



ISSN: 2231-539X

Selection of solvent and extraction method for determination of antimicrobial potential of *Taxus wallichiana* Zucc.

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ABSTRACT

Antimicrobial potential of different plant parts (needle, stem and bark) of Himalayan yew (*Taxus wallichiana* Zucc.) has been investigated with particular reference to selection of solvents and extraction methods. Two extraction methods (maceration and soxhlet), seven solvents (methanol, ethanol, acetone, chloroform, ethyl acetate, di chloro methane and petroleum ether), and 3 groups of microorganisms (bacteria, actinobacteria and fungi) were considered for detection of antimicrobial activity. While qualitative estimations were done using agar well diffusion method, quantitative analysis was based on dilution method. All the plant part showed significant activity against all 3 groups of microorganisms in qualitative bioassays; maximum being in case of needles. Among solvents, ethanolic extract of needles (maceration) showed highest antibacterial activity (15.33 ± 0.25 mm). Growth of actinobacteria was inhibited maximum (22.0 ± 0.26 mm) by the methanolic extracts of needles (maceration). Ethyl acetate extract of needles (soxhlet) showed higher antifungal activity (8.67 ± 0.23 mm). Antibacterial and antifungal activities were higher in maceration and soxhlet methods, respectively. The most affected group among the test microorganisms was bacteria which may be due to their prokaryotic organization. This was also supported by the low minimum inhibitory concentration (MIC) values. Di chloro methane and petroleum ether did not show any antifungal activity. The antimicrobial activity of various plant parts of *T. wallichiana* varied with respect to the solvent as well as the extraction method. The study will have implications in selection of the use of solvent and the extraction procedure in obtaining the antimicrobial metabolites from various plant parts of *T. wallichiana*.

KEY WORDS: *Taxus wallichiana*, antimicrobials, minimum inhibitory concentration (MIC), solvent, extraction method

Received: April 17, 2018

Accepted: May 20, 2018

Published: May 28, 2018

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INTRODUCTION

Antibiotic resistance phenomenon and development of the side effects due to consumption of microbe derived antibiotics suggested the need for alternate sources for combating the infectious diseases. In this perspective, plant based antimicrobials (derived from medicinal plants, in particular) are increasingly receiving attention for harnessing their potential in production of antimicrobial substances, as safer source of antibiotics. The Himalayan mountain ecosystem is well known for harbouring a plethora of medicinal and aromatic plants along with their domestication and cultivation for commercial purposes [1,2]. Plant extracts from a variety of medicinal plants showed potential antioxidant properties [3,4]. Now a day's, antimicrobial compounds are being utilized in various areas such as medical, pharmaceutical, textile and

dairy industries, in food-based products, cosmetics, personal care products, etc.

Taxus wallichiana Zucc. (English name: Himalayan Yew; Hindi name: Thuner; family: Taxaceae) is well recognized as a medicinally important evergreen tree that grows under temperate locations of Indian Himalaya. In the Indian subcontinent, the species grows in the northern hemisphere with its distribution in the hills of northern Jammu & Kashmir, Himachal Pradesh, Uttarakhand and the states in northeast namely Meghalaya, Nagaland, Arunachal Pradesh, and Manipur, at an altitude range of 1800-3300 m. The species has received considerable attention on account of its existing exploitation for the extraction of the drug (taxol) from its bark [5-7]. *T. wallichiana* is also known for its various ethanomedicinal uses [8] such as the leaf paste is used in treatment of asthma and bronchial disorders.

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Tea, made out of the stem bark of Himalayan yew, has been popular in Himalayan tribal communities for curing cold, cough and hypertension. The species is also known as source of antioxidants [4]. However, the plant species still needs to be highlighted for its antimicrobial potential.

Optimization of plant extracts for any activity, such as production of antimicrobials, is primarily important before isolation of antimicrobial compound(s). Several investigators have worked on the selection of suitable solvents for extraction along with the type of extraction method for assessing bioactive compounds including antimicrobials from different medicinal plants/parts [9-12]. Such reports are lacking in case of *T. wallichiana*, therefore, the focus of the present study is on the selection of solvent and extraction method in view of achieving maximum antimicrobial potential of the plant species. Therefore, the aim of the present study is to investigate the antimicrobial potential of *T. wallichiana* with respect to three major groups of microorganisms (bacteria, actinobacteria and fungi) considering seven solvents and two extraction methods.

MATERIALS AND METHODS

Study Site and Sample Collection

Plant samples were collected from Jageshwar area in District Almora (29°35' -29°39' N and 79°59' -79°53' E) of Uttarakhand, India. Herbarium of plant needle was submitted to herbarium record of G. B. Pant National Institute of Himalayan Environment and Sustainable Development, Kosi-Katarmal, Almora, Uttarakhand, India (Voucher number: GBPI 5050). The collected plant parts (needle, stem and bark) were washed, air dried and converted into fine powder for further experimental work.

Chemicals and Microorganisms

Solvents

Ethanol, methanol, acetone, chloroform, ethyl acetate, dichloro methane (DCM), and petroleum ether (PET) from Merck, India.

Microbiological media

Tryptone yeast extract agar (TYE agar), Potato dextrose agar (PDA) from Hi-media, Mumbai, India.

Test microorganisms

Bacteria: 2 Gram +ve = *Bacillus subtilis* (NRRL B-30408) and *B. megaterium* (MCC3124); 4 Gram -ve = *Pseudomonas chlororaphis* (MCC2693), *P. palleroniana* (MCC2692), *Escherichia coli*, and *Serratia marcescens* (MTCC4822).

Actinobacteria: *Nocardia tenirefensis* (MCC2012), and *Streptomyces* sp. (MCC2003).

Fungi: *Trichoderma viride* (ITCC4402), *Paecilomyces variotii* (ITCC3710), *Aspergillus niger* (ITCC2546), *Fusarium oxysporum* (ITCC4219), *F. solani* (ITCC 5017), *Pythium afertile* (ITCC4217), *Trametes hirsuta* (MTCC11397), *Phytophthora* sp., *Alternaria alternata* (ITCC807), and *Penicillium purpurogenum* (ITCC3684).

These test microorganisms were taken from the microbial culture collection, established in the Microbiology Lab of the Institute (GBPNIHESD). Accession numbers shown in the parentheses have been allocated by the National/International depositories: NRRL (Northern Regional Research Laboratory, Agricultural Research Service Patent Culture Collection, United States Department of Agriculture, Illinois; MCC (Microbial Culture Collection, National Centre for Cell Science, Pune, India); MTCC (Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India); ITCC (Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi, India).

Extraction

Needle, bark and stem were extracted through maceration and soxhlet methods, separately, using seven selected solvents (methanol, ethanol, acetone, chloroform, ethyl acetate, PET and DCM).

Maceration

2 g of plant sample (needle, bark, and stem, separately) was mixed in different solvents, separately, in a ratio of 1:5 (dry powder: solvents). The mouth of conical flask was sealed with the para- film. Samples were macerated using rotary shaker (Remi) at 160 rpm for 24 h.

Soxhlet

2 g of plant sample was placed in thimble and extracted with selected solvent, separately, in soxhlet extraction unit (MAC). Extraction was repeated until the sample extract became of color less.

Bioassays for Determination of Antimicrobial Potential of *T. wallichiana* Extracts

Plate based bioassays

For qualitative estimation of antimicrobial potential of *T. wallichiana* extracts, agar plate based bioassays were performed using disc diffusion method. Bacterial and actinobacterial culture suspensions were prepared in TYE agar while fungal culture suspension was prepared in PDA. 100 µl of all the test organisms (separately) were spread uniformly on the respective agar surface (TYE agar plates for bacteria and actinobacteria, and PD agar plates for fungus) with the help of a glass spreader. Sterilized 5 mm filter paper (Whatman No. 1) discs were placed over the agar surface with the help of sterile forceps. 15 µl of extract was loaded over the agar disc. The plates

were then incubated at 25 °C. The results were recorded on the basis of zone of inhibition (mm) after 24 h for bacteria and 120 h for actinobacteria and fungi. All the experiments were performed in triplicate.

Quantitative Estimation/Minimum Inhibitory Concentration (MIC)

MIC was determined following Clinical and Laboratory Standard Institute methodology [13]. Bacterial and actinobacterial culture suspensions were prepared in TYE while fungal cultures were prepared in PD broth. For determination of MIC, 1 ml extract was diluted using different concentration ranging from 100 to 1000 µg/ml, 1 ml test organism and 8 ml broth was taken in sterile test tube, and then incubated at 25 °C for 24 h for bacteria and 120 h for actinobacteria and fungi. Control was prepared in two sets, one containing broth medium and test organism while the other containing broth medium and extract. After 24 h, the MIC values were recorded on the basis of the lowest concentration showing absence of growth in the tubes. The test was further confirmed by plating on TYE agar and PDA medium.

Statistical Analysis

The data was expressed as the means ± standard errors (SE) from experiments, performed in triplicate. Statistical significance was determined using student's t-test. A *p* value <0.05 was considered as significant. Homogenizing grouping of all microorganisms was done separately using Duncan test in SPSS version 20.

RESULTS

Extraction Yield with Reference to Extraction (Soxhlet and Maceration) Methods

Figure 1 shows the extract yield of different plant parts of *T. wallichiana* (needle, stem and bark) extracted by different solvents and extraction methods. The extraction yield was recorded highest in stem in soxhlet ethanolic extract, while it was at par in case of acetone and methanol in soxhlet method. The extraction yield in bark was recorded highest in ethanolic extract in soxhlet and lowest in PET extract of maceration method. In needles, the yield was estimated higher in soxhlet methanolic and acetone extracts in comparison to the other extracts and it was recorded lowest in chloroform extract obtained by maceration method. Among all the plant parts of *T. wallichiana*, including the needle, stem and bark extracts following the maceration and soxhlet methods, the highest extract yield was found in needle extracts obtained by soxhlet method. Higher extract yield with polar solvents indicated towards the presence of more polar molecules in *T. wallichiana*. Low extract yield in ethyl acetate in comparison to other polar solvent was probably due to the poor dielectric constant. The extract weight of different part of *T. wallichiana* used were significantly different at *p* < 0.029.

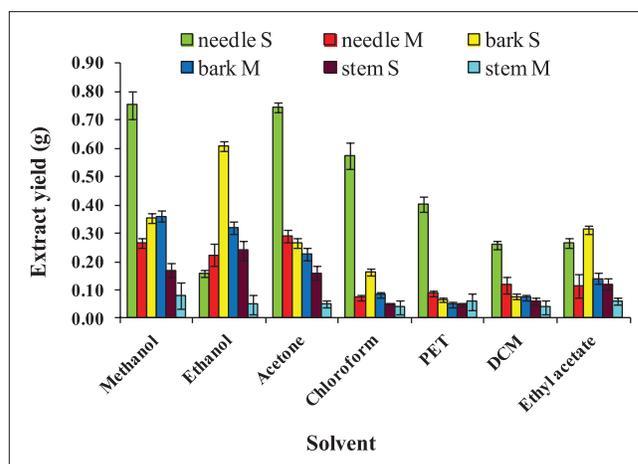


Figure 1: Extract yield of stem, needle and bark of *Taxus wallichiana*. S = soxhlet, M = maceration, PET= petroleum ether, DCM =di chloro methane extract

Antimicrobial Activity in Plant (Needle, Stem and Bark) Extracts

Qualitative plate based bioassays

Needle, bark and stem extracts of *T. wallichiana* showed antimicrobial activity against all the three groups of microorganisms viz. bacteria, actinobacteria and fungi. Tables 1-3 and Figure 2 represent the antimicrobial activity of needle, bark and stem extracts, separately, in both the extraction methods. While all the solvents were effective in revealing the antibacterial activity in *T. wallichiana* extracts, PET and DCM showed selectivity in this aspect. Further, these two solvents (PET and DCM) did not show any activity against actinobacteria and fungi. Amongst bacteria, species of *Bacillus* were the most affected ones in terms of showing inhibition by *T. wallichiana* extracts, while *E. coli* was the least affected. On the basis of Duncan test used for homogeneous grouping of microorganisms separately, out of the two test species of *Bacillus*, *B. subtilis* showed higher inhibition in case of ethanolic extract of macerated needles and *B. megaterium* in case of methanolic extract of macerated needles. The inhibition of *E. coli* in macerated needle acetone extract, *S. marcescens* in macerated needle and bark methanolic extracts, *P. chlororaphis* in macerated needle and bark ethanolic extracts and *P. palleroniana* in soxhlet needle methanolic extract was at par. The actinobacterial species, *N. tenirefensis* and *Streptomyces* sp., were highly inhibited in macerated methanolic needle extract and macerated needle and bark methanolic extracts, respectively.

Among 10 test fungal species, only 5 (*A. niger*, *F. oxysporum*, *F. solani*, *P. variotii* and *T. hirsuta*) got affected by the *T. wallichiana* extracts. Species of *Alternaria*, *Penicillium*, *Phytophthora*, *Pythium* and *Trichoderma* were not at all affected by the *T. wallichiana* extracts. The fungal species namely *A. niger*, *F. oxysporum*, and *F. solani* were inhibited maximally in soxhlet needle ethyl acetate extract. Likewise, *P. variotii* was maximally affected in macerated needle ethanolic extract and *T. hirsuta* in bark ethanolic extract. Figures 3-5 present the comparative

Table 1: Antimicrobial activity of *T. wallichiana* needle

Microorganism	Zone of inhibition (mm)									
	Methanol		Ethanol		Acetone		Chloroform		Ethyl acetate	
	Sox	Mac	Sox	Mac	Sox	Mac	Sox	Mac	Sox	Mac
	Gram+ve									
<i>B. subtilis</i>	12.00±0.32	14.7±0.32	8.80±0.28	15.33±0.25	5.00±0.35	13.33±0.18	6.00±0.22	6.0±0.22	2.46±0.36	-
<i>B. megaterium</i>	8.33±0.42	14.2±0.36	11.33±0.27	13.47±0.25	5.67±0.37	12.43±0.43	5.40±0.32	5.4±0.32	2.34±0.34	-
	Gram -ve									
<i>E. coli</i>	8.33±0.20	9.00±0.34	6.34±0.44	7.30±0.38	6.67±0.28	10.40±0.43	2.70±0.20	2.7±0.20	2.14±0.15	2.67±0.37
<i>P. chlororaphis</i>	8.95±0.39	10.32±0.19	8.00±0.51	12.40±0.20	7.00±0.25	8.56±0.43	2.70±0.37	4.7±0.37	5.00±0.16	6.32±0.43
<i>P. palleroniana</i>	10.11±0.26	9.37±0.26	8.00±0.57	8.33±0.39	8.24±0.47	8.67±0.31	3.27±0.24	5.2±0.24	6.33±0.51	7.33±0.33
<i>S. marcescens</i>	9.00±0.29	15.20±0.56	11.00±0.23	12.67±0.44	10.33±0.45	13.98±0.34	7.90±0.31	2.9±0.31	4.50±0.38	5.33±0.26
	Actinobacteria									
<i>N. tenirefensis</i>	22.00±0.26	22.00±0.26	7.33±0.25	10.67±0.31	11.00±0.45	7.00±0.21	5.00±0.30	5.0±0.20	2.50±0.58	8.45±0.33
<i>Streptomyces</i> sp.	13.00±0.43	16.34±0.33	8.33±0.39	11.33±0.25	5.67±0.34	7.67±0.17	11.00±0.27	11.00±0.37	6.50±0.47	7.00±0.31
	Fungi									
<i>A. niger</i>	4.33±0.39	6.76±0.31	7.20±0.43	7.67±0.43	6.34±0.56	6.34±0.34	3.00±0.44	3.0±0.37	8.00±0.33	2.76±0.09
<i>F. oxysporum</i>	2.37±0.32	3.81±0.32	2.30±0.28	5.78±0.20	-	-	-	-	8.67±0.23	7.84±0.21
<i>F. solani</i>	4.45±0.20	2.33±0.31	3.33±0.23	5.32±0.26	3.17±0.37	5.65±0.26	4.30±0.21	4.3±0.21	6.40±0.05	2.42±0.26
<i>P. variotii</i>	4.32±0.45	5.76±0.42	6.12±0.07	6.67±0.35	5.40±0.35	6.40±0.26	3.30±0.26	3.3±0.26	3.50±0.06	2.98±0.37
<i>T. hirsuta</i>	4.00±0.40	4.27±0.20	3.00±0.25	3.00±0.25	-	-	-	-	-	-

(Sox=soxhlet, Mac=maceration)

Table 2: Antimicrobial activity of *T. wallichiana* bark

Microorganism	Zone of inhibition (mm)									
	Methanol		Ethanol		Acetone		Chloroform		Ethyl acetate	
	Sox	Mac	Sox	Mac	Sox	Mac	Sox	Mac	Sox	Mac
	Gram+ve									
<i>B. subtilis</i>	7.00±0.24	14.32±0.32	8.00±0.24	15.33±0.36	5.00±0.15	13.49±0.18	6.00±0.22	6.0±0.22	4.46±0.36	4.76±0.33
<i>B. megaterium</i>	10.33±0.32	13.74±0.16	9.33±0.28	13.97±0.25	5.67±0.17	13.33±0.43	5.40±0.32	5.4±0.32	4.34±0.34	5.46±0.36
	Gram -ve									
<i>E. coli</i>	6.00±0.24	9.30±0.34	5.00±0.42	7.00±0.38	5.67±0.23	10.00±0.13	2.70±0.20	2.7±0.20	2.14±0.32	2.67±0.38
<i>P. chlororaphis</i>	8.67±0.34	10.32±0.29	6.00±0.28	12.00±0.20	6.00±0.35	8.00±0.43	4.70±0.37	4.7±0.37	5.00±0.16	6.32±0.41
<i>P. palleroniana</i>	7.57±0.32	9.60±0.24	8.00±0.37	7.33±0.39	8.67±0.27	8.32±0.31	5.27±0.24	5.2±0.24	6.33±0.35	7.33±0.35
<i>S. marcescens</i>	10.00±0.39	15.00±0.36	10.00±0.28	12.67±0.44	9.33±0.33	13.67±0.14	2.90±0.31	2.9±0.31	4.50±0.38	5.33±0.22
	Actinobacteria									
<i>N. tenirefensis</i>	18.70±0.36	20.00±0.27	7.43±0.35	10.67±0.31	10.00±0.45	7.00±0.28	5.00±0.30	5.0±0.33	3.50±0.58	8.45±0.28
<i>Streptomyces</i> sp.	11.00±0.43	16.00±0.31	9.52±0.39	11.33±0.27	7.67±0.34	7.27±0.37	11.00±0.37	11.00±0.27	6.50±0.47	7.00±0.21
	Fungi									
<i>A. niger</i>	4.23±0.33	6.76±0.33	7.00±0.28	7.43±0.29	6.56±0.56	6.0±0.24	3.00±0.14	3.0±0.37	6.13±0.33	1.76±0.26
<i>F. oxysporum</i>	2.97±0.34	3.21±0.34	2.67±0.38	4.00±0.22	3.54±0.24	3.87±0.24	2.67±0.24	2.23±0.24	7.84±0.21	6.67±0.25
<i>F. solani</i>	4.45±0.25	2.33±0.24	4.73±0.34	5.32±0.24	3.67±0.37	5.65±0.26	4.30±0.31	4.3±0.21	6.00±0.05	2.32±0.36
<i>P. variotii</i>	3.97±0.41	5.76±0.43	6.00±0.27	6.57±0.32	5.33±0.35	6.00±0.26	3.30±0.26	3.3±0.26	3.00±0.06	1.98±0.31
<i>T. hirsuta</i>	4.20±0.42	3.67±0.24	4.10±0.25	5.08±0.24	-	-	-	-	-	-

(Sox=soxhlet, Mac=maceration)

assessment of all the plant part extracts in different solvents and extraction methods in terms of their inhibitory effect on 3 groups of microorganisms viz. bacteria, actinobacteria and fungi. In general, *T. wallichiana* extracts, made in DCM and PET, showed very low antibacterial activity and no antifungal activity. *T. wallichiana* extracts in macerated ethanolic, methanolic and acetone showed good antibacterial and antiactinobacterial activity, soxhlet ethyl acetate, methanolic and ethanolic extract showed good antifungal activity, indicated by the formation of zone of inhibition. Ethyl acetate extract showed good inhibition activity against species of *Fusarium* (*F. oxysporum* and *F. solani*). Furthermore, the aqueous *T. wallichiana* extracts did not show any antimicrobial activity.

Overall, both the extraction methods (soxhlet and maceration) were effective in revealing the antimicrobial activity. However,

if compared on the basis of the groups of the microorganisms, maceration extracts were better in terms of exhibiting inhibition of bacteria and actinobacteria, while soxhlet was better in case of the species of fungi. Among the solvents, macerated ethanolic needle extract showed highest antibacterial activity (15.33 ± 0.25 mm), macerated methanolic needle extract showed highest actinobacterial (22.0±0.26 mm), and soxhlet ethyl acetate needle extract was best in exhibiting high antifungal activity (8.67±0.23 mm).

Quantitative estimation

The MIC was performed using five solvents i.e. chloroform, ethyl acetate, acetone, ethanol and methanol, out of which the acetone, ethanol and methanol extracts showed good results against all the group of test microorganisms. The

Table 3: Antimicrobial activity of *T. wallichiana* stem

Microorganism	Zone of inhibition (mm)									
	Methanol		Ethanol		Acetone		Chloroform		Ethyl acetate	
	Sox	Mac	Sox	Mac	Sox	Mac	Sox	Mac	Sox	Mac
	Gram +ve									
<i>B. subtilis</i>	6.33±0.25	7.30±0.25	8.33±0.27	7.00±0.37	6.67±0.26	7.34±0.39	4.62±0.44	5.32±0.37	-	-
<i>B. megaterium</i>	7.33±0.39	8.00±0.38	8.66±0.43	9.00±0.26	7.89±0.25	8.54±0.44	3.53±0.26	6.00±0.39	-	-
	Gram -ve									
<i>E. coli</i>	8.00±0.39	8.70±0.44	8.33±0.28	8.67±0.43	8.67±0.25	9.30±0.22	7.02±0.39	9.00±0.20	6.87±0.24	7.00±0.35
<i>P. chlororaphis</i>	7.67±0.22	8.00±0.28	6.40±0.34	6.33±0.51	5.26±0.25	5.30±0.28	4.96±0.27	5.30±0.23	4.71±0.18	4.00±0.20
<i>P. palleroniana</i>	7.00±0.24	7.70±0.42	8.50±0.26	8.77±0.57	5.37±0.21	6.04±0.22	6.08±0.46	6.70±0.26	4.97±0.39	4.67±0.51
<i>S. marcescens</i>	8.67±0.20	9.30±0.22	8.70±0.47	9.00±0.20	7.36±0.32	7.70±0.29	3.22±0.34	3.30±0.20	4.39±0.46	4.50±0.39
	Actinobacteria									
<i>N. tenirefensis</i>	9.33±0.36	10.00±0.25	8.52±0.26	8.00±0.20	5.09±0.26	5.00±0.26	2.56±0.23	2.72±0.13	2.31±0.36	2.67±0.16
<i>Streptomyces</i> sp.	9.00±0.34	9.71±0.38	8.91±0.31	8.37±0.39	7.33±0.38	8.00±0.45	2.85±0.21	3.00±0.26	2.14±0.29	3.00±0.48
	Fungi									
<i>A. niger</i>	3.00±0.39	4.30±0.25	3.30±0.34	4.43±0.23	-	2.30±0.36	-	-	-	-
<i>F. oxysporum</i>	-	-	-	-	-	-	-	-	-	-
<i>F. solani</i>	2.47±0.20	1.70±0.25	2.03±0.27	3.00±0.33	-	-	-	-	-	-
<i>P. variotii</i>	2.67±0.45	3.30±0.28	4.70±0.29	3.67±0.26	-	2.00±0.36	-	-	-	-
<i>T. hirsuta</i>	-	-	-	-	-	-	-	-	-	-

(Sox=Soxhlet, Mac=maceration)

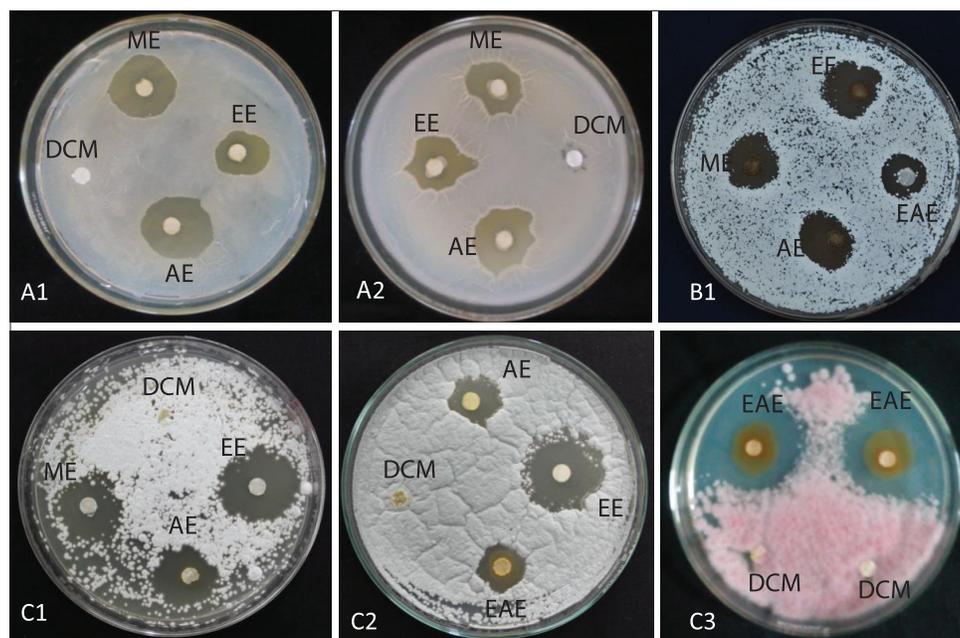


Figure 2: Antimicrobial activity of *T. wallichiana*: (A) Antibacterial activity, (B) Antiactinobacterial activity, and (C) Antifungal activity. DCM=di chloro methane extract, ME=methanolic extract, EE=ethanolic extract, AE=acetone extract, EAE=ethyl acetate extract

most affected group among the three was bacteria, which is probably due to their prokaryotic organization. This was also supported by the low MIC values. Different microorganisms showed variable response towards these different extracts. The results of MIC of the needle, stem and bark extracts are presented in Tables 4-6.

DISCUSSION

In the present investigation, extracts of various parts of *T. wallichiana* have been evaluated for determination of their antimicrobial activity against three major groups

of microorganisms bacteria (Gram -ve and Gram +ve), actinobacteria and fungi. The selection of seven different solvents used in this study was based on their polarity and dielectric constant which, starting from the lower dielectric constant values, can be arranged as follows: di chloro methane (1.3) < petroleum ether (4.3) < chloroform (4.3) < ethyl acetate (6) < acetone (20.7) < ethanol (24.6) < methanol (32.7). As depicted in results, extraction yield with polar solvents was higher in comparison to non-polar solvents. These variations may be due to the difference in their polarity as well as dielectric constant, which play vital role in the solubility of phytochemical compounds in respective

Table 4: Minimum inhibitory concentration (MIC) of *T. wallichiana* needle extracts

Microorganism	MIC (µg/ml)									
	Methanol		Ethanol		Acetone		Chloroform		Ethyl acetate	
	Sox	Mac	Sox	Mac	Sox	Mac	Sox	Mac	Sox	Mac
	Gram+ve									
<i>B. subtilis</i>	400	150	400	300	400	150	600	550	900	-
<i>B. megaterium</i>	300	200	300	200	400	200	400	350	900	-
	Gram -ve									
<i>E. coli</i>	400	400	600	600	400	300	800	800	900	850
<i>P. chlororaphis</i>	400	400	400	300	500	650	200	500	700	700
<i>P. palleroniana</i>	300	600	400	400	300	650	600	300	800	600
<i>S. marcescens</i>	300	200	300	300	600	200	750	600	700	800
	Actinobacteria									
<i>N. tenirefensis</i>	100	200	400	300	200	600	700	600	900	300
<i>Streptomyces sp.</i>	200	200	500	350	400	600	500	350	700	600
	Fungi									
<i>A. niger</i>	700	600	700	800	700	600	500	800	900	900
<i>F. oxysporum</i>	700	600	800	300	-	-	700	-	700	600
<i>F. solani</i>	800	800	900	500	500	500	500	700	700	800
<i>P. variotii</i>	800	700	500	500	500	600	700	700	800	850
<i>T. hirsuta</i>	700	450	900	800	-	-	-	-	-	-

(Sox=soxhlet, Mac=maceration)

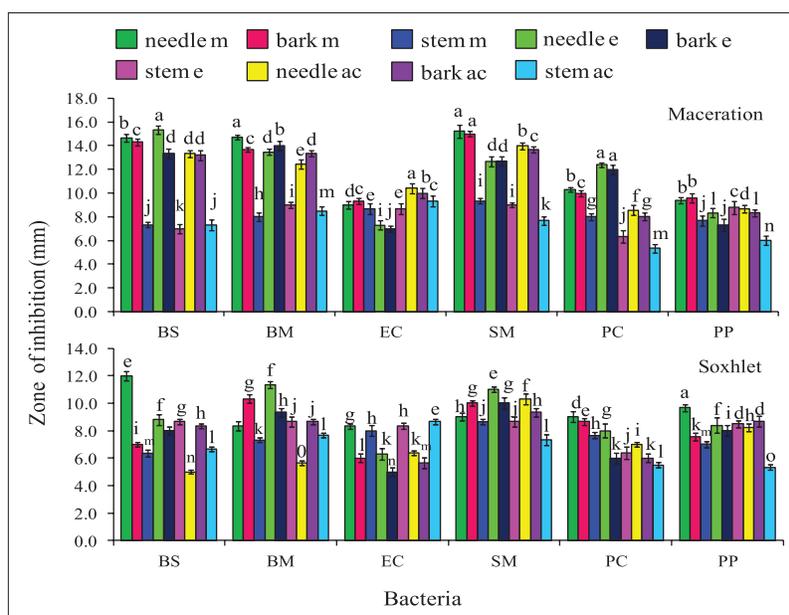


Figure 3: Antibacterial activity of needle, stem and bark of *T. wallichiana*. Methanol= m, ethanol= e, acetone= ac, *B. subtilis*=BS, *B. megaterium*=BM, *E. coli*= EC, *S. marcescens*= SM, *P. chlororaphis*= PC, *P. palleroniana*= PP. Homogenize grouping done with respect to antibacterial activity by Duncan test is denoted by the alphabets a-o

solvents. Therefore, this result confirms the effect of solvent system on the extract yield that consequently confirms the richness of this plant species (*T. wallichiana*) in polar substances [14,15].

The results in the present study coincide with the earlier report of Patel *et al.* [16] on the antimicrobial activity of *T. baccata* needles which was demonstrated through the extracts made in different solvents. In the cited study, ethanol extracts of *T. baccata* showed highest inhibition activity against the selected test bacteria as also in the case of the present study. This preliminary investigation

shows that all the polar solvent extracts, such as ethanol, methanol, acetone, ethyl acetate, were active against the tested microorganisms while the non-polar solvent extracts were not effective in inhibiting the microbial growth. These results are also supported by the previous findings of Sati *et al.* [11] and Nisar *et al.* [17]. Contrary to these results in various plant species, the extracts of *Crossandra infundibuliformis* prepared in PET have been reported with higher antifungal activity [18]. An earlier study carried on *T. wallichiana* needles growing in North-western Frontier Province, Pakistan, reported the absence of antibacterial activity in methanol fractions of the various plant parts [19].

Table 5: Minimum inhibitory concentration (MIC) of *T. wallichiana* bark extracts

Microorganism	MIC ($\mu\text{g/ml}$)									
	Methanol		Ethanol		Acetone		Chloroform		Ethyl acetate	
	Sox	Mac	Sox	Mac	Sox	Mac	Sox	Mac	Sox	Mac
	Gram+ve									
<i>B. subtilis</i>	300	200	500	250	500	700	400	400	400	800
<i>B. megaterium</i>	400	300	200	200	500	700	800	500	800	700
	Gram -ve									
<i>E. coli</i>	500	600	600	600	600	800	800	800	800	400
<i>P. chlororaphis</i>	400	600	600	400	400	700	700	400	400	650
<i>P. palleroniana</i>	500	500	500	600	600	800	800	400	400	600
<i>S. marcescens</i>	300	500	500	300	300	500	500	500	500	750
	Actinobacteria									
<i>N. tenirefensis</i>	400	600	600	300	300	300	300	700	700	700
<i>Streptomyces</i> sp.	600	600	600	600	600	400	400	600	600	700
	Fungi									
<i>A. niger</i>	900	850	900	700	700	650	700	900	-	-
<i>F. oxysporum</i>	-	700	-	900	-	700	-	900	-	-
<i>F. solani</i>	800	900	800	600	900	800	900	950	700	700
<i>P. variotii</i>	800	700	700	700	800	600	800	950	-	-
<i>T. hirsuta</i>	900	900	800	900	-	-	-	-	-	-

(Sox=soxhlet, Mac=maceration)

Table 6: Minimum inhibitory concentration (MIC) of *T. wallichiana* stem extracts

Microorganism	MIC ($\mu\text{g/ml}$)									
	Methanol		Ethanol		Acetone		Chloroform		Ethyl acetate	
	Sox	Mac	Sox	Mac	Sox	Mac	Sox	Mac	Sox	Mac
	Gram+ve									
<i>B. subtilis</i>	500	600	600	700	700	950	950	700	-	-
<i>B. megaterium</i>	600	600	600	700	700	950	950	700	-	-
	Gram -ve									
<i>E. coli</i>	650	600	600	650	650	900	900	800	800	650
<i>P. chlororaphis</i>	650	650	650	600	600	850	850	850	850	800
<i>P. palleroniana</i>	600	650	650	650	650	800	800	800	800	700
<i>S. marcescens</i>	750	600	600	700	700	900	900	900	900	700
	Actinobacteria									
<i>N. tenirefensis</i>	450	300	700	600	600	950	950	800	800	800
<i>Streptomyces</i> sp.	450	300	700	600	600	900	900	850	850	800
	Fungi									
<i>A. niger</i>	850	800	800	800	-	700	-	-	-	-
<i>F. oxysporum</i>	-	-	-	-	-	-	-	-	-	-
<i>F. solani</i>	900	900	800	800	-	700	-	-	-	-
<i>P. variotii</i>	950	750	850	850	-	800	-	-	-	-
<i>T. hirsuta</i>	-	-	-	-	-	-	-	-	-	-

(Sox=soxhlet, Mac=maceration)

Demonstration of the antibacterial activity in the methanolic extracts of all the plant parts (needles, stem and bark) of *T. wallichiana* belonging to Indian Himalayan region, in the present study, appears to be an indicative of the influence of different climatic conditions.

Among the two extraction methods used, the soxhlet method resulted in higher yield in comparison to the maceration method. Interestingly, the antimicrobial activity was recorded higher in macerated extracts. This could be attributed to the higher solubility of extractable bioactive components such as phenols, flavanols, tannin and flavonoids, having antimicrobial potential. These results were also supported by the earlier findings of Felhi *et al.* [15,20]. The cited reports have shown that extraction in different solvents influence the extraction

yield of total phenol content along with the antioxidants and antimicrobial activity. The variation in the extraction yield could also be due to the difference in polarity of the solvents used which plays a key role in increasing the solubility of phytochemical compounds [21,22].

Significant variation in MIC recorded in all the extracts demonstrated towards the variable contribution of the method of extraction and selection of solvent as well as the type of test microorganisms used. Also, variation in MIC of different plant extracts may arise from variation in their chemical constituents and their volatiles indicating the presence of one or more chemical moieties in the crude extract contributing towards the antimicrobial activity. This is proven by the varying results showing highest antifungal

activity in needle ethyl acetate extract (soxhlet), and highest antibacterial activity in leaf ethanolic extract (maceration). Ethyl acetate soxhlet needle extracts are likely to contain

the compounds with the capability to target eukaryotic cells, while ethanolic macerated needle extract composition is suitable to target prokaryotic cells which needs to be further explored [23,24].

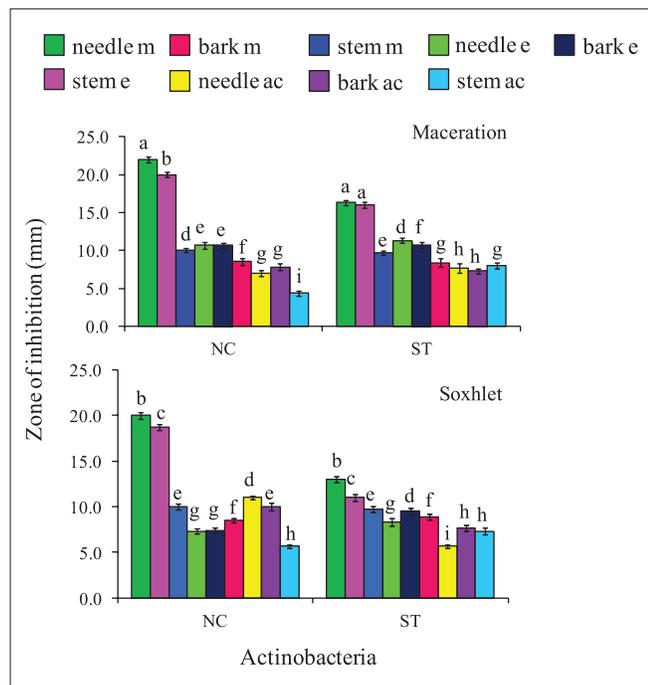


Figure 4: Antiactinobacterial activity of needle, stem and bark of *T. wallichiana*. Methanol= m, ethanol= e, acetone= ac, *N. tenrefensis*= NC, *Streptomyces* sp.= ST. Homogenize grouping done with respect to antiactinobacterial activity by Duncan test is denoted by the alphabets a-i

On the basis of the results obtained, it is concluded that among the various plant parts, needles of *T. wallichiana* possess relatively higher antimicrobial activity. As needle is renewable part of the plant, its utilization for harnessing the antimicrobial potential is recommended. For further isolation of the antibacterial and antifungal compounds, maceration and soxhlet methods are also recommended, respectively. Higher antimicrobial activity achieved in the polar solvents is indicative of the presence of the antimicrobial activity in the polar compounds. On the other side, the resistance to the *T. wallichiana* extracts in fungi, such as *Trichoderma*, is indicative of the mutualistic interaction and the possibility of its use as biopesticide.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

ACKNOWLEDGEMENTS

Authors are grateful to Director GBPNIHESD for extending the facilities and National Mission on Himalayan Studies (Ministry of Environment, Forest & Climate Change, Govt. of India, New Delhi) for financial support.

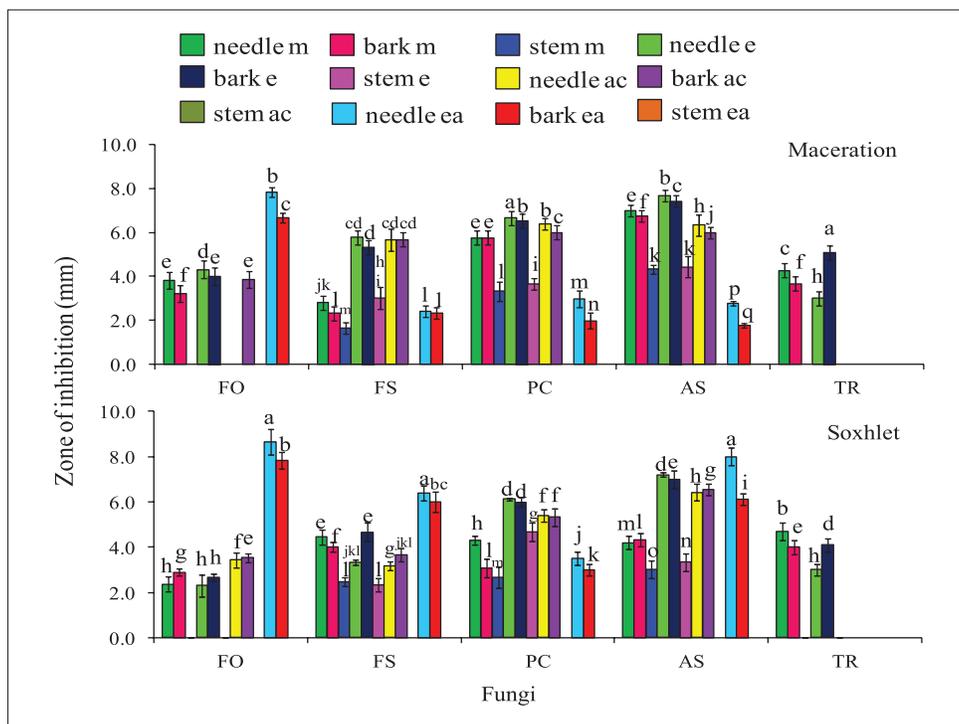


Figure 5: Antifungal activity of needle, stem and bark of *T. wallichiana*. Methanol= m, ethanol= e, acetone= ac, ethyl acetate= ea, *F. oxysporum*= FO, *F. saloni*= FS, *P. variotti*= PC, *A. niger*= AS, *T. hirsuta*= TR. Homogenize grouping done with respect to antifungal activity by Duncan test is denoted by the alphabets a-q

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