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Original Research Article (Experimental)

Evaluation of antimicrobial potential of successive extracts of *Ulmus wallichiana* Planch

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ABSTRACT

Background: The plant *Ulmus wallichiana* Planch. is found in hills of Uttarakhand, India. Bark of *U. wallichiana* is commonly used as traditional healer for bone fracture of animals as well as human beings and also used as wound healer remedy.
Objective: The present study was designed to evaluate antimicrobial potential of various extracts of *U. wallichiana* bark.
Materials and methods: Soxhlet extraction method was used for preparation of different extracts viz. petroleum ether, chloroform, ethyl acetate, ethanol and aqueous. Antioxidant activity was determined by DPPH and FRAP assay method. *In vitro* antimicrobial activity was evaluated using agar well diffusion method.
Results: Ethyl acetate extract exhibited the highest significant antioxidant activity. Antibacterial activity was performed against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and *Pseudomonas aeruginosa.* Amongst the various extracts tested, only ethyl acetate exhibited highest zone of inhibition as compared to other extracts and greater than standard drug. Chloroform extract also showed moderate zone of inhibition. Antifungal activity was evaluated against *Aspergillus fumigates* and *Aspergillus flavus.* The ethyl acetate extract showed maximum zone of inhibition as compared to other extracts. Chloroform extract showed mild antifungal activity. Chloramphenicol and nystatin were used as a positive control as antibacterial and antifungal agent respectively. Furthermore, the highest percentage of phenolic and flavonoid compounds was estimated in ethyl acetate extract.
Conclusion: The ethyl acetate extract of *U. wallichiana* showed the highest antimicrobial activity, and should be further investigated for isolating active compound(s) responsible for antimicrobial activity.

1. Introduction

In the current scenario, there have been increasing antibiotic resistant strains of clinically important pathogens, which have led to the emergence of new bacterial strains that are multi-resistant [1]. The non-availability and high cost of new generation antibiotics with limited effective span have resulted in increase in morbidity and mortality [2]. Therefore, there is an urgent need to look out for new substances from other sources with proven antimicrobial activity. Consequently, this has led to the search for more effective antimicrobial agents of plant origin, with the aim of discovering potentially useful active phytoconstituents that can serve as a source and template for the synthesis of new antimicrobial agents [3].

The plant *Ulmus wallichiana* Planch. (family: Ulmaceae) found in western Himalayan regions of India, is one of the richest emporiums for medical taxa. Traditionally, *U. wallichiana* has been used in the treatment of digestive tract diseases [4]. Bark of this tree is commonly used for bone fracture healing of animals as well as human beings as folk medicine in Uttarakhand (India) [5]. Bark paste of the plant is mentioned in drugs with the potential of wound healing [6]. Other uses of the plant in public domain include treatment of health related disorders with osteoporosis. Leaves are used as fodder for sheep and goats in Jammu and Kashmir (India) [7].

Phytochemically, the plant *U. wallichiana* contains aliphatic hydrocarbon and triterpenes (Masoodi et al., 2013). Some other allelopathic compounds identified are alnulin, betulin, caffic acid,

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catechol, lupenol, ferulic acid, scopoletin and vanillin [8]. A flavonol 2S, 3S-Aromadendrin-6-C-β-D-glucopyranoside [9], ulmoside A, B and Naringenin-6-C-beta-glucopyranoside are also reported in the bark [10]. Despite a long history of use of *U. wallichiana* as a traditional medicine for the treatment of various ailments, especially for it's wound healing property, the plant has never been subjected to antimicrobial activity studies. Thus, it was considered worthwhile to evaluate *U. wallichiana* for antimicrobial potential.

2. Material and methods

2.1. Plant material

The plant material (*U. wallichiana*) was collected from near, Budhar, Budhakedar Nath Ghansali, Tehri Garhwal, Uttarakhand (India). The plant was identified and authenticated at the herbarium of Botanical Survey of India, Dehradun (India) vide reference no. BSI/NRC Tec/Herb (Ident.)/2016-17/455.

2.2. Preparation of extracts

The dried parts of the plant *U. wallichiana* (barks) were collected, and then pulverized through a mechanical grinder. The powdered material was dried in hot air oven at moderate temperature. The powdered material was subjected to successive Soxhlet extraction by solvent in increasing polarity viz. petroleum ether, chloroform, ethyl acetate, methanol and then macerated with water. After that, extract was concentrated and stored at 4 °C until further use in the equipment.

2.3. Determination of total phenolic and flavonoid content

2.3.1. Determination of total phenolic content

One mg/ml of plant extracts were prepared in methanol and then, diluted with 10 ml of distilled water. Then, 1.5 ml of Folin Ciocalteu reagent was added and allowed to incubate at room temperature for 5 min. 4 ml of 20% w/w sodium carbonate solution was added in each test tube and then, further adjusted with distilled water up to the mark of 25 ml, agitated and left to stand for 30 min at room temperature. Absorbance of test samples was measured at 765 nm using spectrophotometer against blank (distilled water) [11].

The phenolic content was calculated with the help of standard curve equation and the formula is given below (Ayurvedic pharmacopeia of India, 2008).

$$\text{Total phenolic content}(\%\ \text{w/w}) = \frac{\text{GAE} \times \text{V} \times \text{D} \times 10^{-6}}{\text{w}} \times 100$$

where

GAE – Gallic acid equivalent (micro gram/ml)
V – Total volume of sample (ml)
D – Dilution factor
W – Sample weight (gm)

2.3.2. Determination of total flavonoid content

The total flavonoid content of all extracts of *U. wallichiana* was estimated according to the aluminum chloride method as follows: Aliquots of extracts 1 mg/ml solution was taken and the diluted standard solution (0.5 ml) was separately mixed with 1.5 ml of methanol (95%), 0.1 ml of aluminum chloride (10%), 0.1 ml of 1 M (potassium acetate) and 2.8 ml of distilled water. Absorbance at 415 nm was recorded after 30 min of incubation against blank (distilled water). The concentration of flavonoid in the test samples was calculated and expressed as mg quercetin equivalent/g of sample [12].

The total flavonoid content was expressed in milligrams of rutin equivalent per gram of extracts.

Percentage of total flavonoid content calculated in mg/g as:

$$\text{Total flavonoid content}(\%\text{w/w}) = \frac{\text{RE} \times \text{V} \times \text{D} \times 10^{-6}}{\text{w}} \times 100$$

where

RE – Rutin equivalent (micro gram/ml)
V – Total volume of sample (ml)
D – Dilution factor
W – Sample weight (gm)

2.4. Antioxidant activity

2.4.1. DPPH assay

Free radical scavenging activity was measured by the spectrophotometric method. Stock solution of DPPH (2, 2 di-phenyl 1-picryl hydrazyl) (15 mg in 10 ml methanol) was prepared such that 75 μl of it in 3 ml of methanol gave an initial absorbance of 0.9. Decrease in the absorbance in presence of sample extract at different concentration (0.2, 0.4, 0.6, 0.8 and 1 ml) was noted after 15 min. IC_{50} was calculated from % inhibition [13].

2.4.1.1. Protocol for DPPH free radical scavenging activity

1. Preparation of stock solution of test sample: 100 mg test sample was dissolved in 100 ml methanol to get 1000 μg/ml solution.
2. Dilution of test solution: 1, 2, 3, 4 and 5 stock were taken and diluted up to 10 ml methanol to get 200, 400, 600, 800 and 1000 μg/ml solution.
3. Preparation of DPPH solution: 15 mg of DPPH was dissolved in 10 ml of methanol. The resulting solution was covered with aluminum foil to protect from light.
4. Estimation of DPPH scavenging activity:
   a) 75 μl of DPPH solution was taken and the final volume was adjusted to 3 ml with methanol, absorbance was taken immediately at 517 nm for control reading.
   b) 75 μl of DPPH solution and 50 μl of the test sample of different concentrations were put in a series of volumetric flasks and final volume was adjusted to 3 ml with methanol.
   c) Absorbance at zero time was taken in UV–visible at 517 nm for each concentration.
   d) Final decrease in absorbance of DPPH with sample of different concentrations was measured after 30 min at 517 nm.
   e) 75 μl of DPPH and 50 μl of methanol were used as a negative control and single methanol blank.

Table 1
Yield of various extracts of *U. wallichiana*.

S. no.	Extract	Yield (% w/w)
1	Petroleum ether	3.8
2	Chloroform	3.56
3	Ethyl acetate	4.40
4	Ethanol	5.0
5	Water	4.90

Table 2
Total phenolic and flavonoid content in different extracts of *U. wallichiana*.

S. no.	Plant extracts	% Total phenolic content (mg/g)	% Total flavonoid content (mg/g)
1.	Ethyl acetate extract	12.835%	7.895%
2.	Chloroform extract	7.102%	2.977%

f) Percentage inhibitions of DPPH radical by test compound were determined by the following formula:

$$\%\ \text{Reduction} = \frac{\text{Control absorbance} - \text{Test substance}}{\text{Control substance}} \times 100$$

g) The determined percentage inhibitions were used against the concentration of the sample to build a regression fit.

h) Calculation of IC_{50} value: Determined with the help of regressed line equation.

2.4.2. FRAP assay

The total antioxidant potential was determined using the ferric reducing antioxidant power (FRAP) assay according to the method of Benzie and Strain [14] with slight modification. The assay was based on the reducing power of a compound (antioxidant). Briefly, 200 µl extracted samples were mixed with FRAP reagent (3 ml) in test tubes and were vortexed. Blank samples were prepared for all samples of extracts. Both samples and blank were incubated in water bath for 30 min at 37 °C and, absorbance of the samples was determined against blank at 593 nm. A series of stock solution at 50, 100, 200, 400 and 800 µg/ml were prepared ($r^2 = 0.9912$) using ascorbic acid as standard curve. The values obtained were expressed as µg of ascorbic acid equivalent per gram of freeze dried sample. Increased absorbance of the reaction mixture correlates with greater reducing power.

2.5. Antimicrobial activity [15]

2.5.1. Agar well diffusion method

2.5.1.1. Preparation of agar media. Nine and half g Mueller-Hinton agar (MHA) was suspended in a 500 ml conical flask and 250 ml distilled water was added. Then, it was heated on hot plate with frequent agitation until it completely dissolved. Then, media was sterilized in an autoclave at 121 °C for 1 h.

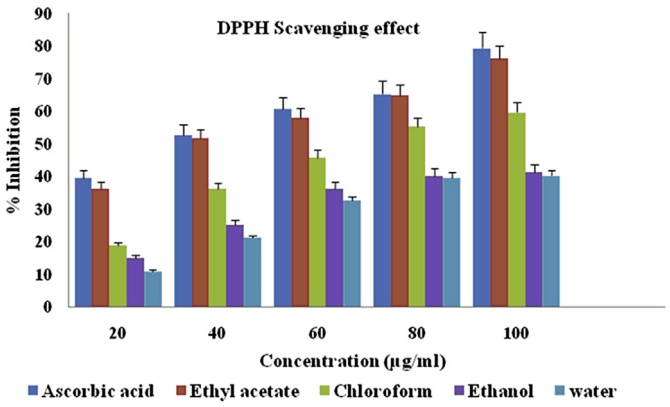

Fig. 1. DPPH scavenging activity after (30 min). Absorbance mean λ max = 517 nm.

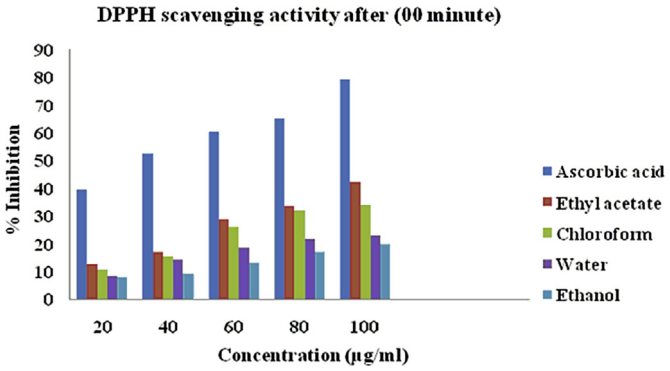

Fig. 2. DPPH scavenging activity after (00 min). Absorbance mean λ max = 517 nm.

2.5.1.2. Procedure. Twenty five ml of MHA was poured into a sterile Petri dish and allowed to solidify. Fifty µl of bacterial inoculums were spread on the solidified MHA media using sterile spreader. In each of these plates two wells (5 mm diameter) were punched into the agar using sterile cork borer. Then, working concentration of 100 mg, 150 mg, 200 mg and 250 mg dilutions were prepared from 500 mg/ml of stock solution of each extracts and 150 µl of each extract was separately added into wells and allowed to diffuse at room temperature. Equal volume of alcohol was used as a negative control, and chloramphenicol and nystatin were used as a positive control as antibacterial and antifungal agent respectively. Plates were incubated for 24 h at 37 °C and the diameter (mm) of clear zone of inhibition was recorded and measure with the help of radius scale.

2.6. Statistical analysis

In the present study all assays were performed in triplicate, and the results were calculated as the mean ± SD. The statistical significance between phenolic content and antioxidant activity values of the extracts was carried out using the Excel program. $p \leq 0.05$ was considered statistically significant.

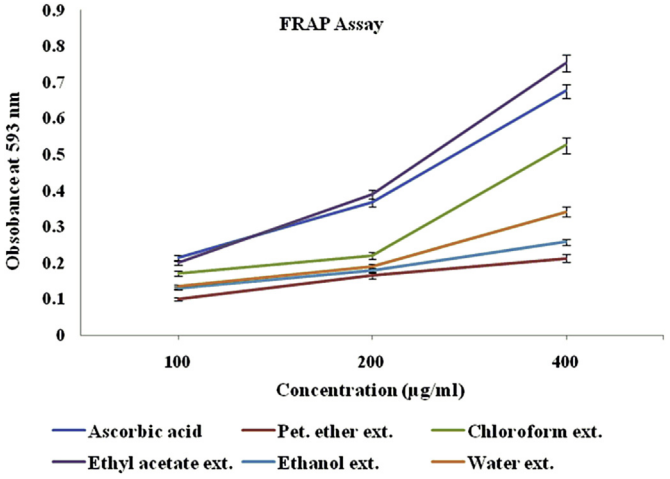

Fig. 3. Antioxidant activity of different extract of *U. wallichiana* by *FRAP* assay method at 593 nm.

Table 3
Antibacterial activity of various extracts of *U. wallichiana*.

S. no.	Plant extract	Concentration (μg/ml)	Zone of inhibition (in mm)					
			Escherichia coli	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Aspergillus fumigates*	*Aspergillus flavus*
1	Ethyl acetate	25	nd	nd	nd	nd	nd	nd
		50	10	16	10	11	7 mm	6 mm
		100	31 mm	55 mm	32	34	22 mm	15 mm
2	Chloroform	25	nd	nd	nd	nd	nd	nd
		50	8 mm	5 mm	2 mm	2 mm	nd	4
		100	25 mm	18	14	15	4 mm	11 mm
3	Ethanol	25	nd	nd	nd	nd	nd	nd
		50	nd	nd	nd	nd	nd	nd
		100	nd	nd	nd	nd	nd	nd
4	Water	25	nd	nd	nd	nd	nd	nd
		50	nd	nd	nd	nd	nd	nd
		100	15 mm	nd	nd	nd	nd	nd

nd = not detectable.

3. Results

3.1. Yield of extracts

Yield of various extracts of *U. wallichiana* using different solvent in increasing polarity are presented in Table 1.

3.2. Standardization of plant extract

3.2.1. Estimation of total phenolic and flavonoid content of *U. wallichiana*

Estimated percentage of total phenolic and flavonoid content of methanol and chloroform extract of *U. wallichiana* are reported in Table 2.

3.3. Determination of antioxidant activity of different extracts of *U. wallichiana*

Results of DPPH scavenging activity of different extracts after 30 min and after 00 min are shown in Figs. 1 and 2. Findings of FRAP assay are reported in Fig. 3.

3.4. In-vitro antimicrobial activity of different extracts of *U. wallichiana*

Measurement of zone of inhibition of various extracts and standard drugs are presented in Table 3.

4. Discussion

Many naturally occurring compounds found in plants, herbs and spices have been shown to possess antimicrobial activity and serve as a source of antimicrobial agents against pathogens [16]. Many infectious diseases represent an important cause of morbidity and mortality worldwide. Therefore, the development of new antimicrobial agents for the treatment of microbial infections is of increasing interest. The main objective of the present study was to evaluate the ability of the *U. wallichiana* bark extract to inhibit the growth of pathogenic bacteria and fungus with and without antibiotics and non-antibiotic drugs. The present study was emphasized on evaluation of antioxidant and antimicrobial potential of *U. wallichiana*.

It has been reported that chemical constituents of the plants like flavonoids and phenolic compounds possess a variety of activities including antimicrobial activity. In the present study, phenolic and flavonoid contents of ethyl acetate and chloroform extract of *U. wallichiana* were identified and quantified. Antioxidant activity was determined by DPPH and FRAP assay method. Ethyl acetate extract showed better antioxidant activity as compared to other extracts (Figs. 1–3).

In vitro antimicrobial activity of *U. wallichiana* was evaluated using agar well diffusion method. Antibacterial activity was performed against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and *Pseudomonas aeruginosa*. Ethyl acetate extract showed better zone of inhibition as compared to all other extracts. Chloroform extract exhibited moderate zone of inhibition. Ethyl acetate extract showed greater zone of inhibition as compared to standard drug chloramphenicol (Table 3). Antifungal activity was evaluated against *Aspergillus fumigates* and *Aspergillus flavus*. Again, amongst all extracts tested, ethyl acetate showed maximum zone of inhibition as compared to other extracts. Chloroform extract showed mild antifungal activity (Table 3). Standard drugs chloramphenicol and nystatin were used as a positive control as antibacterial and antifungal agent respectively while solvents which were used for extraction were used as negative control (Table 4).

Table 4
Antimicrobial activity of control/standard drugs and solvents.

S. no.	Stain	Standard drug/solvent Zone of inhibition (diameter in mm)				
		Standard antibacterial (Chloramphenicol)	Standard antifungal (Nystatin)	Ethyl acetate	Chloroform	Ethanol
1	*Escherichia coli*	25 mm	–	5	5	6
2	*Bacillus subtilis*	34 mm	–	6	5	5
3	*Pseudomonas aeruginosa*	24 mm	–	5	5	5
4	*Staphylococcus aureus*	25 mm	–	2	4	2
5	*Aspergillus fumigates*	–	34 mm	8	5	nd
6	*Aspergillus flavus*	–	20 mm	6	6	nd

nd = not detectable, Concentration of standard drugs chloramphenicol and nystatin was taken 50 μg/ml.

5. Conclusion

In the present study, the ethyl acetate extract of the plant exhibited significant antimicrobial activity, better than standard drug. Phytochemical screening of ethyl acetate extract indicated the presence of mainly phenolic and flavonoid compounds. These active constituents could be responsible for antimicrobial activity of *U. wallichiana*. Furthermore, the total phenols and flavonoids were also estimated in this bioactive extract of the plant. Based on the findings of this study future prospects of the current investigations suggested that ethyl acetate extract of the plant should be further analyzed to isolate the specific antimicrobial principle(s) present in the plant.

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Conflict of interest

None.

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