



# Bio-enrichment of phenolic, flavonoids content and antioxidant activity of commonly used pulses by solid-state fermentation

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## Abstract

Phytochemicals in pulses are good source of natural antioxidants but present in bound form and conventional processing methods are not enough for release of these phenolic compounds. So, in this research, microbial treatment of pulses named red lentil, cowpea, pigeon pea, urad and mung bean was done to investigate the fermentation response on release of polyphenolics and antioxidant compounds. Results obtained showed that phenolic and flavonoids contents along with the antioxidant potential of all the pulses increased under fermentative effect of *Aspergillus awamori* at concentration of  $1 \times 10^{-6}$  spores/ml. The effect of fermentation on increased amount of phenolics and antioxidants recorded as in order i.e. pigeon pea > urad > mung bean > cowpea > red lentil. A significant ( $p < 0.01$ ) correlation was accomplished among total phenol, flavonoid contents with radical scavenging activity which was observed maximum in pigeon pea i.e.  $r^2 = 0.955$  and  $r^2 = 0.976$ , respectively. Likewise significant positive correlation ( $p < 0.01$ ) between total phenolic and flavonoids compounds with three different enzymes (i.e.  $\alpha$ -amylase, glucosidase and xylanase) had justified the role of microbial enzymatic secretions in liberation of phenolic, flavonoids and antioxidant components during solid state fermentation.

**Keywords** *Aspergillus awamori* · SSF · Phenolics · Flavonoids · Antioxidants

## Abbreviations

SSF	Solid state fermentation
TPC	Total phenolic content
TFC	Total flavonoid content
GAE	Gallic acid equivalent
HPLC	High performance liquid chromatography

## Introduction

Pulses are consumed worldwide as an essential source of protein, fibers, iron, minerals and vitamins. Pulses improve diet quality by increasing nutrient intake. Keeping in view, nutritional properties of pulses, the 2010 U.S. Dietary Guidelines for Americans recommends more frequent consumption of lentils, dry peas and beans [1]. Increasing popularity of pulses has led Food and Agriculture Organization to

designate the year 2016 as the International year of pulses. Apart from nutritive value, pulses are rich sources of phytochemicals and bioactive compounds. These polyphenolic compounds of pulses possess antioxidant and anti-carcinogenic effects, indicating that pulses may have significant anti-cancer effects [2]. Various studies stated that phenolic compounds like phenolic acids and flavonoids present in plants are responsible for their antioxidant potential [3–10]. Plant based food sources have phenolic compounds bounded with carbohydrates, proteins and fibers. The quantity of restricted phenolics found in wheat, maize, rice and oats were 90, 87, 71 and 58%, respectively [11].

Milling, dehulling, soaking, germination, thermal processing and chemical polishing etc. were the methods used generally to enhance the nutritional quality of cereals and pulses. Later, biological processing methods like fermentation and enzymatic treatments were tried. Results of various studies indicated that fermentation is a promising approach for preparation of foods. Fermentation helps to minimize the non-digestible carbohydrates, produce active substances, increases the pool of essential amino acids, vitamins as well as minerals and also enhance the overall quality, digestibility, taste and aroma of the food [12–14]. In fermentation, microorganisms convert the chemical composition of raw materials by releasing these cross-linked phenolic

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compounds. According to Bhanja et al. [15, 16], this may be due to hydrolytic action of different enzymes released during fermentation. Many researchers found that amount of phenolic compounds in fermented substrates were higher as compared to the non-fermented samples [17–19].

Thus based on above knowledge, the plan of current investigation was to examine the impact of solid-state fermentation (SSF) in release of total phenolic, flavonoids and free radicals scavenging activity of commonly used five pulses i.e. red lentil, cowpea, pigeon pea, urad and mung bean. GRAS fungus *Aspergillus awamori* was used to ferment pulses and role of  $\alpha$ -amylase, xylanase and  $\beta$ -glucosidase in release of polyphenols was investigated. Finally, both unfermented and fermented ethanol extracts of pigeon pea only were screened for presence of phenolic acids by HPLC.

## Materials and methods

### Materials

#### Microbial strain and chemicals used

The GRAS fungus i.e. *A. awamori* (MTCC 548) was procured from Institute of Microbial Technology, Chandigarh and used for fermentation. Ethanol, methanol, and hexane were purchased from Merck and Qualigens while all the other chemicals and media constituents were of AR or GR grade and procured from Hi Media and Sigma Aldrich. Glassware's used were of Borosilicate.

#### Collection of substrate

Commonly used five pulses as shown in Table 1 and Fig. 1 were selected as substrates and collected from following Institutes.

**Table 1** List of collected substrates is as follows

S. no.	Common name	Botanical name	Variety name	Source of collection
1.	Mungbean	<i>Vigna radiata</i>	MH-421	CCSHAU, Hisar
2.	Cow pea	<i>Vigna unguiculata</i>	KBC-2	Central State Seed Farm, Hisar
3.	Urad	<i>Vigna mungo</i>	UH-1	CCSHAU, Hisar
4.	Pigeon pea	<i>Cajanus cajan</i>	PARAS	CCSHAU, Hisar
5.	Lentil	<i>Lens culinaris</i>	GARIMA	CCSHAU, Hisar

## Methods

### Preparation of inoculum

Potato dextrose agar (PDA) was used to revive and culture the *A. awamori* strain. At the start of every individual experiment, the plates were inoculated and incubated at 25 °C for 120 h. Further, suspension of fungal spore was prepared in sterilized cellular grade water. This suspension was used for inoculation.

### Mode of solid-state fermentation

Individual pulse was measured 50.0 g and impregnated with 50.0 ml Czapek-dox medium [Monopotassium phosphate (1.0 g/l), NaNO sodium nitrate (2.5 g/l), potassium chloride (0.5 g/l) and magnesium sulfate (0.5 g/l)] at room temperature overnight in 500 ml conical flask. The extra media was discarded, sterilization of both fermented as well as non-fermented substrates (pulses) were done in autoclave at 121 °C for 15 min. Further substrate was allowed to cool down at room temperature and a suspension of fungal spores at concentration of  $1 \times 10^{-6}$  spores/ml (10% w/v) was sprinkled. Substrates were mixed properly with inoculated spores and incubated for 6 days at 30 °C. After 17 h of inoculation, the extra heat produced during fermentation was released everyday by shaking the fermented mass. The control samples (non-fermented) of each substrate were not inoculated with spore solution and used as it is.

### Production of extract

Subsequently at usual intervals of 24 h, the fermented product from flask was removed and dried in oven at 60 °C. All the non-fermented (control) as well as fermented samples were crushed to very small sized particles. Further, hexane (1, 5 w/v, 5 min, thrice) at room temperature was used for defatting of each powdered substrates. Later, to evaporate hexane absolutely samples were dried open in air for 24 h and the defatted substrates were reserved at – 20 °C till another course of action. Further, using reserved defatted powder, extracts were prepared according to conditions (i.e. 54% ethanol at 61 °C for 64 min) as optimized by

Pigeon pea (*Cajanus cajan*)Urad (*Vigna mungo*)Mung bean (*Vigna radiata*)Cow pea (*Vigna unguiculata*)Red lentil (*Lens culinaris*)

**Fig. 1** Pulses used as substrates i.e. pigeon pea, urad, mung bean, cowpea and red lentil

Liyana-Pathirana and Shahidi [20]. Finally total phenolic content and antioxidant activities were tested.

### Enzyme assays

#### Extraction of enzymes

At regular 24 h interval, the biomass obtained after fermentation was dissolved in water (1:10 w/v) and the mixture of enzymes obtained was filtered and the liquid part was assayed for presence of enzymes involved in carbohydrate bond cleavage viz  $\alpha$ -amylase, xylanase and  $\beta$ -glucosidase.

#### $\alpha$ -Amylase assay

The  $\alpha$ -amylase activity was estimated by following protocol of Miller [21]. The extracted enzyme mixture was properly diluted and 0.5 ml of it was mixed with 0.5 ml of 0.2 M acetate buffer (pH 5.0) and 1.0 ml of 1% soluble starch to prepare reaction mixture which was further incubated for

10 min at 50 °C. The action of  $\alpha$ -amylase was determined spectrophotometrically by the measurement of the glucose amount liberated from starch at 575 nm. The total volume of enzyme that releases one micromole of reducing sugar (glucose) per min under the assay conditions is known as One unit (U) of amylase activity. Outcome of experiment were shown as U/gds.

#### Xylanase assay

The action of xylanase enzyme was tested by combining properly diluted enzyme source (0.5 ml), acetate buffer having molarity 0.2 M with pH 5.0 (0.5 ml) and 1 ml xylan (from birchwood; Sigma, St. Louis, USA) having concentration 1.0% (w/v). The experiment mixture prepared above was nurtured at 50 °C for 10 min and reaction was terminated by adding DNS solution (2.0 ml) as well as furthermore boiling the tube in a water bath for 10 min. Total quantity of xylose liberated was calculated at 575 nm in spectrophotometer [21]. Under the given assay conditions, One unit of xylanase

enzyme activity was explained as the volume of enzyme that releases 1.0  $\mu\text{mol}$  of xylose per minute and outcomes were represented as U/gds.

### $\beta$ -Glucosidase assay

A reaction was carried out by mixing 0.25 ml enzyme extract, 0.25 ml acetate buffer (100 mM, pH 5.0) and 0.5 ml of 5 mM para-nitrophenyl-beta-D glucoside (pNP $\beta$ G) for estimation of  $\beta$ -glucosidase activity. Further this mixture was treated at 50 °C for 30 min and 1.0 ml of 1 M sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) solution was supplemented to halt the process. The quantification of *p*-nitrophenol was done spectrophotometrically by measuring absorbance at 400. One unit (U) of  $\beta$ -glucosidase action is explained as the amount of  $\beta$ -glucosidase enzyme that frees 1.0  $\mu\text{mol}$  of *p*-nitrophenol in 1 min under the assay conditions and final observations were represented as Unit per gram dry substrate.

### Total phenolic content

The TPC of extracts were estimated via executing the procedure of Singh et al. [22] where 1 ml of Folin–Ciocalteu reagent and 0.8 ml of 7.5% sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) was added to 0.2 ml of ethanol extracted samples. After suitably mixed, components of above reaction mixture were granted to rest for 30 min at room temperature. Readings of absorbance from spectrophotometer at 765 nm were used to calculate TPC while using gallic acid as standard. Outcomes were represented as gallic acid equivalent (GAE) mg/g dry weight of extract.

### Total flavonoid content

The method given by Ordonez et al. [23] was used to determine TFC wherein Quercetin works as reference for calculation of TFC. A fraction of 0.5 ml ethanolic extract was combined with 0.5 ml of 2%  $\text{AlCl}_3$  and incubated at room temperature for 1 h. The absorbance was noted at 420 nm. Outcomes were represented as Quercetin equivalent (QE) mg/g dry weight of extract.

### Radical scavenging ability determination

#### DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging experiment

This experiment was done by applying Brand-Williams [24] procedure of DPPH free radical estimation. DPPH solution with 0.1 mM concentration in methanol was prepared and 2 ml of it was poured to 0.2 ml extract. Mixture was incubated for 15 min in dark and the spectrophotometric absorbance was noted at 517 nm. The scavenging ability of fermented

and non-fermented preparations against DPPH radical were served as VCEAC (Vitamin C equivalent antioxidant capacity) in  $\mu\text{mol/g}$  pulse substrate by using L-ascorbic acid (vitamin C) as the reference.

#### ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) assay

In this assay, procedure of Re et al. [25] was pursued to calculate antioxidant activity. A combination of ABTS<sup>+</sup> (7.6 mM) and potassium persulphate solution (2.6 mM) was prepared in 5.0 ml of condensed water and left to stay still for 12–16 h in absence of light at room conditions. Later on, the experiment preparation was made by adding 1.0 ml of above radical mixture in 60 ml of water. Concisely, 20  $\mu\text{l}$  extract was mixed with 2.0 ml of ABTS solution and was allowed to react at room temperature for 1 min. The spectrophotometer was used to measure absorbance at 734 nm and L-ascorbic acid acted as the positive control. Finally, the ABTS radical neutralizing ability was estimated by measuring the vitamin C equivalent antioxidant capacity (VCEAC) in  $\mu\text{mol/g}$  pulse substrate.

#### HPLC estimation of phenolic acids

Presence of phenolic compounds was detected by using high performance liquid chromatography. HPLC was performed by using Shimadzu HPLC. The outcomes were attained and prepared using Shimadzu LC-solution version 6.42 software for data collection, calculation and processing. Test samples (20  $\mu\text{l}$  injection volume) were passed through phenomenex C-18 column (4.6  $\times$  250 mm) packed with 5  $\mu\text{m}$  diameter particles. 2% (v/v) acetic acid (solvent A) and methanol/ acetonitrile (40/15, v/v) mixture (solvent B) was run with 0.6 ml/min flow rate under the following gradient program: 0–8 min (70% A), 8–19 min (60% A) and 19–30 min (50% A).

#### Statistical analysis

The set of experiments were repeated thrice to calculate the mean values and the standard deviations from the data obtained from three separate experiments. The paired sample *t* test was performed by using SPSS statistics viewer 16.0 in order to carry out analysis of variance. MS Excel was used to calculate Co-relation coefficient ( $r^2$ ) for estimation of rapport between two variables.

## Results and discussion

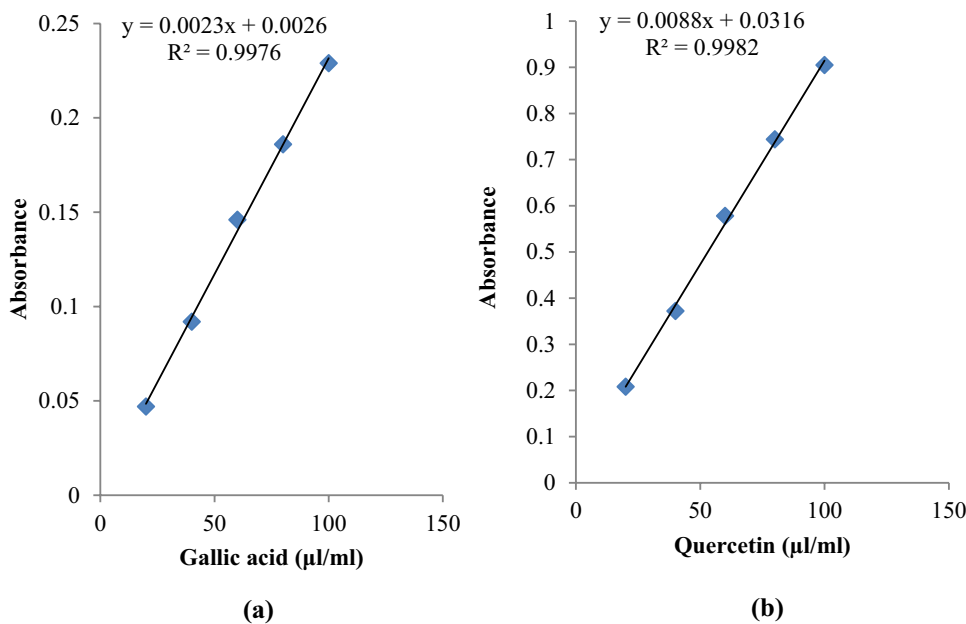
### Total phenolic content and flavonoids content

Gallic acid standard curve equation  $y = 0.0023x + 0.0026$  was used to calculate total phenolic content (GAE  $\mu\text{M/g}$ )

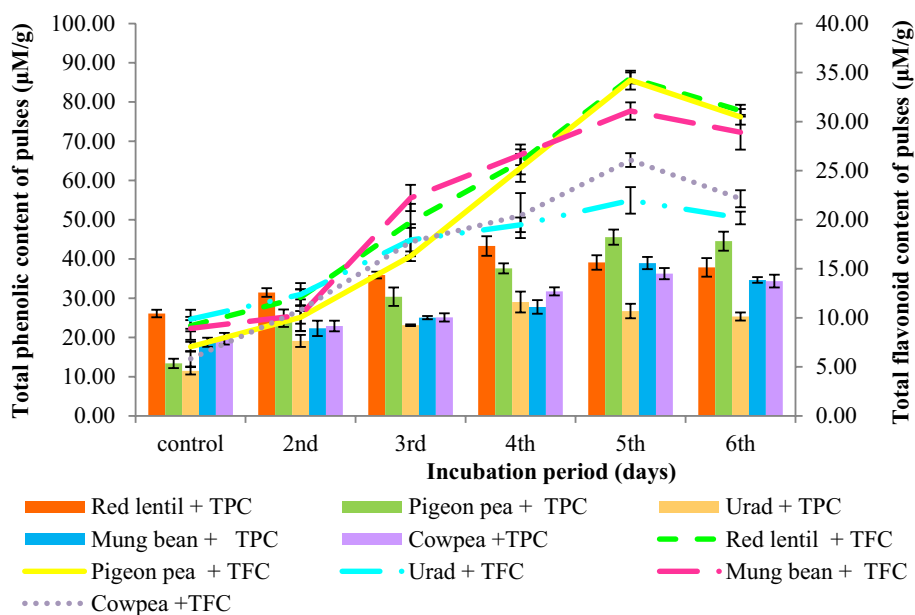
of ethanol extracts of fermented and unfermented pulse samples (Fig. 2a). Results in Fig. 3 showed that fungus *A. awamori* is significantly ( $p < 0.05$ ) capable of increasing total phenolic level of all pulses. SSF imparts maximum effect on pigeon pea where it increased TPC level from  $13.38 \pm 1.20$  (non-fermented) to  $45.55 \pm 1.94 \mu\text{M/g}$  GAE (fermented) on day 5 of fermentation while the extracts of red lentil exhibited a lowest increase in TPC value i.e.  $26.10 \pm 0.97$ – $43.31 \pm 2.49 \mu\text{M/g}$  GAE on 4th day of fermentation (Fig. 3). Similarly, TPC level of urad ( $11.49 \pm 0.94$ – $29.02 \pm 2.66 \mu\text{M/g}$  GAE) on 4th day of

incubation and mung bean ( $18.78 \pm 1.16$ – $38.95 \pm 1.56 \mu\text{M/g}$  GAE) on 5th day of treatment also enhanced significantly under fermentative effect (Fig. 3). The overall comparison for the outcome of fermentation effect on TPC level of pulses was observed as pigeon pea > urad > mung bean > cow-pea > red lentil. Phytochemical composition of pulses vary and this may be due to the reason that the outer structures of grains, particularly the pericarp seed coat and aleurone layers contain much higher levels of phytochemicals such as phenolic compounds, phytosterols, tocots, betaine and folate, than the germ and endosperm [26]. Apart from this,

**Fig. 2** a Standard curve of gallic acid b Standard curve of quercetin



**Fig. 3** Total phenol and flavonoid content of pulses fermented with *A. awamori* (Error bar represents SD,  $n = 3$ )





the polyphenolic composition of cereals is affected by varietal differences along with crop year and production site [27]. Duhan et al. [28] demonstrated the increase in phenolic compounds and antioxidant activity after SSF of wheat.

The standard equation  $y = 0.0088x + 0.0316$  obtained with quercetin was used for calculation of flavonoids content of ethanol extracts of pigeon pea (Fig. 2b). As shown in Fig. 3, the flavonoids content of all pulses enhanced along with the progress of fermentation. Among five pulses, the maximum effect of SSF on TFC was observed in pigeon pea where flavonoids level jumped from  $7.05 \pm 0.50 \mu\text{M/g QE}$  (control) to  $34.23 \pm 0.96 \mu\text{M/g QE}$  on 5th day of fermentation. Overall, around two–five times elevation in TFC was observed in extracts of pulses under fermentative effect. Even the minimum fermentation effect as registered in urad daal was found more than double ( $21.97 \pm 1.35 \mu\text{M/g QE}$ ) the value of non-fermented extract ( $9.86 \pm 0.97 \mu\text{M/g QE}$ ) of the same (Fig. 3). Although fermentation was found to cause significant increase in flavonoids levels but after a certain period of time (post 5th day) this effect start decreasing in all pulses and this may be due to depletion of nutrients and lack of surface for growth on substrate ultimately reduces the metabolic activity of the organism. Nazarni et al. [29] also observed increase in total flavonoids content from ethanolic and ethyl acetic extract of tigarun flower from  $3.61 \pm 0.07$  to  $5.83 \pm 1.00 \text{ mg QE/g}$  and  $1.96 \pm 0.88$  to  $3.41 \pm 0.57 \text{ mg QE/g}$ , respectively after fermentation.

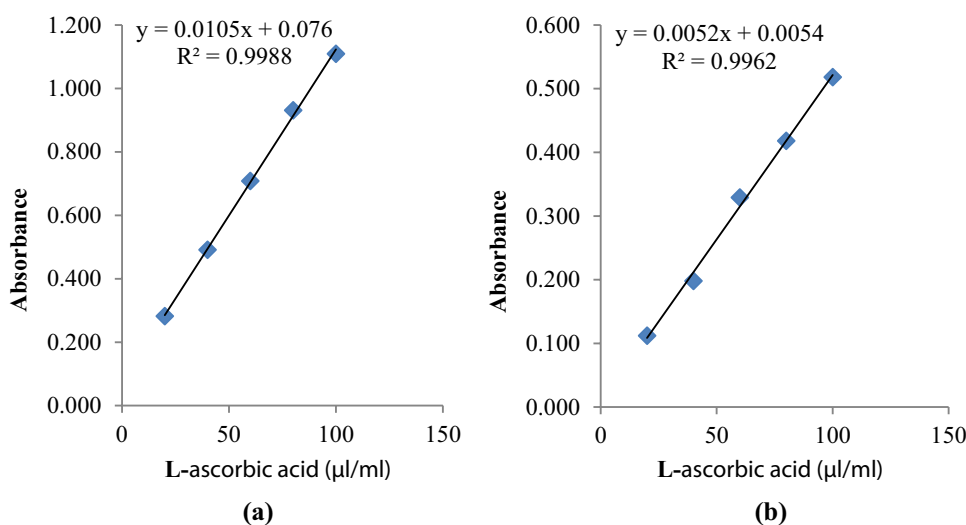
### Antioxidant activity

The DPPH and ABTS radicals scavenging ability of pulse ethanolic preparations were estimated as Vitamin C equivalent antioxidant capacity (VCEAC). In DPPH assay, VCEAC was calculated from equation  $y = 0.0105x + 0.076$ ,  $R^2 = 0.9988$  obtained from standard graph of L-ascorbic

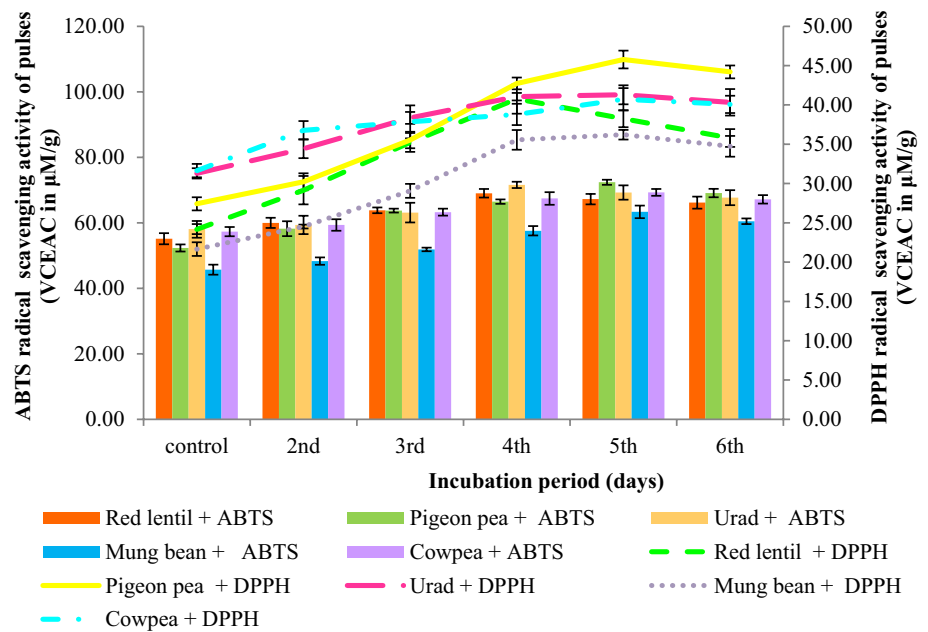
acid (Fig. 4a). As represented in Fig. 5, it was found that fermentation potentially enhanced both DPPH and ABTS scavenging capacity of all pulses. Among the extracts of various pulses examined, the ethanol extract of fermented pigeon pea showed maximum DPPH value i.e.  $45.78 \pm 1.13 \mu\text{M VCEAC/g}$  but when compared to control (non-fermented extracts), red lentil showed the highest increase in DPPH scavenging capacity i.e. from  $27.42 \pm 0.84$  (non-fermented) to  $45.78 \pm 1.13 \mu\text{M VCEAC/g}$  (fermented extracts) (Fig. 5). Earlier in 1999, Knorr observed that the allocation of antioxidant activity in black soybeans relies on the assay procedure, like DPPH and FRAP methods showed that the 90% antioxidant activity of the soybean was contributed by the seed coat while the ORAC protocol shows that the seed coat and dehulled part of the soybean participate evenly to the antioxidant capacity [30]. Similarly Xiao et al. [31] observed that addition of *Cordyceps militaris*-fermented chickpea improved texture, specific volume, colour, sensory and antioxidant properties (FRAP, Reducing power, DPPH and ABTS scavenging) of wheat bread.

In case of ABTS assay, equation  $y = 0.0052x + 0.0054$  obtained from standard graph of L-ascorbic acid was used for VCEAC calculation (Fig. 4b). In Fig. 5, pigeon pea reflects maximum impact of fermentation on ABTS scavenging ability as the VCEAC value was improved from non-fermented extract i.e.  $52.31 \pm 1.08$  to fermented extracts  $72.39 \pm 0.78 \mu\text{M/g VCEAC}$  on 5th day of incubation. While red lentil holds maximum VCEAC value i.e.  $69.02 \pm 1.32 \mu\text{M/g}$  on 4th day of fermentation (Fig. 5). Similar to DPPH, fermentation effectively enhance ABTS scavenging capacity of ethanol extracts in all pulses. Our results were supported by studies of Saharan et al. [32] where both the ABTS and DPPH scavenging properties of wheat, oat, rice, sorghum and maize were enhanced after SSF with *A. oryzae*. Similarly Sadh et al. [33] reported significant increase in DPPH and ABTS

**Fig. 4** a Standard curve of L-ascorbic acid for DPPH assay  
b Standard curve of L-ascorbic acid for ABTS assay



**Fig. 5** ABTS and DPPH radical scavenging property of pulses fermented with *A. awamori* (Error bar represents SD, n = 3)



antioxidant activity in ethanolic extract of seim seed fermented with *A. awamori* and *A. oryzae* on 3rd and 4th day of fermentation, respectively.

Various studies suggested that antioxidant activity of plants is a result of its phenolic composition [34]. Like Singh et al. [35] discovered that stronger antioxidant activity of fermented soybean products was related to markedly higher contents of phenolic acids, flavonoids, and aglycone isoflavone. Similarly, Mastura et al. [36] found a good correlation ( $p < 0.001$ ) between total phenolic contents of organic and inorganic bean samples with antioxidant properties. In present study, total phenol content, total flavonoids content, DPPH and ABTS radical scavenging activity of pigeon pea was correlated. As depicted in Table 2, when TPC was compared with radical scavenging ability, the maximum correlation was observed between TPC and DPPH i.e.  $r^2 = 0.955$  followed by TPC and ABTS ( $r^2 = 0.976$ ) in red pigeon pea. On the other hand, TFC relation with radical scavenging ability was found highest with DPPH in pigeon pea i.e.  $r^2 = 0.977$ , while TFC showed good correlation with ABTS

in cow pea ( $r^2 = 0.935$ ). Overall results of correlation showed that both TPC and TFC of pigeon pea extract exhibited high level of interconnection with antioxidant assays. Almost similar correlation has been observed in other pulses also (Table 2). Sousa and Correia [37] also studied the relationship between phenolic content, antioxidant and anti amylolytic activities in pineapple and guava residues after *Rhizopus oligosporus* mediated solid-state bioprocessing. Similarly in other studies, it has been reported that pulses containing high levels of phenolic material and exhibited high levels of antioxidant activity [38, 39].

### Mode of enzymatic action in discharge of phenolic compounds in SSF

In pulses, phenolic are highly reactive and bind reversibly as well as irreversibly with proteins, leading to lower digestibility and bioavailability of amino acids [40]. Microorganisms secrete enzymes which were screened for their enzyme activity, like Kumar et al. [41, 42] screened *Aspergillus* spp.

**Table 2** Correlation ( $r^2$  value) between total phenolic content, total flavonoid content and antioxidant activity of pulses extracts obtained after SSF with *A. awamori*

Pulses name	$r^2$ value			
	TPC vs DPPH	TFC vs DPPH	TPC vs ABTS	TFC vs ABTS
Red lentil	0.944*	0.726	0.973*	0.783
Pigeon pea	0.955*	0.977*	0.976*	0.935*
Urad	0.950*	0.971*	0.865**	0.828**
Mung bean	0.786	0.952*	0.947*	0.928*
Cowpea	0.811**	0.899**	0.952*	0.967*

\* $p < 0.01$  (highly significant)

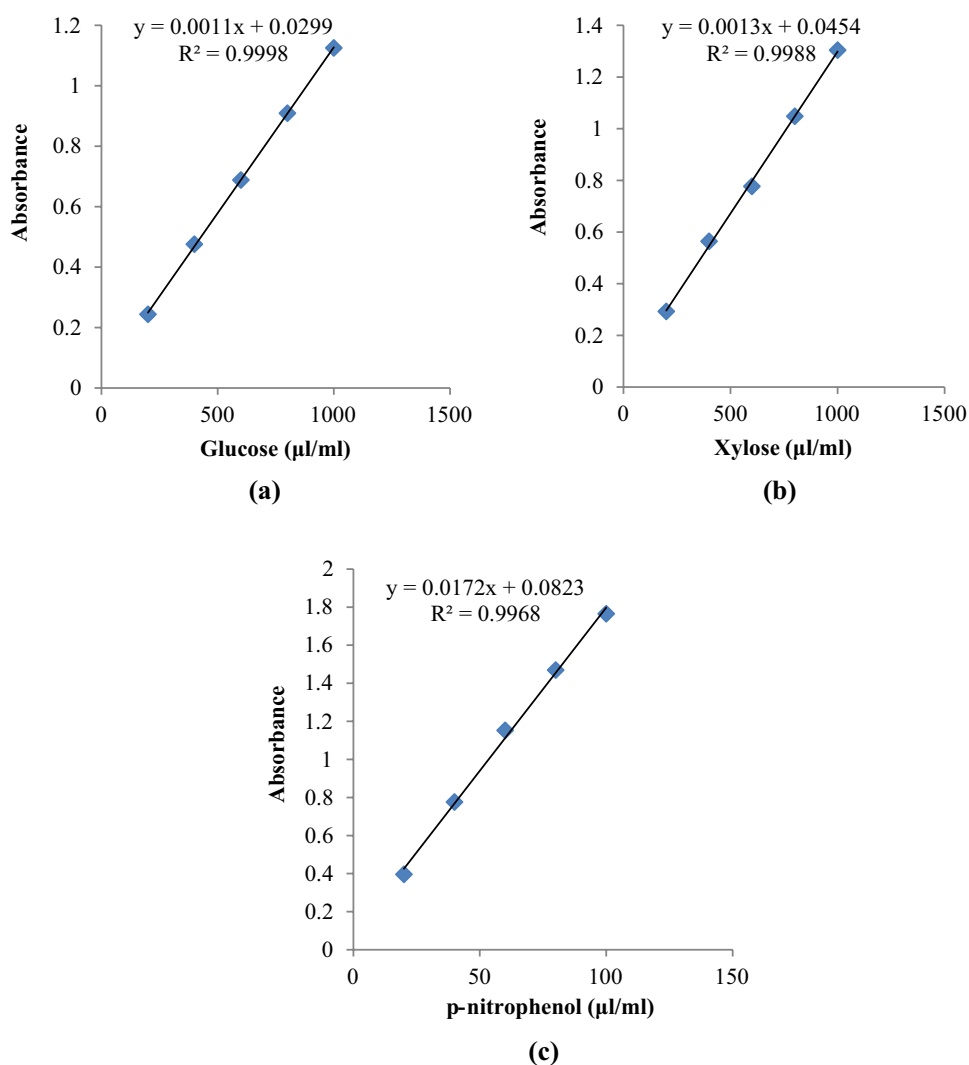
\*\* $p < 0.05$  (significant)

for amylase enzyme activity. In fermentation, these secreted enzymes release these bound phenolic in free form, convert complex proteins to digestible proteins (provide more amino acids for absorption on proteolysis) and thus enhance phenolic, flavonoid content, palatability and digestibility of fermented pulses. Hence, the role of  $\alpha$ -amylase, xylanase and glucosidase enzymes in freeing of phenolic, flavonoids and antioxidants during SSF was studied. For this, the amount of amylase, xylanase and glucosidase enzymes produced was calculated by using standard curve for glucose, xylose and *p*-nitrophenol, respectively (Fig. 6). All the three enzymes  $\alpha$ -amylase, xylanase and glucosidase released by *A. awamori* were significantly ( $p < 0.05$ ) entangled in the improvement of polyphenols during SSF of pulses (Fig. 7). As shown in Fig. 7a, the highest  $\alpha$ -amylase activity during SSF was observed in cowpea where it increased from  $9.22 \pm 2.40$  (control) to  $50.23 \pm 2.87$  U/gds (fermented extracts) on 4th day of fermentation followed by pigeon pea, mung bean, urad, and least was found in red lentil

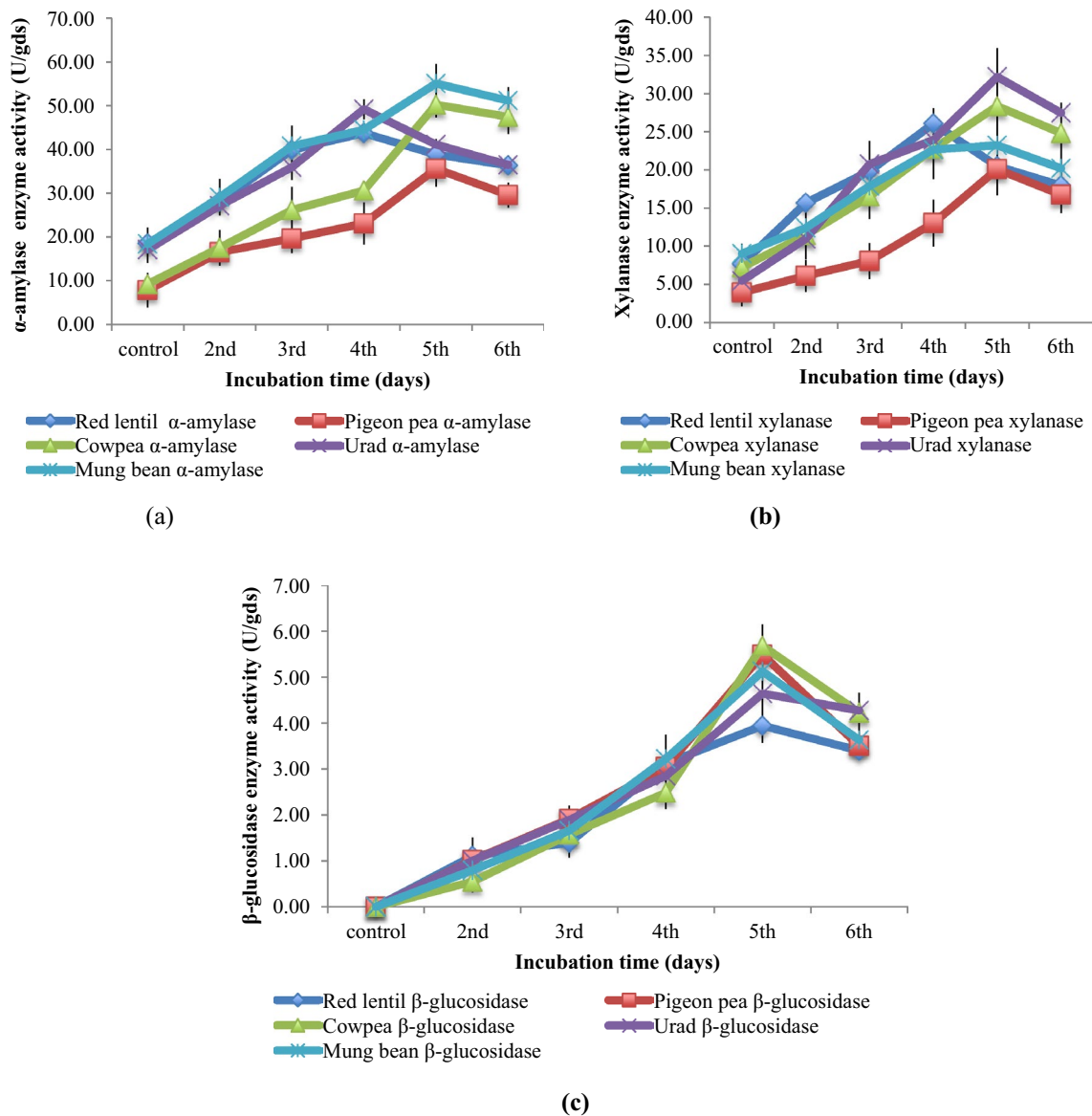
( $18.52 \pm 1.81$ – $43.72 \pm 2.70$ ) on 4th day of SSF. In xylanase assay, maximum enzyme activity was also recorded in urad ( $5.46 \pm 1.16$ – $32.19 \pm 3.77$ ) on 5th day of fermentation and pigeon pea ( $3.94 \pm 1.82$ – $20.10 \pm 3.44$  U/gds) followed by cow pea, red lentil and mung bean (Fig. 7b). On the other hand,  $\beta$ -glucosidase activity was found highest in pigeon pea ( $5.49 \pm 0.64$  U/gds) followed by cow pea ( $5.68 \pm 0.47$  U/gds) and lowest in red lentil ( $3.94 \pm 0.13$  U/gds) on 5th day of fermentation each (Fig. 7c).

The co-relation coefficient ( $r^2$ ) was measured between enzyme activity and TPC to check out the relationship among them (Table 3). Maximum correlation i.e.  $r^2 = 0.959$  was observed between TPC and  $\alpha$ -amylase activity of urad while pigeon pea also showed best correlation (i.e.  $r^2 = 0.947$ ) for xylanase activity with TPC. Correlation between TPC and  $\beta$ -glucosidase activity was found highest i.e.  $r^2 = 0.946$  in mung bean. Similarly TFC was correlated with enzyme assays and results obtained showed that xylanase and TFC of mung bean are highly correlated as  $r^2 = 0.985$  (Table 3).

**Fig. 6** a Standard curve of glucose in amylase assay b Standard curve of xylose in xylanase assay c Standard curve of *p*-nitrophenol in  $\beta$ -glucosidase assay







**Fig. 7** Effect of enzymes **a** α-amylase **b** xylanase **c** β-glucosidase released by *A. awamori* on pulses fermented at different incubation days

Effective correlation trends were observed with α-amylase activity and TFC in urad ( $r^2=0.959$ ), β-glucosidase and TFC in red lentil ( $r^2=0.951$ ). This proves involvement of these enzymes accordingly in liberation of phenolic metabolites during fermentation. Similarly Lee et al. [43] studied the effect of enzymatic hydrolysis by removal of phenolic compounds using a novel laccase from yeast *Yarrowia lipolytica*. Sadh et al. [19, 44, 45] also stated the role of enzymes in abolition of phenolic and antioxidants metabolites. In another study, Wang et al. [46] treated the guava leaves with different enzymes i.e. xylanase, cellulase, β-glucosidase and complex-enzymes. Results based on different enzyme-assisted extraction showed that xylanase-based extraction has no useful effect in recovery of the soluble phenolics

and flavonoids (free and conjugate form) but cellulase or β-glucosidase-based extraction improves the release of insoluble-bound phenolic and flavonoids.

### HPLC based detection of phenolic acids

As the maximum TPC was observed in pigeon pea, so it was selected for HPLC analysis. To study the effect of fermentation on release of phenolic acids, the ethanol extracts of fermented and non-fermented samples of pigeon pea were screened for presence of gallic acid, quercetin and ferulic acid by high performance liquid chromatography. Gallic acid, quercetin and ferulic acid showed peaks with retention time 3.720, 5.287 and 9.370 min respectively.

**Table 3** Correlation ( $r^2$  value) between total phenolic content, total flavonoid content and enzymes activities of pulses extracts obtained after SSF with *A. awamori*

$r^2$ value						
Pulses name	TPC vs $\alpha$ -amylase	TFC vs $\alpha$ -amylase	TPC vs xylanase	TFC vs xylanase	TPC vs $\beta$ -glucosidase	TFC vs $\beta$ -glucosidase
Red lentil	0.917*	0.585	0.932*	0.467	0.764	0.951*
Pigeon pea	0.949*	0.930*	0.947*	0.983*	0.891**	0.940*
Urad	0.959*	0.779	0.810**	0.985*	0.708	0.896**
Mung bean	0.889**	0.949*	0.720	0.935*	0.946*	0.896**
Cowpea	0.937*	0.905*	0.983*	0.973*	0.926*	0.873**

\* $p < 0.01$  (highly significant)

\*\* $p < 0.05$  (significant)

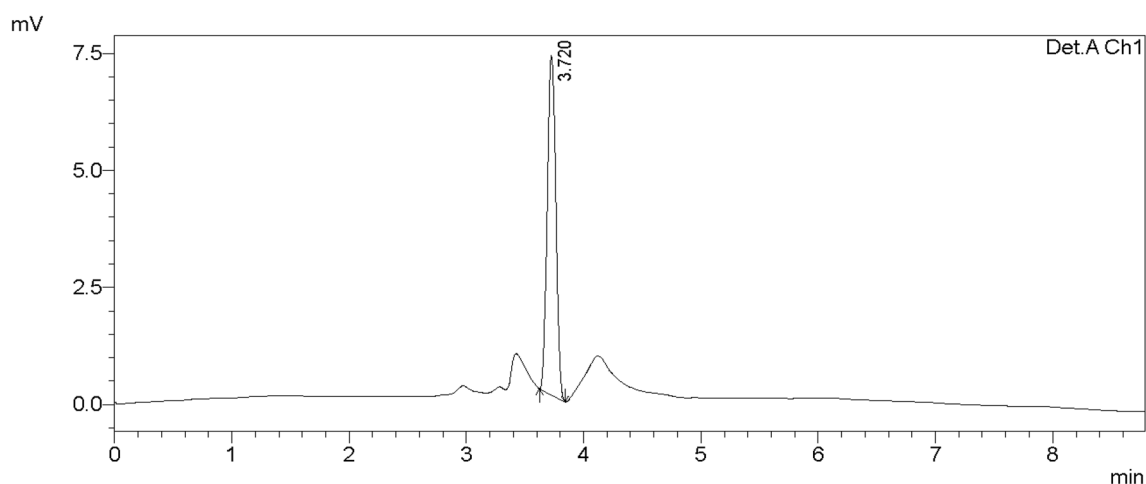
\*\*\*Others (less significant)

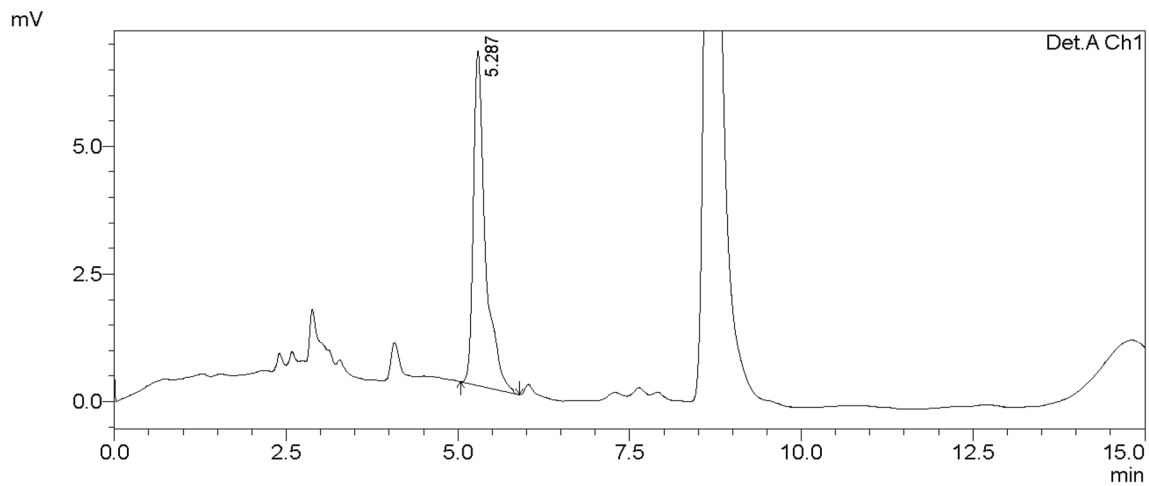
Each standard was measured at 1  $\mu\text{g/ml}$  of solution and Figs. 8, 9, 10 represent their chromatograms.

The HPLC profile of non-fermented pigeon pea sample showed the presence of gallic acid at retention time 3.760 with relative percent area 15.541, quercetin at retention time 5.265 with relative percent area 2.875 and ferulic acid at retention time 9.156 with relative percent area 0.079 (Fig. 11). However, HPLC chromatogram of fermented pigeon pea treated with *A. awamori* (5th day) showed the presence of gallic acid at retention time 3.731 with relative percent area 17.525, quercetin at retention time 5.338 with relative percent area 2.513 and ferulic acid at retention time 9.084 with relative percent area 0.279 (Fig. 12). From Fig. 12, it was also revealed that few peaks are remaining unidentified due to unavailability of the standard. HPLC analysis estimated that polyphenolic compounds were released by biochemical activity of *A. awamori* in response surface methodology (RSM).

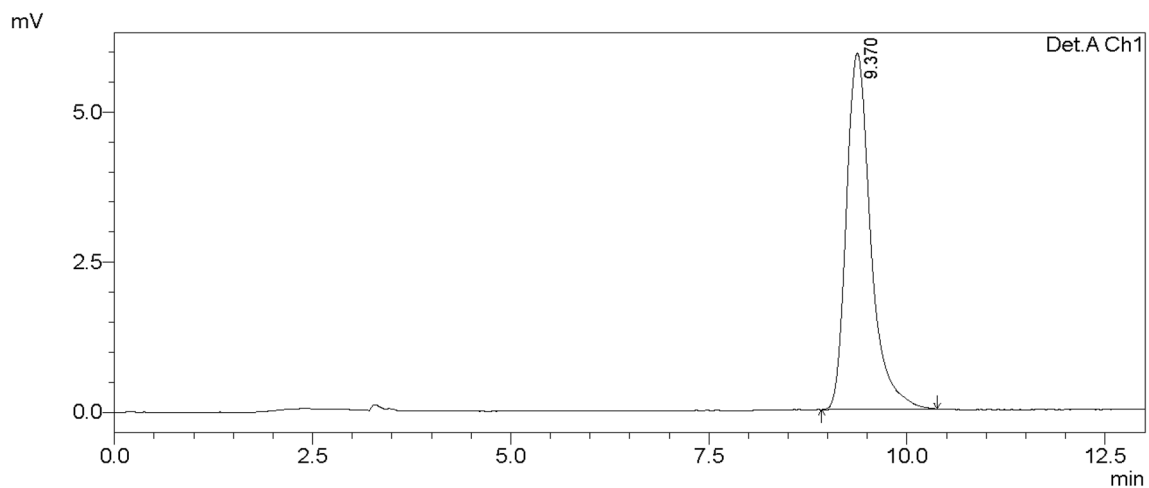
Results of HPLC clearly indicated that gallic acid, quercetin and ferulic acid were enhanced significantly in pigeon pea after fermentation with *A. awamori* (pigeon pea) as compared to the non-fermented samples. Similarly, coffee pulp and wheat bran extracts showed enriched antioxidant capacity as well as increase in amount of other phytochemicals such as chlorogenic acid and *p*-coumaric acid [47–49], while the polyphenols of tea particularly rutin, a leading flavonoid amount reduced after fermentation [50].

Sumino et al. [51] also reported the presence of gallic acid in the fruits of *Ardisia colorata*. Phenolic compounds from fermented paddy straw also contained high amount of gallic acid (0.434 mg/g) as compared to other compounds rutin (0.124 mg/g), quercetin (0.202 mg/g) [52]. Biswas et al. [53] also quantified gallic acid content of the ethanolic extracts. Similarly, Sawant et al. [54] also reported the determination of gallic and ascorbic acid in the extracts of *Phyllanthus embelica* and also identified the phenolic compounds in the extracts for the first time. Moreover, it can be

**Fig. 8** HPLC chromatogram of standard gallic acid (99.5%)



**Fig. 9** HPLC chromatogram of standard quercetin (99.5%)



**Fig. 10** HPLC chromatogram of standard ferulic acid (99.5%)

concluded from the study that the amount of polyphenols and phenolic acids is affected either by extraction process or the fermentation time that results in increased concentration of free polyphenolic compounds.

## Conclusion

Total phenolic and flavonoids content are increased in the fermented samples which also increase the antioxidant potential of all the tested pulses as compared to non-fermented ones. This was further confirmed by the correlation

between total phenolic, flavonoids content and their antioxidant activity in fermented samples of all the pulses. The same effect was reinforced by the activities of the enzymes viz.  $\alpha$ -amylase,  $\beta$ -glucosidase and xylanase which are associated with extraction of polysaccharide linked polyphenolic compounds. Hence, it can be concluded that fermentation is an effective tool to enhance the amount of bioactive compounds in fermented pulses as equated to non-fermented one. So, food products prepared via fermentation of these pulses have good nutritive value and can be involved in daily diet to protect body from degenerative diseases.

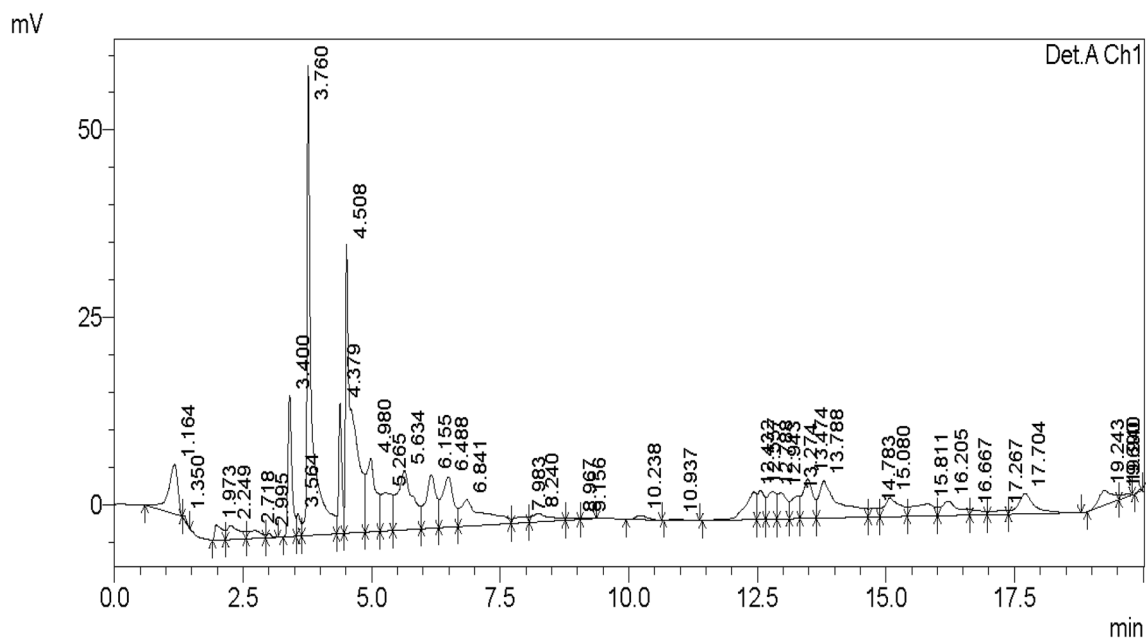


Fig. 11 HPLC chromatogram of ethanol extract non-fermented/control sample of pigeon pea

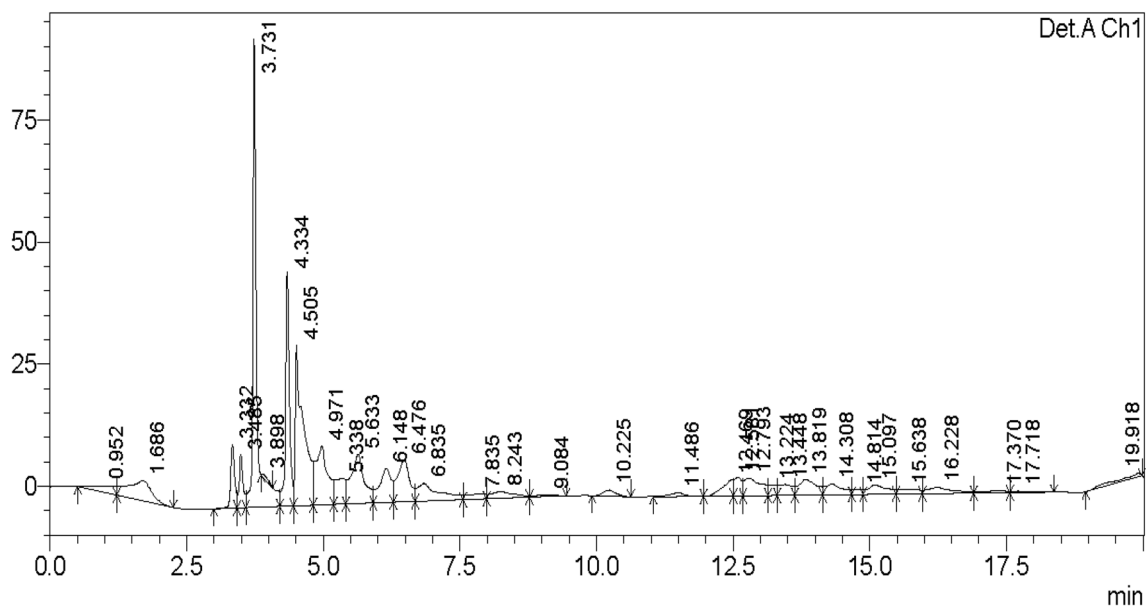


Fig. 12 HPLC chromatogram of ethanol extract of pigeon pea fermented with *A. awamori*

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