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Research Article

ANTIOXIDANT AND ANTI-INFLAMMATORY EFFECTS OF *PHASEOLUS VULGARIS* L. SEEDS ETHANOL EXTRACT: AN IN-VITRO STUDY

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ABSTRACT

Aim: The aim of the present study was to find the antioxidant and anti-inflammatory potential of *Phaseolus vulgaris* L. seeds ethanol extract using in-vitro models. Method: The antioxidants effects of *Phaseolus vulgaris* L. seeds ethanol extract was determined using free radical scavenging assay, reducing power assay, hydrogen peroxide radical scavenging and metal chelating activity. Inhibition of protein denaturation, anti-proteinase action and membrane stabilization methods which includes heat induced haemolysis, hypotonicity-induced haemolysis and anti-lipoxygenase activity were used to resolve the power of anti-inflammatory agents. IC_{50} values are calculated by linear regression method. Different concentrations (100 - 500 µg/ml) and (20 - 100 µg/ml) of *P.vulgaris* ethanol extract and reference standards respectively, were used to evaluate the antioxidant and anti-inflammatory activities. Results: The result suggests that the *Phaseolus vulgaris* L. seeds ethanol extract possesses antioxidant and anti-inflammatory activities when compared with standard drugs. Conclusion: From the study it is concluded that various phytoconstituents flavonoids and other related polyphenols present in *Phaseolus vulgaris* L. seeds ethanol extract are responsible for the antioxidant and anti-inflammatory activities.

Keywords: *Phaseolus vulgaris*, antioxidant, anti-inflammatory

INTRODUCTION

Phaseolus vulgaris L.(family: Fabaceae)also knowns as Bean, Common bean, Kidney bean, Haricot bean, Wax bean[1] is native to Tropical America and now widely cultivated throughout the tropics and temperate regions of the world including India and Pakistan[2]. Beans are highly nutritive and comparatively economical protein food. The phytochemical literature survey of *Phaseolus vulgaris* showed the presence of various phytoconstituents like anthocyanins[3], brassinosteroids[4], caffeic acid[5], catechic and gallic acid[6], coumestrol, daidzen[7], delphinidin[8], equol[7], ferulic acid[5], galactomanans[9], gallic acid, genistein[7], hemagglutinins[9], kaempferol[7], lectins[10], malvidin[8], myrecitin glycoside[11], para coumaricacid[5], petunidin[8], phaseolamin[12], phaseolin[8], para hydroxybenzoic acid[7], phytic acid[13], phytohaemagglutinin[10], proanthocyanidins[14], proanthocyanins[3], quercetin[7], robinin[5] and vanillic acid[7]. Traditionally, it is used as folk medicines for the treatment of acne, bladder, burns, cardiac, carminative, depurative, diabetes, diarrhoea, diuretic, dropsy, dysentery, eczema, hiccups, itch, kidney, resolvent, rheumatism, sciatica, and tenesmus. Various extracts of *P.vulgaris* have been assessed pharmacologically, showed antidepressant[15], analgesic[16], antiobesity[17], Cardiovascular disease[17], antimutagenic[18], antibacterial[19], antitubercular[19], anticancer[20], antidiabetic[21] , antifertility[22], anti-inflammatory[16], anti Parkinson[23], anti-oxidant[24], antifungal[25], antiviral[25], antiproliferative[26], hypolipidemic[27], α-amylase inhibitor[28] activities.

Plant belonging to family fabaceae are esteemed to be agreeable sources of antioxidants due to the presence of polyphenol compounds[29]. A major cause of cell damage or death is oxidative stress, caused by the accumulation of reactive oxygen species(ROS) in animal tissues which leads to progress of miscellaneous cancers and other diseases. Antioxidants have the ability to dismutate ROS which are produced by the oxidation processes in various cells in human beings and also by other external factors such as pollution, radiation and some dietary habits[30]. Inflammation is one of the body's nonspecific internal defensive system, tissue damage caused by burns due to heat, radiation, bacterial or viral invasion showed the similar response that results from the tissue reaction to an accidental cut[31].A survey of literature indicated no systemic approach has been made to evaluate the antioxidant and anti-inflammatory potential of *Phaseolus vulgaris* L. seeds ethanol extract. Therefore, the aim of this study involves determination of antioxidant and anti-inflammatory activity of *Phaseolus vulgaris* L. seeds ethanol extract by in-vitro method.

MATERIALS AND METHOD

Collection and identification of plant material

The seeds of *Phaseolus vulgaris* L. (family: Fabaceae) were purchased from local market Hisar, Haryana, India. The seeds were taxonomically identified by Dr. Sunita Garg, Scientist, CSIR-NISCAIR, New Delhi, India. A voucher specimen was deposited in the herbarium of NISCAIR (*P.vulgaris*; No. NISCAIR/RHMD/Consult/-2017/3112-61-3).

Preparation of crude extract

The coarsely powdered seeds (1 kg) were subjected to extraction with ethanolin a Soxhlet apparatus for 16 hours. The extract was dried and kept in a desiccator for further use.

Preliminary Phytochemical Screening

The extract obtained was subjected to qualitative tests for identification of various phytoconstituents in the plant according to standard procedures[32,33].

Assessment of in-vitro Antioxidant activity

Free radical scavenging assay

The reaction mixture (3.0 ml) consisted of 1.0 ml of2,2-dipheyl-1-picrylhydrazyl (DPPH)in methanol (0.3 mM), 1.0 ml of *Phaseolus vulgaris* ethanol extract (PVEE) dissolved in DMSO with a concentration range of (20-100 µl/ml) and 1.0 ml of methanol. It was incubated for 10 min in dark, then the absorbance was measured at 517 nm[34].

Gallic acid was taken as positive control. The percentage of inhibition was calculated using the formula:

Inhibition (%) = (A0 – A1 / A0) X 100

Where; A0= absorbance of control, A1 = absorbance of test

Reducing power assay

Different concentration range (20-100 µg/ml) of PVEE were prepared in dimethyl sulfoxide (DMSO). To 1 ml of diluted solutions, 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (30 mM) were added and incubated at 50°C for 20 min. Thereafter, 2.5 ml of trichloroacetic acid (600 mM) was added to the reaction mixture, centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) were mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride (6 mM) and absorbance was measured at 700 nm. Ascorbic acid was taken as positive control[35].

Hydrogen peroxide radical scavenging

A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (50mM, pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. PVEE (20-100 µg/ml) respectively in DMSO were added to hydrogen peroxide and absorbance at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was taken as positive control. The percentage of hydrogen peroxide scavenging was calculated as follows[35]:

% Scavenged (H_2O_2) = (A0 – A1 / A0) X 100

Where; A0 is the absorbance of control and A1 is the absorbance of test.

Metal chelating activity

0.1ml of the PVEE in the concentration range of (20-100 µg/ml) separately were added to a solution of 0.5 ml ferrous chloride (0.2 mM). The reaction was initiated by the addition of 0.2 ml of ferrozine (5 mM) and incubated at room temperature for 10 min and then the absorbance was measured at 562 nm. Disodium EDTA was taken as positive control[35,36]. The percentage inhibition of ferrozine- Fe2+ complex formation was calculated as:

[(A0- As)/As] × 100

Where A0 was the absorbance of the control, and As was the absorbance of the extract or disodium EDTA (positive control).

Anti-inflammatory activity

Inhibition of albumin denaturation

1ml of different concentrations of PVEE ranging from (100 - 500 µg/ml) and Aspirin (100 - 500 µg/ml) as a reference standard was mixed with 1% aqueous solution (1ml) of bovine albumin fraction by adjusting pH using small amount of 1N HCl, incubated at 37 ºC for 20 min, denaturation was induced by keeping the reaction mixture in a water bath at 51 º C for 20 min, after cooling the samples the turbidity was measured at 660nm[37,38]. The experiment was performed in triplicate. The Percentage inhibition of protein denaturation was calculated as follows:

Percentage inhibition = (Abs Control –Abs Sample) X 100/ Abs control

Where, Abs = absorbance.

Antiproteinase action

The reaction mixture (2 ml) was containing 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml PVEE of different concentrations (100 - 500 µg/ml). Aspirin (100 - 500 µg/ml) was used as a reference standard. The mixture was incubated at 37 ºC for 5 min and in a water bath then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. 2 ml of 70% perchloric acid was added to arrest the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank[38,39]. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated.

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

Membrane stabilization

Human Blood

The blood was collected from a healthy human volunteer who had not taken any NSAIDS for 2 weeks prior to the experiment and collected in heparinzed vacutainer. The blood was washed three times with 0.9% saline and centrifuged simultaneously for 10 minutes at 3000 rpm. The packed cells were washed with 0.9% saline and a 40% v/v suspension made using isotonic phosphate buffer which was composed of 154mM NaCl in 10mM Sodium Phosphate Buffer at pH 7.4 used as Stock erythrocyte or RBC suspension.

Heat induced haemolysis

Take a test tube having the reaction mixture (2ml) consisted of 1 ml PVEE of different concentrations (100 - 500 µg/ml) and 1 ml of 10% RBCs suspension and saline was taken as control while Aspirin (100 - 500 µg/ml) was used as a reference standard. The reaction mixture was incubated in water bath at 56 ºC for 30min. The tubes were cooled under running tap water, after incubation. The centrifugation process of the reaction mixture was carried out at 2500 rpm for 5 min and the absorbance of the supernatants noted at 560 nm[38,40]. The experiment was performed in triplicates for all the test samples. The Percentage inhibition of Haemolysis was calculated as follows:

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

Hypotonicity-induced haemolysis

1ml of phosphate buffer, 2ml of hyposaline and 0.5ml of human red blood cell suspension were separately mixed with various concentrations of PVEE (100-500µg/ml), reference sample, and control. The standard drug Diclofenac sodium (100-500µg/ml) was treated similar to test sample. Incubation process was carried out at 37 ºC for 30minutes and centrifuged at 3000rpm. The supernatant liquid was decanted and the haemoglobin content was estimated by using a spectrophotometer at 560nm[41]. The percentage haemolysis was calculated by assuming the haemolysis produced in the control as 100%.

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

Anti-lipoxygenase activity

Linoleic acid as substrate and lipoxidase as enzyme were used for assessing the anti-lipoxygenase activity. PVEE (100-500µg/ml) were dissolved in 0.25ml of 2M borate buffer pH 9.0 and added 0.25ml of lipoxidase enzyme solution (20,000U/ml) and incubated for 5 min at 25 ºC. After which, 1.0ml of linoleic acid solution (0.6mM) was added, mixed well and absorbance was measured at 234nm. Indomethacin (100-500µg/ml) was used as reference standard[40]. The percent inhibition was calculated from the following equation:

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

A dose response curve was plotted to determine the IC_{50}values. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity. All tests and analyses were run in triplicate and averaged.

Statistical analysis

Results are expressed as Mean ± SD, n=3. The difference between experimental groups was compared by One Way Analysis of Variance (ANOVA) followed by Dunnett's Multiple comparison test using the software Graph Pad Instat. Linear regression analysis was used to calculate IC50 value. The differences were adjudged to be statistically significant when $P<0.05$.

RESULTS AND DISCUSSION

The preliminary phytochemical screening of ethanol extract of seeds showed the presence of polyphenols, saponins, glycosides, tannins, tri-terpenoids and flavonoids. These phytoconstituents are responsible for many types of medicinal activities like antioxidant, anti-inflammatory etc[42].

Free radical scavenging assay

A significant free radical scavenging impact on DPPH was shown by *Phaseolus vulgaris* L. seeds ethanol extract, it results was concentration dependent (20-100 µg/ml) but standard showed the higher scavenging effect. Inhibition percentage of gallic acid and PVEE was represented in table no.1. IC_{50} values are 34.17 µg/ml and 68.11 µg/ml of gallic acid and *Phaseolus vulgaris* L. seeds ethanol extract. Despite the fact that the DPPH radical searching capacities of the concentrate were not as much as that of standard, the investigation demonstrated that the concentrates have the proton-giving capacity and could fill in as free radical inhibitors or foragers, acting conceivably as essential antioxidant agents. The abatement in absorbance of the DPPH radical caused by cell reinforcement was because of the searching of the radical by hydrogen gift. Outwardly, it is observable as a shading change from purple to yellow. Essentially, a higher DPPH radical-scavenging movement was related with a lower IC_{50} esteem. DPPH radical is a generally utilized substrate for quick assessment of antioxidant agent action in light of its solidness in the radical frame and effortlessness of the test.

Reducing Power Assay

The diminishing limit of compounds could fill in as pointer of potential antioxidant agent property. The reducing power was measured by reductive capacity of antioxidant agent and it was assessed by the change of ferric to ferrous oxide within the sight of the ethanol extract. PVEE showed great diminishing force was outlined in (table no.1). High reducing power was demonstrated by low IC_{50}. Ethanol extract showed dose dependent reducing power. The IC_{50} values were found to be 24.303 µg/ml and 64.48 µg/ml of ascorbic acid and PVEE.

Hydrogen peroxide radical scavenging

Every living cell made hydrogen peroxide (H_2O_2) which is an outcome of breath. It is inconvenient and must be expelled when it is created in the cell. The age of even low levels of H_2O_2 in organic frameworks might be imperative. Catalase enzyme was made in cells to evacuate H_2O_2. Diverse plant materials demonstrate distinctive measures of catalase movement. Hydrogen peroxide searching action relies on the phenolic substance of the concentrate, which can give electrons to H_2O_2 and in this manner killing it in to water. The *Phaseolus vulgaris* L. seeds ethanol extracts showed dose dependant results for H_2O_2 scavenging effect. Increased H_2O_2 radical scavenging effect was indicated by low estimation of IC_{50} value. The IC_{50} was figured as 80.75 µg/ml and 37.56 µg/ml of PVEE and ascorbic acid individually. Along these lines, the present investigation exhibited the noteworthy cell antioxidant action of the concentrate analysed.

Metal chelating activity

Ferrozine can quantitatively form complexes with Fe2+. In the presence of other chelating agents or antioxidants, the complex formation is disrupted, with the result that the purple colour of the complexes decreases. The chelating ability of ferrous ion is shown in table 1 which is dose dependent. The extract showed maximum inhibition, 51.23±0.46% at 100 µg/ml concentration. The IC_{50} was calculated as 93.693 µg/ml and 42.42 µg/ml of PVEE and EDTA respectively.

Table 1: Results of antioxidant activity of PVEE

Conc.(µg /ml)	% Inhibition±SEM							
	Free radical scavenging assay		Reducing power assay		Hydrogen peroxide radical scavenging		Metal chelating activity	
	Gallic acid	PVEE	Ascorbic acid	PVEE	Ascorbic acid	PVEE	EDTA	PVEE
10	42.95±0.26[a]	32.25±0.16[a]	45.88±0.33[a]	35.37±0.47[a]	39.25±0.28[a]	28.25±0.52[b]	41.20±0.19[a]	32.25±0.12[a]
20	44.36±0.39[a]	35.46±0.82[a]	47.87±0.26[a]	38.08±0.36[a]	45.28±0.13[a]	31.05±0.85[a]	45.26±0.16[a]	34.52±0.71[b]
40	51.39±0.72[b]	40.28±0.26[a]	54.85±0.14[a]	45.16±0.45[a]	51.25±0.58[a]	36.04±0.69[a]	49.52±0.47[a]	39.52±0.26[a]
60	59.34±0.12[a]	48.95±0.28[b]	61.69±0.18[a]	52.77±0.26[b]	59.23±0.42[a]	45.25±0.46[a]	54.96±0.96[a]	42.52±0.28[a]
80	65.26±0.56[a]	52.95±0.48[a]	67.25±0.24[b]	56.79±0.24[a]	62.96±0.16[a]	50.26±0.35[a]	59.62±0.25[a]	47.25±0.79[b]
100	70.36±0.43[a]	59.85±0.82[a]	72.3±0.38[a]	62.13±0.15[a]	69.25±0.84[a]	55.23±0.41[a]	62.63±0.42[a]	51.23±0.46[a]
IC_{50}	34.17	68.11	24.303	64.48	37.56	80.75	42.42	93.69

All values are expressed as mean±SEM (n=3), The extracts groups are compared by Student t test with the gallic acid, ascorbic acid and EDTA respectively, a denotes $P < 0.01$, b denotes $P < 0.05$.

Anti-inflammatory activity

Inhibition of albumin denaturation

Secondary (2º) and tertiary structure (3º) of proteins are lost additionally, outside pressure or compound, for example, strong acid or base, a concentrated inorganic salt, an organic solvent or heat are responsible for denaturation of biological functions. Denaturation of proteins caused inflammation it is a very much reported case. Protein denaturation due to thermal induction shown by various anti-inflammatory drugs viz. salicylic acid, flufenamic acid and phenylbutazone in dose dependent manner. The ethanol extract of plant seeds showed inhibitory action on protein denaturation. Maximum inhibition percentage, (79±1.08%) and (77±1.15%) was estimated at 500 µg/ml concentration for Aspirin as standard and PVEE. The IC_{50} values were found to be 183.14 µg/ml and 238.14 µg/ml of aspirin and PVEE respectively, demonstrated in table 2.

Anti-proteinase action

Proteinases have been involved in ligament responses. Neutrophils are known to be a rich wellspring of serine proteinase and are limited at lysosomes. It was already detailed that leukocytes proteinase assume an imperative part in the improvement of tissue harm amid provocative responses and critical level of insurance was given by proteinase inhibitors. PVEE shows maximum percentage inhibition at 500 µg/ml 57±1.62%, which is slightly lower than standard 59±0.95%. The IC_{50} values were found to be 416.83 µg/ml and 456.19 µg/ml of aspirin and PVEE respectively, showed in table 2.

Membrane stabilization

The membrane stabilization of human red blood cells has been utilized as a technique to consider the invitro anti-inflammatory movement in light of the fact that the erythrocyte layer is practically equivalent to the lysosomal layer and its adjustment suggests that the concentrate may well settle lysosomal layers. Adjustment of lysosomal is vital in constraining the inflammatory reaction by keeping the arrival of lysosomal constituents of actuated neutrophil, for example, bacterial compounds and proteases, which brings about additional tissue irritation and harm upon additional cellular discharge.

Heat induced haemolysis

PVEE has been checked at various concentrations which is most effective 57±1.85% and 71±1.18% at 400 and 500 µg/ml for inhibiting the heat induced haemolysis, protect significantly ($p<0.05$) the erythrocyte membrane against lysis induced by heat while 310.76µg/ml and 358.82µg/ml was noted as IC_{50} for aspirin and PVEE respectively, showed in table 2.

Hypotonicity-induced haemolysis

The extract was effective to protect the erythrocyte membrane against lysis induced by hypotonic solution. Diclofenac sodium as a standard and PVEE showed a significant ($p<0.05$) protection 70±1.46% and 65±1.27% of membrane damage against hypotonic solution. IC_{50} was found to be 326.41 µg/ml and 363.55 µg/ml for standard and PVEE, illustrated in table 2.

Anti-lipoxygenase activity

Lipoxygenase (LOXs) are delicate to cell reinforcements and a large portion of their activity may comprise in restraint of lipid hydroperoxide arrangement because of lipidoxy scavenging or lipid peroxy-radical shaped in course of catalyst peroxidation. This can restrict the accessibility of lipid hydroperoxide substrate important for the synergist cycle of LOX. Indomethacin used as a standard and PVEE for anti-lipoxygenase activity which showed a significant anti-lipoxygenase inhibition 79±1.66% and 62±1.19% at 500 µg/ml concentration. IC_{50} was found to be 275.3 µg/ml and 384.8 µg/ml for indomethacin and PVEE, showed in table no.2. The PVEE extracts have potential to inhibit the lipoxygenase enzyme activity.

Table 2: Results of anti-inflammatory activity of PVEE

Conc. (µg/ml)	% Inhibition±SEM									
	Protein denaturation		Proteinase inhibitory action		Heat induced haemolysis		Hypotonicity induced haemolysis		Lipoxygenase inhibitory action	
	Aspirin	PVEE	Aspirin	PVEE	Aspirin	PVEE	Diclofenac Sodium	PVEE	Indo-methacin	PVEE
100	43±0.23[a]	38±0.71[a]	20±0.92[a]	15±0.37[a]	24±0.21[a]	19±0.24[a]	26±0.41[a]	21±0.84[a]	15±0.49[a]	12±0.33[a]
200	51±1.15[a]	45±0.33[a]	26±0.24[b]	22±0.86[a]	33±0.11[a]	25±0.75[a]	38±0.66[a]	35±0.55[a]	23±0.28[a]	24±0.49[a]
300	61±0.85[a]	56±0.28[a]	37±0.77[a]	31±0.49[a]	50±0.39[a]	38±1.12[a]	46±0.31[a]	41±0.39[a]	42±0.62[a]	31±0.58[a]
400	68±0.18[a]	64±0.74[a]	49±0.42[a]	43±0.61[a]	61±0.85[a]	57±1.85[a]	56±0.37[a]	54±0.54[a]	57±0.91[a]	49±0.55[a]
500	79±1.08[a]	77±1.15[a]	59±0.95[a]	57±1.62[a]	75±0.94[a]	71±1.18[a]	70±1.46[a]	65±1.27[a]	79±1.66[a]	62±1.19[a]
IC_{50}	183.14	238.14	416.83	456.19	310.76	358.82	326.41	363.55	275.3	384.8

All values are expressed as mean±SEM (n=3), The extracts groups are compared by Student t test with the aspirin, diclofenac sodium and indomethacin respectively, a denotes $P < 0.01$, b denotes $P < 0.05$.

The results obtained from our studies on PVEE have shown a potential effect of antioxidant anti-inflammatory activities.

CONCLUSION

Taking everything into account, show consider uncovered the in-vitro antioxidant and anti-inflammatory effects of *Phaseolus vulgaris* L. seeds ethanol remove. These exercises might be because of the nearness of polyphenolic mixes, for example, alkaloids, flavonoids, tannins, steroids, and phenols.This indicates that plant seed extract can be use for the treatment of various diseases related to inflammation and oxidative stress.In-vivo studies needs to be performed to confirm the claim. Further studies like isolation, identification and characterization of the pharmacologically potent moiety responsible for these activities are under progress in our research lab.

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