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Original Article

Antioxidant Potential of a Toxic Plant *Calotropis procera* R.Br.

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ABSTRACT

Background: *Calotropis procera* R.Br. (Family, Apocynaceae) is a toxic herb which was known to have great importance in the Ayurveda. The plant is anthelmintic; relieves strangury; cures ulcers; the ashes act as an expectorant. The plant was also known to be used in the treatment of jaundice from ancient times. Studies have shown that it has cardio-protective property in Myocardial Infarction. The present study was focused on evaluating the anti-oxidant activity of the leaves by *in vitro* models. **Methods:** The anti-oxidant activity in different extracts and fractions of the leaves viz. total alcoholic and water extracts; petroleum ether, ethyl acetate, butanol and water fractions were evaluated for antioxidant properties using various *in vitro* models like free radical scavenging activity, hydroxyl radical scavenging activity, nitric oxide scavenging activity, hydrogen peroxide scavenging activity, reduction of ferric ions and lipid peroxidation assay; and total antioxidant capacity was established. **Results:** Antioxidant potency was found to be highest in butanol fraction, water fraction and aqueous alcoholic extract. **Conclusions:** The antioxidant property of different extracts and fractions of the leaves were studied *in vitro* and results have shown that they are potent antioxidant in nature.

Key words: *Calotropis procera*, Free Radicals, ROS, DPPH, Antioxidant.

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INTRODUCTION

Free radicals have been claimed to play an important role in affecting human health by causing several chronic diseases, such as cancer, diabetes, aging, atherosclerosis, hypertension, heart attack and other degenerative diseases. These free radicals are generated during body metabolism.[1] They induce damage to biomembranes, proteins and DNA. Lipid peroxidation, which involves a series of free radical mediated chain reaction processes, is also associated with several types of biological damage. Therefore, much attention has been focused on the use of antioxidants, especially natural antioxidants to inhibit ROS production and protect from damage due to ROS.[2] Exogenous intake of antioxidants can help the body scavenge free radicals effectively.[1]

The medicinal properties of plants have been investigated, in the light of recent scientific developments, throughout the world due to their potent pharmacological activities and economic viability. Antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, anti-bacterial or antiviral activities to a greater or lesser extent. Antioxidant compounds are found to be useful in treatment of diabetes so the attention has been focused to natural products, especially to phenols, because of antioxidant activity of phenolic acids, tannins, flavonoids.[2] Nowadays, there is a noticeable interest in antioxidants, especially in those which can prevent the presumed deleterious effects of free radicals in the human body, and to prevent the deterioration of fats and other constituents of foodstuffs.[1]

*Calotropisprocera*R.Br. (Family, Apocynaceae) is a moderate evergreen herb found more or less throughout India in warm dry places, Afghanistan, Iran, Arabia, Egypt and throughout African continent.[3] It is an erect shrub usually 1.8-2.4 m high (sometimes growing much. higher in arid places); young parts clothed with white cottony tomentum; bark soft, spongy. Leaves subsessile, usually 5.7-15 × 4.5-8.2 cm. (exceptionally 23 by 15 cm.), broadly ovate, ovate oblong, elliptic or obovate, with a short abrupt acumination. Flowers in umbellate cymes purple in colour.[4]

The plant secretes poisonous milky latex. Even though it is toxic, the use of the plant in various diseases has been known to be widespread since ancient times. It was found to be laxative, anthelmintic; used in conditions *viz*. strangury; ulcers; the ash in cough. The leaves are applied hot to the abdomen to cure pain. The flower was used as tonic, appetizer, stomachic and in treatment of piles and asthma (Ayurveda). In the, Gold Coast, the leaves are used to cure swollen legs and also wounds caused by rusty nails. The leaves are said to cure catarrh, being warmed first and then the juice is dropped into the nose. This causes the patient to sneeze, which relieves the accumulation of mucous material. The fresh root is used as a toothbrush, and is considered by Pathans to cure toothache. The medicinal properties of the plant are similar to those of *C. gigantea* and the plant is used in diseases of spleen, liver and abdomen.[3-5]

As no reports have been established on its antioxidant potency in detail, the study has been focused on determining its total antioxidant capacity and further establishing its monograph. Different extracts and fractions of the plant leaves were prepared and the phytochemical investigation was carried out and further *in vitro* modals were employed in evaluating its antioxidant potency.

MATERIALS AND METHODS

Chemicals and Reagents

DPPH, egg phospatidylcholine, β-carotene, and γ-linoleic acid were obtained from Sigma Chemical Co. (St.Louis, MO, USA). BHT, ascorbic acid, Tween-40, deoxy-d-ribose, TCA, and TBA were obtained from Hi-Media Labs (Mumbai, India). 1,10-o-phenanthroline, $FeCl_3$, ammonium molybdate, and sodium dithionite were obtained from Ranbaxy Fine Chemicals (New Delhi, India). Phenylhydrazine and Folin-Ciocalteau's phenol reagent were obtained from BDH Products (UK). Silymarin was a kind gift from Dr. Chidambaramurthy K.N., Texas A&M University, Texas, USA. All other chemicals used were of analytical grade. The solvents used for extraction were from Ranbaxy Fine Chemicals (New Delhi, India). The UV-Visible spectrophotometric values were recorded in JASCO UV-500 Spectrophotometer.

Collection and identification of plant material

Leaves of *C. procera* R.Br. were collected during December 2007 in Mysore city outskirts. The plant was identified and authenticated by Dr. M.N.Naganandini, Asst. Professor, Dept. of Pharmacognosy& Phytochemistry, JSS College of Pharmacy, Mysore. Herbarium specimen of *C. procera*, bearing voucher number KMCP01 was deposited in the Department of Pharmacognosy, JSS College of Pharmacy, Mysore, India.

Preparation of extracts and fractions

The powdered leaves of *C.procera* were extracted with 70% aqueous alcohol by cold maceration for 72 h. The extract was separated from marc by filtration using muslin cloth. The drug was extracted 3 times with a gap of 3 days each. The filtrates were pooled and concentrated to syrupy liquid under reduced pressure using Super fit Rotary vacuum evaporator at 60°C and preserved in well closed container at 4-8°C until use. Syrupy liquid was cooled and added with one liter of distilled water and subjected to fractionation using Petroleum ether (CPPE), ethyl acetate (CPPEA) and butanol (CPB). The fractions were dried in dessicator *in vacuo.*The extract was used directly for assessment of antioxidant capacity through various chemical assays.

Preliminary phytochemical screening

Preliminary phytochemical screening was carried out for different extracts and fractions of the plant to know the nature of constituents.

Standard Procedures were adopted for the preliminary phytochemical screening. Test for Sterol,[17-19] Test for Tri-terpenes,[18,19] Test for Saponins,[18,19] Tests For Alkaloids,[19,20] Tests For Carbohydrates,[18-21] Test For Tannins,[18,19] Tests For Flavonoids,[22] and Tests for lactones[18,23] were carried out and the results are given in Table 1.

In vitro Free Radical Scavenging and Antioxidant Screening

Free radical scavenging activity by DPPH Method

Free radical scavenging potentials of the extracts and fractions were tested against a methanolic solution of DPPH. Antioxidants react with DPPH and convert it toα,α-diphenyl-β-picryl hydrazine. The degree of discoloration indicates the scavenging potentials of the antioxidant extract. The change in the absorbance produced at 517 nm has been used as a measure of antioxidant activity.[6]

Preparation of sample solutions of extracts/fractions

Sample stock solutions (2 mg/ml) of CPPE, CPEA and CPB fractions of *C.procera* leaves were prepared. CPWF and CPWE were first dissolved in 5 ml of distilled water, added with 20 ml of ethanol, filtered and the filtrate was used for the experiment. All were diluted to final concentrations 50-1000 μg/mL in ethanol. 5 to 50 μg of ascorbic acid and 10 to 80 μg of BHT were taken as standards.5 ml of methanolic solution of DPPH was added to all dilutions, shaken well and the mixture was allowed to stand at room temperature for 20 min. The control was prepared as above without extract. The readings were read at 517 nm using methanol as blank. Scavenging activity was expressed as the inhibition percentage calculated using the following formula,

% Anti radical activity [(C- S)/C] X 100

Where C is control absorbance, S is the sample absorbance. Each experiment was carried out in triplicate and results are given in the Table 2.

Nitric Oxide (NO^-) scavenging activity

Nitric oxide reacts with O_2 to produce stable products, nitrates and nitrite through intermediates NO_2, N_2O_4 and N_3O_4. It is estimated by using the Griess reagent. In the presence of test compound (scavenger), the amount of nitrous acid will decrease. The extent of decrease will reflect the extent of scavenging, which is measured at 546 nm.[7]

Preparation of sample solutions of extracts/fractions

Different extracts/fractions were prepared in the same manner as that in case of DPPH activity. Ascorbic acid was used as standard from 25 to 125 μg. Volume was adjusted to 500 μl with alcohol and 500 μl of SNP in PBS was added and incubated at 25^0C for 180 min. 1 ml of Griess reagent was added. The absorbance was immediately measured at 546 nm. The Nitric Oxide scavenging activity was calculated from the formula.

% Nitric oxide scavenging activity = [(C- S)/C] X 100

Where C is control absorbance, S is the sample absorbance. Each experiment was carried out in triplicate and results of various extracts/fractions and standard are listed in the Table 4.

Reduction of ferric ions

Fe^{2+} reacts rapidly with 1,10-*O*-phenanthroline and forms a stable red colored complex. Extracts reacts with Fe^{3+} to reduce and convert it to Fe^{2+}. The degree of coloration indicates the reduction potential of the extracts. The change in the absorbance produced at 510 nm has been used as a measure of Ferric ions reducing activity. The reduction is measured taking Sodium dithionite instead of the extract and considered as equivalent to 100% reduction of all the ferric ions present.[8-10]

Preparation of sample solutions of extracts/fractions

The reaction mixture containing o-phenanthroline (0.5mg), ferric chloride (0.2 mM) and test compound (extracts/fractions of different concentrations 20, 40, 60, 80, 120 μg/mL) in a final volume of 5 ml was incubated for 15- 20 min at ambient temperature. The absorbance at 510 nm was measured. In another set, sodium dithionite (0.3 mM) was added instead of the extract and the absorbance was taken as equivalent to 100% reduction of all the Fe^{3+} present. BHT, Ascorbic acid and Silymarin were used as standards. Table 6 depicts the Fe^{3+} reduction capacity of each extract/fraction.

Total antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH. The green color is measured at 695 nm. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.[7,11]

Preparation of sample solutions of extracts/fractions

Different extracts/fractions (2 mg/ml) in ethanol and standard ascorbic acid (1 mg/ml) in distilled water were prepared. An aliquot of 0.1 ml of sample solution was combined in an Eppendorff tube with 1.9 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and a blank containing only 1.9 ml of reagent was incubated in a thermal block at 95°C for 90 min. The samples were cooled to room temperature; the absorbance was measured at 695 nm against blank. The antioxidant capacities are expressed as equivalents of ascorbic acid. The experiment was conducted in triplicate and values are expressed as ascorbic acid equivalents in mg per gram of extract (mean ± SD) in Table 8.

Antioxidant assay using β CLAMS

Linoleic acid which is an unsaturated fatty acid gets oxidized by ROS produced by oxygenated water. The products formed will initiate the β-Carotene oxidation, which leads to discoloration. This is prevented by antioxidants hence decrease in extent of discoloration indicates the activity i.e. more the discoloration, less will be the antioxidant activity and vice versa.[12-13]

Table 1: Preliminary phytochemical screening of *C. procera*

Chemical Tests	*Aq. Alc.*	*Pet. Ether*	Ethyl acetate	Butanol	Water fraction	Water extract
Tests for Sterols						
a)) Salkowski test	+	+	-	-	-	-
b) Liebermann-Burchard	+	+	-	-	-	-
Tests for Triterpenes						
a)Salkowski test	-	-	-	-	-	-
b) Liebermann-Burchard test	-	-	-	-	-	-
Tests for Saponins						
a) Foam test	-	-	-	-	-	-
b)Haemolysis test	-	-	-	-	-	-
Tests for Alkaloids						
a) Wagner's test						
b) Mayer's test	-	-	-	-	-	-
c)Dragendorff's test	-	-	-	-	-	-
d) Hager's test	-	-	-	-	-	-
Tests for Carbohydrates	-	-	-	-	-	-
a) Fehling's test						
b)Molisch's test	+	-	+	+	+	-
Tests for Tannins	+	-	+	+	+	+
a) Ferric chloride test						
b) Gelatin test	-	-	+	+	+	+
c) Vanillin-HCl test	-	-	-	-	-	-
d) Match stick test	-	-	-	-	-	-
Tests for Flavanoids	-	-	-	-	-	-
a)Shinoda test						
b) Ferric chloride test	+	-	+	+	-	-
c) Lead acetate test	+	-	+	+	+	+
d) Zinc-HCl test	+	-	+	-	+	+
e)NaOH test	+	-	+	+	+	+
f)NaOH- HCl test	+	-	+	+	+	-
Tests for Lactones /Cardiac Glycosides	+	-	+	+	+	+
a)Legal's test	+	+	+	+	+	+
b)Baljet's test	+	+	+	+	+	+

Table 2: DPPH Free radical scavenging activity of different extracts and fractions of *C. procera*

IC_{50} in µg/ml	CPALC		CPPE		CPEA		CPB		CPWF		CPWE	
9.09	3.10	±1.06	4.98	±1.37	4.08	±1.43	9.63	±1.0	5.07	±2.80	3.5	±1.02
18.18	8.35	±1.35	7.25	±1.24	8.58	±2.30	15.55	±0.69	8.09	±1.73	5.36	±0.63
36.36	11.91	±2.80	12.36	±1.50	15.52	±0.78	24.24	±2.53	9.61	±0.98	7.50	±1.83
54.54	16.94	±1.48	16.28	±1.20	23.63	±0.91	33.04	±2.54	11.53	±2.90	8.76	±1.31
72.73	22.12	±0.93	20.08	±0.67	30.10	±2.73	43.57	±0.63	14.31	±1.04	10.48	±0.87
109.09	28.19	±1.85	25.55	±1.04	38.23	±1.67	58.94	±3.06	21.61	±1.30	13.30	±2.64
145.45	32.34	±1.71	31.13	±1.92	45.98	±1.00	72.24	±1.77	24.64	±1.63	15.10	±3.82
181.82	42.95	±2.50	37.10	±1.58	53.18	±1.36	83.94	±1.52	28.14	±3.69	17.53	±1.91
IC_{50} in µg/ml	215 ± 15^{PR}		248 ± 12^{PR}		159.09±4.54		96.64±1.22		335 ± 15^{PR}		620 ± 20^{PR}	

Results given are mean percentage DPPH Radical scavenging activity. IC_{50} values given are in µg/ml mean±SD, n=3

Composition of emulsion

5 mg β-carotene, 40 mg linoleic acid and 400 mg Tween-40 were mixed in 1 ml of chloroform and chloroform was removed under vacuum using the rotavapour at not more than 45^0C. This emulsion was mixed with 20 ml water & 80 ml of oxygenated water.

Preparation of sample solutions of extracts/fractions

2 mg/ml solution of different extracts/fractions and BHT standard in ethanol was used for experiments.50-300 µl of all extracts/fractions were added to separate tubes and volume was made up to 0.3 ml with ethanol. 0.7 ml of distilled water and 3 ml of β-Carotene linoleic acid emulsion was added to each tube. Absorbance was taken at 470 nm at Zero time and tubes were placed at 50^0C in water bath. Measurement of absorbance was continued at an interval of 15 min, till the color of β-Carotene disappeared in the control reaction (t = 180 min). A blank without β-Carotene emulsion and a control without extract was used. Each experiment was carried out in triplicate. The antioxidant activity (%AA) of extracts was evaluated in terms of bleaching of β-Carotene using the following formula. The results are shown in Table 9.

$$\% \text{ AA} = 100[1- (A^0 – A^t)/ A^o 0 – A^o t)]$$

Where, % AA = Antioxidant activity, $\mathbf{A^O}$ = Zero time absorbance of sample,

$\mathbf{A^t}$ = Absorbance of sample after incubation for 180 min, $\mathbf{A^0\,0}$ = Zero time absorbance of control, $\mathbf{A^t\,0}$ = Absorbance of control after incubation for 180 min.

The results were expressed as % antioxidant activity±SD.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging was measured by studying the competition between deoxy-*d*-ribose and sample extracts for hydroxyl radicals produced by Phenyl hydrazine.[12,14] The extent of deoxy-*d*-ribose degradation was measured by TBARS method of Ohkawa *et al.*[15]

Preparation of sample solutions of extracts and fractions

All the extracts/fractions of different concentrations 10 to 80 µg (1 mg/ml) and Gallic acid, standard in the concentration range of 0.5 to 4 µg were freshly prepared in distilled water by sonication and clear filtrate was used. Each reaction mixture containing the sample or standard dilution with 2.8 mM deoxy-*d*-ribose and phenyl hydrazine 0.2 mM was incubated for 2 h at 37^0C in BOD incubator. To each test tube was added 1 ml of 2.8% TCA containing 1% TBA and heated in boiling water bath for 20 min. and cooled. The absorbance was read at 532 nm. Percentage hydroxyl radical scavenging activity was calculated by the formula.

$$\% \text{ Hydroxyl radical scavenging activity} = [(C- S)/C] \times 100$$

Each experiment was carried out in triplicate and the results are given in Table 10.

Lipid peroxidation assay

In this assay, peroxidation of egg phospatidylcholine liposomes is induced by $FeCl_3$ and ascorbic acid as reducing agents. ·OH radicals generated by mixing Fe^{3+} and ascorbate attack the egg phospatidylcholine liposomes. This leads to the formation of (Malondialdehyde) MDA and other aldehydes, which form a pink chromagen with TBA absorbing at 532 nm.[16]

Preparation of sample solutions of extracts and fractions

1 mg/ml stock solution of extract/fraction was prepared in distilled water by sonication for 7 mins and filtered. Clear filtrate is used for the following experiment. Egg phospatidylcholine (20 mg) in chloroform (2 ml) was dried under vacuum in a rotary evaporator to give a thin homogenous film, and further dispersed in normal saline (5 ml) with a vortex mixer. The mixture was sonicated to get a homogeneous suspension of liposomes. Lipid peroxidation was initiated by adding 0.05 mM ascorbic acid to a mixture containing liposome (0.1 ml), 150 mM potassium chloride, 0.2 mM ferric chloride, extract/fraction (20 to 100 µg/ml) in a total volume of 0.5 ml. Gallic acid and Silymarin (1 to 8 µg/ml). The reaction mixture was incubated for 40 mins at 37ϒC. After incubation, the reaction was terminated by adding 1 ml of ice cold 0.25 M sodium hydroxide containing 20% w/v TCA, 0.4% w/v of TBA and 0.05% w/v BHT. After heating at 80ϒC for 20 min, the samples were cooled. The pink chromogen was extracted with 2 ml of butan-1-ol, and the absorbance of the upper organic layer was measured at 532 nm. % Anti lipid peroxidation activity was calculated by the formula,

$$\% \text{ Anti lipid peroxidation activity} = [(C- S)/C] \times 100$$

Where C is the absorbance of the control and **S** is the absorbance of the sample. Each experiment was carried out in triplicate and results were expressed as % Antioxidant activity±SD

Each experiment was carried out in triplicate and the results are given in Table 11.

RESULTS

Preliminary phytochemical screening

Free radical scavenging activity

As shown in Table 2, among the different fractions and extracts of *C. procera,* butanol fraction showed highest DPPH radical scavenging activity with an IC_{50} of 96.64±1.22 µg/ml followed by ethyl acetate fraction (IC_{50} 159.09±4.54 µg/ml). CPALC, CPPE, CPWF and CPWE were not able to show 50% free radical scavenging activity even at highest concentration tested (181.82 µg/ml). The activity ranging from weak to strong occurred in the order: CPWE < CPWF < CPPE < CPALC < CPEA < CPB.

NO scavenging activity

As shown in Table 4, butanol fraction of *C. procera* was found highly active in scavenging NO· compared to other fractions and extracts. CPALC, CPPE, CPWF and CPWE failed to show 50% NO· scavenging activity even at 1000 µg/ml concentration. IC_{50} value of CPB and CPEA were found to be 500.15±20.02 µg/ml and 805.24±94.02 µg/ml respectively. CPB had shown the highest NO· Scavenging activity followed by CPEA. NO· scavenging activity from weak to strong in the order: CPWE< CPWF< CPPE< CPALC< CPEA< CPB.

Table 3: DPPH Free radical scavenging activity of BHT and Ascorbic acid

Conc. in µg/ml	Ascorbic acid		Conc. in µg/ml	BHT	
4.54	42.96	±1.06	4.54	31.66	±0.46
9.09	49.32	±0.37	9.09	52.18	±1.56
13.63	75.93	±0.86	13.63	77.08	±1.12
18.18	94.70	±0.33	18.18	83.07	±1.20
22.72	95.41	±0.39	22.72	88.39	±0.93
			27.27	90.67	±0.44
IC_{50} in µg/ml	**4.91±0.36**			21.88±2.1	

Results given are mean percentage DPPH Radical scavenging activity. IC_{50} values given are in µg/ml mean±SD, n=3

Table 4: NO. Free radical Scavenging activity of different extracts and fractions of *C. procera*

Conc. in µg/ml	CP ALC		CP PE		CP EA		CP B		CP WF		CP WE	
100	9.26	±3.51	9.00	±2.53	9.43	±2.07	18.90	±3.66	10.34	±3.20	9.13	±2.76
200	15.19	±2.93	14.18	±3.60	15.94	±1.91	26.36	±2.72	13.04	±3.35	12.05	±3.41
300	18.38	±3.71	17.75	±1.79	25.91	±2.95	34.05	±2.70	15.21	±3.74	12.99	±2.92
400	24.83	±2.39	20.95	±2.24	30.46	±1.15	44.94	±1.81	18.06	±3.25	14.99	±3.47
600	30.01	±1.78	25.72	±0.85	40.88	±2.46	56.92	±2.86	23.64	±1.76	17.66	±3.43
800	35.10	±3.00	31.58	±2.41	50.31	±3.91	72.57	±0.94	28.14	±3.34	18.96	±3.53
1000	44.58	±1.77	37.93	±2.15	54.86	±3.08	81.00	±2.85	33.25	±4.59	20.91	±2.96
IC_{50} in µg/ml	1076.7±63.5[PR]		1313.3±32.2[PR]		805.24±94.02		500.15±20.2		1723.3±258.1[PR]		2183±76.3[PR]	

Results given are mean percentage Nitric oxide scavenging activity

Table 5: NO.Free radical Scavenging activity of Ascorbic acid

Conc. in µg/ml	% NO. scavenging activity	
25	33.53	±2.17
50	42.56	±2.58
75	46.23	±2.10
100	59.76	±4.16
125	69.56	±0.53
IC_{50} in µg/ml	83.80±3.17	

IC_{50} values given are in **µg/ml** mean±SD, n=3

Table 6: Ferric ion reduction activity of different extracts and fractions of *C. procera*

Conc. in µg/ml	CPALC		CPPE		CPEA		CPB		CPWF		CPWE	
20	9.82	±0.97	7.63	±1.28	19.09	±0.89	20.14	±2.35	7.91	±1.05	3.81	±0.46
40	12.00	±1.36	9.81	±0.81	22.35	±0.32	32.46	±1.61	9.00	±0.96	4.38	±1.07
60	16.65	±1.47	11.72	±0.13	32.73	±1.54	49.07	±2.02	14.18	±0.75	3.55	±0.52
80	22.89	±0.61	15.26	±0.39	38.44	±0.92	64.03	±1.15	15.28	±0.97	5.74	±1.00
120	27.53	±1.21	17.20	±1.38	50.74	±2.49	86.45	±4.96	17.98	±0.29	7.63	±0.22
IC_{50}in µg/ml	235	**±13.46[PR]**	455	**±128.9[PR]**	115	±14.2	60	±12.48	425	**±14.25[PR]**	975	**±10.48[PR]**

Results given are mean percentage Ferric ion reduction activity. IC_{50} values given are in µg/ml mean±SD, n=3

Table 7: Ferric ion reduction activity of standards

Conc. in µg/ml	BHT		Silymarin		Conc. in µg/ml	ASC	
10	7.51	±1.05	13.71	±0.61	2	18.87	±0.64
20	11.55	±0.74	20.16	±0.59	4	26.63	±2.06
40	12.11	±1.06	33.90	±1.62	6	38.72	±5.62
60	14.25	±0.33	50.27	±2.24	8	48.66	±5.09
80	16.67	±0.35	63.16	±1.13	10	60.95	±1.43
IC_{50} in µg/ml	ND		67.28	±5.64		7.83	±0.73

Results given are mean percentage Ferric ion reduction activity. IC_{50} values given are in µg/ml mean±SD, n=3

Table 8: Total antioxidant capacity of different fractions of *C. procera*

Extract/ Fraction	CPALC	CPPE	CPEA CPWE	CPB	CPWF
IC_{50}	161.11±12.73	130.56±19.25	116.67±16.67 113.89±12.73	344.44±20.97	19.44±20.97
BHT			400.00±22.05		
SILY			197.22±4.81		

Results given are mean percentage ascorbic acid equivalent value in mg/gm of extract

Table 9: Antioxidant activity of different extracts/fractions of *C.procera* in β-CLAMS method

Conc. in μg/ml	CPALC		CPPE		CPEA		CPB		CPWF		CPWE	
25	41.26±	3.31	34.65±	2.73	37.31±	0.65	38.60±	4.07	35.32±	1.82	38.64±	1.28
50	52.49±	1.78	38.61±	2.42	40.60±	2.40	45.22±	1.30	44.54±	3.98	39.94±	1.77
100	60.44±	3.51	43.89±	2.85	47.20±	2.37	53.14±	1.26	62.38±	5.32	55.78±	2.34
150	68.32±	2.18	49.86±	3.76	52.51±	2.99	62.39±	1.82	74.25±	5.29	65.70±	1.97
IC_{50}	45.87±	3.6	155.9±	17.18[PR]	124.9±	24.36	83.25±	4.01	65.89[PR]	12.35	82.32	5.67[PR]

PR- Projected IC_{50} value. IC_{50} values given are in μg/ml mean±SD. Results given are mean percentage anti-lipid peroxidation activity

Table 10: Hydroxyl radical scavenging activity of different extracts/fractions of *C.procera*

Conc. in μg/ml	CPALC		CPPE		CPEA		CPB		CPWF		CPWE	
10	10.96±	1.20	8.06±	3.71	11.58±	0.86	18.98±	3.33	21.09±	3.48	2.40±	2.00
20	22.13±	0.36	11.46±	1.76	22.32±	0.78	31.63±	1.38	41.53±	2.14	2.70±	1.94
40	32.01±	1.58	30.39±	1.71	31.21±	1.60	40.07±	1.74	51.71±	3.54	5.39±	0.93
60	50.11±	2.28	46.51±	4.02	45.66±	2.06	58.67±	1.87	58.09±	2.88	9.83±	2.52
80	68.78±	1.34	63.97±	3.37	53.45±	10.67	71.40±	2.40	71.96±	1.19	16.33±	3.20
IC_{50}in μg/ml	59.77±	2.26	59.99±	5.99	75.43±	13.1	50.7±	4.82	39.56±	5.62	224.33±	26.01[PR]

PR- Projected IC_{50} value. IC_{50} values given are in μg/ml mean±SD. Results given are mean percentage hydroxyl radical scavenging activity.

Table 11: Anti Lipid Peroxidation activity of different fractions of *C.procera*

Conc. in μg/ml	CPALC		CPPE		CPEA		CPB		CPWF		CPWE	
20	3.02±	0.40	5.70±	1.02	3.28±	0.59	4.38±	1.19	12.67±	0.85	13.19±	0.88
40	19.24±	1.59	13.38±	0.30	18.37±	4.46	32.50±	1.70	18.43±	1.16	16.54±	0.78
60	34.49±	1.01	23.35±	1.58	43.07±	2.09	78.71±	1.66	34.01±	0.92	19.21±	1.35
80	56.12±	1.54	34.10±	1.36	40.00±	8.93	89.91±	1.20	64.22±	0.81	24.04±	0.34
IC_{50} in μg/ml	74.38±	12.77	104±	4.87 [PR]	65.2±	1.15	47.19±	4.07	70.79±	9.11	205.67±	21.08[PR]

PR- Projected IC_{50} value. IC50 values given are in μg/ml mean±SD.

Reduction of Ferric ions

As illustrated in Table 6, in case of CPB fraction showed highest ferric ion reduction activity followed by CPEA. Their IC_{50} values being 60 and 115 μg/ml respectively. Other extracts and fractions were not able to show 50% reduction of ferric ions as compared to Sodium dithionite even at maximum tested concentration of 120 μg/ml. Ferric ion reduction activity was increased in the order: CPWE< CPWF< CPPE< CPALC<CPEA< CPB.

[PR]- Projected IC_{50} value. IC_{50} value not detected at the highest concentration tested. So it was determined by extrapolating the graph. IC_{50} values given are in μg/ml mean ± SD, n = 3.

Total antioxidant capacity

The value obtained here is indicated as ascorbic acid equivalent value in mg/gm of extract. The results are shown in the Table 8.The highest total antioxidant capacity was found in butanol fraction (344.44±20.97 mg/gm) and water extract showed least (113.89±12.73 mg/gm) total antioxidant capacity. The total antioxidant capacity of different fractions/ extracts of *C. procera* from lowest to highest was present in the order CPWE< CPWF< CPEA < CPPE < CPALC < CPB.

β-CLAMS method

As given in Table 8, water fraction and aqueous alcoholic extract of *C. procera* exhibited high antioxidant activity compared to other fractions.

The antioxidant activity ranging from weak to strong occurred in the order: CPPE <CPEA< CPB< CPWE< CPALC< CPWF. IC_{50} values of CPWF and CPALC were found to be 45.87 and 65.89 μg/ml respectively.

Hydroxyl radical scavenging activity

As shown in Table 9**,** water fraction of *C. procera* has shown strong hydroxyl radical scavenging property followed by butanol fraction compared to other fractions and extracts. IC_{50} values of CPWF and CPB fraction were found to be 39.56 and 50.7 μg/ml respectively. The hydroxyl radicals scavenging activity ranging from weak to strong occurred in the order: CPWE< CPEA< CPPE< CPALC< CPB< CPWF.

Lipid peroxidation activity

As shown in Table 9**,** butanol fraction of *C. procera* is much ahead of all other fractions in inhibiting lipid peroxidation. CPB IC_{50} value was found to be 47.19 μg/ml. Anti-lipid peroxidation activity ranging from weak to strong found in the order: CPWE< CPPE< CPALC< CPWF< CPEA< CPB.

DISCUSSION

Free Radical Scavenging Activity

In very recent years, potent free radical scavengers have attracted a tremendous interest as possible therapeutics against free radical mediated diseases. Free radicals are constantly generated *in vivo*. When an imbalance between free radical generation and body defense mechanisms occurs, oxidative damage will spread over all the cell targets (DNA, lipids, and proteins) and has been reported that a series of human illness can be linked to this damaging action free radicals. Many phenolics, such as flavonoids and tannins have been found to possess potent antioxidant and free radical scavenging activity.[17,18] CP found to contain flavonoids as tested in the preliminary phytochemical screening. Besides flavonoids, CP also found to contain glycosides and sterols.

Total phenolic content of *C. procera,* from low to high concentration was found in the order: CPWF< CPPE< CPWE< CPALC< CPB< CPEA. But DPPH radical scavenging activity was not similar to the above order. The DPPH radical scavenging activity ranging from weak to strong occurred in the order: CPWE< CPWF< CPPE< CPALC< CPEA< CPB. TPC of CPB (GAE 96.83±2.13) and CPEA (GAE 97.97±1.40) were very close. But DPPH radical scavenging activity of CPEA (IC_{50} 159.09 μg/ml) and CPB (IC_{50} of 96.64 μg/ml) differ to a large extent. Other fractions not shown IC_{50} values even at highest concentration used.

CPWE and CPWF showed less percentage free radical scavenging activity compared to other extracts though these fractions tested positive for flavonoids. TPC of these fractions and extracts was found to be low. Very few, less potent phenolics might be extracted into water fractions and water extracts.

NO Scavenging Activity

Ethyl acetate and butanol fractions showed highest NO·scavenging activity. This is because of high phenolic content of ethyl acetate and butanol fractions.

Ferric ion Reduction Activity

Butanol and ethyl acetate fractions showed more potent Ferric ion reducing activity compared to the other fractions and extracts. Again this is due to presence of potent phenolics in these fractions. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant activity by breaking the free radical chain by donating a hydrogen atom.[19]

Total antioxidant activity was carried out to understand the antioxidant capacity of the extracts and fractions in the current study. Because this method gives reliable results with variety of samples (plant lipid-soluble extracts, vegetable oils, butter, pharmaceutical and cosmetic preparations, human serum, etc.). On the other hand, it gives the total antioxidant capacity of a sample irrespective of nature and mechanism of actions of drugs.[11]

The total antioxidant activity of extracts and fractions can be well understood by their chemical nature. Butanol fraction of *C. procera;* showed strong total antioxidant capacity which can be comparable to that of well known standards like Silymarin[21] and BHT.[20]

Butanol fraction tested positive for flavonoids and found to contain high TPC. Many phenolics, such as flavonoids and tannins reported with strong antioxidant capacity and their capacity to donate rapidly a hydrogen atom to radicals.[17,18] So the activity of extracts and fractions can be attributed to the presence of polyphenols.

β-Clams method

The mechanism of bleaching β-Carotene is a free radical mediated phenomenon resulting from hydroperoxides formed from linoleic acid. β-Carotene in this system undergoes rapid discoloration in the absence of an antioxidant. In presence of antioxidants, β-Carotene retains its color. This forms basis of this particular assay.[13]

Aqueous alcoholic extract of *C. procera* showed a very strong antioxidant activity compared to standards. In this activity it is an exception that pet ether fraction also showed strong activity. The reasons could be, the antioxidant capacity of a compound is dependent upon reaction media. Pet ether fractions contain lipophilic components. These tend to show good activity in the system which is rich with lipophilic substances like β carotene and linoleic acid. Constituents present in the fractions which showed remarkable activity in other free radical scavenging mechanisms may not be highly reactive in lipophilic media. The selected plant fractions/extracts can be used as natural antioxidants instead of BHT which is synthetic and use of BHT is said to be unsafe and their toxicity is a problem of concern.[22]

Hydroxyl radical scavenging activity

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cell.[23]

Water fraction of *C. procera;* scavenged ·OH radicals strongly. ·OH radical scavenging activity of Gallic acid was very high and IC_{50} was found to be 3.25 μg/ml.

It is found that flavonoids and tannins are potent ·OH radical scavenging agents. But in case of *C. procera* water fraction showed marked hydroxyl radical scavenging activity. Reason may be, the fractions contain high amount of carbohydrates as explained above which are having similar action as that of mannitol which is strong hydroxyl radical scavenger.[24]

Lipid peroxidation activity

The lipid peroxidation has been broadly defined as the oxidation deterioration of polyunsaturated lipids. MDA is the major product of lipid peroxidation and is used to study the lipid peroxidation process.[25] In biological systems, malondialdehye (MDA) is very reactive species and takes part in the cross linking of DNA, with protein and also damaging the liver cells. Determination of lipid peroxidase content was carried out indirectly by means of derivatizing MDA with TBA at high temperature and acidic condition.[26] Butanol fraction of *C. proceras*howed antilipid peroxidation activity when compared to other fractions. This is again attributed to high TPC of CPB.

H_2O_2 scavenging activity

H_2O_2 in phosphate buffer has the $\lambda_{max}$of 230 nm. In control tubes the absorbance will be only due H_2O_2. In presence of extracts the reduction of absorbance at 230 nm indicates scavenging or breakdown of H_2O_2. When breakdown of H_2O_2 occurs, there will be reduction of absorbance at 230 nm. The maximum concentration tested in case of all the fractions and extracts was 20 µg/ml. If more concentration is taken, absorbance at 230 nm goes beyond 1. To keep absorbance below 1 the low concentration was used. No fraction could show 50% activity at the above mentioned concentration. Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (–SH) groups. Hydrogen peroxide can cross cell membranes rapidly. Once enter inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate.[20]

CONCLUSION

Present work was undertaken to evaluate the antioxidant potency of leaves of *C. procera* R.Br. The antioxidant property of different extracts and fractions of the leaves were studied *in vitro* and results have shown that they are moderate antioxidant in nature. The antioxidant potency reveal that the leaves of *C. procera* R.Br. are worth for further chemical isolation and pharmacological investigations. Two important aspects to note in this study are, CP, its latex and all its parts are toxic in nature in one or the other way though the plant parts are used in Ayurveda and in folk medicine. In traditional and folk medicine the leaves are used in fresh. But we have used the dried leaves. There is a pressing need to study the differences in phytochmeical nature and pharmacological efficacy of CP plant parts and when used fresh and dried.

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CONFLICT OF INTEREST

No conflict of interest are declared.

ABBREVIATIONS

%: Percentage; **% AA**: Antioxidant activity; °C: Degree celcius; **A^0 0**: Zero time absorbance of control; **A^O**: Zero time absorbance of sample; **ASC:** Ascorbic acid; **A^t**: Absorbance of sample after incubation for 180 min; **A^t 0**: Absorbance of control after incubation for 180 min; **BHT**: Butylated hydroxy toluene; **C:** Control; **cm**: Centimeter; **CPALC:** Alcoholic extract of *Calotropis procera*; **CPB**: Butanol fraction of *Calotropis.procera;* **CPEA:** Ethyl acetate extract of *Calotropis procera;* **CPPE:** Petroleum ether extract of *Calotropis procera;* **CPWE:** Water extract of *Calotropis procera;* **CPWF:** Water fraction of *Calotropis procera;* **DPPH**: α,α-diphenyl-β-picryl hydrazyl; **Fe^{2+}**: Ferrous ion; **Fe^{3+}**: Ferric ion; **$FeCl_3$**: Ferric chloride; **GA:** Gallic acid; **gm**: Gram; **IC_{50}:** 50% Inhibitory concentration; **m**: Meter; **M**: Molar; **mg**: Milligram; **min**: Minute; **ml**: Millilitre; **mM**: Milli molar; **Mo**: Molybdenum; **nm**: Nanometer; **$NO^·$**: Nitric oxide radical; **O_2**: Oxygen; **$^·$OH**: Hydroxyl radical; **ROS**: Reactive Oxygen Species; **S:** Standard; **SD**: Standard Deviation; **SILY:** Silymarin; **t**: Time; **TBA**: Thiobarbituric acid; **TBARS**: Thiobarbituric acid Reactive substances; **TCA**: Trichloroacetic acid; **w/v**: Weight per volume; **w/w**: Weight per weight; **α**: Alpha; **β:** CLAMS: **β**: Carotene Linoleate Model System; **β:** Beta; **γ:** Gamma; **µg/ml:** Microgram per milliliter; **µl:** Microlitre.

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PICTORIAL ABSTRACT

SUMMARY

- *Calotropis procera* R.Br. (Family, Apocynaceae) is a moderate evergreen herb found more or less throughout India in warm dry places. Even though the plant secretes poisonous milky latex; it has been used to treat various ailments since ancient times in Ayurveda. It was used mainly as laxative, anthelmintic activity, in strangury, treat ulcers, piles, asthma etc. The pharmacognostical evaluation has been established earlier, but no reports have been established on its antioxidant potency. Therefore the plant was selected to study its total antioxidant capacity and further establishing its monograph. Different extracts and fractions of the plant leaves were prepared and the phytochemical investigation was carried out and further *in vitro* models were employed in evaluating its antioxidant potency.
- From the various results of *in vitro* Antioxidant studies, the butanol fraction, water fraction and aqueous alcoholic extract showed the highest antioxidant potency. From the reports established in the phytochemical screening, Carbohydrates, Tannins, Flavonoids and Lactones were present in the water fraction; Sterols, Carbohydrates, Flavonoids and Lactones were present in the Aqueous alcoholic extract; Carbohydrates, Tannins, Flavonoids, and Lactones were present in butanol fraction. Antioxidant activity was mostly shown by active constituents like phenolic acids, tannins, flavonoids etc. which is already known. Since the above extracts and fractions contain these phytochemicals, It could be the reason for them to show the potent antioxidant activity. The mechanism for antioxidant activity is probably mediated through free radical scavenging activity.
- These results can be useful for any pharmacological studies further and in validating the various ethnobotanical uses of this plant. Also leaves of *Calotropis procera* can be also a source in drug discovery and in preparations of many herbal formulations for its various biological properties.

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