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ANTIOXIDANT, CYTOTOXIC AND PHYTOCHEMICAL ASSESSMENT OF LEAF EXTRACTS OF GOLDEN TRUMPET (*ALLAMANDA CATHARTICA* L.)

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ABSTRACT

Medicinal plants have been used since time immemorial for a number of diseases. Phytochemical analysis of medicinal plants gives an insight towards its medicinal potential. In the present study leaf extracts of one flowering plant *Allamanda cathartica* was explored for its phytochemical, cytotoxic and antioxidant activity. On phytochemical analysis Flavonoids and saponins were present in chloroform, acetone and methanol extracts. Terpenoids was present in hexane, chloroform and acetone extract. Tannin was present in fresh, acetone and methanol extracts. Cardiac glycoside was present in hexane, acetone and methanol extract. Highest concentration of phenolic was found in the acetone extract of the plant 124.23µg(GAE)/ g followed by methanol extract with 92.07µg(GAE)/ g. Highest concentration of flavonoids was found in the acetone extract of the plant 605.50µgQE/g followed by chloroform extract with 307.16µgQE/g. Cytotoxic activity was significant in chloroform extract 97.97% at the dose 200µg/ml followed by hexane extract which showed 77.5 % activity at the highest dose. Remaining extracts showed only mild activities against brine shrimp mortality assay. Highest FRAP value was obtained in methanol extract followed by acetone extract. However extracts showed mild activity in DPPH radical scavenging assay. Results indicated in the study points towards a significant medicinal potential of leaf extracts of *Allamanda cathartica.*

KEYWORDS: Anti oxidant activity, Phytochemical analysis, Brine shrimp mortality assay.

INTRODUCTION

Allamanda cathartica is a flowering shrub belonging to family Apocynaceae. In some tropical countries leaves, roots and flowers are used as laxative and emetic in traditional medicine. Paste of roots is applied on insect bites. The plant is used for curing acute abdominal pain. It has also been used in folk remedies to treat liver tumors, jaundice, splenomegaly and malaria. Some species showed activity against carcinoma cells, pathogenic fungi and HIV.[1] Wound healing activity of the plant was also reported.[2]

Phytochemical analysis of medicinal plants gives an insight into the class of compounds present in the extracts of plants.[3] Secondary metabolites like alkaloids, flavonoids and Terpenoids have been used traditionally as medicine in a number of ailments from cancer, hepatoprotective and digestive activities.[4] Brine shrimp assay has a correlation with anti cancer activity,[5] where as antioxidant activity of the plant gives remedial action of plant products against oxidative stress.[6] The main purpose of study was to analyze the biological potential of leaves of *Allamanda cathartica* using phytochemical, antioxidant and cytotoxic assays as biological parameters.

MATERIALS AND METHODS

Collection and processing of plant materials

Fresh leaves of *Allamanda cathartica* were collected from medicinal germplasm garden of Regional Plant Resource Center (RPRC), Bhubaneswar. Leaves were washed with running tap water to remove dust and impurities and were dried in shade. Later leaves were made into fine powder which was used for serial solvent extraction with different solvents like Hexane, Chloroform, Acetone, and Methanol on the basis of their increasing polarity. After extraction the extract was concentrated using Bucchi(R-200) Rota vapour under vaccum at 45-50ºC.

Phytochemical analysis: All the tests were conducted following the standard protocols.[7]

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

Dragendroff's test: To 1ml of leaf extract 2ml of 1% HCl was added followed by boiling for few minutes, after boiling 2-3 drops of Dragendroff's reagent was added and sample was observed for reddish brown precipitate.

Wagner's test: To 1ml of leaf extract 1ml of 1% H_2SO_4 was added followed by few drops of wagner's reagent. Formation of precipitate depicts the presence of alkaloids.

Mayer's test: To 1ml of leaf extract 2ml of 1% HCl and mayer's reagent was added drop wise and was observed for the formation of precipitate.

Test for Flavonoid: To 2.5 ml of leaf 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.

Test for Anthraquinone: To 1ml of leaf extract 2ml of 5% of KOH was added and was observed for pink colouration.

Test for Saponin: To 1ml of leaf extract 2ml of $NaHCO_3$ was added solution was shaken to form the foam.

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. Positive results form reddish brown ring.

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 sides of the test tube. Presence of cardiac glycoside is determined by reddish brown ring.

Test for Tannin: It was 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 or green colour.
- **Method B:** To 1ml of extract 500µl of lead acetate was added which gives yellow colour if tannin is present.

Test for Starch: To 1ml of extract 500µl of iodine was added, which results in blue coloration.

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 content (TPC) of the crude extracts of leaves was determined using the standard method.[8] To 0.5 ml of test sample (gallic acid or leaf extract), 1.5 ml (1:10 v/ v diluted with distilled water) Folin Ciocalteau reagent was added and allowed to stand for 5

min at 22ºC. After 5 min incubation, 2.0ml of 7.5% of sodium carbonate was added. These mixtures were incubated for 90 min in the dark after slight shaking. Finally absorbance of different samples were measured at 725nm using spectrophotometer. The phenolic content was calculated as gallic acid equivalents GAE/ g on the basis of standard curve of gallic acid. The results were expressed as Gallic acid equivalents (GAE)/ g of the plant material. All the determinations were carried out three times.

Determination of total flavonoid content

The total flavonoid content (TFC) of Allamanda leaves was determined by using the aluminium chloride assay.[9] An aliquot (0.5 ml) of extracts were taken in different test tubes then 2ml of distilled water was added followed by the addition of 0.15 ml of sodium nitrite (5% $NaNO_3$, w/ v) and allowed to stand for 6 min. Later 0.15 ml of aluminium trichloride (10% $AlCl_3$) was added and incubated for 6 min, followed by the addition of 2 ml of sodium hydroxide (NaOH, 4% w/ v) and volume was made upto the 5ml with distilled water. After 15 min of incubation the mixture turns to pink whose absorbance was measured at 510 nm using a spectrophotometer. Distilled water was used as blank. The TFC was expressed in mg of quercitine equivalents (QE) per gram of extract. All the experiments were conducted in triplicates.

Bioevaluation of solvent extracts

Two biological activities were studied First was cytotoxic activity and another was antioxidant activity.

Cytotoxic activity (Brine shrimp lethality test)

Brine shrimp eggs were incubated for 48 hrs(3.6gm of black salt in 200ml distilled water) to get the desired growth of the larvae for biological evaluation. Stock solution of different extracts were prepared at a concentration of 10mg/ml, cytotoxic assay we carried out at 3 doses 50, 100, 200mg/ml for each dose level 3 replicates were used. Motility, readings were taken every hour up to 4 hrs. Motility was graded as below.

4+ = high motility, 3+ = motile, 2+ = sluggish, 1+ = slow and Nil = no activity at all

After 24 hrs, live larvae in the control and experimental samples were counted and percentage inhibition was calculated 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 studies)

To detect antioxidant activity, qualitative 2, 2-Diphenyl1-picrylhydrazyl(DPPH) assay[10] was conducted. About 5µl of each sample was loaded on the TLC sheet and the chromatograms were developed in following solvent systems.

a) Ethyl acetate : methanol : water (40:4.5:4) [EMW] (polar neutral)
b) Chloroform: ethyl acetate:formic acid (5:4:1) [CEF] (intermediate polarity/acidic)
c) Benzene: ethanol: ammonium hydroxide (90:10:1) [BEA] (Non polar/basic)

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

$$DPPH+AH \rightarrow \quad DPPH-H+A^{-}$$

(purple colour) (yellow colour)

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-picryl hydrazyl) (mol. Wt. 394.33) solution was prepared. 4mg DPPH was weighed and dissolved in 10ml methanol and incubated for 30 minute in dark. The concentration of stock solution was done by serial dilution method starting from concentration 500µg/ml, 250 µg/ml, 125 µg/ml, 62.25 µg/ml, 31.12 µg/ml, 15.6 µg/ml, 7.8 µg/ml. All the tests were conducted in triplicates. 1ml of each sample was taken in the test tube and volume was made up to 4ml by methanol. 500µl DPPH solution was added to each test-tube and stirred thoroughly before incubation of 30 minutes. Ascorbic acid was used as reference standard and dissolved in methanol to make solution with the same concentration. Control sample was prepared containing the same volume without any extracts and reference ascorbic acid.

Optical density (OD) was measured at 517 nm in spectrophotometer. The percentage radical scavenging activity was calculated from the following formula:

$$\text{Percentage radical scavenging [DPPH]} = [(Ac - As) \div Ac] \times 100$$

Where Ac was the absorbance of control and As was the absorbance of sample.

Frap Assay (Estimation of total antioxidant activity)

Total antioxidant activity was measured by ferric reducing antioxidant (FRAP) assay of Benzie and Strain.[11]

RESULTS AND DISCUSSION

The phytochemical tests depicted a number of secondary metabolites in the different extracts (hexane,chloroform,acetone and methanol). Out of nine phytochemical screened, five were present in various solvent extracts.These were flavonoids, tannin, saponins, terpenoids and cardiac glycosides(Table 1). Alkaloids, anthraquinones, starch and phlobotanin were absent in all the samples. Acetone extract showed 5 numbers of secondary metabolites followed by methanol which contained 4. Fresh sample showed only tannins. Solvent extracts of *Allamanda cathrtica* showed the presence of medicinally important phytochemicals like flavonoids, sapponis, tannins and terpenoids. Flavonoids are well known for their pharmacological properties like antioxidant, cytotoxic and have been reported to reduce growth of tumors in mice.[4] Terpenoids are a large class of organic hydrocarbons made up of isoprene units and are associated with a number of biological activities like antiviral, antiparasitic and antibacterial activities.[12] The phenolic content was calculated as Gallic acid equivalents GAE/ g on the basis of standard curve of Gallic acid. Highest concentration of phenolics was found in the acetone extract of the plant i.e. 124.23µgGAE/g (Fig 1). The total flavonoids content (TFC) of Allamanda leaves was determined by using the aluminum chloride assay through spectrophotometry. The TFC was expressed in mg of quercetin equivalents (QE) per gram of extract. Highest concentration of flavonoids was found in the acetone extract of the plant i.e. 605.5µgQE/g (Fig 2). Hexane extract was totally devoid of flavonoids. Thus, presence of these molecules is indeed an indication of biological activity of leaf extracts.

Table 1: Phytochemical analysis of fresh and solvent extracts of Allamanda cathrtica.

Phytochemicals	Fresh sample	Hexane	Chloroform	Acetone	Methanol
Alkaloid	-	-	-	-	-
Flavonoid	-	-	+	+	+
Anthraquinone	-	-	-	-	-
Saponin	-	-	+	+	+
Terpenoids	-	+	+	+	-
Cardiac glycosides	-	+	-	+	+
Tannin	+	-	-	+	+
Starch	-	-	-	-	-
Phlobatannin	-	-	-	-	-

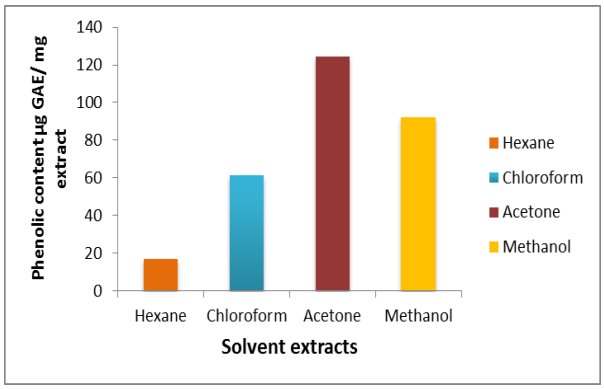

Fig1: Phenolic contents.

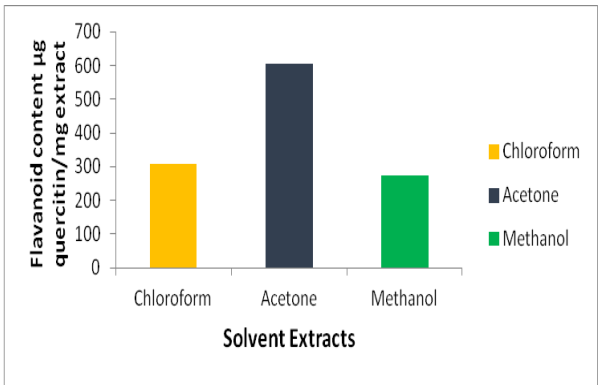

Fig.2 Flavanoid contents.

Bioevaluation of extracts of *Allamanda cathartica*

For biological evaluation two parameters were selected these were cytotoxic activity using brine shrimp assay and antioxidant assays both qualitative (TLC based) and quantitative analysis (DPPH radical scavenging and FRAP assay).

Cytotoxic activity using brine shrimp assay

All the extracts were tested in three doses (50, 100, 200µg/ml). Cytotoxic activity was found highest in chloroform extract (97.97%) at the dose 200µg/ml followed by hexane extract which showed 77.5% activity at the higher dose. Remaining extracts showed only mild activities against brine shrimp mortality assay (Fig 3). Brine shrimp assay has been reported by a number of researchers related to anti- cancer activity.[13] Previously this test was considered as primary test for screening anticancer activity. Amongst all the extracts Chloroform extract showed remarkable activity in this assay and hence same extract is a potential candidate for anti inflammatory, anticancer and anti tumor activities.

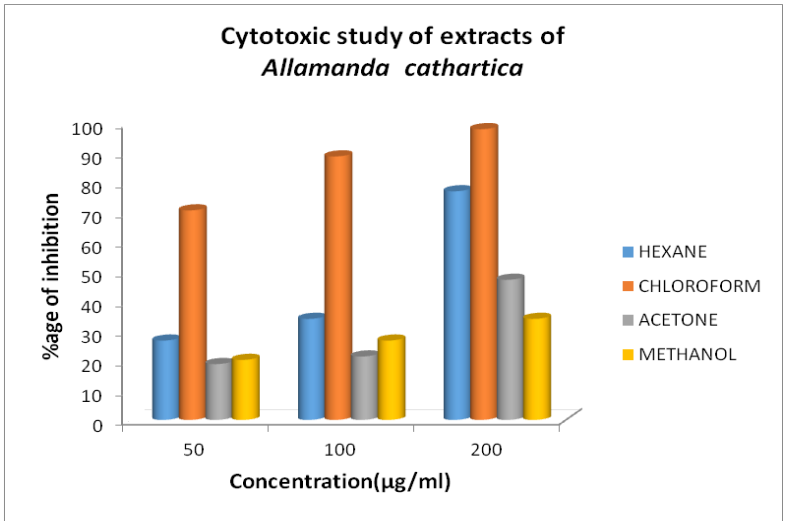

Fig 3: Cytotoxic activity in different doses of different extract of *Allamanda cathartica*.

Antioxidant activity of extrcts of Allamanda Cathartica

Antioxidant activity of *Allamanda Cathartica* was evaluated by one qualitative and two quantitative tests.

TLC based qualitative antioxidant assay

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. Number of yellow bands correspond to the number of antioxidant molecules in the sample. On the basis of TLC, maximum number of antioxidant bands were obtained in Chloroform extract in solvent EMW(Table 2).

Table 2: Qualitative antioxidant assay of solvent extracts of *Allamanda catharatica.*

Samples	Solvents	No. of Bands	R_f Values
Ascorbic Acid	BEA	0	-
	CEF	1	0.15
	EMW	0	-
Hexane	BEA	2	0.05,0.1
	CEF	1	0.5
	EMW	1	0.22
Chloroform	BEA	8	0.05,0.1,0.16,0.27,0.35,0.42,0.51
	CEF	8	0.15,0.18,0.62,0.8,0.86,0.93
	EMW	10	0.48,0.53,0.76,0.78,0.87,0.92
Acetone	BEA	1	0.08
	CEF	4	0.06,0.1,0.18,0.91
	EMW	8	0.08,0.22,0.35,0.4,0.43,0.58,0.55,0.92
Methanol	BEA	0	-
	CEF	1	0.08
	EMW	4	0.08,0.17,0.37,0.45

Quantitative antioxidant assays

The antioxidant activity of different extracts of *Allamanda cathartica* was analyzed with DPPH, a stable free radical. As DPPH picks up one electron in the presence of free radical scavenger, the absorption decrease and the resulting discoloration related to the number of electrons gained. In case of FRAP assay donation of an electron to free radicals results in conversion of $Fe^{3+}$to $Fe^{2+[}$. In TLC based assay a number of yellow bands were observed in almost all the extracts, but in quantitative tests antioxidant activity (Fig 4 and 5) was only mild as compared to the standard Ascorbic acid. This could be due to the fact that molecules in an extract could act synergistically or antagonistically to one another.[14]

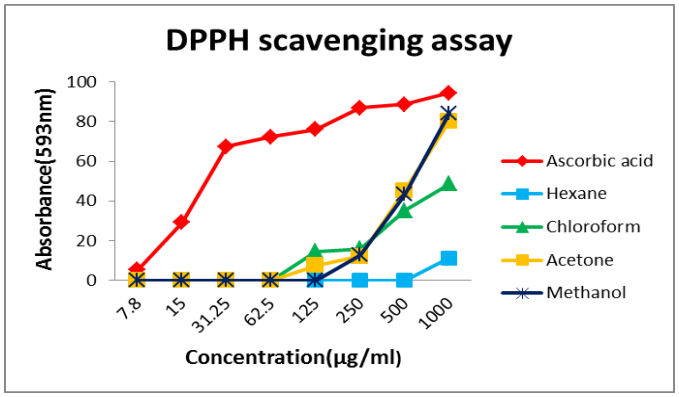

Fig 5: DPPH assay of Solvent extracts of *Allamanda Cathartica.*

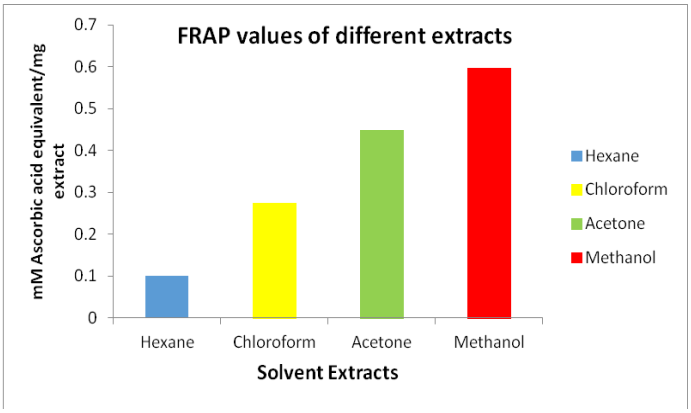

Fig 6: FRAP values of different extracts.

CONCLUSION

Leaf extracts of the flowering plant *Allamanda cathartica* were found to be promising as they possessed medicinally important class of compounds like Terpenoids, flavonoids and sapponin. Although they showed mild antioxidant activity yet chloroform extract of the leaves showed significant cytotoxic activity and hence plant is a potential candidate for anti inflammatory, anticancer and anti tumor activity.

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