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Research Article

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Antibacterial, antifungal, insecticidal and phytotoxic activities of leaves of *Pinus wallichiana*

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ABSTRACT

The crude extract fractions of Pinus wallichiana leaves including n-hexane, ethyl acetate, chloroform, aqueous and residue were studied for antibacterial, antifungal, insecticidal and phytotoxic activities. All fractions exhibited no antibacterial activity against the tested bacterial strains. In case of antifungal bioassay, only n-hexane (F1) fraction showed minimum inhibition at the concentration of 25 µg/mL against Microsporum canis. The ethyl acetate fraction displayed activity against Tribolium castaneum, Rhyzopertha dominica and Callosobruchus analis. The other fraction showed activities against selected insects. In case of phytotoxic assay, all fractions showed concentration dependent activities. Then-hexane(F1), ethyl acetate (F2),chloroform (F3),water (F4) and residue (F5) fractions showed good phytotoxicity at 500µg/ml concentration and moderate activity at 50µg/ml. In short, the results showed that the plant is medicinally important.

Key words: Antifungal, antibacterial, phytotoxic, insecticidal activities, *Pinus wallichiana* leaves

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INTRODUCTION

In Pakistan, five species of genus *Pinus*namely *Pinus wallichiana, Pinus halepensis, Pinus brutia, Pinus roxburghii* and *Pinus geradiana* are widely available [1].The stem of *Pinus roxburghii* yields transparent oleo-resin having pungent and bitter taste. The oil obtained by the distillation of oleo-resin, are utilized as insecticides, disinfectants and in fireworks, ointments, plasters, chewing gums, varnishes, hair fixing and nail polishing etc [2]. The resin is also applied to cure boils and combat gastric troubles [3-4]. Resins are utilized in synthetic rubber and chewing gums [5]. The pine resins are employed to treat rheumatism and for the treatment of smallpox and syphilis [6]. Different parts of the *Pinus* plants are recommended to treat colds, coughs, influenza, bronchitis, tuberculosis and as antiseptic, diaphoretic, rubefacient, diuretic, stimulant and febrifuge [7-8]. Resins are generally sweet, pungent, thermogenic, oleagenous and antiseptic. The resins act as diuretic, purgative, emmenagogue and expectorant actions, inflammations, asthma, chronic bronchitis, piles, diseases of the liver and spleen, urinary discharges, earache, toothache, tuberculosis, lumbago, scabies and epilepsy. The gum has shown good effect in diseases of the vagina and uterus [9].

Pinus wallichiana (P. wallichiana), is known as 'Chil' (Pashto), Kairo (Urdu) and bluepine (English). The plant is widely spread in various regions of Pakistan such as Rawalpindi, Islamabad, Baltistan, Basho forest, Skardu,

Hazara, Bara Gali, Changlagali, Mukhspori and Kashmir. This specie is also distributed in Afghanistan and Nepal [1]. A large number of compounds, such as phenolic acids, ferulic acid, glucosides and *p*-coumaric acid have been reported from *P. wallichiana* [10]. The earlier reported literature reveals that leaves of *Pinus wallichiana* contains *β*-sitosterol*β*-sitosterol-3-*O*-*β*-d-glucopyranoside, 5-hydroxy-7-methoxy-2-(4-methoxyphenyl)-4H-chromen-4-oneandoleanolic acid [11-15].The plant also contains anthocyanidins [16-18].

Keeping in view the medicinal importance of the genus, an effort was made to evaluate the various fractions of the crude extract of leaves of *P. wallichiana* for various biological activities including antifungal, antibacterial, phytotoxic and insecticidal.

EXPERIMENTAL SECTION

Plant collection

The leaves of *Pinus wallichiana* were collected from the Thandiani, Abbottabad, Khyber Pakhtunkhwa province of Pakistan, in June, 2010. The taxonomic identification of the plant was done at the Department of Botany, Hazara University, Mansehra. A voucher specimen was deposited in the herbarium of the Department. The leaves of the plant were air-dried under shade for one month at room temperature. The dried plant material was powdered and kept in polyethylene bags for further study.

Extract preparation

The powdered leaves, 15 kg were soaked in ethanol with occasional shaking at room temperature for one week and filtered. The process was repeated thrice time. The combined filtrates were evaporated under reduced pressure at temperature at45^{o}C. The concentrated ethanolic extracts (2.2kg) were mixed with distilled water and fractionated with different solvents namely *n*-hexane, chloroform, ethyl acetate and methanol etc. to get different fractions (F1 to F5). These fractions were assessed for their antifungal, antibacterial, insecticidal, phytotoxic and cytotoxic potentials.

Anti-bacterial assay

In this bioassay study six fungal and six bacterial strains were selected to be used. The bacterial strain used were classified as *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, *Shigella flexenari* (clinical isolate), *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 *and Salmonella typhi* ATCC 19430. The fungal strains selected for this study were identified as *Trichophyton longifusis* (clinical Isolate), *Candida albicans* ATCC2091, *Aspergillus flavus* ATCC 32611, *Microsporum canis* ATCC 11622, *Fusarium solani* 11712 and C*andida glaberata* ATCC 90030. All these strains were maintain on agar slant at 4^{o}C and the slant was allowed to activate at a temperature of 37 ^{o}C for 24 hours on nutrient agar (NA), for both bacteria and fungi, before any screening is carried out. The microorganisms; *B. subtilis* ATCC 6633, *E. coli* ATCC 25922, *S. flexenari (clinical isolate), S. aureus* ATCC 25923 *P. aeruginosa* ATCC 27853 *and S. typhi* ATCC 19430 were used for evaluation of antibacterial activity. The organisms were stored in Muller hantin agar in the refrigerator at 4^{o}C prior to subculture. Antibacterial testing was carried out on the already developed agar well diffusion method to study the potency of the extract fractions of *P. wallichiana* leaves. Broth media were prepared and the test organisms were transferred to the broth media from agar plate and were grown at 37 ^{o}C for 24 hours. After 24 hours 25 ml of MHA were poured into each petri plate and cooled in sterile condition. The fresh culture was prepared from day old culture, after solidification of MHA in plate, 0.6 ml of fresh culture of test organism were poured on to MHA. Wells of 6 mm diameter were digged in to the medium by using sterile borer and 22 mg of different fractions of the extract of *P. wallichiana* leaves were used against each organism. DMSO and standard antibiotic (imipenum) were added into other wells. The plates were kept in sterilized inoculation chambers for 1 hour to facilitate diffusion of the antimicrobial agent into the medium. The plates were then incubated at 37 ^{o}C for 24 hours and the diameters of the zone of inhibition of microbial growth were measured in millimeters [19].

Antifungal assay

The microorganisms; *Trichophyton longifusis*, *Candida albicans* ATCC 2091, *Aspergilus flavus* ATCC 32611, *Microsporum canis* ATCC 11622, *Fusarium solani* 11712 and *Candida glaberata* ATCC 2091 were used for antifungal assay. All these strains were maintained on agar slant at 4^{o}C, the slant was allowed to activate at a temperature of 37 ^{o}C for duration of 3-4 days on nutrient agar (NA), for fungi, before any screening is carried out. The crude extract fractions were dissolved in DMSO (24 mg/ml) and sterile medium (5 ml) was placed in a test tube and inoculated with the sample solution (400μg/ml) which was then kept in a slanting position at room temperature

for overnight. The tubes were inoculated by a piece of fungus (4 mm diameter) from seven day old culture. The samples were then incubated for 7 days at 28 ^{o}C and the fungal strain starts growth on the slant. The growth inhibition was observed and percentage growth inhibition was determined by calculating with reference to the positive control by applying the formula

$$\% \text{ Inhibition} = \frac{\text{100-linear growth and test (mm)}}{\text{linear growth in control (mm)}} \times 100$$

Amphotericin B and miconazole were used as standard antibiotics [20].

Insecticidal assay

Different fractions were tested against various insects *viz, Rhyzopertha dominica, Tribolium castaneum* and *Callosobruchus analis*. The sample for test was prepared by adding 20 mg of crude extract fractions with 2 ml acetone which was immediately kept in Petri dish covered with the filter papers. Later 24 hours, ten insects were retained in every plate which was incubated at 27 ^{o}C for 24 hours in 50% humid environment of growth chamber. The insects were kept to stand without food for 24 hours after which the mortality number was calculated. The activity was examined as percent mortality. Permethrin (239.5 µg/cm^2) was used as a reference insecticide in this experiment, while acetone was used as negative control [21-22].

Phytotoxicity assay

In this bioassay, various crude extract fractions were tested against *Lemna minor* [23-25]. Stock solutions (20 mg/mL) of various extracts were diluted in order to obtain a final concentration of 500, 50 and 5 µg/mL, respectively. Each flask was then mixed to a 20 mL medium sized 10 plants, each one containing rosette of three fronds. In this experiment Paraquat (0.015 µg/mL) was used as a standard growth inhibitor. All flasks were permitted to keep in growth cabinet for a total duration of seven days. Afterward, the inhibition percentage was calculated with reference to the negative control.

RESULTS AND DISCUSSION

In the recent era, there is an increasing interest of the scientific community to develop new therapeutic agents form natural resources, especially the plants. Medicinal plant base products and drugs are more preferred than modern allopathic drugs, due to their long-term side effects. The comparatively safe and cheap medicinal therapeutic agents from plants, appeared as an important alternative for controlling various microbial diseases, especially in cases of resistant. In view of the above, different fractions of the ethanolic extract of *P. wallichiana* leaves were evaluated for their biological activities. The results are described below:

Anti-bacterial assay

All fractions exhibited no antibacterial activity against the selected bacterial strains (**Table-1**)**.**

Table-1: Anti-bacterial activity of the leaves extract fractions of *Pinus wallichiana* leaves

Microorganism	Zone of Inhibition (mm)					
	Imipenem (Standard drug)	F1	F2	F3	F4	F5
Escherichia coli	35	-	-	-	-	-
Bacillus subtilis	36	-	-	-	-	-
Shigella flexenari	36	-	-	-	-	-
Staphylococcus aureus	43	-	-	-	-	-
Pseudomonas aeruginosa	32	-	-	-	-	-
Salmonella typhi	40	-	-	-	-	-

Key words: F1=n-hexane, F2= ethyl acetate, F3=chloroform, F4= aqueous, F5=residue

Antifungal assay

All the examined fractions (**Table-2**) showed no bioactivity against selected fungal strains except *n*-hexane (F1) which showed minimum inhibition at concentration of 25 (*µg*/mL**)** bioactivity against *M. canis.*

Table-2: Antifungal activity of the leaves extract fractions of *Pinus wallichiana* leaves

Fungal species	Minimum Inhibitory Concentration (µg/mL)					
	Miconazole	F1	F2	F3	F4	F5
Trichophyton longifusis	70.08	-	-	-	-	-
Candida albicans	110.8	-	-	-	-	-
Microsporum canis	98.4	25	-	-	-	-
Fusarium solani	73.10	-	-	-	-	-
Candida glabrata	110.8	-	-	-	-	-
	Amphotericin B					
Aspergillus flavus	20	-	-	16	-	-

Key words: F1 = n-hexane, F2 = ethyl acetate, F3 = chloroform, F4 = aqueous, F5 = residue

Insecticidal assay

The isolated fractions were evaluated for their insecticidal activities (**Table -3**). The *n*-hexane fraction (F1) showed 20 % activity against *R. dominica*, while the ethyl acetate (F2) showed 20 % activities against all the strains. The chloroform (F3) revealed 20 % activity against *R. dominica*, while the aqueous (F4) showed 20 % and 40 % activity against *R. dominica* and *C. analis* and the residue (F5) showed no activities against *C. analis*.

Table-3: Insecticidal activity of the leaves extract fractions of *Pinus wallichiana* leaves

Insect	% Mortality						
	+ve control	-ve control	F1	F2	F3	F4	F5
Tribolium castaneum	100	0	-	20	-	-	-
Rhyzopertha dominica	100	0	20	20	20	20	-
Callosobruchus analis	100	0	-	20	-	40	-

Key words: F1 = n-hexane, F2 = ethyl acetate, F3 = chloroform, F4 = aqueous, F5 = residue

Phytotoxic assay

The various extract fractions of leaves of *P. wallichiana* were evaluated for phytotoxicity. The fractions (F1-F5) showed significant phytotoxicity activity at 500μg/ml concentration, while at low concentration 50μg/ml and 5μg/ml showed moderate activity except chloroform (F3) which showed no moderate activity (**Table-4**).

Table-4: Phytotoxic activity of the leaves extract fractions of *Pinus wallichiana* leaves

Concentrations	% Growth regulation						
	Paraquat (0.015µg/mL)	-ve control	F1	F2	F3	F4	F5
500 μg/ml	100	0	100	75	60	30	75
50 μg/ml	100	0	60	10	05	10	25
5 μg/ml	100	0	05	05	-	05	05

Key words: F1 = n-hexane, F2 = ethyl acetate, F3 = chloroform, F4 = aqueous, F5 = residue

CONCLUSION

The current study established that the extract fractions of *Pinus wallichiana* leaves have insecticidal and phototoxic activities. The plant is frequently used for the treatment of various diseases. This data strongly supports that broad research should be carried out to isolate phytochemical constituents conscientious for insecticidal and phytotoxic.

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