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ANTIFUNGAL SCREENING OF *ANNONA SQUAMOSA* AND *SPONDIAS PINNATA* AQUEOUS LEAF EXTRACT ON 3 FUNGAL GENERA ISOLATED AND IDENTIFIED FROM *ORYZA SATIVA* ROOT AND SEED

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ABSTRACT

Through the study, we isolated and identified five pathogenic fungal genera from Oryza sativa roots and seeds which cause serious ill-effects to life. These were identified as Rhizopus, Aspergillus, Cylindrocarpon, Penicillium and Curvularia. Preliminary phytochemical screening of aqueous leaf extracts of Annona squamosa and Spondias pinnata were conducted and their actions as antifungal agent on 3 fungal genera were determined by disc diffusion method. Among the screened phytochemicals, Spondias pinnata shows maximum types of secondary metabolites in aqueous extract. Cylindrocarpon, Penicillium and Curvularia were used as test organism. The extracts having antimicrobial activity has varied diameter of inhibition zone among tested organisms. Spondias pinnata leaf has antifungal activity against Cylindrocarpon, Penicillium and Curvularia with zone of inhibition of 12mm, 11mm and 5mm respectively. Annona squamosa has antifungal activity against Penicillium and Curvularia with zone of inhibition of 8mm and 5mm and no markable activity against Cylindrocarpon. The highest level of antifungal activity was recorded in Spondias pinnata which was active against all tested organisms that may be due to the different types of phytochemicals.

KEY WORDS

Annona squamosa, antifungal screening, disc diffusion, phytochemicals, plant extract, Spondias pinnata.

INTRODUCTION

Almost all plants, from minute members of algae to giant forest trees, are attacked and destroyed by fungi. Many fungi are saprobes, which absorb nutrients from living hosts. Still others are mutualists living in intimate associations with other organisms that benefit both partners [1]. The negative role in causing innumerable plant and human fungal diseases and their positive role in maintaining the fertility of the soil is certainly beyond anybody's expectations [2]. Fungal diseases may result even into a catastrophe if allowed to run their course unchecked. Rhizosphere which is a thin layer of soil immediately surrounding plant roots has a large number of microorganisms such as bacteria, fungi, protozoa and algae coexist [3]. Application of chemical fertilizers and pesticides cause chemical residues in agricultural products and the ecological environment imbalance. Currently the search for natural products with novel uses, particularly related to pest management is very active. Plant extracts with antimicrobial properties contain a spectrum of secondary metabolites such as alkaloids, saponins, glycosides, tannins flavonoids and terpenoids. They were synthesized in all plant parts, such as bark, leaves, stem, root, flower, fruit, seeds etc. The concentration of these bioactive compounds in each plant species depends on the environmental conditions and pathosystem [4,5]. The negative environmental impacts of pesticide are increasing every day. For this reason, alternative methods of reducing pesticide are being developed. One of the effective

methods is to use plant extracts which provides natural anti-fungal substances. Antifungal substances which are obtained from plants have no side effects against environment thus, giving a significant advantage in the field of medicine.

MATERIALS AND METHODS

Plant materials used for the study includes *Oryza sativa* seed and root for fungal isolation and leaves of *Annona squamosa* Linn and *Spondias pinnata* (Linn.f.) Kurz for phytochemical and antifungal screening. Seeds and roots of *Oryza sativa* were collected from Moncompu Rice Research Station, Alappuzha District of Kerala.

1.Isolation of fungi:

Seeds and roots of *Oryza sativa* (Paddy) were inoculated to PDA medium for fungal isolation.

Systematic position of *Oryza sativa* (Paddy).

Class: Monocotyledons

Series: Glumaceae

Family: Gramineae

Oryza sativa is extensively cultivated in countries like India, Philippines, and Thailand, which is a tall, annual or perennial herb that grows to a height of 50-150 cm. Leaves are narrow and flat. Rice grain is rich in starch and also contains fat, minerals, and vitamins.

Composition of Potato Dextrose Agar (PDA) Medium [6]

Ingredients- Grams / litre

Potatoes infusion from - 200.00

Dextrose -20.00

Agar-20.00

For the preparation of media suspend 44.0 grams in 100 ml distilled water. Heat to boiling and dissolve the medium completely. Sterilize by autoclaving at 15 lbs. pressure (121^0 C) for 15 minutes. Medium was supplemented with 0.5 milligram of tetracycline per ml to inhibit the bacterial growth and then the medium was transferred to sterile petriplate. After the solidification of medium, petriplates were used for inoculation.

Inoculation of *Oryza sativa* seeds and roots to PDA medium

The collected seeds and root pieces were surface sterilized and extreme growing tips of roots and seed coat removed seeds were inoculated in each sterilized petriplates (4 seeds and root pieces /plate) at equal distance. Total six plates were inoculated. Then the plates were sealed with parafilm and incubated at room temperature for fungal growth.

Isolation, identification and pure culture development: Most of the fungal growth was initiated on 4^{th} and 5^{th} day of inoculation. The identification was done by observations and findings of the pure culture on the basis of their colony morphology and spore characteristics [7, 8]. The growing colonies of fungus were observed in different colours. Pure culture was obtained by sub-culturing three times [9]. Identification of different fungi was done with the help of slides prepared by direct mount method from the culture. For preparation, the grown fungi were mounted on a slide, stained with Lacto phenol blue to detect fungal structures [10]. The slides were examined under microscope and photographed.

2. Phytochemical screening of plant materials: Phytochemical screening was done on the aqueous leaf extracts of selected plants for the presence of different bioactive components such as, saponins, flavonoids, tannins, steroids, anthraquinones, qunines, phenols, proteins, coumarins and alkaloids [11].

Collection of plant material and extract Preparation:

Fresh leaves of *Annona squamosa* and *Spondias pinnata* were collected from Poomkavu region of Pathanamthitta, Kerala and were identified with the help of standard herbarium available in department of Botany Catholicate College, Pathanamthitta. Collected leaves were surface sterilized using distilled water. 15 gram of leaves were ground in a mortar and pestle with 10 ml of distilled water. The extract was then filtered using Whatman filter paper. This filtrate was used for further phytochemical analysis.

Preliminary phytochemical screening of aqueous extracts of selected plants:

(a)Test for tannins: Few drops of 1% ferric chloride solution were added to 2 ml of the extract. Occurrence of a blue –black, green or blue green precipitate indicated the presence of tannins

(b) Test for saponins (Frothing test): 5 ml of the aqueous extract was shaken vigorously with 20 ml of distilled water. Formation of persistent froth indicated the presence of saponins.

(c)Test for phenols: Few drops of alcoholic ferric chloride solution were added to the aqueous extract. Formation of violet, bluish green or bluish black colour indicated the presence of phenols.

(d)Test for steroids (Salkowski test): Few drops of concentrated sulphuric acid were added to a little amount of extract and were shaken for few minutes; the

development of red or brown colour indicated the presence of steroids.

(e)Test for Terpenoids: To 5 ml extract 2 ml of chloroform and 3 ml of sulphuric acid were mixed. Formation of reddish brown colour indicated the presence of terpenoids.

(f)Test for coumarins (Colour test): A little of extract was mixed with methanol or ethanol on adding alcoholic potassium hydroxide or sodium hydroxide gives a yellow colour which disappears on adding concentrated hydrochloric acid.

(g)Test for protein detection (Xanthoprotein test): A small amount of the extract was added with 0.5 ml of concentrated nitric acid. Appearance of white or yellow precipitate revealed the presence of protein.

(h)Test for quinines: To the extract, sodium hydroxide was added. Formation of blue, green or red colour indicated the presence of quinines.

(i)Test for Anthraquinones (Bortragers test): The extract was shaken with aqueous ammonia. Formation of pink, violet or red colour indicated the presence of anthraquinones.

(j)Test for flavonoids: 1ml of aqueous sodium hydroxide was added to 1ml of test solution. Formation of yellow colour indicated the presence of flavonoids.

3. Antifungal screening through Disc diffusion method [12].

Fresh aqueous leaf extracts were tested invitro for anti-fungal activity by the standard disc diffusion method against the isolated fungal pathogens such as *Curvularia, Penicillium* and *Aspergillus* which were isolated from seed and root of *Oryza sativa*. Disc containing extract (3 mg) were placed on the petriplate which was uniformly streaked with pure culture of isolated fungus. These plates were then sealed with parafilm and kept at room temperature to allow maximum diffusion of the extract.

The extracts having antimicrobial activity inhibited the growth of the microorganisms and a clear distinct zone of inhibition was visualized surrounding the discs. The intensity of antifungal activity of the extracts were determined by measuring the diameter of zone of inhibition expressed in mm [13]. Diameter of inhibition was measured and graded as '+', if there was zone below 6 mm, '++' if the zone ranged from 6-10 mm and '+++' it was above 10 mm.

RESULTS

Through the study, we isolated and identified five pathogenic fungal genera such as *Rhizopus, Aspergillus, Cylindrocarpon, Penicillium* and *Curvularia* (Table:1). Preliminary phytochemical screening of leaf extracts and its action as antifungal agent on 3 fungal genera were determined by measuring the diameter of zone of inhibition of pathogenic fungi (Table: 2 & 3).

Table 1: List of fungus and its colony morphology isolated from *Oryza sativa* seed and root.

Sl. No.	Plant part inoculated	Name of the fungus	Subdivision	Colony morphology
1	*Oryza* seed	*Rhizopus*	Zygomycotina	White
		Aspergillus	Ascomycotina	Yellow brown
		Cylindrocarpon	Ascomycotina	Orange
		Penicillium	Ascomycotina	Dark green
		Curvularia	Deuteromycotina	Velvety black
2	*Oryza* root	*Aspergillus*	Ascomycotina	Yellow brown
		Penicillium	Ascomycotina	Dark green

Table 2: Preliminary phytochemical screening of leaf extracts of *Annona squamosa* & *Spondias pinnata*

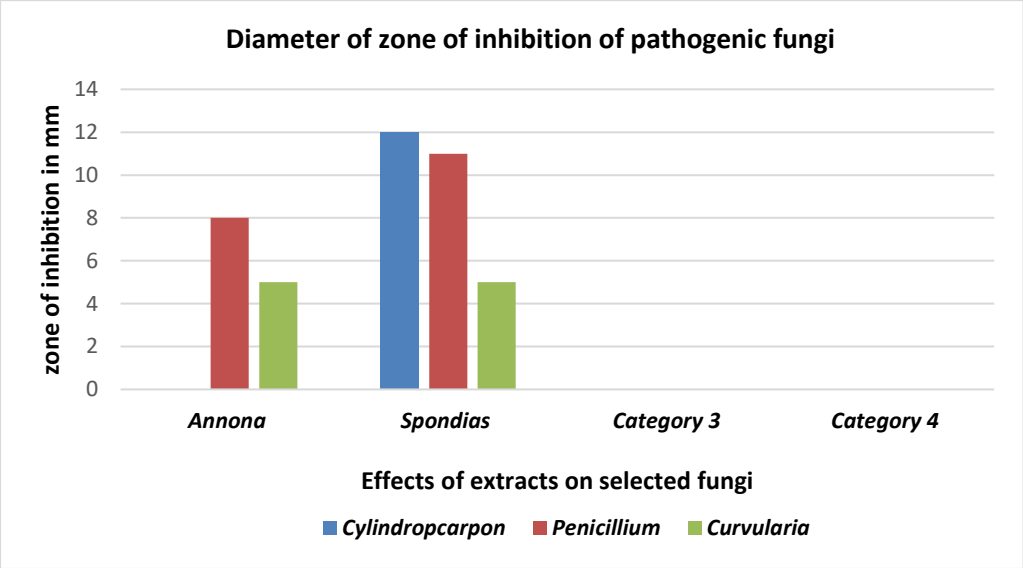
Sl no.	Phytochemicals	*Annona squamosa*	*Spondias pinnata*
1	Flavonoids	_	+
2	Saponins	+	+
3	Terpenoids	_	_
4	Phenols	_	_
5	Tannins	+	_
6	Coumarins	_	+
7	Steroids	+	+
8	Proteins	_	+

9	Anthraquinones	_	_
10	Quinine	_	_

Table3: Antifungal activity of leaf extracts showing diameter of zone of inhibition of pathogenic fungi.

Leaf Extract	Fungus	Effect	Zone of inhibition ((diameter in mm)
	Cylindrocarpon	_	_
Annona squamosa	*Penicillium*	++	8
	Curvularia	+	5
	Cylindrocarpon	+++	12
Spondias pinnata	*Penicillium*	+++	11
	Curvularia	+	5

Diameter of inhibition was measured and graded as '+' if there was zone below 6 mm, '++' if the zone ranged from 6-10 mm and '+++' if it was above 10 mm.



Graph 1: Diameter of zone of inhibition of pathogenic fungi

DISCUSSION

The present investigation of *"Antifungal screening of Annona squamosa and Spondias pinnata aqueous leaf extract on 3 fungal genera isolated and identified from Oryza sativa root and seed"* analyses the diversity of endogenous fungi from *Oryza sativa roots and seeds.* We isolated 5 genera of fungus from root and seed of *Oryza sativa* plant. The fungi isolated from *Oryza* seed were *Rhizopus, Aspergillus, Cylindrocarpon, Penicillium* and *Curvularia*. And the fungi isolated from *Oryza* root includes *Aspergillus* and *Penicillium* (Table:1). Among the screened phytochemicals, *Spondias pinnata* shows maximum types of secondary metabolites in aqueous extract (Table:2).

Cylindrocarpon, Penicillium and *Curvularia* were used as test organism for checking antifungal properties of leaf extracts through disc diffusion method. The extracts having antimicrobial activity has varied diameter of inhibition zone among tested organisms. *Annona squamosa* has antifungal activity against *Penicillium* and *Curvularia* with zone of inhibition of 8mm and 5mm and no markable activity against *Cylindrocarpon. Spondias pinnata* leaf has antifungal activity against *Cylindrocarpon, Penicillium* and *Curvularia* with zone of inhibition of 12mm, 11mm and 5mm respectively. Highest level of antifungal activity was recorded in *Spondias pinnata* which was active against all tested organisms that may be due to the different types of phytochemicals (Table no:3 & Graph :1).

CONCLUSION

The use of plants as medicine by humans has coevolved with the history of man. Even the modern allopathic medicine has its roots in traditional medicine; the present study has witnessed the phytochemical, antifungal potential of leaf extracts of *Annona*

squamosa and Spondias pinnata and shows commentable result against the tested organisms. From the above study, it was also evident that *Oryza sativa* seeds and roots were frequently associated with fungal spp.

The diversity of phytochemicals among plant material was well established. The different phytoconstituents present in the plants are responsible for their antimicrobial activity. Plant metabolites are strong candidate to combat the emerging issues of drug resistance. Numerous studies are progressing in all parts in this direction and the present results are also *at par* with the available reports by various researchers. The diverse spectrum of metabolites like aldehyde, sugar, protein, cardiac glycoside, flavanoid, alkaloid, phenol, tannin and coumarin was reported from these plants in literature. These active principles may have acted alone or in combination to inhibit the growth of fungal and bacterial strains.

The capacity of chemists to modify a molecular structure is almost unlimited, but the capacity to create new structure with therapeutic properties has been found to be limited. Plants offer thousands of new molecules. Also, the chemist can intelligently manipulate these active principles in to nanoparticles of wide biological spectrum. However, more detailed investigations including clinical trials are essential.

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