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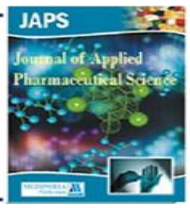

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Antioxidant, Cytotoxicity and Polyphenolic Content of *Calotropis procera* (Ait.) R. Br. Flowers

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ABSTRACT

Oxidative stress has been implicated with the pathology of many diseases such as inflammatory conditions, cancer, diabetes and aging. In view of that an attempt was made to evaluate free radical scavenging activity, cytotoxic activity and polyphenolic content of methanolic extract of *Calotropis procera* flowers.Free radical scavenging activity was estimated using *in vitro* models like 1,1,-diphenyl-2- picryl hydrazyl (DPPH), hydroxyl radical, hydrogen peroxide radical, reducing power and ferric thiocyanate method. Cytotoxicity was analysed following MTT assay using Hep2 and Vero cell lines and polyphenols were estimated using standard methods. Two way ANOVA was used for statistical analyses. The methanol extract of *C. procera* at 500 µg/ml showed better scavenging activity in ferric thiocyanade method (83.63 %) with the lowest IC_{50} of 100 µg/ml followed by hydrogen peroxide, hydroxyl radical scavenging and least activity was found to be present in DPPH assay (50.82 %). The extract had 100 % cytotoxicity on Hep2 cell lines. Flavonoids were found in greater amount than phenols and found to be had higher correlation with antioxidant activities. It was suggested that the flowers of *C. procera* possess *in vitro* antioxidant, cytotoxic activities and thus having much therapeutic value because of their polyphenolic content.

Key words: Antioxidant, *Calotropis procera*, cytotoxicity, polyphenol.

INTRODUCTION

In an aerobic environment, all animals and plants require oxygen and hence reactive oxygen species (ROS) are ubiquitous. It is already established that excessive generation of ROS is involved with structural alterations of cellular molecules leading to cytotoxicity and cell death. This eventually results in a variety of biological phenomena such as mutation, carcinogenesis, aging, radiation or UV exposure, inflammation, ischemia-reperfusion injury, atherosclerosis, diabetes mellitus and neurodegenerative disorders (Yoshikawa *et al*., 2000). Antioxidants play a significant role in the prevention of diseases and do have a capacity to reduce oxidative stress by chelating trace elements or scavenging free radicals and protecting antioxidant defenses (Banerjee and Dasgupta, 2005). The present study was planned to examine the antioxidant and free radical potential of *Calotropis procera* (Ait.) R. Br. flowers.

MATERIALS AND METHODS

The flowers of *C. procera* collected from in and around of Coimbatore district, Tamilnadu, India. The flowers were shade dried and powdered to pass through 100 mesh sieve. 50 g of powder was extracted with 300 ml of methanol using soxhlet apparatus at 35°C for 72 hrs.

Free radical scavenging activity

DPPH assay (Burits and Bucar, 2000)

The hydrogen atom or electron donating abilities of the compounds were measured from the bleaching of the purple-coloured methanol solution of 2, 2-diphenyl-1-picryl hydrazyl (DPPH). This spectrophotometric assay uses the stable free radical, DPPH as a reagent. One thousand microlitres of diverse concentrations (20-500 µg/ml) of the extracts in ethanol were added to 4 ml of 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. The DPPH radical scavenging effect was calculated as inhibition of percentage (I %) using to the following formula:

$$I\ \% = (A_{Blank} - A_{Sample}/A_{Blank})$$

where, A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. The values of inhibition were calculated for various concentrations of the extract. Tests were conceded out in triplicate.

Determination of hydroxyl radical (OH-) scavenging activity (Halliwell *et al*., 1987)

Stock solution of EDTA (1mM), $FeCl_3$ (10mM), ascorbic acid (1mM) H_2O_2 (10mM) and deoxyribose (10mM) was prepared in distilled de- ionized water. The attempt was performed by adding up 0.1ml EDTA, 0.01ml of $FeCl_3$, 0.1ml H_2O_2, 0.36 ml deoxyribose, 1ml of sample extract (20-500 µg/ml) dissolved in distilled water, 0.33ml of phosphate buffer (50 mM, pH 7.4) and 0.1ml of ascorbic acid added. The mixture was incubated at 37^{o}C for 1 hour. A 1.0 ml of incubated mixture was mixed with 1.0 ml of 10% trichloro acetic acid and 1.0 ml of 0.5% thiobarbituric acid (in 0.025M NaOH containing 0.025% BHA) to urbanized the pink color measured at 532nm. The hydroxyl radical scavenging activity is reported as per cent inhibition (I %) of deoxyribose sugar dilapidation and was calculated as

$$I\% = (A_{Blank} - A_{Sample}/A_{Blank}) \times 100$$

Where A_{blank} is the absorbance of control and A_{sample} is the absorbance of test.

Reducing power assay (Oyaizu, 1986)

0.5 ml of sample with different concentrations (20-500 µg/ml) was mixed with 0.5 ml of a 0.2 M phosphate buffer (pH 6.6) and 0.5 ml of a 1% potassium ferricyanide solution. The mixture was incubated in a water bath at 50ºC for 20 min. Subsequently, 0.5 ml of a 10 % (w/v) trichloroacetic acid solution was added, and the mixture was then centrifuged at 3000 rpm for 10 min. Finally, 0.5 ml of the supernatant layer solution was mixed with 0.5 ml of distilled water and 0.1 ml of 0.1% ferric chloride, and the absorbance of the reaction mixture was measured at 700 nm. Three replicates were made for each test sample. Increased absorbance of the reaction mixture indicated increased reducing power of the sample.

Hydrogen peroxide scavenging activity (Ruch *et al*., 1989)

A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Extracts (20-500 µg/ml) in methanol were added to a H_2O_2 solution (0.6 ml, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution contained the phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging was calculated as:

$$H_2O_2 \text{ scavenging effect (\%)} = A_{control} - A_{sample} / A_{control} \times 100$$

where $A_{control}$ is the absorbance of the control, and A_{sample} is the absorbance in the presence of the sample or standards.

Total antioxidant activity determination by ferric thiocyanate method (FTC) **(**Mitsuda *et al*., 1996**)**

For preparation of stock solutions, 10 mg of methanol extract was dissolved in 10 ml water. Then, the solution, which contained different concentrations of stock solution (20 and 500 µg/ml) or standard samples (20-500 µg/ml) in 2.5 ml of potassium phosphate buffer (0.04 M, pH 7.0), was added to 2.5 ml of linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7.0). The mixed solution (5 ml) was incubated at 37°C in a polyethylene flask. The peroxide level was determined by reading the absorbance at 500 nm in a spectrophotometer.

Statistical analyses

The results of these investigations are means and SD of three measurements. Differences between groups were tested by two-way ANOVA. In the assessment of the antioxidant potential, Spearman correlation coefficient (r^2) was used. Linear regressions were also calculated. The P values of <0.05 were considered significant.

Determination of cytotoxicity (Tian *et al*., 2003)

For the assessment of the anticancer activity of the studied plant part, the following cancer cell lines were used: Vero (monkey kidney cell lines) and Hep-2 (human epithelial carcinoma cell line). The cell lines were purchased from Amla research institute, Trichur for MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. Cells were grown in RPMI-1640 medium at 37^{o}C under 5 % CO_2 in a humidified incubator. Cells were harvested, counted (3×10^4 cells/ml), and transferred into a 96-well plate, and incubated for 24 hrs prior to the addition of test compounds. Serial dilutions of test samples were prepared by dissolving compounds in DMSO followed by dilution with RPMI-1640 medium to give final concentration at 50, 25, 12.5, 6.25, 3.125, 1.5, 1.0 and 0.1 mg ml^{-1}. Stock solutions of samples were prepared. Samples at 10 µl and cell lines at 90 µl were incubated for 72 hrs. MTT solution at 5 mg/ml was dissolved in 1 ml of Phosphate Buffer Solution (PBS), and 10 µl of it was added to each of the 96 wells. The wells were wrapped with aluminum foil and incubated at 37ºC for 4 hrs. The solution in each well containing media, unbound MTT and dead cells were removed by suction and 150 µl of DMSO was added to each well. Then the

Table 1: Effect of methanolic extract of C. procera flowers on different antioxidant models

Concentration µg/ml	Percentage of inhibition (I%)									
	DPPH scavenging activity		OH radical scavenging activity		FTC method		Reducing power		H_2O_2 scavenging	
	C. procera flowers	Control (BHT)	*C. procera* flowers	Control (BHT)	*C. procera* flowers	Control (BHT)	*C. procera* flowers	Control (BHT)	*C. procera* flowers	Control (BHT)
20	14 ns	23	18.5	10.41	26.12	35.47	18*	17.3*	15.61	45.02
40	21.7	44.07	30.43	22.21	36.35	46.31	27.41*	28.62*	26.22	65.02
100	32.2	68.41	48.14	31.37	50.90	65.20	39.62*	40.89*	37.91	72.0
200	51.22	84.62	54.70	40.68	74.54	84.32	53.81	44.57	48.32	84.14
500	59.87	72.48	65.43	64.33	83.63	99.01	64.43	51.19	69.25	91.25
SED	4.513		0.791		0.591		0.604		0.849	
IC_{50} µg/ml	195		115		100		153		251	

Values are mean of triplicates
ns- non significant, others significant at P<0.01

plates were shaken and optical density was recorded using a micro plate reader at 540 nm. Distilled water was used as positive control and DMSO as solvent control. Controls and samples were assayed in duplicate for each concentration and replicated three times for each cell line. The cytotoxicity was obtained by comparing the absorbance between the samples and the control. The values were then used to iteratively calculate the concentration of plant extracts required to cause a 50 % reduction (IC_{50}) in growth (cell number) for each cell lines.

Polyphenol estimation

Total flavonoids determination

Aluminum chloride colorimetric method was used for flavonoids determination (Chang *et al*., 2003). The flower extract (0.5 ml of 1:10 g ml^{-1}) in methanol was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It was kept at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a double beam Perkin Elmer UV/Visible spectrophotometer. The calibration curve was made by preparing quercetin solutions at concentrations 12.5 to 100 µg ml^{-1} in methanol.

Total phenols determination

Total phenols were determined by Folin Ciocalteu reagent (McDonald *et al*., 2001). A dilute extract of each plant extract (0.5 ml of 1:10 g ml^{-1}) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined by colorimetry at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg L^{-1} solutions of gallic acid in methanol : water (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg g^{-1} of dry mass), which is a common reference compound.

Estimation of Tannins (Van Burden and Robinson, 1981)

500 mg of the samples was weighed into a 50 ml plastic bottle. 50 ml of distilled water was added and shaken for 1hr in a mechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the mark. This 5 ml of the filtered was pipetted out into a test tube and mixed with 2 ml of 0.1 M $FeCl_3$ in 0.1 N HCl and 0.008 M potassium ferro cyanide the absorbance was measured at 120 nm with in 10 min.

RESULTS

DPPH scavenging activity

DPPH has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances. The method has been used extensively to predict antioxidant activities because of the relatively short time required for analysis. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen donating antioxidant due to the formation of the non radical form DPPH-H by the reaction (Gulcin, 2006). Methanolic extract showed a concentration dependent DPPH radical (Table 1). The inhibition percentage was ranges between 14 to 59.87 with the IC_{50} value of 195 µg/ml.

Hydroxyl radical scavenging assay

Hydroxyl radicals are the major active oxygen species causing lipid oxidation and enormous biological damage (Vankar *et al*., 2006). The percentage of hydroxyl radical scavenging was also significantly increased with the increasing concentrations of flower extract (Table 1). They found to possess the IC_{50} value about 115 µg. BHA was used as standard compound. The extract exhibited similar to that of standard.

Total antioxidant activity determination by ferric thiocyanate method

Lipid peroxidation has been defined as the biological damage caused by free radical that formed under oxidative stress. The inhibitions of activities against lipid peroxidation in linoleic acid can be evaluated by FTC method. *C. procera* flowers and standard compound exhibited effective antioxidant activity. At the different concentrations, the effects of *C. procera* flowers and BHT on lipid peroxidation of the linoleic acid emulsion are shown in

Total reductive capability by Fe^{3+} - Fe^{2+} transformation

In this assay, the yellow colour of the test solution changes to various shades of green and blue colour depending upon the reducing power of each antioxidant samples. The reducing

capacity of the extract may serve as a significant indicator of its potential antioxidant activity. The presence of reductants such as antioxidants substances in the antioxidant samples causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm.

For the measurements of the reductive ability, the Fe^{3+}-Fe^{2+} transformation was investigated in the presence of *C. procera* flower extract using the method of Oyaizu (1986). Like the antioxidant activity, the reducing power of flower extract and BHA increased with increasing concentration. At the different concentrations, flower extract showed higher activities than the control (Table 1) and these differences were statistically significant ($p < 0.05$).

Hydrogen peroxide scavenging activity

Hydrogen peroxide can be formed *in vivo* by many oxidase enzymes such as superoxide dismutase. It can cross membranes and may slowly oxidize a number of compounds. The ability of *C. procera* flower extract to scavenge hydrogen peroxide was determined according to the method of Ruch *et al*. (1989) and is shown in Table 1 and compared with that of BHA as standard and the highest IC_{50} was estimated as 251 µg. There was a statistically significant correlation between those values and the control ($p < 0.01$).

Cytotoxicity

The results of cytotoxic activity of methanolic extract of *C. procera* flowers on Vero and Hep 2 were represented in Table 4. The extract showed maximum activity on Hep 2 cells than Vero cells at higher concentration and it exhibited toxicity only on Hep 2 cells at lower concentration. Following treatment with the extracts for 24 hrs, the cells lost their morphology and showed cell aggregation, cell roundening and finally the 100 % inhibition was observed at the concentration of 50 mg, 25 mg and 12.5 mg.

Polyphenolic content and their correlation with antioxidants

The scavenging properties of *C. procera* flowers are often associated with their flavonoid, phenol and tannin which have the ability to form stable radicals (Vankar *et al*., 2006).

Phenolic compounds are known as powerful chain-breaking antioxidants. As shown in Table 2, the total phenolics in crude extracts of *C. procera* flowers were determined and expressed as GAE, flavonoids and tannins were estimated as QE. The estimated amount of phenols, flavonoids and tannins present in methanolic extract of *C. procera* flowers were 5.2 mg/g, 7.8 mg/g and 4.2 mg/g respectively.

Table 2. Quantitative estimation of phytochemicals.

S. No.	Bioactive compound	Quantity
1.	Phenols	5.2 ± 0.37 mg/g of GAE
2.	Flavonoids	7.8 ± 0.43 mg/g of QE
3.	Tannins	4.2 ± 0.40 mg/g of QE

Values are mean of triplicates ± SD
GAE- Gallic acid equivalent
QE - Quercetin equivalent

The phenols and flavonoids exhibited a positive linear correlation with the antioxidant activities studied. Compared to phenols, flavonoids showed more correlation and it was represented by regression value (Table 3). Flavonoids had maximum correlation with hydrogen peroxide scavenging activity (R_2= 0.975) and phenol exhibited more linear regression with reducing power (R_2= 0.949). All the antioxidant parameters studied were significantly correlated ($P<0.05$).

Table 3. Multiple correlation analysis of methanolic extract of *C. procera* flowers.

Concentration (mg)	% of inhibition			
	Vero	LC_{50}	Hep 2	LC_{50}
50	100		100	
25	100		100	
12.5	83		100	
6.25	78	3.5 mg	85	1.5 mg
3.125	46		62	
1.5	27		51	
1			22	
0.1	-		-	

Table 4. Cytotoxicity of methanol extract of *C. procera* flowers on Hep 2 and Vero cell lines.

	Phenols	DPPH	Reducing power	Hydroxyl scavenging	H_2O_2	FTC	Flavonoids
Phenols	1*						
DPPH	0.881*	1*					
Reducing power	0.949	0.972*	1*				
Hydroxyl scavenging	0.946*	0.902*	0.966*	1*			
H_2O_2	0.909*	0.983*	0.972*	0.941*	1*		
FTC	0.933*	0.963*	0.992*	0.938*	0.943*	1*	
Flavonoids	0.971*	0.939*	0.970*	0.969*	0.975*	0.938*	1*

Significance at $P<0.01$

DISSCUSSION

Free radicals are chemical entities that can exist separately with one or more unpaired electrons. The generation of free radicals can bring about thousands of reactions and thus cause extensive tissue damage. Lipids, proteins and DNA are all susceptible to attack by free radicals (Sreejayan, 1997). Antioxidants may offer resistance against oxidative stress by scavenging the free radicals.

The results of the present study showed that the methanolic extract of *C. procera* flowers exhibited the high radical scavenging property and cytotoxic activity. The effectiveness of the flowers might be due to the hydroxyl groups existing in the phenolic compounds chemical structure (Pourmoard *et al*., 2006) that can provide the necessary component as a radical scavenger. A potent scavenger of free radicals may serve as a possible preventive intervention for the diseases (Gyanfi *et al*., 1999). The present study suggests that the flowers of *C. procera* might be a potential source of natural antioxidants.

Phenolic acids, flavonoids and tannins are the most commonly found polyphenolic compounds in plant extracts (Wolfe *et al*., 2003). The antioxidant activity of phenolics plays an important role in the absorption or neutralization of free radicals (Basile *et al*., 1999). Both the antimutagenic and anticarcinogenic activity of polyphenols is mostly due to their

antioxidant activity, which inactivates direct mutagens/ carcinogens and inhibits the activation of indirect mutagens/ carcinogens extracellularly. Polyphenols also enhance the level of cellular antioxidative system and induce the cytochrome P-450 resulting in detoxifying the activity of carcinogens intracellularly (De Flora, 1988).

CONCLUSION

The antioxidant properties of plant extracts were evaluated in a variety of model systems using several different indices to ensure the effectiveness of such antioxidant materials. The results obtained in this study clearly showed that *C. procera* flowers have powerful antioxidant activity against various antioxidant systems *in vitro*; moreover, these extracts can act as cytotoxic agents. Hence it can be used as easily accessible source of natural antioxidants in pharmaceutical applications.

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