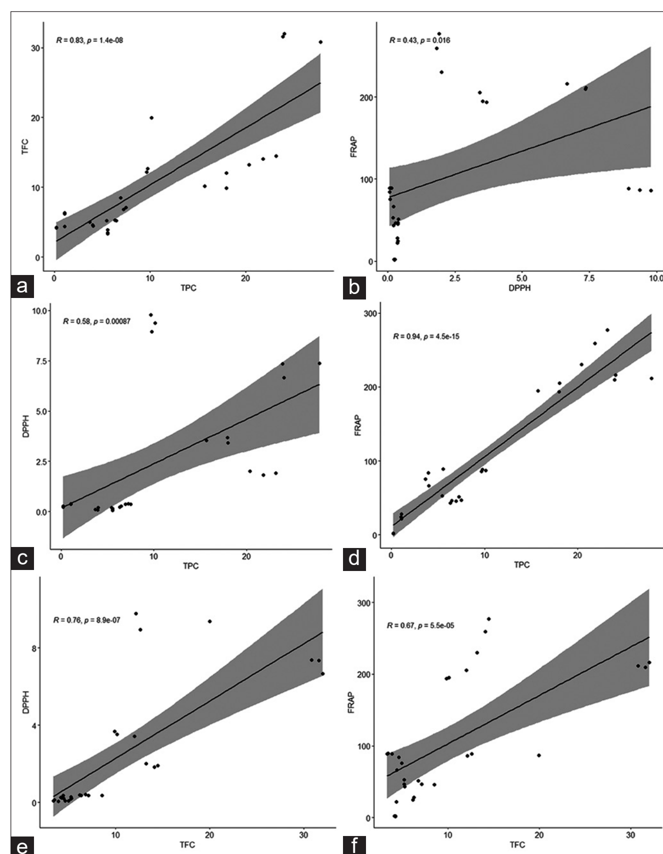

Figure 1: Simple linear correlation of total phenolic content (TPC) with total flavonoid content (TFC) (a), DPPH with FRAP (b), TPC with DPPH (c) and FRAP (d), TFC with DPPH (e) and FRAP (f). R = value Pearson's correlation coefficients, ρ = significance.

4. CONCLUSION

In conclusion, the results of the present study demonstrated that fermentation extraction by *S. cerevisiae* plays some important roles in increasing antioxidant polyphenol content in *O. stamineus* leaves. Fermentation with *S. cerevisiae* on *O. stamineus* can increase phenolics and flavonoids, thereby increasing antioxidant activity. This study shows that dry leaf extract of *O. stamineus* leaves has been known as a good source of antioxidants, and the extraction method with fermentation is a better method to increase the antioxidant activity of *O. stamineus*.

5. ACKNOWLEDGMENTS

The authors would like to thank the technical and administrative staff at the Tropical Biopharmaca Research Center and Biochemistry Department Laboratories at IPB University during the research implementation.

6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as

per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. FUNDING

The research was funded by the Ministry of Research, Technology, and Higher Education, Indonesia (Project code: 18809/IT3.D10/45 PT.01.03/P/B/2023).

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

11. PUBLISHER'S NOTE

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How to cite this article:
Chandra MA, Syamsu K, Ambarsari L, Fatimah N, Nurcholis W. Increasing polyphenol antioxidant in *Orthosiphon stamineus* Benth leaves with fermentation extraction by *Saccharomyces cerevisiae* ATCC-9763. J App Biol Biotech. 2024;12(1):111-116. DOI: 10.7324/JABB.2024.149026