



Improving the nutritive value of olive pomace by solid state fermentation of *Kluyveromyces marxianus* with simultaneous production of gallic acid

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Abstract

Purpose Olive oil industry is a growing industrial sector in Mediterranean countries. Unfortunately, about 30–35% of total olive weight is discarded as olive pomace (OP), a highly environmental-polluting solid waste. OP is mostly used for composting and production of animal feed; nevertheless, the high oil content, phenolic compounds and fiber contents in addition to its low protein content represent major obstacles for both applications. So, the present study was conducted to evaluate the role of solid state fermentation (SSF) in OP using *Kluyveromyces marxianus* NRRL Y-8281 yeast on the chemical composition and tannin content of OP in a trial for its bioconversion into a value-added animal feed.

Methods Chemical composition, nutritive and energetic values, tannin and gallic acid contents of unfermented and fermented olive pomace were investigated.

Results The fermentation altered the chemical composition of OP, so that crude fiber was decreased by 8.56%, while crude protein, fat and carbohydrate contents were increased by 2.74, 2.63 and 3.57%, respectively. Moreover, the effect of fermentation on cell wall constituents, gross energy, digestible energy, total digestible nutrients and digestible crude protein was evaluated. Furthermore, HPLC analysis revealed that *K. marxianus* mediated fermentation of OP resulted in a sharp decrease in tannin content by 96.75% with 2.8 times increase in gallic acid concentration.

Conclusion SSF of OP by *K. marxianus* does not only eliminate the environmental pollution resulting from its accumulation, but also presents a new eco-friendly valorization technique which leaves OP with an altered chemical composition allowing its use as animal feed or compost.

Keywords Olive pomace · Solid state fermentation · *Kluyveromyces marxianus* · Nutritive value · Gallic acid

Introduction

Olive oil industries play an important economic role in Mediterranean countries. Considering a three phase decanter system (the most widely employed method for virgin olive oil production), two major by-products are generated, a solid residue (30%) known as olive pomace (OP) (also known as orujo and olive cake (OC)) which consists of olive skin, pulp

and pit, in addition to significant residual oil content, and a liquid waste (50%) known as olive mill waste water (also known as alpechin) which consists of olive fruit tissue water, washing water and water added for centrifugation (Borja et al. 2006; Dermeche et al. 2013).

It is estimated that the world annual production of OP is 2,881,500 tonnes/year (Nunes et al. 2016). This represents a huge problem for olive oil producing countries, since this vast amount of waste cannot be stored in its original form for extended time due to its high water and significant oil contents that cause rapid deterioration and rancidity often after 4–5 days of its production, as a result, OP is spread on farm lands resulting in serious negative environmental impact regarding soil, underground water and air problems (Brlak et al. 2012).

Three phase OP is a heterogeneous matrix with a chemical composition that differs greatly among different

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producers (Christoforou and Fokaides 2016). OP is quite rich in water (30–50%) and contains a substantial amount of residual oil (2–4%) (Azbar et al. 2004). It is also characterized by high fiber (cellulose, hemicellulose, lignin and pectines) and low protein contents. In addition, it is rich in fat, mostly polyunsaturated fatty acids (Ramachandran et al. 2007; Molina-Alcaide and Yáñez-Ruiz 2008; Lafka et al. 2011). The ash content ranges between 1.7 and 4% and major elements present in OP are potassium followed by calcium, magnesium and sodium (Aliakbarian et al. 2011). Despite its variable composition, fibers followed by fat and protein remain the main constituents of OP (Christoforou and Fokaides 2016). OP is a ligno-cellulosic organic material which has low digestibility and energy content in addition to having high tannin content (Al-Masri and Guenther 1999).

Several practices (in particular, composting and animal feed production) have been introduced for disposal and utilization of OP aiming to reduce the environmental-polluting load with concomitant production of value-added bio-products (Cossu et al. 2013).

Low soil organic matter can lead to limited soil fertility and productivity followed by soil degradation. Thus, the periodic addition of organic fertilizers is strongly recommended to restore soil productivity (López-Piñero et al. 2011). Generally, natural fertilizers are more preferred than synthetic ones. Having significant contents of organic matter, nitrogen, potassium and phosphorus, OP is considered as a potent organic amendment that improves many soil properties, restores crops productivity and improves the soil nutrient level (Abu-Zreig and Al-Widyan 2002). Raw OP can be applied directly to soil as an organic amendment (Kavdir and Killi 2008). However, the residual oil contained in it results in increased soil hydrophobicity and decreased water retention and infiltration rate. Moreover, due to the high content of phenolic and phytotoxic compounds, utilization of raw OP as soil amendment for long time results in accumulation of toxicants in soil with detectable negative effects on plants in addition to land, ground water and surface water contamination (Lopes et al. 2016).

Livestock production in Mediterranean countries is constricted by the scarcity and fluctuating quantity and quality of the feed supply to animals. Agro-industrial residues can serve as alternative sources for livestock providing an economic and eco-friendly method for their disposal and recycling (Molina-Alcaide and Yáñez-Ruiz 2008). However, utilization of OP as animal feed additive is limited due to its low protein content, high phenolic compounds content and high fiber content which renders OP unpalatable and poorly digestible for ruminants. Moreover, the significant amount of energy contained in OP represented by the cellulose (14–26%) is locked in ligno-cellulosic complex and thus it is inaccessible to ruminal microbes for degradation.

Consequently, pre-treatment of OP before animal consumption is necessary to eliminate its toxicity and upgrade its nutritional value (Weinberg et al. 2008). Bioconversion of OP through solid state fermentation (SSF) may represent an excellent pre-treatment strategy (Neifar et al. 2013). SSF is defined as the microbial growth on solid support in the lack or near absence of free water. However, the support must possess enough moisture to sustain microbial growth and metabolism (Pandey 1991). Several researches dealing with SSF of OP using fungal strains have been conducted allowing its use as animal feed additive for both ruminants (cattle, sheep, goats and camels) and poultry (Neifar et al. 2013; Fadel and El-Ghonemy 2015).

The aim of the present work was to evaluate the role of SSF in OP using *Kluyveromyces marxianus* NRRL Y-8281 yeast on the chemical composition and tannin content of OP in a trial for its bioconversion into a value-added animal feed.

Materials and methods

Olive pomace waste

OP was provided during its harvesting season by a local olive-pressing factory (three phase decanter system), located in Al-Arish, North Sinai, Sinai Peninsula, Egypt. It was stored at 4 °C till used.

Chemicals

Gallic acid was obtained from Bio Basic Canada INC (Markham Ontario, Canada). Code No. GB0476. Tannic acid was obtained from Panreac AppliChem (Castellar del Valles, Barcelona, Spain). Code No. 141065. All other chemicals and reagents used in this study were of analytical grade and purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Microorganism

The generally regarded as safe (GRAS) yeast, *Kluyveromyces marxianus* NRRL Y-8281 used in this study was obtained from Agricultural Research Service, Peoria, Illinois, USA.

Adaptation and inoculum preparation of the microorganism

The yeast strain was streaked on YME medium with agar (Wickerham 1951) for 48 h at 30 °C. The stock culture was stored at 4 °C and sub-cultured every 4 weeks, then stored at 4 °C. A loop of the culture was inoculated in 50 ml of sterile

inoculum medium (composed of the stock medium without agar), then incubated on controlled incubator shaker (New Brunswick Scientific, USA) at 150 rpm for 24 h at 30 °C.

Solid state fermentation

For SSF, an aliquot of 1 ml of each inoculum containing about 10^8 cells/ml was 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 at static incubator for 48 h at 45 °C (Fathy et al. 2017).

Chemical analysis of unfermented (UFOP) and fermented (FOP) olive pomace

Chemical analysis of unfermented and fermented OP was done as described by AOAC (1997) method.

The moisture, ash, crude protein (CP) and crude fat contents of UFOP and FOP were determined in accordance with the AOAC (1997). Crude protein determination involved the use of routine Kjeldhal nitrogen assay ($N \times 6.25$). Total carbohydrate content was determined using the phenol–sulfuric acid method of Dubois et al. (1956). Cell wall constituents including neutral detergent fiber (NDF), acid detergent fiber and acid detergent lignin were determined according to Goering and Van Soest (1970) and Pv et al. (1991). Hemicellulose was calculated as the difference between NDF and ADF, whereas cellulose was the difference between ADF and ADL. Gross energy (kcal/kg DM) was calculated according to Blaxter (1968) where, each g crude protein = 5.65 kcal, g fat = 9.40 kcal and g (crude fiber and carbohydrate) = 4.15 kcal. Digestible energy (kcal/kg DM) was calculated according to NRC (1977) where, digestible energy (DE) = gross energy \times 0.76. Total digestible nutrients (%) was calculated according to NRC (1977) where, total digestible nutrients % = digestible energy/44.3. Digestible crude protein (%) was calculated according to NRC (1977) where, digestible crude protein (%) = $0.85 X1 - 2.5$. Where $X1$ = crude protein% on DM basis.

High-performance liquid chromatography analysis

Preparation of unfermented and fermented culture filtrates

Culture filtrates were prepared by extracting the samples with eightfold (v/w) acetate buffer (0.02 M, pH 5.5) by shaking (200 rpm) at 30 °C for 60 min. The resultant slurry was centrifuged at $10,070 \times g$ for 15 min at 4 °C. Finally, the extracts were collected and considered as a source of tannic and gallic acids.

Analysis of unfermented and fermented culture filtrates

Tannic and gallic acids were estimated in both UFOP and FOP culture filtrates using HPLC technique. Aliquots were withdrawn and filtered through a 0.2 μ m membrane then subjected to HPLC analysis.

HPLC system specifications

All samples were analyzed by HPLC system Agilent 1100 series (Waldborn, Germany) equipped with variable wave length detector (G1314A), G1311A quaternary pump, G1322A degasser, G1329A thermostated autosamples and Zorbax 300SB C18 column (Agilent Technology, USA). Ten microliters of each sample and standard were injected and eluted at room temperature with water: methanol (80:20) as a mobile phase at a flow rate of 1 ml/min. Detection was carried out at 272 nm. Standard solutions (3 mg/ml) and (2 mg/ml) for tannic and gallic acid, respectively, were analyzed to compare results.

Data statistical analysis

Data were expressed as mean \pm standard error and analyzed statistically using independent sample *t* test for comparison between the fermented and unfermented olive pomace groups. Differences were considered significant at $P < 0.05$.

Results and discussion

The chemical composition of UFOP and FOP was estimated and presented in Table 1. The moisture content of UFOP was found to be 35% which was approximately similar to the ratio of 30% reported by Uribe et al. (2013). Also,

Table 1 Chemical analysis of unfermented (UFOP) and fermented (FOP) olive pomace

| Item | Pomace | |
|---|-------------------------------|-------------------------------|
| | UFOP | FOP |
| Moisture content (%) | 35.00 \pm 0.12 ^b | 35.84 \pm 0.05 ^a |
| Chemical analysis (%) on dry matter basis | | |
| Organic matter | 93.52 \pm 0.12 | 93.90 \pm 0.19 |
| Crude protein | 10.05 \pm 0.02 ^b | 12.79 \pm 0.11 ^a |
| Crude fiber | 42.60 \pm 0.11 ^a | 34.04 \pm 0.06 ^b |
| Fat content | 13.62 \pm 0.05 ^b | 16.25 \pm 0.03 ^a |
| Carbohydrate content | 27.25 \pm 0.06 ^b | 30.82 \pm 0.01 ^a |
| Ash content | 6.48 \pm 0.05 ^a | 6.10 \pm 0.06 ^b |

Values are given as mean \pm standard error of three batches

Mean bearing different superscripts in the same row are significantly different ($P < 0.05$)



the crude protein was found to be 10.05% on dry matter basis which is close to the ratio 9.5% reported by Fadel and El-Ghonemy (2015). Uribe et al. (2014a) stated crude protein ratio of 7.64%, while Neifar et al. (2013) reported lower protein content (6.5%). In addition, the crude fiber was found to be 42.6% on dry matter basis. This result is in accordance with that conducted by Uribe et al. (2013). Crude fiber ratios of 40, 40.72 and 33% were reported by Ramachandran et al. (2007), Brlek et al. (2012) and Fadel and El-Ghonemy (2015), respectively. Besides, the crude fat was found to be 13.62% on dry matter basis which was higher than that reported by Uribe et al. (2013), (2014a) and Fadel and El-Ghonemy (2015) stating crude fat ratio of 8.19, 10.31 and 5.7%, respectively. But, this result was lower than that reported by Vera et al. (2009) who recorded a ratio of 16.5%. Moreover, the carbohydrate content of UFOP was found to be 27.25% on dry matter basis which was lower than the carbohydrate content (77.99%) reported by Uribe et al. (2014b). Finally, the ash content was found to be 6.48% on dry matter basis. This result is in accordance with that reported by Uribe et al. (2014a), (2014b) and (2015), and nearly in the same range of Neifar et al. (2013) and Fadel and El-Ghonemy (2015) who reported 7% ash content, while Uribe et al. (2013) reported 5.47% ash content. Cell wall constituents (NDF, ADF, ADL, hemicellulose and cellulose) values were found to be 56.91, 48.28, 8.74, 8.63 and 39.54%, respectively (Table 2). Neifar et al. (2013) reported ratios of 59, 45, 31, 14.1 and 14.3% for NDF, ADF, ADL, hemicellulose and cellulose, respectively. And, ratios of 62 and 48% were estimated for NDF and ADF, respectively, by Fadel and El-Ghonemy (2015).

The variation in the chemical composition of OP reported by different authors can be attributed to the difference in olive variety, cultivation conditions (cultivation soil, geography of cultivation soil and use of pesticides and fertilizers), climatic conditions, harvesting conditions (the degree of ripening, harvesting time and system) and the applied oil extraction process (Doymaz et al. 2004; Brlek et al. 2012;

Uribe et al. 2013, 2014a, 2014b, 2015; Christoforou and Fokaides 2016).

Bioconversion of OP into a better-quality feed using SSF could be an alternative to chemical and physical treatment for enhancing its quality for microbial fermentation in rumen. SSF of OP using *K. marxianus* resulted in an increase in crude protein by 2.74%, changing it from 10.05% for UFOP to 12.79% for FOP. This can be attributed either to the increased biomass or the secretion of enzymes into the fermented medium (Fadel and El-Ghonemy 2015). The increased protein content suggests that the treated substrate is a good protein source for livestock (Neifar et al. 2013; Fadel and El-Ghonemy 2015). Also, a decrease of ash and crude fiber content by 0.38 and 8.56%, respectively, was observed after fermentation. The reduction in the crude fiber content indicates an increased digestibility of the resultant substrate (Neifar et al. 2013). In addition, the biodegradation of OP using *K. marxianus* resulted in an increase in carbohydrate content by 3.57% comparing to UFOP. The increased carbohydrate content can partially be attributed to the degradation of polysaccharide compounds which was associated with an increase in reducing sugars (Neifar et al. 2013). Increased carbohydrate content is an additional advantage besides the reduction of fiber content in the improvement of biomass quality through biological treatment as reported by Jahromi et al. (2011). Moreover, the crude fat was increased by 2.63% over the UFOP which is similar to the finding of Fadel and El-Ghonemy (2015) when growing *Aspergillus oryzae* FK-923 on OP.

Results in Table 2 showed that FOP expressed less cell wall constituents than UFOP as exhibited by the decrease of NDF, ADF, ADL and cellulose by 9.87, 16.16, 16.59 and 16.06%, respectively. Fadel and El-Ghonemy (2015) reported a reduction in NDF and ADF by 28.06 and 32.08%, respectively. While, Neifar et al. (2013) reported a reduction in NDF, ADF and ADL by 23, 14 and 11%, respectively,

Table 2 Cell wall constituents of unfermented (UFOP) and fermented (FOP) olive pomace

| Constituent (%) | Pomace | |
|-------------------------------|---------------------------|---------------------------|
| | UFOP | FOP |
| Neutral detergent fiber (NDF) | 56.91 ± 0.01 ^a | 51.29 ± 0.63 ^b |
| Acid detergent fiber (ADF) | 48.28 ± 0.13 ^a | 40.48 ± 0.05 ^b |
| Acid detergent lignin (ADL) | 8.74 ± 0.02 ^a | 7.29 ± 0.11 ^b |
| Hemicellulose | 8.63 ± 0.08 ^b | 10.81 ± 0.07 ^a |
| Cellulose | 39.54 ± 0.03 ^a | 33.19 ± 0.06 ^b |

Values are given as mean ± standard error of three batches

Means bearing different superscripts in the same raw are significantly different ($P < 0.05$)

Table 3 Energetic and nutritive values of unfermented (UFOP) and fermented (FOP) olive pomace

| Item | Pomace | |
|--------------------------------------|---------------------------|---------------------------|
| | UFOP | FOP |
| Energetic values | | |
| Gross energy (kcal/kg DM) | 4747 ± 3.28 ^b | 4942 ± 7.08 ^a |
| Digestible energy (kcal/kg DM) | 3608 ± 2.49 ^b | 3756 ± 5.38 ^a |
| Nutritive values | | |
| Total digestible nutrients (TDN) (%) | 81.44 ± 0.56 ^b | 84.78 ± 0.12 ^a |
| Digestible crude protein (DCP) (%) | 6.04 ± 0.01 ^b | 8.37 ± 0.10 ^a |

Values are given as mean ± standard error of three batches

Means bearing different superscripts in the same raw are significantly different ($P < 0.05$)

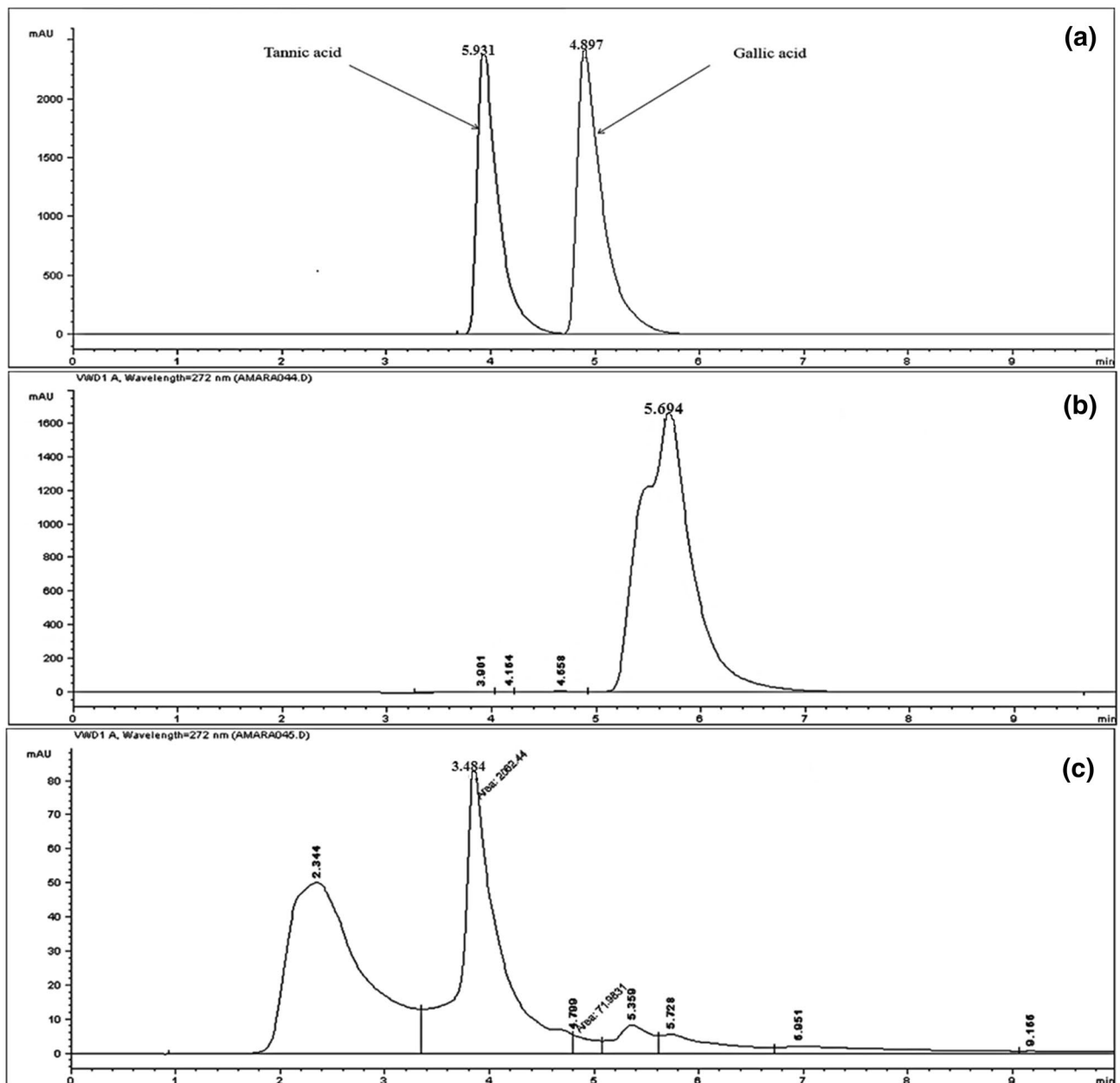


Fig. 1 Typical HPLC chromatograms of tannic acid and gallic acid standards (a), UFOP filtrate (b) and FOP filtrate (c)

when OP was utilized as a solid support for the SSF of *Fomes fomentarius*.

Table 3 shows the energetic and nutritive values of UFOP and FOP. The gross energy of UFOP was found to be 4747 (kcal/kg DM) which was higher than the value 4200 (kcal/kg DM) reported by Fadel and El-Ghonemy (2015). Results showed that fermentation of OP by *K. marxianus* increased the gross energy up to 4942 (kcal/kg DM). While, Fadel and El-Ghonemy (2015) conducted an increase up to 4320 (kcal/kg DM) after fermentation. In addition, results showed that fermentation of OP by *K. marxianus* increased the digestible

energy (from 3608 to 3756 (kcal/kg DM), total digestible nutrients (from 81.44 to 84.78%) and digestible crude protein (from 6.04 to 8.37%).

Tannins are the second most abundant group of phenols in nature and they are considered as a plant's secondary metabolites, as they do not have a direct role in plant metabolism. Instead, they have several important biological activities, such as defense against microbial attacks and protection of plant tissues from being attacked by insects and herbivores due to their unflavored taste (Scalbert 1991).



Tannins are uniquely characterized from other phenolic compounds by their ability to precipitate proteins from solutions to other macromolecules such as starch, cellulose, and minerals through formation of strong complexes with them (Lekha and Lonsane 1997; Aguilar et al. 2007). This property is the major source of tannin's undesirable effects in nutrition. Their ability to bind to macromolecules including proteins and minerals prevents their digestion and absorption and leads to inhibition of digestive enzymes (Rodríguez-Durán et al. 2011). Moreover, tannins are responsible for the astringent and bitter taste of fruits, juices and wines which reduces the food intake. In addition, the consumption of large amounts of tannin is associated with development of some forms of cancer (Jana et al. 2014). However, the consumption of low concentrations of tannin in feed is associated with an increase in nitrogen assimilation in ruminants showing higher growth rates and milk production (Belmares et al. 2004; Aguilar et al. 2007). When ingested, tannin can prevent diarrhea, inflammation, diuretics and cancer. In addition, they can be used for the treatment of poisoning caused by heavy metals due to their metal chelating capacity. Moreover, they are considered as potent antimicrobial agents (Jana et al. 2014).

For confirmation of OP tannin degradation by *K. marxianus* fermentation and release of gallic acid in the fermented medium, both UFOP and FOP filtrates were subjected to HPLC analysis. It is well-known that HPLC analysis is very efficient in demonstrating differences in chemical constituents of samples. HPLC profile of OP tannin bioconversion to gallic acid by *K. marxianus* fermentation is shown in Fig. 1a–c. Figure 1a represents the HPLC pattern of tannic acid and gallic acid standards. HPLC profile of UFOP and FOP is shown in (Fig. 1b, c). Using tannic acid and gallic acid as standards, HPLC data analysis showed revealed that tannic acid concentration in UFOP represented 1154.0 µg/gds, while in FOP, tannic acid represented 37.49 µg/gds. Also, gallic acid concentration in FOP was equal to 61.0 µg/gds comparing to 21.57 µg/gds in UFOP. From these results, it can be concluded that 96.75% of total tannins contained in UFOP were hydrolyzed and degraded producing gallic acid increasing its concentration by 2.8 times as a result of *K. marxianus* fermentation (Fig. 1a–c). The degradation of OP tannin during fermentation by *K. marxianus* can be attributed to the secretion of tannase enzyme produced when OP was used as a sole carbon source for solid state cultivation of *K. marxianus* (Fathy et al. 2017).

Conclusion

Regarding the reduction of tannin content and the increase of gallic acid concentration in addition to the positive effect of fermentation on the chemical composition of OP through increasing protein, carbohydrate and energy contents with

decreasing fiber content, SSF of OP by *K. marxianus* is considered as a suitable technique for bioconversion of OP into a value-added animal feed. However, further research work is necessary to optimize the improvement of the chemical composition and to verify the effectiveness of this degraded substrate on the performance of live animals.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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