



## Antibacterial, antifungal, insecticidal, cytotoxic and phytotoxic activities of the crude extracts of *Taxus wallichiana* Zucc twigs

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### ABSTRACT

This study was proposed to investigate the in-vitro antifungal, antibacterial, insecticidal, cytotoxic and phytotoxic potential of ethanolic extract of twigs of *Taxus wallichiana* Zucc. Traditionally, this plant is used in folk medicines in Pakistan, for the treatment of various microbial infections. The crude sub fractions of ethanolic extracts of the plant were tested against six bacterial and six fungal strains using hole diffusion and macro-dilution methods, respectively. The n-hexane and ethyl acetate fractions showed 15 and 24 mm zones of inhibition against *Staphylococcus aureus*, while the chloroform fraction exhibited 35 mm inhibition against *Shigella flexenari*. In case of antifungal activity, both the chloroform and methanol fractions showed minimum inhibitory concentration at the concentration level of 15 µg/mL against *Microsporum canis*, while ethyl acetate fraction exhibited antifungal activity against *Candida albicans* and *Candida glaberata* at 27 and 13 µg/mL concentrations, respectively. The rest of the fractions showed no antifungal activity. The cytotoxicity assay revealed that all the fractions have activity against the shrimp. The n-hexane, chloroform and methanol fractions were found active against *Rhizopertha dominica*, *Callos bruchuanalis* and *Tribolium castaneum*. In case of phytotoxicity assay, all fractions showed significant activity at the concentration of 500 µg/ml.

**Keywords:** *Taxus wallichiana* Zucc, antifungal, antibacterial, insecticidal activity, phyto toxicity, cytotoxicity, twigs.

### INTRODUCTION

The Himalayan yew belongs to family (Taxaceae) and botanically classified as *Taxus wallichiana* Zucc (*T. wallichiana*). It is a evergreen medium sized tree, widespread on each side of the Himalaya, extending from Afghanistan to Burma. It is known as Birmi in Punjabi and European yew in English. In Pakistan it is found in Chitral, Hazara, Murre, Kuram and Poonch [1-2]. Traditionally *T. wallichiana* is used for the treatment of various ailments and coloring different materials. It is also use as antispasmodic and given to the patients of haemoptysis, epilepsy, asthma and in other spasmodic infectious conditions. The *T. wallichiana* fruits are used as sedative, antispasmodic and emmenagogues [3]. The leaves are often used for indigestion, epilepsy, nervousness, hysteria and prescribed as aphrodisiac. Another specie of the genus i.e. *Taxus cupidata* parts are used to treat kidney disorders, diabetes and diuresis in China [4]. *T. wallichiana* contains various chemical constituents including ill-defined alkaloids known as taxine [5-6]. Taxine is found in almost all parts of the *T. wallichiana*. An acid, known as

winterstein has also reported from *T. wallichiana* [7-8]. The uncommon compounds which included molting hormones [9-10], alkaloids [11] and specifically antitumor taxane [12] are also reported from *T. wallichiana* plant. Different ecdysones [10], lignins [13] taxane and terpenoids [12] have also been investigated from the plant.

## EPERMENTAL SECTION

### Plant material

Plant materials (twigs) were collected from the Thandiani region, Abbottabad, Khyber Pakhtunkhwa province of Pakistan in May, 2009. Taxonomic identification of the plant was done at the Department of Botany, Hazara University, Pakistan. A voucher specimen was deposited in the herbarium of the Department. The twigs of the plant were air-dried under shade for two consecutive months at room temperature. The dried plant materials were later powdered and stored in a polyethylene bags for further studies.

### Extraction

The dried and powdered twigs, 20 kg were soaked in ethanol with occasional stirring at room temperature for a period of one week. After filtration the filtrates were evaporated under reduced pressure at temperature below 50°C. The process was repeated thrice. The concentrated ethanol extracts of twigs (2 kg) were obtained. The crude extract was suspended in water and fractionated with different solvents namely *n*-hexane, chloroform, ethyl acetate, methanol and residue etc., to obtain different fractions A to E. These fractions were studied for antifungal, antibacterial, insecticidal, phytotoxic and cytotoxic activities.

### Fungal and bacterial strains

The sub fractions were tested for antimicrobial activities against six fungal and six bacterial strains. Bacterial strains used in the study were *Escherichia coli* ATCC 25922, *Bacillus Subtilis* ATCC 6633, *Shigella flexenari* (clinical isolate), *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC27853 and *Salmonella typhi* ATCC 19430. The fungal strains include *Trichophyton longifusus* (clinical isolate), *Candida albicans* ATCC 2091, *Aspergillus flavus* ATCC 32611, *Microsporium canis* ATCC 11622, *Fusarium solani* 11712 and *Candida glaberata* ATCC 90030. They were maintained on agar slants at 4°C. The bacterial strains were allowed to activate at 37°C for 24 h on nutrient agar (NA) and fungal strains on Sabouraud glucose agar (SGA), prior to any screening.

### Determination of antibacterial activity

The antibacterial tests were done by the hole diffusion method using a cell suspension of about  $1.5 \times 10^6$  CFU/mL, obtained from a Macfarland turbidity standard No. 0.5 [14].

The suspension was standardized by adjusting the optical density to 0.1 at 600 nm using Shimadzu, UV-VIS Spectrophotometer [15]. Holes of 6 mm diameter were then bored on the Mueller Hinton agar (MHA) plate (8 mm thick) and were filled with 50  $\mu$ L of sub fractions or standard drug(s). The inoculated plates were incubated at 37°C for 24 hours. Antibacterial activity was investigated by measuring the diameter of the zone of growth inhibition around the holes. The assay was repeated three times and then the mean diameter was calculated. In these bioassays imipenem, miconazole and amphotericin B were used as standard antibiotics for comparison.

### Determination of antifungal activity

In this method, extracts (10 mg/mL) were dissolved in DMSO and were allowed to serially dilute with sterile water in microplates in a laminar flow cabinet. An equal volume of actively growing cultures of the test fungi were mixed to the different wells and cultures were allowed to grow overnight in 100% humid environment at 37°C. At morning, tetrazolium violet was mixed to all the wells and the growth was observed by a violet color of the culture. The lowest concentration of the test solutions, which cause inhibition of growth, was taken as the minimum inhibitory concentration (MIC). In this bioassay, amphotericin B and miconazole were used as positive controls.

### Estimation of cytotoxic activity

In this experiment, a shallow rectangular plastic dish (22 x 32 cm), filled with artificial sea water was used. The sea water was obtained from commercial salt mixture, mixed with double distilled water. The Brine shrimp (*Artemia salina* leach) eggs were allowed to hatch in the dish. The dish was allowed to unequally partition by using an artificial perforated device. About 50 mg of the eggs were made sprinkled in to large compartment which becomes darken. The minor compartment was exposed to the ordinary light. After two days duration, Nauplius were collected and removed by a pipette from lighted side. Testing fraction was prepared by dissolving 20 mg of each compound in

dimethylformamide (DMF) (02 ml). Three different stock solutions i.e., 550, 50, and 5 mg/mL were transferred to 9 vials (three for every dilution were used for each test sample) with one vial containing DMF, was kept stored as a control. The solvent was allowed to evaporate by keeping overnight. Later on, after two days, when the shrimp larvae were ready, 1 mL of sea water and 10 shrimp were mixed to each vial (30 shrimps/ dilution) with a volume adjusted with sea water to 5 mL per vial. After a total duration of 24 hours, the numbers of survivors were made to count using standard procedure [16-18].

#### Estimation of insecticidal activity

The insecticidal bioassay was done by direct contact application of the test fractions using filter paper [19]. In this bioassay, 3 mL of all extract/fractions (1 mg/mL) were applied to the filter papers of (90 mm diameter). After making dried, each of this filter paper was kept in a separate petri dish along 10 adults of each of *Tribolium castaneum*, *Rhyzopartha dominica* and *Callosobruchus analis*. After duration of 24 hours, 10 test insects were placed in each plate and incubated at 27°C for 24 hours with 50% relative humidity in growth chamber. These entire insects were allowed to stand in the absence of food for 24h after which the mortality number was counted. The results were recorded as percent mortality, calculated with reference to the positive and negative controls. In this bioassay, permethrin (235.71 $\mu$ /cm<sup>3</sup>) was used as a reference insecticide, while acetone with test insects were used as negative controls (Table 4).

#### Determination of phytotoxic activity

In this study, the crude extract fractions were tested against *lemna minor* [19]. Stock solutions of extracts (10 mg/mL) were diluted to get final concentrations of the extracts as 500, 50, and 5 $\mu$ g/mL. The tested was conducted in triplicate. Nine sterilized flasks, three for each concentration, were used. To each flask was then added 20 mL medium and 10 plants, each one containing rosette of three fronds. Parquet was used as a standard growth inhibitor. All flasks were plugged with cotton and kept in the growth cabinet for 7 days. Afterward, the number of fronds per flask were counted and their growth regulation in percentage was calculated by the given formula:

$$\text{Growth regulation (\%)} = \left[ \frac{100 - \text{Number of fronds in test sample}}{\text{Number of fronds in negative control}} \right] \times 100$$

## RESULTS AND DISCUSSION

In Pakistan, *T. wallichiana* plant is traditionally used for the treatment of various diseases especially against microbial infections. In this study, different fractions of the ethanolic extract of *T. wallichiana* twigs were checked for antibacterial, antifungal, insecticidal, cytotoxic and phytotoxic activities.

The crude sub fractions of ethanolic extract of the plant were tested against six bacterial including *Escherichia coli* ATCC 25922, *Bacillus Subtilis* ATCC 6633, *Shigella flexenari* (clinical isolate), *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC27853 and *Salmonella typhi* ATCC 19430 and the results are expressed as inhibition zones (mm) in Table 1. Both the *n*-hexane and ethyl acetate fractions showed 15 and 24 mm zones of inhibition against *Staphylococcus aureus*, while the chloroform fraction exhibited 35 mm inhibition against *Shigella flexenari*. Rest of the fractions showed no activity against the tested microorganisms.

The antifungal activity of the extracts was checked for six fungal strains namely *Trichophyton longifusus* (clinical isolate), *Candida albicans* ATCC 2091, *Aspergillus flavus* ATCC 32611, *Microsporium canis* ATCC 11622, *Fusarium solani* 11712 and *Candida glaberata* ATCC 90030. The results of the assay are expressed as minimum inhibitory concentration (MIC) and are given in Table 2. The data reveals that both the chloroform and methanol fractions are active against *Microsporium canis* having 15  $\mu$ g/mL minimum inhibitory concentration, while ethyl acetate fraction also showed antifungal activity against *Candida albicans* and *Candida glaberata* at the concentrations of 27 and 13  $\mu$ g/mL, respectively. The rest of the fractions show no antifungal activity.

For cytotoxic activity, all crude extract fractions of the plant were tested against *Lemna minor*. and the results are expressed as % mortality. The cytotoxicity assay (Table 3), reveals that all the fractions have cytotoxic activity against Brine shrimp.

The insecticidal bioassay was conducted by direct contact application method for the test fractions against *Tribolium castaneum*, *Rhyzopertha dominica* and *Callosobruchus analis*. The results are presented in **Table 4**. The data showed that n-hexane, chloroform and methanol fractions are active. The n-hexane fraction showed 40 and 30% mortality against *Rhyzopertha dominica* and *Callosobruchus analis*, respectively. While chloroform and methanol fractions exhibit 20 and 27% mortality against *Callosobruchus analis* and *Tribolium castaneum*, respectively.

Phytotoxicity assay was carried out for all the extracts against *Lemna minor*. Parquet was used as a standard growth inhibitor. The results are expressed as % growth regulation (**Table 5**). All fractions show phytotoxic activity. The activity was found to be concentration dependent. The fractions having 500 µg/ml concentration showed the highest activity against *Lemna minor*, while moderate activity was observed for the fractions having 50 µg/ml concentrations. Only, the n-hexane, ethyl acetate and residue fractions showed no activity at the concentration level of 5 µg/ml.

**Table 1: Antibacterial activities of the extract fractions of *Taxus wallichiana* twigs**

Microorganism	Zone of Inhibition (mm)					
	Imipenem (drug)	A	B	C	D	E
<i>Escherichia coli</i>	35	-	-	-	-	-
<i>Bacillus subtilis</i>	36	-	-	-	-	-
<i>Shigella flexneri</i>	36	-	35	-	-	-
<i>Staphylococcus aureus</i>	43	15	-	24	-	-
<i>Pseudomonas aeruginosa</i>	32	-	-	-	-	-
<i>Salmonella typhi</i>	40	-	-	-	-	-

**Key words:** A = n-hexane, B = chloroform, C = ethyl acetate, D = methanol, E = residue

**Table 2: Antifungal activities of the extract fractions of *Taxus wallichiana* twigs**

Fungal species	Minimum Inhibitory Concentration (µg/mL)					
	Miconazole	A	B	C	D	E
<i>Trichophyton longifusus</i>	70.08	-	-	-	-	-
<i>Candida albicans</i>	110.80	-	-	27	-	-
<i>Microsporum canis</i>	98.40	-	15	-	15	-
<i>Fusarium solani</i>	73.10	-	-	-	-	-
<i>Candida glabrata</i>	110.80	-	-	13	-	-
	Amphotericin B					
<i>Aspergillus flavus</i>	20	-	-	-	16	-

**Key words:** A = n-hexane, B = chloroform, C = ethyl acetate, D = methanol, E = residue

**Table 3: Brine shrimp cytotoxicity of the extract fractions of *Taxus wallichiana* twigs**

Extract	Concentration (µg/ml)	% Mortality
n-Hexane	1000	43.4
	100	23.5
	10	16.4
Chloroform	1000	45.4
	100	25.5
	10	16.4
Ethyl acetate	1000	46.8
	100	34.3
	10	12.5
Methanol	1000	45.4
	100	32.2
	10	14.4
Residue	1000	44.3
	100	24.4
	10	15.6

**Key words:** A = n-hexane, B = chloroform, C = ethyl acetate, D = methanol, E = residue

**Table 4: Insecticidal activities of the extract fractions of *Taxus wallichiana* twigs**

Insect	% Mortality						
	+ve control	-ve control	A	B	C	D	E
<i>Tribolium castaneum</i>	100	0	0	0	0	10	0
<i>Rhizopertha dominica</i>	100	0	40	20	0	0	0
<i>Callosbruchus analis</i>	100	0	30	0	0	27	0

**Key words:** A = n-hexane, B = chloroform, C = ethyl acetate, D = methanol, E = residue

**Table 5: Phytotoxic activity of the extract fractions of *Taxus wallichiana* twigs**

Conc. of sample (µg/ml)	% Growth regulation						
	Paraquat (0.015 µg/ml)	-ve control	A	B	C	D	E
500	100	0	85	85	75	5 0	25
50	100	0	05	25	08	21	05
5	100	0	-	05	-	05	-

**Key words:** A = n-hexane, B = chloroform, C = ethyl acetate, D = methanol, E = residue

## CONCLUSION

The present study confirmed that the extract fractions of *Taxus wallichiana* twigs have antibacterial, antifungal, insecticidal and phytotoxic activity. The plant is frequently used for the treatment of various diseases. This data strongly supports that extensive research should be conducted to isolate phytochemical constituents responsible for antifungal, insecticidal and phytotoxic activities to elaborate the hidden medicinal potential of the plant.

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