Evaluation of *Lawsonia inermis* Leaf Extracts for Their *in vitro* Fungitoxicity Against Certain Soilborne Pathogens

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Abstract

Screening of Lawsonia inermis *leaf extracts for their fungitoxicity was carried out* in vitro *against some selected soil-borne phytopathogens,* Rhizoctonia solani, Pythium aphanidermatum *and* Macrophomina phaseolina. *Of the three solvent extracts studied, cold-water extract at 10% concentration showed a maximum of 70% inhibition of the mycelial growth of* R. solani. *Methanol extract at 2.5% concentration completely inhibited* P. aphanidermatum *while the same at 10% proved effective against* M. phaseolina *by 60% when compared to cold water and petroleum ether extracts. Petroleum ether extract was ineffective against* R. solani *and* M. phaseolina. *The amount of total phenols was 4.5 mg/g of fresh leaf tissue. Reverse phase high performance liquid chromatographic analysis of the cold-water extracts revealed the presence of four different absorption peaks of which only two compounds could be identified i.e. tannic acid and catechol.*

Keywords: *Lawsonia inermis, Rhizoctonia solani, Pythium aphanidermatum, Macrophomina phaseolina,* fungitoxicity

Introduction

Soil-borne pathogens cause serious diseases of crops at various stages leading to a greater extent of losses in their production. Though synthetic fungicides are effective, they are relatively expensive and leave harmful residues on plants. Being non-biodegradable and highly toxic, they pollute environmental ecosystems. Higher plants have reservoirs of biodegradable secondary metabolites (Fawcett and Spencer, 1970) that are reported to inhibit various phytopathogenic fungi (Pandey and Pant, 1997). The use of plant products in disease control seems to be a logical approach besides the use of chemicals and biological agents. Hence, a study was undertaken with a view to assess the antifungal activity of *Lawsonia inermis* (Lythraceae) leaf extracts in cold water, methanol and petroleum ether 40-60°C against *Pythium aphanidermatum* (Edson) Fitpatrick, *Macrophomina phaseolina* (Maublance) Ashby and *Rhizoctonia solani* Kuhn that are some of the soil-borne phytopathogens using food poisoning technique(Grover and Moore, 1962). Preliminary screening and identification of various phenolic compounds in the leaf extracts was also carried out.

Materials and methods

Preparation of leaf extracts and test for their fungitoxicity

Cold water leaf extract (1:1 w/v) was prepared by homogenizing 10 g of fresh leaves with 10 ml of distilled water followed by filtering through muslin cloth. Filtrate was then centrifuged at 5000 rpm for 10 min and the supernatant collected was made upto a final volume of 10 ml. Methanol and petroleum ether leaf extracts (1:1 w/v) were prepared using soxhlet extraction method, where 10 g of air dried powdered leaf material was extracted at 50°C for 8 h in 300 ml of the respective solvents. Later the extracts were evaporated to 10 ml, filtered through sterilized microbial filters (0.4 µm) and stored in refrigerator as stock solution. The filtrates were then separately mixed aseptically with molten PDA (potato dextrose agar) medium to have final concentration of 2.5, 5 and 10% (e.g. medium with 2.5% plant extract was prepared by adding 0.5 ml of leaf extract to 19.5 ml of medium) and poured immediately into the Petri plates. PDA with the respective solvents served as control. Fungal cultures were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. Fungal mats cut to a size of 4 mm were inoculated at the center of all the Petri plates. Streptomycin 1 g/l was added to PDA stock medium to check any bacterial growth. Each experiment was replicated thrice and incubated at 26°± 2°C until the mycelial growth in the controls reached the edge of the petri plates. Fungal growth in each plate was recorded by measuring its diameter. The mean value of mycelial growth inhibition was taken into account for calibration of per cent inhibition at various concentrations.

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The per cent inhibition of the mycelial growth was calculated using the formula given below:

$$\text{Per cent inhibition} = 1 - \frac{\text{Diameter of treated colony}}{\text{Diameter of control colony}} \times 100$$

Thin-layer chromatographic analysis (TLC)

The TLC plates (20 x 20 cm) were coated with 1 mm thickness of silica gel G (Experiment- Merck). After air-drying the slurry on TLC plates in open for 2 h, the silica gel was activated by heating the plates in an oven at 110°C for 2 h. Using micro pipette, 25 µ l of the leaf extracts were applied on the plates and were run separately for about 90 min in different solvent systems like hexane, chloroform: hexane (9:1), chloroform: acetic acid (9:1), and n- butanol: acetic acid: water (4: 1: 5) varying in polarity. The plates were then sprayed separately with the following chromogenic reagents: Vanallin–sulphuric acid for the presence of phenols, higher alcohols and steroids, and Folin–Ciocalteau reagent for the presence of phenols (Ashworth, 1969). The TLC plates were also observed under visible and ultra violet (UV) light. The retention factors (Rf) of the spots developed on the TLC plates were recorded.

Reverse phase – high pressure liquid chromatography (RP-HPLC)

Phenolic compounds in aqueous leaf extract were separated and identified using Shimadzu RP-HPLC (OC-R4A chromatopac) with C18 column. The pressure maximum was set to 400 psi; with a flow rate of 1 ml/min. UV wavelength used for detection was 280 nm. Two pumps, A consisting of 100% HPLC grade methanol and B consisting of 15% methanol in 3% formic acid that was degassed for one hour were used to run the leaf extracts. Microprocessor controller maintained the mobile phase consisting of A: B (15: 85). Cinnamic acid, gallic acid, hydroxy–benzoic acid, tannic acid, catechol, pyrogallol, ferulic, salicylic, orcinol and resorcinol were chosen as standards.

Estimation of total phenols

Total phenols were quantified using Folin-Ciocalteaus reagent with catechol as standard and the absorbance was recorded at 650 nm (Malick and Singh, 1980).

UV spectrophotometry

The UV absorption maxima of the water extracts was recorded between a range of 200 to 700 nm using Beckman UV visible DU – 64 spectrophotometer.

Results and Discussion

in vitro fungitoxicity of *L. inermis* leaf extracts

Various levels of inhibition were exhibited by the *L. inermis* leaf extracts against the three fungi tested. Cold-water extract at 10% concentration proved to be effective against *R. solani* mycelial growth when compared to methanolic and petroleum ether extracts showing a maximum inhibition of 70% (Fig 1). This was contradictory to the findings of Kurucheve *et al.*,(1997) who reported that the cold water extracts of *L. inermis* at 10% concentration inhibited the growth of *R. solani* only by 48.8%. Methanolic extract at

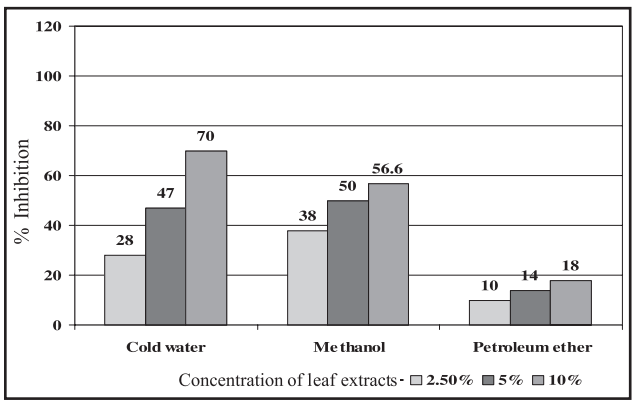

Figure1. Inhibition of *R. solani* by *L. inermis* leaf extracts

2.5% concentration completely inhibited the mycelial growth of *P. aphanidermatum* showing the susceptibility of the fungi to the very low concentration of methanolic extract. While, cold water and petroleum ether extracts at 10% concentration could inhibit only upto 64% and 55% respectively (Fig 2). Maximum inhibition of *M. phaseolina*

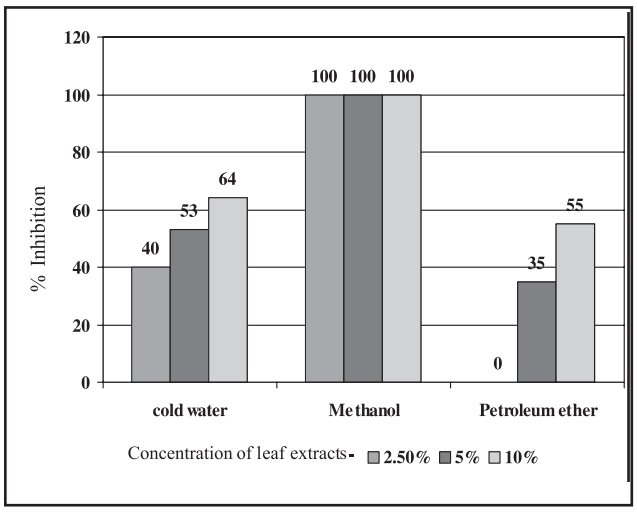

Figure 2. Inhibition of *P. aphanidermatum* by *L. inermis* leaf extracts

i.e. 60% was seen in the methanolic extract at 10 per cent concentration level followed by cold water extract (44%). But an inhibition of only by 7.82% on *M. phaseolina* mycelial growth by aqueous leaf extract of *L. inermis* was reported (Thiribhuvanamala and Narasimhan, 1998) that is far below the value observed in the present study. petroleum ether extract exhibited very poor inhibition (2.2%) of *M. phaseolina* even at 10% concentration (Fig 3). Probably the quantitative and qualitative differences in the fungitoxic principles extracted from the leaves of *L. inermis* by various solvents and the susceptibility of the fungi to these may be

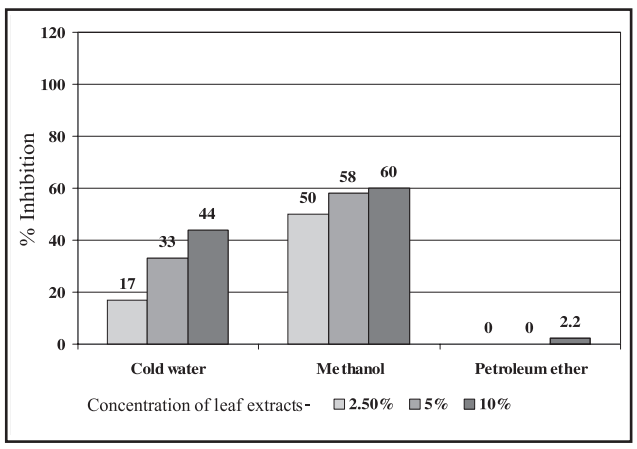

Figure 3. Inhibition of *M. phaseolina* by *L. inermis* leaf extracts

one of the many factors responsible for the variations in the mycelial growth inhibition of the fungi studied.

Preliminary separation and screening of phytochemical components

When the TLC plates developed in chloroform: acetic acid (9:1) were sprayed with Folin-Ciocalteau reagent, three spots with Rf values 0.43, 0.22 and 0.1 in cold water extract, three in methanolic extract (Rf 0.94, 0.86 and 0.48) and two in petroleum ether (Rf 0.94 and 0.86) were seen revealing the presence of phenolic compounds. Spots with Rf values 0.94 and 0.86 were found to be common in both petroleum ether and methanolic extract showing the extractability of these compounds in both the solvents. In the petroleum ether extract, a specific spot with Rf 0.92 that was also seen under visible light and two spots in the cold water extract with Rf values 0.48 and 0.71 were observed when the TLC plates developed in chloroform: hexane (9:1) and hexane solvent systems respectively were sprayed with Vanillin-sulfuric acid. These spots could be either steroids or phenolics. The spots mentioned above with Rf values 0.92 and 0.48 were super imposable with the Rf values 0.94 and 0.48 obtained in the methanolic extract developed in chloroform: acetic acid (9:1) solvent system and sprayed with Folin-Ciocalteau reagent indicating them to be phenolic compounds. While the spot (Rf 0.71) reveals the presence of steroids as it was specifically seen with Vanillin-sulfuric acid reagent but not with Folin-Ciocalteau reagent treatment and this needs further conformation. Various phytochemical constituents such as terpenes, phenolics and steroids from hexane and ethanol extracts of two *Eucalyptus* sp were screened and identified using TLC (Zidah *et al.*, 2000). Qualitative and quantitative analysis of phenolic acids in *G. biloba* leaves was carried out using TLC and HPLC (Ellnain and Zgorka, 1999). Cold water extract in n-butanol: acetic acid: water (4:1:5) solvent system exhibited three fluorescent spots (Rf 0.62, 0.76 and 0.88) under UV light and three spots (Rf 0.5, 0.63 and 0.83) under visible light. Natarajan and Lalithakumari (1987) also identified three fluorescent spots in the leaf extracts of *L. inermis* after running the TLC plates in n-butanol: acetic acid: water (4:1:5). They also recorded two characteristic peaks one at 236nm and another at 272 nm corresponding to lawsone in the U.V spectrophotometric analysis of cold water extract. Where as in the present study, a peak was recorded at 265 nm but the compound was not characterized. In 100g of fresh leaves, the amount of total phenols extracted in water was quantified to be 0.45g.

RP-HPLC identification of phenolic compounds

In the RP-HPLC analysis of aqueous leaf extract, four peaks with retention times (Rt) 4.51, 5.99, 7.19 and 17.78 min were observed. Rt values 4.51 and 5.99 were super imposable with the Rt of standards for tannic acid (4.67 min) and catechol (6.05 min), respectively indicating the presence of tannic acid and catechol in detectable amounts. However, the two other peaks with Rt 7.19 and Rt 17.78 correlated with none of the standards used and therefore could not be identified (Fig 4). No characteristic peaks in the sample that corresponded with the retention time of the standards of cinnamic acid, gallic acid, pyrogallol, ferulic, salicylic, hydroxy–benzoic acid, orcinol and resorcinol were noticed indicating their absence or their presence in non detectable amounts. To confirm this further studies are needed to be carried out. Presence of high amounts of phenolic acids was reported in chickpea resistant to fungal infections (Mohammadi and Daivedi, 1991). Sorghum grain resistant to fungal attack contained both a greater variety and larger amounts of the phenolic acids and unknown compounds than did susceptible varieties (Hahn *et al.*, 1983). *In vitro* antifungal activities of phenolic compounds were reported by many authors (Mandavia *et al.*, 1997; Venkatachalm and Jayabalan, 1995). Reports on 24 beta-Ethylcholest-4-en-3 beta-ol from the roots(Gupta *et al.*, 1992), a naphthoquinone from stem bark (Gupta *et al.*,1993) and tannins from roots (Bakkali *et al.*, 1997) of *L. inermis* have already been published. Thus, in the present study it could be concluded

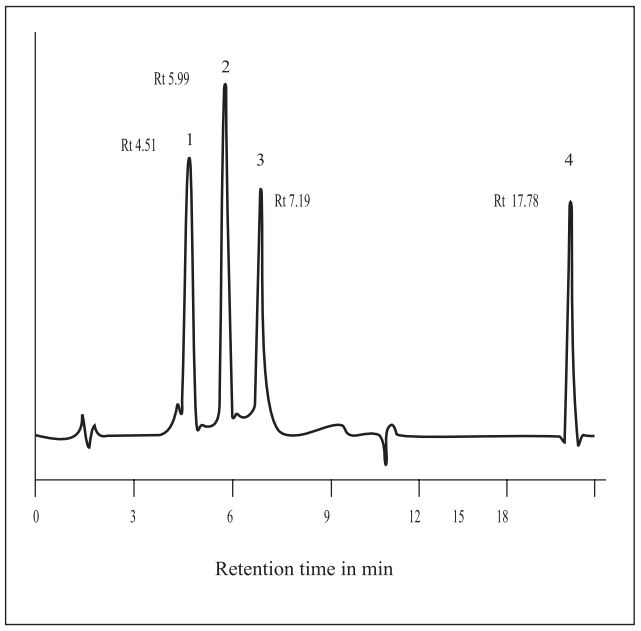

Figure 4. RP-HPLC studies with cold water extracts of *L. inermis* leaves

1.Tannic acid (Rt of standard- 4.67min)

2.Catechol (Rt of standard- 6.05min)

* Rt- Retention time in minutes

** The above values are an average of 3 runs

that the presence of phenolic compounds in the *L. inermis* leaf extracts along with other phytochemical components resulted in the *in vitro* fungal inhibition. However, these need to be tested *in vivo* to determine their effectiveness in controlling diseases caused by these fungi.

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