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Interactive study of phytochemicals and their antioxidant efficiencies in Mungbean (*Vigna radiata* L.)

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Abstract
Mungbean seeds grown in India were evaluated for total phenolic content, hydrophilic and hydrophobic phenols, o-dihydric phenols, flavonoids and antioxidant potential (free radical scavenging capacity by DPPH, total antioxidant activity using ferric thiocyanate assay). Analysis of variance revealed significant differences in phytochemical constituents in different methanolic extracts. Results concluded that phenolic contents in seed parts as well as in cooked cotyledons correlated ($p<0.05$) significantly with antioxidant properties in most of the extracts. Hence, the methanolic extracts of seed coat showed highest phenolics (68.62 mg $GAEg^{-1}$) and highest scavenging efficiency against DPPH (0.61 mg/ml) as a consequence. The present study demonstrated mungbean as a legume enrich with high bioactive constituents as well as antioxidant potential and thus serve as future nutraceutical source.

Keywords: Mungbean, bioactive constituents, antioxidant, phenols

1. Introduction
Consumer's interest in natural products instead of synthetically produced commodities has grown considerably in recent years. Health-conscious nutritionists have long recommended natural products including spices and herbs in dishes to add flavour [1].Free radicals such as superoxide ions (O_2^-), hydroxyl (OH^-) and nitric oxide radicals (NO^-) are naturally produced in the body through normal metabolism of carbohydrates, amino acids and fats. Overproduction of free radicals can result in oxidative stress, a deleterious process that damages the cell structure. Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. There are two basic categories of antioxidants, namely, synthetic and natural. During recent years consumers have been more concerned about the addition of synthetic additives to food and the two most commonly used antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have shown DNA damage induction [2]. Therefore, there is an increasing interest in natural food additives, such as spices or spice extracts, which can function as natural antioxidants besides seasoning the food.
Pulses have a different range of natural phytochemicals that have antioxidative potential, tone of detoxification enzymes, impact on immune system and fall of inflammation, antibacterial and antiviral effects. They are also an excellent source of nuetraceutical constituents such as fibre, phytic acid and polyphenols such as flavonoids, isoflavones, lignans and tannins. Among the different pulses grown in the country, the respective share of production for green gram (*moong*) alone is 9 per cent [3]. Mungbean (*Vigna radiata* L.) or green gram is a tropical legume and its seeds are primarily used for food purposes. The potential use of mungbean protein hydrolysate prepared from tryptic hydrolysis as an antioxidative hydrolysate and as a carrier for anticancer asiatic acid is applicable on human hepatocellular liver carcinoma cells. In perspective of nutritional benefits and nutraceutical attributes of mungbean, characterization and compositional analysis of its seed are of great importance. All the legumes need to be processed before consumption to improve their palatability and nutritional properties. Among thermal processing, dehydration appears to be a healthy alternative to preserve the structure, improving the nutritional and sensorial quality of legumes. Hence, the objective of this study was to evaluate the profile and content of phenolic compounds and their relation with the antioxidant capacity as affected by processing like dehulling, cooking etc. in '*satya'* variety of mungbean grown in Haryana (India).

2. Materials and Methods

The present investigation was conducted in the MA & UUP Section, Department of Genetics & Plant Breeding, Chaudhary Charan Singh Haryana Agricultural University, Hisar (Haryana).

2.1 Extract preparation

For experimental analysis, healthy seeds of mungbean *var*. satya were provided by the Pulses section, Department of Genetics and Plant Breeding, CCS Haryana Agricultural University (India). After sorting damaged seeds, a major portion of seeds from each variety was de-husked to obtain seed coat and dal. Dehusked dal was divided into two sets. The first set was kept for extraction which constitutes intact dehusked seeds and the second part was cooked using distilled water in 1:10 (w/v). Thus, we obtained four samples of mungbean, 100 gm each for further analysis. Powdered samples were extracted separately by refluxing for six hours using methanol.

2.2 Determination of total phenolic content

Determination of total phenolic content was done by Folin-Ciocalteu reagent using gallic acid as standard [4].To a 50 ml volumetric flask 1.0 ml extract, 1.0 ml Folin–ciocalteau reagent and 2.0 ml of Na_2CO_3 (20%w/v) were added and mixed and final volume was made to 50 ml. The mixture was allowed to settle for 30 minutes and then centrifuged at 6000 rpm for 5-7 minutes. After centrifugation, the solution was measured colorimetrically at 730 nm using Shimadzu UV-Vis spectrophotometer (UV-2600). A blank was also prepared by following same aforementioned procedure without asample. After multiplication with the dilution factor, the concentration of phenolic content was expressed as equivalent to milligrams of gallic acid per gram of extract (mg $GAEg^{-1}$) by using the standard plot.

2.3 Determination of hydrophilic and hydrophobic phenolic contents

50 ml of crude extract was fractionated to obtain constituting hydrophilic and hydrophobic components by mixing the extract with deionized water and n-butanol (100ml each) in separating funnel as per Wettasinghe's method [5].The mixture was then allowed to stand until separate layers visible. Separated layers were then concentrated using rotavapor at 40°C temperature. After measuring the weight of each fraction, the phenolic content of each fraction was determined as per previous method.

2.4 Preparation of standard curve of o-dihydric phenols

o-dihydric phenols in methanol extracts were estimated by Arnow's method [6].using catechol as standard. 0.4 ml of extracted solutions were added to 1 ml 0.5N HCl, 1 ml Arnow's reagent and 2 ml 1N NaOH. The final volume was made to 10 ml using double distilled water. The intensity of resulting orange red color was measured colorimetrically at 515 nm using aspectrophotometer.

2.5 Determination of flavonoids

The aluminum chloride colorimetric assay [7], with modification was used. Briefly, 1 ml of diluted (1:4) extracts were added. A blank solution using doubled distilled water was prepared. Then 0.3 ml 5% $NaNO_2$ was added to the testing samples, followed by 0.3 ml 10% $AlCl_3$, 2 ml of 1M NaOH and the total volume was made10ml with dilution and mixed thoroughly. Then the absorbance was measured at 510 nm against blank. Total flavonoid contents were expressed as mg catechin equivalent per gram of the extract (mg CAE/g).

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

The antioxidant response of extracts against DPPH free radical was estimated [8]. Methanol treated extracts were dried completely and weighed. The dried mass of methanol extracts was redissolved in required volume of methanol to make the stock solution (1 mg/ml). Different concentrations (0.1-1 mg/ml) were made by appropriate dilutions with 100% methanol from the stock solution. In extracts of different concentrations, 2.0 ml of DPPH solution (0.025 g L^{-1} in 50% methanol) was added and the mixture was shaken and absorbance was measured at 515 nm at every 5 minutes interval until the reaction subsided. After 2 hours, the percent absorbance was declined corresponding to the percentage of DPPH scavenged which was an expression of antioxidant activity. By using Microsoft Excel Software, a quadratic regression equation ($y = ax^2+bx+c$) was obtained. By putting y = 50% in the equation $y = ax^2+bx+c$; it was converted to the form $ax^2+bx+c = 0$. IC_{50} was calculated from the equation $ax^2+bx+c = 0$ by using the formula:

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

where, $x = IC_{50}$ (mg/ml)

Calculation

The calculation for DPPH scavenged (% $DPPH^*_{sc}$) was done by following formulae:

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

Where $A_{control}$ and A_{sample} represents the absorbance of control and sample. Based on the results obtained after calculation a graph was made by plotting percent DPPH free radical scavenging activity or inhibition percentage (y-axis) against extract concentration (x-axis).

2.7 Ferric thiocyanate (FTC) method

The FTC method [9] was used for the evaluation of antioxidant activity of the extracts under study. After making required dilutions, samples were mixed with 5 ml of linoleic acid emulsion and final volume was madeto 10 ml using 0.2 M phosphate buffer (pH 7.0) and incubation was done at 37°C for 96 hours (4 days). After incubation aliquots of 0.1 ml were drawn from the incubated mixture after 24 hours interval and mixed with 30% ammonium thiocyanate, 20 mM ferrous chloride in 3.5% HCl and final volume was made to 10 ml with 75% ethanol and allowed to stand for 10 minutes. The color developed was measured colorimetrically at 500 nm using aspectrophotometer. By using quadratic regression equation ($y = ax^2+bx+c$) calculations were done as described earlier. A control mixture was prepared simultaneously following same procedure without the test sample.

Calculation: observed antioxidant activity was expressed as:
Antioxidant activity (%) = {1- (increase in abs. of sample/ increase in abs. of control)} x 100

2.8 Statistical analysis

Four replications of each sample were used for statistical analysis for minimizing random experimental error. Values obtained were expressed as mean± S.E. Both one way and

two-way analysis of variance (ANOVA) and F-test were carried out to assess significant differences in between means ($p<0.05$). Correlation analyses of polyphenolic composition and their antioxidant activities were carried out using Pearson correlation program in Online Statistical Analysis (OPSTAT www.hau.ernet.in).

3. Result and Discussion

Extract yield of all the four treatments of pulse crop did not varied widely. Extract yield of seed coat, raw dal, cooked dal and whole seed extracts of mungbean were 6.99, 6.43, 6.36 and 6.59 g/100g, respectively.

3.1 Estimation of total phenols in mungbean extracts

In mungbean extracts, it was observed that phenolic content was highest in seed coat (68.62 mg $GAEg^{-1}$). Corresponding value for total phenolics in raw dal, cooked dal, whole seed extracts was 41.37, 34.30, 55.02 mg $GAEg^{-1}$. For 100g sample, total phenols (in terms of gallic acid equivalents) were observed as 0.47g in seed coat extract, 0.26g in raw dal extract, 0.21g in cooked dal extract and 0.36g in whole seed extract (Fig.1)

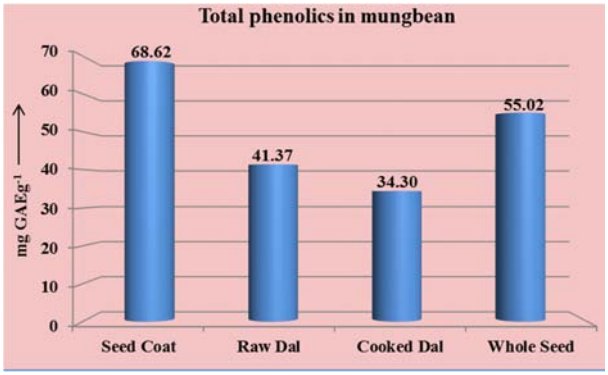

Fig 1: Total phenolics (mg $GAEg^{-1}$) in various extracts of mungbean

3.2 Estimation of ortho - dihydric phenolic content in mungbean extracts

In mungbean extracts, the methanolic extract of seed coat showed maximum o-dihydric phenolic content (43.95 mg $COEg^{-1}$) followed by whole seed extract (21.75 mg $COEg^{-1}$), raw dal extract (15.35 mg $COEg^{-1}$) and minimum in cooked dal extract (2.07 mg $COEg^{-1}$) (Fig. 2). Hence, order for o-dihydric content in different extracts of mungbean follows: seed coat > whole seed > raw dal > cooked dal

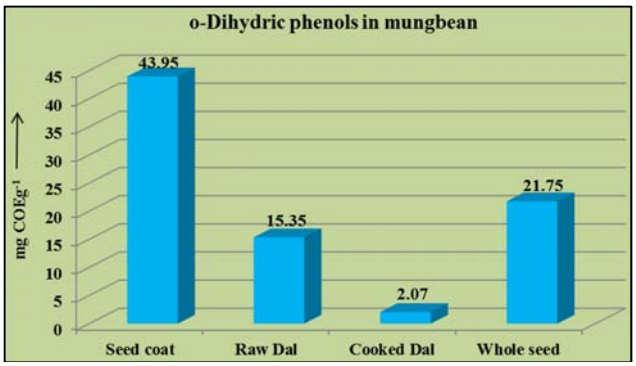

Fig 2: o-Dihydric phenolic content (mg $COEg^{-1}$) in various extracts of mungbean

3.3 Estimation of hydrophobic and hydrophilic phenolic content in mungbean extracts

Hydrophobic and hydrophilic phenols of all the four treatments of mungbean varied widely. In seed coat extracts, hydrophobic and hydrophilic phenols were 34.15 mg $GAEg^{-1}$ and 34.47 mg $GAEg^{-1}$ which exhibited 50% and 50% of total phenolics respectively. In case of raw dal and cooked dal extract hydrophobic phenols were (34% of total phenol) 14.02 mg $GAEg^{-1}$ and (27% of total phenol) 9.15 mg $GAEg^{-1}$ while hydrophilic phenols were (66% of total phenol) 27.35 mg $GAEg^{-1}$ and (73% of total phenol) 25.15 mg $GAEg^{-1}$ respectively. Similarly, in whole seed extract 31% and 69% of total phenols was constituted by hydrophobic and hydrophilic phenols. For 100g sample, highest amount of hydrophobic and hydrophilic phenols was present in seed coat extract i.e. 0.238g and 0.240g respectively (Table 1).

3.4 Estimation of flavonoid content in mungbean extracts

In mungbean extracts, the methanolic extract of seed coat showed maximum flavonoid content (4.78 mg $CAEg^{-1}$) followed by whole seed extract (1.28 mg $CAEg^{-1}$), raw dal extract (1.07 mg $CAEg^{-1}$) and minimum in cooked dal extract (0.13 mg $CAEg^{-1}$). Graphical representation for total flavonoid content (mg $CAEg^{-1}$) in mungbean extracts.

Table 1: Total hydrophobic and hydrophilic phenolic content (mg $GAEg^{-1}$) in mungbean extracts

Sr. No.	Character	Extract	(mg $GAEg^{-1}$ extract d.w.b.)					(mg GAE/100g sample d.w.b.)
			R_1	R_2	R_3	R_4	Mean	
1.	Hydrophobic phenolic content in mungbean	Seed Coat	32.20	35.00	35.40	34.00	34.15	238.70
2.		Raw Dal	13.50	14.40	15.00	13.20	14.02	90.14
3.		Cooked Dal	8.60	9.40	9.40	9.20	9.15	58.19
4.		Whole Seed	17.40	17.00	17.20	17.40	17.25	113.67
	SE(d)		0.60					
	CD at 5%		1.32					
	CV%		4.56					
		Extract	(mg $GAEg^{-1}$ extract d.w.b.)					(mg GAE/100g sample d.w.b.)
			R_1	R_2	R_3	R_4	Mean	
1.	Hydrophilic phenolic content in mungbean	Seed Coat	33.30	34.00	35.00	35.60	34.47	240.49
2.		Raw Dal	27.40	28.00	26.20	27.80	27.35	175.86
3.		Cooked Dal	26.20	24.20	25.80	24.40	25.15	159.95
4.		Whole Seed	38.60	37.20	37.20	38.00	37.75	248.77
	SE(d)		0.62					
	CD at 5%		1.38					
	CV%		2.85					

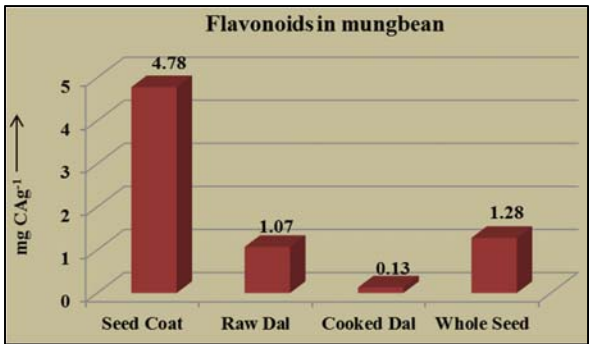

Fig. 3: Flavonoid content (mg $CAEg^{-1}$) in various extracts of mungbean

3.5 Evaluation of antioxidant activity in mungbean extracts

a) Evaluation of DPPH free radical scavenging activity

The corresponding IC_{50} values to scavenge DPPH• radical were 0.61, 0.83, 0.83, 0.69 mg/ml of the extract. The methanolic extract of seed coat showed maximum antioxidant activity in terms of radical scavenging capacity. Graphical representation for IC_{50} values in mungbean extracts is given in Fig. 4. The % of inhibition or antioxidant activity of methanolic extracts in ascending order is: cooked dal (64.60) < raw dal (65.00) < whole seed (70.00) < seedcoat (74.50).

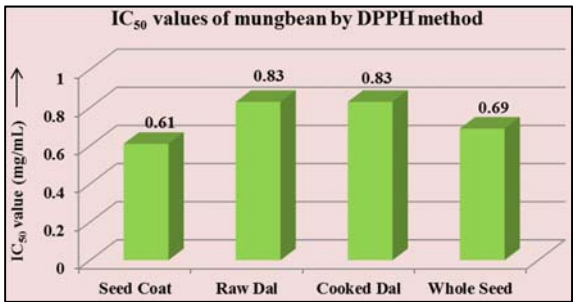

Fig. 4: IC_{50} values of mungbean extracts by DPPH free radical scavenging method

b) Evaluation of antioxidant activity by ferric thiocyanate (FTC) method

The maximum antioxidant activity exhibited by methanolic extracts of seed coat, raw dal, cooked dal and whole seed were 65.00%, 55.00%, 51.80% and 62.00% respectively. The corresponding IC_{50} values were 0.73, 0.87, 1.07, 0.82 mg/ml of the extract. The methanolic extract of seed coat showed maximum antioxidant activity. Graphical representation for IC_{50} values in mungbean extracts is given in Fig. 5.

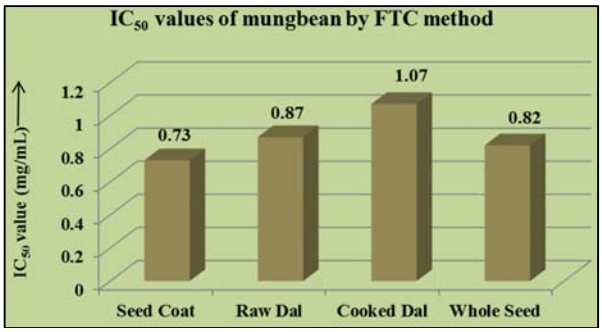

Fig 5: IC_{50} values of mungbean extracts by ferric thiocyanate (FTC) method

3.6 Relationship between various phytochemicals and antioxidant activities of mungbean extracts

The corresponding correlation values obtained for seed coat extract is shown in table 2. Total phenols were found to be highly correlated with flavonoid (r =0.985*) and with IC_{50} value by DPPH method (r =0.984*). The correlation between hydrophilic phenolic content and IC_{50} value by DPPH method was also positive and highly significant as r=0.956*. No significant correlation was analysed between IC_{50} values obtained by both the antioxidant activity methods (table 2).

The corresponding correlation values obtained for raw dal extract is shown in table 3. Total phenols were highly significant with o-dihydric phenols and IC_{50} value by FTC method with correlation coefficient value 0.965*, 0.952* respectively. There was a significant correlation observed between hydrophilic phenol and hydrophobic phenol (r =0.955*). A high and positive correlation was observed between IC_{50} value by DPPH method and flavonoid (r =0.972*). The correlation between IC_{50} value by DPPH method &IC_{50} value by FTC method was also positive and highly significant as r=0.992** Rest of the correlation values obtained for raw dal extract were found to be non-significant (Table 3).

Table 2: Correlation coefficient (r) between polyphenolic contents and their antioxidant activities observed in seed coat extract of mungbean

	Total Phenolics	o-Dihydric phenol	Hydro-philic phenol	Hydro-phobic phenol	Flavonoid	DPPH (IC_{50})	FTC (IC_{50})
Total Phenolics	1.000						
o-Dihydric phenol	0.170^{NS}	1.000					
Hydrophilic Phenol	0.917^{NS}	0.096^{NS}	1.000				
Hydrophobic phenol	0.832^{NS}	0.225^{NS}	0.542^{NS}	1.000			
Flavonoid	0.985^{*}	0.336^{NS}	0.888^{NS}	0.841^{NS}	1.000		
DPPH (IC_{50})	0.984^{*}	0.032^{NS}	0.956^{*}	0.744^{NS}	0.944^{NS}	1.000	
FTC (IC_{50})	0.793^{NS}	0.674^{NS}	0.800^{NS}	0.557^{NS}	0.871^{NS}	0.736^{NS}	1.000

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

Table 3: Correlation coefficient (r) between polyphenolic contents and their antioxidant activities observed in raw dal extract of mungbean

	Total Phenolics	o-Dihydric phenol	Hydro-philic phenol	Hydro-phobic phenol	Flavonoid	DPPH (IC_{50})	FTC (IC_{50})
Total Phenolics	1.000						
o-Dihydric phenol	0.965^{*}	1.000					
Hydrophilic Phenol	0.449^{NS}	0.200^{NS}	1.000				
Hydrophobic phenol	0.402^{NS}	0.626^{NS}	0.955*	1.000			
Flavonoid	0.839^{NS}	0.770^{NS}	0.574^{NS}	0.135^{NS}	1.000		
DPPH (IC_{50})	0.920^{NS}	0.826^{NS}	0.659^{NS}	0.118^{NS}	0.972^{*}	1.000	
FTC (IC_{50})	0.952^{*}	0.855^{NS}	0.663^{NS}	0.141^{NS}	0.934^{NS}	0.992^{**}	1.000

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

Table 4: Correlation coefficient (r) between polyphenolic contents and their antioxidant activities observed in cooked dal extract of mungbean

	Total Phenolics	o-Dihydric phenol	Hydro-philic phenol	Hydro-phobic phenol	Flavonoid	DPPH (IC_{50})	FTC (IC_{50})
Total Phenolics	1.000						
o-Dihydric phenol	0.973*	1.000					
Hydrophilic phenol	-0.278NS	-0.054NS	1.000				
Hydrophobic phenol	0.931NS	0.824NS	0.609NS	1.000			
Flavonoid	0.971*	0.995**	0.046NS	0.820NS	1.000		
DPPH (IC_{50})	0.962*	0.925NS	0.255NS	0.891NS	0.952*	1.000	
FTC (IC_{50})	0.959*	0.944NS	0.164NS	0.854NS	0.970*	0.996**	1.000

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

The respective correlation values obtained for cooked dal extract is shown in table 4 Total phenols were highly significant with o-dihydric phenols, flavonoids, IC_{50} value by DPPH method and IC_{50} value by FTC method with correlation coefficient value 0.973*, 0.971*, 0.962* and 0.959* respectively. There was a negative correlation observed between hydrophilic phenol, total phenols and o-dihydric phenol (r = -0.278) and (r = -0.054). A high and positive correlation was observed between o-dihydric phenol and flavonoid (r =0.995**). Similarly corresponding correlation coefficient values for relationship between flavonoid and both antioxidant activities were 0.952* and 0.970* respectively. IC_{50} values by both the method were also highly correlated (r =0.996**). Rest of the correlation values obtained for cooked dal extract were found to be non-significant (Table 4).

The respective correlation values obtained for whole seed extract is shown in table 5. Total phenols were highly significant with o-dihydric phenols, hydrophobic phenols, flavonoids, IC_{50} value by DPPH method and IC_{50} value by FTC method with correlation coefficient value 0.956*, 0.987*, 0.999**, 0.951* and 0.953* respectively. Hydrophobic phenols were highly significant with flavonoids, IC_{50} value by DPPH method and IC_{50} value by FTC method with correlation coefficient value 0.986*, 0.983* and 0.961* respectively. The correlation between flavonoid content and IC_{50} by FTC method was also positive and highly significant as r=0.960*. A positive non-significant correlation (r =0.902) was analysed between IC_{50} values obtained by both the antioxidant activity methods (table 5).

Table 5: Correlation coefficient (r) between polyphenolic contents and their antioxidant activities observed in whole seed extract of mungbean

	Total Phenolics	o-Dihydric phenol	Hydro-philic phenol	Hydro-phobic phenol	Flavonoid	DPPH (IC_{50})	FTC (IC_{50})
Total Phenolics	1.000						
o-Dihydric phenol	0.956*	1.000					
Hydrophilic Phenol	0.915NS	0.986*	1.000				
Hydrophobic phenol	0.987*	0.898NS	0.844NS	1.000			
Flavonoid	0.999**	0.958*	0.922NS	0.986*	1.000		
DPPH (IC_{50})	0.951*	0.827NS	0.746NS	0.983*	0.944NS	1.000	
FTC (IC_{50})	0.953*	0.878NS	0.866NS	0.961*	0.960*	0.902NS	1.000

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

These results indicated that different phenolic substances had different degrees of contributions to the overall antioxidant activities. Furthermore, the mixture of phenolic substances in the extract may have synergistic effect, which is affected by varying test conditions. In addition, it is increasingly apparent that phenolics can display various biological activities (anticancer, prevention of cardiovascular diseases) through a number of molecular mechanisms and that not all of these activities could be directly related. Some raw dal and cooked dal extracts were also having non-significant correlations between total phenolics and antioxidant activities. The results on legumes are in agreement with an earlier report [10] in which no significant correlation was observed between the total phenolic content and antioxidant activity among 92 plant extracts. The lack of correlation could be due to different responses of different phenolic compounds in different assay systems. Some research workers who investigated different crops also reported that there might not be a significant correlation between flavonoids content and antioxidant activity [11, 12, 13].

4. Conclusion

The suitable processing treatment for of legumes before consumption is a very important aspect to achieve the maximum concentration of desired phytoconstituents in suitable extracts. Dehulling and cooking are the important factors as the nature of phytoconstituents such as phenols and flavonoids present in pulses may be altered, which affects the antioxidant activity of pulse seeds. In this context, it was concluded that phytochemical constituents and antioxidant activity were dependent on the processing method. The results concluded that the Mungbean extract may be valuable natural antioxidant sources and are potentially applicable in both medicine and healthy food industry.

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