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Praveen Onial
Chemistry-Division Forest Research Institute, Dehradun

Amit Pandey
Pathalogy-Division Forest Research Institute, Dehradun

MSM Rawat
Chemistry-Department, Hemwati Nandan Bahuguna Garhwal University, Srinagar

Rameshwar Dayal
Chemistry-Division Forest Research Institute, Dehradun

Correspondence:
Praveen Onial
Chemistry-Division Forest Research Institute, Dehradun

In-vitro antifungal assessment of fruits pericarp of *Terminalia chebula* against fungus of forestry origin

Praveen Onial, Amit Pandey, MSM Rawat, Rameshwar Dayal

Abstract
The *in vitro* fungi toxicity of *Terminalia chebula* fruits pericarp was tested against three pathogenic moulds in the laboratory. An initial screening of affectivity of different solvent extracts was done by in-vitro antifungal activity. The antifungal activity was determined by food poison technique for three test organisms viz *Fusarium solani* (seedling), *Aspergillus niger* (seeds), *Aspergillus flavus* (seedling).The MIC evaluated at three different concentrations. Cent per cent mortality was recorded in 3% in methanol extract against *Aspergillus flavus* and *Fusarium solani*. Methanol sample gave the best result with 100% average percent mortality. The results of the study demonstrate a new chemical utilization approach of the fruits pericarp of *T. chebula* towards the development of biofungicides in the management of above fungi of common occurrence in Indian forestry.

Keywords: *Terminalia chebula*, antifungal activity, fungus, *Fusarium solani* (seedling), *Aspergillus niger* (seeds), *Aspergillus flavus* (seedling), mortality, Minimum Inhibition Concentration (MIC).

1. Introduction
Availability of quality planting material, disease free management of plantations and preservation of wood under storage and use are amongst the key elements required for enhanced and improved forest productivity. Management of pathogenic and wood decay fungi is one of the serious concerns in Indian forestry. It is estimated that nearly 50-60% seeds are lost due to fungal deterioration while still on forest floor and during storage. About 70% mortality of seedlings in nursery is caused by fungal diseases [1]. Enormous amount of synthetic pesticides for biomass production are used world over. However, inappropriate and indiscriminate use of the synthetics has put human and animal health at risk and causing adverse effects in the environment. The resistance of the pathogens to synthetic fungicides and only few formulations available for the particular fungus makes it necessary to discover new classes of antifungal and compounds that inhibit their resistant mechanisms. This has led to the search for therapeutic alternatives, particularly among medicinal plants and compounds isolated from them that are used for their empirically antifungal properties [2]. In search of eco-friendly and coherent alternative to chemical fungicides, fungicides of biological origin (bio- fungicides) was found as one of the best option for the management. Many plant products have been successfully exploited as fungicides. Neem (*Azadirachta indica*) and Vach (*Acorus calamus*) have shown great promise in controlling fungus. And based on this background, the fungicidal activity of fruit pericarp of *Terminalia chebula*, (Hindi-Harra), Trade-Myrobalan, a tree under the family Combretaceae and 15-24 m. in height and 1.5-2.4 m. in girth, with a cylindrical bole of 4-9 m., found throughout the greater parts of India, the fruits has been utilized for the study against the *Fusarium solani* (seedling), *Aspergillus niger* (seeds), *Aspergillus flavus* (seedling). The mature fruits were collected during January-April by shaking the trees, and are dried in thin layers, preferably in shade, and graded for marketing. The raw Myrobalans are graded under different trade names, selection being based upon their solidness, colour and freedom from insect attack. The dried flesh surrounding the seed is rich in tanning (av., 30-32%) whose content considerably varies with the different grades of myrobalans from different areas.

Terminalia chebula is a multiproduct crop par excellence providing a number of ecosystem services. *T. chebula* has been a subject of extensive investigations, and distribution, chemistry, biological activities of the compounds isolated from different parts have been published [3, 4, 5, 6, 7].

The study was aimed to assess the *in vitro* fungi toxic effects of the extracts and fractions

obtained from the fruits pericarp of *T. chebula* against three pathogenic fungi such as *Fusarium solani* (seedling), *Aspergillus Niger* (seeds), *Aspergillus flavus* (seedling), causing diseases in seeds, seedling and trees respectively. The fruits of *T. chebula* are important by-products of plantations, remain underutilized and may provide potential fungi toxic properties. The feasibility of utilization of the abundantly available fruits of *T. chebula* towards the development of biofungicides in the management of above fungi of Indian forestry was examined.

2. Materials and Methods

A voucher specimen (No. 156864-65 7/3/2008) was deposited in the Herbarium of Systematic Botany Branch, Botany Division of the Forest Research Institute, New Forest, Dehradun. The plant was confirmed and authenticated by them. The fruits collected from the Forest Research Institute, Dehradun campus were shade dried and the pericarp was separated, powdered (80 mesh) followed by extraction with solvents of increasing polarity (Pet. ether, Chloroform, Methanol and aq. Methanol) and finally dried under reduced pressure using rotary evaporator. The pathogenic fungi *Aspergillus niger* (seeds), *Fusarium solani* (seedling), *Aspergillus flavus*, were obtained from the culture library of Forest Pathology Division, F.R.I. Dehradun. Cultures of each of the fungi were maintained on potato dextrose agar (PDA) medium and were stored at 4 ^{0}C.
The fungal assay was carried by using standard poison food technique [8] against fungi *Fusarium solani, Aspergillus niger, Aspergillus flavus*. Antifungal activities against *Aspergillus niger* (seeds), *Fusarium solani* (seedling), *Aspergillus flavus*, have been demonstrated for the first time against the target fungus of forestry importance.
The assay was carried out by using following:
A. Culture media preparation
B. Assay methods
C. Fungal strains
D. Concentration of essential oil

2.1 Culture Medium (Potato Dextrose Agar Medium)

Composition of potato dextrose agar (for 1000 ml of water) consisted of potato (200g), glucose (20g) and agar (15g). In 200g of potato dextrose and agar powder (Hi media) water put in 1000 ml of water, and boiled until dissolved completely. Then the solution was filtered with cheese cloth, and all liquid was squeezing out and was poured into conical flask and plugged with cotton. Then the solution of culture media prepared in flask, and plugged with cotton was sterilized in an autoclave at 121 °C and 151 lbs pressure for 15 minutes.

2.2 Assay Methods

Poison food technique was employed for screening of different extracts and pure compounds for their antifungal activity against the target fungi. A culture of test fungi was grown on potato dextrose agar (PDA) medium. Extracts and the Compounds were dissolved in the same solvents to make desired concentrations (x% = 1%, 2% and 3%) for screening. PDA was supplemented with the concentrated extract to make x %. This medium was poured in the culture plates under sterile conditions. Then a small disc of 6mm of mycelia was cut with sterile borer (0.1cm) and transferred aseptically upside down at the centre of the petri plate. Appropriate controls were also made by adding solvent in the same concentrations as added in the treated. Solvents were added to check the inhibitory effect of solvents on the growth of the fungi. The cultured plates were incubated at 25 ± 1°C and the radial growth of the fungal colonies was measured after seven days of incubation. Three replicates were maintained. The antifungal activity was evaluated by measuring the relative growth of fungus in treatment vis-à-vis control. The percent growth inhibition over control was measured by using formula of Vincent and minimum inhibitory concentration of the extract/pure compounds was determined for the concentration conferring 100% fungal growth inhibition.

I= C-T/C X 100

Where, **I** is growth inhibition percent
C is colony diameter (mm) in control
T is colony diameter in test.

2.3 Fungal Strains

Three fungi were used for assay namely *Fusarium solanii* (FRI No. 1137), *Aspergillus niger*, and *Aspergillus flavus*.

2.3.1 ***Fusarium solani*** is a filamentous fungus in the genus *Fusarium*, and the anamorph of Haematonectria haematococca. Commonly isolated from soil and plant debris. The fungus has a worldwide distribution, but its frequency as a medically important pathogen is not fully known. Aside from keratitis, it is an infrequent cause of fungal infections but remains the most common disease-causing fungus in its genus.

2.3.2 ***Aspergillus niger*** is a fungus and one of the most common species of the genus *Aspergillus*. It causes a disease called black mold on certain fruits and vegetables such as grapes, onions, and peanuts, and is a common contaminant of food. It is ubiquitous in soil and is commonly reported from indoor environments, where its black colonies can be confused with those of Stachybotrys (species of which have also been called "black mould") [9], (Samson R.A. *et al.*, 2001). Some strains of *A. niger* have been reported to produce potent mycotoxins called ochratoxins[10], other sources disagree, claiming this report is based upon misidentification of the fungal species. Recent evidence suggests some true *A. niger* strains do produce ochratoxin A [9, 10, 11]. It also produces the isoflavone orobol.

2.3.3 ***Aspergillus flavus*** is a saprotrophic and pathogenic [12] fungus with a cosmopolitan distribution[13]. It is best known for its colonisation of cereal grains and legumes. Post-harvest rot typically develops during harvest, storage, and/or transit. A. flavus infections can occur while hosts are still in the field (pre-harvest), but often show no symptoms (dormancy) until post-harvest storage and/or transport. In addition to causing pre-harvest and post-harvest infections, many strains produce significant quantities of toxic compounds known as mycotoxins, which when consumed are toxic to mammals[14].
A. flavus is also an opportunistic human and animal pathogen causing aspergillosis in immunocompromised individuals[15].

2.4 Concentration of Extracts and Compound

The experiment was set up by using different concentration of extracts and fractions. Concentrations used were 1%, 2% and 3%. All the experiment was done in UV room under sterile conditions.

2.5 Preparation of Plant Extracts

Fruits were collected from the trees planted in the campus of the F.R.I., Dehradun. The fruits were shade dried for one week

and the fruits pericarp was separated. The fruits pericarp powder (80 mesh), (100g) was then extracted with petroleum-ether, choloroform, and methanol in a soxhlet apparatus. All the extracts were filtered through Whatman filter paper no.1 and then concentrated over rotatory evaporator at reduced pressure. A yield of 0.50% from petroleum ether, 1.83% from chloroform, 2.15 from acetone, and 26.5% from methanol solvent system were obtained. The residue and the fractionated samples were weighed and tested for the antifungal activities against *Aspergillus Niger* (seeds), *Fusarium solani* (seedling), *Aspergillus flavus*.

2.6 Screening of Susceptible Fungi

One gram of each of the dried evaporated solvent extract of all the solvents was dissolved in 10ml of the respective solvent. 500µl of each solvent extract was amended with 15µl of PDA medium before solidification. The medium having only solvent served as control. Test fungi were inoculated and after incubation at 25 ± 2 ^{0}C for two days the percent inhibition of mycelia growth was determined. The colony diameter was measured in millimeter. For each treatment three replicates were maintained, (Figure I, photo plate 1& 2).

2.7 Minimum Inhibitory Concentration (M.I.C.)

Minimum inhibitory concentration of effective solvent extract was determined by standard poison food technique [3] (Barazani V.O. *et al.*, 2003). Three different concentrations of effective extract 100mg/ml, 250mg/ml and 500mg/ml and 10mg/ml of standard were taken for the assay. The plates were filled with 50ul each of controls and test concentrations using a micro titre pipette. The plates were allowed to diffuse at room temperature for two hour. The plates were then incubated at 25 ^{0}C for two days and the zone of inhibition was measured.

$$\textbf{M.I.C. (\%)} = \frac{\textbf{CONTROL} - \textbf{TREATMENT}}{\textbf{CONTROL}} \quad \textbf{X 100}$$

(50%) Inhibition = O.K.
More then (80%) Inhibition = Excellent

3. Results & Discussion

Cent per cent mortality was recorded at 3.0 % concentrations of methanol extract and fractionated sample (methanol extract after chloroform refluxion) for *Fusarium solani* and at 2% concentration for *Aspergillus flavus.* The extracts could not show results against the *Aspergillus niger* (Fig.1, photo-plate I). Hence, the methanol and fractionated sample can be best utilized as biofungicides against *Fusarium solani* and *Aspergillus flavus.*

4. Conclusion

The present investigation reveals that *Terminalia chebula* fruits pericarp possessing fungicidal properties. Since growth of *Fusarium solani* and *Aspergillus flavus* was suppressed by more than 50% at 2% concentration. Our results clearly demonstrate that the extract & fractionated material of the fruit pericarp of *T. chebula* are effective in their antifungal action with 75% mortality in case of *Fusarium solani* and 100% mortality in case of *Aspergillus flavus* at 2% MIC. Cent percent mortality was observed against *Fusarium solani* and *Aspergillus flavus* at 3% MIC. This study has highlighted *Terminalia chebula* fruit pericarp as a potential antifungal agent against *Fusarium solani* and *Aspergillus flavus* under in vitro conditions. It is concluded that antifungal activity of methanolic fruit pericarp extract of *T.chebula* would be helpful in interacting various kinds of plant diseases caused by the fungus. Methanol extract and fractionated sample (methanol extract after chloroform refluxion) have been demonstrated for the first time to poses fungicidal activity against the target fungus of forestry importance. Thus, a new chemical utilization approach of the fruit pericarp of *T.chebula* was shown in perspective of the value added utilization of agro forest biomass residues in search for safer and effective new alternative to petrochemical-derived and synthetic products.

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