Enhancing antioxidant activity of olive pomace with reinforcing its phenolic compounds by fermentation

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Background and objective
Valorization of olive pomace (OP) by solid-state fermentation of the generally regarded as safe yeast, *Kluyveromyces marxianus* was reported to enhance its antioxidant activity. However, a detailed identification of bioactive compounds present in unfermented OP and fermented OP was not clarified. The aim of the present study was to identify the major classes of bioactive compounds, and alteration in the phenolic profile after fermentation to explain the enhanced therapeutic activity of OP after fermentation.
Materials and methods
Methanolic extracts of unfermented OP and fermented OP were subjected to qualitative preliminary phytochemical analysis. Moreover, both extracts were subjected to high-performance liquid chromatography analysis using different phenolic compounds' standards. Also, total antioxidant capacity of both extracts was evaluated.
Results
Simple phenols, tannin, phlobatannins, flavonoids, steroids, terpenoids, cardiac glycosides, reducing sugars, alkaloids, carbohydrates, sterols, and triterpenes are the major phytochemical classes present in both extracts. While both extracts lack saponin, anthraquinones, free amino acids, free cholesterol, and polyuronides. High-performance liquid chromatography analysis confirmed that fermentation of OP by *K. marxianus* led to a sharp increase in rutin, vanillin, and cinnamic acid contents by 69.22, 39.35, and 31.40%, respectively. This was accompanied with 22.78, 7.07, and 5.81% increase in quercetin, catechin, and syringic acid contents, respectively. While gallic, caffeic, and coumaric acid contents were decreased after fermentation by 59.24, 55.25, and 53.96%, respectively. Methanolic extracts of unfermented OP and fermented OP showed a maximum total antioxidant capacity of 144.81±1.47 and 187.57±4.00 mg_{VCE}/l at a concentration of 10 and 6 mg/ml, respectively.
Conclusion
Solid-state fermentation of OP with *K. marxianus* strongly affected its total antioxidant capacity by increasing its content of several bioactive compounds.

Keywords:
Kluyveromyces marxianus, olive pomace, phenolic compounds, phytochemical screening, solid-state fermentation, total antioxidant capacity

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Introduction

Olive pomace (OP) is the solid waste retained after olive oil extraction. It represents a serious environmental problem to Mediterranean countries as it is produced in large quantities over a short period during its harvesting season. Despite its hazardous effects to environment, OP is a rich source of bioactive compounds that can serve as valuable therapeutic raw materials. These OP-derived bioactive compounds find their applications in different industrial sectors such as in food, pharmaceutical and cosmetics industries [1]. To achieve sustainable olive oil industry, cheap, innovative eco-friendly techniques for recovery of phenolic compounds from OP are highly encouraged such as microwave and supercritical fluid-assisted extraction. However, enzyme-assisted extraction of phenolic compounds from OP was reported to give higher yields [2].

Solid-state fermentation (SSF) is defined as the microbial growth on solid support in the absence or near absence of free water. SSF is a green technology that gains great attention in the field of extraction of bioactive compounds from agroindustrial wastes.

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Unlike traditional extraction techniques, SSF does not only extract bioactive compounds, but also valorize the solid residue used for fermentation due to the interplay of the enzymes secreted by the microorganism in the fermented medium leading to increased concentration of bioactive compounds in the pomace used as solid support [3].

Valorization of OP by SSF of the generally regarded as safe (GRAS) yeast, *Kluyveromyces marxianus* NRRL Y-8281 was first reported by Mahmoud *et al.* [4]. It was proved to enhance the antioxidant and anticancer activities of OP against different cancer cell lines and to enhance its chemical composition [5]. However, identification of major classes of bioactive compounds present in both methanolic extracts of unfermented olive pomace (UFOPME) and fermented olive pomace (FOPME) in addition to alteration in the phenolic profile after *K. marxianus* fermentation is strongly needed to explain enhanced antioxidant activity of OP acquired after fermentation.

Materials and methods

Materials

Olive pomace waste

OP (picual variety) was provided during its harvesting season by a local olive-pressing factory that apply a three-phase decanter system for olive oil extraction, located in Al-Arish, North Sinai, Sinai Peninsula, Egypt. It was stored at −20°C till used.

Microorganism

The GRAS yeast, *K. marxianus* NRRL Y-8281 used in this study was obtained from Agricultural Research Service, Peoria, Illinois, USA.

Methods

Culture maintenance and inoculum preparation

The yeast strain was grown in test tubes containing a stock slant medium having a composition of 10.0 g/l glucose, 5.0 g/l peptone, 3.0 g/l yeast extract, 3.0 g/l malt extract, and 20.0 g/l agar [6]. After autoclaving at 121°C for 20 min and cooling, slants were inoculated with the organism and incubated at 30°C for 48 h and then stored as a stock culture at 4°C. For inoculum preparation, the surface of the agar stock culture was scraped with a platinum needle and resuspended in sterile 50 ml of inoculum medium (composed of the same contents of the stock slant medium except the agar) in 250 ml Erlenmeyer flasks, and then incubated at 30°C on a controlled incubator shaker (New Brunswick Scientific, Enfield, Connecticut, USA) at 150 rpm for 24 h.

Solid-state fermentation

For SSF, suspension aliquots of 1 ml (containing about 10^8 cells/ml) were inoculated in 250 ml Erlenmeyer flasks containing 5 g of sterilized OP (sterilized at 121°C for 20 min at 15 psi). Incubation was done in a static incubator for 48 h at 45°C [4].

Preparation of phenolic-rich extracts

Sample preparation

Both unfermented olive pomace (UFOP) and fermented olive pomace (FOP) were dried in oven at 35°C. The dried samples were then ground using an electrical blender to get powder. The resulted powder was sieved through a sieve and then stored at −20°C till use [4].

Polyphenol extraction

Phenolic-rich extracts of both UFOP and FOP were prepared by methanol (1 : 10 w/v) using a shaking water bath (100 rpm) at 50°C for 2 h. Extracts were filtered through Whatman No. 1 filter paper; then concentrated under vacuum at 30°C till complete dryness. Concentrated extracts were stored at −20°C till use [4].

Extracts reconstitution

Both extracts were reconstituted to obtain different extract concentrations (2, 4, 6, 8, and 10 mg/ml) using methanol.

Identification of the bioactive constituents of extracts

Preliminary phytochemical analysis

UFOPME and FOPME were subjected to qualitative phytochemical screening for the identification of different classes of bioactive compounds (namely, simple phenols, tannins, phlobatannins, saponin, steroids, terpenoids, cardiac glycosides, reducing sugars, anthraquinones, alkaloids, amino acids, cholesterol, carbohydrates, polyuronides, flavonoids, sterols, and triterpenes) present in the extracts using the standard methods described by Harborne [7] and Evans and Trease [8].

High-performance liquid chromatography analysis

High-performance liquid chromatography (HPLC) analysis was carried out using an Agilent 1260 series. The separation was carried out using C18 column (4.6 mm×250 mm i.d., 5 μm). Ten microliters of each sample and standard was injected and eluted at room temperature (25°C). The mobile phase consisted of 2% acetic acid (A) and acetonitrile (B) at a flow rate of 0.8 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (90% A), 0–15 min (45% A), 15–17 min (20% A),

17–18 min (90% A), and 18–20 min (90% A). The multiwavelength detector was checked at 280 nm. Standard solutions of catechin (200 μg/ml), syringic acid (40 μg/ml) and cinnamic acid (20 μg/ml), gallic acid (60 μg/ml), caffeic acid (60 μg/ml), rutin (200 μg/ml), coumaric acid (40 μg/ml), vanillin (40 μg/ml), and quercetin (160 μg/ml) were analyzed to compare results. Ten microliters was used as an injection volume for all samples and standard solutions. The column temperature was maintained at 25°C.

Assessment of total antioxidant capacity of extracts

To a volume of 0.1 ml of samples, 1 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was added. The tube is capped and incubated in a boiling water bath at 95°C for 90 min. After cooling the sample to room temperature, the absorbance was measured at 695 nm against blank. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent was used for the sample. Results were expressed as vitamin C (mg_{VCE}/L) equivalents [9].

Statistical analysis

Data were reported as mean±SD for three different batches and were analyzed statistically using independent samples *t* test to detect statistically significant differences between phenolic compounds' concentrations of UFOPME and FOPME. Differences were considered significant at significance level *P* value less than 0.05, while two-way analysis of variance/Bonferroni-adjusted pairwise comparisons were used to detect statistically significant differences between antioxidant activity of UFOPME and FOPME at different extract concentrations. Differences were considered significant at significance level *P* value less than 0.05 and 0.025, respectively. Statistical analysis was carried out using the SPSS 16.0 Program (IBM Inc., Chicago, Illinois, USA).

Results and discussion

Identification of the bioactive constituents of extracts

Preliminary phytochemical analysis

Both UFOPME and FOPME were subjected to qualitative preliminary phytochemical analysis and the results are shown in Table 1. Results showed that simple phenols, tannin, phlobatannins, flavonoids, steroids, terpenoids, cardiac glycosides, reducing sugars, alkaloids, carbohydrates, sterols, and triterpenes are the major phytochemical classes present in both extracts. Obied *et al*. [10] reported the presence of simple phenols, flavonoids, carbohydrates, sterols, and triterpenes with absence of alkaloids and cardiac glycosides from OP extracts. Also, reducing sugars have been detected and quantified in OP [11].

Table 1 Qualitative preliminary phytochemical analysis of unfermented olive pomace methanolic extract and fermented olive pomace methanolic extract

Phytochemical class	UFOPME	FOPME
Simple phenols	+	+
Tannin	+	+
Phlobatannins	+	+
Flavonoids	+	+
Carbohydrates	+	+
Steroids	+	+
Sterols and triterpenes	+	+
Terpenoids	+	+
Alkaloids	+	+
Cardiac glycosides	+	+
Reducing sugars	+	+
Anthraquinones	–	–
Saponins	–	–
Amino acids	–	–
Cholesterol	–	–
Polyuronides	–	–

FOPME, fermented olive pomace methanolic extract; UFOPME, unfermented olive pomace methanolic extract. (+) indicates the presence of phytochemical class, (–) indicates the absence of phytochemical class.

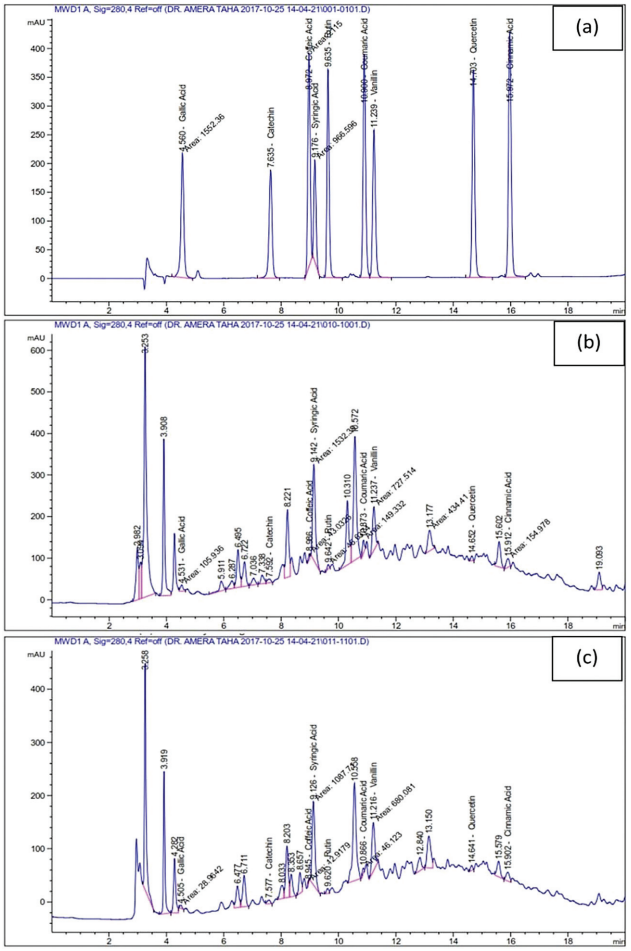
On the other hand, qualitative preliminary phytochemical screening results of both extracts showed absence of anthraquinones, saponins, amino acids, cholesterol, and polyuronides. This agrees to some extent with Obied *et al*. [10], who reported absence of anthraquinones and saponins from olive waste extracts with the presence of polyuronides.

The presence of different therapeutic phytochemical classes in both UFOPME and FOPME makes them potential antioxidant and therapeutic agents; however, a more detailed identification of individual therapeutic compounds are still required.

High-performance liquid chromatography analysis

For more detailed identification of individual compounds present in both UFOPME and FOPME, the HPLC technique was applied using different phenolic compound standards including gallic acid, caffeic acid, syringic acid, cinnamic acid, coumaric acid, rutin, vanillin, quercetin, and catechin (Fig. 1a). These compounds have been chosen due to their known antioxidant and anticancer efficiencies, which can give a glance about the previously reported antioxidant and anticancer activities of both OP extracts. All these compounds were detected in both

Figure 1



Typical HPLC chromatograms of phenolic compound standards (a), unfermented olive pomace methanolic extract (UFOPME) (b), and fermented olive pomace methanolic extract (FOPME) (c). HPLC, high-performance liquid chromatography.

extracts by different concentrations because of microbial conversion of OP. HPLC chromatograms of UFOPME and FOPME (Fig. 1b and c, respectively) show that fermentation of OP with *K. marxianus* led to a small increase in syringic acid (5.81%) and catechin (7.07%) contents. This was accompanied by a moderate increase in quercetin (22.78%), cinnamic acid (31.40%), and vanillin (39.35%) contents. Moreover, a large increase in rutin content (69.22%) was observed after fermentation. On the other hand, gallic acid, caffeic acid, and coumaric acid contents were sharply decreased after fermentation by 59.24, 55.25, and 53.96%, respectively (Table 2).

Exact concentration of phenolic compounds in both extracts is presented in Table 2. Differences between UFOPME and FOPME, for three different batches for each, were found to be statistically significant at ($P<0.05$, $n=3$) using independent samples t test for

Table 2 Concentration of different phenolic compounds in unfermented olive pomace methanolic extract and fermented olive pomace methanolic extract

	Concentration (μg/gds)	
Compound name	UFOPME	FOPME
Gallic acid	98.88±0.30 [a]	40.30±0.90 [b]
Catechin	270.68±0.12 [a]	289.80±8.75 [b]
Caffeic acid	29.48±0.05 [a]	13.19±0.10 [b]
Syringic acid	1531.45±9.54 [a]	1620.43±20.96 [b]
Rutin	112.49±4.95 [a]	190.36±18.35 [b]
Coumaric acid	59.35±0.98 [a]	27.3±0.030 [b]
Vanillin	395.04±8.32 [a]	550.48±11.89 [b]
Quercetin	95.68±3.60 [a]	117.48±10.65 [b]
Cinnamic acid	26.05±0.02 [a]	34.24±0.64 [b]

Data was represented as mean±SD of three different batches. FOPME, fermented olive pomace methanolic extract; UFOPME, unfermented olive pomace methanolic extract. Means bearing different letters are significantly different from each other ($P<0.05$) as indicated by independent samples t test.

gallic acid [58.58; 95% confidence interval (CI), 57.06–60.10, t (4)=106.738, $P<0.001$]; catechin [−19.12; 95% CI, −33.15 to −5.09, t (4)=−3.784, $P=0.019$] ; caffeic acid [16.29; 95% CI, 16.11–16.47, t (4)=254.397, $P<0.001$]; syringic acid [−88.98; 95% CI, −125.89 to −52.07, t (4)=−6.692, $P=0.003$]; rutin [−77.87; 95% CI, −108.34 to −47.40, t (4)=−7.096, $P=0.002$]; coumaric acid [32.05; 95% CI, 30.47–33.63, t (4)=56.32, $P<0.001$]; vanillin [−155.44; 95% CI, −178.70 to −132.18, t (4)=−18.55, $P<0.001$]; quercetin [−21.80; 95% CI, −39.82 to −3.78, t (4)=−3.36, $P=0.028$], and cinnamic acid [−8.19; 95% CI, −9.22 to −7.16, t (4)=−22.15, $P<0.001$].

These findings agree with Zhao *et al.* [12] who reported the presence of gallic acid (0.045 mg/g sample), caffeic acid (0.044 mg/g sample), vanillin (0.329 mg/g sample), *p*-coumaric acid (0.097 mg/g sample), rutin (1.360 mg/g sample), and cinnamic acid (0.019 mg/g sample) in 70% methanol extrac of defatted OP. In addition, Višnjevec *et al.* [13] reported the presence of cinnamic acid (340 mg/kg d.w.) as well as rutin (48 mg/kg d.w.), vanillin (23 mg/kg d.w.), and caffeic acid (26 mg/kg d.w.) in the methanolic extract of OP. Also, Morsi *et al.* [14] detected similar compounds in the methanolic extract of OP. Reported concentrations were 0.37 mg/g dried defatted pomace for syringic acid, 0.15 mg/g dried defatted pomace for gallic acid, 0.31 mg/g dried defatted pomace for catechin, 0.31 mg/g dried defatted pomace for caffeic acid, 0.55 mg/g dried defatted pomace for *p*-coumaric acid, 0.27 mg/g dried defatted pomace for rutin, and 0.34 mg/g dried defatted pomace for quercetin. Besides, rutin (19.83 mg/100 g d.w.) was detected in OP

methanolic extract in addition to gallic acid (7.65 mg/100 g d.w.), caffeic acid (32.38 mg/100 g d.w.), and *p*-coumaric acid (5.55 mg/100 g d.w.) [15]. Moreover, Malapert *et al.* [16] detected caffeic acid (68.00 mg hydroxytyrosol equivalent/L) and *p*-coumaric acid (17.70 mg hydroxytyrosol equivalent/L) in OP extract, whereas Alhamad *et al.* [17] detected caffeic acid (1732.30 mg/100 g d.w.), syringic acid (64.30 mg/100 g d.w.), catechin (261.40 mg/100 g d.w.), and gallic acid (359.20 mg/100 g d.w.). Rutin presence in OP aqueous extract was reported by Tapia-Quirós *et al.* [18].

Fluctuation of individual compounds concentration between authors is attributed to different factors including the olive cultivar, the method applied for phenolic-rich extract preparation, the solvent used for extraction and/or reconstitution, and HPLC assay conditions [19]. Alteration of phenolic compound concentration after fermentation of OP was reported before. Pasten *et al.* [15] reported a decrease of caffeic acid concentration by 42, 100, 100, and 30% after OP fermentation with the fungal strains *Beauveria bassiana*, *Rhizodiscina cf. lignyota*, *Fusarium flocciferum*, and *Aspergillus niger*, respectively. This was accompanied with a decrease of vanillin concentration by 88, 88, 78, and 62% by the four fungal strains in the same sequence. Moreover there was a decrease of catechin by 100% for all tested strains.

Also, Mandal *et al.* [20] reported alteration of OP phenolic profile after compositing, such that gallic acid was decreased from 359.20 to 316.20 mg/100 g d.w.; catechin was decreased from 261.40 to 232.40 mg/100 g d.w.; syringic acid was decreased from 64.30 to 55.10 mg/100 g d.w.; and caffeic acid was decreased from 1732.30 to 1642.30 mg/100 g d.w. by fermentation.

Assessment of total antioxidant capacity of extracts

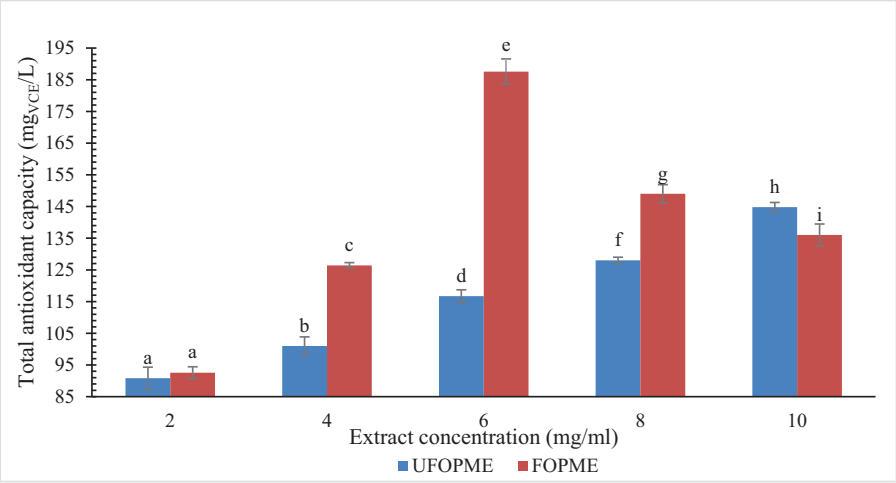
Total antioxidant capacity of UFOPME and FOPME was assessed to detect the effect of alteration of the phenolic profile on the extracts' antioxidant activity.

Total antioxidant capacity of UFOPME and FOPME at different concentrations is shown in Fig. 2. UFOPME showed a gradual, concentration-dependent increase in total antioxidant capacity reaching its maximum (144.81±1.47 mg_{VCE}/L) at a concentration of 10 mg/ml. On the other hand, FOPME showed a bell-shaped total antioxidant capacity as a response to increasing extract concentration with a peak of 187.57±4.00 mg_{VCE}/L at a concentration of 6 mg/ml.

A statistically significant interaction between fermentation and extract concentration on total antioxidant capacity was tested using the two-way analysis of variance test. There were no outliers; residuals were normally distributed ($P>0.05$) and there was homogeneity of variance ($P=0.535$). Three different batches for each extract were used ($n=3$).

Results showed that fermentation [$F(1, 20)=537.275$, $P<0.001$, partial $\eta^2=0.964$], extract concentration [$F(4, 20)=522.877$, $P<0.001$, partial $\eta^2=0.991$] as well as

Figure 2



Total antioxidant capacity of different concentrations of unfermented olive pomace methanolic extract (UFOPME) and fermented olive pomace methanolic extract (FOPME). Data was represented as mean±SD of three different batches. Means bearing different letters are significantly different from each other as indicated by two-way analysis of variance/Bonferroni-adjusted pairwise comparisons ($P<0.05$ and 0.025, respectively).

the interaction between the two independent factors [F (4, 20)=207.394, $P<0.001$, partial $\eta^2=0.976$] significantly affect the total antioxidant capacity. The mean difference of total antioxidant capacity between UFOPME and FOPME was found to be statistically significant at concentrations of 4 mg/ml [25.430; 95% CI, 20.991–29.869, F (1,20)=142.828, $P<0.001$, partial $\eta^2=0.877$]; 6 mg/ml [70.860; 95% CI, 66.421–75.299, F (1,20)=1108.977, $P<0.001$, partial $\eta^2=0.982$]; 8 mg/ml [21.000; 95% CI, 16.561–25.439, F (1,20)=97.400, $P<0.001$, partial $\eta^2=0.830$] and 10 mg/ml [8.763; 95% CI, 4.325–13.202, F (1,20)= 16.961, $P=0.001$, partial $\eta^2=0.459$]. While the mean difference between both extracts at a concentration of 2 mg/ml [1.760; 95% CI, -2.679 to 6.199, F (1,20)= 0.684, $P=0.418$, partial $\eta^2=0.033$] was not statistically significant. An analysis of simple main effects for extract concentration was performed.

There was a statistically significant difference in total antioxidant capacity for UFOPME at different concentrations [$F(4,20)=202.497$, $P<0.001$, partial $\eta^2=0.976$], as for FOPME [$F(4,20)=527.774$, $P<0.001$, partial $\eta^2=0.991$]. All pairwise comparisons were run for each simple main effect with reported 95% confidence intervals using statistical significance receiving a Bonferroni adjustment and being accepted at the P value less than 0.025 level. All concentrations were significantly different from each other for both extracts.

This assay is based on the ability of the sample to reduce Mo (VI) to Mo (V), so the activity of compounds is ruled by their reduction potential [21]. The half peak reduction potential (Ep/2) is a suitable parameter for representing the reducing activity of phenolics. The lower the reduction potential, the higher the antioxidant activity of compounds. Ep/2 values of quercetin, catechin, and rutin are 0.03, 0.16, and 0.18 mV, respectively, showing the highest activity to quercetin [22].

Also, results reported by Yakovleva *et al.* [23] showed that phenolic compounds can be ordered as quercetin<gallic acid<caffeic acid<*p*-coumaric acid according to their Ep/2 values, reflecting higher antioxidant activity in the reversed order. In the same context, according to Olszowy [24], the reduction potential can order flavonoids, phenolic acids, and catechin in the order of quercetin<caffeic acid<catechin<*p*-coumaric acid, indicating higher reducing power (antioxidant activity) to flavonoids.

Thus, the finding that FOPME has higher total antioxidant activity than UFOPME agrees with electrochemical behavior studies published before since the increased content of flavonoids and catechins reflected higher antioxidant activity.

Conclusion

SSF of OP using the GRAS yeast, *K. marxianus* is a promising technique for valorization of OP into value-added, phenolic-rich product with enhanced therapeutic activity.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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