



EFFECTS OF JUSTICIA WYNAADENSIS EXTRACTS ON SPORE GERMINATION OF PATHOGENIC AND STORAGE FUNGI

SULLIMADA UTHAIAH PONNAMMA* AND KIRGANDUR MANJUNATH

*Department of Microbiology and Biotechnology,
Bangalore University, Bangalore, India.*

ABSTRACT

The potential risk to health with the use of chemical fungicides in/on foods has necessitated the search for natural alternatives, such as those from plants. The present investigation evaluates the *in vitro* antifungal effects of the aqueous and methanolic extracts of *Justicia wynaadensis* (Nees) T. Anders at concentrations of 1mg/ml, 5mg/ml and 10mg/ml against certain pathogenic- and storage-fungi, by assaying for the germination of spores by the slide germination test. It was observed that the most susceptible organism to the aqueous extract at the minimum concentration tested (1mg/ml) was *R. stolonifer* (97% inhibition), followed by *P. chrysogenum* (42.9% inhibition) and *F. fusariodes* (26.7% inhibition), whilst the conidia/spores of all the fungal species tested were susceptible to 1mg/ml of the methanolic extract compared to the control. Thus, there is significant potential for the extract of *J. wynaadensis* to be used for crop protection and as a food preservative, especially as a 'non-toxic preservative'.

KEYWORDS: *Justicia wynaadensis*, antifungal, spore germination, slide germination, Kodagu, plant extract.



SULLIMADA UTHAIAH PONNAMMA *

Department of Microbiology and Biotechnology, Bangalore University, Bangalore, India.

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INTRODUCTION

Food losses represent a waste of resources used in production such as land, water, energy and inputs; producing food that will not be consumed leads to unnecessary CO₂ emissions in addition to loss of economic value of the food produced.¹ Food losses take place at production, post-harvest and processing stages in the food supply chain.² Microbiological damage by fungi and bacteria is one of the causes for losses in the post-harvest food chain. Also, consumption of mycotoxins produced by fungi in food stuffs and feed could cause mycotoxicoses, and especially of concern when they present themselves as carcinogens. In 1993, the International Agency for Research on Cancer (IARC) assessed and classified Aflatoxin B₁ and naturally occurring mixtures of aflatoxins as human carcinogens.³ In order to minimize mold growth as well as to reduce the risk of mycotoxin contamination in food supplies, improvements in their storage and handling is required. The use of chemicals to inhibit fungal growth in/on foods has not been accepted very well by the consumers, thus driving the attention of researchers toward natural alternatives, such as those from natural products. Research into plant derived fungicides for their possible applications to control plant pathogenic fungi is being intensified as these have enormous potential to inspire and influence modern agrochemical research.⁴ Phenols and phenolic glycosides, cyanogenic glycosides, saponins, sulphur compounds are some of the constitutive plant compounds reported to have antifungal activity.⁵ *Justicia wynaadensis* (Nees) T. Anders, a scandent herb, belongs to Acanthaceae and is common in forests of Irpu, Kodagu, south-west India.⁶ Aqueous extract of its stem and leaves are used in the preparation of a dessert by the natives of Kodagu district, Karnataka, India exclusively during monsoons (wherein the herb is locally called 'Madduthoppu') as it is said to possess maximum medicinal properties during the season. The use of this plant by the Kuruchiar tribes of Tirunelli forest, Wayanad district, Kerala as an external application over rheumatic swellings⁷ and cellular cholesterol lowering properties⁸ of the extract have been reported. The plant is said to possess catalase and peroxidase activity; also polyphenols and flavonoids have been identified and estimated.⁹ Methanol extract of the plant when subjected to GC-MS analysis identified twenty four compounds.¹⁰ TLC-Bioautography guided screening for compounds inhibitory to *Klebsiella pneumoniae* from the methanolic extract of *J. wynaadensis*, followed by GC-MS analysis of the active fractions identified phytol, saturated (myristic acid, palmitic acid, stearic acid) and unsaturated fatty acids (linoleic acid).¹¹ The objective of the present study was to evaluate the in vitro antifungal effects of the extracts of the aerial parts of *Justicia wynaadensis* against certain pathogenic fungi and storage fungi by assaying for the germination of spores by the slide germination test.

MATERIALS AND METHODS

Collection of the plant material: The aerial parts of

Justicia wynaadensis plant was collected from Kodagu district of Karnataka on the 18th day of the 'Kakkada' month, as it is locally called, which generally falls during July-August; it was identified and authenticated at National Ayurveda and Dietetics Research Institute Bangalore, as *Justicia wynaadensis* (Nees) T. Anders belonging to the family Acanthaceae. The plant is a scandent herb with leaves that are elliptic-lanceolate, acuminate, glabrous and produce inflorescence that are slender, axillary and terminal, on long spikes bearing pinkish flowers; common in forests of Irpu, Kodagu, South-West India.⁶ *Preparation of the extract:* The dried plant material was crushed by hand and extracted successively with petroleum ether, chloroform, acetone, methanol and water in a Soxhlet extractor by continuous hot percolation. After filtration through muslin cloth, the extracts containing the organic solvents were evaporated to dryness at room temperature, while the aqueous extract was oven dried at 50°C. The percent extractives for methanol and aqueous extract were significantly higher compared to the other extracts, hence were considered for the study. Cultures used: *Alternaria* spp., *Aspergillus flavus*, *Aspergillus niger*, *Cladosporium cladosporioides*, *Curvularia* spp., *Fusarium fusarioides*, *Penicillium chrysogenum*, *Rhizopus stolonifer*, *Trichoderma viridae*. All fungal cultures barring *Alternaria* and *Curvularia* were identified at Agharkar Research Institute, India. All test fungi were isolated from soil and air samples. The cultures were periodically subcultured onto Martin's Rose Bengal Agar medium. *Preparation of test solutions:* A stock solution of a concentration of 100mg/ml each of methanol extract and aqueous extract of *Justicia wynaadensis* was prepared in DMSO separately. From the stock, working concentrations of 10mg/ml, 5mg/ml and 1mg/ml was prepared in potato dextrose broth. *Preparation of Inoculum and test procedure:* For the spore germination assay, the fungi were subcultured onto Potato Dextrose Agar (PDA). Following growth for a period of ten days, the various fungal spores were aseptically transferred into sterile Eppendorf tubes containing 50µl of working test solutions, using 1.3mm inoculation loop. The spore concentration was carefully adjusted to give 20-25 spores per microscopic field, under 45X objective lens. Suitable controls containing potato dextrose broth and solvent (DMSO) were maintained along with the experimental sets. Two replications were prepared for each test solution and for each test fungus. The contents were vortexed, mounted onto a hemocytometer and incubated at 28°C for 24 hours in a moist chamber. The hemocytometer was observed with a 45X objective under a binocular light microscope and checked for germination of fungal spores. The hemocytometer facilitates the counting of spores. Germination was recorded for 100 spores per replication under several microscopic fields. The spores were considered germinated when the germ tube length was 1.5 times the spore diameter¹⁰. The percentage inhibition of spore germination compared with that of the corresponding control was calculated using the formula given below:

$$\% \text{ Inhibition} = \frac{(\text{No. of spores germinated in control}) - (\text{No. of spores germinated in treatment})}{\text{No. of spores germinated in control}} \times 100$$

RESULTS AND DISCUSSION

Percentage Inhibition of fungal spores using the aqueous extract of *Justicia wynaadensis*.

Table 1 shows the results of the spore germination test using the aqueous extract. Germination of the conidia/spores of *Rhizopus stolonifer*, *Fusarium fusariodes*, *Aspergillus flavus*, *Cladosporium cladosporioides* and *Aspergillus niger* were completely inhibited at 10mg/ml of the aqueous extract while those of *Rhizopus stolonifer* and *Fusarium fusariodes* were inhibited at 5mg/ml. Compared to the control, germination of some of the fungi was promoted by the aqueous extracts such as those of *Trichoderma viridae* at all three concentrations tested, *Penicillium chrysogenum* at 5mg/ml and 10mg/ml, and *Alternaria* spp. at 1mg/ml. The most susceptible organism to the aqueous extract at the minimum concentration tested (1mg/ml) was *R. stolonifer* (97% inhibition), followed by *P. chrysogenum* (42.9% inhibition) and *F. fusariodes* (26.7% inhibition). The spores of *R. stolonifer* produced small germ tubes at 1mg/ml, became large and spherical at 5mg/ml and elliptical at 10mg/ml. Very few conidia of *F. fusariodes* were observed at 5mg/ml, while at 10mg/ml, no conidia could be seen, indicating lysis. The germ tube of *C. cladosporioides* was highly distorted and in a zigzag manner when treated with 5mg/ml of the extract, while at 10mg/ml the conidia became large and spherical resembling protoplasts. *Curvularia* spp. was more tolerant to the extract at all the concentrations tested, when compared to the other fungi.

Percentage Inhibition of fungal spores using the methanol extract of *Justicia wynaadensis*.

Table 2 shows the results of the spore germination test using the methanolic extract. The fungi tested responded differently to the varied concentrations (1mg/ml, 5mg/ml and 10mg/ml) of the methanolic extracts. From the results, it is interesting to note that the conidia/spores of all the fungal species showed some degree of susceptibility at the minimum concentration of 1mg/ml of the methanolic extract compared to the control. *R. stolonifer* was inhibited at all the three concentrations tested. The germination of conidia of *A. flavus* was completely inhibited at 10mg/ml. It is observed that the germination of *T. viridae* was promoted at 5mg/ml and 10mg/ml of the extract. The most susceptible organism to the methanolic extract at the minimum concentration tested was *R. stolonifer*, followed by *A. flavus* and *Curvularia* spp. respectively. Methanolic extract is better for the inhibition of the conidia of *P. chrysogenum* compared to the aqueous extract, while inhibition of conidial germination of *F. fusariodes* is better with the

aqueous extract. Spores of *R. stolonifer* were the most susceptible to the methanolic extract; a similar effect was observed with the aqueous extract. It was interesting to note that germination of conidia of *Aspergillus flavus*, a fungi capable of Aflatoxin B₁ production, was completely inhibited with 10mg/ml of both aqueous and methanolic extract of *J. wynaadensis*.

The search for ecofriendly natural fungicides from plant extracts as an alternative to chemical fungicides can be observed from numerous reports. Studies carried out on the inhibitory activity of the extracts of the Indian spice plant, *Cinnamomum zeylanicum*, against the pathogenic demataceous moulds, *Alternaria solani* and *Curvularia lunata* showed that the acetone and chloroform extract of the bark completely inhibited the germination of *C. lunata* and *A. solani* spores at 50µg/ml; also, the inhibitory effect was maximum with acetone and chloroform extract, and minimum with the aqueous preparation.¹¹ Extracts of 15 plants of Bangladesh were tested against the fruit rot pathogens wherein 100%, 87% and 82% inhibition of the germination of the spores of *Rhizopus artocarp*, *Fusarium oxysporum* f. sp. capsici and *Alternaria tenuis* respectively was observed with the aqueous leaf extracts of *Azadirachta indica*.¹² Similarly, 100% inhibition of the spores of *A. niger* was observed at 40 µl/ml concentration, while 93% and 91% was observed against *A. fumigatus* and *A. flavus* respectively, in a study on the effect of *Origanum vulgare* essential oil on spore germination of three species of *Aspergillus*.¹³ In a preliminary investigation using medicinal plant extracts, varying degrees of antifungal and Aflatoxin B₁ inhibitory activities were reported against *Aspergillus flavus*.¹⁴ In another study¹⁵, germination of the spores of *Colletotrichum gloeosporioides* was promoted with the extracts of *Erythrina americana*, *Ricinus communis*, *Azadirachta indica* and *Schinus molle* compared to the control. Such effects were observed in *Trichoderma*, *Penicillium chrysogenum* and *Alternaria* at varied concentrations in our study. This variation in susceptibility to antifungal compounds may result from differential abilities in metabolism of the compounds resulting in detoxification by the enzymes of the pathogen.¹⁶ It could also be that one or more phytochemicals identified^{10,11} in the extracts of *Justicia wynaadensis* contain stimulatory substances favoring the germination of the spores of the aforesaid fungi, while at the same time exhibiting inhibitory effects on *Alternaria* spp., *Aspergillus flavus*, *Aspergillus niger*, *Cladosporium cladosporioides*, *Curvularia* spp., *Fusarium fusariodes*, *Penicillium chrysogenum* and *Rhizopus stolonifer* at the concentrations tested.

Table 1
Percentage Inhibition of germination of fungal spores
using the aqueous extract of *J. wynaadensis*

Fungal Type	Percent Germination *Control	Percentage Inhibition		
		Conc. of extract		
		1mg/ml	5mg/ml	10mg/ml
<i>Alternaria</i> spp.	7	**	71.4	28.6
<i>Aspergillus flavus</i>	90	17.8	94.4	100
<i>Aspergillus niger</i>	98	13.3	93.9	100
<i>Cladosporium cladosporioides</i>	93	20.4	46.2	100
<i>Curvularia</i> spp.	52	3.8	9.6	44
<i>Fusarium fusariodes</i>	15	26.7	100	100
<i>Penicillium chrysogenum</i>	7	42.9	**	**
<i>Rhizopus stolonifer</i>	35	97	100	100
<i>Trichoderma viridae</i>	6	**	**	**

*number of spores germinated out of 100 counted

** increase in no. of germinated spores compared to control

Table 2
Percentage Inhibition of fungal spore germination
using the methanol extract of *J. wynaadensis*

Fungal Type	Percent germination *Control	Percentage Inhibition		
		Conc. of extract		
		1mg/ml	5mg/ml	10mg/ml
<i>Alternaria</i> spp.	84	16.7	42.9	76.2
<i>Aspergillus flavus</i>	23	52.2	91.3	100
<i>Aspergillus niger</i>	98	12.2	98	98
<i>Cladosporium cladosporioides</i>	89	34.8	70.8	92.1
<i>Curvularia</i> spp.	46	39.1	73.9	89.1
<i>Fusarium fusariodes</i>	22	9	18	22.7
<i>Penicillium chrysogenum</i>	34	35.3	6.3	6.3
<i>Rhizopus stolonifer</i>	20	100	100	100
<i>Trichoderma viridae</i>	6	16.7	**	**

*number of spores germinated out of 100 counted

**increase in no. of germinated spores compared to control

CONCLUSIONS

In conclusion, the aqueous and methanolic extracts of *Justicia wynaadensis* are inhibitory to the germination of most of the spores/conidia of the tested fungal forms. Of particular interest are the inhibitory effects against Aflatoxin B₁ producer *Aspergillus flavus* and against *Rhizopus stolonifer*. Thus, there is significant potential for the extract to be used in the management of post-harvest fungal infestation of storage crops, crop protection against fungal pathogens, and as a food preservative, especially a 'non-toxic preservative', since the extracts have been incorporated for centuries in the preparation of desserts by the local population. Both the aqueous and the methanolic extracts did not affect the

germination of *Trichoderma viridae* spores suggesting that the fungi is able to overcome the inhibitory properties of the plant extracts possibly by detoxification or by tolerance or by evasion altogether. Thus, there is scope to study the resistance mechanisms of the fungi that may pave the way for the development of novel strategies towards the control of phytopathogens. Also the extracts of this plant may be used to promote germination of *Trichoderma* spores when used as a bioinoculant and/or as a biocontrol agent, thus serving a dual purpose.

CONFLICT OF INTEREST

Conflict of interest declared none.

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Reviewers of this article

Dr S.Thippeswamy Ph.D

Faculty,
Dept of Microbiology,
University college of Science,Tumkur
University,Tumkur,Karnataka



Asst.Prof.Dr. Sujata Bhattachary

Assistant Professor, School of Biological
and Environmental Sciences, Shoolini
University, Solan (HP)-173212, India



Prof.Dr.K.Suriaprabha

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