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Research Article
Anti-microbial and Anti-oxidant Properties of *Cunninghamia lanceolata*

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Abstract

Objective: In the present study, an attempt was made to evaluate antimicrobial and antioxidant potential of alcoholic plant extract (APE) of a selected plant *Cunninghamia lanceolata* (*C. lanceolata*) to understand the phytochemical properties of the APE. The APE was also screened for the presence of flavonoids, proteins and phenolic compounds, which are the major constituents responsible for efficient antimicrobial and antioxidant activities. **Materials and Methods:** Plant tissue extract was prepared from the selected plant *Cunninghamia lanceolata.* Various phytochemicals studies were performed to check the flavonoids, phenolics and protein contents by the spectrophotometry method. Antioxidant properties were analyzed by the DPPH as well as by H_2O_2 assay. Further antimicrobial activities against the selected bacterial and fungal pathogens were also checked by the spread plate methods. Standard deviation (SD) about the mean was calculated from data obtained for 3 replicates for each of the parameters studied. **Results:** Qualitative analysis of crude APE of *C. lanceolata* showed proteins (17.7 mg mL^{-1}), flavonoids (2.35 mg mL^{-1}) and phenols (0.19 mg mL^{-1}). The crude APE of *C. lanceolata* was revealed that maximum zone of clearance was observed against *Klebsiella pneumoniae* (23.6 mm) with (minimum inhibitory concentration) MIC value was 0.36 mg mL^{-1} out of the selected bacterial strains while maximum antifungal activity (zone of clearance) was observed against *Aspergillus niger* (18.5 mm). DPPH and H_2O_2 retain the scavenging activity with 85.1 and 51.7%, respectively. While ferrous ion chelating activity of APE of the *C. lanceolata* was observed with 81.5%. **Conclusion:** The APE of *C. lanceolata* was found a potential source of natural antioxidant as well as strong antimicrobial agent which have high content of protein, flavonoids and phenolics.

Key words: *Cunninghamia lanceolata*, antimicrobial activity, antioxidant activity, minimum inhibitory concentration, phenolics

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The rapid emergence of multiple drug resistant strains of pathogens to presently used antimicrobial agents has generated an urgent intensive search for new medicines or anti-microbial agents from medicinal plants[1]. Medical communities are now trying to resolve the problems by the use of plant-based medicines[2,3]. Plants (nature gift) provide us biologically active compounds which are used routinely as remedies for various human diseases. Although medicinal plants produce slow recovery but their therapeutic effects are miraculous. Herbal medicines have been accepted universally and have an impact on both human health and international trade[4]. Even in foreign countries, use of plants and phyto-medicines has increased dramatically in the last two decades[5]. Present study is based on medicinal uses of a conifer i.e., *Cunninghamia lanceolata* which has great economic value and used primarily for timber and paper production. *Cunninghamia lanceolata* is an evergreen conifer found naturally in the sub-tropical region of Central-Southern China. *Cunninghamia* genus is traditionally said to contain two similar species, *C. lanceolata* and *C. konishii*, often referred to as the China fir and Taiwan fir, respectively[6,7]. In country, *C. lanceolata* has been primarily used as timber but its leaves and branches been treated as waste. Phytochemicals present in the leaves and branches have many important medicinal properties[8]. Volatile constituents of the wood from *C. konishii*, contain several terpenoids, including, α-cedrene, β-cedrene, α-terpenol and cedrol, which show antimicrobial activities[9]. The essential oils extracted from plant *C. lanceolata* exhibited cytotoxic activity against human lung, liver and oral cancer cells and also exhibited antimicrobial activities mainly against Gram positive bacteria and yeast[8]. Konishiol from *C. konishii* showed cytotoxic activity against human solid tumor cell lines[10]. The essential oils of *C. konishii* wood have been shown to have strong anti-fungal activities against wood decay fungi and plant pathogenic fungi[11].

This study focuses on the antimicrobial and antioxidant activity of the *C. lanceolata*, showing pharmaceutical activity. Currently used antimicrobial drugs become resistant to available antibiotics. The purpose was to find out the efficient natural antimicrobial molecule to combat the disease causing pathogens.

MATERIALS AND METHODS

Collection of plant: The collection of fresh leaves of plant was done from the rural village in district Shimla, Himachal Pradesh (India) located at 31.61°N 77.10°E in the Himalayan region. The altitudinal range of these areas lies between 1000-2500 m above mean sea level. The climate of the selected area was cool, the region receives moderate rainfall during monsoon of which the bulk was received during the months of July, 2015. *In vitro* studies on fresh leaves of plant *Cunninghamia lanceolata* was done at Department of Biotechnology, Himachal Pradesh University, Shimla, India.

Preparation of plant tissue extracts: Fresh plant material (leaves) was washed under running tap water, air dried and then homogenized to fine powder. The powdered preparations were stored in airtight glass vials at 4°C till further use. For preparing tissue extracts, 1.0 g of air-dried powder was placed in 10 mL of methanol in a conical flask, plugged with cotton and the same was kept on a rotary shaker at 200 rpm (shaking) for 48 h. The extract was filtered through Whatman filter paper No. 1 and the filtrate was centrifuged at 10,000 rpm at 4°C for 15 min. The supernatant was collected and completely evaporated at room temperature. Left over APE (alcoholic plant extract) residue was dissolved in PBS (0.05 M phosphate buffer saline, pH 7.2) and was sterilized by filtration (0.22 µm, Millipore syringe filter). The filter-sterilized preparation of APE obtained was stored in a freezer -70°C in airtight vials for further studies to test anti-bacterial and anti-fungal activities.

Screening of phytochemical in the tissue extracts of *C. lanceolata:* The plant extract prepared was used for screening of phytochemical and other biologically active compounds. Phytochemical analysis was carried out according to the standard methods. The extract was screened for the presence of flavonoids, proteins and phenolic compounds.

Test for flavonoids: Shinoda test was used for flavonoids analysis. In this test, the plant extract was treated with 5 mL of 95% ethanol followed by addition of hydrochloric acid (12 N). On addition of 0.5 g of magnesium in the plant extract/mixture, the color changed to pink which showed the presence of flavonoids in plant extract[12,13].

Total flavonoids content were determined in the sample extracts by reaction with sodium nitrite, followed by the development of coloured flavonoids-aluminum complex formation using aluminum chloride by following 0.15 mL of $NaNO_2$+0.15 mL of $AlCl_3$+plant extract at different concentration. After 6 min, 1 mL of 1 M NaOH was added and total volume was made 10 mL with distilled water followed by mixing of solution and monitoring spectrophotometrically at 510 nm.

Test for phenolic content: The total phenolic content in the each APE was assayed as described previously[8] using tannic acid as a standard molecule. Total phenolic content was measured by Folin-Ciocalteau method. The APE was dissolved in the distilled water and then 0.5 mL of Na_2CO_3 prepared was added in each test tube. After 5 min, 0.25 mL of Folin-Ciocalteau phenol reagent (diluted in 1:1) was added in each test tube. Absorbance at 700 nm was taken and average was calculated.

Test for protein content: Various concentration of BSA (1, 5, 7.5 and 10 µg mL^{-1}) was prepared in distilled water. One hundred microliters of plant extract was added in each of the above preparations to separate tubes. Then, 2 mL of Bradford reagent was added to each tube and after 2 min absorbance was taken at 595 nm. It is a colorimetric protein assay based on an absorbance shift of dye CBB (coomassie brilliant blue) R-250 in which under acidic conditions, the red form of dye is converted to its blue form to bind the protein being assayed.

Antioxidant activities

Free radical scavenging activity: DPPH stable free radical method is an easy, rapid and sensitive way to analyze the anti-oxidant activity of a specific compound/plant extracts[14]. Hundred millimolar of stock solution of DPPH (4 mg/10 mL) was diluted to 1: 10 then, 0.5 mL of this solution was added at different concentration of APE. Equal amount of distilled water was also added to make final volume 1.0 mL, after 30 min of incubation followed by absorbance at 517 nm. The radical scavenging activity was measured as a decrease in the absorbance of DPPH. The calibration curve was prepared by using ascorbic acid as standard/ reference molecule. The degree of discoloration of violet color of DPPH radical, as it gets reduced, indicated the radical scavenging potential of the APE[15]. The DPPH radical scavenging activity was estimated by using the following formula:

$$\text{Scavenging of APE (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Decreases in absorbance were observed due to radical scavenging activity of APE through the donation of hydrogen to form the stable DPPH molecule resulting color change from purple to yellow.

Hydrogen peroxide scavenging activities: APE of various concentrations was added to H_2O_2 solution (1 mL). The solution of H_2O_2 (200 mM) was prepared in PBS (pH 7.4). APE at the concentration of (100-1000 µg mL^{-1}) was added to H_2O_2 solution (1 mL). A_{230} values were determined after 10 min incubation at 37°C against a reagent blank containing phosphate buffer without H_2O_2 [16,17].

Antimicrobial activity by well diffusion method: Anti-bacterial activities of the APE preparations were tested using well diffusion method against selected bacterial strains (*Salmonella typhii, Staphylococcus citreus, Salmonella paratyphi, Staphylococcus epidermidis, Staphylococcus aureus, Salmonella typhimurium, Escherichia coli, Shigella flexneri, Pseudomonas aeruginosa, Enterobacter faecalis, Klebsiella pneumoniae and Streptococcus citreus*). Thirty microliters of each of the bacterial culture (OD_{600} = 1.0) was spread over Mueller Hinton agar plate. Wells were cut and 80 µL of the APE was loaded against above mentioned each bacterium. The plates were incubated at 37°C for 24 h for the bacterial growth to appear. The plates were observed for the zone of clearance around the wells. The zone of clearence(s) around the well (mm) including the well diameter was recorded[18]. The observations were taken in three different directions in all 3 replicates and the average values ± SEM was tabulated. Values less than 8 mm for the tested fern extracts were considered as not active against microorganisms[19].

Minimum inhibitory concentration (MIC) against selected bacterial strains: Microdilution method was used to determine the minimum inhibitory concentrations (MICs) of anti-microbial agents. The lowest concentration of an antimicrobial agent that inhibits the visible growth of microorganismis was referred as the MIC. The MIC assay was performed in a 96-well micro-titre plate against 10 bacterial strains (*Salmonella typhi*, *Staphylococcus citreus*, *Salmonella paratyphi*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Enterobacter faecalis, Klebsiella pneumoniae* and *Streptococcus citreus*). The plate was incubated at 37°C for 24 h.

Anti-fungal activity of APE of *C. lanceolata*: Anti-fungal activity of APE against 2 fungal strains (*Aspergillus niger* and *Fusarium oxysporum*) was also tested using well diffusion technique.

RESULTS

Qualitative analysis (primary phytochemical investigation) of APE of *C. lanceolata*: Chemical tests were employed in the

preliminary phytochemical screening for various secondary metabolites and revealed excellent presence of proteins, flavonoids and phenols in that order (Table 1). Qualitative analysis of APE of *C. lanceolata* revealed (Table 1) abundance of proteins (17.7 mg mL^{-1}), flavonoids (2.35 mg mL^{-1}) and phenols (0.19 mg mL^{-1}).

Anti-microbial activity

Anti-bacterial activities of APE of *C. lanceolata:* When APE was tested for anti-bacterial activity against 10 pathogenic bacteria using well diffusion technique (Fig. 1), the APE preparation of *C. lanceolata* showed average zone of clearance of 23.6, 23.0 and 20.0 mm against *Klebsiella pneumoniae* (Gram negative bacterium), *Enterobacter faecalis*, *E. coli* (Gram negative bacterium) and *S. aureus* (Gram positive bacterium), respectively (Table 2).

MIC against selected bacterial strains: APE of *C. lanceolata* (11.6 mg mL^{-1}) showed prominent and marked MIC values 0.36, 0.72 and 0.72 mg mL^{-1} against *K. pneumoniae, E. coli* and *S. aureus,* respectively (Table 3).

Anti-fungal activity of APE: Anti-fungal activity of APE against 2 fungal strains (*Aspergillus niger* and *Fusarium oxysporum*) was also tested using well diffusion technique. The antifungal activity of APE of *C. lanceolata* showed marked and prominent average zone of inhibition against *Aspergillus niger* (18.5 mm) and *Fusarium oxysporum* (9.0 mm).

Antioxidant assay of APE

Free radical scavenging activity (DPPH activity): The 2, 2-diphenylpicryl-1-picryl-hydrazyl (DPPH) radical scavenging activities in the APE were measured according to the DPPH method. The radical scavenging activity was measured as a decrease in the absorbance of DPPH or increase (%) in activity hence, scavenging activity (%) was observed to be 85.1% (Table 4).

Hydrogen peroxide scavenging activity of APE: H_2O_2 scavenging (%) activity was observed in APE of *C. lanceolata* (51.7%; Table 4).

Metal ion chelating activity (ferrous ion chelating activity): The metal ion chelating activity was measured as a decrease in the absorbance of DPPH or increase (%) in activity hence, best chelating (%) activity (81.5%) was observed in APE of *C. lanceolata* (Table 5).

Major biochemical constituents of APE of *C. lanceolata:* The protein content (1.27 mg mL^{-1}) was found in APE of *C. lanceolata.* Flavonoid content (2.35 mg mL^{-1}) was observed in APE of *C. lanceolata.* The highest phenolic content (0.19 µg mL^{-1}) occurred in *C. lanceolata* (Table 4).

Table 1: Major biochemical constituents of selected APE of *C. lanceolata*

Biochemical constituents	*Cunninghamia lanceolata*	Concentration (mg mL^{-1})
Proteins	+++	17.7
Phenols	+++	2.35
Flavonoids	++	0.19

$^{++}$Presence of phytochemical, $^{+++}$Excellent presence of phytochemical

Table 2: Anti-bacterial activity of APE of *C. lanceolata* against a selected panel of pathogenic bacterial strains

Test organism	*Cunninghamia lanceolata* Zone of clearance (mm)
Gram negative bacteria	
Klebsiella pneumoniae	23.6
Escherichia coli	23.0
Salmonella typhi	21.8
Pseudomonas aeruginosa	19.3
Salmonella paratyphi	18.6
Proteus vulgaris	12.9
Shigella flexneri	12.6
Gram positive bacteria	
Staphylococcus aureus	20.0
Staphylococcus citreus	18.3
Staphylococcus epidermis	15.6

Table 3: MIC of APE (11.6 mg mL^{-1}) of *Cunninghamia lanceolata* against selected bacterial strains

	Concentrations of plant extract in different wells								
Microorganisms	11.6	5.8	2.9	1.45	0.72	0.36	0.18	0.09	MIC value (mg mL^{-1})
Klebsiella pneumonia	-	-	-	-	-	+	+	+	0.36
Escherichia coli	-	-	-	-	+	+	+	+	0.72
Staphylococcus aureus	-	-	-	-	+	+	+	+	0.72
Salmonella typhi	-	-	-	+	+	+	+	+	1.45
Pseudomonas aeruginosa	-	-	-	+	+	+	+	+	1.45
Salmonella paratyphi	-	-	+	+	+	+	+	+	2.9
Staphylococcus citreus	-	+	+	+	+	+	+	+	5.8
Staphylococcus epidermis	+	+	+	+	+	+	+	+	11.6
Proteus vulgaris	+	+	+	+	+	+	+	+	>11.6
Shigella flexneri	+	+	+	+	+	+	+	+	>11.6

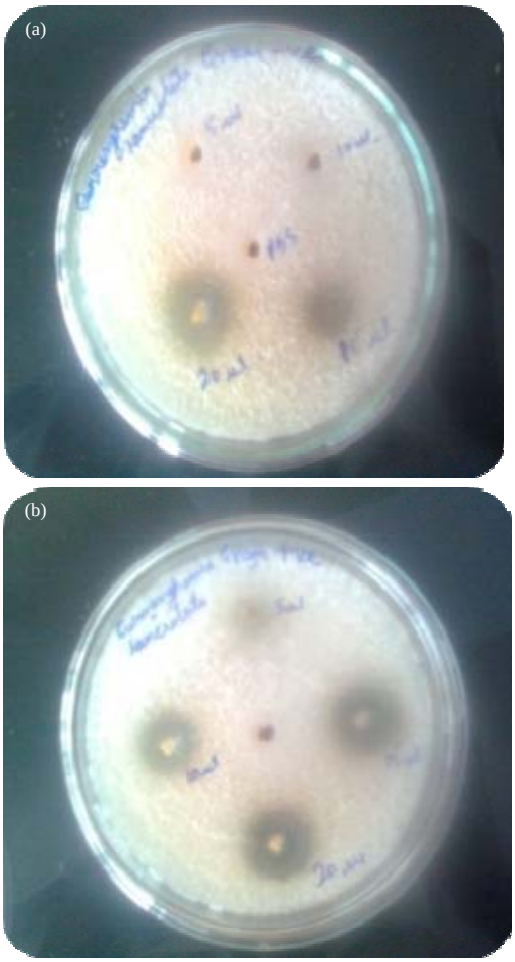

Fig. 1: Zone of clearance of APE of *C. lanceolata* (a) *Salmonella typhi* and (b) *S. epidermidis*

Table 4: Biochemical constituents of APE of *C. lanceolata*

Biochemical tests (absorbance)	APE of *C. lanceolata* Mean±SEM	Content (mg mL^{-1})
Protein (A_{595})	0.576±0.016	1.27
Flavonoids (A_{510})	0.821±0.271	2.35
Phenol (A_{700})	0.653±0.015	0.19

Table 5: Antioxidant activity of APE of *C. lanceolata*

Anti-oxidant assay (absorbance)	Activity (%)
DPPH scavenging activity (A_{517})	85.1
H_2O_2 scavenging activity (A_{230})	51.7
Ferrous ion chelating activity (A_{562})	81.5

Table 6: Haemolytic activity in APE (11.6 mg mL^{-1})

	A_{414}		
Alcoholic plant extract	Control	Test Mean±SEM	Haemolytic activity (%)
Cunninghamia lanceolata	0.582	0.46±0.235	13.0

Haemolysis test of APE on human RBCs: In the APE of *C. lanceolata*, the haemolysis of target human RBCs (Blood group 'O' Rh positive) was observed to be ~ 13% (Table 6).

DISCUSSION

Present study is an approach to evaluate antibacterial, MIC, antifungal, antioxidant potential of APE of selected plant *C. lanceolata* and to understand the importance of phytochemical properties of the APE and its haemolytic activity. As the search for new anti-microbial agents intensifies, APE may provide attractive alternate source(s) of molecules as antimicrobial agents. The result of the present study clearly showed that, amongst a variety of phytochemicals possessed by plants, the APE of *C. lanceolata* may be used in antimicrobial, antioxidant, anticancer as well as bio-control agents. Recently, many pharmaceutical innovations are developed to find and separate the secondary metabolites from the plants for designing the new antimicrobial medicines. It will help to produce new medicines with fewer side effects, less cost, affordable and more effective in the treatment of various infectious diseases in future. Today most of the researches about *C. lanceolata* have focused on planting and eco-physiology in agro-forestry but medicinal aspects are also very important. *C. lanceolata* in this country has been primarily used as timber but its leaves and branches been treated as waste. Phyto-chemicals present in the leaves and branches have many important medicinal properties[20,21].

In the present study, APE of *C. lanceolata* was tested against 10 pathogenic bacterial strains involved in various diseases in human being and other animals and APE of *C. lanceolata* was found effective against all the pathogenic bacteria that are causative agent(s) of diseases like typhoid (*Salmonella typhimurium*), paratyphoid (*Salmonella paratyphi*), pneumoniae (*Klebsiella pneumoniae*), common persistent urinary tract infections (*Pseudomonas aeruginosa*), urinary stones/urinary tract infections (*Proteus mirabilis*), shigellosis or diarrhea (*Shigella flexneri*), traveler's diarrhea (*Escherichia coli*) and oral/dental infections (*Streptococcus mutans*) or constituent common skin micro flora (*Staphylococcus aureus* and *Streptococcus epidermidis*), maximum zone of inhibition was found against *Klebsiella pneumoniae* (23.6 mm), *Escherichia coli* (23.0) and *Staphylococcus aureus* (20.0 mm). There is obvious need to find newer anti-microbial compounds as wide spread use of anti-microbial drugs continue to cause significant increase in resistant bacteria, particularly resistant Gram-positive organisms. Currently, Gram positive bacteria have been emerging as one of the most important hospital and community pathogens in world-wide manner[22].

Phytochemical screening of medicinal plants is very important in identifying new sources of therapeutically and industrially important compounds[23]. In this study, The APE of

C. lanceolata showed best anti-fungal activity against *Aspergillus niger* and *Fusarium oxysporum*, respectively. Same results were also recorded from essential oils of *C. konishii* wood which have strong antifungal activities against wood decay fungi and plant pathogenic fungi[11]. In addition to this, MIC was also recorded against different pathogenic bacteria and APE of *C. lanceolata* (11.6 mg mL^{-1}) showed best results against *Klebsiella pneumoniae* (MIC 0.36 mg mL^{-1}). When APE was tested for anti-oxidant activity, best % scavenging activity in DPPH and H_2O_2 assay was observed 85.1 and 51.7%, respectively. While ferrous ion chelating activity detected in the APE of *C. lanceolata* was 81.5%. The result of the present study showed that the APE of *C. lanceolata* contain highest amount of flavonoids and phenolic compounds which exhibited the greatest antioxidant activity[8]. The occurrence of flavonoid and terpenoids content in the plants may be one of the reasons for their antibacterial activity[24]. Bioflavanols isolated from the ethanol extract of branches and leaves of *C. lanceolata* act as analgesics and anti-inflammatory[8]. Flavanoids isolated from *C. lanceolata* have anti-cancerous activities against liver and kidney tumors[25]. A flavonoid naming quercetin have anti-obesity activity, it can reduce insulin resistance in animals without fat accumulation in the muscles[26].

Present study implied the applications of *C. lanceota* plant for the medicinal use. These preliminary analyses suggest that *C. lanceota* plant contains potentially health-protective phytochemical compounds with a potent source of natural antioxidants and antibacterial activities that may be clinically promising. Moreover, studies are needed to confirm these results at molecular level as molecular characterization of active constituents of *C. lanceolata* or will be achieved by fractionation on suitable matrices followed by structural elucidation from NMR/mass spectrometry etc. Further, *in vivo* studies will be done on a mouse animal model to confirm these results.

CONCLUSION

The isolation and purification of therapeutic potential compounds from *C. lanceolata* could be used as an effective source against bacterial and fungal diseases in humans. The present study provides evidence that the extract of *C. lanceolata* is a potential source of natural antioxidant.

SIGNIFICANCE STATEMENTS

This study discovers the biological properties of *Cunninghamia lanceolata* that can be beneficial to fight with the disease causing pathogens. The potentially health-protective photochemical compounds with an effective source of natural antimicrobial and antioxidants can be clinically promising. This study will help the researcher to uncover the critical areas of medicinally important plants or herbs in Western Himalayans regions that many researchers were not able to explore. Thus a new theory on natural antimicrobial and antioxidants may serve as a benchmark for the chemical synthesis of active semi-synthetic analogs.

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