Research Article
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Antioxidant and phytochemical analysis of volatile oil and extracts of *Pinus wallichiana*

Abstract

The present study aims to explore the chemical constituents and antioxidant potential of Pinus wallichiana essential oil and extracts. Chemical composition of the essential oil was analyzed by GC-MS and result showed that the oil contain three major monoterpene compounds viz. α-pinene (48.6%), β-pinene (45.6%) and limonene (5.6%). Antioxidant potential of Pinus wallichiana was analyzed by three basic methods; DPPH radical scavenging method, FRAP assay and Fe2+ ion chelating activity. Among leaf and bark extracts, best radical scavenging, activity was determined by aqueous extract (IC50 values 20.83±0.8µg/ml) of bark followed by its methanol extract (IC50 value 25.9±1.6µg/ml). The aqueous extract of bark also exhibited better reducing and chelating activity than leaf extracts. Essential oil showed moderate radical scavenging and chelating activity but negligible reducing activity. Phytochemical analysis of extracts determined that the aqueous and methanol extracts of bark contain rich amount of poly phenol and flavonoids, in comparison to the leaf extracts. A significant correlation between the antioxidant activity and these phytoconstituents of the extracts has been observed. The results of the present study concluded that the bark of Pinus wallichiana is a potential source of active antioxidant constituents that could be further explored and exploited for numerous commercial applications.

Keywords: Pinus wallichiana, antioxidant, phytochemicals

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Introduction

Oxidative stress and their adverse effect on human health has become a serious problem. Under stress conditions, there is increase in production of reactive species viz., hydrogen peroxide (H_2O_2), superoxide ion (O_2), and hydroxide radical (OH) during cellular respiration than enzymatic antioxidants (e.g., superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase) and non-enzymatic antioxidants (e.g., ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, carotenoids, and flavonoids). This imbalance between reactive oxygen species production and the antioxidant defence could cause damage to cellular bio-molecules like carbohydrates, proteins, lipids and DNA, that may leads to metabolic and genetic alteration.[1] A lack of antioxidants could facilitates the development of degenerative diseases,[2] including cardiovascular diseases, cancers,[3] neurodegenerative diseases, Alzheimer's disease[4] and inflammatory diseases.[5]

Medicine plants are effective in treating various diseases caused by oxidative stress. The medicinal properties of plants are because to their biologically active metabolites like phenolic and flavonoid compounds, which are potential antioxidants.[6,7] Positive correlation between these phytochemicals in plant extracts and antioxidant activity has been reported b many researchers.[6,8,9]

Pinus wallichiana commonly called Blue pine or *Kail*, is evergreen coniferous tree native to Himalaya, grows in mountains at altitudes of 1800–4300m. it yields superior timber and especially used as joinery wood. The wood yields oleoresin (by tapping) which by distillation furnishes turpentine and rosin. It is a source of numerous valuable bye products, such as gases, light and heavy tar oils, turpentine pitch, pyroligneous acids, etc. The residue of wood is withdrawn as charcoal. *Pinus wallichiana* essential oil produced by distillation is used for medical purposes. Earlier, some efforts were made to use leaves by reducing them to fibre which was used for weaving into medicinal underclothing, for the manufacture of coarse matting, resembling coconut mating, for surgical dressings. During the recent past, in many rural areas of Kashmir, blue pine wood pieces were used to obtain a dark brown, viscous and sticky substance, called *killam*. This substance was traditionally applied by farmers on their arms and legs to protect them from insects (*Khase*) while working in water logged paddy fields. *Killam* would stick firmly; thereby protecting the exposed parts from insect bites (*Khase*), etc.[10] So keeping the immense traditional importance of *Pinus wallichiana* in view, the present study aims to explore the phytochemicals and antioxidant properties of different parts of *Pinus wallichiana.*

Material and methods

Plant material and Preparation of extracts

Spines and bark of *Pinus wallichiana* was collected from China region of Bhaderwah, Jammu and Kashmir. Identification of the plant material was done an expert taxonomist of Department of Botany, University of Jammu, Jammu. Plant material (leaf and bark separately) was shade dried was powdered in an electronic grinder for the preparation of extracts. Three types of extracts were prepared in three different solvents *viz.,* water, methanol and chloroform. 200g of dried powder was extracted thrice for each solvent. Resulting extracts were pooled, filtered and evaporated by using rotary vacuum evaporator. The slurry obtained was finally lyophilized to dried powder.[11]

Isolation and chemical analysis of essential oil

Fresh spines of *Pinus wallichiana* were subjected to hydro distillation in a Clevenger type apparatus for 3-4h for isolation of essential oil. The extracted oil was dried by adding anhydrous sodium sulphate and stored at low temperature.[12] Chemical composition of the isolated essential oil was analyzed at Indian Institute of Integrative Medicine (CSIR, India), Canal Road, Jammu, India. System used for analysis is GC-MS 4000 (Varian, USA) with a Varian CP-SIL 8CB column (30m×0.32mm i.d., 1µm film thickness). Injector temperature was 230°C. Oven temperature program used was holding at 60°C for 5min, heating to 250°C at 3°C/min. Helium as a carrier gas used at a constant flow of 1.0 ml/min and an injection volume used was 0.20µl. The Mass spectrometer scan parameters included electron impact ionization voltage of 70 eV, a mass range of 40–500m/z. The components present in essential oil were analyzed by comparing their mass spectra with the compounds available in NIST05 (version 2.0) library.[13]

DPPH radical scavenging activity

Antiradical activity of the extracts and essential oil was determined by DPPH method. Essential oil was analyzed by method given by Bozin et al.[14] 1ml of different concentrations of the test sample was mixed with 1ml of a DPPH (90μM, in methanol), and final volume was made to 4ml with methanol. The mixtures were kept at 25°C in the dark for 1hour. The absorbance was measured at 517nm using a UV-VIS spectrophotometer.

Free radical scavenging activity of *Pinus wallichiana* extracts was determined by DPPH method given by Abe et al.[15] with some modifications. 1ml of DPPH (0.5mM in methanol) solution was mixed to 2 ml sample and to this 2ml of 0.1 M sodium acetate buffer (pH 5.5) was added. The mixtures were well shaken and kept at room temperature in the dark for 30min. The absorbance was measured at 517nm. The radical scavenging activity was calculated as a percentage of DPPH radical discoloration, using the equation:

$$\%Radical\ Scavenging\ Activity = \left[\left(A_0 - A_S\right)A_0\right] \times 100$$

Where, A_0 is the absorbance of the control and A_S is the absorbance of the test compound.

FRAP assay

Reducing power of *Pinus wallichiana* extracts was analyzed by FRAP assay according to the method followed by Li et al.[16] FRAP reagent was prepared in acetate buffer (300mM) by adding 10mM 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ) solution in 40mM HCl and 20mM $FeCl_3$ solution in proportion of 10:1:1 (v/v), respectively. FRAP reagent was prepared fresh at the time of use. 50µl of the sample was added to 1.5ml of the FRAP reagent and after 3-4min, absorbance was measured at 593nm. A standard curve was prepared by using $FeSO_4$ (100-2000µM) and the result was expressed as µmol Fe (II)/gm dry weight of extract.

Chelation activity

The chelating power of the *Pinus wallichiana* Extracts on ferrous ions was determined by the method given by Dinis et al.[17] Test sample (500µl) and 740µl of methanol were added to 20µl of 2mM $FeCl_2$. The reaction was initiated by the addition of 40µl of 5mM ferrozine into the mixture. The reaction mixture was incubated at room temperature for 10min and finally the absorbance was measured at 562nm. The ratio of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the equation:

$$\%\ chelating\ power = \left[\left(I_0 - I_S\right)I_0\right] \times 100$$

Where, I_0 is the absorbance of the control and I_S is the absorbance of the test compound.

Total phenols and flavonoids in extracts

Total phenolic content in the extracts was estimated by Folin-Ciocalteu method.[18] 0.5ml of extract was mixed with 0.5ml of 1N Folin-Ciocalteu reagent and 1ml of 20% Na_2CO_3. After 10 min of incubation, the absorbance was measured at 750nm Gallic acid was used as standard compound to estimate total phenolic content and was expressed in mg GAE/g of the extract.

Total flavonoid content of the extracts was estimated by $AlCl_3$ colorimetric method.[19] Plant extracts were diluted with distilled water to a volume of 3.5ml and added 150µl of a 5% $NaNO_2$ solution. After 5min, 300µl of 10% $AlCl_3$ solution was added. After 6min, 300µl of 1M NaOH and 550µl of distilled water were added. The mixture was well shaken and absorbance was measured at 510nm in UV-VIS spectrophotometer. Quercetin was used as standard compound to estimate total flavonoid content in extracts and was expressed in mg GAE/g of the extract.

Results and discussion

Hydro-distillation of fresh needles of *Pinus wallichiana* yielded 1.2% light yellow coloured essential oil. Chemical composition of the essential oil was analyzed by Gas Chromatography and Mass Spectrometry (GC-MS). Analysis showed the presence of three major components α-pinene (48.6%), β-pinene (45.6%) and limonene (5.6%) accounting for 99.8% of total components of the essential oil. All the three compounds found in essential oil belong to monoterpene class of terpenoids. Dar et al.[20] also analyzed the chemical composition of the essential oil of *Pinus wallichiana* growing in high altitudes of Kashmir, India. They found diverse range of compounds in essential oil but still in agreement that β-pinene (46.8%) and α-pinene (25.2%) are the major components of the essential oil. The difference in chemical composition might be due to spatial variation as the plant samples are from two different regions of Jammu and Kashmir.

Antioxidant activity of *Pinus wallichiana* was analyzed by three different methods *viz.,* DPPH radical scavenging activity, metal ion chelating activity and FRAP assay. The results of antioxidant activity have been given in Table 1. The DPPH radical scavenging activity was determined on the basis of concentration providing 50% inhibition. Among the leaf and bark extracts, highest antiradical potential was observed in aqueous and methanol bark extracts with IC_{50} values 20.83±0.8µg/ml and 25.9±1.6µg/ml respectively. Chloroform extract of both leaf and bark have shown negligible radical scavenging activity. Essential oil isolated from leaf part showed moderate radical scavenging activity with IC_{50} values 514.4±5.8µg/ml.

The ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ, was determined in FRAP assay. Reducing potential of *Pinus wallichiana* extracts and essential oil was expressed in µmol Fe (II)/g dry weight of plant material. Strongest antioxidant activity

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to reduce the Ferric ions (Fe^{3+}) was observed in bark aqueous extract (3495±11.3µmol Fe (II)/g) followed by bark methanol extract (2530±6.3µmol Fe (II)/g) like in case of radical scavenging activity. All leaf extracts and essential oil exhibited very poor reducing activity in comparison to the bark extracts.

Table 1 Total Phenols, flavonoids and antioxidant potential of *Pinus wallichiana* extracts

Extract	TPC (mg GAE/g dry weight)	TFC (mg QE/g dry weight)	DPPH Assay (IC50 in µg/ml)	FRAP Assay (µmol FeII /g)	%age Chelation capacity at 500µg
(Leaves) Methanolic	30.8±1.1	-	260.4±3.2	111.6±3.2	57±1.2
Aqueous	40±1.1	10±0.5	285.3±4.1	86.6±1.3	19±0.6
Chloroform	10±0.4	-	-	26.6±2.1	34±0.4
Essential oil	-	-	514.4±5.8	-	16±0.3
(Bark) Methanolic	330±6.4	520±12.a7	25.9±1.6	2530±6.3	59±1.8
Aqueous	447±9.6	610±11.2	20.83±0.8	3495±11.3	44±1.4
Chloroform	30±0.5	-	-	245±3.3	29±0.9

Data presented as mean±standard deviation; TPC, Total phenolic content; TFC, Total flavonoid content; GAE, Gallic acid equivalent; QE, Quercetin equivalent.

Chelating property on metal ions is one of important mechanism of antioxidant activity. Metal ion chelating capacity was determined at 0.5mg/ml concentration. Methanol extract of both leaf and bark of *Pinus wallichiana* showed a better chelating effect on ferrous ions (59±1.8% & 57±1.2%). Aqueous extract of bark also showed moderate chelating capacity with 47% at 0.5mg/ml. Essential oil and chloroform extracts determined poor chelating capacity of fe^{2+} ions. Biological properties of phenolic and flavonoid compounds have already been known universally. Table 1 also represented total phenol and total flavonoid content in leaf and bark extracts of *Pinus wallichiana*. Highest amount of poly phenols and flavonoids was observed in aqueous extract of bark (447±9.6mg GAE/g; 610±11.2 QE/g dry weight, respectively) followed by methanol extract of bark (330±6.4 mg GAE/g; 520±12.7 QE/g dry weight). Leaf extracts possess comparatively less poly phenol and flavonoid content. Among all the extracts, least content of poly phenol and flavonoid was observed in chloroform extracts of both leaf and bark. Many studies have shown that the antioxidant activities in the plants are associated with their phytochemicals contents. Naeem et al.[21] reported that the methanol extract of *Pinus wallichiana* (bark) growing in Kuldana, Murree (Pakistan), contained a wide variety of flavanols in considerable amounts. Dar et al.[20] analyzed that the essential oil isolated from *Pinus wallichiana* growing in Kashmir (J&K), has strong antiproliferative potential against five human cancer cell lines and good radical scavenging activity on DPPH radicals. Maimoona et al.[22] also reported antioxidant potential and phytochemicals in different extracts of *Pinus wallichiana* growing in Pakistan and found a positive correlation between the antioxidant properties and total flavonoid content. This may be due to their redox properties of the phenolic and flavonoids that make them good reducing, scavenging and chelating agent.[18,23] The results also revealed that there is significant correlation between the antioxidant activity (Reducing activity, Radical scavenging activity, and chelating power) and the phenolic/flavonoid content of *Pinus wallichiana* extracts. Therefore, it is possible that these phytochemical compounds are the major contributor for the antioxidant activity of the *Pinus wallichiana*.

Conclusion

In conclusion, the present study explored the phyto constituent and antioxidant properties of *Pinus wallichiana* growing in Bhaderwah region of Jammu & Kashmir (India). GC-MS analysis showed that its essential oil is a rich source of α-pinene and β-pinene and possess moderate antioxidant activity. Bark of *Pinus wallichiana* determined strong antioxidant activity and can serve as a valuable source of natural antioxidant. A significant correlation between antioxidant activity and TPC/TFC were found indicating phenolic and flavonoids compounds are the major contributor of antioxidant potential of *Pinus wallichiana.*

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Conflicts of interest

Author declares that there are no conflicts.

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