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PHYTOCHEMCIAL SCREENING AND BIOLOGICAL EVALUATION OF THE *CALOTROPIS GIGANTEA MILL* FLOWERS

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ABSTRACT

Calotropis Gigantea is a member of the Apocynaceae family is used in folk medicine for different types of diseases. The purpose of the present study is to investigate the anti oxidant activity of the ethanolic extract of Flowers of *Calotropis Gigantea Mill*. The anti microbial, analgesic and anti inflammatory activity antioxidant activity of extract was done by using the DPPH method against the standard ascorbic acid, anti microbial activity with ofloxacin and analgesic anti inflammatory activity using Paw oedema volume method. Here the extract shows activity at higher concentration against the standard ascorbic acid.

KEYWORDS: Calotropis Gigantea, anti microbial, anti inflammatory, anti oxidant activity, DPPH activity.

INTRODUCTION

C. gigantea is a common wasteland weed and commonly known as giant milk weed. This plant is a native of Bangladesh, Burma, China, India, Indonesia, Malaysia, Pakistan, Philippines, Thailand and Sri Lanka. The plant has oval, light green leaves, milky stem and clusters of waxy flowers that are either white or lavender in colour. C. gigantea is frequently available in India and used for several medication purposes in traditional medicinal system.[1] Most recently C. gigantea is scientifically reported for several medicinal properties viz. the flowers are reported to possess analgesic activity[2], antimicrobial and cytotoxic activity.[3]

Leaves and areal parts of the plant are reported for anti-diarrhoeal activity4, anti-Candida activity[5] and antibacterial activity[6], antioxidant activity.[7] Roots are reported to contain anti-pyretic activity[8], cytotoxic activity[9], antimicrobial activity[10], insecticidal activity[11], wound healing activity[12], CNS activity[13] and pregnancy interceptive properties.[14] Latex of the plant is reported to contain purgative properties, procoagulant activity[15], wound healing activity[16] and antimicrobial activity.[17] Stem was reported to possess hepatoprotective effects.[18] The present review is focused an overall outline of the medicinal properties and biomolecules of C. gigantea and its future prospects for the further scientific investigation for the development of effective therapeutic compounds.

MATERIALS AND METHODS

Drying and size reduction: Bark part of the two plants were carefully shade dried for 15 days. To ensure complete dryness they were kept in hot air oven at 45˚C for 5 minutes. Then they are subjected to size reduction to make powder by using mechanical grinder. The crushed mass of bark was then carried out for the process of extraction.

Extraction procedure[12]

1. 800gms of the air-dried powdered plant material extracted with ethanol in soxhlet extractor.
2. Soxhalation of leaf powder with ethanol for 24hrs to obtain the product.
3. Then the dried marc is extracted with water by decoction.
4. Concentrate the extract by distilling of the solvent and then evaporating to dryness on the water-bath.

Preliminary Phytochemical Analysis[13,14,15,16]

Test for Tannins

1. A small portion of extract was treated with 5% ferric chloride solution. Appearance of green to blue color was taken as a positive test for tannins.
2. Small portion of extract was treated with lead acetate. Appearance of creamy precipitate was considered as a positive test for tannins.

Test for Alkaloids

1. **Mayer's Test:** The Extract to be tested is treated with few drops of dilute 2N HCL and 0.5 ml Mayer's reagent .White precipitate was obtained which confirm the presence of alkaloids.

2. **Wagner's Test:** The extract is treated with few drops of 2N HCL and 0.5 ml Wagner's reagent. Brown flocculent precipitate was obtained which confirm the presence of alkaloids.
3. **Hager's Test**: The extract is treated with few drops of dilute 2N HCL and 0.5 ml Hager's reagent. Yellow colored precipitate was obtained which confirms the presence of alkaloids.

Test for Steroids

1. **Salkowski reaction:** To 2ml.of extract, add 2ml of chloroform and 2ml.conc.H_2SO_4. Shake well, Chloroform layer appears red and acid layer shows greenish yellow fluorescence.
2. **Liebermann-Burchard reaction:** Mix 2 ml extract with chloroform. Add 1-2ml.aceteic anhydride and 2 drops of conc.H_2SO_4 from the side of test tube.
3. **Libermann's reaction:** mix 3ml.extract with 3ml. acetic anhydride. Heat and cool. Add few drops conc. H_2SO_4. Blue color appears.

Tests for Glycosides

1. **Borntrager's test:** About 50mg of extract was hydrolysed with 2ml of concentrated HCl for 2hrs on water bath and filtered. To 2ml of filtrate hydrolysate, 3ml of $CHCl_3$ was added and shaken. $CHCl_3$ layer was separated and 10% NH_3 solution was added. Formation of pink colour indicates the presence of anthraquinone glycosides.
2. **Baljet's test:** The alcoholic or aqueous extract test solution is treated with sodium picrate. Appearance of yellow to orange colour indicates the presence of glycosides.
3. **Keller-Kiliani test:** About 2ml of test solution is treated with few drops of ferric chloride solution and mixed and then sulphuric acid containing ferric chloride solution is added, it forms two layers. Appearance of lower layer in reddish brown and upper layer in bluish green indicates the presence of glycosides**.**

Test for Saponins

Foam's test: A small amount of dry extract was boiled with water and allowed to cool. It was then shaken vigorously for a minute. The formation of persistent honey comb like froth was considered as a positive test for saponins.

Test for Sugars

1. **Molisch's test:** It was performed for the presence of carbohydrates. 1 ml of 10% alcoholic solution of α-napthol was added to the extract and mixed. Then 1ml of concentrated sulphuric acid was carefully poured along the sides of the test tube violet ring formed at the junction which is considered positive test for carbohydrates.
2. **Fehling's test:** 5ml of solution of extract was heated with equal volumes of Fehling's solution A & B. Transition of color from blue through green to reddish orange confirms the presence of reducing sugars.
3. **Benedict's test:** 5 ml of solution of the extract was heated with 5 ml of Benedict's reagent. A green, yellow or orange red precipitate was considered as a positive test for reducing sugars.

Test for Proteins

1. **Biuret test:** A small portion of extract was treated with Biuret reagent.
2. **Xanthoprotein test:** Mix 3ml. T.S. with 1ml.conc. H_2SO_4. White precipitate is formed. Boil. Solution turns black or brownish due to Lead sulphide formation.

Pharmacological evolution[17-27]

Anti microbial evolution[28]

Test Organisms Bacterial strains were obtained from National Chemical Laboratories (NCL), Pune and Microbial Type Culture Collection (MTCC), Chandigarh. The strains used for the present study were Staphycococcus aureus (NCIM 2079), Bacillus subtilis (NCIM 2063), Escherichia coli (NCIM 2931, Proteus vulgaris (NCIM 2027).

Procedure

The antimicrobial activity of the extract was assessed by disc diffusion method. Nutrient agar medium was prepared and sterilized by an autoclave. In an aseptic room, they were poured into a petridishes to a uniform depth of 4 mm and then allowed to solidify at room temperature. After solidification, the test organisms, Escherichia coli, Bacillus subtilis, Staphylococcus aureus, and Proteus vulgaris were spread over the media with the help of a sterile swab socked in bacterium and is used for antibacterial study. The ethanolic extract residues were dissolved in dimethyl sulfoxide (DMSO) to produce a concentration of 100, 250,500 µg/disc and used for the study. Ofloxacin 5 µg/disc was used as the standard. Then the sterile filter paper discs (6mm) having a capacity to hold 10 µl of extracts were immersed in definite concentration of plant extracts and placed over the solidified agar in such a way

that there is no overlapping of the zone of inhibition. Plates were kept at room temperature for half an hour for the diffusion of the sample into the agar media. The organism inoculated petridishes were incubated at 37 °C for 24 hours. After the incubation period is over, the zone of inhibition produced by the samples and standard were measured. All tests were performed in triplicate.

Analgesic and Anti inflamamtory activity

Analgesic activity

Tail-Fick Test

The basal reaction time of each mouse was determined using tail-withdrawal response when one-third of the tail was immersed in water bath at 51˙C.[11] The cutoff time for immersion was 180 s. The reaction time was evaluated 30, 60, 90, 120 and 240 min after oral administration of extracts, distilled water or acetylsalicylic acid.

Formalin Test

The method used in our study was similar to that described previously.[12] Twenty microliter of 5% formalin was injected subcutaneously into the right hind paw of mice. The time (in seconds) spent in licking and biting responses of the injected paw was taken as an indicator of pain response. Responses were measured for 5 min after formalin injection (early phase) and 20–30 min after formalin injection (late phase). Swertia Chirata root M extracts (0.5 and 1.0 g/kg, i.p.) were administered 60 min before formalin injection. Indomethacin (10 mg/kg, i.p.) was administered 30 min before formalin injection. Control group received the same volume of saline by oral administration.

Anti-inflammatory activity

Carrageenan induced hind paw edema in rats Paw edema was produced in rats by carrageenan following the methods of Winter et al. (1962) respectively.[13] Male rats eighing 100–120 g were divided into groups of six animals. A volume of 0.05 ml of 1% carrageenan in normal saline solution (NSS) in 0.2M carbonate buffer was injected intradermally into the plantar side of the right hind paw of the rat. Test drugs and vehicle were given 1 h prior to carrageenan injection. Paw volumes were measured using a plethysmometer (model 7150, Ugo Basile, Italy) before as well as 1, 3 and 5 h after carrageenan, injection. Results obtained were compared with those obtained from there

Antioxidant activity by DPPH method[29]

Antioxidant behaviour of the extracted compound was measured *in vitro* by the inhibition of generated stable 2,2-diphenyl- 1-picrylhydrazyl (DPPH) free radical. Methods vary greatly as to the generated radical, the reproducibility of the generation process, and the end point that is used for the determination. The DPPH solution was prepared by dissolving accurately weighed 22 mg of DPPH in 100 ml of ethanol. From this stock solution, 18 ml was diluted to 100 ml with ethanol to obtain 100µM DPPH solutions. The sample solution was prepared by accurately weighed 2.1 mg of each of the compounds and dissolved in 1 ml of freshly distilled DMSO separately to obtain solutions of 2.1 mg/ml concentration and the standard solution of was prepared by accurately weighed 10.5 mg of α-Tocopherol and dissolved in 1 ml of freshly distilled DMSO to get 10.5 mg/ml concentration.

A different concentration of extract was prepared by the addition of ethanolic solution of DPPH radical. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm against the corresponding blank solution. The final concentration of the samples and standard α-Tocopherol solutions used is 100µg/ml. The percentage scavenging DPPH radical inhibitions were calculated by using the following formula:

$$\text{DPPH radical scavenging activity } (\%) = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100$$

Where, Abs control was the absorbance of DPPH radical and ethanol, Abs sample was the absorbance of DPPH radical and sample/standard.

The scavenging activity was expressed in terms of IC50, the concentration of the samples required to give a 50% reduction in the intensity of the signal of the DPPH radical. The results were done at least in triplicate.

RESULTS

Table: 1 Phytochemical constituents present in total ethanolic extract of *Calotropis Gigantea.*

Tests	*Calotropis Gigantea*
1. Tannins	+
2. Alkaloids	-
3. Steroids	+
4. Glycosides	
Cardiac	+
Anthraquinone	-
Saponin	-
Flavanoids	+
Coumarins	-
5. Sugars	+
6. Protiens	+

(+) - **Indicates the presence of the phyto constituent**

(-) - **Indicates the absence of the phyto constituent**

Anti microbial activity

Analgesic activity

Table 1: writhing test.

Group	Traetment	N	Route of administration	Dose mg/kg	No.fo writches	Inhibition writhing response
1	Control	6		-	49.06±4.08	
2	Aspirin	6	300	i.p	12.05±2.14	90
3	Flower of Calotropis Gigantea	6	50	i.p	20.65±2.10	40
4	Flower of Calotropis Gigantea	6	100	i.p	18.85±1.78	65
5	Flower of Calotropis Gigantea	6	150	i.p	8.68±1.14	85

Mean = S.E.M. of 6 animals. ** = P≤0.001= highly significant. Group II,III, IV, and V compared with Group I.

		Alcoholic extract of *Calotropis Gigantea*			Ofloxacin		
		Zone of inhibition in mm					
		100µg/ml	250mg/ml	500µg/ml	100µg/ml	250mg/ml	500µg/ml
Name of the organisms	Staphylococcus aureus	10	14	18	12	20	26
	Bacillus subtilis	8	16	20	14	18	24
	Escherichia coli	6	18	22	12	16	25
	Proteus vulgaris	12	14	20	12	14	22
Control	DMSO	-	-	-	-	-	-

Anti-inflammatory activity

Table2 Carrageenan –induced paw edema method.

Group	n	Dose (mg/kg)	Pawvolume increase(ml)			Inhibition (%)		
			1hr	3hr	5hr	1hr	3hr	5hr
Control	6		0.36±0.07	0.69±0.05	0.82±0.03			
Aspirin	6	300	0.10±0.02**	0.21±0.02**	0.27±0.03**	72	70	67
Calotropis Gigantea	6	100	0.22±0.04*	0.47±0.01*	0.51±0.02*	39	32	38
Calotropis Gigantea	6	150	0.18±0.03*	0.39±0.01*	0.42±0.04*	50	43	49
Calotropis Gigantea	6	200	0.07±0.01**	0.24±0.02**	0.35±0.02**	81	65	57

n = 6 animals in each group. * = p≤0.01 (significant).** = p≤0.01(highly significant) control groups, which received vehicle only

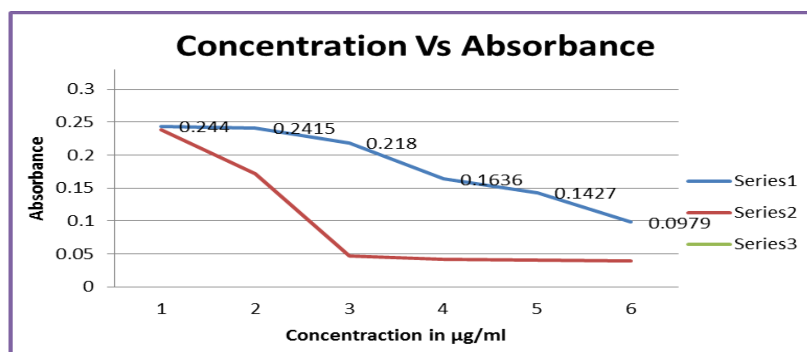
Anti oxidant activity

Table 2: Anti oxidant activity of alcoholic extract of *Calotropis Gigantea.*

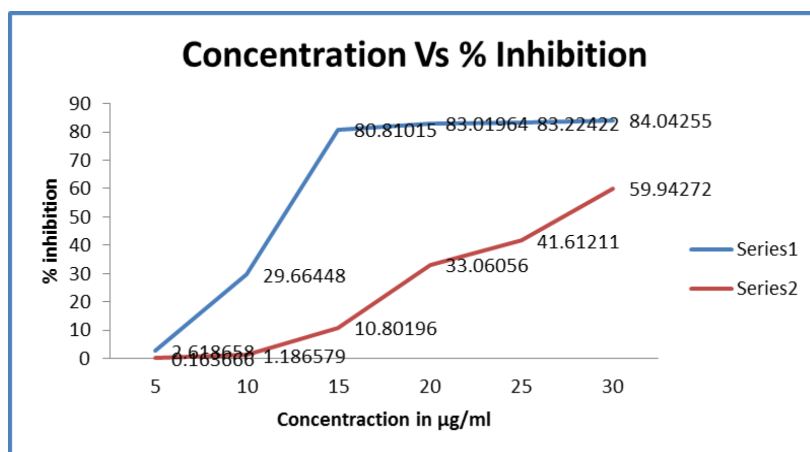
Concentration (µg/ml)	Ascorbic acid (Abs)	Alcoholic extract of *Calotropis Gigantea* (Abs)
5	0.2380	0.244
10	0.1719	0.2415
15	0.0469	0.218
20	0.0415	0.1636
25	0.0410	0.1427
30	0.0390	0.0979
Control	0.2444	

Table 3: % inhibition of alcoholic extract of with *Calotropis Gigantea* ascorbic acid

Concentration (µg/ml)	Ascorbic acid (% Inhibition)	Alcoholic extract of *Calotropis Gigantea* (% Inhibition)
5	2.618658	0.163666
10	29.66448	1.186579
15	80.81015	10.80196
20	83.01964	33.06056
25	83.22422	41.61211
30	84.04255	59.94272



Graph-1: Concentration Vs Absorbance.



Graph-2: concentrations Vs % Inhibition.

DISCUSSION

The present results reveals that the alcoholic extract shows the activity less than the standard. The extract was diluted with concentration of 100 µg/ml, 250µg/ml, 500µg/ml. In that the extract with concentration of 500µg/ml shows the significance activity than the remaining concentrations.

Analgesic activity of administration of Calotropis Gigantea Flower ethanolic extract at the dose level of 100, 150 and 200 mg./kg b. wt. to the rats produced weak effect on the writhing induced by the injection of 0.6% acetic acid when compared with the aspirin (300mg/kg) by 79% while the treated group with Calotropis Gigantea Flower ethanolic extracts inhibited the writhing by 40%, 65%, 85% respectively(table 1). The ethanolic extract of Calotropis Gigantea Flower (100- 200 mg/kg) produced inhibition of formalin induce biphasic pain response (neurogenic and inflammatory pain) in rats. The analgesic effect of this fraction occurred predominately during the II phase; 200 mg dose level was more efficient in the late phase. Anti-inflammatory activity of Calotropis Gigantea root, The inhibitory activity on carrageenan induced rat hind paw edema, caused by the subplanatar administration of Calotropis Gigantea Flower ethanolic extract, at various assessment times after carrageenan injection are tabulated. the standard drug aspirin, a cyclooxygenase inhibitor, at the dose of 300mg/kg body weight exhibited significant ($p \leq 0.01$) edema inhibition.

Calotropis Gigantea Flower ethanolic extract at doses of 50,100,150 mg/kg boy weight also possessed significant ($p \leq 0.001$) inhibitory effect on carrageenan induced paw edema at all recorded times. This increase was observed at 1 hr. and was maximum at 5hr. after administration of carrageenan in the vehicle group.

The Alcoholic extract of *Calotropis Gigantea* tested for antioxidant activity by using DPPH Assay method. Here the results were compared with the standard Ascorbic acid. The result reveals that the extract shows results less than the standard. The concentration of the extract was taken in to 5-30 µg/ml. The % of inhibition shows that the up to 30µg/ml. The % inhibition is therefore it shows more activity than compare with other concentrations.

CONCLUSION

Based on the above results concluding that the Alcoholic extract of the *Calotropis Gigantea* shows better anti microbial activity against the standard drug ofloxacin, analgesic and anti inflammatory activity better activity when compare with the standard compound Indomethacin, antioxidant activity when compare with the standard ascorbic acid.

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