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PHYTOCHEMICAL SCREENING, ANTIOXIDANT AND CYTOTOXIC ACTIVITY OF *CLERODENDRUM SIPHONANTHUS*

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ABSTRACT

Four solvent leaf extracts of *Clerodendrum siphonanthus* were prepared and their phytochemical, Antioxidant and Cytotoxic activities was evaluated. Solvent extracts showed the presence of Tannins and Alkaloids. Qualitative and quantitative antioxidant assay was carried out by using TLC based antioxidant assay and DPPH free radical scavenging assay, Ferric reducing anti–oxidant power assay (frap assay) respectively. In TLC based antioxidant assay most of the bands observed in chloroform extract followed by ethyl acetate and Methanol extracts in BEA solvent. In FRAP assay, ethyl acetate and Methanol extracts exhibited mild activity. In DPPH assay ethyl acetate and chloroform extract have effective antioxidant activity. Hexane extract showed cytotoxic potential with 100% activity at highest dose administered.

KEYWORDS: Phytochemical, antioxidant, cytotoxic, *Clerodendrum siphonanthus*, DPPH assay, FRAP assay.

INTRODUCTION

Clerodendrum siphonanthus is basically an ornamental flower belonging to family Verbenaceae which consists of a number of medicinally important plants. Studies on the extracts of different species of the genus *Clerodendrum* have been carried out by a number of researchers round the globe.[1] Roots and leaf extracts of C. *indicum*, C. *phlomidis*, C. *serratum*, C. *trichotomum*, C. *chinense* and C. *petasites* have been used for the treatment of rheumatism, asthma and other inflammatory diseases.[2,3] Plant species such as C. *indicum* and C. *inerme* were used to treat coughs, serofulous infection, buboes problem, venereal infections, skin diseases and as a vermifuge, febrifuge and also to treat Beriberi disease.[4] Repellent response of *Clerodendrum viscosum* to the larvae and adults of flour Beetle, *Tribolium confusum* has also been studied by Scientists.[5] The commonly reported useful parts of the plant for therapeutic uses are Root and Leaves (Ayurvedic Medicinal Plants website). The antifungal activity of the ethanolic extract of leaf of C. *viscosum*, has also been reported.[6] The free radical scavenging potential of the roots of C. *viscosum* was also studied reported by researchers.[7] Thus it is expected from the member of same genus that it might also be harbouring such medicinal properties. Keeping all the above in view the selected plant was explored for biological activity using antioxidant, cytotoxic and phytochemical parameters.

MATERIALS AND METHODS

Collection and processing of plant materials

Whole plant samples of *Blumea lacera* were collected from the campus of Regional Plant Resource Center (RPRC), Bhubaneswar. Samples were thoroughly washed under tap to remove dust and soil particles, dried in shade and were made into fine powder using mechanical grinder of Lexus make. Moisture content of the plant was calculated by using the following formula

$$\textbf{Moisture content (\%)} = \frac{Fw - Dw}{Fw} \times 100$$

Where, Fw = Fresh weight of plant sample
Dw = Dry weight of powdered plant sample

Solvent extraction was done by soxhlet extraction method. Percentage yield of all the four extracts was also calculated using the following formula.

$$\textbf{\% of yield} = \frac{Extract\ weight}{Powdered\ Weight} \times 100$$

Phytochemical analysis of solvent extracts

Phytochemical analysis was conducted using standard protocols.[8] Brief methodology for phytochemical analysis of the different metabolites was as follows

1. **Test for Alkaloids:** Alkaloid tests were done by using 3 different reagents.

- **Dragendroff's test** - To 1ml of extract 2ml of 1% HCl was added and then boiled for few minutes, after boiling 2-3 drops of dragendroff's reagent was

added & sample was observed for reddish brown precipitate.

- **Wagner's test -** To 1ml of extract 1ml of 1% H_2SO_4 was added followed by few drops of wagner'sreagent. Formation of precipitate depicts the presence of alkaloids.
- **Mayer's test -** To 1ml of extract, 2ml of 1% HCl and mayer's reagent was added dropwise and was observed for the formation of precipitate.

2. **Test for Flavonoid:** To 2.5 ml of extract, 1 ml of 10% NaOH was added. From the side of the test tube, drops of conc. HCl were added. Yellow colour turns to colourless which indicates presence of flavonoids.
3. **Test for Anthraquinone:** To 1ml of extract 2ml of 5% of KOH was added and was observed for pink colouration.
4. **Test for Saponin:** To 1ml of extract 2ml of $NaHCO_3$ was added and on shaking forms lather.
5. **Test for Terpenoids:** To 1ml of extract 400µl of chloroform and 4-5 drops of conc.H_2SO_4 was added from the walls of the test tube. A reddish brown ring indicates the presence of terpenoids.
6. **Test for Cardiac glycoside:** To 2.5ml of extract 2ml of glacial acetic acid**,** few drops of $FeCl_3$ and conc.H_2SO_4 was added from the walls of the test tube. Presence of cardiac glycoside is determined by reddish brown ring.
7. **Test for Tannin:** It can be observed by two methods.

- **Method A -** 1ml of extract was boiled and few drops of $FeCl_3$ were added to it. The sample was observed for blue, black, green colour.
- **Method B -** To 1ml of extract 500µl of lead acetate was added which gives yellow colour.

8. **Test for Starch:** To 1ml of extract 500µl of iodine was added, which results in blue coloration.
9. **Test for Phlobatannin**: To 1ml of extract 1% HCl was added and boiled, formation of precipitate occurs on positive test.

Determination of total phenolic content

The total phenolic contents of the plant was determined with some modification as described by standard protocols.[9] Extracted samples of *Clerodendrum siphonanthus* (100µl) was mixed with 750µl of Folin Ciocalteu reagent (10 fold dilution with distilled water) and incubated at 25ºC for 5 min. Then 750µl of sodium carbonate (60 g/L) solution was added to the mixture. Following 90 min incubation at 25ºC , absorbance was measured at 725 nm using UV-visible spectrophotometer. The total phenolic content was measured using a standard curve of gallic acid at 0.02 – 0.1 mg/mL concentrations. Total phenolic content was calculated for each sample and expressed as milligrams of gallic acid equivalent per 100 mL of sample.

Cytotoxic activity

Brine shrimp *(Artemia salina*) mortality assay

Cytotoxic activity study was carried out by brine shrimp lethality assay using standard protocols[10] Here simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. Brine shrimp (*Artemia salina*) eggs were hatched in artificial sea water, which was prepared using black salt 3.6 gm/ 200 ml distilled water. The eggs were incubated for 48 hours at temperature of about 28° C to get the desired growth of the larvae for biological evaluation. For each dose level 3 replicates were used. To each test tube of negative control, positive control and extracts, 20 numbers of brine shrimp larvae were taken and volume was made up to 10ml by adding salt water. Cytotoxic assay was carried out at four doses 25, 50, 100 and 200µg/ml. Motility assessment of larvae was conducted at each hour up to four hours.

Motility readings were graded as below.

4+ = high motile, 3+ = motile, 2+ = sluggish, 1+ = slow, Nil = no activity

After 24 hrs, the number of survived larvae in the control and experimental tubes were counted. From this data, the percentage (%) of inhibition of the brine shrimp was calculated for each concentration using the following formula:

$$\text{Percentage Inhibition} = \frac{\text{No of larvae in control} - \text{No of larvae in experimental}}{\text{No of larvae in control}} \times 100$$

Antioxidant activity

Qualitative Analysis (TLC based antioxidant assay)

TLC is one of the most widely used and potent technique to resolve mixture of plant compounds. It is also called DPPH (2,2-Diphenyl 1-picrylhydrazyl) assay. TLC sheet (Silica gel 60F_{254}, Merck company, Germany) was used as stationary phase. The developed TLC plate was sprayed with 0.2% DPPH in methanol as an indicator as per the standard protocol.[11]

The presence of antioxidant compounds were detected by yellow spots against a purple background on the TLC sheet sprayed with 0.2% DPPH in methanol.

DPPH+AH ——› DPPH–H+A^-

(Violet color) (Yellow color)

Three types of solvents were prepared for TLC chromatography technique.

1. BEA – Benzene: Ethanol: Ammonium hydroxide (90:10:1) [Non polar/Basic]

2. CEF – Chloroform: Ethyl acetate: Formic acid (5:4:1) [Intermediate polarity/Acidic]

3. EMW – Ethyl acetate: Methanol: Water (40:4.5:4) [Polar/Neutral]

Qualitative screening of the constituents in each of the plant extract of *Blumea lacera* for antioxidant activity was done by TLC analysis. The precoated TLC sheets

were activated at 100^0C for 2 minutes. The samples were spotted with the help of micro tips leaving 1cm from the bottom of the sheet. After drying of sheets DPPH solution was sprayed. Yellow bands on purple background represent the antioxidant bands of the extract.

Rf values of all the antioxidant bands were calculated using the following formula.

Retardation factor (R_f)

$$= \frac{\text{Distance travelled by the compound}}{\text{Total Distance travelled by the solvent}}$$

Quantitative anti-oxidant Analysis

Quantitative analysis was done by two popular methods as follows;

DPPH free radical scavenging assay

For DPPH free radical scavenging assay 1mM DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was prepared by adding 4mg of DPPH dissolved in 10ml methanol. DPPH assay was done by serial dilution method starting from concentration of plant extracts (7.8 µg/ml, 15.62 µg/ml, 31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500µg/ml, 1000µg/ml) was prepared in methanol. 1ml of each sample was taken in the test tubes and 500µl of DPPH solution was added. For control, each test tube contained 1ml methanol and 500µl DPPH**.** Samples were incubated for 30 minutes at room temperature in dark. All the samples were taken in triplicate and complete set of experiment was repeated three times. Optical density (OD) was measured at 517nm in spectrophotometer [12]. The percentage of free radical scavenging activity was calculated from the following formula

Percentage free radical scavenging [DPPH] = [(Ac – As) ÷ Ac] × 100

Where, Ac = Absorbance of control and As = Absorbance of sample.

FRAP ASSAY (Estimation of total antioxidant activity)

Total antioxidant activity is measured by ferric reducing antioxidant power (FRAP) assay as per the standard protocols.[13] Spectroscopic method is based upon the ability of antioxidants to reduce Fe^{+3} to Fe^{+2} in the presence of TPTZ, forming an intense blue Fe^{+2}-TPTZ complex with an absorption maximum at 593 nm. The decrease in absorbance is proportional to the antioxidant present. The FRAP reagent (300 mM Acetate buffer pH 3.6: 40 mM Dilute HCl: 10 mM TPTZ: 20 mM $FeCl_3.6H_2O$ in the ratio of 10: 1: 1) was prepared and then incubated at 37^oC in a water bath for 10 minutes. Absorbance of FRAP reagent was taken at 0^{th} minute (t_0) which was the control of the experiment. Ascorbic acid was taken as standard. A total of 100 µL of sample/standard and 300µl of distilled H_2O was then added to the FRAP reagent and incubated at 37°C for 4mins (t4). A reagent blank was prepared as described above but 100µl of distilled H2O was added instead of test sample. Duplicate test tubes were taken and absorbance was measured at 593nm. Ascorbic acid was taken as standard and 1.0mM to 0.1mM concentration of standard was prepared for the FRAP assay and based on the observations a standard curve was plotted. A number of dilutions of each sample extract were tested allowing dose response curves to be produced. The FRAP values were expressed in mmol Ascorbic acid equivalents (AAE).

RESULTS AND DISCUSSIONS

Moisture content of *Clerodendrum siphonanhus* was 51.10% percent and as can be seen in Table 1, methanol extract showed maximum yield suggesting that polar molecules outnumber the non polar.

Table 1: Percentage of yield.

Extract	Weight of sample	% of yield
Hexane	1.35gm	2.5%
Chloroform	4.30gm	8.2%
Ethyl acetate	3.14gm	6.01%
Methanol	22.57gm	43.19%

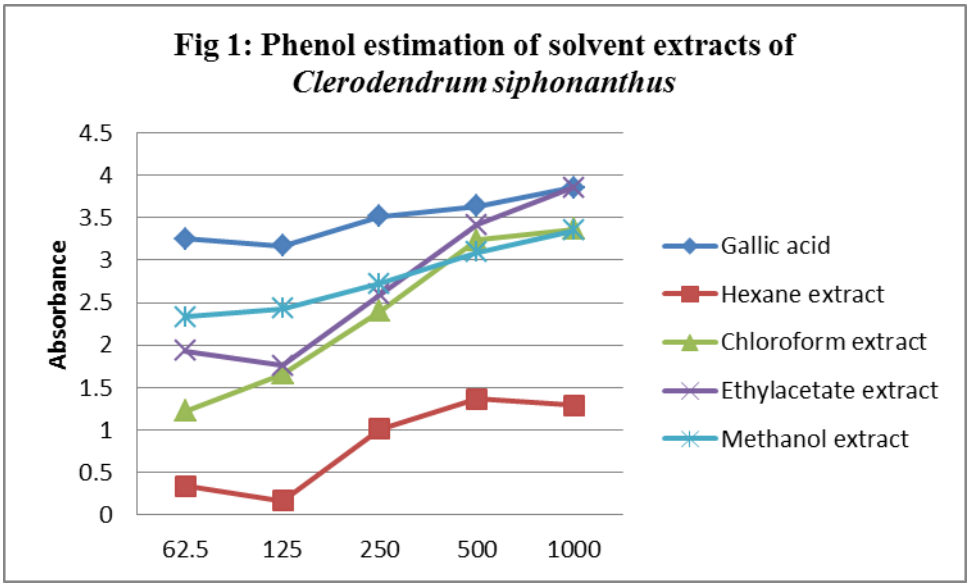
Phytochemical screening of leaf extract of *Clerodendrum siphonanthus*

Presence of alkaloids was predominant as it was found positive in all the three tests. Tannin was present in all the extract except hexane. All other phytochemicals were not detected. However when total phenolics content was estimated (Fig.1), ethyl acetate extract showed similar absorbance when compared to the standard Gallic acid at the highest dose. At highest concentration of 1000mg, chloroform and methanol extract showed equivalent activity but was less than the standard. Presence of alkaloids in the extracts clearly indicate the medicinal potential of this ornamental plant as a number of alkaloids isolated from plants have been used as antibacterial, antiviral and antifungal agents[14].

Table 2: Phytochemical analysis of leaf extracts of *Clerodendrum siphonanthus.*

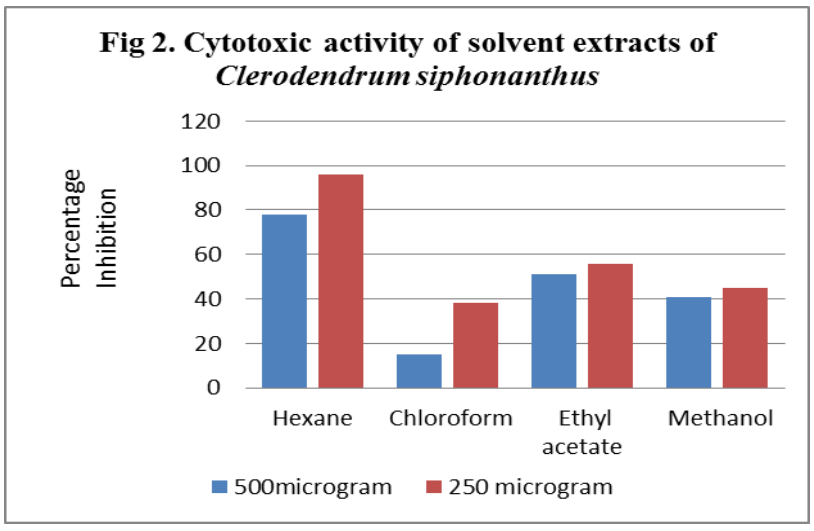
Phytochemical	Hexane extract	Chloroform extract	Ethyl acetate extract	Methanol extract
Alkaloids				
Mayer's test	+	+	+	+
Wagner's test	+	+	+	+
Dragendroffs test	+	+	+	+
Flavonoids	-	-	-	-

Anthraquinone	-	-	-	-
Saponin	-	-	-	-
Tannin	-	+	+	+
Terpenoids	-	-	-	-
Phlabotanins	-	-	-	-
Cardiac glycoside	-	-	-	-



Cytotoxic activity

As can be observed from Fig 2, all the extracts showed dose dependent activity. A highly significant activity was obtained in hexane extract which was 98% at the highest dose of 500 microgram and 79% at 250 microgram dose. This needs further exploration for isolating active principles. The activity against brine shrimp larvae is an important parameter to assess the anticancer activity as both the activities are co related.[15]



Antioxidant activity

As can be observed in Table 3, all the extracts showed the presence of antioxidant bands with maximum number of antioxidant bands in Chloroform extract in BEA solvent. However, in case of Qualitative DPPH radical scavenging assay, methanol extract showed better antioxidant potential but was less than the standard Ascorbic acid(Fig 2). In FRAP assay (Fig 3) three extracts chloroform, ethyl acetate and methanol extract showed similar activity but was less than the pure compound Ascorbic acid. This could be due to the fact that extracts are a combination of a number of molecules which either mask one another's activity or enhance one other's activity. As at higher doses antioxidant activity of standard and other extracts was marginally different so same needs further elaboration.

Thus, overall study has provided lead for further explorative work in the form of a potent hexanc extract with significant cytotoxic activity and three extracts for their antioxidant potential. Presence of alkaloids in the extracts is further indication of medicinal potential of the ornamental plant *Clerodendrum siphonanthus*.

Table 3: TLC based antioxidant assay of solvent extracts of *Clerodendrum siphonanthus.*

Solvent extracts	SOLVENT	No. of Anti-oxidant bands	Rf values
HEXANE	BEA	1	0.3
	CEF	1	0.31
	EMW	1	0.29
CHLOROFORM	BEA	8	0.75, 0.57, 0.50, 0.48, 0.39, 0.32, 0.25, 0.20
	CEF	4	0.66, 0.26, 0.21, 0.16
	EMW	4	0.64, 0.39, 0.32, 0.24
ETHYL ACETATE	BEA	5	0.70, 0.40, 0.36, 0.31, 0.25
	CEF	5	0.55, 0.40, 0.35, 0.17
	EMW	3	0.65, 0.44, 0.34
METHANOL	BEA	4	0.70, 0.40, 0.37, 0.31
	CEF	5	0.74, 0.63, 0.42, 0.35, 0.28
	EMW	2	0.79, 0.74

Fig 3: DPPH assay of solvent extracts of *Clerodendrum siphonanthus*

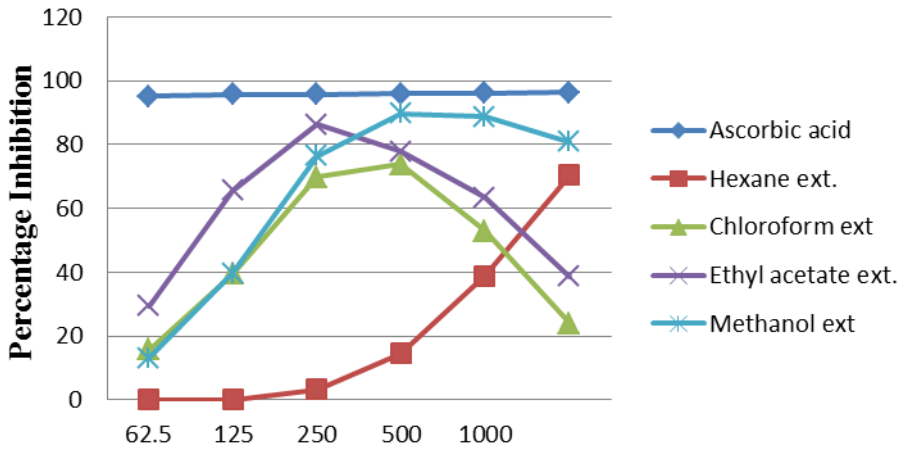
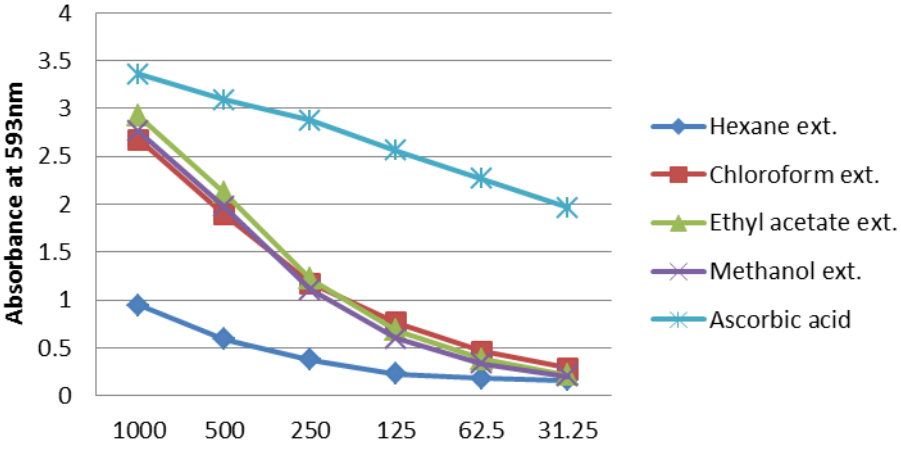


Fig 4: FRAP Assay of solvent extracts of *Clerodendrum siphonanthus*



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