Vol. 9/2 (2009) 228 - 234

JOURNAL OF NATURAL REMEDIES

In-vitro free radical scavenging activity of ethanolic extract of leaves of *Cajanus cajan* (L.) Millsp

T. Ghosh [1]*, T. K. Maity [2], A. Bose [1]

1. Institute of Pharmacy and Technology, Salipur, Cuttack district, Orissa - 754202.

2. Department of Pharmaceutical Technology, Jadavpur University, Kolkata - 700032.

Abstract

Objective: To study the *in vitro* antioxidant activity of the ethanolic extract of leaves of *Cajanus cajan* Linn. Materials and methods: The antioxidant activity, reducing power, nitric oxide scavenging activity, superoxide radical scavenging activity, anti lipid peroxidation activity and total phenolics of the ethanolic extract of *Cajanus cajan* were studied *in vitro* as parameters to investigate its antioxidant potential. Results: The reducing power of the extract was found to be concentration dependant. The absorbance increased with increasing amount of the sample. The antioxidant activity, nitric oxide scavenging activity, superoxide radical scavenging activity and anti lipid peroxidation activity were also concentration dependant with IC_{50} value being 324.59 µg/ml, 46.42 µg/ml, 35.24 µg/ml and 338.02 µg/ml respectively. The activities were found to be comparable with the reference drugs. Amount of total phenolics was found to be 39.8 µg of pyrocatechol equivalent per mg of extract. Conclusion: The results obtained in the present study indicate that ethanolic extract of *C. Cajunus* is a potential source of natural antioxidants.

1. Introduction

The role of free radical reactions in disease pathology is well established, suggesting that these reactions are necessary for normal metabolism but can be detrimental to health as well including outcome of various diseases like diabetes, immunosupression, neurodegenerative diseases and others [1, 2]. Free radicals lead to cellular necrosis [3], which is implicated in a membrane pathophysiological condition, including atherosclerosis, rheumatoid arthritis as well as toxicity of many xenobiotics [4]. Lipid peroxidation is one of the major factors causing deterioration of foods during storage and processing. Although there are some synthetic antioxidant compounds, such as butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA), which are commonly used in processed foods, it has been reported that these compounds are responsible for liver damage and carcinogenesis [5, 6]. Therefore the development and utilization of more effective antioxidants of natural origin are desired.

* Corresponding author
Email: tghosh75@yahoo.co.in

Cajanus cajan (L.) Millsp. (*Fabaceae*) is a perennial woody shrub with strong stems, freely branching, root system deep and extensive with a taproot, distributed throughout India. In India, the young leaves are applied for the treatment of sores and help to expel bladderstones. Leaf juice is used for jaundice [7], toothache, dysentery, heart disease, bronchitis and as anthelmintic [8]. The plant has been reported to show hypoglycaemic activity [9]. The plant is reported to contain β-sitosterol, stigmasterol, flavanone such as cajaflavanone, lupeol, α and β amyrins, a new anthraquinone viz. cajaquinone [10]. Flavonoids are natural products, which have been shown to possess various biological properties related to antioxidant mechanism [11]. As *C. cajan* contains flavonoids it is thought worthwhile to investigate the *in vitro* free radical scavenging potential of the leaves of *Cajanus cajan.*

2. Materials and Methods

2.1. Plant material

The plant was identified by the taxonomists of Botanical Survey of India, Howrah. After authentication, fresh leaves were collected in bulk from young matured plants from the rural belt of Salipur, Orissa, India during early summer, washed under running tap water to remove adhering dirt, shade dried and finally milled in to coarse powder by a mechanical grinder.

2.2. Preparation of the extract

The powdered plant material (400 g) was defatted with petroleum ether (60-80°C) and then extracted with 1.5 litre of ethanol (95%) in a soxhlet apparatus. The solvent was then removed under reduced pressure, which obtained a greenish-black sticky residue (yield: 15.5 % w/w with respect to dried plant material).

2.3. Animals

In vitro anti lipid peroxidation study was carried out using liver from a Wistar albino rat (150 g) of male sex. The animal was housed in a polyacrylic cage (38 × 23 × 10 cm) and maintained under standard laboratory conditions. The rat was allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The procedure described was reviewed and approved by the Institutional Animal Ethical Committee.

2.4. In vitro free radical scavenging activity

2.4.1. Antioxidant activity

The antioxidant activity was determined according to the thiocyanate method (Gulchin *et al*, 2002) [12]. 1 ml of different concentrations of the extract of *C. cajan* was added to linoleic acid in potassium phosphate buffer (2.5 ml, 0.04 M, pH 7.0). The mixed solution was incubated at 37°C. The peroxide value was determined by reading the absorbance at 500 nm, after reaction with ferrous chloride and thiocyanate after two hours of incubation. The solutions without added extracts were used as blank samples. Percent inhibition was calculated from the formula:

$$\% \text{ inhibition} = (A_c - A_t)/ A_c \times 100$$

where A_c and A_t are absorbance of control and test samples respectively.

2.4.2. Amount of total phenolic compounds

The method of Singleton *et al*, 1999 [13] was followed. 0.1ml of 10mg/ml aqueous solution of the extract was diluted with 46 ml of distilled water in an Erlen meyer flask. Afterwards, 1 ml of Folin – Ciocalteu Reactive (FCR) was added into this mixture followed by addition of 3 ml of Na_2CO_3 (2%) after 3 min. Subsequently, mixture was shaken on a shaker for 2 h at room temperature and then absorbance was measured at 760 nm. The concentration of total phenolic compounds was determined as micrograms of

pyrocatechol equivalent by using the following equation that obtained from the standard pyrocatechol graph.

Absorbance = 0.001 x Pyrocatechol (μg) + 0.0033.

2.4.3. Reducing power

The reducing power of the ethanolic extract of *C. cajan* was determined according to the method of Oyanzu, 1986 [14]. Accurately weighed 10 mg of the extract in 1 ml of distilled water was mixed in to the mixture of 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50°C for 20 min. Following incubation, 2.5 ml of 10% trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1 %) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated the increased reducing power.

2.4.4. Assay for superoxide radical scavenging activity

The assay for superoxide radical scavenging activity was performed as per standard procedure [15, 16]. The reaction mixture contained 50 mM phosphate buffer (pH 7.6), 20 μg riboflavin, 12 mM EDTA and 0.1 mg/ml of NBT (nitro blue tetrazolium), all added in chronological sequence. Reaction was started by illuminating the reaction mixture containing different concentrations of the sample extract for 90 sec and then measuring the absorbance at 590 nm. Ascorbic acid was taken as the positive standard.

Table 1. Antioxidant activity of ethanolic extract of leaves of *Cajanus cajan*

Sample	Concentration (μg/ml)	% inhibition	IC_{50}	r*
	100	15.19 ± 1.76		
	200	35.62 ± 2.25		
Ethanolic Extract	300	46.87 ± 3.69	324.59	0.9994
	400	56.83 ± 3.27		
α-tocopherol			311.94	0.9699

n =3, Values are Mean ± S.D. r* – regression co- efficient.

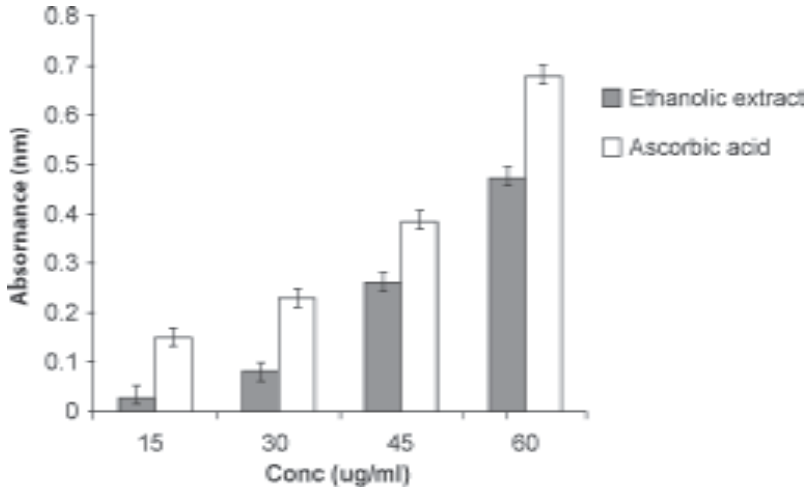

Table 2. Reducing activity of ethanolic extract of leaves of *Cajanus cajan*

Concentration	Absorbance	
(mg/ml)	Ethanol extract	Ascorbic acid
15	0.025 ± 0.003	0.150 ± 0.007
30	0.082 ± 0.006	0.229 ± 0.008
45	0.262 ± 0.011	0.385 ± 0.026
60	0.473 ± 0.028	0.678 ± 0.035

Table 3. *In vitro* NO radical scavenging activity of ethanolic extract of leaves of *Cajanus cajan*

Sample	Concentration (µg/ml)	% inhibition	IC_{50}	r*
	4.17	4.75 ± 0.47		
	8.33	22.98 ± 2.27		
Ethanolic Extract	16.67	29.43 ± 2.47	46.42	0.9934
	33.33	42.46 ± 3.20		
	100.00	64.64 ± 3.40		
Ascorbic acid			15.02	0.9861

n=3, Values are Mean ± S.D. r* – regression co- efficient.

Table 4. Superoxide radical scavenging activity of ethanolic extract of leaves of *Cajanus cajan*

Sample	Concentration (µg/ml)	% inhibition	IC_{50}	r*
	11.67	6.42 ± 0.53		
	20.83	25.22 ± 2.28		
Ethanolic Extract	41.67	63.11 ± 2.10	35.24	0.9906
	83.33	81.25 ± 4.65		
Ascorbic acid			34.24	0.9868

n=3, Values are Mean ± S.D. r* – regression co- efficient.

Table 5. *In vitro* anti lipid peroxidation activity of ethanolic extract of leaves of *Cajanus cajan*

Sample	Concentration (µg/ml)	% inhibition	IC_{50} (ìg/ml)	r*
	150	18.24 ± 1.46		
	300	45.42 ± 3.09		
Ethanolic Extract	450	63.15 ± 3.36	338.02	0.9981
	600	70.41 ± 3.92		
α-tocopherol			133.86	0.9947

n=3, Values are Mean ± S.D. r* – regression co- efficient.

2.4.5. Assay for nitric oxide scavenging activity

The method of Bagul *et al.*, 2003 [17] was followed. For the experiment, sodium nitropruside (10 mM) in phosphate buffered saline was mixed with different concentrations of the extract dissolved in methanol and incubated at room temperature for 2½ h. The same reaction without the sample but equivalent amount of methanol served as control. After incubation period, 0.5 ml of Griess reagent was added. Absorbance of the chromophore formed was measured at 546 nm. Ascorbic acid was used as positive control.

The procedure is based on the principle that, Sodium nitropruside solution spontaneously generates nitric oxides, which reacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Scavengers of nitric oxides compete with oxygen leading to reduce production of nitrite ions.

2.4.6. $FeCl_2$-ascorbic acid stimulated lipid peroxidation in liver homogenate [18]

The Wister albino rats weighing 175-200 g were killed by decapitation and their liver tissues were quickly removed. A 2 g portion of liver tissue was sliced and then homogenized with 10 ml of 150 mM KCl Tris-HCl buffer (pH 7.2). The reaction mixture was composed of 0.25 ml of liver homogenate, 0.1 ml of Tris-HCl buffer (pH 7.2), 0.05 ml of 0.1 mM ascorbic acid, 0.05 ml of 4 mM $FeCl_2$ and 0.05 ml of various concentrations of the extract were incubated for 1 hr at 37°C. Appropriate controls were used to eliminate any possible interference with the thiobarbituric acid assay. After incubation 2 ml of 0.6% thiobarbituric acid (TBA) were added to 0.5 ml of incubated solution and shaken vigorously. The mixture was heated for 30 min in a boiling water bath. After cooling 5 ml of n-butanol was added and the mixture was again shaken vigorously. The n-butanol layer was separated by centrifugation at 1000 g for 30 min and the absorbance was measured at 532 nm.

3. Results and Discussion

Preliminary phytochemical screening [19] of the extract gave positive tests for presence of flavonoids, cardiac glycosides and alkaloids.

Although oxygen is essential for life, its transformation to reactive oxygen species (ROS), may provoke uncontrolled reactions. Such challenges may arise due to exposure to radiation, chemical etc. Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and some other mechanism [20].

Table 1 reveals that the extract possesses significant antioxidant activity with IC_{50} value being 324.59 (μg/ml) and is comparable with the reference drug α-tocopherol.

Table 2 shows the reductive capacity of the ethanolic extract of *C. cajan* as compared with ascorbic acid. For measurements of the reductive ability, we investigate Fe^{+3} to Fe^{+2} transformation in the presence of ethanolic extract using the method-discussed earlier. The reducing capacity of the compound may serve as a significant indicator of its potential antioxidant activity [21]. The reducing power of the ethanolic extract of *C. cajan* was found to be concentration dependant.

Phenols are very important plant constituents because of their free radical scavenging ability due to their hydroxyl groups [22]. The phenolic compounds may contribute directly to antioxidant action. In ethanolic extract of *C. cajan* (1 mg) 39.8 μg pyrocatechol equivalent was detected. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans [23].

Nitric oxide (NO) exhibits numerous physiological properties and it is also implicated in several pathological states [24]. Ethanolic extract of *C. cajan* significantly decreased with IC_{50} value 46.42 µg/ml, in a dose- dependent fashion, the concentration of nitrite after spontaneous decomposition of sodium nitropruside, indicating that the ethanolic extract may contain compounds that are able to scavenge nitric oxide (Table 3). However, the specificity of this assay has been questioned since nitrite is one final product of the reaction of nitric oxide with oxygen, through intermediate such as NO_3, N_2O_4, N_2O_3 [25]. Therefore the decrease in the nitrite production could also be due to interaction of the extract with other nitrogen oxides [26].

The *in-vitro* superoxide radical scavenging activity is measured by riboflavin/light/NBT (Nitroblue tetrazoline) system reduction. The method is based on generation of superoxide radicals by auto oxidation of riboflavin in the presence of light. The superoxide radical reduces NBT to a blue colored formazone that can be measured at 560-nm [27]. The capacity of the ethanolic extract of *C. cajan* to inhibit the colour to 50% is measured in terms of IC_{50}. We have observed that the extract reduced the absorbance in a dose dependent manner, and the IC_{50} value was calculated to be 35.24 µg/ml from the curve, which is comparable with the reference drug (Table 4).

In vitro lipid peroxidation in liver homogenate can proceed in a non-enzymatic way. The process is induced by ascorbate in the presence of Fe^{2+}/Fe^{3+}, and it has been reported that Fe^{2+} and ascorbic acid stimulated lipid peroxidation in rat liver microsomes and mitochondria. $FeCl_2$-ascorbic acid induced *in vitro* lipid peroxidation study reveals that the extract has significant anti lipid peroxidation potential with IC_{50} value of 338.02 µg/ml, which is comparable with the reference drug - tocopherol (Table 5).

The present study indicates that ethanolic extract of *C. cajan* leaves possess good free radical scavenging activity. Further study regarding the isolation and characterisation of the active constituents responsible for the antioxidation activity *in vitro* is currently under progress in our laboratory.

4. Acknowledgement

The authors are thankful to the management of Institute of Pharmacy and Technology, Salipur and the authorities of Jadavpur University for providing necessary facilities to carry out the present research work.

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