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Antioxidant potential of pod coats extracts of cluster bean and their efficacy in stabilizing soybean oil

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Abstract
Present investigation was conducted to determine phenolic composition and antioxidant activity of pod coat extracts of cluster bean and their effect on tocopherols and carotenoids content of crude soybean oil. Pod coats were extracted with solvents of varying polarity (acetone, ethyl acetate and chloroform). Extract yield as well as concentration of different phytoconstituents varied in different solvent. Highest yield was observed in acetone extract (5.44%). Total phenolics, flavonoids and tannins content were also highest in acetone extract (24.18±0.12 mg $GAEg^{-1}$, 10.77±0.09 mg $CAEg^{-1}$ and 2.48±0.03 mg $TAEg^{-1}$). Tocopherols were highest in ethyl acetate extract (7.10±0.04 mg/g). Acetone extract showed highest DPPH free radical scavenging activity i.e. 77.43%. Tocopherols (33.04 mg/100g of oil) and carotenoids content (20.48 mg/kg of oil) were highest in the soybean oil sample stabilised with acetone extract (2000ppm) of pod coat of cluster bean at the end of incubation period.

Keywords: Antioxidant activity, flavonoids, phenols, tocopherols and tannins

1. Introduction
Antioxidants are food additives which are used to protect food products from oxidative deterioration caused by free radicals. In ancient times, spices were used for flavour as well as preservation. Several synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) are used for preservation of food but they may be harmful for human consumption as many recent publications have indicated their possible toxic effects on human health as well as environment [1]. With rapidly increasing awareness about food additive safety, alternative antioxidants of natural origin has gained interest. Various plant extracts and essential oil have been screened for natural antioxidants.
Legumes being a rich source of quality proteins and micronutrients, hold an important place in daily diet of Indians. Incorporation of legumes in cereal based diet improves overall nutritional status as well as prevents protein calorie malnutrition [2]. Intake of legumes is associated with a number of positive health benefits such as anticarcinogenic, antiatherogenic, hypoglycaemic and hypocholesterolemic potential which are known to be linked with polyphenolic compounds and dietary fibres present in legumes [3]. Clusterbean (*Cyamopsis tetragonoloba* L.) is also popular as guar. It is a bumper crop of leguminosae family. It has been used as vegetable in India and Pakistan from hundreds of years. It is chiefly cultivated in the subtropical and semi arid region of north-west India (mainly in Rajahstan) and south-east Pakistan [4]. Guar seed is very popular in various industries due its galactomannan rich endosperm. Guar galactomannan is also known as guar gum, is used as a viscosity enhancer for both food and nonfood purposes. Fresh or dry forage of guar is also used as cattle feed. It is used as cover crop and green manure in agriculture [5]. Young tender pods of guar are eaten as a vegetable or snacks and the mature seeds can be consumed during food shortage. Hence present study is an attempt to determine the antioxidant potential of pod coat extracts of cluster bean and effect of addition of these extracts on oxidative quality of crude soybean oil.

2. Materials and Methods
2.1 Solvent extraction
The threshed pods of cluster bean were collected from the experimental field of CCS Haryana Agriculture University, Hisar, Haryana. Pod coats were separated from grass, stones and other plant parts and then grinded. The powdered samples were extracted with petroleum ether (60-80°). 100g dried defatted powdered sample of each crop were then extracted separately by the soxhlet method using ethyl acetate, acetone and chloroform for 8h.

These extracts were analysed of total phenols, tocopherols, flavonoids, tannins and DPPH free radical scavenging activity.

2.2 Estimation of total phenolic content

The total phenolic content was determined by Folin-Ciocalteu method using gallic acid as standard [6]. For estimation of total phenolics in various extracts, 1 ml extract was taken in a 100mL flask. Then 1 mL Folin-Ciocalteu reagent (1:1) and 2.0 mL of Na_2CO_3 (20%, w/v) was added, content was mixed and diluted to make final volume was made up to 50.0mL with double distilled water. The mixture was kept undisturbed for 25 minutes and then centrifuged at 6000 rpm for 8-10 minutes. Supernatant was taken and its absorbance was noted at 730 nm and total phenolic content was expressed in terms of gallic acid equivalent (mg $GAEg^{-1}$ extract).

2.3 Determination of flavonoids

The aluminium chloride colorimetric method was used for estimation of flavonoids [7]. Diluted extract (1mL) was taken in a test tube. To the extract, 5% $NaNO_2$ (0.3 mL) was added. After 5 minutes 10% $AlCl_3$ (0.3 mL) was added. Immediately, 1M NaOH (2 mL) was added and content was shaken vigorously for few seconds and then diluted with distilled water to make final volume 10 mL. Reddish pink colour obtained was measured at 510 nm against reagent blank. Flavonoids content of samples was expressed as catechin equivalent (mg $CAEg^{-1}$ extract).

2.4 Determination of tocopherol content

Total tocopherols were estimated by using α-tocopherol as standard according to method of Philip [8].

Reagents

2,2-Dipyridyl reagent (0.07% in 95% ethanol)
$FeCl_3.6H_2O$ reagent (0.02% in 95% ethanol)
Toluene
Ethanol

Method

Aliquots of 10, 20, 30, 40, 50 and 60 ppm solution of α-tocopherol in ethanol or extract were taken in different flask and ethanol was added to make to make a volume of 8mL then 1mL 2,2-dipyridyl reagent was added to this mixture, followed by 1mL $FeCl_3.6H_2O$ reagent. Resulting mixture was shaken for 10-15 seconds and then absorbance was measured at 520 nm. Same procedure was followed for the assessment of total tocopherols content in crude soybean oil samples taking 1g oil in place of extract.

2.5 Determination of tannin content

Tannin content was determined by Pearson method with slight modification using tannic acid as standard [9].

Reagents

Folin–Denis reagent
Tannic acid
Saturated sodium carbonate solution

Preparation of Folin-Denis reagent

A mixture containing 50g of sodium tungstate ($Na_2WO_4.2H_2O$), 10g of phospho molybdic acid, 350 mL of water, 25 mL of 85% phosphoric acid. Mixed the content till almost all the solids were dissolved and then refluxed gently for 3h. The contents were cooled, diluted to 500 mL and filtered. The normality of the reagent was determined by titration with 1N NaOH using phenolphthalein as indicator.

Method

One mL of standard tannic acid solution (0.01, 0.02, 0.03, 0.04, 0.05, and 0.06 mg/mL) or extract was taken in separate test tubes and then 1mL of Folin–Denis reagent was added to each test tube. After 5 minutes, 2.5 mL of saturated sodium carbonate solution was added. Final volume was made to 10 mL with double distilled water and mixed thoroughly. Thereafter mixtures were incubated at room temperature (approximately 30ºC) for 30 minutes and resulting blue colour was measured at 760 nm.

2.6 2,2'-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

Antioxidant activity of the extracts was expressed in terms of DPPH free radical scavenging activity [10]. Briefly 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 mg of extract were taken in different flasks. Then 2.5 mL of 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH: $0.025gL^{-1}$ in methanol) was added and total volume was made to 10 mL with methanol. Content were mixed by vortex for 1-2 minutes and then placed in spectrophotometer Spectronic 20 (Milton Roy Company). The absorbance was measured after every 15 minutes at 517 nm, as the reaction reached at plateau (time at steady state) gradually. The antioxidant activity was expressed as the percentage of decrease of the absorbance after 2 hours, relative to the control, corresponding to the percentage of DPPH scavenged.

Calculation

The percentage of DPPH scavenged (% $DPPH^*_{sc}$) was calculated using

$$\%\ DPPH\ ^*_{sc} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where $A_{control}$ is the absorbance of control and A_{sample} is the absorbance of the sample.

2.7 Addition of various extracts in soybean oil

The calculated amount of butylated hydroxy anisole (BHA) as well as extract was added to crude soybean oil samples taken in different beakers. The mixture was homogenised thoroughly. Samples were prepared in triplicate and incubated at 50^0C for 28 days. Control sample was also prepared. Required quantity of the samples was removed periodically and studied for tocopherols and carotenoids content.

2.8 Determination of carotenoid content in oil samples

Carotenoid content was determined by the method of Vasconcellous [11].

Reagent

Cyclohexane

Method

Oil sample (0.5g) was taken in 100 mL conical flask. The oil sample was dissolved in cyclohexane (2.5% w/v) and the absorbance was read at 417 nm and carotenoid content was calculated by using formula:

$$\text{mg carotene}/\text{kg oil} = \frac{(\text{absorbance at 417 nm}) \times \text{sample volume in mL}}{0.204 \times (\text{sample weight in g})}$$

2.9 Statistical analysis
Three replicate determinations of each sample were taken to minimize experimental error. Values were expressed as mean ± S.E. Correlation between phenolic constituents and antioxidant activity was calculated using Pearson correlation program in Online Statistical Analysis (OPSTAT www.hau.ernet.in).

3. Results and Discussion
3.1 Yield percentage, Total phenols, flavonoids, tocopherols and tannins
In cluster bean pod coat extracts, highest yield was obtained with acetone i.e. 5.44% however there is not much difference in extract yield of ethyl acetate (4.28%) and chloroform (4.03%). Extracts yield, total phenolics, flavonoids, tocopherols and tannins content of pod coat extracts of cluster bean are presented in Table 1. Total phenolics content (mg GAE/g) in pod coat of cluster bean was found to be highest in acetone extract i.e 24.18±0.12 followed by ethyl acetate (19.30±0.23) and chloroform (13.54±0.11). Similar trend was observed in flavonoids content. It was highest in acetone extract (10.77±0.09 mg CAE/g) followed by ethyl acetate (8.47±0.05 mg CAE/g) and then chloroform extract (6.20±0.07 mg CAE/g). Tocopherols content (mg/g) in cluster bean was maximum in ethyl acetate extract (7.10±0.04) followed by acetone (6.45±0.04) and chloroform (4.09±0.12) extracts. Very low tannin content was found in pod coats. Acetone extract of cluster bean had highest tannin content i.e. 2.48±0.03 followed by ethyl acetate (2.12±0.06) and lowest in chloroform extract (1.95±0.07).

Table 1: Phytochemical constituents of various pod coat extracts of clusterbean and pigeonpea

S. No	Phytochemical Constituents ↓ / Extracting Solvent →	Acetone extract	Ethyl acetate Extract	Chloroform Extract
1.	Yield (g/100g)	5.44±0.02	4.28±0.01	4.03±0.03
2.	Total Phenolics (mg GAE/g)	24.18±0.12	19.30±0.23	13.54±0.11
3.	Flavonoids (mg CAE/g)	10.77±0.09	8.47±0.05	6.20±0.07
4.	Tocopherols (mg/g extract)	6.45±0.04	7.10±0.04	4.09±0.12
5.	Tannin content (mg TAE/g)	2.48±0.03	2.12±0.06	1.95±0.07
6.	IC_{50} value of DPPH method (mg/mL)	0.23	0.34	0.63

Values are mean of three replicate determinations ± standard error
mg GAE/g- milligrams gallic acid equivalent/g of the extract (d.w.b.)
mg CAE/g- milligrams catechin equivalent/g of the extract (d.w.b.)
mg TAE/g- milligrams tannic acid equivalent/g of the extract (d.w.b.)

Extract yield, total phenols, flavonoids and tannins generally increase with increase in polarity of solvents used for extraction and similar trend was observed in current investigation. Lower yield of chloroform extract may be due to lower concentration of methylated derivatives of various phenols, terpenoids and sterols in pod coats. Various phenolic compounds like phenolic acids, flavonoids and tannins, found to have diverse biological properties such as anti-carcinogenic, anti-inflammatory and anti-atherosclerotic properties which might be linked to their antioxidant potential [12]. Litrature reports reveal the presence of various phenolic acids such as cholorogenic acid, sinapic acid, gallic acid, caffeic acids as well as flavonoids myricetin and Kaempferol in different cultivars of guar [13]. Results of present investigation are in close agreement with some previous studies [14,15].

3.2 DPPH free radical scavenging activity
Antioxidant activity was measured by DPPH method and presented in Fig. 2. DPPH is a considerably stable radical which displays maximum absorption at 517 nm. It reacts with antioxidant compounds present in extract and changes from 2,2-diphenyl-1-picrylhydrazyl to 2,2-diphenyl-1-picrylhydrazine. The maximum free radical scavenging activities exhibited by acetone, ethyl acetate and chloroform extract of cluster bean were 77.43%, 74.63% and 58.46% respectively, at the concentration of 0.7 mg/mL of the extract. The corresponding IC_{50} values to scavenge $DPPH^{\bullet}$ radical were 0.23, 0.34, and 0.63 mg/mL of the extract. Among three extracts, highest DPPH free radical scavenging activity of acetone extract might be due to higher phenolic and flavonoids content. The structure activity relationship (SAR) of flavonoids had been analyzed by Van Acker *et al.* (1996) [16].

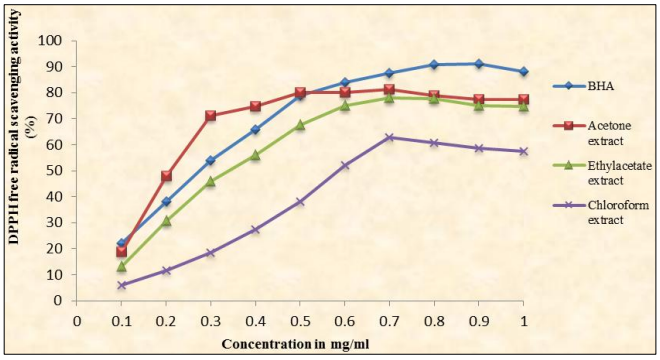

Fig 1: DPPH free radical scavenging activity (%) of BHA (standard) and pod coat extracts of clusterbean

In the second part of our study, crude soybean oil samples were supplemented with various pod coat extracts at 1000 and 2000ppm concentration. BHA was used as standard for comparison. BHA was added in crude soybean oil at 100 and 200ppm concentration. These crude soybean oil samples were kept at 50°C for a period of 28 days. Then effect of addition of various extracts as well as BHA on oxidative quality of oil was determined periodically in terms of tocopherols and carotenoids content.

3.3 Relationship between phytoconstituents and antioxidant activity of cluster bean pod coat extracts
To determine the role of various phytochemicals in DPPH free radical scavenging activity, correlation was calculated

and presented in Table 2. Correlation coefficients (r) were obtained between total phenols, flavonoids, tocopherols, tannins and IC_{50} value of DPPH method of different extracts. Correlation studies showed that there is a strong and highly positive correlation between total phenols and flavonoids with correlation coefficient r = 0.999**. Phenols showed positive correlation (r = 0.968*) with tannins as well. However phenols were negatively correlated with IC_{50} value of DPPH method (r = - 0.979*). Flavonoids have shown significant positive correlation with tannins (r = 0.980*) while negative correlation with IC_{50} value of DPPH method (r = - 0.967*). Rest of the correlation were found to be non- significant. Statistically significant correlation ($p<0.05$) was reported between polyphenolic content and antioxidant capacity values [17]. Reports revealed that significant positive correlation (p = 0.01) between total phenolics and total antioxidant activity values of acetone extracts (80% v/v) of fababean, broad bean, azuki bean, red lentil, green lentil and pea seeds [18].

Table 2: Correlation coefficient (r) between polyphenolic contents and their antioxidant activities observed in pod coat extracts of cluster bean

	Total phenols	Flavonoids	Tocopherols	Tannins	IC_{50} DPPH
Total phenols	1.000				
Flavonoids	0.999**	1.000			
Tocopherols	0.776^{NS}	0.742^{NS}	1.000		
Tannins	0.968*	0.980*	0.594^{NS}	1.000	
IC_{50} DPPH	-0.979*	-0.967*	-0.889^{NS}	-0.897^{NS}	1.000

*Significant at 5% level, ** significant at 1% level, NS= Non- Significant

3.4 Effect on carotenoids content of crude soybean oil

Carotenoids like α-carotene, β-carotene and β-cryptoxanthin have pro-vitamin A activity. Uptake of carotenoids reduces the risk of cardiovascular and degenerative diseases, macular degeneration, cataracts as well as particular type of carcinomas [19]. Due to the presence of double bonds in carbon chain, carotenoids easily undergo oxidation or isomerisation (cis-trans) during processing and storage, thus losses colour as well as biological activity. Many factors which influences the stability of carotenoids such as type and physical form of carotenoids, presence of metal, light, oxygen, severity of heat, and food matrix etc. [20]. Carotenoids content of all the soybean oil samples decreased regularly with increasing incubation period (Figure 2.). Carotenoids content of control sample decreased from 30.96 mg/kg to 13.59 during incubation. Sample stabilised with BHA (200ppm) showed least decline in carotenoids content as it was 23.19mg/kg at the end of incubation while it was 23.19 mg/kg in case of BHA (100ppm).

The carotenoids content of different samples stabilised with cluster bean pod coat extracts at the end of incubation period was in following order:

BHA (200ppm) > BHA (100ppm) > ACE (2000ppm) > ACE (1000ppm) > EAE (2000ppm) > CFE (2000ppm) > EAE (1000ppm) > CFE (1000ppm) > Control

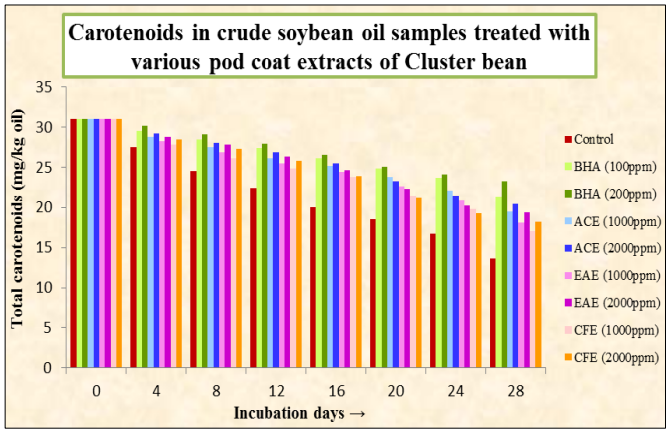

Fig 2: Total carotenoids in crude soybean oil samples stabilised with BHA and different extracts of pod coat of cluster bean

3.5 Effect on tocopherols content of crude soybean oil

Seed oils are rated as richest source of natural tocopherols. α-Tocopherol can react with singlet oxygen and produce hydroperoxide which decompose to give secondary oxidation products like tocopherolquinone and epoxytocopherol quinine [21]. During oxidation of lipids, tocopherols present in oil gradually undergo peroxidation. Tocopherol content in all the samples decreased during incubation (Figure 3). Tocopherols content (mg/100g) of control decreased from 38.25 (at zero time) to 24.87 (28th day). At the end of storage period, tocopherols content in crude soybean oil sample supplemented with cluster bean pod coat extracts was in the following order:

BHA (200ppm) > BHA (100ppm) > ACE (2000ppm) > ACE (1000ppm) > EAE (2000ppm) > EAE (1000ppm) > CFE (2000ppm) > CFE (1000ppm) > Control

Tocopherol content was higher in samples treated with acetone extract. All extracts were more effective at 2000ppm concentration than 1000ppm. This could be due to higher phenolic content of cluster bean extracts. Both tocopherols and carotenoids themselves act as natural antioxidants hence their concentration was not much affected during storage under accelerated condition (50°C) in present study. However carotenoids content showed large decrease than tocopherols.

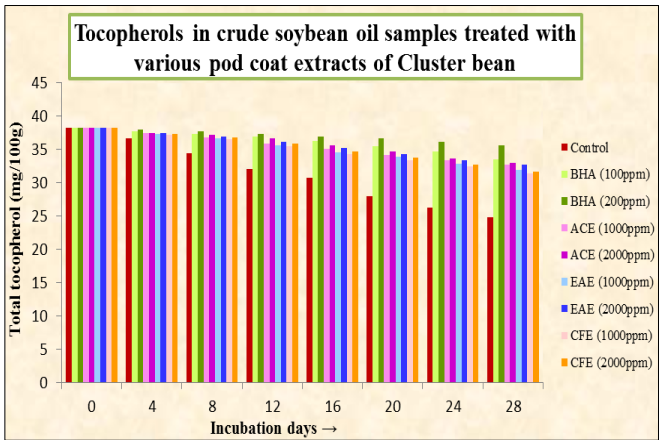

Fig 3: Tocopherols content in crude soybean oil samples stabilised with BHA and different extracts of pod coat of Cluster bean

4. Conclusion

It is evident from results of present study that pod coats of cluster bean contains significant amount of phenols,

flavonoids, tocopherol and posses remarkable antioxidant potential. Pod coat extracts of cluster bean lowered the rate of oxidative degradation of carotenoids and tocopherols hence they can be used as natural antioxidants in food industry.

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