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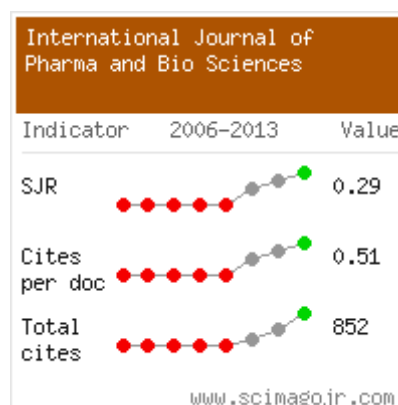
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PHYTOCHEMICALS, *IN VITRO* ANTIOXIDANT ACTIVITY AND PROXIMATE COMPOSITION OF SEEDS OF *ENTADA PHASEOLOIDES* LINN. MERRILL.

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ABSTRACT

In the present study, the chemical composition and *in vitro* antioxidant activity of methanol extract of seeds of *Entada phaseoloides* were analyzed by various *in vitro* antioxidant models viz. DPPH radical FRAP and ABTS assay along with the evaluation of IC₅₀ value. Screening of the phytochemical constituents and high performance liquid chromatography (HPLC) analysis of the methanol extract of *Entada phaseoloides* were performed for identification of some of the functional compounds present in the seeds. The methanol extract of *Entada phaseoloides* exhibited a significant ascending trend of percentage inhibition with maximum activity at 60µg/ml and 80µg/ml concentration for all the models of free radical scavenging activity. The results showed high amount of phenolic and flavonoids, confirmed by HPLC analysis, and thus it might be an alternative to the synthetic antioxidants available in the market. The chemical composition analysis revealed a high quantity of protein, carbohydrate, fat and minerals. The results demonstrated that the seeds of *Entada phaseoloides* exhibits an excellent antioxidant activity with high level of nutrients and thus presents a great potential as a source of highly nutritious potent natural antioxidants.

KEYWORDS: Antioxidant activity, chemical composition, phytochemical constituents, *Entada phaseoloides*, HPLC



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INTRODUCTION

Antioxidants play an important role in human health and nutrition, as they are known to protect the body against reactive oxygen species (ROS)¹. Furthermore, ROS play a cardinal role in the etiology of numerous diseases. In recent years, there is an increasing interest in finding antioxidant phytochemicals, because they can inhibit the propagation of free radical reactions, protect the human body from diseases². Polyphenols constitute a large group of naturally occurring substances in the plant kingdom, which include flavonoids. The plant phenolics are commonly present in fruits, vegetables, leaves, nuts, seeds, barks, roots and in other plant parts. These substances have considerable interest in the field of food chemistry, pharmacy and medicine due to a wide range of favourable biological effects including antioxidant properties. The antioxidant property of phenolics is mainly due to their redox properties. Hence, there has been increasing interests in natural antioxidants and their potential health benefits. Now a day's numerous crude extracts and pure natural compounds have been reported to possess antioxidant properties³. In folk medicine, barks, stems, leaves, and seeds of *E. phaseoloides* have been used to treat haemorrhoids, stomach ache, toothache, spasm, gastritis, parotitis, and lymphadenitis⁴. In India, the boiled seeds of gila bean are consumed by Karbi tribes of Assam and Oceanic group of tribes such as Onges and Great Andamanese. The soaked seed kernels are roasted/boiled and eaten by northeast tribal sections such as Garo, Khasi, Naga and Kanikkars of Tamil Nadu and Kerala⁵. The half-ripened seeds are used as a coffee substitute in South America⁶. The species has been used medicinally in Malaysia, the Philippines and Java. The seeds are considered as tonic, emetic and anthelmintic⁷. It possesses a wide range of ethnopharmacological properties contributed by phytochemical constituents of which most abundant are saponins, diterpenes, triterpenes and phenolics compounds. The saponin extracted from seed kernels of *E. phaseoloides* shows significant activity against Walker 256

carcinoma in rats⁸. The extract was found to possess anti-inflammatory property as it acts on proliferative phase of inflammation⁹. In addition to this, it possesses antiarthritic¹⁰, antidiabetic and hypolipidemic activities¹¹, antiulcer¹², antitoxicity¹³, anticomplement and antimicrobial¹⁴ and also possesses molluscicidal activities (crude and processed product of *Entada phaseoloides*)¹⁵. Literature survey shows that the seeds of *Entada phaseoloides* are very high in protein, carbohydrates and lipids contents¹⁶. Local people in many places consume seeds as food, but so far, no specific study has been done in detail. Hence, an effort was made to study the nutritive value and antioxidant activity of *Entada phaseoloides* seeds both as food and as feed additives along with the evaluation of IC₅₀ and determination of the phytochemical constituents, total phenolic and flavonoids.

MATERIALS AND METHODS

Plant materials

The dried seeds of *Entada phaseoloides* are purchased from local market and identified by taxonomist (Dr. I. C. Barua) and the voucher specimen of the dried seeds has been kept at the herbarium of Department of Agronomy, Assam Agricultural University, Jorhat-785013, Assam.

Extracts preparation

Dried seeds powder (1kg) was soaked in methanol (3L) in a round bottom flask (5L) and the mixture was left over night at room temperature. Next day the mixture was stirred mechanically at room temperature once daily for 3 days. Then filtered off the solid residue and concentrated the filtrate under vacuum at 50^o C using rotary evaporator. Again, the solid residue was dipped in sufficient amount of methanol for 3 days and is repeated three times in order to avoid any wastage of extract. The concentrated extract had been dried in water bath for 1 hour at 50^o C and finally 200 g of crude extract was obtained achieving a percentage yield of 20.

Chemical composition

Proximate composition was determined in accordance with standard methods¹⁷. It constitutes different classes of nutrients viz. carbohydrates, protein, fat, crude fibre, ash and moisture as well as caloric value. The moisture content was determined by drying transversely cut seeds in an oven at 80⁰C for 24 hr and has been expressed on a percentage basis. The air-dried samples were powdered separately in a Willy mill to 60-mesh size and stored in screw-capped bottles at room temperature for further analysis. The ash content was determined by heating the dried sample in a silica dish at 600⁰C for 6 hr or at 550-600⁰C for 12 h in a muffle furnace on percentage basis. The crude protein content was calculated by multiplying the nitrogen content by a factor of 6.25 i.e. (N x 6.25). The nitrogen value, a precursor for protein, was determined by micro Kjeldahl method, involving digestion, distillation and finally titration of the sample. Crude fat content was determined using Soxhlet. The percentage of organic matter was calculated out by subtracting the percentage of ash from one hundred (100). Percentage of carbohydrate content was calculated out as: Carbohydrate (%) = [100- (% Crude protein+% Crude fat + % Crude fibre + % Total Ash)]. The calorific value was estimated using "Atwater factor" i.e. by multiplying crude protein, crude lipid and carbohydrate by factor of 4, 9 and 4, respectively¹⁸. Calcium and iron content was determined by digesting 100mg of ground seed with a mixture of concentrated nitric acid (2ml), 60% perchloric acid (0.8ml) and concentrated sulphuric acid (1ml). After cooling, the digest was diluted with 10ml of deionised distilled water, filtered with What man No. 42 filter paper and the filtrates were made up to 20ml in a glass volumetric flask with deionised distilled water. All the minerals except phosphorus were analysed from a triple acid-digested sample by an atomic absorption spectrophotometer–GBC-932AA. The phosphorus content in the triple acid digested extract was determined colorimetrically¹⁹. Results were expressed as a percentage (g/100 g) on dry matter basis and represent the mean of three determinations ± SEM.

Phytochemical analysis

Standard screening tests was carried out to detect the presence of different phytoconstituents such as steroids, saponins, tannins, glycosides, diterpenes, triterpenes, alkaloids, phenolics and flavonoids^{20, 21}.

Inhibition concentration (IC₅₀) value

IC₅₀ represents the amount of sample (µg extract/ml) necessary to scavenge free radicals by 50%. The IC₅₀ values were calculated by extrapolating from linear regression analysis.

Determination of total phenolics and flavonoids content.

Total phenolic content of the extract was determined by the modified Folin–Ciocalteu method²². Briefly, an aliquot of the extract was mixed with 9 ml of distilled water in 25 ml volumetric flask. To that, Folin-Ciocalteu reagent (1ml) was added and mixed. After an incubation period of 5 min, 7% Na₂CO₃ (10ml) was added and the volume was diluted upto 25 ml. The mixture was kept for 90 min at room temperature before the absorbance was measured at 765 nm using UV-Vis Spectrophotometer against a blank containing all the reagents, except the polyphenol solution, which had been substituted with distilled water. Total phenolic content was expressed as mg/g tannic acid equivalent using the following equation based on the calibration curve: $y = 0.1216x$ (R²=0.9365), where x is the absorbance and y is the Gallic acid equivalent (mg/g). The flavonoid content was determined spectrophotometrically using a method based on the formation of a flavonoid-aluminum complex with some modifications²³. Briefly, aliquots of the extracts were added to volumetric flasks containing 4 ml of distilled water and to this, 0.3 ml NaNO₂ (5%) was added. After 5 min, 10% AlCl₃ (0.3ml) and 2 ml of 1M NaOH were added and the volume was made upto 10 ml. The absorbance was read at 420 nm UV-Vis spectrophotometer. Total flavonoid content was calculated as quercetin (mg/g) using the following equation based on the calibration curve: $y=0.0255x$ (R²=0.9812), where x is the

absorbance and y is the quercetin equivalent (mg/g). The concentration of total flavonoid content was expressed as mg/g of quercetin equivalent.

DPPH (2, 2-diphenylpicrylhydrazyl) Radical Scavenging Activity

In order to evaluate the free radical scavenging activity of the test samples, the change in optical density by DPPH radical was assessed

$$\text{DPPH Scavenged (\%)} = [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}}) / \text{Abs}_{\text{control}}] \times 100$$

Where $\text{Abs}_{\text{control}}$ is the absorbance of the control reaction and Abs_{test} is the absorbance of the test sample.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was carried out according to Benzie and Strain with certain modifications²⁵. FRAP solution (2.8 ml) containing 300 mM acetate buffer, 10 mM TPTZ solution and 20 mM FeCl_3 solution were added to 0.15 ml of different concentrations (3, 9, 20, 40, 60, 80, 100, 110 $\mu\text{g/ml}$) of the plant extracts. The mixture was incubated at 37^o C for 30 min in the dark and absorbance was measured at 593 nm. The results were expressed in μM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalent from a standard calibration curve.

ABTS (2, 2-azinobis-3- ethylbenzothiazoline-6-sulfonic acid) Assay

The free radical-scavenging activity was determined by ABTS radical cation

$$\% \text{ inhibition} = [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}}) / \text{Abs}_{\text{control}}] \times 100$$

High performance liquid chromatography-Diode array detection (HPLC-DAD) analysis

The extracts were analyzed to detect and quantify the presence of the gallic acid and quercetin in the methanol extract with the aid of HPLC system equipped with binary gradient pump, column heater and degasser online, photodiode array detector and Chromeleon Software. Separation was achieved using a reversed phase column, C18 (4.6 \times 250 mm, 4 μm), PROD, ACCLAIM at temperature of 25 $^{\circ}\text{C}$. DAD detection was employed in the wavelength range between 210 and 500 nm

by standard method²⁴. The sample extracts were diluted with methanol to give different concentrations of the plant extracts (1, 3, 6, 9, 20, 40, 60, 80 $\mu\text{g/ml}$). Then 0.2 ml DPPH was added to 2.8 ml extracts at various concentrations and incubated at 37 $^{\circ}\text{C}$ for 30 min. Absorbance was read at 517 nm. Ascorbic acid was used as reference standard. Percentage inhibition was calculated as

decolorization assay²⁶. ABTS was dissolved in water (7 μM) to get the stock solution. ABTS radical cation ($\text{ABTS}^{+\cdot}$) was produced by reacting ABTS stock solution with 2.45 μM potassium persulfate (final concentration) and kept in the dark at room temperature for 12–16 h before use. The samples containing the ABTS solution were diluted with distilled water to an absorbance of 0.700 ± 0.02 at 734 nm and equilibrated at 30^o C. Reagent blank reading was taken. After addition of 3.0 ml of diluted ABTS solution, the absorbance was read exactly 6 min after initial mixing. The results were expressed in terms of TEAC (Trolox equivalent antioxidant capacity) and are calculated out from standard curve. The percentage inhibition was calculated as

(273 nm). Samples were dissolved in the corresponding solvent of the extract at the concentration of 10 mg/mL. The volume of sample injected was 20 μL using an L-7200 auto-sampler. The mobile phase was a mixture of Methanol: Acetonitrile: Water (60:20:20 v/v) and 0.1% O-phosphoric acid: Acetonitrile (400:600 v/v) and the flow rate was 1 mL/min. The elution system was in isocratic mode.

Statistical analysis

The data were presented as the mean (\pm SEM) of triplicate tests. The graphs were prepared

using Graph Pad Prism 6 (version 6.03). Statistical analysis was performed with two-way analysis of variance (ANOVA) followed by LSD.

RESULTS AND DISCUSSION

Chemical composition

Based on proximate analysis, the seeds were found to have 91.55±0.82% Dry matter and 8.45±0.35% Moisture, 97.50± 0.64% organic matter and 2.50±0.10% total ash. The seed flour has a higher amount of fat, crude protein and carbohydrates i.e. 8.30±0.166%, 24.06±0.53% and 61.18±1.30% respectively. Earlier findings stated that seed kernels contain 256.7 g/kg crude protein, 108.1 g/kg lipid, 27.3 g/ kg ash and a high content of carbohydrate (585.7 g/kg)¹⁶. The total crude fibre and the nitrogen free extractives (NFE) content were found to be 3.96 ± 0.18% and 61.18 ± 0.75% respectively. The high contents of NFE enable the seeds of this creeper as a good source of energy. The calorific value of *Entada phaseoloides* was calculated out as 415.66 ± 8.80 Kcal/100g DM. The seeds are also found to be a good source of minerals especially calcium, phosphorus and iron comprising of 1.5± 0.02%, 0.77 ± 0.017% and 0.0332 ± 0.002 respectively (Table 1).

Preliminary phytochemical screening

Phytochemical screening reveals the presence of alkaloids, diterpene, triterpene, glycosides, steroids, saponins, phenolics, flavonoids in higher amounts and tannins in trace amount (Table 2).

Total phenolic and flavonoid compounds

The aluminium chloride colorimetric method uses wavelength scan of the complexes of the sample and standard with aluminum chloride showed that the complexes formed by flavonoids (quercetin) with C-3 or C-5 hydroxyl group²⁷ revealing total flavonoids content in the extract. Flavonoids have good antioxidant potential and play some important pharmacological roles against diseases, such as cardiovascular diseases, cancer, inflammation and allergy, so they are considerable in human nutrition and health.

Phenols and polyphenols present in the methanol extract contribute significantly to the total antioxidant capacity of the seeds. Total phenol and total flavonoid content was found to be high in the MEEP seeds i.e. 0.281±0.02 mg Gallic acid/g of dry seed and 0.137±0.07 mg Quercetin/g of dry seed respectively. Similar findings also reported high total phenolic content (TPC, 245.59, 240.22, 240.03 and 117.0 mg of Gallic acid equivalent (GAE)/gm of dried extract) in the crude extract, carbon tetrachloride and aqueous soluble fractions of both bark and seed respectively²⁸.

DPPH radical scavenging activity

The DPPH assay is based on the spectrophotometric measurement of the DPPH concentration change resulting from the reaction with an antioxidant. DPPH is a stable nitrogen-centered free radical, the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation. It was found that the DPPH radical scavenging activities of extract increased with increasing concentration. IC₅₀ for DPPH radical-scavenging activity was 10.04 µg/ml and that of Ascorbic acid, 7.81 µg/ml, respectively. Factors like stereo-selectivity of the radicals or the solubility of the extract in different testing systems have been reported to affect the capacity of extracts to react and quench different radicals²⁹. The DPPH radical scavenging activity of the extract was found almost similar to the Standard Ascorbic acid at different concentrations (Figure 1). Strong DPPH radical scavenging activity was reported with IC₅₀ value of 3.24, 1.55 and 3.6 µg/ml in the crude extract of bark of *E. phaseoloides*; and its chloroform and aqueous soluble fractions respectively²⁸.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay measures the ability of the plant extracts to reduce ferric to ferrous at low pH causing the formation of ferrous-tripyridyltriazine complex. The antioxidant ability of the plant extracts were presented in terms of mM Fe²⁺. The MEEP exhibited good ferric reducing property at various concentrations and

its antioxidant activity is directly proportional to its concentration (Figure 2).

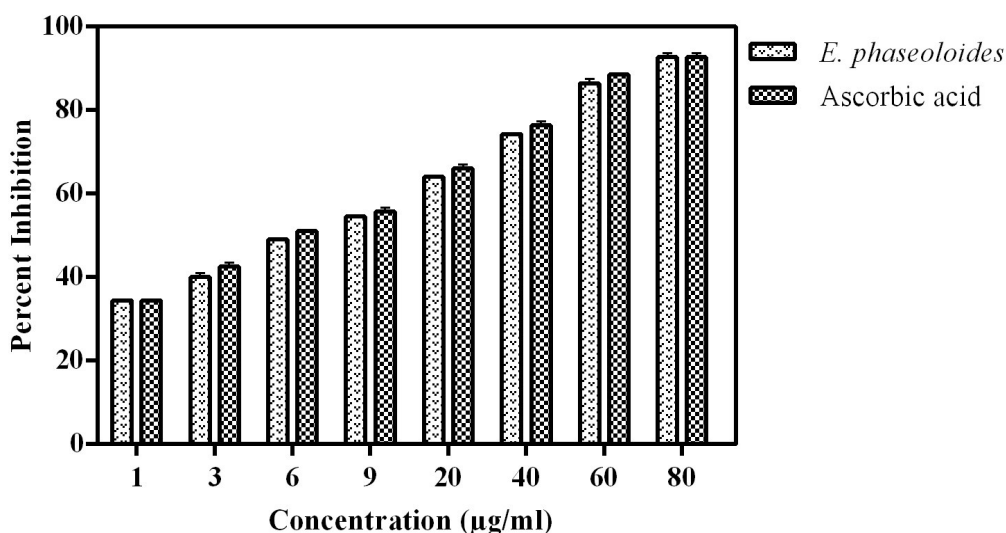
ABTS (2, 2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) Assay

ABTS is a blue chromophore produced by the reaction between ABTS and potassium persulfate. Addition of the plant extract to this pre-formed radical cation reduced it to ABTS in a concentration-dependent manner. The results were compared with trolox standard curve ($R^2=0.9586$) and the activity was found to be increased from 20 TEAC to 131.4 TEAC at a concentration of 1-9 $\mu\text{g/ml}$ (Figure 3) and therefore the result demonstrates that the extract is a potent antioxidant. The ethanol extract of stems of *Entada phaseoloides* was earlier reported to be a good source of natural antioxidants³⁰.

HPLC-DAD Analysis

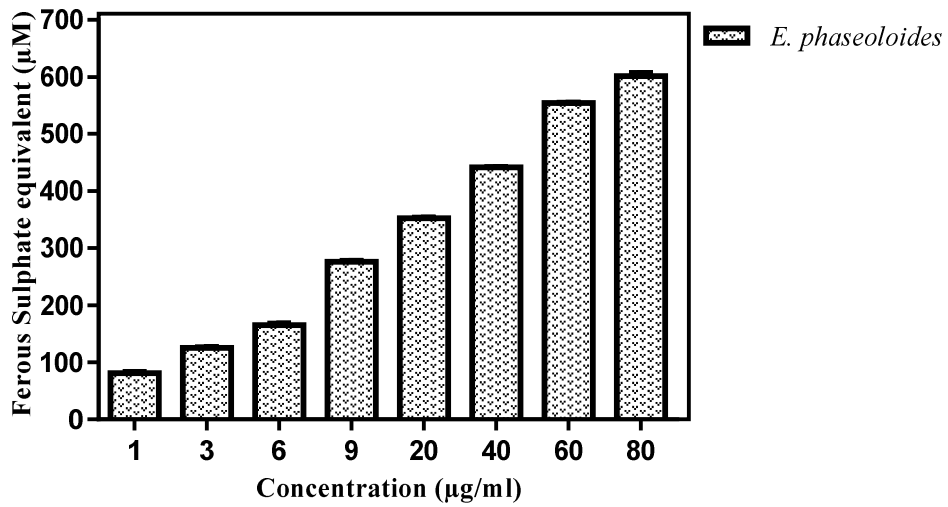
To confirm the HPTLC profile, HPLC-DAD was performed. All the conditions of reversed-phase HPLC-DAD were appropriate for the characterization of the extract. The chromatographic analysis revealed the presence of gallic acid [Figure 4(a)] and quercetin [Figure 4(b)] in the extract. As presented in figure 4(a), out of seven peaks, peak four was assigned to Gallic acid with an RT of 3.137 min, and height (mAU) of 242.25 ± 0.8 . Similarly in figure 4(b), out of fifteen peaks, peak eighth was assigned to be quercetin, with an RT of 3.343 min and height (mAU) of 58.63 ± 0.40 . Gallic acid was present in greater amount as compared to quercetin. A positive correlation has been found between antioxidant activity and total phenolics & total flavonoid content. Terpenoids have anti-inflammatory, antioxidant and neuroprotective activities³¹. Thus, the antioxidant potential exhibited by the extract was possibly due to the high polyphenolic content in the extract.

Figure 1
DPPH radical scavenging activity of methanol extract of *Entada phaseoloides*



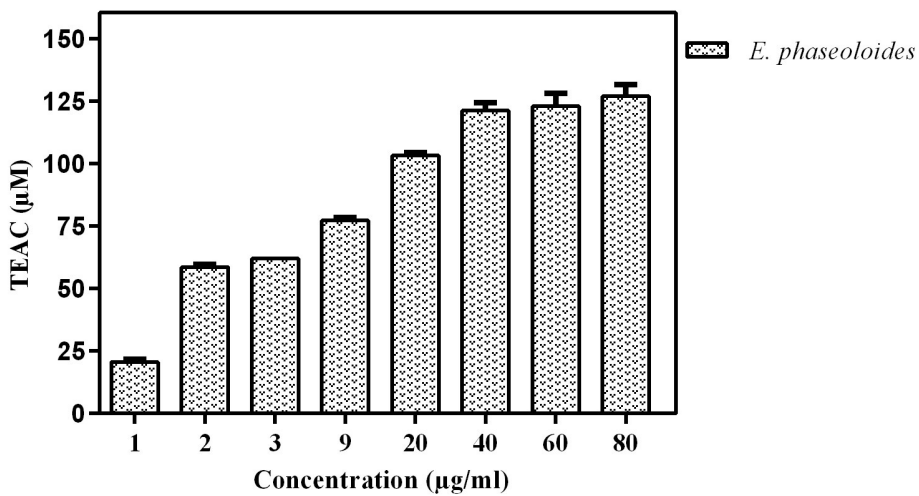
Bars represent percentage inhibition (Mean \pm SEM, N=3) that do not differ significantly (* $P < 0.05$) between groups

Figure 2
FRAP Assay of methanol extract of *Entada phaseoloides*



Bars represent reductive ability (Mean \pm SEM, N=3) that do not differ significantly (*P < 0.05) between groups
The FRAP values were expressed as $\mu\text{M FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalent from a standard calibration curve

Figure 3
ABTS Assay of methanol extract of *Entada phaseoloides*



Bars represent reductive ability (Mean \pm SEM, N=3) that do not differ significantly (*P < 0.05) between groups
The ABTS values were expressed as TEAC (Trolox equivalent antioxidant capacity).

Figure 4(a)
HPLC profile of the methanol extract of Entada Phaseoloides showing Gallic acid peak

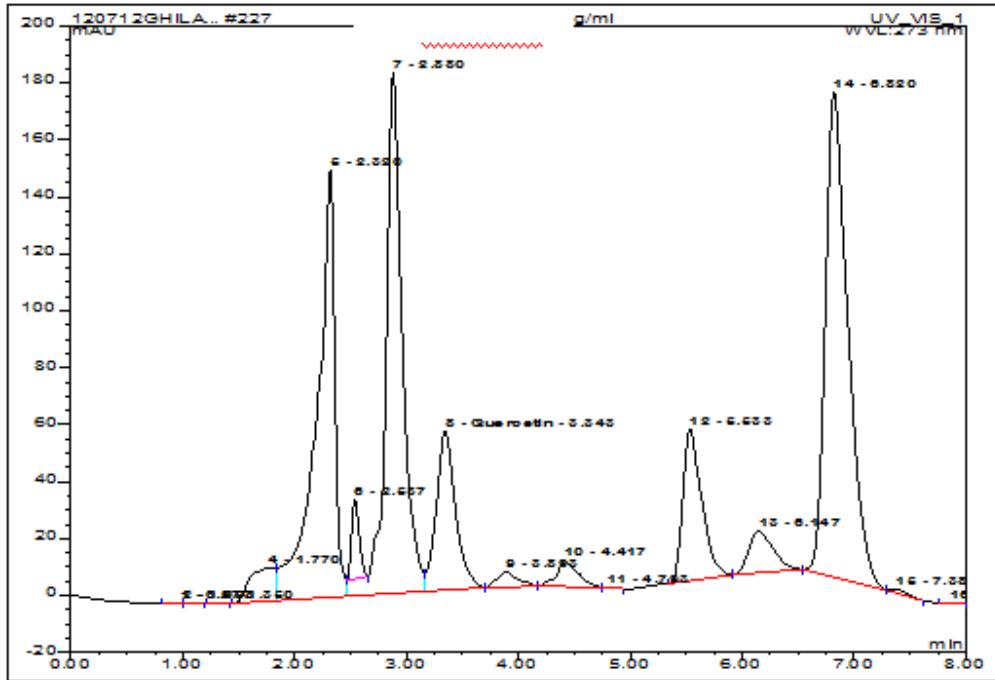


Figure 4(b)
HPLC profile of the methanol extract of Entada Phaseoloides showing Quercetin peak

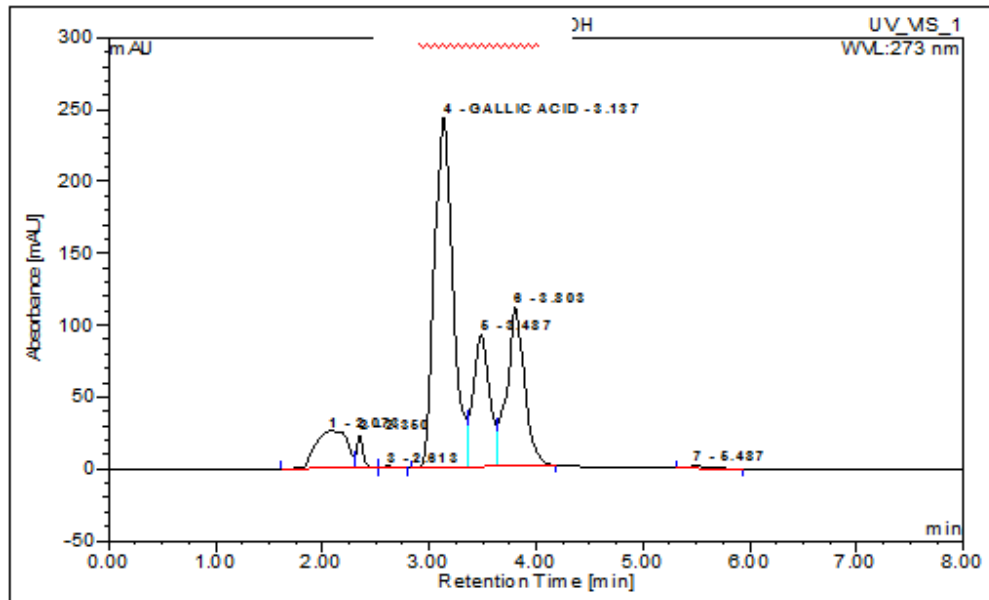


Table 1
Chemical composition of the seeds of *Entada phaseoloides* (L.) Merrill.

Components	<i>Entada phaseoloides</i>
Proximate constituents (g/100g DM)	
Moisture	8.45±0.35
Crude protein (N× 6.25)	24.06±0.53
Crude lipid	8.30±0.17
Crude fibre	3.96±0.18
Total Ash	2.50±0.10
Carbohydrates (NFE)	61.18±1.30
Calorific value (Kcal/100g DM)	415.66±8.80
Minerals (mg/100g DM)	
Calcium	1500±23.09
Phosphorus	770±16.92
Iron	33.20±1.89

Table 2
Phytochemicals present in methanol extract of seeds of *Entada phaseoloides* (L.) Merrill

Tests	<i>Entada phaseoloides</i>
Steroids	+++
Phenolics	+++++
Tannins	+
Saponin	+++++
Flavonoids	++++
Glycosides	+++
Diterpenes	+++
Triterpenes	+++
Alkaloids	+++

Note: Sufficiently present (+++++), moderately present (+++), present in trace amount (+).

Table 3
Chromatographic profile of methanol extract of seeds of *Entada phaseoloides*

HPLC Analysis				
Peak	RT value	Peak area (mAU)	Amount (ppm)	Assigned substance
4	3.317	242.25±0.80	0.016	Gallic acid
8	3.343	58.63±0.40	0.009	Quercetin

CONCLUSION

Based on the results obtained, it can be concluded that the seeds of *Entada phaseoloides* is a good source of phenolics and flavonoids. The seeds exhibit high level of nutrients such as protein, fats, carbohydrates, crude fiber, calorific values, calcium, phosphorus and iron besides displaying antioxidant property. The availability and consumption of protein in most of the developing countries remain inadequate due to population explosion and urbanization, and results in Protein Energy Malnutrition (PEM). So the seeds can be used as a cheap protein supplement to minimize the problems

associated with protein deficiency. At the same time, it can be used as a source of natural antioxidants as an alternative to synthetic antioxidant as there is a strong need for effective antioxidants from natural sources. Therefore, the seeds of *Entada phaseoloides* may be recommended for large-scale consumption as an alternative potential source of protein and natural antioxidant particularly in the developing countries.

CONFLICT OF INTEREST

Conflict of interest declared none.

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