

Antifungal activity of fruit extracts of *Flueggea leucopyrus* Willd. Against phytopathogenic fungi *Aspergillus* spp.

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Abstract: To study the phytoconstituents and antifungal activity of *Flueggea leucopyrus* fruit extracts of hexane, chloroform and methanol were tested against plant pathogenic fungi. The preliminary phytochemical analysis of the fruit extracts revealed the presence of oil in chloroform extract, oil and flavones in hexane extract, flavones, phenol and almost all the tested compounds in the methanol extract except coumarin and alkaloids. The TLC profile of the tested extracts showed two spots each (0.2, 0.4; 0.2, 0.5; 0.2, 0.6) for hexane, chloroform and methanol extracts respectively. Among the three extracts, the methanol extract showed significant activity by inhibiting the growth (*Aspergillus fumigatus* (23.3 mm), *A. flavus* (17.3 mm) and *A. niger* (16 mm)). The secondary metabolic compounds like flavones, oil, phenol, quinones, saponins, sugar and tannins. The findings revealed that the phytoconstituents in the fruit extracts is highly resistant and can be used as a potential source for the development of new antifungal compounds. The preliminary phytochemical study was analyzed of the fruit extractions.

Keywords: *Flueggea leucopyrus*, Phytoconstituents, TLC profile, Antifungal activity, *Aspergillus* spp.

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I. Introduction

The overzealous and indiscriminate use of most of the synthetic fungicides has created different types of environmental and toxicological problems. Recently, in different parts of the world, attention has been paid towards exploitation of higher plant products as novel chemotherapeutants in plant protection. The popularity of botanical pesticides is once again increasing and some plant products are being used globally as green pesticides^[1]. The exploitation of natural products to control decay and prolong storage life of perishables has received more and more attention. Biologically active natural products have the potential to replace synthetic fungicides^[2]. Plants have the ability to synthesize aromatic secondary metabolites, like phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins^[3]. The components with phenolic structures, like carvacrol, eugenol and thymol, were highly active against pathogens. These groups of compounds show antimicrobial effect and serves as plant defense mechanisms against pathogenic microorganisms^[4]. Plant extracts and essential oils show antifungal activity against a wide range of fungi^[5].

The antifungal activity of the ethanolic extracts of ten Argentinean plants used in native medicine is reported. The volatile antimicrobial substance allicin (diallyl thio sulphinate) is effectively controlled seed-borne *Alternaria* spp. in carrot, *Phytophthora* leaf blight of tomato and tuber blight of potato as well as *Magnaporthe* on rice and downy mildew of *Arabidopsis thaliana*^[6]. Application of plant products especially essential oils is a very attractive method for controlling post-harvest diseases. Essential oil extracted from lemon grass (*Cymbopogon* spp.) post-harvest anthracnose of mango fruit^[7]. Extracts of *Larrea divaricata*, *L. cuneifolia* and *Zuccagnia punctata* displayed remarkable activity in the assays against the majority of the test fungi. In addition to the former plants *Prosopanche Americana* also inhibited yeast growth^[8].

F. leucopyras belonging to Euphorbiaceae family is commonly known in Sri Lanka by 'Katupila' and in Gujarat by 'Humri' is a plant used by the indigenous people for the treatment of wounds and cancer in Sri Lanka. Extracts of leaves exhibited *in vitro* broad spectrum antimicrobial activities. Till date there is no pharmacognostical scientific work has been done on its leaf and stem^[9]. The chemical components of *Flueggea microcarpa* are extensively investigated and the occurrence of bergenin as a major constituent in the plant was reported^[10]. The antifungal activity of bergenin (2β- D-glucopyranosyl 4-o- methyl gallic acid and lactone occurring isocoumarin^[11] isolated from *F. microcarpa*^[12] against several plant pathogenic fungi also carried out. The *Securinega* alkaloids are a group of compounds isolated from the plants of *Securinega*, *Phyllanthus* and *Flueggea* genera. Most of these compounds exhibit a wide range of biological activities such as GABA receptor antagonist, antimalarial and antibacterial activity^[13].

Antifeedant and larvicidal activities of hexane, chloroform and ethyl acetate extracts of *Flueggea leucopyrus* were studied against 3rd instar larvae of *Earias vittlla* [14]. *Securinega leucopyrus* (*F. leucopyrus*) pharmacognostical, phytochemical and antimicrobial activities are not reported yet. Hence, the present study is aimed to investigate the efficacy of the solvent extracts of fruit of *F. leucopyrus* against fungal pathogens *Aspergillus* spp. isolated from mango leaves.

II. Materials and Methods

Collection of plant materials

Fresh plant fruit of *Flueggea leucopyrus* Willd were collected from the natural habitat (Fig.1) of Bentharam halli Village, Krishnagiri during the month of August and September 2012. Taxonomic identification of the plant was carried out with the help of the flora of presidency of madras [15]. The fruits were washed thoroughly three times with water and once with distilled water. The fruits were shade dried and powdered. The powdered samples were sealed in separate polythene bags, until the time of extraction.

Preparation of plant extracts

The dried fruit materials were pulverized into fine powder using a grinder (mixer). About 40gm of powdered fruit was extracted successively with 200 ml of hexane (62-66°C), chloroform (60-62°C) and methanol (56-60°C) in Soxhlet extractor until the extract was clear. The solvent extracts were evaporated by using rotary vacuum evaporator (Model: PBV – 7D) and the resulting pasty form extracts were stored in refrigerator at 4°C for further use [16].



Figure 1. Habit and fruits of *Flueggea leucopyrus* Willd.

Qualitative analysis

Preliminary and qualitative phytochemical screening tests were conducted on the fruit extracts of *F. leucopyrus*. All the extracts such as hexane, chloroform, and methanol extract were tested with standard methods [17-18]. The presence and absence of phytoconstituents tested were recorded as present (+) or absent (-).

Thin Layer Chromatography (TLC)

A slurry of the adsorbent (Silica gel G) was prepared with water (1:2). Dried and clean glass plates (20 cm x 5 cm) were laid in a row as a template, the suspension was poured into Stahl TLC spreader, which was adjusted to 0.25 mm thickness and coated in a single passage on the spreader over them. The prepared plates were allowed to air-dry and placed in an Oven at 110° C for 30 min after drying towards activation. Then, they were transferred into a dust free chamber. The application of spots was done using capillary tubes about 2 cm above the bottom of the plate. Chromatograms were detected with vanillin-H₂SO₄ reagent (1 gm vanillin dissolved in 100 ml H₂SO₄ heated at 110° C after spraying).

Isolation of epiphytic fungi

Infected plant materials were thoroughly washed in running tap water, then surface sterilized [19]. The selected leaf and stem segments were immersed in 0.1% mercuric chloride solution for 60 sec followed by rinsing with sterile distilled water three times for 10 sec and allowed to surface dry under sterile conditions. After drying, each leaf and stem segment were cut into approximately 0.5 cm squares and placed on petri plates containing potato dextrose agar medium (PDA) supplemented with streptomycin (100 mg/L) to suppress bacterial growth. Petri plates were incubated at 30° C in a light chamber for up to one week. They were monitored every day for growth of fungal colonies. Fungi growing out from the samples were subsequently transferred onto fresh PDA plates. The procedure of transferring to fresh PDA plates was carried out several times in order to isolate pure colonies.

Identification of the epiphytic fungi

The epiphytic fungal isolate were identified based on the morphological characteristics. The morphological examination was performed by observing the fungal colony and the characteristics of the spores. The fungal isolate was grown on PDA at 30° C for 7-9 days, and the conidia of the isolate were examined under the microscope. Slide culture technique also used to observe the morphology of the fungal isolate [20].

Antifungal assay

The paper disc diffusion method was used to determine the antifungal activity of the extracts [21]. Sterile discs were impregnated with 60 µl of each extract at concentration of 100 mg/3ml. A 100 µl of fungal culture/spore was spread onto the surface of potato dextrose agar medium. Immediately, fungal extract discs and positive and negative control discs were placed onto the surface of the potato dextrose agar plate by using sterile forceps. Amphotericin - B (100 units/disc), Clotrimazole (10 mcg/disc), Ketoconazole (10 mcg/disc) were used as positive control. Paper disc treated with 50% methanol was used as negative controls. The plates were incubated at 30° C for 48-72 hrs. The millimeter of inhibition zone around each of the disc was measured at the end of the incubation time. Experiments were performed in triplicate and the antifungal activity was expressed as the average of millimeters of the inhibition zone produced by the test extracts.

III. Results

Preliminary phytochemical analysis of various solvent extract of fruit of *F. leucopyrus* revealed the presence of oil in chloroform, oil and flavones in hexane, flavones, oil, phenol and almost all the tested compounds in the methanol except coumarin and alkaloids (Table 1). On spraying with Vanillin- Sulphuric acid reagent and heating the plate at 105° C for ten minutes showed each two spots at Rf value is (0.2, 0.4; 0.2, 0.5; 0.2, 0.6) for all the extracts respectively (Table 2). The antifungal activity of the fruit extracts of hexane, chloroform and methanol were tested against plant pathogenic fungi *Aspergillus flavus*, *A. fumigatus* and *A. niger* isolated from mango infected leaf tissues of mango plant (Fig. 2).

Table 1. Preliminary phytochemical screening of *F. leucopyrus* fruit extracts.

S. No	Test	Hexane	Chloroform	Methanol
1	Alkaloids	-	-	-
2	Coumarins	-	-	-
3	Flavones	+	-	+
4	Oil	+	+	+
5	Phenol	-	-	+
6	Quinones	-	-	+
7	Saponins	-	-	+
8	Sugar	+	-	+
9	Tannins	+	-	+

The antifungal activity of hexane extract showed maximum inhibition zone of 22.3 mm against *A. niger*, 14.3 mm against *A. fumigatus* and 8.3 mm against *A. flavus*. Hexane extract showed an effective antifungal activity when compared with control antifungal agent (Amphotericin-B (16.3 mm, 12 mm and 12.3 mm) and Clotrimazole (20.3 mm, 25.6 mm and 25.3 mm) *Aspergillus niger*, *Aspergillus fumigatus* and *Aspergillus flavus* respective strains). Chloroform extract displayed refusal anti-fungal activity against all the tested plant pathogenic fungi (Table 3).

Table 2. TLC profile of *F. leucopyrus* fruit extracts.

Mobile phase	Rf value at different solvent extracts		
	Hexane	Chloroform	Methanol
Benzene Acetic acid water (7: 3:1)	0.2 , 0.4	0.2, 0.5	0.2, 0.6

The methanol extract showed significant antifungal activity against *A. fumigatus* (23.3 mm), *A. flavus* (17.3 mm) and *A. niger* (16 mm) were documented. Methanolic extract showed an effective antifungal activity when compared with control antifungal agent (Amphotericin-B 12 mm, 12.3 mm, 16.3 mm). Methanolic extract exhibit significant antifungal activity and the result also near to the zone produced by the (Clotrimazole 25.6 mm, 25.3mm, 20.3 mm and Ketoconazole 29.6 mm, 26.3 mm, 24.6 mm) control antifungal agents. There was absence of zone production by the methanolic control. Among the three extracts, the methanol extract showed potent antifungal activity by inhibiting the growth of all the tested plant pathogenic fungi (Fig. 3).

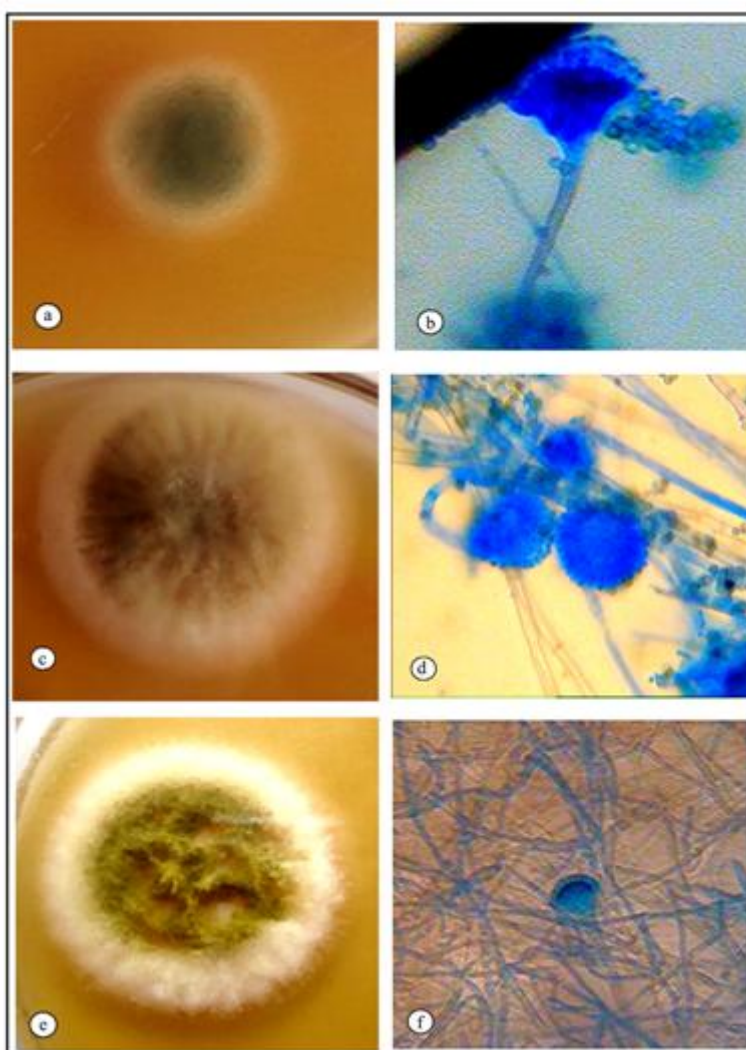


Figure 2. Colony morphology and sporulating structure of *Aspergillus* fungus. a-b. *A. fumigatus*, c-d. *A. flavus*, e-f. *A. niger*.

Table 3. Antifungal activity of different solvent extracts of *F. leucopyrus* fruit by disc diffusion method.

S. No	<i>Aspergillus</i> fungus	Zone of inhibition (mm)					
		Solvents			Positive controls		
		HE	CH	ME	AM	CL	KE
1.	<i>A. flavus</i>	8.3 mm	—	17.3 mm	12.3 mm	25.3 mm	26.3 mm
2.	<i>A. fumigatus</i>	14.3 mm	—	23.3 mm	12 mm	25.6 mm	29.6 mm
3.	<i>A. niger</i>	22.3 mm	—	16 mm	16.3 mm	20.3 mm	24.6 mm

HE-Hexane, CH-Chloroform, ME-Methanol, AM-Amphotericin, CL-Clotrimazole, KE-Ketoconazole.

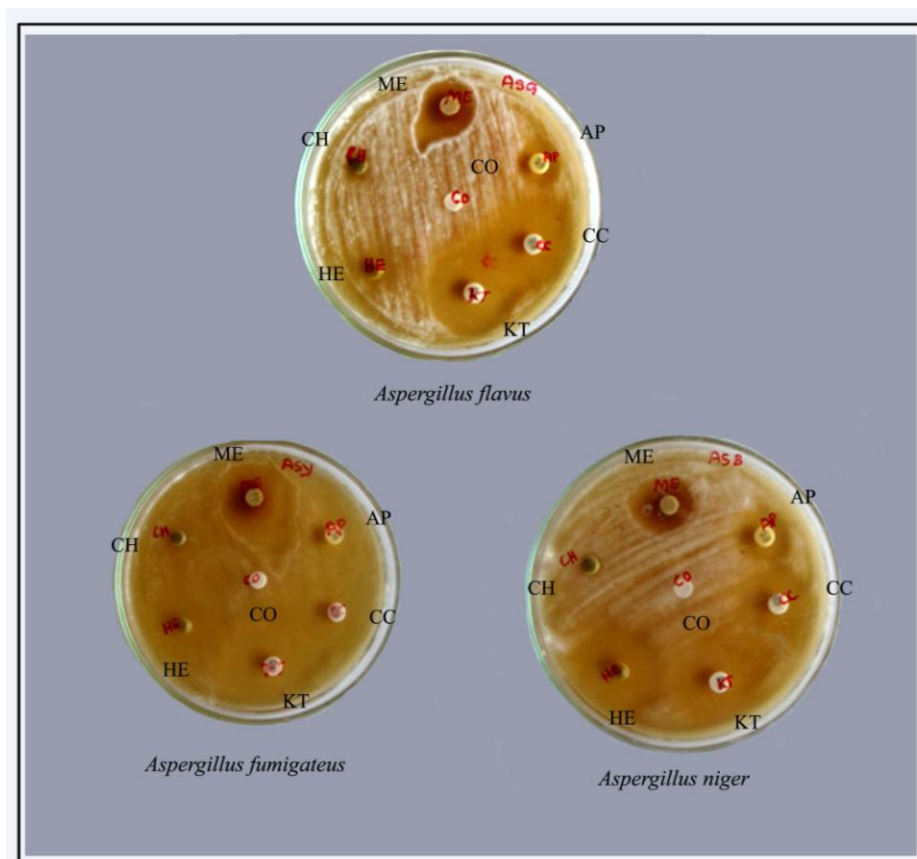


Figure 3. Effect of *F. leucopyrus* fruit extraction on *Aspergillus* fungus by disc diffusion method. **HE**-Hexane, **CH**-Chloroform, **ME**-Methanol, **AP**-Amphotericin, **CC**-Clotrimazole, **KT**-Ketoconazole, **CO**-Negative control. In the present study, the methanolic extract exhibited significant antifungal activity against plant pathogenic fungi. The results revealed that the phytoconstituents in the fruit extracts may be responsible for the significant antifungal activity exhibited by the extracts and can be used as potential source for the development of new antifungal compounds.

IV. Discussion

Various phytochemical compounds detected from the fruit of *F. leucopyrus* are known to have beneficial importance in medicinal sciences. Recently, a number of studies have been carried out on the phytochemistry of plants across the world [22-23]. Application of *Securinega leucopyrus* (Katupila/Humari) is a commonly used plant in the management of acute and chronic types of wounds in Sri Lankan folklore medicine [24]. As per our preliminary phytochemical studies, among hexane, chloroform and methanol extract, the methanol extract revealed the presence of maximum number of phytoconstituents such as flavones, oil, phenol, quinones, saponins, sugar and tannins. The presence or absence of the phyto-constituents depends on the solvent used for the extraction and the physiological property of the fruit. Flavonoids obtained from the flowers of *Nerium oleander* were found to have high activity against *Staphylococcus aureus*, *S. albus*, *Klebsiella* sp., *Candida albicans* and *Aspergillus niger* and moderate activity against *Pseudomonas* and *Proteus* sp. [25]. At lower concentrations tannins inhibit the growth of microorganisms and act as anti-fungal agents at higher concentrations they act by coagulating the protoplasm of the microorganism [26]. Saponins are used as mild detergents and in intracellular histochemical staining. In medicine, they are used for treating hypercholesterolemia, hyperglycemia and weight loss, and have antioxidant, anticancer, anti-inflammatory and antifungal properties [27].

Hexane extracts were tested against plant pathogenic fungi. It produced maximum inhibition zone of 22.3 mm against *Aspergillus niger*, 14.3 mm against *A. fumigatus* and 8.3 mm against *A. flavus*. The methanol extract showed significant antifungal activity against *A. fumigatus* (23.3 mm), *A. flavus* (17.3 mm) and *A. niger* (16 mm) were documented. Methanolic extract showed an effective activity when compared with control antifungal agent (Amphotericin-B, 12 mm, 12.3 and 16.3), methanolic extract exhibit significant antifungal activity and the result was also nearer to the zone produced by the (Clotrimazole 25.6 mm, 25.3mm and 20.3 mm, Ketoconazole 29.6 mm, 26.3mm and 24.6mm) control antifungal agents. There was no zone produced by the methanolic control. Similar results were reported [28] that methanol extract of fruit of *Achyranthes*

tetracantha exhibited broad spectrum antibacterial, antifungal activity against *Trichophyton rubrum*, *Microsporum gypseum* and *Penicillium marneffeii*. Garcia *et al.*,^[29] studied antifungal activity of eighteen plant extracts from nine traditional Mexican medicinal plants to test against two dermatophytes fungal species (*Trichophyton mentagrophytes* and *Trichophyton rubrum*), one non-dermatophyte (*Aspergillus niger*) and one yeast (*Candida albicans*).

The strongest effect was manifested by the hexane extracts from *Eupatorium aschenbornianum* and *Sedum oxypetalum*, as well as the methanol extracts from *Lysiloma acapulcensis* and *Annona cherimolia*. Aqueous extract of *Acacia nilotica*, *Achras zapota*, *Datura stramonium*, *Embllica officinalis*, *Eucalyptus globules*, *Lawsonia inermis*, *Mimusops elengi*, *Peltophorum pterocarpum*, *Polyalthia longifolia*, *Prosopis juliflora*, *Punica granatum* and *Syigium cumini* have recorded significant antifungal activity against one or the other *Aspergillus* species tested. *Aspergillus flavus* recorded high susceptibility and hence solvent extracts viz., petroleum ether, benzene, chloroform, methanol and ethanol extracts of all the twelve plants were tested for their antifungal activity against it.

Among the solvent extracts tested, methanol gave more effective results than ethanol, chloroform, benzene and petroleum ether, except for *Polyalthia longifolia*, where petroleum ether extract recorded highly significant antifungal activity than other solvent extract^[30]. Similar result was observed in our studies. The crude extract of hexane, chloroform and methanol were screened for antifungal activity. Among the three extracts, the methanol extract showed potent antifungal activity by inhibiting the growth of all the tested plant pathogenic fungi of *Aspergillus* spp.

Plants have developed natural defense mechanisms to protect themselves long before the man played an active role in protecting them. It is known that plants synthesize a variety of bioactive compounds in plant tissues as secondary metabolites that have antifungal activity to stop or inhibit the development of mycelia growth, inhibition of germination or reduce sporulation of fungal pathogens, each of these groups presented variable mechanisms of action, for example, the toxicity of polyphenols in microorganisms is attributed to enzyme inhibition by oxidation of compounds^[31].

Prithviraj *et al.*,^[32] isolated the compound Bergenin (2-β- D-glucopyranosyla 4-o- methyl gallic acid δ lactone) naturally occurring isocomarine from *Fluggea microcarpa* which have the antifungal activity against several plant pathogenic fungi. From our TLC profile studies, identified two spots in each extracts, that may be separate compounds and which may be responsible for the observed antifungal activity. However further studies are needed to determine the chemical identity of the bioactive compounds responsible for the observed antifungal activity in *F. leucopyrus*. Natural plant-derived fungicides may be a source of new alternative active compounds, in particular with antifungal activity. The high proportion of active extracts in the assayed species, selected according to available ethno botanical data, corroborates the validity of this approach for the selection of plant species in the search for a specific activity.

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