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Antioxidant Activity of Phenolic Components from n-Butanol Fraction (PC-BF) of Defatted Flaxseed Meal

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ABSTRACT

Flaxseed (*Linum usitatissimum* L.) is the richest source of phenolic compounds. Owing to high content of phenolics, defatted flaxseed meal exhibited broad spectrum of bioactivities. In the present study antioxidant potential of selectively isolated PC-BF was evaluated by various methods such as total antioxidant activity, reducing power, DPPH free radical, hydrogen peroxide and nitric oxide scavenging activity. Preliminary phytochemical screening reveals only presence of phenols and carbohydrates in PC-BF. UV spectra indicated the presence of phenolic compounds other than earlier reported phenols and flavonoids in n-butanol fraction. PC-BF showed 225 μg of total phenolic content per milligram of PC-BF. Significant antioxidant activity close to standard antioxidant BHT and BHA was found in PC-BF. This study will enhance the chemoprotective potential of defatted flaxseed meal.

Key words: Flaxseed, n-butanol fraction, UV spectra, phenolic components, flavonoids, antioxidant activity

INTRODUCTION

An important field of research today is the control of 'redox' status with the properties of food and food components (Dimitrios, 2006). Plants constitute richest source of natural antioxidants to counteract Reactive Oxygen Species (ROS). Reactive oxygen species are continuously generated inside the human body as a result of contact with excess of exogenous chemicals in our ambient environment and/or due to a number of `endogenous metabolic processes involving redox enzymes (Ebrahimzadeh *et al.,* 2008). Hence, natural antioxidants due to their radical scavenging ability are considered as possible protection against many chronic diseases as well as lipid peroxidation (Gulcin *et al.*, 2007).

The consumption of flaxseed (*Linum usitatissimum* L.) and its products are being encouraged because of the metabolic effects of its major components α-linolenic acid (ALA), soluble fiber and lignan (Oomah *et al.*, 1997). Flaxseed due to its high content of ALA and lignan prevents oxidative lung damage, inflammation and fibrosis (Lee *et al.*, 2009). Furthermore, other phenolic compounds in flaxseed such as p-coumaric acid and ferulic acid glucosides were found to possess antioxidant properties and are special interest in dermatology (Yuan *et al.*, 2008). In addition to

antioxidant potential, lignan Secoisolariciresinol diglucoside (SDG) and other phenolic compounds reveal various bioactivities like phytoestrogenic, anticarcinogenic and cardioprotective (Johnsson *et al.*, 2002).

Antioxidant activities of four major fractions of ethanolic extract of flaxseed meal was studied in a α-carotene-linoleate model system. Out of four major fractions separated according to their maximum UV absorption at 280 nm, fraction I of phenolic compounds with maximum UV absorption at 290 nm was most active (Amarowicz *et al.*, 1993). Flaxseed meal is the prosperous source of bioactive phenolic acids. Flaxseed was reported to contain 8-10 g kg^{-1} total phenolic acids, about 5 g kg^{-1} of esterified phenolic acids and 3-5 g kg^{-1} of etherified phenolic acids and variations in phenolic acid content largely attributed to seasonal effects (Oomah *et al.*, 1995). Reported phenolic acids in flaxseed were p- coumaric, o- coumaric, ferulic, p-hydroxybenzoic, gentisic, vanillic, sinapic acids in free and /or bound form (Babrowski and Soulski, 1984; Kozlowska *et al.*, 1983). By now various methods have been used for extraction of SDG and phenols from flaxseed such as extraction with ethanol; dioxane (1:1, v/v), ethanol or methanol; water (50-100%), 95% ethanol; dioxane (1:1, v/v), 70% methanol; water, 80% methanol, 1, 4-dioxane; ethanol (1:1, v/v) on alkaline treatment (Beejmohun *et al.*, 2007). Besides, phenols were also extracted with absolute methanol (Oomah *et al.*, 1995). On further fractionation of phenolic extract of defatted flaxseed meal, phenylpropanoid glucosides such as linusitamarin, linocinamarin, daucosterol (Luyengi *et al.*, 1993) and Flavonoids like herbacetin 3, 8-O-diglucoside, herbacetin 3,7-O-dimethyl ether, kaempferol 3,7-O-diglucoside and (–) pinoresinol diglucoside and SDG with their antioxidant potential (Qiu *et al.*, 1999) were reported in saturated n-butanol of defatted flaxseed meal. However, there is no any report on remaining phenols and their antioxidant potential of n-butanol fraction of defatted flaxseed meal. Hence, the present study was undertaken to isolate and evaluate the antioxidant potential of PC-BF.

MATERIALS AND METHODS

Chemicals and reagents: Methanol, n-hexane, n-butanol, solvent ether were purchased from Qualigen (India). 1, 1-Diphenyl-2-Picryl-Hydrazyl (DPPH), linoleic acid were purchased from Sigma Chemical Co. (St Louis, MO, USA). Ferric chloride, ferrous chloride, Tween-20, Butylated Hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT), sodium hydrogen phosphate, sodium dihydrogen phosphate and phosphoric acid were purchased from Molychem (Mumbai, India). Folin-Ciocalteau reagent, gallic acid, sodium carbonate, hydrochloric acid, sodium nitroprusside, N-(1-naphthyl) ethylenediamine dihydrochloride, sodium hydroxide, sodium chloride and hydrogen peroxide were purchased from Merck (India). All these chemicals and reagents were of analytical grade.

Defatted flaxseed meal and extraction: A double pressed flaxseed cake powder obtained from "Omega-3 oil unit, under NAIP-ICAR Component-3, Sangamner, M.S., India" in year 2010, was used for extraction. Residual oil from flaxseed meal was removed by n-hexane (1:4, w/v). One kilogram dried defatted flaxseed powder was extracted with 10 L methanol for 3 h at 60°C in a soxhlet apparatus. Methanol extract was concentrated in a rotavapour at 60°C under vacuum. Then the dried residue was further partitioned with n-butanol: water (1:1, v/v). n-Butanol fraction was separated from aqueous fraction and concentrated in rotavapour at 80°C under vacuum. Dried n-butanol residue was dissolved in minimum quantity of methanol and precipitated with solvent ether (1:5, v/v), yields 1.213 g of brown colored sticky precipitate of phenolic components (PC-BF). PC-BF was finally dissolved in methanol for further characterization.

Preliminary phytochemical screening: Preliminary qualitative phytochemical screening of PC-BF in methanol for the presence of carbohydrates, reducing sugars, phenols, flavonoids, saponins, cardiac glycosides, cynogenic glycosides, alkaloids and terpenoids were carried out by using standard described procedures (Okigbo *et al*., 2009; Shrivastav *et al*., 2009).

UV-Visible spectra: A UV-Visible spectrum of PC-BF in methanol was recorded on UV-VIS spectrophotometer (Chemito UV2100, India).

Total antioxidant activity determination: Total antioxidant activity of the PC-BF was determined according to the thiocyanate method (Mokbel and Hashiga, 2005) with some modification. A portion of 2 mL (1 mg mL^{-1}) PC-BF in methanol was mixed with 2.1 mL linoleic acid (2.51%, in methanol), 4 mL of 0.05 M phosphate buffer (pH 7.0) and 1.9 mL of water. Mixture in a vial with screw cap was placed at 40°C in the dark. To 0.1 mL of this mixture 2.7 mL methanol (75%) and 0.1 mL ammonium thiocyanate was added. After addition of 0.1 mL of ferrous chloride (0.02 M, in 3.5% HCl) to the reaction mixture, the absorbance at 500 nm of the resulting red solution was measured at several time intervals until control reached the maximum value. The inhibition of lipid peroxidation in percent was calculated by the following equation:

$$\text{Inhibition of lipid peroxidation (\%)} = [(A_o - A_1)/A_o] \times 100$$

where, A_o was the absorbance of the control incubated with linoleic acid but without sample and A_1 was the absorbance of PC-BF/BHT or BHA.

Free radical scavenging activity: Free radical scavenging activity of PC-BF in methanol was measured by 1, 1-Diphenyl-2-Picryl-Hydrazil (DPPH) method described by Shimada *et al*. (1992) with some modification. One milliliter of 0.1 mM solution of DPPH in methanol was added to 0.5 mL of PC-BF (1 mg mL^{-1}) in methanol at different concentration (50-250 μg). After 30 min absorbance was measured at 517 nm. The percentage of DPPH inhibition was calculated by using equation:

$$\text{Inhibition (\%)} = [(A_o - A_1)/A_o] \text{x} 100$$

where, A_o was the absorbance of the control and A_1 was the absorbance of PC-BF /BHT or BHA.

Reducing power: The reducing power of PC-BF was determined by the method described by Oktay *et al*. (2003). Different concentrations (50-250 μg) of PC-BF in methanol (1 mg mL^{-1}) and reference standard BHT, BHA in 1 mL of distilled water were mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanate (2.5 mL). The mixture was incubated at 50°C for 20 min and then trichloroacetic acid (10%, 2.5 mL) was added to the mixture. This was centrifuged at 3000 rpm for 10 min. The upper layer of the reaction mixture (2.5 mL) was mixed with 2.5 mL distilled water and 0.5 mL $FeCI_3$ (0.1%). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Scavenging of hydrogen peroxide: The ability of PC-BF in methanol to scavenge hydrogen peroxide was determined according to the method of Ruch *et al*. (1989). A solution of hydrogen peroxide (2 mM) was prepared in phosphate buffer (pH 7.4). 200 μL concentration of PC-BF

dissolved in methanol was added to a hydrogen peroxide solution (0.6 mL). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of PC-BF and standard compounds was determined by equation:

$$\% \text{ Scavenged } H_2O_2 = [(A_o - A_1)/A_o] \times 100$$

where, A_o was the absorbance of the control and A_1 was the absorbance of PC-BF /BHT or BHA.

Nitric oxide-scavenging activity: Nitric oxide scavenging activity of PC-BF was determined according to the procedure described by Kumaran and Karunakaran (2006). Sodium nitroprusside (10 mM) in phosphate-buffered saline was mixed with 100 µL concentration of PC-BF dissolved in methanol and incubated at room temperature for 15 min. The same reaction mixture, without the PC-BF but with an equivalent amount of methanol, served as control. After the incubation period, 0.5 mL of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. BHT and BHA were used as standards.

Determination of total phenolic contents: A total phenolic content from PC-BF in methanol was determined using slightly modified Folin-Ciocalteu method (Elzaawely *et al*., 2005). Two microliter of PC-BF in the methanol (1 mg L^{-1}, w/v) was diluted to 5 L with distilled water and added 0.5 mL Folin-Ciocalteu (diluted 1:10, v/v) in it followed by 2 mL of Na_2CO_3 (7.5%, v/v) solution. The solution was mixed and allowed to stand for 30 min. Absorption was recorded at 765 nm. The total phenolic content was expressed as gallic acid equivalents in micrograms per milligram of PC-BF.

Statistical analysis: Experimental results were expressed as mean of measurements and their corresponding standard deviations. The data were analyzed by an analysis of variance ($p<0.05$). Results were processed by computer programmes: Excel (2003) and Statistical software Instat.

RESULTS AND DISCUSSION

Preliminary phytochemical screening: Preliminary phytochemical analysis of PC-BF reveals only presence of carbohydrates and phenols. Positive test to the carbohydrates, could be due the presence of phenolic glycosides.

UV-Visible spectra: UV-Visible spectra of PC-BF in methanol were measured by scanning wavelength from 190 nm to 700 nm. Figure 1 reveals the UV spectra of PC-BF in methanol with maximum absorption between 290 to 360 nm. These results could confirm presence of phenolic acids, flavonones and/or flavanones (Naczk *et al*., 1992). The resultant specific maximum absorption range also implies that lignans and their derivatives could be absent in PC-BF, as it exhibited UV absorption between 270-290 nm (Amarowicz *et al*., 2006). Moreover, the earlier reported flavonoids in n-butanol fraction such as herbacetin 3, 8-O-diglucoside (maximum UV absorption at 225, 272 nm), (–) pinoresinol diglucoside (maximum UV absorption at 237, 286 nm), (–) pinoresinol diglucopyranoside (Maximum UV absorption at 237, 286 nm) Qiu *et al*., (1999), could be not in attendance within the PC-BF. Thus, UV spectra indicated the presence of phenolic acids in PC-BF. However, further detail spectroscopic study is needed to confirm it.

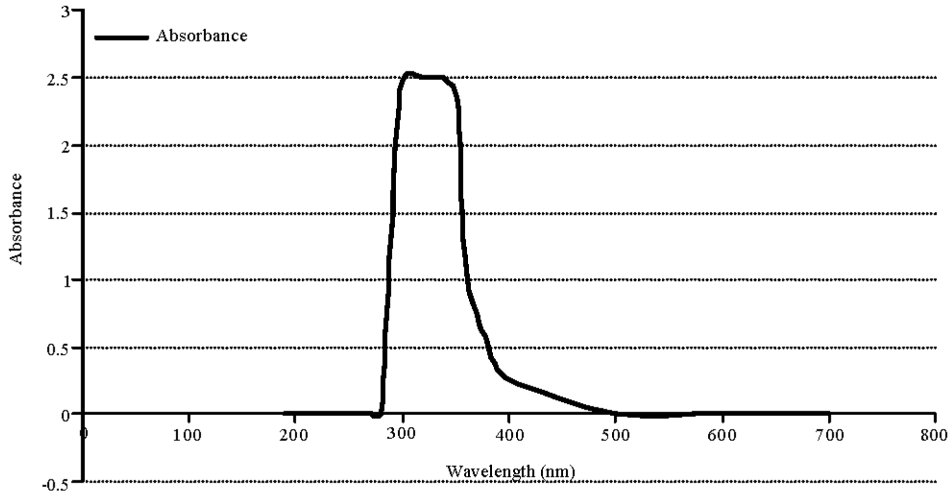

Fig. 1: UV Spectra of PC-BF in methanol. (PC-BF: Phenolic components from n-butanol fraction)

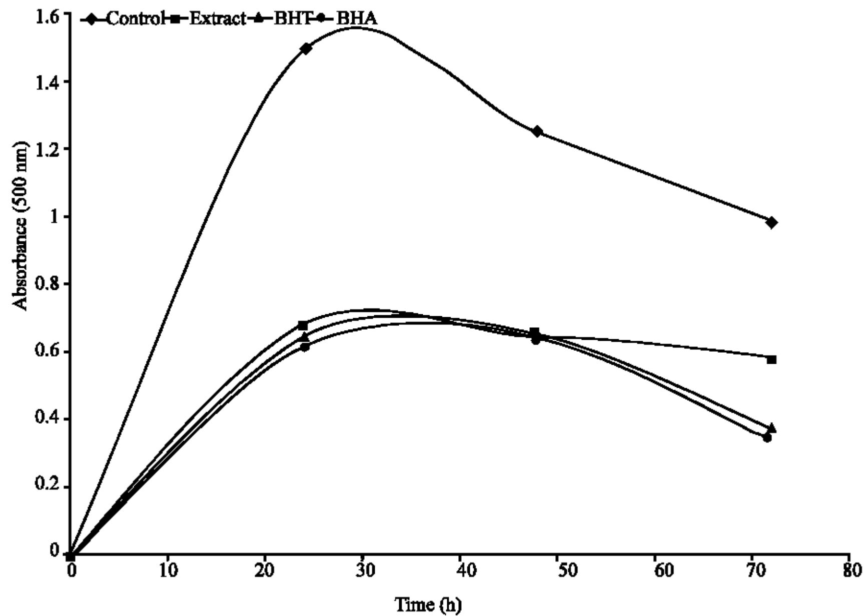

Fig. 2: Total antioxidant activity of PC-BF, BHT and BHA (1 mg mL^{-1}) was determined by the thiocyanate method at different time intervals. (PC-BF: Phenolic Components from n-Butanol Fraction, BHT; Butylated hydroxyanisole and BHT; Butylated hydroxytoluene)

Total antioxidant activity: Figure 2 shows the total antioxidant activity at concentration of 1 mg mL^{-1} of PC-BF, BHT and BHA. Control reached its maximum absorption at 24 h and decreased thereafter due to oxidation of linoleic acid generating linoleic acid hydroperoxides. The difference between PC-BF and control was statistically significant ($p<0.05$). PC-BF showed antioxidant activity close to the standards, BHT and BHA at same concentration. The percentage inhibition of PC-BF, BHT and BHA at concentration 1 mg mL^{-1} on linoleic acid peroxidation were 54.44±0.024, 56.78±0.024 and 58.73±0.023%, respectively.

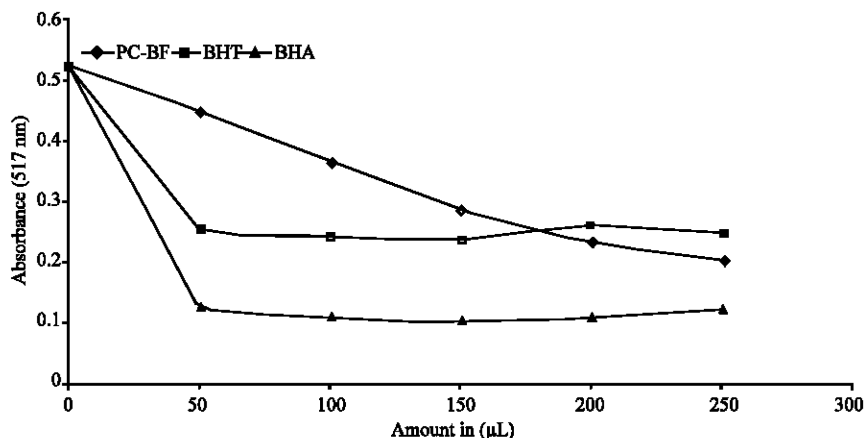

Fig. 3: Free radical scavenging activity of PC-BF, BHT and BHA at various concentrations (50, 100, 150, 200 and 250 µL). PC-BF; Phenolic components from n-butanol fraction, BHT; Butylated hydroxyanisole and BHT; Butylated hydroxytoluene.

Table 1: Comparison of free radical scavenging, Nitric oxide scavenging and Hydrogen peroxide scavenging activity of PC-BF and standard antioxidants BHT, BHA (PC-BF: Phenolic Components Fromn-Butanol Fraction, BHT: Butylated hydroxyanisole and BHT: Butylated hydroxytoluene)

	DPPH free radical scavenging activity (%) at 100 µL	Nitric oxide scavenging activity (%) at 100 µL	Hydrogen peroxide scavenging activity (%) at 200 µL
PC-BF	30.16±0.80[a]	24.41±0.39	25.52±0.075
BHT	89.50±0.50	90.14±0.70	79.80±0.12
BHA	93.54±0.56	92.25±0.71	37.62±0.12

[a] Value represent the mean of three replicates ± S.D

Free radical scavenging activity: Figure 3 reveals the concentration dependent free radical scavenging activity of PC-BF, BHT and BHA. The reduction capability of DPPH radical was determined by the decrease in its absorbance at 517 nm induced by antioxidants. Owing to rapid hydrogen acceptable ability of DPPH, it reacts with antioxidants and gets converted into 1, 1-diphenyl-2-picrylhydrazine and hence shows decrease in absorbance (Yuan *et al.*, 2008). PC-BF exhibited significantly higher activity than the control ($p<0.05$). At all concentrations PC-BF showed the moderate free radical scavenging activity than BHT and BHA. Table 1. shows percentage of free radical scavenging activity of PC-BF, BHT and BHA at 100 µL concentration.

Reducing power: Figure 4 shows the reducing power abilities of PC-BF, BHT and BHA. The reducing power of PC-BF, BHT, BHA increases with increase in concentration. The reducing power of a compound is related to its electron transfer ability and may therefore, serve as an indicator of its potential antioxidant activity (Elzaawely *et al.*, 2005). However, antioxidant activity has been attributed to different mechanisms and reactions such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging (Ozen, 2009). The reducing power activity of PC-BF showed higher activity than the control and the difference was statistically significant ($p<0.05$). The reducing power of PC-BF, BHT and BHA showed the following order: BHA>BHT>PC-BF.

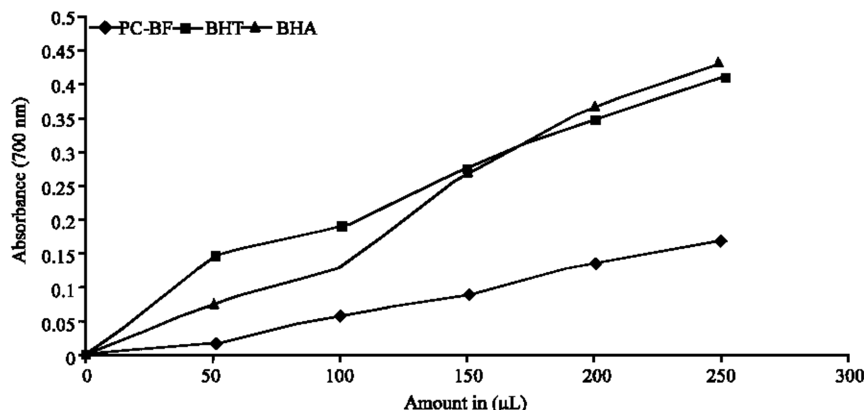

Fig. 4: Reducing power of PC-BF, BHT and BHA at various concentrations (50, 100, 150, 200 and 250 µL). (PC-BF: Phenolic components from n-butanol fraction, BHT: Butylated hydroxyanisole and BHT: Butylated hydroxytoluene)

Scavenging of hydrogen peroxide: The ability of hydrogen peroxide scavenging of PC-BF, BHT and BHA was determined at 200 µL concentration. Hydrogen peroxide has strong oxidizing properties. It can be formed in vivo by many oxidizing enzymes. It can cross membranes and may slowly oxidize a number of compounds Table 1. reveals percentage of hydrogen scavenging activity of PC-BF, BHT and BHA at 200 µL concentration. Hydrogen peroxide itself is not very reactive, however it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells (Gulcin *et al*., 2010).

Nitric oxide-scavenging activity: Table 1 also exhibits percentage of nitric oxide scavenging activity of PC-BF, BHT and BHA at 100 µL concentration. The plant/plant products may have the property to counteract the effect of Nitric Oxide (NO) formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health. PC-BF exhibited comparatively moderate nitric oxide scavenging activity than BHT and BHA.

Determination of total phenolic contents: Phenolic components are very important plant constituents with scavenging ability because of its hydroxyl group. PC-BF in methanol was exhibited 225±0.025 µg of total phenolic content per milligram of PC-BF. However, there was no general correlation between phenolic content and antioxidant activity (Peschel *et al*., 2006).

CONCLUSION

In this investigation, we have used simple novel approach for isolation of phenolic components other than earlier reported phenolics such as lignans, flavonoids in n-butanol fraction of defatted flaxseed meal and demonstrated their antioxidant potential. The result underline that in all studied models PC-BF exhibited different levels of antioxidant activity, close to standard antioxidants BHT and BHA. Preliminary phytochemical screening reveals only presence of phenols and carbohydrates in PC-BF. UV spectra confirmed the presence of phenolic compounds other than earlier reported phenols in n-butanol fraction. Phytochemical screening and characteristic UV spectra indicated

phenolic acids, phenolic glycosides might be present in PC-BF. However, further investigation on isolation, purification and characterization of individual phenolic component and their in vivo antioxidant activities and in different antioxidant mechanisms are warranted. Antioxidant activity of these discriminating phenolic compounds showing maximum UV absorption between 290 to 360 nm of n-butanol fraction will contribute the overall chemopreventive potency of flaxseed meal along with the other phenolic compounds.

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