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EVALUATION OF *NYCTANTHES ARBORTRISTIS* FOR ANTIFUNGAL ACTIVITY USING *ASPERGILLUS FLAVUS AND ASPERGILLUS NIGER* AS TARGET EXPERIMENTAL MODELS

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ABSTRACT

Nyctanthus arbortristis leaf extracts were subjected to antifungal activity against *Aspergillus flavus* and *Aspergillus niger*. Four leaf extracts were prepared these were hexane, chloroform, ethyl acetate and methanol extracts. Three methods were used to assess the antifungal activity these were radical growth method, agar diffusion method and biomass reduction. Reduction in aflatoxin content was also observed using UV-vis spectropotometer. Amongst all the extracts ethyl extract exhibited good activity in all the methods. Ethyl acetate extract was able to reduce the aflatoxin content by 42% when compared with control samples. At higher dose of 1mg/ml same extract inhibited the growth of fungus (A.flavus) by 60% and 40% in *Aspergillus niger* on day 2 in radical growth method.

KEYWORDS: *Nyctanthus arbortristis,* Antifungal activity, *Aspergillus flavus*, *Aspergillus niger*, medicinal plant.

INTRODUCTION

Nyctanthes arbortristis is commonly known as harsingar, it flowers at night and also referred as night flowering jasmine.[1] Plant is well known in Ayurvedic medicines for eliminating fever and other ailments[2] Major medicinal uses of this plant are anti- helmintic, anti-pyretic, laxative, anti- rheumatic and also as sedatives[3] Phytochemicals like tannin, carotene, glucose, flavonoid, oleanic acid, essential oils etc. have been reported for significant hair tonic, anti-viral, anti malarial, anti-bacterial, anti-inflammatory, anti-fungal and anti-oxidant activities.[4] As the medicinal plant has already shown anti- inflammatory activity hence was picked up for exploring antifungal activity against *Aspergillus flavus* and *Aspergillus niger*.

Aspergillus flavus is a soil pathogen which produces aflatoxins, it contaminates human and animal food and results in a number of diseases like liver cancer and aspergillosis and lung inflammations.[5] It also destroys grains, ligumes and tree nuts. Aspergillus niger is also known as black fungus and cause black mould disease[6] Consumption of secondary metabolites of these causes serious health conditions like growth retardation, liver inflammation, heart and kidney damage leading to death.[7]

Medicinal plants are being used from ages for the treatment of a number of diseases hence in this study popular medicinal plant *Nyctanthes arbortristis* was explored for antifungal potential and its effect on the secondary metabolite aflatoxin.

MATERIALS AND METHODS

Collection and Processing of Plant Materials

Leaves of *Nyctanthes arbortristis* were collected from the medicinal garden of Regional Plant Resource Centre (RPRC), Bhubaneswar. Leaves were weighed and washed with running tap water to remove dust and impurities followed by shade drying under room temperature for about 4-5 days and were grinded coarsely by using grinder and the moisture content of the leaves was calculated by using the following formula:

$$\text{Moisture content (\%)} = \frac{\text{Weight of fresh leaves - weight of dried leaves}}{\text{Weight of fresh leaves}} \times 100$$

Solvent extraction was done by using maceration process. 200 grams of powdered leaf sample was taken in a beaker and was subjected for serial extraction with different solvents like Hexane (0.1), Chloroform (4.1), Ethyl acetate (4.4), and Methanol (5.1) on the basis of their increasing polarity, stirred well and kept overnight. Next day filtration was done using whatman filter paper. The process was repeated thrice for each solvent. Extracts were concentrated under vacuum by using Bucchi rotary evaporator R-200 at 45-50˚C depending upon the boiling points of the solvents used.

Percentage of Yield

Concentrated extracts were transferred to screw cap vials and extract yield was calculated by using the formula -

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

All the extracts were stored in screw capped vials for further use.

Antifungal activity of solvent extracts

A. By radical growth method: The antifungal properties of the extracts were determined by using the modified radical growth method[8] Each leaf extracts was prepared in DMSO (20mg extract/1ml) and spread over solidified PDA plates in different doses of 0.25, 0.5 and 1mg/ml. Centre of plates was inoculated with single spore of fungus. Plates were kept for incubation at 32˚C. Each assay was conducted in triplicate. Radical growth was measured on alternative days starting from day 2 to day 14 until the entire plate gets covered with fungal mat. Fungal mats of all the samples were dried and weighed to estimate the biomass inhibition by the solvent extracts of the plants using the following formula.

$$\text{Percentage Inhibition} = \frac{\text{Wt of control - Wt of extract mat}}{\text{Wt of control}} \times 100$$

B. By agar well diffusion method: Stock solutions of all the leaf extracts were prepared in DMSO (20mg extract/1ml). Spore suspension (1×10^6/100 µl) was spread evenly over each PDA plates, Different doses 0.25, 0.5 and 1mg/ml was poured in the wells cut in plates. Control and experimental samples were kept for incubation at 32˚C for 72 hours. Zone of inhibition was measured for every sample. Biomass inhibition was calculated at the end when the plate gets covered with fungal mats. Each assay was performed in triplicates and experiment was repeated thrice for each solvent extract.

C. Isolation and estimation of aflatoxin content and estimation of biomass of the control and experimental samples: For isolation of aflatoxin standard protocols were used.[9] Sabouroud liquid broth medium of control (without extract) and experimental samples (with solvent extracts) were inoculated with fungal spores ($1X10^6$). On day 15th of inoculation, samples were deactivated by autoclaving. Samples were filtered for removing the fungal mats, biomass was weighed for every extract and percentage inhibition was calculated. After removing the fungal mats from the broth by filtration, culture filtrate was transferred to a separating funnel, extracted with equal volume of chloroform, shaken for about 30 minutes and allowed to stand for 30 minutes. The organic phase was separated and was left for drying at room temperature. The dried filtrates obtained, contains the crude aflatoxin content. Crude aflatoxin of control and experimental sample was estimated by taking absorbance at 400 nm using microplate reader, Percentage inhibition was calculated comparing with the control samples. Fungal mats of all the samples were dried and weighed to estimate the biomass inhibition.

RESULTS AND DISCUSSIONS

Moisture content of *Nyctanthes arbortristis* was 60.04 percent and yield of methanol extract was highest amongst the solvent extracts followed by chloroform, ethyl acetate and hexane (Table1).

Table 1: Yield of solvent extracts *Nyctanthes arbortristis.*

Solvent Extracts	% of Yield
Hexane	1.65%
Chloroform	8.26%
Ethyl acetate	5.108%
Methanol	19.36%

Antifungal activity of solvent extracts

The radical growth is expressed in terms of percentage of growth inhibition. Growth inhibition was observed highest on 2nd day i.e. 60% in ethyl acetate leaf extracts of *Nyctanthes arbortristis* at 1mg/ml doses, while other extracts showed mild growth inhibition as compared to the *A.flavus* (control) (Table2). Whereas in *A.niger* (control) treated plates highest inhibition was also observed in ethyl acetate with 41.4% at 1mg/ml doses on 2nd day observation (Table3).

Table 2: Evaluation of N. arbortristis extract on A. flavus using radical growth method.

Solvent extracts	Dose (mg/ml)	Growth inhibition(Mean ± SD)				
		Day-2	Day-4	Day-6	Day-8	Day-10
Hexane	0.25	10±0.41	0.2±0.76	NI	1.3±0.77	NI
	0.5	10±0.41	0.2±0.76	NI	1.3±0.77	NI
	1	10±0.41	0.2±0.76	NI	1.3±0.77	NI
Chloroform	0.25	20±0.76	7.5±0.98	1.5±1.02	1.3±0.89	NI
	0.5	25±0.78	7.5±0.98	1.5±1.02	2.0±1.44	NI
	1	25±0.76	5.0±0.49	2.3±2.08	2.0±1.42	NI
E.acetate	0.25	25±0.76	5.0±0.49	2.3±0.98	2.0±0.98	2.3±0.97
	0.5	35±0.88	10±1.4	5.4±0.76	3.3±0.87	4.7±1.24
	1	60±1.79	15±1.6	6.9±0.41	6.0±1.79	5.8±0.41
Methanol	0.25	5±0.48	2.5±0.49	NI	1.3±0.98	NI

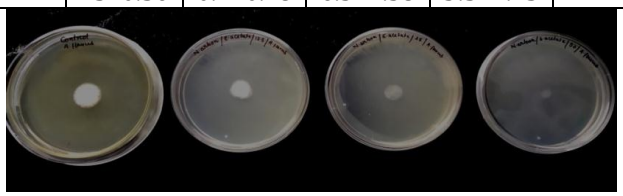
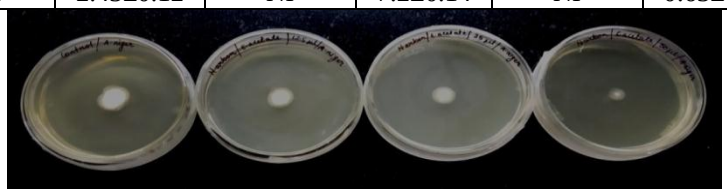
	0.5	10±0.41	5.0±0.48	4.6±2.09	3.3±0.85	NI
	1	15±0.56	0.2±0.78	6.9±1.98	9.3±1.23	NI
Ethyl acetate extract DAY 2	 **Control 0.25mg/ml 0.5 mg /ml 1mg/ml**					

Table 3: Effect of *N. arbortristis* solvent extracts on radical growth of *A. niger*.

Solvent extracts	Dose (mg/ml)	Growth inhibition(Mean ± SD)				
		Day-2	Day-4	Day-6	Day-8	Day-10
Hexane	0.25	2.4±0.03	7.5±0.15	2.4±0.99	1.91±0.18	NI
	0.5	2.4±0.03	7.5±0.15	2.4±0.76	NI	0.63±0.12
	1	2.4±0.03	11.11±0.13	2.4±0.76	NI	NI
Chloroform	0.25	14.6±1.66	15±1.22	15.7±0.82	10.71±0.09	5.66±0.09
	0.5	28.1±1.52	17.5±1.36	15.7±0.82	29.16±0.08	6.91±0.16
	1	31.7±1.68	20±1.08	13.6±0.97	29.16±0.08	6.91±0.16
E.acetate	0.25	28.1±1.52	20±1.04	15.2±1.08	18.7±1.09	5.66±0.09
	0.5	31.7±0.15	20±1.02	20±0.96	20±0.15	8.17±0.03
	1	41.4±0.04	22.5±0.28	20±0.21	22.5±0.35	11.94±0.05
Methanol	0.25	7.3±0.14	5±0.12	4±0.16	3.22±0.09	1.88±0.07
	0.5	12.19±0.15	2.5±0.15	7.2±0.14	0.64±0.15	0.63±0.12
	1	2.43±0.12	NI	7.2±0.14	NI	0.63±0.12
Ethyl acetate extract Day 2	 **Control 0.25mg/ml 0.5 mg /ml 1mg/ml**					

Biomass reduction followed by radical growth method

Biomass reduction is the final outcome of the effect of extracts on the fungus. As can be seen(Table4) in the case of methanol extract effect of the extract was negligible i.e, less than 6%, whereas in case of ethyl acetate extract which was most active on day 2 was successful in reducing the biomass upto 67% in A. flavus whereas 54% in A. niger. Thus the damage caused to the fungus was of irreversible type. This makes ethyl acetate a potent candidate for detailed antifungal activity.

Table 4: Biomass reduction in A. flavus and A. niger after radical growth.

Sample	Dose (mg/ml)	*Aspergillus flavus* *Biomass* %Mean ± SD	*Aspergillus niger* *Biomass* %Mean ± SD
Hexane	**0.25**	8.36 ± 2.24	0.59 NIL
	0.5	11.49 ± 2.39	8.05 ± 1.52
	1	14.79 ± 1.39	16.56 ± 0.02
Chloroform	**0.25**	21.15 ± 1.07	16.71 ± 1.52
	0.5	36.82 ± 1.10	23.43 ± 1.54
	1	46.84 ± 2.57	33.43 ± 1.45
E. acetate	**0.25**	35.94 ± 0.76	23.88 ± 0.41
	0.5	52.74 ± 0.41	41.49 ± 0.41
	1	67.41 ± 0.41	54.02 ± 0.02
Methanol	**0.25**	4.65 ± 2.57	NIL
	0.5	5.18 ± 2.41	NIL
	1	5.59 ± 2.41	5.59 ± 2.41

Agar well diffusion method

Zone of inhibition was observed in ethyl acetate extracts of *N.arbortristis* at 1mg/ml doses against *A.flavus* as well as in *A.niger* (Fig 3) on day 3. In both the cases zone was more than 10mm which is considered as active.[10] As the plant extracts are a combination of large number of

molecules hence they were tested at higher dose of 1mg/ml to 0.25mg per ml, However standard antifungals like fluconazol and Nystatin[11] are active at a lower dose of 5microgram/ml to 250 microgram /ml. Biomass reduction was also conducted in which chloroform extract and ethyl acetate extracts were able to reduce the biomass 15-54% in different doses(Table 5)

Control E.acetate Control E.acetate

Fig. 1: Zone of inhibition created by ethyl acetate extract of *N.arbortristis* on *A. flavus* and A.niger at highest dose of 1mg/ml.

Table 5: Effect on A.flavus and A. niger on Biomass by N. arbortristis extracts after Agar well diffusion method.

Solvent Extract	Dose (mg/ml)	*Aspergillus flavus* Mean ± SD	*Aspergillus niger* Mean ± SD
Hexane	0.25	NI	NI
	0.5	NI	NI
	1	NI	NI
Chloroform	0.25	15.58 ± 2.45	17.87 ± 0.45
	0.5	21.89 ± 2.14	20.33 ± 0.28
	1	28.60 ± 2.14	22.66 ± 0.25
E. acetate	0.25	29.77 ± 0.041	27.84 ± 0.57
	0.5	42.08 ± 0.032	33.54 ± 0.52
	1	54.93 ± 0.028	42.22 ± 0.12
Methanol	0.25	NI	NI
	0.5	1.10 ± 2.87	NI
	1	2.04 ± 2.36	NI

Effect of extracts on aflatoxin content and biomass in liquid broth medium

Absorbance of crude aflatoxin isolated from extracts was compared with the extracts as can be seen in Fig 2. Absorbance is indirectly proportional to the amount of aflatoxin. All the polar extracts were able to reduce the presence of aflatoxin. Even biomass reduction (Table 6) was obtained in the extracts (Chloroform, ethyl acetate and methanol) which were able to reduce the aflatoxin content. Thus overall it can be concluded that ethylacetate extract of the plants holds promise as per antifungal activity is concerned.

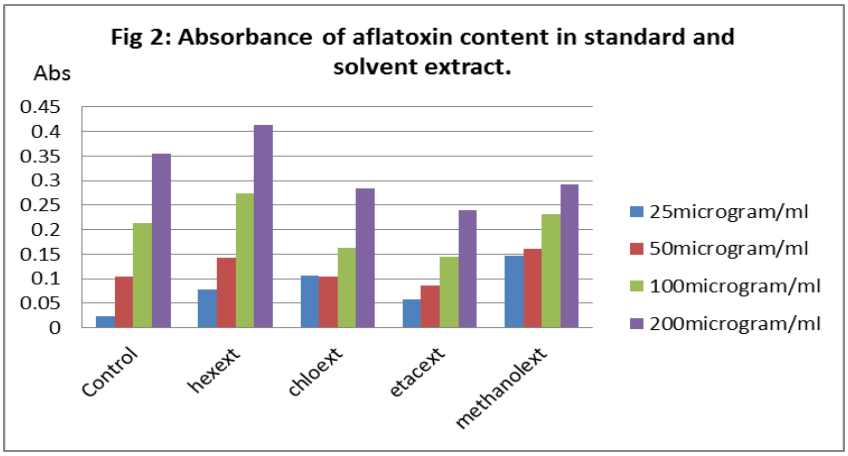


Table 6: Biomass reduction in liquid broth medium.

Solvent extract	Biomass reduction A.flavus % Inhibition	Biomass reduction A.niger % Inhibition
Hexane	NIL	NIL
Chloroform	44.35	35.08
Ethyl Acetate	54.58	42.52
Methanol	23.96	25.43

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REFERENCES

1. Mishra V, Shukla A, Pandeti S, Barthwal MK, Pandey HP, Palit G, Narender T. Arbortristoside-A and 7-O-trans-cinnamoyl-6β-hydroxyloganin isolated from Nyctanthes arbortristis possess anti-ulcerogenic and ulcer-healing properties. Phytomedicine, 2013; 20(12): 1055-63. doi: 10.1016/j.phymed.2013.04.010.
2. Agrawal J, Pal A. *Nyctanthes arbor-tristis* Linn--a critical ethnopharmacological review. J Ethnopharmacol, 2013; 146(3): 645-58. doi: 10.1016/j.jep.2013.01.024.
3. Godse CS, Tathed PS, Talwalkar SS, Vaidya RA, Amonkar AJ, Vaidya AB, Vaidya AD. Antiparasitic and disease-modifying activity of Nyctanthes arbor-tristis Linn. in malaria: An exploratory clinical study. J Ayurveda Integr Med, 2016; 7(4): 238-248. doi: 10.1016/j.jaim.2016.08.003.
4. Saxena RS, Gupta B, Saxena KK, Srivastava VK, Prasad DN. Analgesic, antipyretic and ulcerogenic activity of Nyctanthes arbor tristis leaf extract. J Ethnopharmacol, 1987; 19(2): 193-200. doi: 10.1016/0378-8741(87)90041-9.
5. Otsuka R, Kushima H, Fujita M, Ishii H. Aspergillus Niger-pulmonary Aspergillosis in an Immunocompetent Woman. Intern Med, 2021; 60(14): 2341-2342. doi: 10.2169/internalmedicine.6057-20.
6. Ghuffar S, Ahmed MZ, Irshad G, Zeshan MA, Qadir A, Anwaar HA, Mansha MZ, Asadullah HM, Abdullah A, Farooq U. First Report of Aspergillus niger causing Black rot of Grapes in Pakistan. Plant Dis., 2020. doi: 10.1094/PDIS-06-20-1390-PDN
7. Linsell CA, Peers FG. Aflatoxin and liver cell cancer. Trans R Soc Trop Med Hyg, 1977; 71(6): 471-3. doi: 10.1016/0035-9203(77)90136-5.
8. El Moussaoui A, Jawhari FZ, Almehdi AM, Elmsellem H, Fikri Benbrahim K, Bousta D, Bari A. Antibacterial, antifungal and antioxidant activity of total polyphenols of Withania frutescens.L. Bioorg Chem, 2019; 93: 103337. doi: 10.1016/j.bioorg.2019.103337.
9. Ling L, Ye ZM, Wang SY, Jin Y, Chen YH, Zheng TL, Zhang LS, Pei XF. [Isolation and Study on the Aflatoxin Genes of Aflatoxin-producing Fungi in Paprika Samples in Chengdu]. Sichuan Da Xue Xue Bao Yi Xue Ban., 2015; 46(5): 684-7.
10. Bhalodia NR, Shukla VJ. Antibacterial and antifungal activities from leaf extracts of Cassia fistula l.: An ethnomedicinal plant. *J Adv Pharm Technol Res*., 2011; 2(2): 104-109. doi:10.4103/2231-4040.82956
11. Teodoro GR, Brighenti FL, Delbem AC, Delbem ÁC, Khouri S, Gontijo AV, Pascoal AC, Salvador MJ, Koga-Ito CY. Antifungal activity of extracts and isolated compounds from Buchenavia tomentosa on Candida albicans and non-albicans. Future Microbiol, 2015; 10(6): 917-27. doi: 10.2217/fmb.15.20.