Research Paper

In vitro Antioxidant and Pharmacognostic Studies of Leaf Extracts of *Cajanus cajan* (L.) Millsp

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***Cajanus cajan* (L.) Millsp is one of the second most dietary legume crops. The leaf extracts may be used as a potential source of natural antioxidant. The ash values, extractive values, total phenolic and flavonoid content, *in vitro* antioxidant activity of various leaf extracts as well as anatomical investigation of *Cajanus cajan* were carried out. Physicochemical parameters such as total, acid-insoluble and water-soluble ash values and moisture content of the leaf powder of *C. cajan* were found to be 9.50%, 1.40 g/100 g, 4.15 g/100 g drug and 6.72%, respectively. Percent yield of acetone, aqueous, ethanol, ethyl acetate and chloroform leaf extracts were 9.0, 10.6, 13.75, 8.7 and 5.8 g/100 g, respectively. Significant amount of phenolic and flavonoid content were observed. The results of the antioxidant activity were found to be concentration-dependent. The IC_{50} values for DPPH assay determined for aqueous and ethanol extracts were 0.69 and 0.79 mg/ml, respectively. Reducing power is increased with increasing amount of concentration in both aqueous and ethanol leaf extracts. The highest hydroxyl radical scavenging activity reached up to 83.67% in aqueous and 78.75% in ethanol extracts and in phosphomolybdenum assay the aqueous extract showed strong antioxidant capacity up to 55.97 nM gallic acid equivalents/g. It was found that the aqueous extract possessed highest antioxidant activity in all the assays tested.The antioxidant characteristics of leaf extracts are possibly because of the presence of polyphenols. Microscopic study showed the presence of collenchyma, fibres, xylem, phloem, epidermis, trichomes, palisade tissue, basal sheath, pith and cortex in leaf, petiole and pulvinus.**

Key words: *Cajanus cajan*, total phenolics, flavonoid, antioxidant, free radicals, hydroxyl radical scavenging activity

Cajanus cajan (L.) Millsp is a perennial member of the family Leguminosae, commonly known as Pigeon pea and red gram (in English); kardis, gandule bean, tropical green pea, kadios, Congo pea, gungo pea, gunga pea, fio-fio, mgbụmgbụ, no-eye pea, *toor dal, arhar dal, togari bele* (Kannada), *thuvaram paruppu* (Tamil), *thuvara parippu* (Malayalam) and *kandi pappu* (Telugu). It is one of the second most important dietary legume crops grown in the tropical regions. When compared with the other grain legumes India contributes about 90% of the world production, ranks sixth position in production and area[1]. Pigeon pea is a multipurpose plant as it is eaten as *dhal* which is rich in proteins. Its leaves are used for rearing silk worms; pods are used as vegetable, green leaves and husk are used as fodder and green manure[2]. The leaves of pigeon pea have been widely used to kill worms, relieve pain and arrest blood, as a *Chinese medicine*[3]. Chemical constituent investigations have indicated that pigeon pea leaves are rich in flavonoids and stilbenes, which are considered responsible for beneficiaries of pigeon pea leaves on human health[4,5]. Boiled leaves are given orally to nullify the effect of intoxication and as a laxative in eastern Rajasthan. The leaf paste is applied for inflammations and oral ulcers[6]. Leaf, seeds and young stems of pigeon pea are used to cure gingivitis, stomatitis and as a tooth brush in some parts of India and Tamil Nadu[7]. Flavonoids were isolated from leaf extracts (isorhamnetin, luteolin, apigenin, quercetin)[4,8] and roots of pigeon pea. Genistein and genistin isoflavonoids isolated from the roots of *C. cajan* were found to possess antioxidant activity[8-10]. Antioxidants are known to possess antiinflammatory, anticardiovascular disease, anticancer and antineurogenerative properties. These are important in biological and industrial processes. Antioxidants are used widely as food additives. To improve flavours various types of spices are added which are well known for their antioxidant capacities since ancient times[11]. Many of the disorders are linked to oxidative stress due to free radicals[12]. Antioxidants possess the ability to protect body from damage caused by free

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radical induced oxidative stress. Many free radical scavenging antioxidants are present within the body, of which many are derived from dietary sources like teas, vegetables and fruits[13]. It is prerequisite to know about pigeon pea plant in view of its anatomical, genetical and morphological for a successful improvement of program. Biological phenomena of pigeon pea are known to some extent but the information regarding anatomical characters is very scanty[14]. Some works have been carried out on the root, stem, leaf, petiole and pulvinus anatomy of pigeon pea[15]. There is limited information regarding the biological activities of *C. cajan* plant. Hence, In view of importance of *C. cajan* as ethanomedicinal plant, the present work was aimed to assess the possible antioxidant activities of acetone, aqueous, ethanol, ethyl acetate and chloroform leaf extracts of pigeon pea through various *in vitro* models and physicochemical studies as well as microscopic studies of leaf, petiole and pulvinus were carried out. Pharmacognostic studies help in identification and standardization of crude drug which is helpful to determine the contaminants present in the plant mixture.

MATERIALS AND METHODS

Plant material:
The leaves of *C. cajan* variety ICP-26 were collected from the field of Department of Biotechnology, Kakatiya University, warangal. The leaves were washed, cleaned and air-dried under shade for about 2-3 months. Fresh leaves of *C. cajan* were used for microscopic studies.

Sample extraction:
The shade dried leaves were ground to a fine powder using a mechanical blender. Fifteen grams of the sample was extracted by maceration with 150 ml of acetone, aqueous, ethanol, ethyl acetate and chloroform, separately. The extracts were then evaporated to dryness at room temperature and were stored for further use.

Determination of physicochemical studies:
The dried and powdered leaf material was subjected to determination of physicochemical parameters such as, acid-insoluble, water-soluble, total ash content and moisture content[16]. Extractive values of various leaf extracts viz., acetone, aqueous, ethanol, ethyl acetate and chloroform were determined[17].

Estimation of total phenolic content:
Total phenolic content of various leaf extracts of *C. cajan* were determined by using Folin-Ciocalteu reagent according to the method of Singleton and Rossi[18]. One millilitre of Folin-Ciocalteu reagent was mixed with 1 ml of the leaf extract. Then 1 ml of saturated sodium carbonate (35%) was added to the mixture after 3 min and the mixture was made up to 10 ml by addition of sterilized distilled water and incubated at room temperature for 90 min in the dark. After incubation, the absorbance was measured at 725 nm against blank. Gallic acid was used as standard and results were showed as mg of gallic acid equivalents (GAE) per gram of dry leaf extract.

Estimation of total flavonoid content:
The total flavonoid content of various leaf extracts of *C. cajan* was determined using the method[19]. A small quantity of various leaf extracts (0.25 ml) were diluted with 1.25 ml of sterile distilled water and 75 µl of 5% sodium nitrite solution were added and incubated for 6 min. Then 150 µl of 10% $AlCl_3.H_2O$ was added and mixed well. Then immediately the absorbance was measured against blank at 510 nm. The flavonoid content was showed as mg of rutin equivalents (RE) per gram of dry extract.

DPPH free radical scavenging activity:
DPPH free radical scavenging activity of dried crude extracts of aqueous and ethanol leaf extracts of *C. cajan* were examined[20]. A small quantity of DPPH (4.3 mg) was dissolved in 3.3 ml of methanol and it was protected from light by keeping the test tubes in dark. For control 3 ml of methanol was taken in a test tube and 150 µl of DPPH solution was added to it and absorbance was taken immediately at 517 nm. Different concentrations of leaf extracts (0.2, 0.4, 0.6, 0.8, 1 mg/ml) and ascorbic acid as standard were taken. To these extracts 150 µl of methanol was added. Then each sample was diluted further with methanol up to 3 ml and 150 µl of DPPH was added to each tube. Then the tubes were kept in dark for 15 min and absorbance was taken at 517 nm using blank as methanol on UV/Vis spectrometer. The IC_{50} values for aqueous and ethanol extracts were estimated. The DPPH activity was calculated using the formula, % inhibition=(absorbance of control-absorbance of test sample)/absorbance of control)×100.

Determination of reducing power:
The reducing power of aqueous and ethanol leaf extracts of *C. cajan* were determined according to the method of Oyaizu[21]. Two and half millilitres of different concentrations of *C. cajan* leaf extracts (0.4, 0.8, 1.2, 1.6, 2 mg/ml) were taken and 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide were taken and mixed well. Then they were incubated at 50^0 for 20 min and 2.5 ml of 10% trichloroacetic acid at 5000 g for 10 min. Later 2.5 ml of supernatant was collected and mixed with 2.5 ml deionised water, 0.5 ml of 0.1% ferric chloride and incubated for 10 min. After incubation, absorbance at 700 nm was measured against blank. Increased absorbance showed enhanced reducing power.

Hydroxyl radical scavenging activity:
Hydroxyl radical scavenging activity of aqueous and ethanol leaf extracts of *C. cajan* were determined using the method of Smirnoff and Cumbes[22]. Three millilitres of reaction mixture contained about 1 ml of 1.5 mM $FeSO_4$, 0.3 ml of 20 mM sodium salicylate, 0.7 ml of 6 mM hydrogen peroxide and different concentrations of leaf extract (250, 500, 750 and 1000 µg/ml). Then the reaction mixtures were kept in water bath at 37° for 1 h. After incubation, the absorbance of the solution was measured at 562 nm. The scavenging activity of hydroxyl radical was calculated using the following: $[1-(A_1-A_2)/A_0]\times100$, where A_1 is absorbance in the presence of extract, A_2 is absorbance without sodium salicylate and A_0 is absorbance of the control.

Phosphomolybdenum assay:
Phosphomolybdenum activities of the acetone, aqueous, ethanol, ethyl acetate and chloroform leaf extracts of *C. cajan* were determined by the method[23]. One millilitre of reagent solution (0.6 M H_2SO_4, 28 mM Na_3PO_4 and 4 mM $(NH_4)_2MoO_4$) and 0.1 ml of sample solution. The samples were incubated at 95° for 90 min by capping them with silver foil. After incubation the tubes were cooled to room temperature and absorbance was measured at 695 nm against blank. Ascorbic acid was used as standard. Total antioxidant activity was expressed nM GAE/gram of dry extract.

Microscopic studies:
The fresh leaves of *C.cajan* were excised from the plants and fixed for at least 24 h in ethanol:chloroform: acetic acid (ECA, 60:30:10) v/v. After fixation, the leaf material was dehydrated by alcohol solution and sections of leaf, petiole and pulvinus were done by free hand transverse section method at a thickness of 9-11 µ[24,25]. The sections done were stained with saffranin and were arranged on to a glass slide[26]. The anatomical sections were observed under compound microscope at a projection of 10X and 40X.

Statistical analysis:
The experiments were performed in triplicate where ever neceessary and the data were statistically analysed as mean±SE. All graphs were plotted using MS Excel® software 2010. The values of correlation coefficient, intercept, slope and standard errors were obtained by non linear and linear regression analysis applying this program.

RESULTS AND DISCUSSION

C. cajan leaf extracts showed varied physicochemical parameters such as total ash content of 9.50%, while water soluble ash is greater than that of acid insoluble ash at 4.15 and 1.40 g/100 g, moisture content 6.72%, respectively (Table 1). These results were supportive with the results of *Portulaca quadrifida*[27]. Ash value is useful in finding out genuineness and purity of drug and the values are significant in quantitative standards[28]. The results of extraction yield, colour, and consistency were presented (Table 2). The extraction yield of various solvents ranged from 5.8 to 13.75 g/100 g and could be ranked from high to low in the order: ethanol>aqueous>acetone>ethyl acetate>chloroform extracts. Crude extract of *C. cajan* exhibited a wide range of colour. Acetone, ethyl acetate, chloroform and ethanol extracts were dark green, yellowish green, greenish black and green in colour, whereas aqueous is dark brown in colour. Consistency of these extracts varied from sticky (ethyl acetate and acetone), flakes (chloroform), resin (ethanol) and amorphous (aqueous), respectively. Evaluation of physicochemical parameters like ash values is useful in finding out the non physiological and physiological ash

TABLE 1: ASH VALUES OF LEAF POWDER OF *C.CAJAN*

Parameters	Ash values
Total Ash	9.50±0.29
Acid-insoluble ash	1.40±0.09
Water soluble ash	4.15±0.15
Moisture content	6.72±0.21

#Each value in the table is expressed as mean±SEM (*n*=3)

as well as presence of impurities[29]. Pharmacognostic studies basically deal with authentication of commonly used traditional medicinal plants through morphological and physicochemical properties. This helps in identification of adulterants and also to identify controversial and closely related plant species[30].

Total phenolic content of *C. cajan* leaf extract was evaluated by using the Folin-Ciocalteu colorimetric method. The phenolic contents in the analysed leaf extracts ranged from 37.54 to 57.20 mg GAE/g (Table 3). The highest concentration of phenols was assessed in aqueous, acetone and ethanol leaf extracts. Ethyl acetate and chloroform showed considerably smaller concentration of phenols. Similar results have been reported in *Marrubium peregrinum* extracts[31]. The total phenol contents in leaf extracts depend on the type of extract i.e. the polarity of solvent utilized in extraction. Many investigations on qualitative composition of plant extracts revealed the presence of higher concentrations of phenols in the plant extracts using polar solvents[32]. Aqueous and ethanol leaf extracts of *C. cajan* showing highest antioxidant activity have the highest phenolic concentration. Phenols are important constituents of plant due to their scavenging ability on free radicals because of their hydroxyl groups. Thus, the phenol content of plants may lead directly to their antioxidant activity[33].

The concentration of flavonoids in various leaf extracts of *C. cajan* ranged from 29.51 to 49.43 mg RE/g (Table 3). The highest concentration of flavonoid was found in aqueous and ethanol leaf extracts while in ethyl acetate and chloroform leaf extracts lowest flavonoid concentration was assessed. The concentration of flavonoid in aqueous leaf extract was 49.43 mg RE/g which is very similar to the value of ethanol leaf extract. These results are supportive with the *Marrubium peregrinum* extracts[31]. The concentration of the flavonoids depends on the polarity of the solvents used in the preparation of extract[34].

TABLE 2: EXTRACTIVE VALUES OF LEAF EXTRACTS OF *C. CAJAN*

Parameter	Colour	Consistency	Yield
Acetone	Dark green	Sticky	9.0±0.20
Aqueous	Dark brown	Amorphous	10.6±0.26
Ethanol	Green	Resin	13.75±0.32
Ethyl acetate	Yellowish green	Sticky	8.7±0.14
Chloroform	Blackish green	Flakes	5.8±0.06

#Each value in the table is expressed as mean±SEM (*n*=3)

TABLE 3: TOTAL PHENOLIC AND TOTAL FLAVONOID CONTENT OF *C. CAJAN* LEAF EXTRACTS

Name of Extract	Total phenolic content (mg GAE/g dry extract)	Total flavonoid content (mg RE/g dry extract)
Acetone	50.95±0.78	36.17±1.12
Aqueous	57.20±1.37	49.43±0.98
Ethanol	55.03±0.42	48.32±0.27
Ethyl acetate	42.86±1.75	32.11±0.53
Chloroform	37.54±2.30	29.51±1.72

#Each value in the table is expressed as mean±SEM (*n*=3); GAE: gallic acid equivalents, RE: rutin equivalents

To evaluate the free radical scavenging activity, DPPH assay is widely used. At room temperature, DPPH is stable free radical which produces violet solution in methanol. At 517 nm DPPH shows strong absorption band in visible spectrum (deep violet colour)[35]. In the DPPH free radical scavenging activity, aqueous and ethanol leaf extracts were evaluated for their free radical scavenging activity with ascorbic acid as standard. At different concentrations tested 0.2, 0.4, 0.6, 0.8, 1 mg/ml, the scavenging effect of aqueous and ethanol leaf extracts of *C. cajan* were found to be 19.69, 35.75, 47.27, 55.45 and 66.06% in aqueous and 16.36, 31.81, 41.51, 52.12 and 58.48% in ethanol extracts, respectively. The IC_{50} value was calculated for each extract and shown in figs. 1 and 2. The IC_{50} value for aqueous extract is 0.69 mg/ml and ethanol extract is 0.79 mg/ml, respectively. With the increase in the concentration of extract the increase in the scavenging effect was observed. According to our observations, we opine that the strong activity of the extracts is due to the available hydroxyl group present in the substance[35]. From the results of DPPH, the aqueous extract showed highest antioxidant activity compared to the ethanolic extract. In the DPPH system, our results are in contradiction with Wu *et al*[8]. who reported that the antioxidant activity is superior to that of aqueous leaf extract in *C. cajan*. Similarly, 70% methanol extract of *C. cajan* leaves reported that they possess antioxidant and free radical scavenging properties[36]. Whereas highest antioxidant activity examined by DPPH assay was reported in ethanol seed extracts of *C. cajan* by Maneechai *et al*.[37].

The reducing capabilities of aqueous and ethanol extracts of *C. cajan* are shown in fig. 3. Absorbance at 700 nm showed greater reducing power. At different concentrations 0.4, 0.8, 1.2, 1.6 and 2.0 mg/ml, the reducing power of aqueous and ethanol leaf extracts of *C. cajan* were found to be 0.61, 0.96, 1.30, 1.21 and 1.64 for aqueous and 0.58, 0.73, 1.29, 1.18 and 1.57 for ethanol leaf extracts, respectively. The IC_{50}

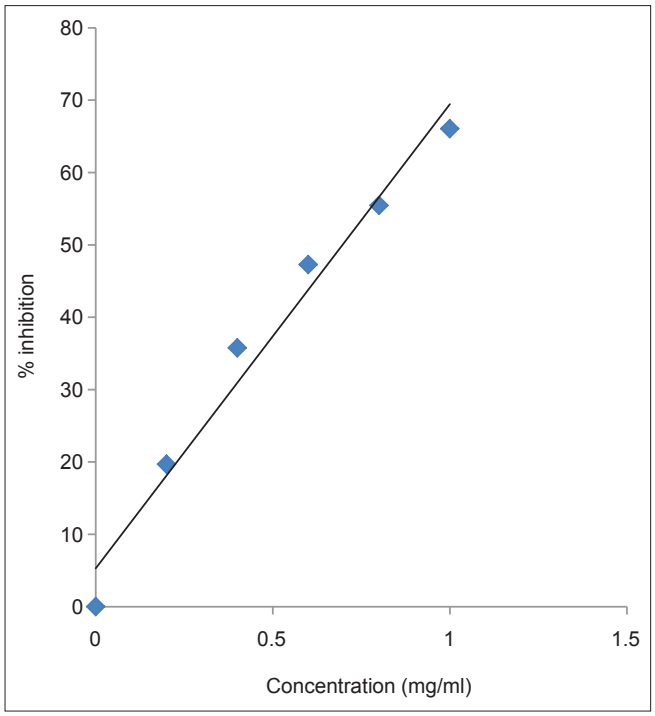

Fig. 1: DPPH assay of aqueous leaf extract of *C. cajan*. Equation of the line is y=64.15x+5.295, R^2=0.973, IC_{50}=0.691692.

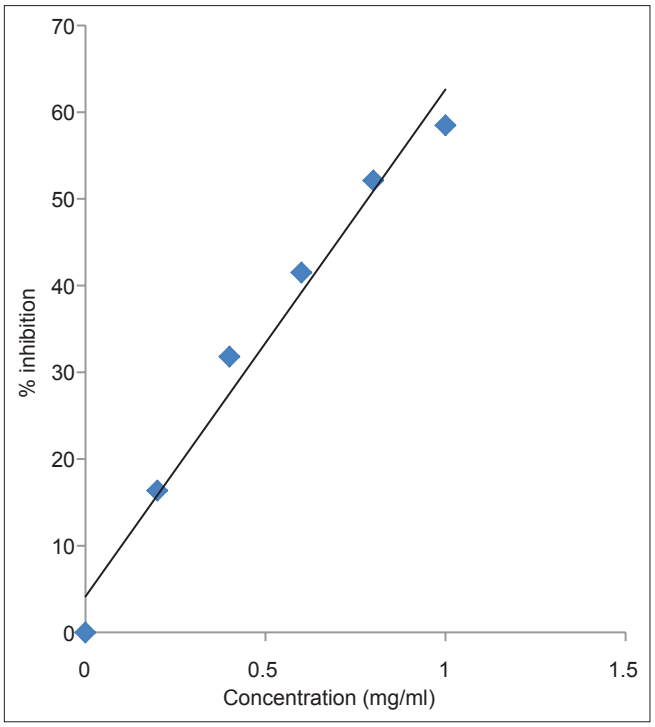

Fig. 2: DPPH assay of ethanol leaf extract of *C. cajan*. Equation of the line is y=58.48x+4.141, R^2=0.975, IC_{50}=0.790186.

values of aqueous and ethanol extracts were found to be 115.9 mg/ml and 145.8 mg/ml, respectively. Reducing power of the extract was found to be concentration dependent. The reducing power is based on the hydrogen donating ability. Reducing power of the compound may serve as a significant indicator of its potential antioxidant activity[38]. The reducing power of the extracts increased linearly with concentration. Similar results have been reported by Gosh et al. in ethanol leaf extracts of *C. cajan*[39].

The hydroxyl radical scavenging activity of aqueous and ethanol leaf extracts of *C. cajan* in various concentrations tested 250, 500, 750 and 1000 µg/ml the scavenging activity were found to be 53.88, 66.32, 70.20 and 83.67% in aqueous and 52.27, 63.05, 67.87 and 78.75% in ethanol leaf extracts, respectively. The IC_{50} values of aqueous and ethanol extracts were found to be 2.4 and 2.6 µg/ml, respectively. In this activity aqueous and ethanol extracts of *C. cajan* increased in dose dependent manner (fig. 4). Similarly, good hydroxyl radical scavenging activity was also reported in legume seed extracts[40]. Oxidative damage to DNA, proteins and lipids is caused by hydroxyl radical[41]. The hydroxyl radical induces severe damage in adjacent biomolecules and is the most reactive of the reactive oxygen species[42]. In the present study, the aqueous and ethanol leaf extract at 250 µg/ml could reach more than 50%, while the best effect (up to 83% and 78% in aqueous and ethanol extracts, respectively) was observed at higher concentration (1000 µg/ml). Hence *C. cajan* leaf extracts can be considered as good scavengers of hydroxyl radicals.

To assess the antioxidant capacity by phosphomolybdenum assay the basic principle implied is the reduction of molybdenum forms a green molybdenum complex by the antioxidant compounds present in the plant extracts which has absorption at 695 nm[43]. The data on total antioxidant capacity observed in acetone, aqueous, ethanol, ethyl acetate and chloroform leaf extracts of *C. cajan* are shown in fig. 5. The antioxidant activity of phosphomolybdenum assay was found in this order: aqueous>ethanol>ethyl acetate>chloroform>acetone. Among the extracts tested, aqueous extract showed highest antioxidant activity in comparison to others.

Leaves are trifoliate. The veins within the lamina are visible to the naked eye. In the midrib region of leaf, the vascular tissue occurs in the ventral half with xylem towards inside and phloem outside. The dorsal part of the midrib consists of fibres, above which a cap of collenchymatous cells are present. A distinct palisade layer is present in the leaf lamina. The simple and glandular hairs are found on

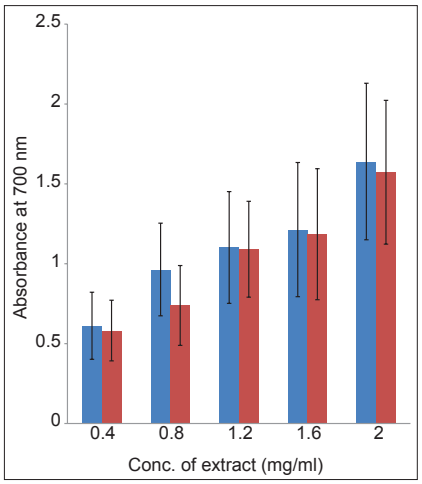

Fig. 3: Reducing power assay of aqueous and ethanol leaf extracts of *C. cajan*.
IC_{50} values are (blue bars) aqueous=115.901 and (red bars) ethanol=145.87.

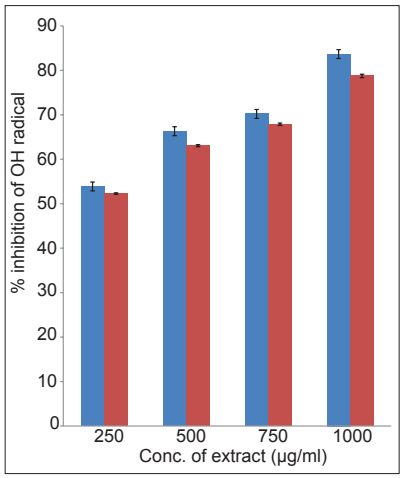

Fig. 4: Hydroxyl radical scavenging activity of aqueous and ethanol leaf extracts of *C. cajan*.
IC_{50} values are (blue bars) aqueous extract=2.4039 and (red bars) ethanol extract =2.639.

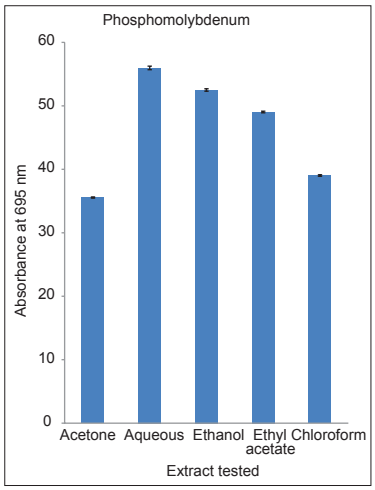

Fig. 5: Phosphomolybdenum assay of various leaf extracts of *C. cajan*.

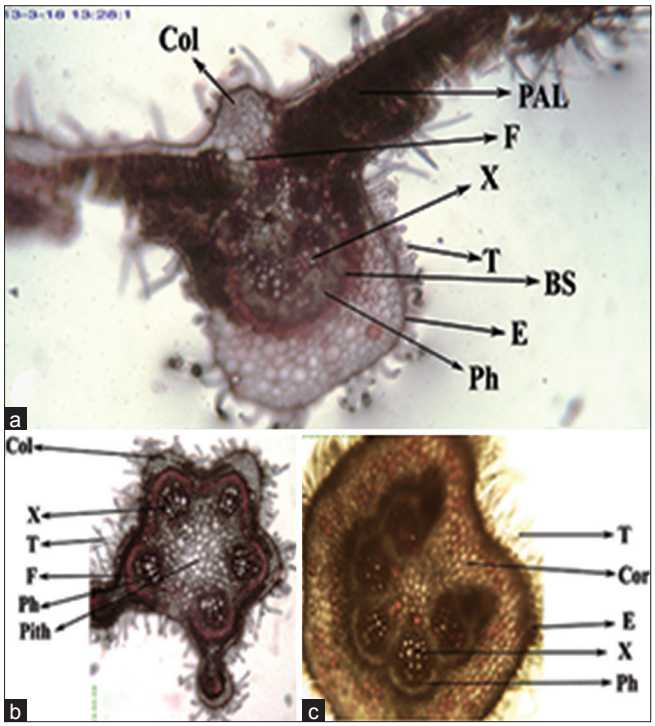

Fig. 6: Microscopic characters of *C. cajan var* ICP-26.
(A) Transverse section of leaf, (B) transverse section of petiole, and (C) transverse section of pulvinus of *C. cajan*. T is glandular trichomes, E is epidermis, Col is collenchyma, PAL is palisade tissue, F is fibres, BS- is basal sheath, Cor is cortex, Ph is phloem and X is xylem and pith.

leaves (fig. 6a). Similar glandular and simple hairs are found on all aerial parts of the plant[15]. The petiole contains number of xylem vessels outside which lies the fibres. The upper surface of the petiole contains two flanges in which vascular bundles are present. Pith is present towards the centre. Trichomes are present surrounding the petiole (fig. 6b). Pulvinus is a swollen region present at the proximal end of the petiole. At the junction of leaflet and petiole pulvinus is found. In the centre of pulvinus, vascular tissue is arranged in a horse shoe shape at the centre, xylem on inside and phloem surrounded by fibres. Pulvinus consists of cortical tissue (fig. 6c). The changes in state on different sides of pulvinus is responsible for the movements of petioles and leaves. The movements of leaves are influenced by intensity of light falling on pulvini[44]. Our results suggest that *C. cajan* leaf extract can be used as an effective and safe source of natural antioxidant that might be helpful in preventing various oxidative stress related diseases, as ethanomedicine and also for production of green drugs commercially.

In the present study, a set of pharmacognostical parameters were conducted on *C. cajan* leaves. These studies revealed the presence of various important bioactive compounds and proved that the plant leaves can also be of medicinal importantance. These results may help in standardization, identification and in carrying out further research in *C. cajan* leaf based drugs which are used in traditional and modern pharmacopoeia.

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