World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 6.129

ASSESSMENT OF ANTIFUNGAL ACTIVITY OF *COMBRETUM ROXBURGHII* SOLVENT LEAF EXTRACTS AGAINST *ASPERGILLUS FLAVUS.*

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Article Received on 14/06/2023 **Article Revised on 04/07/2023** **Article Accepted on 25/07/2023**

ABSTRACT

Combretum roxburghii belongs to a medicinally important family Combretaceae which is used in traditional medicine against inflammation, infection, diabetes, malaria, bleeding, diarrhoea, cancer and treatment of syphilis abdominal pain. In the present study solvent leaf extracts were explored for antifungal activity against *Aspergillus flavus.* Four leaf extracts (hexane, chloroform, ethyl acetate and methanol) were prepared. Four extracts were tested at two doses i.e., 1000µg and 2000µg/ml. Hexane and chloroform extract showed mild activity in radical growth method. Methanol extract showed best activity in agar well diffusion method. Chloroform extract showed 45% and 89% reduction in biomass of the fungus in agar diffusion method and liquid broth medium respectively. Ethyl acetate extract reduced the aflatoxin content by 66% when compared with control samples. Thus all the four extracts had antifungal activity in one way or other.

KEYWORDS: *Combretum roxburghii*, Combretaceae, *Aspergillus flavus*, antifungal activity.

INTRODUCTION

Aspergillus flavus is a saprophytic and pathogenic fungus with a cosmopolitan distribution.[1] It is best known for its colonization of cereal grains, legumes, and tree nuts.[2]Postharvest rot typically develops during harvest, storage, and/or transit. Its specific name *flavus* derives from the Latin meaning yellow, a reference to the frequently observed color of the spores.[3] *A. flavus* infections can occur while hosts are still in the field (preharvest), but often show no symptoms (dormancy) until postharvest storage or during transport.[4] In addition to causing preharvest and postharvest infections, many strains produce significant quantities of toxic compounds known as mycotoxins, which, when consumed, are toxic to mammals.[5] *A. flavus* is also an opportunistic human and animal pathogen, causing aspergillosis in immune compromised individuals and is associated with hepatic cancer.[6] Medicinal plants have been used since ancient times in virtually all cultures as a source of medicines.[7] *Combretum roxburghii* belongs to a large family Combretaceae and family have been used in the treatment of syphilis, abdominal pain, diarrhoea and other ailments.[8] In the present study plant was explored for its antifungal and anti aflatoxigenic activity, results have been discussed.

MATERIALS AND METHODS

Collection and processing of plant materials:
Fresh leaves of *Combretum roxburghii* were collected from medicinal germplasm garden of Regional Plant Resources Centre (RPRC), Bhubaneswar. Then the leaves were weighed in a weighing balance and were washed with running tap water to remove dust and impurities. Samples were complete dried in shade at room temperature for about 1week and then dried leaves were grinded in to fine powder using blender (Lexus make) and transferred into airtight containers with proper labelling for further use.

Moisture content determination:
Weight of leaves was taken twice, firstly after collection and secondly after drying in shade and moisture content of the plant was determined by comparing the weights of fresh and dried leaves.

Moisture content of the leaves was calculated using the following formula.

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

Where Fw = fresh weight of leave sample
Dw = Dry weight of powdered samples

Solvent extraction and processing
Solvent extraction was done using maceration method. 13gm of powdered leaf sample of plant *Combretum*

roxburghii was taken in a beaker. Sample was subjected to serial extraction with 250ml of different solvents like Hexane, Chloroform, Ethyl acetate and Methanol on the basis of their increasing polarity, stirred well and kept overnight. Next day filteration was done using whatsman filter paper. The process was repeated three times for each solvent. Then the collected extracts were concentrated in vacuum under pressure using Buchi (R-200) Rotavapour under vaccum at 45-50˚C depending upon the sample. Concentrated extracts were transferred to screw cap vials and extract yield was calculated by using the formula

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

Antifungal activity of solvent extracts

The antifungal properties of the extracts was determined by these three methods.

A. Radical growth method
B. Agar well diffusion method
C. Biomass reduction

Radical growth method

Antifungal properties of the extracts was determined by standard radical growth method[9] Potato dextrose agar plates were poured, Extract was tested at two doses 1000µg and 2000µg by placing 80 and 160 micro litre of extract in 100ml of media and poured in 4 plates. The plates were inoculated by fungus with the help of sterilized loop in centre of the plate. Plates were kept for incubation at 36˚C. Radical growth was measured on alternative days starting from day 2 to 10. Radical growth of experimental was compared with that of control and positive control. After 10 day biomass reduction was done. Fungal mats of all samples were dried at 100˚C for over night and then weighed to estimate the biomass reduction of the solvent extracts using the following formula.

$$\text{Biomass reduction}(\%) = \frac{\text{Weight of control} - \text{weight of extract mat}}{\text{Weight of control}} \times 100$$

Agar well diffusion method

For Potato dextrose agar (PDA) plates, 15.6gm of Potato dextrose agar (PDA) was dissolved in distilled water and then autoclaved. Media poured in plates. Spore suspension was prepared by taking loopful of fungus stain from a master plate and adjusted with autoclaved distilled water to 1×10^6. 10µl of fungal suspension was spread over solidified PDA plates with the help of L spreader and left for drying. The dried PDA plates were now used for agar well diffusion method. Wells were cut. Extracts (hexane, chloroform, ethyl acetate, methanol) were tested at two doses 1000 and 2000µg/ml by loading 20 and 40µl of extracts in the hole created in the agar plates. Plates were kept for incubation at 32˚C and observed for the zone of inhibition. After one week fungal mats are dried in oven at 100˚C for overnight then percentage of inhibition was calculated by comparing the experimental sample with those of controls.

Isolation and estimation of aflatoxin content and estimation of biomass of the control and experimental samples.

For isolation of aflatoxin standard protocols were used.[10] 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 spectrophotometer. 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 and yield of solvent extracts

Moisture content of the plant (*Combretum roxburghii*) leaves was found to be 76.95%. Yield of methanol extract was highest amongst the solvent extracts followed by hexane, chloroform and ethyl acetate.

Table 1: Yield of different solvent extracts.

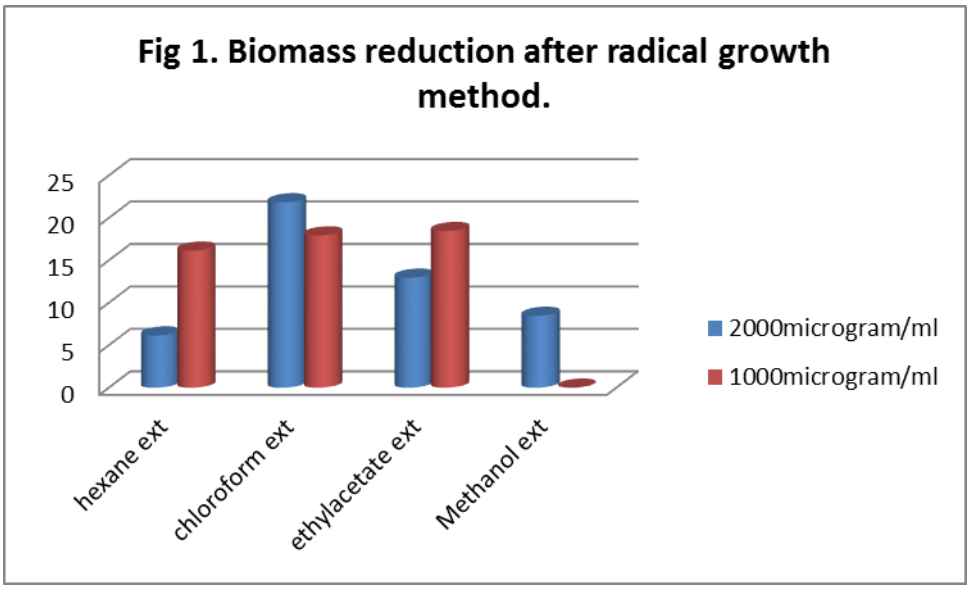
SOLVENT EXTRACTS	PERCENTAGE
HEXANE	2.57%
CHLOROFORM	1.85%
ETHYL ACETATE	9.57%
METHANOL	14.94%

Antifungal activity using radical growth method

As can be observed from Table 2, antifungal activity was more on earlier days where as it reduced with passing time. Thus, it can be assumed that extracts had direct mode of action which diminished with the reduction of available extract in later days. Chloroform extract showed best biomass reduction(Fig1) amongst the solvents, where as radical growth was better in hexane extract.

Table 2: Radical growth activity of solvent extracts.

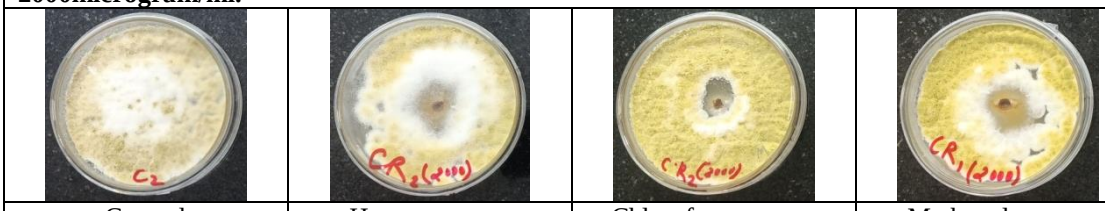
Solvent extract	Dose (in µg)	Day-2		Day-4		Day-6		Day-8		Day-10	
		Growth (in cm) Mean	% of inhibition	Growth (in cm) Mean	% of inhibition	Growth (in cm) Mean	% of inhibition	Growth (in cm) Mean	% of inhibition	Growth (in cm) Mean	% of inhibition
Control		2.37		4.38		6.68		8.14		8.4	
Hexane	2000	1.9	39.86	3.2	26.94	5.87	12.12	7.8	4.17	8.32	
	1000	1.42	19.83	3.7	15.52	5.56	16.76	7.4	8.72	8.55	Nil
Chloroform	2000	1.85	21.94	3.6	17.80	5.92	11.37	6.64	18.42	8.23	2.02
	1000	2.12	10.54	4.07	7.07	6.33	5.23	8.2	Nil	8.67	Nil
Ethyl acetate	2000	2.18	8.01	4.55	Nil	6.44	3.59	7.48	8.10	8.23	2.02
	1000	2.11	10.97	4.2	4.10	6.37	4.64	7.8	4.17	8.32	0.95
Methanol	2000	2	15.61	4.2	4.10	5.83	12.72	8.1	0.49	8.3	1.19
	1000	2.12	10.54	4.02	8.21	6.1	8.68	7.56	7.12	8.25	1.78



Antifungal activity using agar well diffusion method

Except for hexane extract all the extracts showed significant zone of inhibition when compared with control (Fig 2). However, biomass reduction was not promising.

Fig 2: Zone of inhibition by solvent extracts of *Combretum roxburghii* at a dose of 2000microgram/ml.



Control	Hexane extract	Chloroform extract	Methanol extract

Isolation of aflatoxin from liquid medium and anti aflatoxin activity of solvent extracts.

Crude Aflatoxin was isolated by the liquid liquid separation as per the standard protocol.[10] Percentage inhibition was calculated using weight of crude aflatoxin obtained and was compared with the control samples which were devoid of any extract. Similarly percentage inhibition was also calculated spectrophotometerically. Data is presented in Table 3, which shows remarkable inhibition in ethyl acetate extract. Thus, overall the same extract needs further exploration for its inhibitory potential against aflatoxin.

Table 3: Percentage of inhibition in aflatoxin by solvent extracts of *Combretum roxburghii* extracts.

Sample	Dose	Weight of Crude aflatoxin (in gm)	% of inhibition	Absorbance	% of inhibition
Control		0.025		3.072	
Hexane	1000µg	0.015	40%	2.728	11.19%
	2000µg	0.013	48%	2.602	15.29%
Chloroform	1000µg	0.018	28%	0.838	7.61%
	2000µg	0.021	16%	2.481	19.23%
Ethyl acetate	1000µg	0.005	80%	2.436	20.70%
	2000µg	0.011	56%	2.728	11.19%
Methanol	1000µg	0.033	Nil	1.656	46.09%
	2000µg	0.034	Nil	2.227	27.91%

ACKNOWLEDGEMENTS

Authors acknowledge the Forest, Environment and Climate change Department, Government of Odisha for providing funding for the smooth conduct of entire work and also acknowledge Chief Executive, Regional Plant Resource Center, Bhubaneswar, Odisha for providing lab facilities. Authors also acknowledge the Department for funding Junior research fellowship (PR &SM) for the project.

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