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COMPARATIVE ANTIOXIDANT QUALITY EVALUATION OF UNDERUTILIZED /LESS COMMON SOUTH INDIAN LEGUMES

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ABSTRACT

Legumes are the cheapest source of proteins with desirable nutritional qualities like ability to lower serum cholesterol with high fibre content and high concentration of poly-unsaturated fatty acids. In this context, underutilized legumes including tribal pulses viz, *Canavalia ensiformis* (jack bean), *Macrotyloma uniflorum* (horse gram) and *Phaseolus lunatus* (lima bean) were powdered and evaluated for antioxidant property and identification of functional groups of bioactive compounds. Among the legumes studied, jack bean registered higher levels of tannins (0.855 g/100g), flavonoids (0.063 g/100g), alkaloids (0.645 g/100g) and saponins (0.525 g/100g); while horse gram registered higher levels of total free phenolics (1.670g/100g). The antioxidant activity ranged from 0.15µg/mg to 1.02 µg/mg as ascorbic acid equivalents. The free radical scavenging activity of studied legumes with respect to nitric oxide, superoxide, iron (chelating) and reducing power capacity was accounted up to 21.11%, 19.11%, 27.11% & 1.23, respectively. FTIR peaks showed the presence of the compounds like amines, sulfonates, aliphatic organo halogens, and aliphatic and aromatic nitro compounds. Further, mechanistic studies on pharmacological evaluation are needed for commercial exploitation of these legumes as Nutraceuticals.

KEYWORDS*:* Macrotyloma uniflorum, Phaseolus lunatus, Canavalia ensiformis, Antioxidants, FTIR.

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INTRODUCTION

Phytochemicals with antioxidant property are naturally present in food are of great interest due to their beneficial effects on human health as they offer protection against oxidative deterioration[1]. Phenolic compounds, present in legumes with their antioxidative effects on human health, include phenolic acids such as gallic acid, caffeic acid, syringic acid, etc., flavonoids and other polyphenolic compounds. Many researchers indicated that flavonoids are mainly responsible for the antioxidant activity[2, 3, 4.] Antioxidants are radical scavengers which protect the human body against free radicals that may cause pathological conditions such as ischemia, anaemia, asthma, arthritis, inflammation, neuro-degeneration, Parkinson's diseases, mongolism, ageing process and perhaps dementias[5]. An increasing number of studies reveal that dietary antioxidants are capable of blocking neuronal death *in vitro* and many therapeutic properties in animal models of neurodegenerative diseases including Alzheimer's and Parkinson's diseases[6.] The antioxidant activity of dietary polyphenols is considered to be much greater than that of the essential vitamins[7]. Hence, studies on the evaluation and exploitation of phyto-nutrient compounds particularly phenolic acids, flavonoids and high molecular weight tannins of legumes as natural antioxidants have assumed great significance[8]. Legumes are an inexpensive source of proteins with desirable characteristic such as abundance of carbohydrates, ability to lower the serum cholesterol, high fiber, low fat (except oil seeds), high concentration of polyunsaturated fatty acids and a long shelf life[9]. Exploration and exploitation of unconventional legumes are promising methods to fulfil the deficiency of proteins and essential fatty acids in human nutrition as evidenced by studies on some wild legumes [10, 11, 12, 13]. Legumes produce primary and secondary metabolites and other products such as pharmaceuticals, anti - feedants and industrial products [14.] Plant secondary metabolites (PSMs), such as polyphenols exhibits antioxidant, antimutagenic, anticarcinogenic, antiinflammatory, and antimicrobial effects that might potentially be beneficial in preventing diseases and / or protecting the stability of the genome[15]. They can act as reducing agents (free radical terminators), metal ion chelators and singlet oxygen quenchers [16]and thus prevent oxidative damage to biomolecules, such as DNA, lipids and proteins. In this context, the present study has been undertaken to investigate the comparative profile of antioxidant activity and phytochemical properties of the seed extract of certain underutilized and little known South Indian legumes.

MATERIALS AND METHODS

Reagents and chemicals

Gallic acid, tannic acid, quercetin, sulphanilic acid, naphthyl ethylene diamine dihydro chloride (NEDA), ethylene diamine tetra acetic acid (EDTA), riboflavin, O-phenanthroline (Sigma-Aldrich), nitro blue tetrazolium (NBT), Folin-ciocalteu's reagent, ferric nitrate, ammonium molybdate, sodium nitroprusside, ferric chloride, potassium ferric cyanide were purchased from Sigma Aldrich, India. All other chemicals used are of Analytical grade and HPLC grade solvents.

Samples

Mature pods of *Macrotyloma uniflorum* (horse gram), *Phaseolus lunatus* (lima bean) and *Canavalia ensiformis* (jack bean) were purchased from local markets of Madurai, Tamil Nadu, India. After drying in the sun, the pods were thrashed to separate mature seeds. After a thorough cleaning and removal of broken seeds and foreign materials, the seeds were stored in plastic containers at room temperature (25°C) until further use. Dry mature seeds of different species (10 g each) were powdered in a Wiley Mill to 60-mesh size with suitable precaution to avoid contamination of samples.

Extract preparation

Ten grams of each powdered seed samples were shaken separately in ethanol for 72 hrs

on an orbital shaker at room temperature. Extracts were filtered using a Buckner funnel and Whatman No 1 filter paper. Each filtrate was concentrated to dryness under reduced pressure at 40ºC using a rotary evaporator. Each extract was re suspended in methanol to make 50 mg/ml stock solution

Estimation of Total Phenolics

The total phenolic content in the seed extracts were determined with the Folin-ciocalteu's reagent according to Wolfe and co-workers method[17]. The reaction mixture contained: 200 µl of diluted rice bran extract, 800 µl of freshly prepared diluted Folin Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The final mixture was diluted to 7 ml with deionized water. Mixtures were kept in dark at ambient conditions for 2 h to complete the reaction. The absorbance of seed extracts was measured at 765 nm using a spectrophotometer (Amersham BioSciences UltroSpec, 2100Pro, New Delhi). Total phenol content was expressed as gallic acid equivalents (GAE).

Estimation of Total Tannins

Colorimetric estimation of tannins was performed based on the measurement of blue color formed by the reduction of phosphotungstomolybdic acid by tannin like compounds in alkaline solution[18]. A known amount of extract was mixed with 5.0 ml of Folin- Denis reagent (FD) and Na_2CO_3 solution and made up to 100 ml, mixed well and absorbance was read at 760 nm after 30 min using spectrophotometer (Amersham BioSciences UltroSpec, 2100Pro, New Delhi). Total tannin content expressed as mg tannic acid equivalent /100 g of sample (TAE).

Estimation of Total Flavanoids

Total flavonoid content was determined using aluminium chloride ($AlCl_3$) according to a known method[19]. The plant extract (0.1 ml) was added to 0.3 ml distilled water followed by 5% $NaNO_2$ (0.03 ml). After 5 min at 25°C, $AlCl_3$ (0.03 ml, 10%) was added. After further 5 min, the reaction mixture was treated with 0.2 ml of 1 mM NaOH. Finally, the reaction mixture was diluted to 1 ml with water and the absorbance was measured at 510 nm using a spectrophotometer (Amersham BioSciences UltroSpec, 2100Pro, New Delhi). Total flavanoid content was calculated as quercetin equivalents (QE).

Estimation of Total Alkaloids

The total alkaloid contents in the seed samples were measured using 1,10-phenanthroline method[20] with slight modifications. 100mg seed powder was extracted in 10ml 80% ethanol. This was filtered through muslin cloth and centrifuged at 5000rpm for 10 min. Supernatant obtained was used for the further estimation total alkaloids. The reaction mixture contained 1ml seed extract, 1ml of 0.025M $FeCl_3$ in 0.5M HCl and 1ml of 0.05M of 1, 10-phenanthroline in ethanol. The mixture was incubated for 30 min in hot water bath with maintaining a temperature of 70 ± 2ºC. The absorbance of the red coloured complex was measured at 510nm against the reagent blank. Alkaloid contents were estimated and it was calculated with the help of standard curve of colchicines (0.1mg/ml, 10mg dissolved in 10ml ethanol and diluted to 100ml with distilled water).The values were expressed as g.100g-1of dry weight (CE).

Estimation of Total Saponins

The total saponin content in the seed powdered extract was studied by previously established procedure[21]. 20 g of powdered sample was treated with 100 ml of 20% aqueous solution of ethanol, heated over a hot water bath for 4 h at about 55°C with continuous stirring. The mixture was filtered and the residue re-extracted. The combined extracts were reduced to 40 ml over a water bath at about 90°C and the concentrate was transferred into a separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and 60 ml of n-butanol was added to the combined extracts and washed twice with 10 ml of 5% aqueous NaCl. The remaining solution was heated in a water bath, dried in an oven to a constant weight and the saponin content was calculated as g $100g^{-1}$.

Evaluation of Total antioxidant capacity

The total antioxidant capacity of the seed extracts was assessed by phosphomolybdenum method[22]. An aliquot of 0.3 ml of the sample solution was mixed with 2.7 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The absorbance of the test sample was measured at 695 nm. Gallic acid was used as standard. The antioxidant activity was expressed in the samples as ascorbic acid equivalents (mg/g of ethanol extract).

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was measured by the method described by Garrat[23]. The reaction mixture (3ml) containing 2 ml of sodium nitroprusside (10mM), 0.5 ml of phosphate buffer saline (1M) were incubated at 250ºC for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite was pipetted and mixed with 1 ml of sulphanilic acid reagent (0.33%) and allowed to stand for 5 min for completing diazotization. Then 1 ml of naphthylethylene diamine dihydrochloride (1% NEDA) was added, mixed and allowed to stand for 30 min. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions which can be estimated by the use of griess illusively reaction at 540 nm.

Superoxide radical scavenging activity

Measurement of superoxide anion scavenging activity was performed based on the method described by Winterbourne[24]. The assay mixture contained sample with 0.1ml of nitro blue tetrazolium (1.5 mM NBT) solution, 0.2 ml of EDTA (0.1M EDTA), 0.05 ml riboflavin (0.12 mM) and 2.55 ml of phosphate buffer (0.067 M phosphate buffer). The control tubes were also set up where in DMSO was added instead of samples. The reaction mixture was illuminated for 30 min and the absorbance at 560 nm was measured against the control samples. Ascorbate was used as the reference.

Iron chelating activity

The method of Benzie and Strain was adopted for the assay[25]. The reaction mixture containing 1 ml of 0.05% O-Phenanthroline in methanol, 2 ml ferric chloride (200µM) and 2 ml of various concentrations of sample ranging from 10 to 1000µg was incubated at room temperature for 10 min and the absorbance of the same was measured at 510 nm. EDTA was used as a classical metal chelator.

Determination of Reducing Power assay

Reducing activity was carried out by following the method of Oyaizu[26]. Different concentration (1000, 500,250,125 µg /ml) of seed extracts (dry ethanol) were prepared with DMSO and taken in a test tube as triplicates. To test tubes 2.5 ml of sodium phosphate buffer pH 6 and 2.5 ml of 1% potassium ferric cyanide solution was added. These contents were mixed well and were incubated at 50° C for 20 minutes. After incubation 2.5ml of 10% TCA was added and were kept for centrifugation at 3000rpm for 10 minutes. After centrifugation 5 ml of supernatant were taken and to this 5 ml of distilled water was added. To this about 1ml of 1% ferric chlorite was added and was incubated at 35°C for 20 minutes. The absorbance was measured at 700nm using UV spectrophtotometer and the blank was prepared by adding every other solution but without extract and ferric chloride (0.1%) and the control was prepared by adding every other solution but without the extract. Increase in absorbance of the reaction mixture indicates increased reducing power. The experiment was conducted in triplicates and values are expressed as equivalents of ascorbic acid in µg / mg of extract.

FTIR (Fourier Transformer Infra red Spectrophotometer)

Fourier transformer infrared (FTIR) Spectrophotometer was used to identify the characteristic functional groups in the seed extract. A small quantity (5 mg) of the seed extract was dispersed in dry potassium bromide (KBr). The mixture was thoroughly

mixed in a mortar and pressed at a pressure of 6 bars within 2 min to form a KBr thin disc. Then the disc was placed in a sample cup of a diffuse reflectance accessory. The IR spectrum was obtained using Perkin Elmer 2000 Infrared spectrometer (USA). The sample was scanned from 400 to 4000 cm^{-1} for 16 times to increase the signal to noise ratio.

Statistical analysis

All data were reported as the mean ± SD of three measurements.

RESULTS AND DISCUSSION

The data on phytochemical quantitative analysis for the whole legume grains are presented in Table-1. The concentration of phenolic content, tannins, flavanoids, alkaloids and saponins obtained ranged from 1.670–1.226 g GAE/100g; 0.855-0.101 g TAE/100g; 0.638-0.038gQE/100g; 0.645-0.359g CE/ 100g and 0.520-0.117, respectively. While, the highest phenolic content i.e., 1.670 g/100g was registered in the seeds of horse gram, jack bean registered the lowest concentration (1.226 g /100g). The contents of extractable total phenolics and tannins of the dry heated samples were found to be higher than those of raw samples for each solvent. This could be attributed to the solubility of phenolics and other aromatic compounds. The presences of phenolic substances including tannins in horse gram have already been reported in earlier studies [27, 28]. With regard to tannin content, jack bean seeds were found to have higher levels (0.855g/100g), while the lowest concentration of 0.101g/100g was recorded in horse gram. Tannins are oligomeric higher molecular weight polyphenolic compounds occurring naturally in plants. The highest flavanoid content (0.638g/100g) was exhibited by jack bean; whereas the lowest concentration (0.038g/100g) was observed in horse gram. Plant flavonoids have attracted attention as potentially important dietary chemoprotective and antitumour agents. The highest concentration of alkaloids was exhibited (0.645g/100g) by jack bean and while the lowest concentration (0.359g/100g) was recorded in horse gram. Alkaloids affect several metabolic activities in the body and most possess dramatic physiological activities and hence, are widely used in medicine. The highest concentration of saponins content (0.520 g/100g) was recorded in jack bean and while the lowest concentration (0.117 g/100g) was obtained in horse gram. Saponins have both beneficial and adverse effects on human health. Contrary to their hypocholesterolemic property[29], saponins also exhibit hemolytic activity by reacting with the sterols of erythrocyte membrane[30]. The antioxidant activities of the investigated legumes appear to be in the range of 1.02µg/mg, jack bean 0.08µg/mg for lima bean and 0.15 µg/mg horse gram. From the above results it can be inferred that the wild legumes exhibit higher levels of antioxidant activity compared to common legumes.

Radical scavenging activity of legumes

The percentage of scavenging activity, viz., nitric oxide, superoxide, iron chelating and reducing power capacity of studied legumes, was accounted up to 21.11%, 19.11%, 27.11% & 1.23, respectively. The scavenging activity with respect to ascorbic acid equivalents is shown in Table- 2. All the extracts showed significant scavenging activity in a dose dependent manner at a concentration ranging from 125–1000 µg. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitric oxide[31]. Superoxide is a highly reactive molecule that reacts with various substances produced through metabolic processes. Superoxide dismutase enzymes present in aerobic and anaerobic organisms catalyses the breakdown of superoxide radical[32]. It causes lipid peroxidation through the Fenton and Haber-weiss reaction and decomposes the lipid hydroxide into peroxyl and alkoxyl radicals that can perpetuate the chain reactions[33]. This could be due to the presence of relative concentration of bioactive constituents and the mixture of impurities / other nutrients in the extracts. The radical-scavenging abilities of some commonly consumed and underutilized tropical legumes [cowpea (*Vigna unguiculata*), pigeonpea (*Cajanus cajan*) and

African yam bean (*Sphenostylis sternocarpa*)] were studied[14]. Metal chelating capacity was significant since they reduced the concentration of the catalysing transition metal in lipid peroxidation[34]. It was reported that chelating agents, which form α-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential thereby stabilising the oxidised form of the metal ions[35].

Fourier Transform Infrared Spectrophotometer (FTIR)Analysis

The Infrared Spectroscopic (IR) analysis of studied legumes, in a band width ranging from 400 to 4000 cm^{-1}, revealed the presence of different functional group (Table-3). The peaks indicate that the extract of legumes may have the compounds like alcohol, phenols, amines, alkenyl, methylene, sulfonates, aliphatic organo halogen, and aliphatic and aromatic nitro compounds. Further, it also indicates the possible chemical bond and compound type as follows (Fig 6&7), O-H stretching for alcohol (3398.97,3399.83 cm^{-1}), C-H stretching for methylene asymmetric/symmetric (2926.54,2926.26 cm^{-1}), C=C stretching for alkenyl (1658.26,1658.03 cm^{-1}),NO_2 stretching for aliphatic and aromatic nitro compounds (1547.86,1547.51 cm^{-1}) ,O-H bending for phenol or tertiary alcohol(1408.19,1408.06 cm^{-1}).SO_2 stretching for sulfonates(1161.05 cm^{-1}) and C-H bending for vinyl (985.09 cm^{-1}) only found in lima bean and jack bean.C-F stretching for aliphatic fluro compounds were present in only horse gram. C-H stretching for 1, 4- Di substitution para (860.09, 859.99 cm^{-1}), C-I stretching for aliphatic organo halogens (574.61, 573.89 cm^{-1}) were observed in all the legumes. From the above analysis it is concluded that amines, aliphatic and aromatic nitro compounds, bromides, sulfonates and fluorides are the most common compounds in the legumes. The phytochemical composition and antioxidant activity of the legumes might be attributed to the presence of the above mentioned functional groups. The above results are confirmed with Infrared Spectra analysis interpretation, which seems to be a practical approach[36].

Table 1
Quantitative Analysis of phytochemical compounds of Jack bean, Horse gram and Lima bean *

Phytochemical compounds	Jack bean (*Canavalia ensiformis)*	Horse gram (*Macrotyloma uniflorum*)	Lima bean (*Phaseolus lunatus*)
Phenolics[a]	1.226 ± 0.038	1.670 ± 0.097	1.265 ± 0.209
Tannins[b]	0.855 ± 0.025	0.101 ± 0.093	0.205 ± 0.037
Flavanoids[c]	0.638 ± 0.059	0.038 ± 0.065	0.072 ± 0.014
Alkaloids[d]	0.645 ± 0.020	0.359 ± 0.030	0.474 ± 0.011
Saponins	0.520 ± 0.047	0.117 ± 0.049	0.175 ± 0.054

**All the values are mean of triplicate determinations and expressed in g /100 g; a- Gallic acid equivalent; b- Tannic acid equivalent;c-Quercetin equivalent; d- colchicine equivalent's*

Table 2
Scavenging activity of Ethanolic extract of Jack bean, Horse gram and Lima bean (%)

Concentration of solvent extract (µg/mlml)	Nitric oxide scavenging activity			Superoxide radical scavenging activity			Iron chelating activity			Reducing power capacity		
	JB	HG	LB	JB	HG	LB	JB	HG	LB	JB	HG	LB
125	12.23±0.21	4.31±0.01	2.81±0.11	7.53±0.01	2.31±0.01	1.65±0.02	11.23±0.21	3.31±0.01	1.81±0.11	0.32±0.02	0.21±0.02	0.06±0.02
250	15.10±0.12	6.50±0.10	3.30±0.03	9.00±0.02	4.50±0.10	3.10±0.00	14.10±0.12	5.50±0.10	2.30±0.03	0.61±0.02	0.47±0.02	0.13±0.02
500	17.34±0.01	9.95±0.21	6.05±0.10	11.05±0.21	7.95±0.21	5.04±0.13	19.15±0.01	7.95±0.21	4.05±0.10	0.89±0.02	0.71±0.02	0.26±0.02
1000	21.11±0.05	13.16±0.04	7.16±0.02	19.31±0.25	14.16±0.04	9.35±0.30	27.11±0.05	11.16±0.04	9.16±0.02	1.23±0.02	0.98±0.02	0.51±0.02

All the values determined are triplicates of mean ± standard error, Ascorbic acid standard JB-Jack bean; HG-Horse gram; LB-Lima bean

Table 3
FTIR Spectrum of Lima bean, Horse gram and Jack bean

Wave number (cm^{-1})			Type of Bond		Compound type
Lima bean	Horse gram	Jack bean			
3398.97	3399.83	3401.66	O - H ,N-H(s)	Stretching	Amines, Alcohol
2926.54	2926.26	2925.28	C - H ,N-H(s)	Stretching	Methylene, , amine salts, Asymmetric/symmetric
1658.26	1658.03	1658.06	C= C, C=O(s) N-H (b)	Stretching, bending	Alkenyl , amides
1547.86	1547.51	1402.32	NO_2 , P-CH3 (b)	Stretching, bending	Aliphatic and aromatic nitrocompounds, phosphine
1408.19	1408.06	1078.47	O – H, C-F (s) C-Br (s)	Bending, stretching	Phenol or tertiary alcohol, Flurides Aryl Bromides
1161.05	-	859.79	SO_2 , S-O (s)	Stretching	Sulfonates
-	1046.0	571.84	C - F , C-Br (s) C-I (s)	Stretching	Aliphatic fluro compounds, bromides Iodides
985.09	-	547.54	C-H,C-I (s) C-Br (s),C-Cl	Bending. stretching	Vinyl C-H out of plane bend, iodides Bromides
860.09	859.99	529.71	C - H ,C-I (s) C-Br (s),C-Cl	Stretching	1.4- Disubstitution (para). Iodides bromides chloride
547.61	573.89		C - I	Stretching	Aliphatic organo halogen

Figure 1
IR Spectroscopic analysis of lima bean

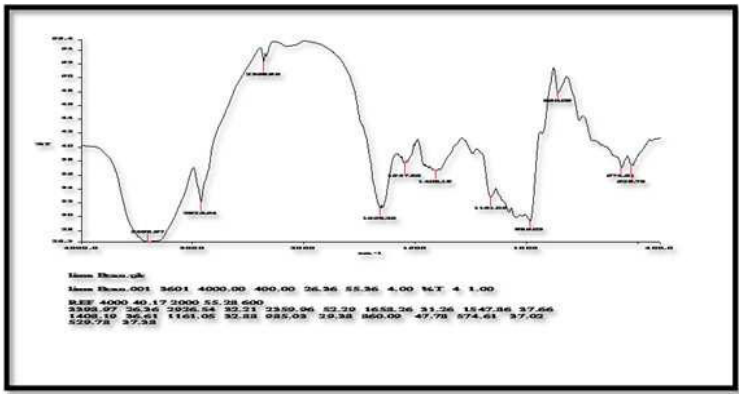


Figure 2
IR Spectroscopic analysis of Horse gram

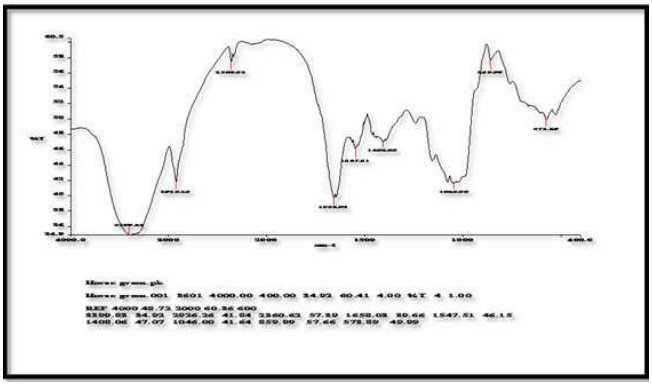
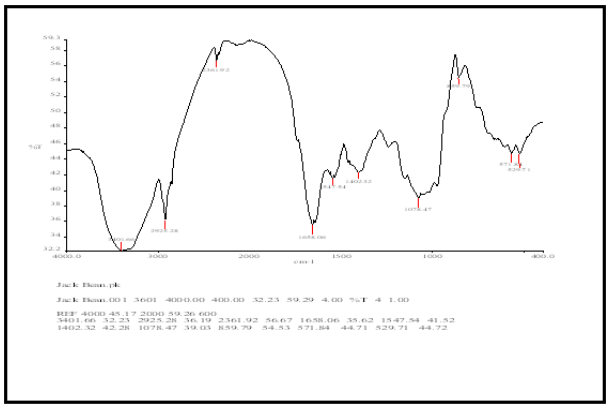


Figure 3
IR Spectroscopic analysis of jack bean



CONCLUSION

Legumes contain varied amounts phytochemical compounds including antioxidants. Antioxidative potential of seed extracts studied here are highly comparable with standards. Most of the functional groups have been identified to be, amines, aliphatic and aromatic compounds, sulfonates and bromides. This data is useful from a clinical nutrition point of view and help in the isolation of bioactive compounds.

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