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IN VITRO HEPATOPROTECTIVE ACTIVITY OF *ALLAMANDA CATHARTICA* L.ON THE BRL3A CELL LINE

Nisha Pothan[1]*, Jyoti Harindran[2]

1. Research Scholar, Department of Pharmaceutical Sciences, Karpagam University, Karpagam, Coimbatore, Tamil Nadu-641021, India.
2. Principal, University College of Pharmaceutical Sciences, Mahatma Gandhi University, Kottayam , Kerala, India

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For Correspondence:

Nisha Pothan

Research Scholar, Department of Pharmaceutical Sciences, Karpagam University, Karpagam, Coimbatore, Tamil Nadu-641021, India.

E-mail:

nisha7925@yahoo.co.in

ABSTRACT

The various phytochemical compounds detected from the flowersand roots are known to have beneficial importance in medicinal sciences .The family Apocynaceae consists of several important medicinal plants with wide range of biological activities, interesting phytochemical constituents. In this study, cytotoxic activity of methanolic and aqueous extracts was prepared from flowers and roots of *Allamanda cathartica* Linn were tested for cytotoxic activity on BRL 3-A cell lines using the MTT assay. MTT assay was used to evaluate the reduction of viability of cell cultures in the presence and absence of the extracts. Cell viability was inhibited to different extents by the extracts. An investigation also carried out to evaluate the *in vitro* hepatoprotective effect of extract of *Allamanda cathartica* L using antitubercular drugs(Isoniazid, Pyrazinamide, Rifampicin) and D (+)-Galactosamine as toxicant and silymarin as standard drug by MTT assay[(3-(4,5 dimethylthiazole –2 yl)-2,5 diphenyl tetrazolium bromide) assay.

INTRODUCTION

The various phytochemical compounds detected from the crude extracts of flowers and roots are known to have beneficial importance in medicinal sciences. Recently, a number of studies have been carried out on the phytochemistry of plants across the world. Ornamental flowers of the family Apocynaceae were selected for phytochemical screening in this study. Various species of this family have a range of traditional uses. Several species are also widely grown for ornamental purposes.The family Apocynaceae consists of several important medicinal plants with wide range of biological activities and interesting phytochemical constituents.[1-4]

Allamanda cathartica L. (Synonyms: Echites verticillata Sessé & Moç, Orelia grandiflora Aublet, *Allamanda grandiflora* (Aublet) Poiret in Lam, *Allamanda hendersonii* W. Bull ex Dombrain.*Allamanda cathartica L* (A. cathartica). commonly known as the Yellow Bell, Golden Trumpet or The Buttercup flower is a genus of tropical shrubs and vines belonging to the family Apocynaceae. Hailing from tropical America, Brazil in particular, this genus was named after 18th century Swiss Botanist Dr. Frederic Allamand, who sent its seeds to Linnaeus, the Swedish Botanist. The word 'cathartica' means purgative[4]. Because of its rapid growth, pruning is often necessary, which can expose gardeners to the toxic sap that causes dermatitis symptoms of rash, blisters, and itch. Although incidence is much less, plant parts are toxic if ingested. All parts contain the toxic iridoid lactone, allamandin[4,5]. The bark, latex and the infusion of its leaves in small doses is cathartic. The decoction of its bark is a hydragogue. In Guyana, its latex is employed as a purgative and for relieving colics. *Allamanda cathartica Linn*. is a perennial shrub used in traditional medicine for treating malaria and jaundice.. The leaf extract of *A. cathartica* was found to promote wound healing in Sprague Dawley rats[12]. The leaves are also the source of many bioactive compounds with anti-inflammatory activity[13]. The flower is also used as a laxative [14]

MATERIAL AND METHODS

Collection and identification of *Allamanda cathartica Linn*

Selection and Collection of plant on the basis of ethno botanical survey, traditional use and literature survey. The mature flower and roots of *Allamanda cathartica Linn* were collected in the morning locally from Pathanamtitta District, Kerala,India, in the month of November . The powdered drug packed in a paper bags & stored in air tight container until use.Identification and Authentication of here by Dr.Elizabeth.T.Mangatt, Professor and Head Dept. of Botany, Marthoma College Thiruvalla, Kerala, India (Voucher. No 138/17/OCT/2013)

Preparation of extract

One kg of powdered parts of *Allamanda cathartica Linn* was taken and 2500 ml of 95% methanol was added. It was refluxed for 2 hours and filtered through muslin cloth while hot. Filtrate was concentrated, evaporated and standardized. The yield was calculated.

PHYTOCHEMICAL ANALYSIS

Preliminary Phytochemical studies of various extract of *Allamanda cathartica Linn* was performed for major classes of constituents like alkaloids, carbohydrates, protein , amino acid,Terpinoids, Saponins, glycosides, steroids, tannins, flavonoid and phenolic compounds according to published standard methods[17] .The extracts of *Allamanda cathartica Linn* subjected to various chemical tests in order to determine the secondary plant constituents presents by employing the use of various methods as follows:

Test for Reducing Sugars; To 2 ml of the extract, Add 5ml of a mixture (1:1) of Fehling's solution IA and Fehling's solution II (B) and for five minutes the mixture was boiled in a water bath. A brick-red precipitate indicated the presence of free reducing sugars

Test for the presence of anthraquinones: To 1ml of the extract was shaken with 10 ml of benzene, filtered and 10 percent ammonia solution added to the filtrate. The mixture was shaken; the presence of a pink, red or violet colour in lower phase indicated the presence of anthraquinones.

Test for Saponins: To 1ml of the extract was dissolved in a 5 ml of distilled water in a test-tube, the test tube was stopperred with a cork and shaken vigorously for 25 seconds and then allowed to stand for 15 minutes. The appearance of frothing which persists on warming indicated the presence of saponins

Test for Flavonoids: To a portion of the extract, a few drops of 10 % ferric chloride solution were added. A green or blue colour indicated the presence of phenols.

Test for Steroids/Terpenes: To 1ml of the extract was dissolved and 2 ml of acetic anhydride and cooled well . Sulphuric acid was carefully added. A color change from violet to blue to green indicated the presence of a steroids

Test for Tannins:To 1ml of extract was dissolved in water followed by a few drops of 10% ferric chloride. A blue –black, green or blue –green precipitate would indicate presence of tannins

Test for Alkaloids:To 1ml of extract was stirred with 2ml of hydrochloric acid on a stem bath; the 1ml portion filtrate was treated with a few drops of Mayer's reagent and a second 1ml portion was treated with Dragendorff's reagent. Turbidity with either of these reagents would indicate the presence of alkaloids[4]

Preparation of solutions:

Toxicants:

a. 10 mg of antitubercular drugs Isoniazid, Rifampicin and Pyrazinamide (1:2:5) were dissolved in 1 ml DMSO and diluted to 10 ml with minimum essential medium. By diluting with water 1000, 500,250 and 125 µg/ml solutions were prepared.

b. 10 mg of D (+)-Galactosamine was dissolved in 1 ml DMSO and diluted to 10 ml with minimum essential medium. By diluting with water 50, 40 and 20 µg/ml solutions were prepared.

c. Silymarin at a concentration of 250µg/ml was used as standard

Sample Solution:

10 mg of *Allamanda cathartica Linn* was dissolved in 1 ml of DMSO and diluted to 10 ml with minimum essential medium. By diluting with water 1000,500,250 and 125 µg/ml solutions were prepared

Cell lines used[15]:

The cell line BRL 3A used for screening hepatoprotective activity of the plant extract was obtained from National Centre for Cell Sciences,Pune, India. Description of cell line is as follows.

Tissue/Organ: Normal,Liver
Strain : Buffalo
Source : Rat
Morphology: Epithelial

Stock cells of BRL3A were cultured in DMEM (Dulbecco's modified eagles medium) supplemented with 10% inactivated Foetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO2 at 37^0C until confluent. The cells was dissociated with TPVG. The stock cultures was grown in 25 cm culture flasks and all experiments was carried out in 96 microtitre plates

Determination of cell viability by MTT Assay

Principle: The ability of the cells to survive a toxicity on the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used.[15]

Procedure:[15]

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 105 cells/ml using DMEM medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37^{o} C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 48 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^{o} C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

% Growth Inhibition = 100 – Mean OD of control group/Mean OD of individual test group X 100

Determination Hepatoprotective activity [16]

The monolayer cell culture was trypsinated and the cell count was adjusted to 1.0 x 10^{-5}cells/ml using medium containing 10% new born calf serum. To each well of the 96 well microlitre plate, the diluted cell suspension of 0.1 ml (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once and 100µl of different drug concentrations was added to the cells in microtitre plate. The plate was then incubated at 37ºC for 3 days in 5% CO_2 atmosphere and microscopic examination was carried out and the observations recorded every 24 hours. After 72 hours, the drug solutions in the wells were discarded and 50 µl of MTT was added to each well. The plates were gently shaken and incubated for 3 hours at 37ºC in 5% CO_2 atmosphere. The supernatant was removed and 50 µl of propanol was added and the plates were gently shaken to solubilise the formazan. The absorbance was measured using a micro plate reader at a wavelength of 540 nm. . The percentage cell viability was determined, based on which the percentage protection offered by test and standard drugs was calculated over the ethanol control

The percentage growth inhibition and percentage cell protection was calculated using the formula

% cell protection = Mean O.D of individual test group /Mean O.D of control group x 100

% growth inhibition = 100 - Mean O.D of individual test group /Mean O.D of control group x 100

Statistical analysis :Data are expressed as means ± SEM. Mean difference between groups were analyzed by Student's' test. P value < 0.001 was considered to be statistically significant

RESULT AND DISCUSSION

PHYTOCHEMICAL ANALYSIS

Preliminary Phytochemical studies of various extracts of *Allamanda cathartica Linn* was performed for major classes of constituents like alkaloids, carbohydrates, protein , amino acid, Terpinoids, Saponins, glycosides, steroids, tannins, flavonoid and phenolic compounds according to published standard methods. Methanolic and Aqueous extracts of *Allamanda cathartica Linn* were tested for cytotoxic activities on on BRL 3-A cell lines. Extracts were prepared from flower and roots of the plant and the yield of extracts were given in *Table 1*.

Table 1. The Percentage yield of *Allamanda cathartica* Linn extracts

Solvent		***% of Yield***	***Appearance***
Methanol extract	Roots	92	Brown
	Flower	157	Brown
Aqueous	Roots	81	Brown
	Flower	134	Brown

Weight (mg) of crude extract per 10 g of fresh plant material

Phytochemical Screening

The phytochemical screening showed (Table 2) that the presence of alkaloids, amino acids, flavonoids, glycosides, proteins, reducing sugars, starch, steroids, tannins, terpenoids etc.

Table 2 :Phytochemical screening on *Allamanda cathartica Linn* with given solvents

Ingredients	***Methanol***		***Aqueous***	
	RE	*FE*	RE	FE
Alkaloids	+	+	+	+
Amino Acids	+	+	+	+
Anthraquinones	-	-	-	-
Flavonoids	+	+	+	+
Glycosides	+	+	+	+
Gums and Mucilage	-	-	-	-
Proteins	+	+	-	+
Reducing Sugars	+	-	+	-
Saponins	-	+	+	+
Starch	+	+	+	+
Steroids	+	+	+	+
Tannins	+	+	+	-
Terpenoids	+	+	+	-

+: Positive result; - : Negative result; RE-Root extract, FE-Flower extract

Cytotoxic Effect of Plant Extracts

Cytotoxicity of extracts *Allamanda cathartica Linn* was determined by MTT assay on BRL 3-A cell lines**.**The results of the cytotoxic activity of crude extracts from roots and flower of are summarized in Table 3.

Table 3: Cytotoxicity of anti tubercular drugs on BRL 3-A cell line by MTT assay

Inhibition of cell viability (%)				
Extract concentration (µg/mL)				
Crude extract	10	100	250	500
ME-R	6.6 ±9.20	13±0.1	24.5±1.0	57.1±1.25
ME-F	16 ±1.60	7±3.0	12.2±3.08	23±3.42
AE-R	2.6 ±3.40	3.1±3.12	2.0 ±3.90	9.6 ±3.60
AE-F	1.16 ±1.44	7.3 ±1.20	0.5 ±0.92	1.6 ±1.22

Values are averages and standard deviations for 3 independent experiments. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level

Determination Hepatoprotective activity

The tetrazolium salt (3-(4, 5dimethylthiazole–2 yl)-2,5 diphenyl tetrazolium bromide) was taken up into the cells and reduced in a mitochondria dependent reaction to yield formazan. This accumulates within the cell, due to the fact that it do not pass through the plasma membrane. The product is librated on solubilisation of the cells, and can be detected and quantified by a colorimeric method. The ability of cells to reduce MTT provides an indication of mitochondrial integrity and activity which was interpreted as a measure of viability cell number. The assay has therefore been adopted for use with cultures of exponentially growing cells. Determination of ability to reduce MTT to the formazan derivative after exposure to test compounds compared to the control, enables the relative protection of test chemicals to be assessed. From dose-response curves were calculated for the toxicants over a range of concentrations, enabling 50% cytotoxic concentration (CTC_{50}) to be calculated. (i.e. concentrations of hepatotoxicant required to reduce cell viability).This concentration of hepatotoxicants was used to test the protective effect of *Allamanda cathartica Linn* . Since approximately 50% inhibition was achieved with 500 µg/ml*(Table 3)* , 50% cytotoxic concentration (CTC_{50})was taken as 500µg/ml for anti tubercular drugs on BRL 3-A cell lines. Since approximately 50 % inhibition was achieved with 40 µg/ml, CTC_{50} was taken as 40 µg/ml for Galactosamine HCl drugs on BRL 3A cell lines. (*Table 4)*

Table 4: Hepatoprotective activity of Allamanda cathartica Linn roots by MTT assay

Sl no	Con. of extract (µg/ml)+ hepatotoxicants(500µg/ml)	% Protection by anti tubercular drugs[a]	% Protection by galactosamine HCl[a]
1	*Allamanda cathartica* Linn 1000 µg/ml + toxicant	86.00	89.39
2	*Allamanda cathartica* Linn 500 µg/ml + toxicant	79.84	72.41
3	*Allamanda cathartica* Linn 250 µg/ml + toxicant	65.64	74.24
4	*Allamanda cathartica* Linn 125 µg/ml + toxicant	50.24	57.40
5	Silymarin 250 µg/ml + toxicant	99.47**	95.13**
6	Only toxicant	45.05*	53.51*
7	Control	100*	100*

[a]All values are mean ± S.E.M. n=6, *P < 0.001 when compared to untreated cells.**P < 0.001 when compared to intoxicated cells

Table 5: Hepatoprotective activity of *Allamanda cathartica* Linn flower by MTT assay

Sl no	Con. of extract (µg/ml)+ hepatotoxicants(500µg/ml)	% Protection by anti tubercular drugs[a]	% Protection by galactosamine HCl[a]
1	*Allamanda cathartica* Linn 1000 µg/ml + toxicant	81.00	83.93
2	*Allamanda cathartica* Linn 500 µg/ml + toxicant	76.74	73.51
3	*Allamanda cathartica* Linn 250 µg/ml + toxicant	69.94	70.34
4	*Allamanda cathartica* Linn 125 µg/ml + toxicant	55.49	53.40
5	Silymarin 250 µg/ml + toxicant	99.47**	95.13**
6	Only toxicant	45.05*	53.51*
7	Control	100*	100*

[a]All values are mean ± S.E.M. n=6, *P < 0.001 when compared to untreated cells.**P < 0.001 when compared to intoxicated cells

Hepatoprotective activity of *Allamanda cathartica Linn* against anti tubercular drugs and galactosamine HCl by MTT assay

The ratio of 1:2:5 anti tubercular drugs isoniazid, rifampicin, pyrazinamide in was used as hepatotoxicant to assess the hepatoprotective effect of *Allamanda cathartica Linn* extract. The percentage protection of plant extract was determined and presented in (Table 5). Since the 50% cytotoxic concentration(CTC_{50})of antitubercular drugs was approximately 500 µg/ml (Table5), this concentration was used to determine the hepatoprotective effect of the drugs against BRL 3-A cell lines by MTT assay. Silymarin at the concentration of 250µg/ml showed highest protection (99.47%). *Allamanda cathartica Linn* flowerextract at 1000µg/ml showed 81% protection followed by 125µg/ml which showed least protection i.e. 55.49%. *Allamanda cathartica Linn* roots extract at 1000µg/ml showed 86% protection followed by 125µg/ml which showed least protection i.e. 50.24%.

Galactosamine HCl was used as hepatotoxicant to assess the hepatoprotective effect of *Allamanda cathartica Linn* extract. The percentage protection of plant extract was determined and presented in Table 4. Since the 50% cytotoxic concentration (CTC_{50}) of galactosamine HCl was approximately 40µg/ml (Table 4), this concentration was used to determine the hepatoprotective effect of the drugs against BRL 3-A cell lines by MTT assay. Silymarin at the concentration of 250µg/ml showed highest protection (95.13%). *Allamanda cathartica Linn* flowerextract at 1000µg/ml showed 83.93% protection followed by 125µg/ml which showed least protection i.e. 53.40% . *Allamanda cathartica Linn* root extract at 1000µg/ml showed 89.39% protection followed by 125µg/ml which showed least protection i.e. 57.40 % .

CONCLUSION

The members of the family Apocynaceae are also known to contain cardioactive glycosides, as in *Allamanda* .Generally, glycosides are non-volatile and serve as defense mechanisms of plants against predation by many microorganisms and herbivores . Flavonoids found in flowers have been referred to as 'nature's biological response modifiers', because of their inherent ability to modify the body's reaction to allergies and viruses; they also posses anti-allergic, anti-inflammatory, anti-microbial and anti-cancer properties . Steroidal compounds are of importance and interest in pharmacy. Synthetic steroids, which are widely used in modern medicine, have a range of side effects. But plant steroids are non-toxic and are known for their cardiotonic, insecticidal and antimicrobial properties. They are also used in nutrition, herbal medicine and cosmetics. Phenols are a class of low-molecular-weight secondary metabolites found in most terrestrial plants. Phenolic compounds are the largest group of phytochemicals and account for

most of the antioxidant activity in plants. At lower concentrations tannins inhibit the growth of microorganisms and act as anti-fungal agents; at higher concentrations they act by coagulating the protoplasm of the microorganism. Saponins are used as mild detergents and in intracellular histochemical staining. In medicine, they are used for treating diseases and weight loss, and have antioxidant effect .The ornamental flowers of *Allamanda cathartica*, studied here can be potential sources of useful drugs with antimicrobial, antidiabetic and anti-inflammatory activities. Since these plants are commonly grown in kerala, and produce a large number of flowers, these flowers can be a cheap source of efficacious drugs. Hence more advanced studies are needed in order to identify the bioactive principles to treat various human ailments.

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