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Der Pharmacia Lettre, 2010, 2(3): 95-100
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-5071
USA CODEN: DPLEB4

In vitro antioxidant activity of *Calotropis gigantea* hydroalcohlic leaves extract

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Abstract

Antioxidants play an important role to protect human against infections and degenerative diseases. The aim of this study is to verify the In-vitro antioxidant properties and to calculate the total polyphenol contents, total tannins, flavonoid contants of hydroalcohlic extract. The powdered crude drugs were extracted with hydro-alcoholic solvent (70:30) by double maceration process. Phytochemical tests of hydro-alcoholic extract reveals the presence of carbohydrate, alkaloid, flavonoid, steroids, protein, amino-acids and tannins. The antioxidant activity of hydroalcohlic extract of Calotropis gigantea leaves (HECGL) was investigated using in-vitro models like DPPH (1,1-Diphenyl-2-Picryl-Hydrazyl) free radical scavenging activity. Reducing power assay using ascorbic acid, nitric oxide scavenging activity using curcumin equivalents. At the same time the phenolic content of the extracts was determined using Folin-Ciocalteau reagent, total tannins and total flavonoids using rutine to evaluate their contribution to total antioxidant activity. HECGL at 400µg/ml concentration showed maximum DPPH radical scavenging activity (85.17%) and for nitric oxide scavenging (54.55%) at 100µg/ml concentration. Reducing power of HACGL was increases with increasing the concentration of extract. Phenolic contents, expressed as gallic acid equivalents 63.08 ±4.17 /100 mg of the dried weight of cg. Tannin 0.52% and Flavonoids 46.97± 1.95 µg mg-1.It is concluded that, this study is to verify the antioxidant properties of hydroalcohlic extract, and to define the total polyphenol contents, flavonoids and tannins in Calotropis gigantea leaves.

Key words: Antioxidant, Free radicals, *Calotropis gigantea*, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) , Nitric-Oxide

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INTRODUCTION

Antioxidant compounds play an important role in preventing or delaying the onset of major degenerative diseases. The physiological role of antioxidant compounds is to scavenge for free radicals. [1] Active oxygen (hydroxyl, peroxylradicals and single toxygen) is highly toxic and an important causative agent of many diseases including cancer, heart disease, cataract and congestive disorders. Antioxidant compounds block the oxidation processes that produce free radicals which contribute towards these chronic diseases and aging. [2,3] Free radicals are highly unstable and reactive especies are capable of damaging molecules such as DNA, proteins and carbohydrates. The body is under constant attack from these freeradicals formed as a consequence of the body's normal metabolic activities. [1] Anti- oxidants therefore play an important role in animal health. Conventional antioxidants improve animal performance during conditions characterized by in- creased tissue oxidant levels such as stress, injury and infection. [4,5] A variety of free radical scavenging antioxidants are found in dietary sources like fruits, vegetables and tea. The purpose of this study was to evaluate the antioxidant activity of HECGL. *Calotropis gigantea* Linn. belongs family Asclepiadacea commonly known as arka and wild growing tropical plant, which possesses number of medicinal properties. It is reported to contain cardiac glycosides, β sitosterol, madrine, saponins, alkaloids, tannins, trisaccharides and flavonols. The plant has been used for various disease conditions, including leprosy, ulcers, tumours and piles. Various pharmacological activities reported like antifertility, anti-inflammatory activity, hepatoprotective activity, antimyocardial infraction activity and antidiarhoeal activity.

MATERIALS AND METHODS

Chemical and Instrument

Ascorbic Acid, Pottasium ferrocynate ($K_3Fe(CN)_6$), TCA, phosphate buffer, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), sodium nitroprusside, Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride in 5% H_3PO_4. Instrument UV spectrophotometer (Shimadzu-UV-1601), centrifuge machine (Eltekresearch centrifuge-TC-4100D).

Plant Material

Leaves of *Calotropis gigantea* were collected and authentified from Department of Pharmacy Barkatullah University Bhopal (India). An Authentication specimen number is BUPH-4024/A.

Preparation of extracts

Approximately 200g of powdered crude drugs were extracted with hydro-alcoholic solvent (70:30) by double maceration process. The macerates were filtered with muslin cloth and concentrated using rotary evaporator. The hydroalcoholic extract yield a brownish green solid residue weighing 2.601 gm (1.73% w/w).

Experimental Methods

Preliminary phytochemical investigation

The preliminary phytochemical screening of the extract was carried out to know the different constituents present in HECGL as per the standard procedures. [6-10]

DPPH Free Radical Scavenging Activity (Table 1)
The free radical scavenging capacity of the extracts was determined using DPPH. DPPH solution (0.004% w/v) was prepared in 95% methanol. HECGL *was* mixed with 95 % methanol to prepare the stock solution (5 mg/ml). Freshly prepared DPPH solution (0.004% w/v) was taken in test tubes and HECGL was added followed by serial dilutions (1 µg to 500 µg) to every test tube so that the final volume was 3 ml and after 10 min, the absorbance was read at 515 nm using a spectrophotometer. Ascorbic acid was used as a reference standard and dissolve in distilled water to make the stock solution with the same concentration (5 mg/ml). Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95 % methanol was served as blank. % scavenging of the DPPH free radical was measured by using the following equation: %Scavenging Activity = (Ac-As)/Ac)×100 Where, Ac is the absorbance of the control reaction and As is the absorbance in the presence of the sample of the extracts. The inhibition curve was plotted for duplicate experiments and represented as % of mean inhibition ± standard deviation. IC_{50} values were obtained by probit analysis. [11,12]

Table 1. DPPH Free Radical Scavenging Activity of HECGL

S.No.	Concentration(µg/m)	Inhibition(%)DPPH
1.	100	52.74
2.	200	59.15
3.	300	63.25
4.	400	68.54
5.	500	71.38

Nitric Oxide scavenging Activity
Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions which were measured by the Griess reaction. Sodium nitroprusside (10 mm) in 0.5M- phosphate buffered saline (PBS) was mixed with 3.0 ml of different concentrations (20-100µg/ml) of the drug dissolved in the suitable solvent systems and incubated at 25 ^{o}C for 150 minutes. The samples from the above were reacted with 0.5 ml of Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride in 5% H_3PO_4) was added. The absorbance of the chromophore formed was measured at 546 nm. The same reaction mixture without the ethanolic extracts of Plants but with equivalent amount of 0.5M phosphate buffer served as control. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test. Curcumin was used as a reference compound. The nitric oxide radicals scavenging activity was calculated according to the equation: *% Inhibition = ((A0-A1) / A0 × 100)* Where A0 was the absorbance of the control (blank, without extract) and A1 was the absorbance in the presence of the extract. [13]

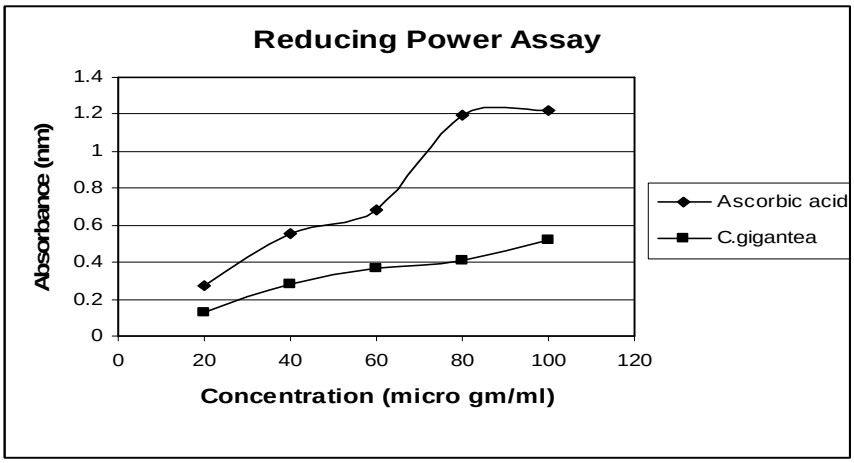
Table 2. Nitric Oxide scavenging Activity of HECGL

Drug	Inhibitory concentration (IC_{50} µg/ml)
	Nitric Oxide
HECGL	106.66
Curcumine	21.16

Reducing Power Assay

The different concentration of the HECGL (5-60 µg/ml) in 1 ml distilled water were mixed with phosphate buffer (2.5 ml, 2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50ºC for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture which was then centrifuged at 3000g (rpm) for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance of the reaction mixture indicated increased reducing power. The absorbance was measured at 700 nm. Ascorbic acid was taken as reference. [14, 15]

Graph 1. Reducing Power determination of HECGL



Reducing power determination of HECGL and standard Ascobic acid

Total Phenolic Contents

The Folin-Ciocalteu reagent assay was used to determine the total phenolics content. The sample (0.2 ml) of different conc. (50-500 µg/ml) was mixed with 2.5 ml of Folin-Ciocalteu's phenol reagent and allowed to react for 5 min. Then, 2 ml of 7.5% Na2CO3 solution was added, and the final volume was made up to 10 ml with deionized water. After 1 h of reaction at room temperature, the absorbance at 760 nm was determined. Gallic acid was used as standard for the calibration curve. [16]

Total Tannin content

Take 1 gm of sample in 100 ml of water, filter, transfer 10 ml of filterate to a conical flask 1 litre capacity, add 750 ml of water and 25 ml of indigosulphonic acid solution & titrate with constant stirring against N/10 $KMnO_4$ to a golden yellow colour. 1 ml of N/10 $KMnO_4$ is equivalent to 0.004157 gm of tannin compounds calculated as tannic acid, run a blank test by titrating 25 ml of indigosulphonic acid in 750 ml of water. [17]

Amount of total flavonoid content

To determination of the total flavonoid content (TFC), 2.5 ml of each extract solution was mixed with 2.5 ml $AlCl_3$ reagent in ethanol 90% and allowed to stand for 40 min at room temperature. After that, the absorbance of the mixture at 415 nm was measured with a spectrophotometer. Ethanol 90% (2.5 ml) plus sample solution (2.5 ml) was used as a blank. Rutin was used as a

reference compound. The TFC for each extarct [as µg rutin equivalents (RE) / mg of extract] was determined on the basis of the linear calibration curve of rutin (absorbance versus rutin concentration). [18]

Statistical Analysis

Experimental results were mean ± SEM of three parallel measurements. Linear regression analysis was used to calculate the IC_{50} value. Student's *t*-test was used for the comparison between two means for the possible significant interrelation. Data were considered statistically significant only when *p* value < 0.05.

RESULTS AND DISCUSSION

Preliminary phytochemical screening of HECGL showed the presence of alkaloids, sterols, triterpenes, saponins, flavonoids, tannins, carbohydrates, cardiac glycosides and amino acids. Shinoda test and thin layer chromatography for flavonoids using mobile phase n-butanol: water: glacial acetic acid (4:5:1) and spraying reagent as ferric chloride solution again confirmed its presence. Table 1 illustrates a significant decrease in the concentration of DPPH radical due to the scavenging ability of both HECGL and ascorbic acid. HECGL at 400µg/mL concentration showed maximum DPPH radical scavenging activity i.e.85.17% where as ascorbic acid at the same concentration exhibited 93.16% inhibition. The IC_{50} values were found to be 20.89µg/mL and 7.58µg/mL for (CG) and ascorbic acid respectively. Table 2. showes the antioxidant activity of hydro alcoholic extract of *C.gigantea* by nitric oxide method. For the measurements of the reductive ability, we investigated the Fe^{3+}- Fe^{2+} transformation in the presence of the HECGL. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant. Reducing power of the selected diluted extract found to be significant ($p < 0.01$) and as good as L-Ascorbic acid (Graph1). The reducing power of HECGL increased with increasing amount of sample. Reducing power of HECGL were found 56.53 mg Ascorbic acid equivalent per gm of extract. Phenolic contents of HECGL were found 63.08 ± 4.17 mg gallic acid equivalent per gm of extract. Total tannin content was found 0.52% expressed as tannic acid in the *CG* powder. Total flavonoid contents of HECGL were found to be 46.97± 1.95 µg mg-1

CONCLUSION

It is well known that free radicals are one of the causes of several diseases. The result from the two in-vitro antioxidant model reveals that the hydroalcohlic extracts of *Calotropis gigantea* leaves (HECGL) had significant antioxidant activity. The exact constituents that show free radical scavenging action are unclear. However, the observed antioxidant activity of HECGL may be due to the presence of tannins and flavonoids found in preliminary phytochemical investigation. Thus, to conclude *Calotropis gigantea* leaves powder extract showed antioxidant activities hence further studies are needed to evaluate the *in-vivo* antioxidant potential of these extracts in various animal models.

Acknowledgement

The work was supported by Department of Pharmacy Barkatullah University Bhopal, India. I would like to thanks Dr.Anupam Kumar Pathak, Head of the Department, Barkatullah University, Bhopal, India.

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