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Original research

Investigating qualitative parameters and antioxidant activity of phenolic compounds extracted from canola meal using solid-state fermentation by *Aspergillus niger*

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ABSTRACT

Canola meal was solid-stately fermented (SSF) by *Aspergillus niger* CBS 120.49 to increase its total phenolic content (TPC) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. In order to obtain this purpose, 90 subfractions of canola meal were fermented at different moistures content (MC) of 40, 50, and 60% and different temperatures of 25, 30, and 35 ºC, followed by extraction within 5 days. Results showed that subfractions with 40% MC at of 30 ºC and 35 ºC with growth of 2982 and 2988 mg/100g had the most TPC in day 5. Moreover, SSF samples with 40% MC and at 35 ºC showed the highest (86.7%) DPPH radical scavenging activity. HPLC analysis showed that some free phenolic acids of SSF-treated samples increased approximately by 4-10 folds as compared to unfermented samples. A significant increase about 120-133 ppm in syringic acid content was found in fermented samples at MC of 40% and temperatures of 30-35 ºC compared to its trace amount in unfermented samples, which is probably due to bioconversion of sinapic acid. It turned out that, a large amount of insoluble phenolic compound of canola meal, treated by *Aspergillus niger* at 5 day SSF, would be released in the form of free acids significantly compared with unfermented sample.

Keywords: Bioconversion; Sinapic acid; Syringic acid; Releasing phenolic

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1. Introduction

Generally speaking, Antioxidants extend the shelf-life of food products, maintain their sensory and nutritional qualities and play a major role in preventing or delaying auto-oxidation (Shahidi et al., 2010; Dehghan-Manshadi et al., 2018). In sufficient amounts, phenolic compounds could be a prominent replacement for synthetic antioxidants. With a highly effective antioxidant activity, they could be consisting of vegetative products (Schmidt et al., 2005).

Canola (*Brassica napus*), is the most important subspecies of Brassicaceae, which is the most cultivated oil seed crop after soybean in the world (Chang et al., 2014). Canola/rapeseed also could rank as the first phenolic acid container among oil seeds, which phenolic acids are major group of phenolic compounds in it. (Amarowicz et al., 2001). The total content of phenolic acids in rapeseed and canola is from 623.5 to 1280.9 mg per 100 g of flour, respectively and from 1542 to 1837 mg per 100 g defatted meal, on a dry weight basis (Amarowicz et al., 2001). Defatted canola meal, consists of 639 to 1837 mg per 100 g of sinapic acid derivatives in different varies (where sinapic acid could account for more than 73 percentages of the free phenolic acids and 80–99 percentages of the total phenolic acids), depending on the oilseed plant variety and the oil processing method (Vuorela et al., 2004; Thiyam et al., 2006).

Sinapin accounts for a large proportion of the esterified phenolic acids as well as about 80% of the total phenolic acids of rapeseed (Kozlowska et al., 1990; Dehghan-Manshadi et al., 2018b). In previous studies which were done for evaluation of antioxidant capacity of alpha-tocopherol compared to some phenolic acids and BHA by DPPH method, the radical scavenging power was specified in this order: caffeic acid < ferulic acid < sinapic acid< alpha-tocopherol <BHA (Thiyam et al., 2013).

Thiyam et al. (2006) showed that sinapin is unlike sinapic acid, and sinapic acid could prevent the formation of hydroperoxides. Therefore, they affirmed that, extracts that were rich in sinapic acid

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had better prevention effects in oxidation of lipids, compared to sinapin extracts.

Phenolic acids exist in free and bound forms. The former are placed in the external part of the pericarp and could be extracted by organic solvents, whereas the latter are connected with various molecules mostly in cell wall structure in the form of ester, ether or acetal bonds (Jamal et al., 2011; Dehghan-Manshadi et al., 2020). Fermentation of plant-based foods is one of the common ways to enhance the antioxidant activity of complex compounds by enzymatic hydrolysis. Therefore, this method could be introduced as an efficient technique with extraction procedure. On the other hand, Vuorela et al. (2004) extracted antioxidants from canola with aqueous methanol, hot water as solvent, and also enzymatically with ferulic acid esterase and ferulic acid esterase which enzymatically extraction indicated the most sinapic acid content. Also, Ferulic acid esterase breaks the linkage in sinapin and results in free choline and sinapic acid.

In Addition to ferulic acid esterase, some other components like esterase, xylanase, cellulose or lipase cause the microorganisms fermentation to hydrolyze glucosides, and also, hydrolysis of plant cell wall matrix or starch cause bounded phenolics to change into free form (Vattem & Shetty, 2003). Therefore, these enzymes act as extractors in plant cell wall. However, using pure enzymatic systems is totally an expensive procedure.

Due to the reasons discussed above, the fermentation of food materials could be used to enhance the antioxidative activity of food products. In addition, sinapin as the main phenolic ester in canola, which constitutes about 80% of the total phenolics, is mainly associated with either hemicellulose or highly branched pectin and other complex polysaccharides. Thus, enzymes produced during fermentation of canola meal cause sinapic acids to be released and consequently cause a dramatic increase in free extracted phenolic acids.

Aspergillus, as some other fungi's like *Schizophyllum*, *Penicillium*, Trichoderma, and *Phanerochaete chrysosporium*, is known as a cellulase producing species (Sun & Cheng, 2002).

Recently, SSF has been taken into consideration for the extraction of antioxidants including phenolics from various plants (Dey et al., 2014). In this context, black *Aspergilla* such as *Aspergillus niger* is the most important microorganism used in industries because of several advantages like having good fermentation potency and high capacity in cell-wall break down by having the ability to produce various phenolic acid esterases (Jamal et al., 2011). Similar to important leasing enzymes like xylanase, arabinose and cellulose, *Aspergillus niger* especially CBS 120.49 strain has been considered in many studies as an important species that produces specific esterase to clave ferulic acid and sinapic acid esters (like sinapin) from plant polymers such as ferulic acid esterase that cause sinapic acid and choline ester in sinapin to be broken (DE Vries et al., 2002).

In one investigation, a mixture of enzymes of ferulic acid esterase, cellulase and xylanase was applied to degradation of oat hulls, which caused 86 % digestibility in cell wall and released phenolic compounds (McKinon et al., 2005). Some studies about fermentation of canola meal by *Aspergillus niger* were done, but they addressed the issue of change in physicochemical properties or upgrading nutritional value of final products (Shi et al., 2016; Shi et al., 2015). Furthermore, some researchers employed canola meal as a substrate to evaluate the potential of this media for lipase or protease production by different *Aspergillus* species (Freitas et al., 2013).

There are many researches about using *Aspergillus niger* for releasing and increasing phenols from bran, beet sugar pulp, etc. To the best of our knowledge, this is the first study investigating the application of *Aspergillus niger* for increasing the extraction efficiency of canola meal phenolic compounds, contrary to Hu et al. (2004), who used solid state fermentation (SSF) of canola meal in 25 days to decrease the phenolic content by *Pleurotus ostreatus*.

The objective of this work was to optimize different parameters of pretreatment of canola meal by SSF in order to increase the release of phenol content and antioxidant activity to find an alternative for the reuse of these residues.

2. Material and Methods

2.1. Materials

Canola seeds (*Brassica napus*) were cultivated in Faculty of Plant Breeding in College of Agriculture & Natural Resources, University of Tehran, Iran. Chemicals were purchased from Merck Chemicals (Germany): PDA (potato dextrose agar) culture, 2,20-diphenyl-1-picryl-hydrazyl (DPPH), Folin-Ciocalteau's, sodium carbonate and phenolic acid standards such as gallic. All other materials like ethanol as organic solvent were of analytical grade and purchased from local markets.

2.2. Canola meal preparation

Canola samples were pulverized with a coffee grinder (IKA-Werke, Germany) and sieved to reduce the particle size of 0.50-0.10 mm, and the powder was defatted with hexane using a Soxhlet apparatus before being sealed in plastic bags and stored at 4 ºC before analysis (Wanasundara et al. 1994).

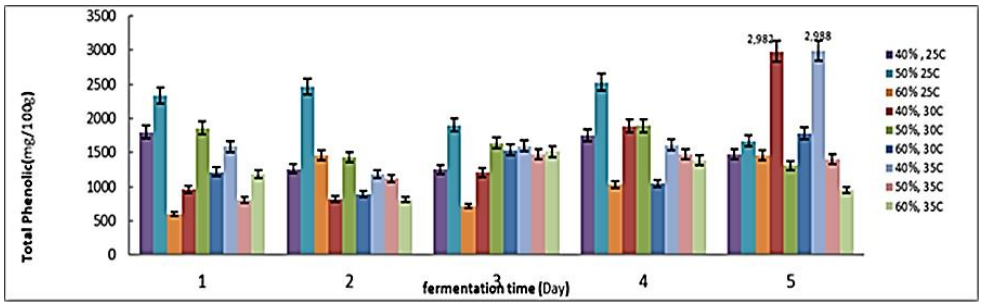

Fig. 1. Phenolic content during 5 days of fermentation under different temperatures and moistures content of media.

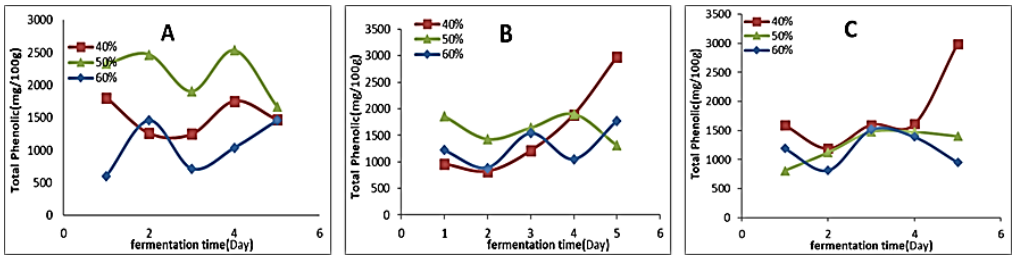

Fig. 2. Total phenol content extracted from fermented canola meals, A: 25 ºC, B: 30 ºC & 35 ºC, temperatures of incubation.

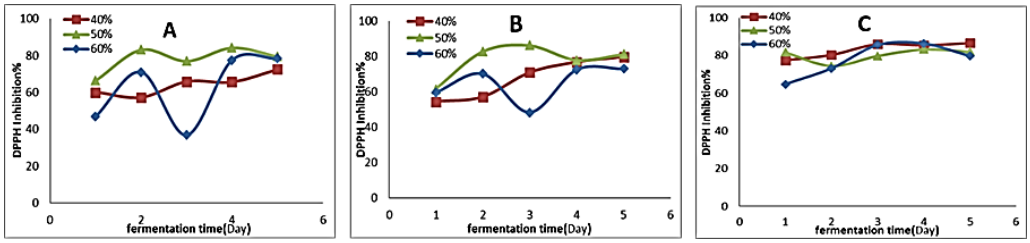

Fig. 3. DPPH Inhibition in extracts of fermented canola meals, A: 25 ºC, B: 30 ºC & 35 ºC, temperatures of incubation.

2.3. *Organism, inoculum preparation*

Aspergillus niger CBS 120.49 was obtained from the Persian Type Culture Collection (PTCC) in Iranian Research Organization for Science and Technology (IROST) (Tehran, Iran). It was inoculated to potato dextrose agar (PDA) and incubated in for 3 to 5 days in 25 ºC. Then, for spore collecting from 3-day old PDA, the culture was submerged with sterile saline containing 0.01% (v/v) Tween 80, and hyphae's were removed from spore's solution by filtration through Whatman filter paper grade 1 with the aid of a sterile Büchner funnel with the force of a vacuum pump. Inoculum was adjusted to a concentration of 3×10^6 spores/ml and was kept at 4 ºC until use.

2.4. *Solid-state fermentation (SSF) and optimization process*

It had been confirmed that the optimum growing of *Aspergillus niger*, would be in range of 40% - 70% MC. Therefore, the MC of medium in 40, 50 and 60% was selected to reach the maximum level of phenolic release (Pinto et al., 2005).

Quantities of 10 g of each defatted canola meal were taken in sealed containers and autoclaved at 121 ºC for 30 min. The sterile medium (after increasing the moisture contents of 40, 50 and 60% by sterile distilled water) was inoculated with 1 mL spore suspension of inoculum containing 3×10^6 spores/mL (as mentioned in part 2.3). The cultures were incubated at 25, 30 and 35 ºC for a period of 120 h. Substrates (6.0 g) were harvested at intervals of 24, 48, 72, 96 and 120 h and extracted with 100 mL ethanol 96% in ultrasonic bath in conditions explained in part 2.3.1. Moreover, control samples for moisture contents of 40, 50 and 60% were extracted under the same procedure. The extracts were collected and kept in 4 ºC until they were used for analytical assays.

2.5. *Extraction method of phenolics*

A portion of fermented canola meal (6.0 g) in each day of fermentation (1 to 5) was extracted with 100 mL of 95% ethanol for 45 min using an ultrasonic bath (Elma, Germany) with a fixed power (550 W) and under temperature of 75 ºC water bath. This procedure was selected using ethanol extraction method of Wanasundara et al. (1994) and modified Teh et al. (2014), procedure in ultrasonic bath, by different temperatures and time of extraction (data have not been shown), and the best results obtained were 45 min and 75 ºC in unfermented samples.

2.6. *Determination of total phenolic content (TPC)*

The measurement method of total phenolic content of ethanolic extract was based on Gutfinger (1981). First, 4.9 mL of distilled water was added to 0.1 ml extract in a 10 ml Erlenmeyer flask. Then 0.5 ml of Folin-Ciocalteau (2 N) was added as reagent to the mixture. After 3 min delay, 1 mL of the sodium carbonate 35 % w/v was added and the solution was brought to a volume of 10 mL with distilled water. Absorption of blank and samples were measured by spectrophotometer in 725 nm. First, the blank was counted as zero, and then the adsorption of extracted samples was measured. The standard curve was drawn based on 0-400 g gallic acid per ml of solution, and results have been reported as gallic acid equivalents (GAE)/100 g of canola meal.

2.7. *DPPH radical scavenging assay*

Antioxidant activity of extracts was measured by DPPH radical scavenging using Brand-Williams et al. (1995) method. The procedure started by mixing of 100 µL of extract with a 2.9 mL of 0.1 mM DPPH˙ methanolic solution. After 30 min that the mixture was placed in a dark room, the adsorption was measured by spectrophotometer at 517 nm against the blank. The inhibition percentage of the DPPH˙ by following equation was measured:

$$\text{Inhibition of DPPH (\%)} = \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \times 100$$

where, absorbance of control is the absorbance of DPPH˙ solution without extract (Karami et al., 2019).

2.8. HPLC analysis

For HPLC, chromatography system including separation unit with PDA detector waters was applied. Data were collected and displayed with Millennium 32 software.

HPLC system equipped with auto sampler injector to inject sample in a 15 cm×4.6 mm pre-column (Eurospher 100-5 C18) with reversed phase matrix (3.5 µm) by waters. Solvents were methanol (solvent A) and distilled water (solvent B) with 1 mL/min the flow-rate movement. Injection condition consisted of 20 µL volume of sample which was injected in constant temperature of 25 °C.

2.9. Statistical analyses

The analysis of data was carried out with SPSS version 25.0 to calculate the significant differences using Tukey multiple-range test at $p < 0.05$.

3. Results and Discussion

3.1. Effects of moisture content, temperature and duration of fermentation on yield of polyphenols and antioxidant capacity of canola seed cake extracts

The phenol content of all samples is shown in Fig. 1. All charts are based on data of TPC and DPPH inhibition% of ethanolic extracts of samples cultured for 5 days in 3 different temperatures (Temp) of 25, 30 and 35 ºC. In addition, each chart in each temperature showed 3 diagrams based on data of TPC and DPPH inhibition of samples grew in triple moisture content (MC) of cultures (40, 50 and 60%).

3.1.1. Effect of moisture content of media in releasing of phenolics

Totally, all the evaluated samples indicated significant mycelia growth on canola meal as culture. Visually, among the tested samples, the constant temperature of 25 ºC gave the worst radial growth results in different MC's, with the latest complete areal expansion and after 72 h of cultivation.

Moreover, samples growing in constant temperature of 25 ºC of incubation generally did not show a good yield in phenolic content of extracts in different moisture contents against other samples. However, as shown in Fig. 2, among these samples growing in different moisture contents of media (40, 50 and 60%), the 50% moisture content of growing had the best yield in releasing phenolics from days 1 to 5, especially in the fourth day, and markedly increased against unfermented samples. This phenomenon of releasing of trapped phenolics in tissue could be correlated with activity of carbohydro-lisases and ligninolytic oxidative enzymes which mostly produced in initial stages of *Aspergillus niger* growth for the nutrients achievement (Ajila et al., 2012).

As shown in Fig. 2B, in 30 ºC, the total phenolics content decreased in the first 24 h in all MC's during the fermentation period. Similar results were previously reported by some researches in the production of phenolics by the SSF in the same condition for different agriculture product wastes fermented by *Aspergillus niger* or other funnies (Dulf et al., 2015; Correia et al., 2004). About this initial fall of TPC, it is suggested that due to the presence of most phenolics in insoluble-bound form and only a small amount in soluble form, these available amounts could be used as carbon source instead of complex carbohydrates by fungi (Dulf et al., 2015; Jamal et al., 2011). This phenomenon may coincide with lack of hydrolase and esterase enzymes in efficient amounts.

Nevertheless, in radial growth, compared to 25 ºC, the samples with different MC's, incubated in constant temperature of 30 ºC in days 1 to 5 have much better results in radial growth and were visible in day one. But concomitant to the growth of microorganisms, the TPC of subfractions, results by changing moisture content were in weak trend, except that total phenols extracted from samples growing in 40% MC demonstrated a significantly higher increase in day fifth against other samples ($p < 0.05$). Previously, some authors reported similar results, with highest hydrolyzing enzyme production by *Aspergillus* strains in 40% MC of media (Freitas et al. 2013). However, in the second place, samples of 50% and 60% MC had a significant increase in total phenols compared to the control sample ($p < 0.05$).

Surprisingly, the total phenols of samples growing in 35 ºC had different treatments and dramatically increased in 40% moisture content extracts of day 3 to 5. Moreover, all samples in this temperature showed the best growth rate in media in all samples except 60 % moisture content. This result is according to study of Jamal et al. (2011) that evaluated the effect of incubation temperature on phenolics production by *Aspergillus niger* inoculated on palm oil mill in five levels 30 to 50 ºC. They also found the highest TPC (941 ± 3.72 GAE mg/l) at 35 ºC.

Table 1. Effect of temperature of growing of *Aspergiluss niger* on total phenol content in canola meal extract in 40% moisture content.

Day	Temperature					
	25 ºC		30 ºC		35 ºC	
	TPC	DPPH Inhibition	TPC	DPPH Inhibition	TPC	DPPH Inhibition
1	1802.5	60.1	961.7	54.5	1589.2	77.5
2	1262.9	57.1	823.7	57.1	1187.6	80.3
3	1250.3	65.9	1206.4	71	1595.4	86.1
4	1752.3	65.6	1890.3	77	1608	85.5
5	1470	72.4	2982	79.9	2988.4	86.7

Table 2. Effect of temperature of growing of *Aspergiluss niger* on total phenol content in canola meal extract in 50% moisture content.

Day	Temperature					
	25 ºC		30 ºC		35 ºC	
	TPC	DPPH Inhibition	TPC	DPPH Inhibition	TPC	DPPH Inhibition
1	2329.5	66.3	1859	61.6	804.8	81.7
2	2467.6	83.0	1432.3	82.9	1118.5	74.6
3	1902.9	76.9	1639.3	86.4	1476.2	79.8
4	2530.3	84.2	1896.6	78.0	1476.2	83.2
5	1670.7	79.4	1313.0	81.4	1401	82.1

Table 3. Effect of temperature of growing of *Aspergiluss niger* on total phenol content in canola meal extract in 60% moisture content.

Day	Temperature					
	25 ºC		30 ºC		35 ºC	
	TPC	DPPH Inhibition	TPC	DPPH Inhibition	TPC	DPPH Inhibition
1	604	46.9	1219	59.7	1187. 6	64.7
2	1457.4	71	886.4	70.3	811.1	73.1
3	717	37	1538.9	48.4	1513.9	85.6
4	1037	77.5	1049.5	72.6	1388.4	86.4
5	1457.4	78.3	1777.4	73.1	949.1	79.8

It is evident from these results that most phenolic production was in day 5 of fermentation in 40% moisture content of media in 30 ºC and 35 ºC of growing with 2982, 2988.36 mg/100g, respectively. Compared to the total phenolic content of control sample, which was in average of 1520 mg/100g, the total phenol increased by fermentation was nearly two folds. Because most canola meal phenols are esterified and insoluble-bound and trapped in cell matrix (about 80–99% of the total phenolic acids, to a large extent are in the form of esters and glucosides, the TPC increasing in fermented canola meal extracts may be attributed to the effects of extensive enzymes those produced to break down cell walls (like phenolic acid esterase) which cause to release bound phenols during fermentation (Vuorela et al., 2004; De Vries & Visser, 2001; De Vries, 2003).

3.1.2. *Effect of temperature of incubation in phenolics release*

The obtained data about the effect of different temperatures on TPC at each constant MC, revealed some remarkable information. So, they are shown again in Tables 1, 2 and 3.

In constant amount of 40% moisture content we could found out that with an increase in the temperature of incubation, we could have a good effect on increasing the production of increasing TPC as well as speed of mold grow. In addition, increase in phenolic acid production in days 4 and 5 was the highest but in constant 50% MC, this trend was reversed and 25 ºC and then 30 ºC samples totally have more production of phenolic acid than 35 ºC, respectively. Furthermore, TPC's in all samples were reduced in the fifth (last) day.

Also, *Aspergillus niger* in day 4 and 5 of growth in 60% moisture content of canola meal as culture media caused the most release of phenolic acids in 30 ºC against 25 ºC and 35 ºC, respectively.

One possible explanation for the difference in results and decrease in total phenol in some days could be the phenolic acid polymerization in some stages of growth probably due to some unwanted stress on the fungus (Vattem & Shetty, 2003; Di Majo et al., 2008). About this phenomenon, Dulf et al. (2015) suggested formation of new insoluble phenolics due to lignification and conjugation of free acids by some enzymes like tannin forming peroxidase could be another reason of decreasing TPC in some stages of SSF. In addition, degrade of some phenolic compounds to consuming as a carbon source may be occurred by *A. niger* when enough nutriment is unavailable (Jamal et al., 2011).

Totally, our results are in agreement with previous studies on enrichment of polyphenolic content and radical scavenging property of some other wastes by using *Aspergillus* species (Machado et al., 2012).

3.2. *Effects of moisture content, temperature and duration of fermentation on antioxidant activity (DPPH inhibition (%))*

Contrary to phenolic content, antioxidant activities achieved by DPPH assay, samples which grew in 25 ºC and MC of 40 to 60% showed a good inhibition of free radicals generated by DPPH though the 50% moisture content of growing had better antioxidant activity (Fig. 3). It was confirmed before that the radical scavenging activity of the phenolic acids in the DPPH system under the tested conditions, sinapic and ferulic acids showed more activity in antioxidant power against other phenolic compounds due to having more OH and CH2OH in their benzoic cyclones (Thiyam et al., 2013). Therefore, this good antioxidant capacity in these samples with lower TPC may refer to the release of more powerful free phenolics.

As seen in diagrams of DPPH inhibition, all three graphs (40, 50 and 60% moisture content) especially 50% MC showed the best results in 30 ºC samples, and totally showed the same trend like 25 ºC graphs and did not have any significant difference ($p < 0.05$). This contradiction may be explained by the fact that the phenolic content is not the only effective case on antioxidant activity of extract and also differences of composition and molecular structure of phenolic compounds could be an important factor (Verma et al., 2009). However, the growing of *Aspergillus niger* totally in 35 ºC (40 to 60% MC) had the best results and the antioxidant capacities

of samples showed meanly the best levels. Furthermore, all samples in 40% moisture content in canola meal caused the most DPPH inhibition in all temperatures (25 ºC to 35 ºC). Individually, the highest antioxidant capacity was about 86.7% regarding 35 ºC which is consistent with the information obtained from the phenolic acid content in 35 ºC and 40% moisture content of day fifth extracts.

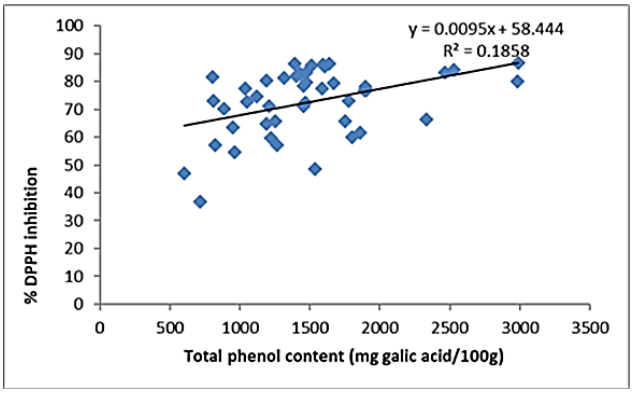

Fig. 4. Relationship between radical scavenging capacity (DPPH assay) and total phenolic content of extracts from solid state fermented.

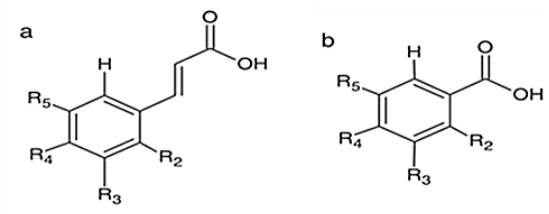

Fig. 5. Structures of the canola and rapeseed phenolic acids. a: Hydroxycinnamic structure, b: Hydroxybenzoic structure (Hall et al., 2001).

3.3. Inter correlations

As it showed in Fig. 4, the antioxidant activity in our subfractions did not have linear correlations with TPC (R^2 = 0.1858, p = 0.3301).

Previously about both of the linear correlation (Salar et al., 2012; Beta et al., 2005) or Nonlinear correlation between total phenolic content and antioxidant activity have been reported (Di Majo et al., 2008; Vattem & Shetty, 2003; Correia et al., 2004). This contradiction as mentioned before could be correlated with the existence of different types of phenolic compounds, which may have different potencies to free radical scavenging in each case as well as different synergies in collected extract in total antioxidant capacity (Safari et al., 2006; Wanasundara et al., 1994).

In addition, sinapic acid has the most antioxidant activity (Due to the presence of two methoxy groups attached to the phenolic ring) in comparison to sinapin and other phenolic compounds in canola meal, and a little amount of sinapic acid (<16%), is in free form (Schmidt et al., 2005; Gaspar et al., 2010).

Overall, it have been reported that sinapic acid could be six times more efficient than Trolox and respectively from more to less may be caffeic (4 times), syringic (1.3 times), ferulic (0.9 times), and protocatechuic acid (0.79 times) (Safari et al., 2006). Thus, the lower amount of TPC in an extract with higher amount of more effective antioxidant may have a good %DPPH inhibition result. In addition, Thiyam et al. (2006) showed that sinapine is unable to stop hydroperoxides which may form in oxidation process compared to sinapic acid. Therefore, our results confirmed the pervious researches that we could increase the antioxidant activity of canola meal extract by increasing the amount of sinapic and other free phenolic acids (Kozlowska et al., 1990; Schmidt et al., 2005; Gasper et al., 2010).

Thus, in our case, one particular reason was due to an increase in free bound phenolic acids which have more antioxidant power than their esterified forms. Therefore, although some samples that do not have a high amount of extracted phenolic compound; surprisingly we have more than 70% DPPH inhibition, which shows a high quality of antioxidant power. Finally, advances in the radical scavenging could be influenced and produced by other byproducts through fermentation. Though this bad correlation between TP and antioxidant capacity may be created by some fungi secondary metabolites that are responsible for the relationship between the antioxidant activity and total phenolics during fermentation (Cai et al., 2011; Mousavi et al., 2013).

Consequently, these results showed fermentation of canola meal in 35 ºC , 40% moisture in 5 day has the best release of phenolics (2988.36 mg gallic acid/100g and 86.7% DPPH Inhibition) in this fermentation conditions.

3.4. HPLC results

3.4.1. Free phenolics distribution

After reviewing the total phenol contents of all samples, two fermented samples showed the highest TPC and/or strongest antioxidant activity selected for next phase of analysis. Therefore, ethanolic subfractions of fermented canola meals in 30 ºC, 40% MC and 35 ºC, 40% MC were analyzed by HPLC. The chromatographic profile of this subfraction was compared with 10 standard samples of phenolic acids (p-coumaric, gallic, cinnamic, caffeic acid, ferulic acids, gensitic acid, syrginic acid, sinapic acid, salicyc acid, and chlorogenic acid).

The chromatograms of the ethanolic subfraction of 30 ºC, 40% MC and 35 ºC, 40% MC were very similar, except the absorption peaks of the 30 ºC, 40% MC fermented sample were lower than that of the 35 ºC, 40% MC.

The results of phenolic acid contents and the elution positions of mixture chromatograms of representative two selected ethanolic subfractions and the unfermented sample are shown in Table 4.

Totally some of the free phenolic acids of samples of 30 ºC, 40% MC and 35 ºC, 40% MC have been increased significantly when compared to unfermented samples. Because of vey near retention time of ferulic and sinapic acids in unfermented samples which contained a small content of free sinapic acid, the obtained peak was not separated. So, we assumed the 14.1 mg/L just as ferulic acid in this sample. About two fermented samples, the peak of sinapic and ferulic acids overlapped, and the amounts were calculated by dividing the peak areas according to retention times of each phenol. It is assumed that each part before and after the retention time in upside and downside trend is mirror. Hence, the results of HPLC analysis indicated that in 30 ºC, 40% MC and 35 ºC, 40% MC, fermented subfraction the free form of ferulic acid content were significantly increased and reached about 5.86 and

4.23 times higher against corresponding phenolic acids of unfermented sample ($p < 0.05$).

Besides, the degradation of bound ferulic acid and maximum level of ferulic acid in the 72 h fermentation l of *Aspergillus niger* on wheat bran have been reported by Hegde et al. (2006).

In addition, these two fermented 30 ºC, 40% MC and 35 ºC, 40% MC subfractions, the free form of sinapic acid content were increased about 5.85 and 7.44 fold against of unfermented sample, respectively.

Surprisingly, the content of syringic acid in fermented 30 ºC , 40% MC and 35 ºC, 40% MC samples were increased about 119.6 and 132.57 ppm compared to trace amounts in unfermented samples. Generally, this phenomenon may be explained by the bioconversion of portion of sinapic acid to syringic acid due to the similar structural subordinates on ring phenol and sinapic acid as dominant (see Table 5 and Fig. 5). This is possible by phenylpropanoid chain cleavage. The same result was obtained by Mukherjee el al. by fungus *Paecilomyces variotii* to conversion of sinapic to syringic acid (Mukherjee et al., 2006). However, 3, 4 dihydroxybenzoic and cinamic acids decreased in fermented samples (See Table 4). The same results were obtained by Jamal et al. (2011) about the fermentation of palm mill by *Aspergillus niger* that in their case the amount of 4-hydroxybenzoic acid decreased about five-fold. This could be explained by oxidation or inter-conversion of molecular structure of phenolic acids (benzoic or cinnamic acid derivatives) (Hegde et al., 2006).

Suddenly, we found 7.32 and 5.43 mg/L Kaepmpherol in 30 ºC, 40% MC and 35 ºC, 40% MC samples known as flavonoid. The interesting fact is that it is known that two major flavonoids of canola meal are kaempferol-3-O- β-D-glucopyranosyl-(1→2)- β -D-glucopyranoside-7-O- β -D-glucopyranoside and kaempferol-3-O-(2-O-sinapoyl)-β-D-glucopyranosyl-(1→2)-β Dglucopyranoside-7-O- β -D-glucopyranoside (Lee et al., 2014; Yang et al., 2015). So, this 5 to 7-fold increase of kaepmpherol could be the result of glycosidase enzymes, especially β-glucosidase produced by our fungi which are capable of releasing phenolic acids. As hypothesized, corresponding to the amount of β-glucosidase, we had the enhancement of this polyphenolic content (Salar et al., 2012). At low concentrations, these compounds also have a bitter taste higher than the minimal tolerance threshold, which are known to be the main constituents of bitter taste in rapeseed. Therefore, decomposition of this compound, in addition to the antioxidant release (kaempferol) in the extract, also could remove the off flavoring of extract and facilitate their usage as the food additive (Hald et al., 2018).

3.4.2. Relationship between free phenolic acids and enzyme production

According to Kozlowska et al. (1983), cell-wall bound phenolics and most phenolics are generally connected with carbohydrates and proteins. In their investigation, nine phenolic acids were found, and sinapic, ferulic, p-coumaric, and o-coumaric acids had the largest amount, sequentially. The structural linkage of canola meal phenolics may be caused by ether linkages with hydroxyl groups of benzene ring of lignin, ester linkages with carbohydrates or carboxylic group of proteins (Acosta-Estrada et al., 2014). Consequently, these phenolics by insoluble bound, before cell wall destruction and hydrolysis of these linkages could not be extracted by conventional methods of extraction (Vuorela et al., 2003).

Consequently, the high level of total phenolic content mentioned before and this high productivity of free phenolic acids in two analyzed samples are the evidences of a high productivity of extent range of enzymes. Phenolic compounds could be some parts of complicated structures such as *di* or polymers which cross linked by esterification with the cell wall composition such as lignin, cellulose, etc. (Zhang et al., 2010). It could be explained that when a high content of leasing degrading enzymes like α-amylase, β-glucosidase, xylanase cellulase, pectinase, ferulic acid esterase, etc., are produced concomitant to the growth of microorganisms, more content of phenolic compound could be extracted easily by ethanol in extraction procedure and cause an increase in total phenolic content (TPC) and antioxidant activity.

The soluble part of phenolic compound are mostly in free form or linked to sugars like some glycosides in [1,2-di-O-sinapoylgentiobiose, 1,2-di-O-sinapoylglucose, feruloyl choline (4-O-8') guaiacyl-di-sinapoyl, and sinapine (4-O-8') guaiacyl-di-sinapoyl] and exist in the cell vacuoles. Whereas after oil extraction, the residue phenolic compounds which due to strict links with the cell walls by hemicellulose or pectin, have remained in canola meal, generally have hydrophobic properties and mostly are in form of in-soluble molecules like sinapine, sinapine (4-O-8') guaiacyl, feruloyl choline (4-O-8') guaiacyl, feruloyl choline (5-8') guaiacyl, ferol-sinapoyl-trihexoside (Shahidi et al., 2015; Schmidt et al., 2014; Yang et al., 2015).

According some investigations it has been reported that when ferulic acid esterase is alongside other cell wall degrading enzymes like xylanase, arabinanase, could increase the releasing of ferulic acid in extract (McKinnon et al. 2005). Therefore, some esterase enzymes could break insoluble-bound forms of phenolic acids trapped within the tissue. It could be concluded that esterase is like ferulic acid esterase aside from releasing of bonded phenols from tissue that may break many of phenolics that are in juice but still are in dimer forms. In our case, this phenomenon caused an increase in some of phenolic acids about 4 to more than ten times in contrast to unfermented samples (see Table 4). The good yield of ferulic acid esterase could be achieved by high level of ferulic, sinapic acids and syringic acid as intermediary products. Given that the syringic acid does not exist as a prominent phenolic in canola, it may be produced from syringaldehyde as intermediate product of initial sinapic acid and finally be oxidized to syringic acid by a complex enzyme system (Mukherjee et al., 2006). So, this proved very good yield of ferulic acid esterase and other lysing enzymes production by *Aspergillus niger* CBS 120.49. Our results are consistent with some articles about production of the ferulic acid esterase and capability of this species. Moreover, with researches that introduced *Aspergillus niger* as fungi which have a very high yield of production of carbohydrate-cleaving (a-amylase, b-glucosidase and xylanase) and other lignin-degrading peroxidases which degrade phenyl rings, increasing the free phenolic content (Schmidt et al., 2014; Jamal et al., 2011; Salar et al., 2012).

So, about the antioxidant activity of these samples, we could conclude that the 35 ºC, 40% MC sample had a better DPPH inhibition% by more powerful radical scavenging because of having more ferulic and sinapic acid against 30 ºC, 40% and unfermented samples.

Table 4. Phenolic acid content after 5 days of fermentation (µg/mL canola meal extract).

Phenolic acid	Unfermented sample	30 ºC 40% MC*	35 ºC 40% MC**
cinnamic acid	2.62	trace	0.35
ferulic acid	6.26	36.70	26.53
sinapic acid	7.84	45.87	58.37
syringic acid	trace	119.59	132.57
Kampferol	trace	7.32	5.43

*Ethanolic extract of fermented canola meal in 30 ºC and 40% moisture content. **Ethanolic extract of fermented canola meal in 35 ºC and 40% moisture content.

Table 2. Example structure of common phenolic acids isolated from rapeseed and canola (Hall et al., 2001).

Phenolic acid	R2	R3	R4	R5
Hydroxycinnamic structure (a)				
caffeic acid	H	-OH	-OH	H
cinnamic acid	H	H	H	H
p-coumaric acid	H	H	-OH	H
ferulic acid	H	-OCH3	-OH	H
sinapic acid	H	-OCH3	-OH	-OCH3
Hydroxybenzoic structure (b)				
gentisic acid	-OH	H	H	-OH
p-hydroxybenzoic acid	H	H	-OH	H
protocatechuic acid	H	-OH	-OH	H
salicylic acid	-OH	H	H	H
syringic acid	H	-OCH3	-OH	-OCH3

4. Conclusion

According to our conclusion, solid state fermentation (SSF) and *Aspergillus niger* CBS 120.49 could act as a good procedure and suitable species to increase total phenol content (TPC) and antioxidant capacity of canola meal. It turned out that the 5-day SSF caused the release of a large amount of soluble and insoluble phenolic compound in the form of free acids, and the best efficiency was in 40% moisture content of media in 30 ºC and 35 ºC of growth in day 5th with 2982, 2988.36 mg/100g. Furthermore, SSF samples in 40% moisture content in canola meal had the best DPPH inhibition in all temperatures (25 ºC to 35 ºC), and the highest was about 86.7% regarding 35 ºC. So, this suggests that the optimum condition for SSF by *Aspergillus niger* CBS 120.49 is 35 ºC and 40% MC to earn maximum amount of TPC, free phenolics and antioxidant capacity which could refer to high quantity and high yield of a wide range of enzymes to release them.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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