

Antioxidant and antibacterial properties of leaves of *Elaeocarpus sphaericus* Roxb. and *Pinus wallichiana* from Uttarakhand region of India

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Abstract

Background: *Elaeocarpus sphaericus* is known for its medicinal and spiritual values in India, since long back. *Pinus wallichiana*, a conifer is known for its timber wood and rich phenolics in the needle. However still, there is no report on the phytoconstituents and biological activities of the leaves of these plants from Uttarakhand region of India. **Aim and Objectives:** To identify the phytochemicals, antioxidant, and antibacterial properties of methanolic and aqueous extracts of leaves of *E. sphaericus* Roxb and *P. wallichiana* for exploring their potential for medicinal use. **Materials and Methods:** Leaves were used for extraction in aqueous and hydro-methanolic solvents through soxhlet method. Extracts were examined for the presence of phenolics and flavonoids. Antioxidant properties were explored by scavenging assays for 2, 2-diphenyl-2-picryl hydrazyl, nitric oxide, H₂O₂. Agar well diffusion assay was applied for the antimicrobial potential of the extracts against pathogenic bacteria. Experiments were done in triplicates, and average values with standard deviations are shown. **Results:** Hydro-methanolic extracts were rich in phenols and flavonoids. *E. sphaericus* extract has shown better antioxidant property than that of *P. wallichiana*. Hydro-methanolic extracts have shown antioxidant potential better than aqueous extracts. Only, hydro-methanolic extracts showed antibacterial activities. *E. sphaericus* have shown better antibacterial activities than that of *P. wallichiana*. **Conclusion:** Hydro-methanolic extracts of *E. sphaericus* and *P. wallichiana* are rich in phenolics and flavonoids and possess good antioxidant and antibacterial properties. Leaf extract of *E. sphaericus* has promising antioxidant and antibacterial potential.

Key words: Antibacterial, antioxidant, blue pine, *Elaeocarpus sphaericus*, phyto-chemical, *Pinus wallichiana*, rudraksha

INTRODUCTION

Plants have been used for its therapeutic values worldwide since ancient period due to effectiveness, low cost, and lesser side effects.^[1,2] The World Health Organization has estimated that about 80% of the world's population is dependent primarily on traditional medicine.^[3,4] The alarming rise in the incidences of antimicrobial resistance^[5,6] and free radical related disorders, e.g., coronary diseases, cancer, diabetes mellitus, and liver disorders^[7,8] have attracted the attention of researchers to look for alternative therapeutics. Plants have the richest potpourri of various compounds which shows a good potential as an antimicrobial agents and antioxidants.^[9] India is an adobe of numerous

medicinally important plants^[10] and Uttarakhand, especially is the land of the treasure of medicinal plants in India.^[11] Several plants are reported to have promising antimicrobial and antioxidant properties.^[12-14] There are several reports from different regions of the globe on the medicinal properties of *Elaeocarpus sphaericus* (syn. *Elaeocarpus ganitrus*).^[15-17] However, very few investigations have been carried out

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on the medicinal properties of *Pinus wallichiana*.^[18,19] It is noteworthy that phytochemical properties of plants may vary depending on their geographical distribution.^[20] Therefore, it seems pertinent to explore the phytochemical and bio-medical properties of these plants from Uttarakhand region.

To the best of our knowledge, this is the first study on the phytochemical, antibacterial, and antioxidant properties of leaf extracts of *E. sphaericus* and *P. wallichiana* from Uttarakhand region of India.

MATERIALS AND METHODS

Plant Material

Leaves of the *E. sphaericus* Roxb. were collected from the Graphic Era University Campus, Clement Town, Dehradun, Uttarakhand, India and needles of *P. wallichiana* were collected from Barkot region of district Uttarkashi of Uttarakhand, India. Plants were authenticated by Dr. Rakesh Mohan Painuly, Lecturer, Department of Botany, Hemwati Nandan Bahuguna Garhwal University, Chauraas Campus, Srinagar Garhwal. Specimen samples have been submitted in the herbarium of Department of Botany, Hemwati Nandan Bahuguna Garhwal University, vide voucher numbers GUH 20720 and GUH 20744 for *E. sphaericus* and *P. wallichiana*, respectively.

Preparation of Plant Extract

Leaves of the plants were dried in shade and finally crushed to dry powder. The 50 g of leaf powder was subjected to soxhlet extraction separately, using hydro-methanolic (70% methanol in water) and aqueous solvents at 50-60°C for 8 h. Extracts were filtered through muslin cloth and evaporated at room temperature to reduce the volume. The concentrated extracts were dissolved either in dimethylsulfoxide (DMSO) for hydro-methanolic extract or in distilled water for aqueous extract. Aqueous extracts were named as RDLA and PWCA for the leaves of *E. sphaericus* and *P. wallichiana*, respectively. Similarly, hydro-methanolic extracts were named as RDLM and PWCM for the leaves of *E. sphaericus* and *P. wallichiana*, respectively.

Chemicals, Reagents, and Growth Media

Chemicals and reagents from standard companies like Sigma-Aldrich, Gurgaon, Haryana, Hi Media, Mumbai, India and Central Drug House (CDH), New Delhi, India were used in the study. 2, 2-diphenyl-2-picryl hydrazyl (DPPH), Ascorbic acid, and DMSO were procured from Sigma, while methanol, ethanol, and general chemicals were procured from CDH. Components of growth media and antibiotics were procured from Hi Media.

Phytochemical Analysis

Saponins, phenol, and flavonoids were analyzed in different extracts following standard protocols based on colorimetric assays.^[21]

Antioxidant Assays

DPPH scavenging assay

DPPH is a stable free radical; scavenging of DPPH from solution by adding the extracts is a good index for assigning an antioxidant function to any test material. DPPH scavenging assay was done following the standard procedure.^[22] Briefly, concentrations of all extracts were set to 1.4 mg/ml after dilutions including ascorbic acid as a standard solution. A control reaction was set using DPPH and methanol only. 25 µl of the plant extract/ascorbic acid and 475 µl of methanol were added in a test tube, to this 2 ml of DPPH was added. Incubation of the reaction was done for 30 min. Discoloration of purple was measured by absorbance (A) at 517 nm using a spectrophotometer. Percentage DPPH scavenging activities were measured using following formula:

$$\text{Scavenging activity (\%)} = \frac{(A \text{ control} - A \text{ sample})}{A \text{ sample}} \times 100$$

Experiments were done in triplicate, and the average value of all three readings along with standard deviation (SD) is shown.

Nitric oxide (NO) scavenging assay

NO scavenging activities of different extracts were performed following standard method.^[23] Ascorbic acid is taken as a known antioxidant for positive control. Similar concentration (1.4 mg/ml) of extracts and ascorbic acid were used for the assay. Reaction without extract was considered as negative control. Each reaction was set by mixing 2 ml of sodium nitroprusside, 0.5 ml phosphate-buffered saline PBS, and 0.5 ml of extract. This mixture was incubated at 25°C for 30 min. Afterward, 0.5 ml of the Griess reagent was added, and the mixture was further incubated for 30 min. Absorbance was measured against reagent blank at 546 nm. The following formula was used for calculating the % scavenging of NO:

$$\text{NO scavenging activity (\%)} = \frac{(A \text{ control} - A \text{ sample})}{A \text{ sample}} \times 100$$

Values were shown as the average of three independent readings along with SDs.

H₂O₂ scavenging assay

H₂O₂ scavenging test was performed following standard procedure.^[24] This test is attributed to the phenolics, which can donate an electron to H₂O₂ and turn it to H₂O, thereby decreasing the concentration of H₂O₂ in the reaction

mixture. Briefly, the concentration of all extracts was made to be 1.4 mg/ml after dilutions including ascorbic acid as standard antioxidant. For each test, 10 μ l of the plant extract was added to 0.6 ml of H₂O₂ solution (40 mM) and total volume was made up to 3 ml using phosphate buffer. The presence of hydrogen peroxide was monitored by taking absorbance at 230 nm following an incubation of 10 min. Control was set using PBS and H₂O₂ solutions only. The percentage of scavenging of hydrogen peroxide of extracts and standard compound was calculated using the following equation:

$$\text{Scavenging activity (\%)} = \frac{(A \text{ control} - A \text{ sample})}{A \text{ sample}} \times 100$$

Each experiment was done in triplicate separately, and average values along with SDs are shown.

Antibacterial susceptibility assay

We tested the antibacterial potential of the extracts against the known pathogenic bacteria. In the present study, we included both types of bacteria, Gram-positive and Gram-negative, e.g., *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Lactobacillus lactis*.

The antibacterial potential was analyzed by agar well diffusion method.^[25] The 100 μ l of log phase bacterial culture (OD_{600nm}: 0.6-0.8) was spread onto the Lysogeny broth agar plate and dried. Wells were bored in the plates with the help of sterile micro tip (diameter-0.5 cm). Five different concentrations (0.21, 0.70, 0.84, 1.05, and 1.40 mg/ml) of RDLM, RDLA, PWCM, and PWCA were analyzed, initially. Higher concentrations (7.17, 23.9, 28.68, 35.85, and 47.8 mg/ml) of selected extracts were also analyzed. The 20 μ l of the each extract was introduced into the separate wells. Ampicillin was used as a standard antibacterial drug for the comparisons (100 mg/ml) and one solvent control (DMSO or water) in separate wells on the same plate. The plates were incubated overnight at 37°C for 18 h. Microbial growth inhibition was determined by measuring the diameter of the zone of inhibition (ZOI) in mm. The experiments were done in triplicate and the mean values \pm SD are shown. The results were compared with the standard antibiotic ampicillin (100 mg/ml).

RESULTS

Phytochemical Property

Qualitative measurements through colorimetric assays have shown the presence of phenols and flavonoids in aqueous and hydro-methanolic extracts of both the plants. Phenols and flavonoids were found significantly higher in hydro-methanolic extracts (RDLM and PWCM) when compared with that of aqueous extracts.

Antioxidant Properties

DPPH scavenging activity

At a concentration of 1.4 mg/ml, all extracts and standard have shown the significant DPPH scavenging activities [Table 1]. As shown in Table 1, RDLM has shown the highest activity (91.28 \pm 0.14%) among all extracts that were the closest to the DPPH scavenging activity of standard, ascorbic acid (95.13 \pm 0.36%). PWCM has shown the second highest activity of DPPH scavenging (45.52 \pm 0.65%). RDLA and PWCA have shown relatively lower activities.

NO scavenging activity

All extracts and standard, at a similar concentration, have shown the significant NO scavenging activities [Table 1]. RDLM has shown the highest activity (44.96 \pm 1.55%) among all extracts while standard ascorbic acid showed maximum activity (68.59 \pm 0.83%). PWCM has shown the second highest NO scavenging activity. Aqueous extracts of both the plants showed relatively lower activities (15.47 \pm 0.95% and 16.82 \pm 1.63 for RDLA and PWCA, respectively).

H₂O₂ scavenging activity

As shown in Table 1, all extracts, and even the standard showed lower H₂O₂ scavenging activities. The activity was found to be 8.52 \pm 0.67% for ascorbic acid. PWCM has shown the highest activity in this assay (15.36 \pm 0.81%) while RDLM has shown the least activity (2.09 \pm 1.10%). Activities for RDLA and PWCA were found to be 9.57 \pm 1.01% and 11.98 \pm 0.62%, respectively.

Table 1: Antioxidant properties of different extracts

Extracts	Concentration (mg/ml)	DPPH scavenging activity \pm SD* (%)	NO scavenging activity \pm SD* (%)	H ₂ O ₂ scavenging activity \pm SD* (%)
RDLM	1.4	91.28 \pm 0.14	44.96 \pm 1.55	2.09 \pm 1.10
RDLA	1.4	26.04 \pm 0.19	15.47 \pm 0.95	9.57 \pm 1.01
PWCM	1.4	45.52 \pm 0.65	42.28 \pm 1.43	15.36 \pm 0.81
PWCA	1.4	20.89 \pm 0.22	16.82 \pm 1.63	11.98 \pm 0.62
Ascorbic acid	1.4	95.13 \pm 0.36	68.59 \pm 0.83	8.52 \pm 0.67

SD: Standard deviation, DPPH: 2, 2-diphenyl-2-picryl hydrazyl, NO: Nitric oxide, RDLM: Rudraksha leaves aqueous, RDLA: Rudraksha leaves methanolic, PWCM: Pinus wallichiana aqueous, PWCA: Pinus wallichiana methanolic

Table 2: Antibacterial activity of RDLM

Extract/ standard	Concentrated (mg/ml/well)	Average ZOI±SD (mm)					
		<i>Ll</i>	<i>Sp</i>	<i>St</i>	<i>Pa</i>	<i>Sa</i>	<i>Ec</i>
RDLM	0.21	0	0	0	0	0	8.33±0.57
RDLM	0.70	13.66±0.57	12.33±1.1	14.33±0.57	13±0	8.66±0.57	12.33±0.57
RDLM	0.84	14.33±0.57	12.66±0.5	15.66±0.57	13.66±0.57	12±1	14±0.57
RDLM	1.05	15.66±0.57	14±1	14±1	15.33±0.57	13±1	15.66±0.57
RDLM	1.40	17.33±0.57	16.66±1	16.66±0.57	17±0	15.66±1.1	17.33±0.57
Ampicillin	100	13.6±0.57	11.33±0.57	18.33±0.57	16.33±0.57	17±1	15.66±0.57

ZOI: Zone of inhibition, SD: Standard deviation, *Ll*: *Lactobacillus lactis*, *Sp*: *Streptococcus pneumoniae*, *St*: *Salmonella typhi*, *Pa*: *Pseudomonas aeruginosa*, *Sa*: *Staphylococcus aureus*, *Ec*: *Escherichia coli*, RDLM: Rudraksha leaves methanolic

Antibacterial activity

RDLM and PWCM exhibited good antibacterial activity in agar well diffusion assays against the bacteria used in this study. Ampicillin (100 mg/ml) is taken as standard antibiotic.

RDLM exhibited antibacterial properties better than ampicillin [Table 2]. At a concentration of 0.70 mg/ml, RDLM has shown a ZOI of 13.66 ± 0.57 mm against *L. lactis*, which was comparable to the ZOI of the standard. At 0.70 mg/ml concentration, RDLM has shown a ZOI of 12.33 ± 1.1 mm against *S. pneumoniae*, which was higher than that of standard (11.33 ± 0.57 mm). RDLM has shown very good antibacterial activity against *E. coli*, even at the lowest concentration (0.21 mg/ml), ZOI was reported to be 8.33 ± 0.57 mm, which was around 53% of standard. Against the bacteria *S. typhi*, *P. aeruginosa*, and *S. aureus*, RDLM showed activities at 1.4 mg/ml concentration, which were nearly similar to that of the standard.

PWCM showed antibacterial activities only against *P. aeruginosa* and *E. coli* at higher concentrations [Table 3]. At the concentration of 47.8 mg/ml, PWCM showed a ZOI of 15.66 ± 0.57 mm and 14.00 ± 0.57 mm against *P. aeruginosa* and *E. coli*, respectively, which were closer to their respective ZOI of standards (16.66 ± 0.57 mm and 14.66 ± 0.57 mm).

Aqueous extracts (RDLA and PWCA) did not show significant antibacterial properties at the concentrations used here.

DISCUSSION

Plants have been used as natural therapeutics for the different ailments of mankind, since prehistoric period. Medicinal properties of plants are due to the presence of phytochemicals (secondary metabolites), known to be involved in plant defense against microbes and insects.^[26] Despite rising trend of synthetic chemical drugs, the contribution of natural products cannot be overlooked for providing the basic skeleton for modern medicines.^[27]

Table 3: Antibacterial activity of PWCM

Extract/ standard	Concentration (mg/ml/well)	Average ZOI±SD (mm)	
		<i>Pa</i>	<i>Ec</i>
PWCM	7.17	10.66±0.57	9.66±0.57
PWCM	23.9	11±0	11±0
PWCM	28.68	11.33±0.57	11±0
PWCM	35.85	12±1	14±0.57
PWCM	47.8	15.66±1.1	14±0.57
Ampicillin	100	16.66±0.57	14.66±0.57

ZOI: Zone of inhibition, SD: Standard deviation, *Pa*: *Pseudomonas aeruginosa*, *Ec*: *Escherichia coli*, PWCM: Pinus wallichiana methanolic

Poly phenols are well-known bioactive compounds of the plants, responsible for biological activities such as antimicrobial, antioxidant, anti-cancer, and others.^[28-30] We reported the presence of phenols and flavonoids in extracts. Hydro-methanolic extracts showed the higher phenols and flavonoids. DPPH and NO scavenging activities of different extracts were in the order of RDLM > PWCM > RDLA > PWCA, probably due to the varying concentration of flavonoids. Flavonoids are known for its antioxidant properties by scavenging the free radicals.^[31] RDLM has shown DPPH scavenging activity, similar to that of ascorbic acid. H₂O₂ scavenging activities were found highest in PWCM and second highest in PWCA, which might be due variations in phytochemicals.

Phenolics and flavonoids are well-known antimicrobial agents.^[32] A rise in flavonoids level is reported in the plants, in response to microbial infections.^[33] In our assays, aqueous extracts did not show the antibacterial activities, probably due to less concentration of phenols and flavonoids as evident from our qualitative assays. Contrary to the PWCM, RDLM showed an antibacterial effect at lower concentrations and on all Gram-positive and Gram-negative bacteria, taken in the study. PWCM acted against *P. aeruginosa* and *E. coli* at higher concentrations only. This could be explained on the basis of lower murein content and thinner cell wall of Gram-negative bacteria. Polyphenols may act by inhibition of replication enzymes, inactivation of toxins, and destabilization of the

cell wall.^[34] Owing to the differences in the cell wall structure and virulence properties, different bacterial pathogens might exhibit variations in the susceptibilities to different antibacterial agents.

In general, hydro-methanolic extracts of the leaves of both the plants have shown the good amount of phenols and flavonoids, due to which they exhibited strong antioxidant and anti-microbial potential. *E. sphaericus* leaf extracts had shown better biological activities than those *P. wallichiana* leaf extracts. Our results of the *P. wallichiana* from Uttarakhand region were in congruence with the earlier reports from Pakistan and Iran.^[18,35,36] In addition, we have shown the promising antibacterial properties in leaf extracts of *P. wallichiana*. Similarly, *E. sphaericus* from the similar geographical area have shown notable antioxidant and antimicrobial properties.

To the best of our knowledge, this is the first report on antibacterial and antioxidant properties of leaf extracts of these plants from Uttarakhand region of India. Further studies are required for the identification of active principals and their bio-medicinal properties to develop an effective and safe herbal therapeutics.

CONCLUSIONS

Aqueous and hydro-methanolic extracts of the leaves of *E. sphaericus* and *P. wallichiana* have shown the presence of phenols and flavonoids. Extracts, especially hydro-methanolic extracts have exhibited strong antioxidant and antibacterial potential. *E. sphaericus* leaf extracts have shown better biological activities than those *P. wallichiana* leaf extracts.

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