

ALLAMANDA CATHARTICA LINN.: EXTRACTION AND PHARMACEUTICAL EVALUATION OF VARIOUS EXTRACTS OF LEAVES AND FLOWERS

MEENAKSHI FARTYAL

Laboratory of Plant Tissue Culture and Secondary Metabolites, Department of Botany, University of Rajasthan, Jaipur, India
Email: mksh35@gmail.com

Received: 22 Jul 2016, Revised and Accepted: 20 Aug 2016

ABSTRACT

Objective: Extraction and evaluation of the antifungal activity of extracts from leaves and flowers of *Allamanda cathartica* (Apocynaceae) Linn.

Methods: Leaves and flowers of *A. cathartica* were collected, dried and extracted by using well-established methods for alkaloids, flavonoids, steroids and crude extracts in polar and non-polar solvents. Extracts were screened for antifungal activity using 'Disc Diffusion Assay' against *Candida albicans* (Yeast), *Aspergillus flavus* and *Tricophyton mentagrophyte* (fungi). Inhibition zone (IZ), Activity index (AI), Minimum inhibitory concentration (MIC), Minimum fungicidal concentration (MFC) and Total activity (TA) were studied. Mean and standard deviation has also been calculated.

Results: *C. albicans* found to be the most susceptible organism while *T. mentagrophyte* found to be resistant. Alkaloid of leaf (IZ= 22 mm, AI= 0.65±0.02, MFC= 0.156 mg/ml, MIC= 0.078 mg/ml, TA= 557.59 ml/g) showed the best activity against *C. albicans*. Pet ether extract (IZ= 12 mm, AI= 0.40±0.02, MFC= 0.312 mg/ml, MIC= 0.156 mg/ml, TA= 114.48 ml/g) and water extract (IZ= 13 mm, AI= 0.43±0.02, MIC= 0.312 mg/ml, MIC= 0.156 mg/ml, TA= 99.36 ml/g) of flower also showed very good activities against *C. albicans*. The range of MFC and MIC found to be 1.25-0.156 mg/ml and 0.625-0.078 mg/ml, respectively.

Conclusion: Results indicate the good antifungal potency of extracts against tested microorganisms. Hence, may be explored for the formation of new antifungal drugs.

Keywords: Antifungal activity, Alkaloid, Flavonoid, Steroid, Polar and non-polar solvents, Disc diffusion assay, Minimum inhibitory concentration, Minimum fungicidal concentration, Total activity

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DOI: <http://dx.doi.org/10.22159/ijcpr.2016v8i4.15272>

INTRODUCTION

The use of medicinal plants as a source for relief from illness can be traced back over five millennia to written documents of the early civilization in China, India and the Near east, but it is doubtless an art as old as mankind. Neanderthals living 60,000 y ago in present day Iraq used plants such as hollyhock. These plants are still widely used in ethnomedicine around the world [1, 2]. Medicinal plants still are a rich source of antimicrobial compounds. There has been an increasing interest worldwide on therapeutic values of natural products. It is believed that the cure, to any debilitating human ailments, may be found, among the world's flora in nature's pharmacy [3]. Phytochemicals have made a significant contribution in maintaining human health. The significance of drugs derived from plants cannot be over emphasized with the recent trend of high percentage of resistance in microorganisms to the present day antibiotics [4]. The effort has been intensified by researcher towards a search for more plant source of antimicrobial agents. In the present study *Allamanda cathartica* has been selected for the study.

Allamanda cathartica (common name Golden trumpet) is a woody climbing evergreen shrub belongs to family Apocynaceae. It is native to Central America and Brazil, cultivated in India for showy flowers, found wild in Karnataka. Various medicinal properties as a good purgative, Antidote for poisoning, inflammation, constipation and ascites are attributed to this plant. Distilled extract of the plant claims to the cure of malignancy, fungal and bacterial diseases, for colic and acute abdominal pain. It is used for jaundice and enlarged spleen resulting from malaria. It is active *in vivo* in mice and *in vitro* against human carcinoma of the nasopharynx. The alcoholic and aqueous extract is hypertensive. It's cathartic (milky sap) possess antibacterial and possibly anticancer activity. The present investigation was undertaken to find out the antifungal potential of extracts of different parts of *A. cathartica* against some fungi.

Alkaloids are known to have pharmacological effects and are used in medications, as recreational drugs or in entheogenic rituals.

Literature indicates that plant alkaloids have considerable biological activity [5, 6]. The presence of Alkaloids, Sterols, and Flavonoids in leaf of *A. cathartica* and their antifungal activity against *Candida albicans* were reported [7].

Flavonoids are potent water-soluble antioxidants and free radical scavengers, which prevent oxidative cell damage and have strong anti-cancer activity [8]. It was reported that flavonoids can improve the blood circulation and lower the blood pressure [9]. Different phytoconstituents such as alkaloids, phenolic compounds, flavonoids, saponins, glycosides, terpenoids, steroids, coumarins, quinones, phytosterols, proteins, and carbohydrates were identified in the aqueous, acetone, petroleum ether, chloroform and ethanol extracts of the flowers *Allamanda cathartica* Linn. [10].

Steroids are frequently used as signaling molecules, represents highly concentrated energy stores, along with phospholipids function as components of cell membranes. Literature related to the antimicrobial activity of steroids of *Allamanda cathartica* was not yet found.

The methanolic extracts of *A. cathartica* showed active inhibition against *Salmonella typhi*, *salmonella paratyphi*, *Streptococcus aureus*, *Klebsiella vulgaris*, *Shigella dysenteriae*, *Shigella boydii* and *Escherichia* [11]. Petroleum ether extract of *A. cathartica* showed good inhibition against *Staphylococcus aureus* (20 mm), *Escherichia coli* (13 mm), *Pseudomonas aeruginosa* (19 mm), *Acinetobacter sp* (20 mm), *Proteus sp* (18 mm) [12]. *Allamanda cathartica* (leaf) extract found to exhibit potential antimicrobial properties against human pathogenic bacteria *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Proteus mirabilis* [13]. Petroleum ether and chloroform extracts of *Allamanda cathartica* exhibited promising antifungal activity [14].

Considering the rich diversity of plants, it is expected that screening and scientific evaluation of plant extracts for their antimicrobial activity may provide new antimicrobial substances. Review of the current literature reveals that no work has been carried out for

extraction and screening of specific compound from selected plant. Hence, in the present work an extraction and screening for antifungal activity of extracts of *A. cathartica* has been undertaken.

MATERIALS AND METHODS

Different parts of *A. cathartica* (leaf and flower) were collected in the month of April to June from the western parts of India (Jaipur, Rajasthan). Plants were identified by senior taxonomist at Department of Botany, University of Rajasthan and voucher specimen no: RUBL 21177 was submitted to the Herbarium, Botany Department, University of Rajasthan.

Preparation of extracts

Alkaloids extraction

Alkaloids were extracted from different parts of the selected plant by well-established method [15]. Finely powdered sample (100g) of plant parts were extracted in 20 ml methanol after shaking of 15 minutes. After filtration; filtrate kept for drying then residual mass were treated with 1% H₂SO₄ (5 ml, 2 times). Extraction was then done in 10 ml. Chloroform (CHCl₃) by using a separating funnel. The organic layer of chloroform was rejected, and the aqueous layer was basified with 30% NH₄OH (pH=9-10). Now again, extraction was done in 10 ml. chloroform and the organic layer of chloroform (lower layer) were collected in a flask, and repetition of the step was done with fresh chloroform. Extracts were then dried in vacuo for further use.

Flavonoid extraction

Selected plant parts were separately washed with sterilized water; shade-dried, and finely powdered using a blender. Each sample was subjected to extraction, following the method of Subramanian and Nagarjan [16]. One hundred grams of each finely powdered sample was soxhlet extracted with 80% hot methanol (500 ml) on a water bath for 24 h and filtered. The filtrate was re-extracted successively with petroleum ether (fraction I), diethyl ether (fraction II), and ethyl acetate (fraction III) using a separating funnel. Petroleum ether fractions were discarded as being rich in fatty substances, whereas diethyl ether and ethyl acetate fractions were analyzed for free and bound flavonoids respectively. The ethyl acetate fraction of each of the samples was hydrolyzed by refluxing with 7% H₂SO₄ for 2 h (for removal of bound sugars) and the filtrate was extracted with ethyl acetate in separating funnel. Ethyl acetate extract obtained was washed with distilled water to neutrality. Diethyl ether (free flavonoids) and ethyl acetate fractions (bound flavonoids) were dried in vacuo and weighed. The extracts were stored at 4 °C and were re-suspended in their respective solvents to get 10 mg/ml concentration for antimicrobial assay.

Steroid extraction

Steroids were extracted from different parts of the selected plant by well-established method [17] after preliminary detection of steroids. Finely powdered sample (100g) of plant parts were extracted in petroleum ether for 2-4hr. After filtration, the residual mass was treated with 15% ethanolic HCl for 4hr. Extraction was then done in ethyl acetate followed by washing in dis. water to neutralize the extract. The neutral extract was then passed over sodium sulfate to remove moisture contents and was dried in vacuo. Chloroform was used for reconstitution of extract, filtered and dried for further use.

Extraction in polar and non-polar solvents

Powder of all the two plant parts (Leaf and flower) were taken in different round bottom flasks in different solvents. 20 g powder was taken in each flask and water, methanol and petroleum ether were used as a solvent. Dried material and solvents were taken in 1:10 ratio. Those were kept at soxhlet unit for 24 h. Then extracts were filtered. The filtrates were subjected to evaporation to obtain dried extract. The percentage yield of each dried plant extract was calculated.

Selected test microorganisms

Three pathogenic bacteria were screened, *Candida albicans* (MTCC no. 183), *Aspergillus flavus* (MTCC no. 277) and *Tricophyton mentagrophyte* (MTCC no. 7687). The pathogens were procured from IMTECH (Chandigarh, Punjab, India). Fungal strains were grown and maintained on Sabouraud Dextrose Agar medium.

Candida albicans is a major model of pathogenic yeast which is found in mouth, throat, intestine and genitourinary tract of human and considered as a common constituent of bowel flora together with many bacterial species e. g. *E. coli*, *S. aureus* and *P. mirabilis*. It lives in 80% of the human population with no harmful effects, although overgrowth results in candidiasis which is often observed in immune-compromised individuals such as patients of cancer, transplant, and AIDS. It is a causal agent of opportunistic oral and genital infections in humans [18]. Superficial and mycosis infections cause local inflammation and discomfort in human beings [19]. Candidiasis also known as 'thrush', which usually occurs in immune compromised people [20].

Aspergillus flavus is the second leading cause of invasive and non-invasive aspergillosis [21]. The presence of *Aspergillus* in the air is a major risk factor for both invasive and allergic aspergillosis [22]. *A. flavus* can cause storage problems in stored grains. It also causes diseases in economically important crops, such as maize and peanuts and produces potent mycotoxins. It can also be a human pathogen, associated with aspergillosis of the lungs and sometimes causing corneal, otomycotic and nasoorbital infections.

Tricophyton mentagrophyte is a cosmopolitan dermatophyte, belonging to a homogeneous group of fungi called the dermatophytes. The organism is found in soil, the floor of swimming pools, hairs of wild boar, cats and dogs, farm animals, foot wears and from human toe webs without clinical lesions. It requires keratin for growth and can cause a variety of cutaneous (hair, nail and skin) infections in humans and animals hence considered to be anthropophilic or zoophilic in nature [23, 24]. It causes dermatophytosis in dogs, cats, cattle and especially in rodents [25-27].

Antimicrobial assay

'Disc Diffusion Assay' was performed for screening [28]. SD agar base plates were seeded with the fungal inoculum (inoculum size 1×10⁷CFU/ml). Sterile filter paper discs of Whatman no.1 (6 mm in diameter) were impregnated with 100 µl of each of the extract of concentration 10 mg/ml to give a final concentration of 1 mg/disc. Discs were left to dry in vacuo so as to remove residual solvent, which might interfere with the determination. Discs with extract were then placed on the corresponding seeded agar plates. Each extract was tested in triplicate along with Ketoconazole (1 mg/disc) for *T. mentagrophyte* and Terbinafine for *C. albicans* and *A. flavus* as standard drugs. The plates were kept at 4 °C for diffusion of extract, thereafter were incubated at 27 °C (*C. albicans* and *A. flavus* for 48 h and *T. mentagrophyte* for 5-7 d). Activity index for each extract was calculated [table 1] by the standard formula viz.

$$\text{Activity index} = \frac{\text{IZ produced by the extract}}{\text{IZ produced by standard}} \quad (\text{IZ} = \text{inhibition zone})$$

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal (MFC) concentration

Minimum inhibitory concentration (MIC) was determined for each plant extract showing antimicrobial activity against the test pathogens. 'Broth microdilution' method was followed for determination of MIC values [29]. Plant extracts were resuspended in acetone (which has no activity against test microorganisms) to make 10 mg/ml final concentration. Two-fold serially diluted extracts were added to broth media of 96-wells of microtiter plates.

Thereafter 100 µl inoculums (1×10⁷ CFU/ml) was added to each well. Fungal suspensions were used as negative control, while broth containing standard drug was used as positive control. Microtiter plates were then incubated at 27 °C for 48 h. Each extract was assayed in duplicate and each time two sets of microplates were prepared, one was kept for incubation while another was kept at 4 °C for comparing the turbidity in the wells of a microplate. The MIC values were taken as the lowest concentration of the extracts in the well of the microtiter plate that showed no turbidity after incubation. The turbidity of the wells in the microtiter plate was interpreted as the visible growth of microorganisms. The minimum fungicidal concentration (MFC) was determined by subculturing 50 µl from each well showing no apparent growth [table 2]. Least concentration of extract showing no visible growth on subculturing was taken as MFC.

Table 1: Antifungal activity of extracts of *Allamanda cathartica* against some pathogenic fungi

Plant part	Extract	Microorganisms					
		<i>C. albicans</i>		<i>A. flavus</i>		<i>T. mentagrophyte</i>	
		IZ(mm)	AI	IZ(mm)	AI	IZ(mm)	AI
Leaf	A1	22	0.65±0.01	-	-	-	-
	S1	7	0.20±0.01	-	-	-	-
	E1	-	-	7	0.23±0.01	-	-
	E2	9	0.26±0.01	-	-	-	-
	P1	-	-	-	-	-	-
	M1	-	-	-	-	-	-
	W1	-	-	-	-	-	-
Flower	A2	8	0.27±0.01	-	-	-	-
	S2	9	0.30±0.01	-	-	-	-
	E1	-	-	7	0.23±0.01	-	-
	E2	-	-	7	0.23±0.01	-	-
	P2	12	0.40±0.01	-	-	-	-
	M2	9	0.30±0.01	-	-	-	-
	W2	13	0.43±0.02	-	-	-	-

(a) A1, A2= Alkaloid extract of respective plant parts, (b) S1, S2= Steroid extract of respective plant parts, (c) E1, E2= Free and Bound flavonoids of respective plant parts, (d) P1, P2 = Pet ether extract of respective plant parts, (e) M1, M2= Methanolic extract of respective plant parts, (f) W1, W2= Water extract of respective plant parts, (g) IZ=Inhibition zone in mm (value: including 6 mm diameter of disc), (h) AI= Activity index (IZ developed by extract/IZ developed by standard), (i) [-]= no activity, (j) ±=SEM

Table 2: MIC and MFC of active extracts of *Allamanda cathartica* against some pathogenic fungi

Plant parts and Extracts Leaf		Flower													
Microorganisms	MIC and MFC	A1	S1	E1	E2	P1	M1	W1	A2	S2	E1	E2	P2	M2	W2
<i>C. albicans</i>	MFC	0.156	1.25	-	0.625	-	-	-	1.25	0.625	-	-	0.312	0.625	0.312
	MIC	0.078	0.625	-	0.312	-	-	-	0.625	0.312	-	-	0.156	0.312	0.156
<i>A. flavus</i>	MFC	-	-	1.25	-	-	-	-	-	-	1.25	1.25	-	-	-
	MIC	-	-	0.625	-	-	-	-	-	-	0.625	0.625	-	-	-
<i>T. mentagrophyte</i>	MFC	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MIC	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(a) A1, A2= Alkaloid extract of respective plant parts, (b) S1, S2= Steroid extract of respective plant parts, (c) E1, E2= Free and Bound flavonoids of respective plant parts, (d) P1, P2 = Pet ether extract of respective plant parts, (e) M1, M2= Methanolic extract of respective plant parts, (f) W1, W2= Water extract of respective plant parts, (g) MIC= Minimum inhibitory concentration, (h) MFC=Minimum fungicidal concentration, (i) [-] = no activity.

Total activity (TA) determination

Total activity is the volume up to which test extract can be diluted without losing the ability to kill microorganisms.

It is calculated by dividing the amount of extract from 1g plant material by the MIC of the same extract or compound isolated and is expressed in ml/g [30] [table 3].

Table 3: Quantity and total activity of crude extracts of *Allamanda cathartica* Linn

Plant part	Extract	Quantity of extract mg/g dwt	Total Activity(ml/g)		
			<i>C. albicans</i>	<i>A. flavus</i>	<i>T. mentagrophyte</i>
			Leaf	A1	43.5
	S1	281.67	450.67	-	-
	E1	61.1	-	98.56	-
	E2	124.70	399.68	-	-
	P1	35	-	-	-
	M1	203.75	-	-	-
	W1	112.5	-	-	-
Flower	A2	674.12	1078.59	-	-
	S2	450	1442.30	-	-
	E1	175	-	280	-
	E2	348.34	-	557.34	-
	P2	17.86	114.48	-	-
	M2	172.23	552.01	-	-
	W2	15.5	99.36	-	-

(a) A1, A2= Alkaloid extract of respective plant parts, (b) S1, S2= Steroid extract of respective plant parts, (c) E1, E2= Free and Bound flavonoids of respective plant parts, (d) P1, P2 = Pet ether extract of respective plant parts, (e) M1, M2= Methanolic extract of respective plant parts, (f) W1, W2= Water extract of respective plant parts, (g) TA= total activity (extract per gm dried plant part/MIC of extract)

RESULTS

Antimicrobial efficacy of secondary metabolites (Alkaloid, steroid and flavonoid) and crude extracts in polar and non polar solvents

(methanol, pet ether, water) were assessed by using IZ, AI (table 1), MIC, MFC (table 2). Quantity of extract per gram of plant material was also calculated (table 3). In present investigation, 14 extracts were tested against three pathogenic organisms, including one yeast

C. albicans and two fungi *A. flavus* and *T. mentagrophyte*. Among all the tested extracts all the three crude extracts of leaf found to be inactive against all the tested pathogens. *T. mentagrophyte* found to be resistant pathogen as no extract showed activity against it. *C. albicans* found to be most susceptible organism as 8 out of 14 extracts showed activity against it. Best activity was observed in alkaloid extract of leaf (IZ= 22 mm, AI= 0.65±0.02, MFC= 0.156 mg/ml, MIC= 0.078 mg/ml, TA= 557.59 ml/g) against *C. albicans*. Pet ether extract of flower (IZ= 12 mm, AI= 0.40±0.02, MFC= 0.312 mg/ml, MIC= 0.156 mg/ml, TA= 114.48 ml/g) and water extract of flower (IZ= 13 mm, AI= 0.43±0.02, MFC= 0.312 mg/ml, MIC= 0.156 mg/ml, TA= 99.36 ml/g) also showed very good activities against *C. albicans*. Bound flavonoid of leaf (IZ= 9 mm, AI= 0.26±0.01, MFC= 0.625 mg/ml, MIC= 0.312 mg/ml, TA= 399.68 ml/g), steroid of flower (IZ= 9 mm, AI= 0.30±0.01, MFC= 0.625 mg/ml, MIC= 0.312 mg/ml, TA=1442.30 ml/g), alkaloid of flower (IZ= 8 mm, AI= 0.27±0.01, MFC= 1.25 mg/ml, MIC= 0.625 mg/ml, TA= 1078.9 ml/g) and methanolic extract of flower (IZ= 9 mm, AI= 0.30±0.01, MFC= 0.625 mg/ml, MIC= 0.312 mg/ml, TA= 552.01 ml/g) showed good activities against *C. albicans* while free flavonoid of leaf (IZ= 7 mm, AI= 0.23±0.01, MFC= 1.25 mg/ml, MIC= 0.625 mg/ml, TA= 98.56 ml/g), free flavonoid of flower (IZ= 7 mm, AI= 0.23±0.01, MFC= 1.25 mg/ml, MIC= 0.625 mg/ml, TA= 280 ml/g) and bound flavonoid of flower (IZ= 7 mm, AI= 0.23±0.01, MFC= 1.25 mg/ml, MIC= 0.625 mg/ml, TA= 557.34 ml/g) showed satisfactory activities against *A. flavus*. Steroid of leaf (IZ= 7 mm, AI= 0.20±0.01, MFC= 1.25 mg/ml, MIC= 0.625 mg/ml, TA= 450.67 ml/g) showed satisfactory activity against *C. albicans*.

Among all the tested extracts, none of the tested extracts showed activity against more than one pathogen. Plant extracts, which had shown activity in diffusion assay, were evaluated for their MIC and MFC values (table 2). The range of MFC and MIC of extracts recorded was 1.25-0.156 and 0.625-0.078, respectively. In present study, lowest MIC value 0.078 mg/ml was recorded against *C. albicans*, indicating significant antimicrobial efficacy of tested extracts. Quantity of extract obtained per gram from plant parts and TA was calculated and recorded (table 3). TA indicates the volume at which extract can be diluted without losing ability to kill microorganisms. High values of TA were observed against *C. albicans* (1078.59 ml/g) followed by *A. flavus* (557.34 ml/g).

DISCUSSION

Extracts from different parts of *A. cathartica* have previously been studied for their antifungal activity but, still, meager work has been carried out as far as the antifungal activity of steroids and crude extracts is concerned. Most of the research has been restricted on the determination of IZ of extracts without calculating AI, MIC, MFC and TA. Determination of MIC and MFC has now become an inevitable step in antimicrobial studies in order to establish their antimicrobial activity so as to explore them at industrial level for the production of drugs, which could replace the existing ones. Hence, most of the studies carried out so far could only reveal their antimicrobial activities, but are not helpful for establishing them as antifungal. The result of the present study indicated that the extracts of both the parts (leaf and flower) of *A. cathartica* have antifungal activity against two of the selected fungus. The results were in agreement with the findings of previous studies. Furthermore, it may help to discover new chemical classes of antifungal that could serve as selective agents for the maintenance of human health and provide biochemical tools for the study of infectious diseases.

CONCLUSION

C. albicans found to be most susceptible organism while *T. mentagrophyte* found to be resistant throughout the study. Best activity was observed in the alkaloid extract of the leaf against *C. albicans*. Pet ether and water extract of the flower were also showed a good level of antifungal activities while other extracts found to have marginal activities. Therefore, extracts of *A. cathartica* will be very beneficial in the studies relevant to the formation of nature based antifungal preparations.

ACKNOWLEDGEMENT

The author is thankful to 'Head' Department of Botany, the University of Rajasthan for providing all necessary facilities and

Prof. Padma kumar for her assistance in language and proofreading of the manuscript.

CONFLICT OF INTERESTS

Declare none

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How to cite this article

- Meenakshi Fartyal. *Allamanda cathartica* linn.: Extraction and pharmaceutical evaluation of various extracts of leaves and flowers. *Int J Curr Pharm Res* 2016;8(4):28-32.