Antioxidant and collagenase inhibitory activity of ether soluble phenolic components of n-butanol fraction (ESP-BF) of flaxseed

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Abstract

The present study was conducted to investigate antioxidant, collagenase inhibitory activity of ether soluble phenolic components of n-butanol fraction (ESP-BF) of flaxseed. Results demonstrated that ESP-BF had potent DPPH radical scavenging (IC$_{50}$ 51.18 μg/ml) and anti-lipid peroxidation (IC$_{50}$ 70.74 μg/ml) activity better than standard, ascorbic acid. ESP-BF also showed a dose dependent increase in reducing power. However, the observed ABTS scavenging activity of ESP-BF was weaker than standard, trolox. ESP-BF showed high concentration of total phenolics than flavonoids. Preliminary phytochemical screening and HPTLC profiling of ESP-BF could exhibit presence of secoisolariciresinol diglucoside (SDG), flavonoids and phenyl propanoids. Furthermore, ESP-BF inhibited collagenase activity and had IC$_{50}$ of 78.80 μg/ml. In conclusion, ESP-BF possesses potent antioxidant and collagenase inhibitory activity, which could be due to its SDG and flavonoids content.

Keywords

Flaxseed, Antioxidant activity, Collagenase inhibitory activity, Phenols, Secoisolariciresinol diglucoside (SDG)

Introduction

Plants produce phenolic compounds for their protection. Plant phenolics generally divided into different types on the basis of their basic structure. It mainly includes simple phenols, phenolic acids, coumarins and isocoumarins, naphthoquinones, xanthones, stilbenes, anthraquinones, flavonoids, lignans, lignins and tannins. There are more than 8000 different known phenolic compounds with diverse structure and activities (Robbins, 2003). Among them, phenolic acids (derivatives of benzoic and cinnamic acids) and flavonoids are more common (Robbins, 2003; Dykes and Rooney, 2007). Accumulating evidences suggest that plant phenols have been receiving great research interest owing to their wide range of health promoting effects. Most of the health promoting effects of phenolics is associated with their antioxidant potential. The antioxidant capacity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers or metal chelators (Demiray et al., 2009).

Certain functional foods are known to exhibit multiple health effects. These days, flaxseed is becoming more popular as functional food owing to its high content of omega-3 fatty acid, alpha-linolenic acid (ALA) and lignan, secoisolariciresinol diglucoside (SDG). Moreover, it is also the richest source of different types of phenolic compounds (Kasote, 2013). These phenolics primarily include SDG, linusitamarin linocinamarin, daucosterol, herbacetin 3, 8-O-diglucoside, herbacetin 3,7-O-dimethyl ether, kaempferol 3,7-O-diglucoside, (−) pinoresinol diglucoside, phenolic acids and tannins. Interestingly, presence of most of these phenolics has been reported in the n-butanol fraction of flaxseed (Luyengi et al., 1993; Qui et al., 1999; Kasote et al., 2011a; Kasote et al., 2011b).

Thus far, SDG is most studied flaxseed phenolic compound pertaining to its in vitro antioxidant potential. SDG reported to have DPPH and hydroxyl radical scavenging, and lipid peroxidation inhibitory activity (Qui et al., 1999; Prasad, 1997; Kitts et al., 1999). Besides this, moderate DPPH radical scavenging potential was shown to flaxseed flavonoids herbacetin 3,8-O-diglucoside, herbacetin 3,7-O-dimethyl ether, kaempferol 3,7-O-diglucoside, and (−) pinoresinol diglucoside (Qui et al., 1999). In our earlier study we were selectively isolated ether insoluble phenolic components such as caffeic acid, tannins and phenolic acid glycosides from n-butanol fraction of defatted flaxseed meal (Kasote et al., 2011a; Kasote et al., 2011b). Flaxseed lignans and flavonoids could be present in ether soluble fraction n-butanol fraction; and they may have potent antioxidant potential jointly rather than individual entity; however, detail study in this context has not been reported.
been undertaken so far. Hence, aim of the present study was to isolate, characterize ether soluble phenolic components of n-butanol fraction (ESP-BF) and evaluate their antioxidant, collagenase inhibitory potential.

Material and Methods

Chemicals

Hexane, methanol, butanol, solvent ether was purchased from molychem, Mumbai, India. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), disodium hydrogen ortho-phosphate, potassium dihydrogen phosphate, sodium chloride, trichloroacetic acid, potassium per sulfate, potassium ferricyanide, ferric chloride, ABTS (2, 2’-azinobis (3-ethylbenzthiazoline-6-sulphonic acid), TPTZ (2, 4, 6-tripyridyl-s-triazine), ascorbic acid, thioarbituric acid (TBA), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), butylated hydroxyl toluene (BHT), quercetin, gallic acid, collagenase enzyme II, and gelatin powder were procured from Sigma-Aldrich, USA.

De-oiled flaxseed meal and extraction of ESP-BF

A double pressed flaxseed cake powder obtained from “Omega-3 oil unit, under project NAIP-ICAR Component-3, Sangamner, MS, India” was used for extraction. Residual oil from de-oiled flaxseed powder was removed by n-hexane (1:4, w/v). The dried flaxseed powder (100 g) was extracted with methanol for 3 hrs at 60°C in a soxhlet apparatus. Methanol extract was concentrated in rotavapour at 60°C under vacuum. The dried residue further partitioned with n-butanol: water (1:1, v/v). n-Butanol fraction was separated out and concentrated in rotavapour at 80°C under vacuum. Dried n-butanol residue was dissolved in minimum quantity of methanol and ether insoluble phenolic compounds were precipitated with solvent ether (1:5, v/v). Solvent ether supernatant was separated, filtered and concentrated in rotavapour under vacuum. The resultant yellowish brown colored residue (0.8 g) was finally dissolved in methanol, and used for further assay as ESP-BF.

Preliminary phytochemical screening

Preliminary qualitative phytochemical screening of ESP-BF was carried out for the presence of carbohydrates, reducing sugars, phenols, flavonoids, saponins, cardiac glycosides, cyanogenic glycosides, alkaloids and terpenoids by using standard described procedures (Okigbo et al., 2009; Shrivastav et al., 2009).

HPTLC analysis

High performance thin layer chromatography (HPTLC) profile of ESP-BF was prepared by using CAMAG HPTLC system. Silica gel plate (Silica gel 60 F254, Merck) was used for HPTLC analysis. 2 µl of ESP-BF was applied onto the plates as 6 mm band, 8 mm from the bottom edge by means of a 50 µL Hamilton microsyringe. The separation was carried by using solvent system butanol: water (5:4, v/v, upper layer). The chromatogram was developed in glass twin-trough chamber (10 cm × 10 cm, with metal lids; CAMAG, Switzerland) previously saturated with mobile phase vapor for 20 min. The development distance was 70 mm. After development, the plate was dried with dryer. Chromatogram was developed at 254 nm for both ESP-BF and standard, SDG.

In vitro anti-oxidant assays

DPPH radical scavenging assay

DPPH radical scavenging assay was performed according to method described by Brand-Williams et al. (1995) with slight modification. Different concentrations (0.01 to 0.1 mg/ml) of ESP-BF and ascorbic acid (standard) were mixed with 5 ml of methanolic DPPH solution (33 mg/l) and the resulting solution was kept in dark at 37°C for 20 minutes, before the absorbance was measured at 517 nm. The percentage radical scavenging activity was calculated from the following formula:

\[
\text{% scavenging [DPPH] = \left[ \frac{A_0 - A_1}{A_0} \right] \times 100}
\]

Where, \(A_0\) was the absorbance of the control and \(A_1\) was the absorbance of ESP-BF/Ascorbic acid.

Total anti-oxidant capacity (ABTS assay)

The method of Re et al. (1999) was adopted for the determination of ABTS activity of ESP-BF. This assay is based on decolorization that occurs when the radical cation ABTS\(^+\) is reduced to ABTS\(^-\) (2, 2’-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid). In brief, the radical was generated by reaction of a 7 mM solution of ABTS in water with 2.45 mM potassium per-sulphate (K\(_2\)SO\(_8\)) (1:1). The mixture was held in dark-ness at 27°C for 16 h (time needed to obtain stable absorbance at 734 nm). After incubation, the radical solution was further diluted with water (1 ml of ABTS reagent + 27 ml distil water) until the initial absorbance value of 0.7 ± 0.005 at 734 nm was reached. For the assay of test samples 980 µl of ABTS\(^+\) reagent was mixed with 20 µl of the sample or standard. Absorbance was taken after 6 min at
The absorbance difference was calculated between initial (0 min.) and 6th min. reading. Trolox (0.01 to 0.1 mg/ml) was used as a standard.

The percentage of scavenging inhibition capacity of ABTS•+ of the ESP-BF was calculated using the following equation and compared with standard, Trolox.

\[
\%\text{ inhibition} = \left[ \frac{(A_0 - A_i)}{A_0} \right] \times 100
\]

Where, \(A_0\) was the absorbance of the control and \(A_i\) was the absorbance of ESP-BF/Trolox.

**Anti-lipid peroxidation assay**

Lipid peroxidation inhibitory effect of ESP-BF was determined according to method described by Pawar et al. (2011). Briefly, goat brain was perfusing with 0.15 M KCl. A mixtures containing 0.5 ml of tissue homogenate, 1 ml 0.15M KCl and 0.5 ml different concentrations of ESP-BF were prepared. Lipid peroxidation was initiated by adding 100 µl of 1 mM ferric chloride and incubated at 37°C for 30 min. The reaction was stopped by adding 2 ml ice-cold 0.25N HCl (containing 15% trichloroacetic acid (TCA), 0.38% TBA and 0.2 ml 0.05% BHT. The reaction mixture was heated at 80°C for 60 min, cooled and centrifuged. Absorbance of the supernatant was measured at 532 nm against a blank, which contained all reagents except brain homogenate and ESP-BF. Identical experiments were performed to determine the normal (without ESP-BF and FeCl\(_3\)) and induced (with ESP-BF) lipid peroxidation level in the tissue. Ascorbic acid was used as a standard. The percentage of anti-lipid peroxidation effect was calculated by the following formula.

\[
\%\text{ALP} = \left[ \frac{A_{\text{FeCl}_3} - A_{\text{en}}}{A_{\text{FeCl}_3} - A_{\text{Normal}}} \right] \times 100
\]

Where, \(A_{\text{FeCl}_3}\): Absorbance of FeCl\(_3\); \(A_{\text{Normal}}\): Absorbance of control reaction; \(A_{\text{test}}\): Absorbance of test reaction containing ESP-BF.

**Total antioxidant activity (FRAP method)**

In addition to ABTS assay, total antioxidant activity was also determined by FRAP (ferric reducing antioxidant potential) method with some modifications (Benzie and strain 1999; Mukherjee et al., 2011). The stock solutions included 300 mM acetate buffer (3.1 g C\(_4\)H\(_7\)Na\(_2\)O\(_4\)•3H\(_2\)O and 16 ml C\(_2\)H\(_2\)O\(_3\)), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM FeCl\(_3\)•6H\(_2\)O solution. The fresh working solution was prepared by mixing 30 ml acetate buffer, 3 ml TPTZ, and 3 ml FeCl\(_3\)•6H\(_2\)O. The temperature of the solution was raised to 37°C before using. ESP-BF (150 µl) was allowed to react with 2850 µl of the FRAP solution for 30 min in the dark condition. The absorbance of colored product (ferrous tripyridyltriazine complex) was measured at 593 nm. FeSO\(_4\) was used as a standard and results were expressed in µM Fe (II)/g dry mass.

**Reducing power assay**

The Fe\(^{2+}\)-reducing power capacity of ESP-BF was performed according to method described by Hazra et al. (2008) with some modification. Briefly, different concentrations (0.01 to 0.1 mg/ml) of ESP-BF in phosphate buffer (0.2 M, pH 6.6) were mixed with potassium hexa-cyanoferrate (0.1%). The mixture was incubated at 50°C for 20 min. 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 FeCl\(_3\) (0.1%) and the absorbance was measured at 700 nm. A higher absorbance of the reaction mixture indicated greater reducing power.

**Collagenase II inhibitory activity**

The collagenase II inhibitory activity was measured according to method described by Sawabe et al. (1998) with a slight modification. Briefly, 50 mM Tris-HCl was added to 10 mg of gelatin powder in such a way that 2 ml of total volume was maintained. In another tube, 40 µl of Collagenase II enzyme was to add various concentrations of ESP-BF. After 15 minute incubation at room temperature, enzyme-ESP-BF mixture was mixed with gelatin solution and re-incubated at 37°C for 30 minute. 2 ml of Ninhydrin solution was added to 200 µl of upper layer of incubated samples. Tubes were kept in boiling for 20 minutes; and the absorbance was at 570 nm after cooling against blank and control. The percentage was calculated by using following formula:

\[
\%\text{ inhibition} = (\text{Control} - \text{Sample})/ \text{Control} \times 100
\]

**Determination of total phenolic content**

Total phenolic content was determined using Folin-Cio-calteu method (Zahin et al., 2009) with a slight modification. Briefly, 0.5 ml of ESP-BF was mixed with 5 ml of Folin-Cio-calteu reagent (1:10 diluted). 4 ml 1M Na\(_2\)CO\(_3\) solution was added to the mixture. After incubation at room temperature for 15 min, the absorbance was measured at 765 nm. The phenolic content was evaluated from a gallic acid standard curve.
Determination of total flavonoid content

The total flavonoid content was determined with aluminium chloride ($\text{AlCl}_3$) method (Asgarirad et al., 2010). 0.5 ml of ESP-BF was added to 1.5 ml of distilled water followed by 0.1 ml $\text{AlCl}_3$ (10%). Reaction mixture was incubated at room temperature in dark after addition of 0.1 ml potassium acetate and 2.8 ml distilled water. The absorbance was measured at 415 nm. The flavonoid content was calculated from a quercetin standard curve and results were expressed as mg/g of dry mass.

Statistical analysis

All data are given as the mean of three replicates ± SD. Results were processed by computer programmes: Excel (2007). The IC$_{50}$ values calculated using ‘$y = mx + c$’ formula.

Results and Discussion

Preliminary phytochemical screening and HPTLC analysis

We developed simple approach for selective isolation of ESP-BF. Preliminary phytochemical screening of ESP-BF showed the presence of carbohydrates, phenols and terpenoids. HPTLC profiling of ESP-BF was carried out to in order to identify its phenolic constituents, and to quantify unique bioactive lignan, SDG. A novel solvent system, n-butanol:water (4:1, v/v) was employed to separate phenolic components of ESP-BF. Fig. 1 showed HPTLC chromatogram of ESP-BF and standard SDG at 254 nm. ESP-BF chromatogram showed 11 different peaks (Fig.1A). SDG showed a single peak at Rf 0.45 (Fig.1B). ESP-BF chromatogram showed the presence of SDG; and its content was 15.19%. Photo-documentation of HPLTC plate under UV at 254 and 366 nm was done in order to know the presence of flavonoids and phenylpropanoids (data is not shown). ESP-BF showed a dark band at 254 nm. At 366 nm ESP-BF exhibited fluorescence, blue, pink, yellow bands, which could confirm the presence of flavonoids and phenylpropanoids. However, further detailed chromatographic studies are essential to support our findings.

In vitro anti-oxidant assays

DPPH radical scavenging assay

Results showed that ESP-BF was effective in reducing the stable radical DPPH to the yellow colored diphenylpicrylhydrazine, indicating that ESP-BF had DPPH radical scavenging activity (Fig. 2A). ESP-BF demonstrated a dose dependent DPPH radical scavenging activity close to standard antioxidant ascorbic acid. IC$_{50}$ values of ESP-BF and standard ascorbic acid in this assay were 51.18 μg/ml and 53.56 μg/ml respectively. The IC$_{50}$ value of the ESP-BF was slightly less than that of the standard.
Total antioxidant capacity (ABTS assay)

The ABTS assay is based on the inhibition of radical cation, ABTS$^+$ by antioxidants (Murali et al., 2011). In ABTS assay, ESP-BF showed potent scavenging effects against ABTS with an IC$_{50}$ value 161.44 μg/ml. Trolox was used as reference standard; had IC$_{50}$ value 126.96 μg/ml. Results of this study confirmed that total antioxidant capacity of ESP-BF was close to standard, Trolox (Fig. 2B).

Anti-lipid peroxidation assay

It has been well established that lipid peroxidation in biological systems is a toxicological phenomenon initiated by ROS; that can lead to various pathological consequences (Hochstein and Atallah, 1988). ESP-BF showed a dose dependent increasing anti-lipid peroxidation potential; and had IC$_{50}$ value 70.74 μg/ml. Anti-peroxidation potential of ESP-BF was compared with standard antioxidant, ascorbic acid (Fig. 2C). The resultant IC$_{50}$ value for ascorbic acid was 126.2 μg/ml. The IC$_{50}$ value of the ESP-BF was less than that of the standard.

Total antioxidant activity (FRAP method)

FRAP is the simple and rapid method, based on the reduction of a ferroin analog, the Fe$^{3+}$/ferricyanide complex to the ferrous form. This reducing capacity could serve as a significant indicator of its potential antioxidant activity (Chung et al., 2002; Gulcin et al., 2010). ESP-BF exhibited well reducing power; and the resultant reducing of ESP-BF was increases with increasing concentration (Fig. 3A).

Gelatinolytic Inhibition of Collagenase II

The agents that inhibit collagenase activity may have beneficial effects for maintaining healthy skin by preventing dermal matrix degradation. It has been demonstrated that certain flavonoids, particularly the flavonols, may prevent collagen breakdown by inhibiting collagenase in inflamed skin as well as photo-aged skin (Sin and Kim, 2005). Results showed that ESP-BF had potent Collagenase II activities. Collagenase II inhibitory potential of ESP-BF was increased with elevating concentration; and showed an IC$_{50}$ of 78.80 μg/ml (Fig. 3B). The observed collagenase inhibitory potential of ESP-BF could be due to its SDG and flavonoids content. However, further details studies are warranted in this regard.

Total phenol and flavonoid contents

Plant phenolics including flavonoids are the major class secondary metabolite possessing potent antioxidant activity. Accumulating evidences suggest that there is direct correlation between antioxidant activity and phenolic content (Valerga et al., 2012; Gao et al., 2011). The resultant total flavonoid and phenol contents of ESP-BF were 76.56 ± 2.5 mg gallic acid equivalent/g of dry mass and 55.17 ± 5.9 mg quercetin equivalent/g of dry mass respectively. ESP-BF appeared to have a higher concentration of total phenols than flavonoids.

Conclusions

In the present study we have first time demonstrated that ESP-BF possess potent different levels of antioxidant and free radical scavenging activities, comparable or higher to those of standard antioxidants such as ascorbic acid and Trolox. HPTLC profiling substantiates the presence of SDG in ESP-BF. The observed antioxidant activities of ESP-BF could be due to its SDG, flavonoid and phenylpropanoid content. Moreover, ESP-BF has strong collagenase inhibitory potential. The findings of this study confirmed that ESP-BF could have potential application in the health and food industry. However, further in vivo studies in the context of safety and efficacy are essential.
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References


