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IDENTIFICATION AND QUANTIFICATION OF SOME NATURAL COMPOUNDS OF *Pinus gerardiana* LEAF EXTRACT AND ITS ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES

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Abstract

Pinus gerardiana (chilgoza) is an evergreen tree, in India with numerous health benefits including anti-septic, anti-fungal, anti-bacterial, antioxidant, anti-inflammatory, anti-viral, antineuralgic, choleretic, diuretic, expectorant, hypertensive properties. The plant is classified as endangered and enlisted in Red Data Book, hence it should be uncovered before its extinction. The leaf part of the plant is extracted and six natural compounds including quercetin, caffeic acid, β-sitosterol, catechin, lupeol and p-coumaric acid were identified and quantified from it. Mass spectroscopy analysis assumed the presence of twenty-one different compounds. The primary identification of the compounds was done from natural product data bases. Secondary identification using HPLC retrieved the presence of nineteen compounds. HPTLC analysis confirmed and quantified the presence of six major compounds.The quantity of catechin (2.86%w/w of extract) was found to be maximum followed by Caffeic acid (2.06%w/w of extract) in the DCM/Methanol leaf extract. Different extracts were analyzed for the antioxidant and antimicrobial activities. Antimicrobial activity was performed using zone of inhibition method for eight different leaf extracts of *Pinus gerardiana*. Among the extracts hydroalcoholic extract showed good potency in terms of zone of inhibition (14.33 ±0.25mm) against S. aureus whereas acetone extract showed good potency (16.01±0.81mm) against S.mutans,ethylacetate extracts showed good potency (16.33±0.47mm) against E. coli, ethylacetate extracts showed good potency (17.35±0.58) against K. pneumoniae and n-hexane extracts showed good potency (16.33 ±0.38) against *P. aeruginosa*. Chlormphenicol was used as standard. Antioxidant activity was done by DPPH method. Ascorbic acid was taken as control in the antioxidant study. IC50 values were calculated from the known protocol. Methanol extract showed good antioxidant activity (51.3 ±3.1µg/ml) whereas n-hexane showed the least (189.5 ±1.30µg/ml).

Keywords: *Pinus gerardiana; extract; Mass spectroscopy; HPLC; HPTLC; Antioxidant; antimicrobial*

Introduction

Pinus gerardiana a small to medium sized evergreen tree, bearing common name "chilgoza or neozapine in India". It's international dissemination is very scanty, sequestered to mountains of eastern Afghanistan, Pakistan, India, and other dispersed regions in the Hindu Kush Himalaya [1].The present scenario of the chilgoza forest has already shriveled to about 2000 hectares in Himachal Pradesh, India and categorized as Endangered enlisted in Red Data Book [2-5].Nuts of Chilgoza (pine nuts) are palatable comprising un-saturatedfatty acids, pharmacologically advantageous for lowering high cholesterol level in human. It also contain vitamins,beta-carotene, thiamin (B1), riboflavon (B2), niacin (B3), pantothenic acid (B5),vitamin B6, folate (B9), vitamin C, vitamin E, vitamin k as well as minerals such as calcium, iron, magnesium, manganese, phosphorus, zinc, potassium andphosphorus as well as other compounds with water, that is why the plant is reputed for curative and nutritional drives[6,7].

*P. gerardiana*seeds, barks, roots have been proclaimed earlier for several pharmacological activities including anti-septic, anti-fungal, anti-bacterial, antioxidant, anti-inflammatory, anti-viral, antineuralgic, choleretic, diuretic, expectorant, hypertensive properties [8-10].Many reports have shown several biomolecules from the natural extract pertinent to different diseases from different parts of the plants, hither from leaf part has never been exposed. In this research, we have reported the leaf extract relevant to biomolecular identification, quantification, and some pharmacology (antimicrobial and antioxidant) associated with it.

Methods

Collection of plant material

Pinus gerardiana leaves part collected from Reckongpeo region of Kinnaur district and Killar region of Chambadistrct of Himachal Pradesh, India respectively. Plant was authenticated from Department of Forest Product, DR YS Parmar University of Horticulture and Forestry, Nauni, Solan, H.P, India and received an herbarium no. 13614

Drying and size reduction of leaves

Plant sample was air dried in the dark room to avoid overlosses of thermolabile and volatile constituents. Various other methods were also employed for drying plant material such as salt drying or silica gel, oven drying and freeze drying.The plant material was reduced to coarse fine powder with the help of mechanical grinder[11].

Selection of solvent

Solvent selection for the extract preparation was done in increasing polarity *viz.* n-hexane, chloroform, ethylacetate, acetone, n-butanol, methanol, hydroalcoholic and water.

Extract preparation

Pinus gerardiana leaves part extraction were done using NCI protocol [12]. Soxhlet assembly was used for the extract preparation. A combination of dichloromethane (DCM)-methanol solvent in the ration (1:1) was taken for the initial extraction. Later on fractionation was done in increasing polarity and dried under vacuum. Eight different extracts were collected such as n-hexane, chloroform, ethylacetate, acetone, n-butanol, methanol, hydroalcoholic and aqueous extract. Extracts were stored in a well closed container, covered with aluminium foil and stored in the refrigerator. The extracts were diluted with the solvent and administered for the *in-vitro* antimicrobial and antioxidant studies.

Liquid chromatography-mass spectroscopy profiling of *Pinus gerardiana* leaves DCM/methanol (1:1) extract

The major chemical constituents reveal in the DCM/methanol (1:1) extract of *Pinus gerardiana* leaveswere identified by using liquid chromatography-mass spectroscopy (LC-MS). The instrument used for LC-MS was MicroTOFQ and the technique used was ESI (Electrospray ionization technique). For the liquid chromatography separation, Phenomenex C18(150 × 4 mm i.d., 5 µ) with single quadrupole mass spectrometry analyzer was used. For the mobile phase, 0.5 percent formic acid–acetonitrile (75:25 percent) was used as. 0.5 ml/min was the flow rate. The solvent had isocratic elution controlled. The temperature of the column was maintained at 30°C. In the positive ion mode,

the MS spectrum was acquired and 50 m/z-1000m/z was scanned. The drying gas (N2) nebulizing pressure was of 25psi, temperature was 350°C and the gas flow rate was 6 ml/min. About 0.6 g of the extract was diluted with methanol and filtered prior to examination with a 0.22 µm nylon filter. For examination, a 5 µl volume of the extract was injected onto the column. By using spectrum database for organic compounds, the mass fragmentations were identified [13].

Antimicrobial assay of different extracts of *Pinus gerardiana*leaves

Collection of Bacterial strains

The bacterial strains were obtained from IMTECH, Chandigarh. Differentbacterial strains are *Escherichia coli***(MTCC-739)**, *Klebsiella pneumonia* **(MTCC-39)**, *Staphylococcus aureus* **(MTCC-737),***Pseudomonas aeruginosa***(MTCC-2453)** and *Streptococcus mutans***(MTCC-890)**. In Mueller Hinton broth, the microbial strains were subcultured and incubated at 37°C for 24 hours before to the experiment.

Antimicrobial activity of plant extracts

The antimicrobial activity of the plant extract was evaluated using the method agar well diffusion [13-15].Turbidity of the sub cultured microorganisms was adjusted using 0.5 McFarland as standard with sterile distilled water (~1.5×108 cells/ml). In triple distilled water Mueller Hinton Agar powder (HiMedia) was prepared. Agar glass plates were prepared and inoculated using spread plate method with the tested microorganisms.Then, the plates were retained for 30 min in the laminar air flow.Cork borer was used to punch 6 mm diameter wells in the seeded agar plates. Then, the extracts prepared were added a constant concentrationof 100 µl/well in the wells. As a positive control the standard antibiotic chloramphenicol (HiMedia) has been used. DMSO was used as a negative control to ensure that the solvent used to dissolve the extract did not display antimicrobial activity. Then, the plates were incubated for 24 hrs.at 37°C. The inhibition zone was measured after incubation, using the zone scale.

Antioxidant assay of different extracts of *Pinus gerardiana*leaves

Radical Scavenging Activity for DPPH

The free radical scavenging activity of hexane, chloroform, ethyl acetate, acetone, n-butanol, methanolic, hydroalcholic and aqueous extracts of DPPH (2,2-diphenyl-2-picrylhydrazyl) was evaluated by known method [16-19]. Ascorbic acid was used as standard. The(radical solution) stock solution, prepared by dissolving 24 mg DPPH in 100 ml methanol, was kept in refrigerator until further application. The working solution of the radical was prepared by diluting the DPPH stock solution with methanol in order to achieve an absorbance of approximately 0.98 (±0.02) at 517 nm.In the test, 3 ml of DPPH working solution was mixed with 100 µl plant extract (1mg/ 1ml) or the standard solution. The absorption was measured at 517 nm for a period of 30 min. The percentage of antioxidant or radical free scavenging activity was calculated using the following formula:

%Antioxidant activity = [Ac-As)/Ac] x 100

where, Ac and As are the control and sample absorbance, respectively. The control contained 100 µl methanol in place of the plant sample.

Qualitative phytochemical profiling of the *Pinus gerardiana* leaves DCM/methanol (1:1) by HPLC

DCM/methanol (1:1) of *Pinus gerardiana*leaves(6mg) extract was dissolved in 10 mL methanol (80%) and further diluted with 25ppm solution. The solution was filtered with syringe filter 0.22 microns. The HPLC system (AgilentTechnologies) consists of an LC-binary pump, diode array detector, EZ-chrom system controller,anda column of Innoval C18 (4.6×250mm). 0.14 g of anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) was added to 900ml of HPLC grade water, and orthophosphoric acid (0.5ml) was dissolved in the above mixture for separation. The mixture volume was make up with water upto1000 ml and then filtered by a membrane filter (0.45 µ). The solution was then placed in a sonicator for 3 minutes after filtration. The prepared solution was considered the solvent of the mobile phase gradient solvent (A) and solvent (B) was used as the Acetonitrile. A sample of 20µl was injected via the SIL-HTC Shimadzu Autosampler. Solvent systemconditions were 20:80 ratios, flow rate 1.5ml/min and a run time of about 45 minutes. The chromatogram at wavelength of 227nm was obtained [13].

HPTLC quantification by HPLC of major constituents *Pinus gerardiana* leaves in DCM/methanol (1:1) extract

The major components of *Pinus gerardiana* leaves DCM/methanol (1:1) extract were quantified using an HPTLC instrument with automatic sample applicator CAMAGLinomat V, TLC scanner III, Camag twin trough chamber10×10 cm, and WinCATS software in present study. The extract (suspended in methanol) andstandard solutions (each 5 µl) (suspended in methanol) were applied in the form of a band havingbandwith a width 8 mm; a distance between 14 mm bands and a constant application rate of 150 nL s-1 using a microsyringe (Hamilton-Bonaduz Schweiz, Linomat syringe, size500 µl) to 60 F254 TLC (10 × 10 cm with a thickness 200 µm) silica gelprecoated plates. TLC plates wereput in a glass developing chamber under the various mobile phases and development was carried out upto 8 cm in an ascending manner. After the development, the densitometric scanning of the air-dried plate was performed by the help of TLC scanner operated inreflectance-absorbance mode, slit dimensions: 6×0.45 at 254 nm. The calibration curve of all thestandards was drawn. The sample and standard spots were applied on TLC plates and thecontents of metabolites were analyzed using regression equation from the calibration plot andexpressed as % w/w [20].

Results

Liquid chromatography-mass spectroscopy profiling of Alcohol:DCM/ 1:1 extract of *P. gerardiana*

We initiated our investigation with mass analysis of the highly polar Alcohol:DCM/ 1:1 extract of *P. gerardiana*. The chemical constituents present in the alcohol:DCM/ 1:1extract were recognized using LC-MS spectroscopy. We observed numerous peaks and analyzation of each peak was done further. When we matched masses of various natural products in the data bases (Dictionary of Natural Product 28.2 and others mentioned in methodology), we found it, most similar with Quercetin, caffeic acid, α-sitosterol, catechin, lupeol, etc. All told, twenty-one compounds were identified and summarized in Table. 1. But the discrepancy of this method was similarity in masses (Masses of α-carotene and lycopene, Vanillic acid and Gallic acid, etc). Further authentication was carried out using HPLC profiling of Alcohol:DCM/ 1:1 extract of *P. gerardiana*.The LC-MS spectra is given below (figure 1).

Qualitative phytochemical profiling of Alcohol:DCM/ 1:1 extract of *P. gerardiana*by HPLC

Qualitative HPLC analysis of alcohol:DCM/ 1:1 extract of *P. gerardiana*was performed using for further confirmation of our previous experiment by LCMS technique. The phytochemical screening was based on observed chromatogram. Ninteen peaks were observed, indicating the presence of nineteen major compounds in the extract. We assumed that the maximum area should corresponds to catechin. The early assumption of similar masses bearing compounds identified from LCMS might be reduced or the instrument was unable to detect them due to less concentrations of those molecules in the mixture. Different peaks, retention time, peak area and area percentage was analyzed (Table 2). We were assured now for the presence of nineteenmajor compounds in alcohol:DCM/ 1:1 leaf extract of *P. gerardiana*. For identification and assurance, we collected all the markers and performed HPTLC analysis further.The HPLCchromatogram is represented in figure 2.

Quantification of major constituents present in *P. gerardiana*by HPTLC

HPTLC analysis of alcohol:DCM/ 1:1 leaf extract of *P.gerardiana*was performed and final assurance was done by matching the spots present in it with the assumed markers. The R_f value of the markers matched with 6 compounds in our herbal extract. We were unable to identify/ quantify other 13 compounds due to non-availability of some markers or might be due to less concentration of those which we have installed the other markers. The identified compounds were quantified using known protocol [21]. The discussions of individual molecules are as follows.

Quercetin quantification by HPTLC in *P. gerardiana*

The Toluene: methanol (7:3, v/v) mobile phase showed sharp peaks consisting with R_f value of 0.65

for Quercetin (Fig A). Quercetin found in the alcohol:DCM/ 1:1 leaf extract of *P. gerardiana* was 1.50 percent w/w of the extract.The HPTLC chromatogram is presented (Figure3).

Quantification by HPTLC of Caffeic acid in *P. gerardiana*

Mobile phase consisting of toluene: ethyl acetate: formic acid (5:4:1, v/v/v) revealed sharp peaks for Caffeic acid with an R_f value of 0.49 (Figure4). Caffeic acid was 2.059 percent w/w of extract found in the extract.Figure 4 shows the HPTLC chromatogram of extract and Caffeic acid.

Quantification by HPTLC of β-Sitosterol in *P. gerardiana*

Mobile phase consisting n-hexane: ethyl acetate (8:2, v/v) showed sharp peaks for β-Sitosterol (Figure5) with an R_f value of 0.61. The β-Sitosterol found in the extract was 0.80 percent w/w.Extract and β-Sitosterol HPTLC chromatogram is shown below (Figure 5).

Catechin quantification by HPTLC in *P. gerardiana*

For Catechin (Figure 6), **t**he mobile phase consisting of toluene: ethyl acetate: formic acid (5:4:1, v/v/v) revealed sharp peaks with R_f value of 0.22. Catechin was found in 2.86 percent w/w in the extract.The extract and Catechin HPTLC chromatogramare presented below (Figure 6).

Lupeol quantification by HPTLC in*P. gerardiana*

The Mobile phase consisting of toluene: ethyl acetate: formic acid (5:4:1, v/v/v) revealed sharp peaks for Lupeol (Figure 7) with R_f value of 0.32. Lupeol was found 0.36 percent w/w in the extract. The HPTLC chromatography of the extract and Lupeol is shown below (Figure 7).

Quantification of *p*-Coumaric acid in *P. gerardiana*by HPTLC

The Mobile phase consisting toluene: ethyl acetate: formic acid (5:4:1, v/v/v) revealed sharp peaks for *p*-coumaric acid with an R_f value of 0.32 (Figure 8). In the extract, *p*-coumaric acid found was 1.84% w/w of DCM/Methanol extract. The HPTLC extract chromatogram and *p*-coumaric acid is presented below (Figure 8).

Antimicrobial results

The prepared eight leaf extracts were evaluated against five bacterial strains at a concentration of 100 µl/well. The standard antibiotic chloramphenicol (HiMedia) was used as a positive control. DMSO was used as a negative control in each agar plate to make sure that the solvent used for dissolving the extract does not show antimicrobial activity. Then, the plates were incubated. After incubation, the zone of inhibition was measured using zone scale method and tabulated (Table 3). Zone of inhibition were measured in millimeter. The graphical representation of the antimicrobial activity is shown in figure 9.

In the showed petri plate, 1,2,3,4,5,6,7,8 areaqueous, *n*-hexane, methanol, chloroform, ethyl acetate, acetone, n-butanol, hydroalcoholic extracts respectively which are brown in color. Here maximum potency for antimicrobial activity was shown by Hydroalcoholic extract (14.33 ± 0.25 mm) and minimum by Chloroform extract (9.60 ± 0.17 mm) against *Staphylococcus aureus.* In case of *Staphylococcus mutans*maximum potency was shown by Acetone extract (16.01 ± 1.81) and minimum by n-hexane extract (8.66 ± 0.20).Against *Escherichia coli*theantimicrobial activity by Ethyl acetate extract (16.33 ± 0.42) and minimum by n-butanol extract (10.33 ± 0.46).Maximum potency for antimicrobial activity shown by n-hexane extract (16.33 ± 0.38) and minimum by aqueous extract (11.66 ± 0.65) against *Pseudomonas areuginosa*bacteria.Whereas as*Klebsiella pneumonia*maximum potency by ethyl acetate extract (17.35 ± 0.58) and minimum by Hydroalcoholic extract (11.33 ± 0.15).(Figure 10-14, Table 3).

Antioxidant result

Eight extracts and positive control (Ascorbic acid) were used for antioxidant activity using DPPH method by known protocol. No extract were shown more potency than ascorbic acid but within all extracts methanolic extract reported maximum potency (51.3±3.1µg/ml) for antioxidant activity and minimum value was observed for hexane extract (189.5±1.3µg/ml). The increasing order of the extracts with respect to antioxidant activity are n-hexane < Aqueous < Chloroform <Hydroalcoholic <Acetone < n-butanol <Ethylacetate< Methanol. A

graphical representation of the antioxidant activity is shown in figure 15.

Discussion

Six natural compounds including quercetin, caffeic acid, β-sitosterol, catechin, lupeol and p-coumaric acid were identified from HPLC, mass spectroscopic analysis and quantified from HPTLC analysis. MS investigationpresumed the occurrence of twenty-one different natural compounds. The primary empathy of the compounds was thru from natural product data bases. Secondary identification using HPLC repossessed the incidence of nineteen different natural compounds. HPTLC analysis established and quantified the presence of six major compounds. The amountof Catechin was found to be maximum, which was 2.86% w/w of DCM/Methanol leaf extractfollowed by Caffeic acid (2.06% w/w of extract). Eight different extracts were analyzed for the antioxidant and antimicrobial activity. Antimicrobial activity was done by zone of inhibition method and antioxidant activity was done by DPPH methodusing Chloramphenicol and ascorbic acid as control respectively.Among the eight different extracts hydroalcoholic extract showed good potency in terms of zone of inhibition), which was 14.33 ± 0.25 mm against *S. aureus* bacterial strain, acetone extract showed good potency (16.01 ± 0.81 mm) against *S. mutans* bacterial strain, ethylacetate extracts showed good potency (16.33 ± 0.47 mm) against *E. coli*, ethylacetate extracts showed good potency (17.35 ± 0.58 mm) against *K. pneumoniae* and n-hexane extracts showed good potency (16.33 ± 0.38) against *P. aeruginosa*. The zone of inhibition for chloramphenicol ranged from 22.36 ±0.40 to24.66 ± 0.46 against five strains of bacteria, which concludes that the leaf extracts of *Pinus gerardiana*possessed good antibacterial activity. Antioxidant activity was done by DPPH method. IC_{50} values were calculated from the known protocol. Methanol extract showed good antioxidant activity (51.3 ± 3.1 µg/ml) and n-hexane showed the least (189.5 ± 1.30 µg/ml). The IC_{50} value of the control (Ascorbic acid) was17.9 ± 2.1 µg/ml, which concludes that the leaf extract is average with respect to antioxidant activities.

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References

1. Chib, SS. Kanauar of the trans-Himalaya. – EssEss Publication, New Delhi, 1978;110.
2. Tandon, JC. Revised working Plan for the Kinnaur and Kochi forests (Upper Sutlej valley). – Himachal Pradesh, 1963; 1961-62 to 1975-76.
3. Singh, R.V., Khanduri, D.C., Lal, K. Chilgoza pine (*Pinus gerardiana*) regeneration in Himachal Pradesh. – Indian Forester, 1973; 3: 126-133.
4. Sehgal, R.N., Sharma, PK. Chilgoza: the Endangered Social Forestry Pine of Kinnaur. – Dr. YS Parmar University of Horticulture and Forestry, Nauni-Solan, India. Technical Bulletin No. FBTI, 1989; 1: 1-8.
5. Dogra, P.D. Gymnosperms of India-II. Chilgoza pine (*Pinus gerardiana*Wall.). Bulletin of the National Botanic Garden, 1964; 109: 446.
6. Brufau, G.,Boatella, J., and Rafecas, M. Nuts: source of energy and macronutrients. *British Journal of Nutrition* 96,2006;S24-S28.
7. Sagrero-Nieves, L. Fatty acid composition of Mexican pine nut (Pinus cembroides) oil from three seed coat phenotypes. *Journal of the Science of Food and Agriculture*,1992; 59, 413-414.
8. Amr, A.R., and Abeer, E.Hypolipideimic and hypocholestermic effect of pine nutsin rats fed high fat, cholesterol-diet.*World Applied Sciences Journal*, 2011; 15, 1667-1677.
9. Lawlees, J. The encyclopaedia of essential oils (Element Books Limited: Boston). Li, H, Huang et al. (2010). Anti-thrombotic activity and chemical characterization of steroidal saponins from Dioscoreazingiberensis CH Wright. *Fitoterapia*, 1992; 81, 1147-1156.
10. Singh, G., Kumar, D., and Dash, A.K. Pinus gerardianaWallichex. D. Don.- A Review, Phytomedicine Plus 2021; 1 (2): 100024.
11. Gopalasaththeeskumar K. Significant role of soxhlet extraction process in phytochemical

Research. *Mintage Journal of Pharmaceutical & Medicinal Sciences*. 2018; 7(1): 43-47
12. McCloud T.G. High throughput extraction of plant, marine and fungal specimens for preservation of biologically active molecules. *Molecules*, 2010; 15:4526-4563.
13. Mari, A.D., Lyon, L. Fragner, P. Montoro, S. Piacente, S. Wienkoop, et al., "Phytochemical composition of *Potentilla anserina* L. analyzed by an integrative GC-MS and LC-MS metabolomics platform," *Metabolomics*, 2013; 9: 599–607.
14. Devi, J., Sharma, S., Kumar, S., Jindal, D.K., Dutta, P.P., Kumar, D. "Transition metal (II) complexes of hydrazones derived from tetralone: synthesis, spectral characterization, in vitro antimicrobial and cytotoxic studies", Research on Chemical Intermediate, 2021; 47: 2433-2467.
15. Kumari M., Kumar, R., Kaur, J., Mehta, SK., Pandey, S.K., Kumar, D., Dash, Ashutosh K., Gupta, N. DL-Valine assisted fabrication of quercetin loaded CuOnanoleaves through microwave irradiation method: Augmentation in its catalytic and antimicrobial efficiencies, Environmental Nanotechnology, Monitoring & Management, 2020, 14 ,100306 .
16. Ahmed, D., Khan, M.M., Saeed, R. Comparative analysis of phenolics, flavonoids, and antioxidant and antibacterial potential of methanolic, hexanic and aqueous extracts from *Adiantum caudatum* leaves. *Antioxidants* 4, 394-409.
17. Kumar, D., Kumar, R., Ramajyam, R., Lee, K.W., Shin, D.S. Synthesis, antioxidant and molecular docking studies of (−)-catechin derivatives, Journal of the Korean Chemical Society, 2021, 65, 106-112.
18. Kumar, K. , Kumar, D., Jindal, D.K., Yadav, M., Sharma, N., Gupta, R. Comparative antioxidant activity of roots and fruits of *Piper sylvaticum* Roxb, J. Compr. Pharm., 2016, 3, 132-135.
19. Getahun, T., Sharma, V., Kumar, D., Gupta, N. Chemical composition, antibacterial and antioxidant activities of essential oils from *Laggeratomentosa* Sch. Bip. ex Oliv. et Hiern (Asteraceae), Turkish Journal of Chemistry, 2020, 44, 1-10.
20. Bhole, R.P., Shinde, S.S., Chitlange, S.S., and Wankhede, S.B. "A High-Performance Thin Layer Chromatography (HPTLC) Method for Simultaneous Determination of Diphenhydramine Hydrochloride and Naproxen Sodium in Tablets," *Analytical Chemistry Insights,* 2015; 10, 47-51.
21. Xie, P. S. "A feasible strategy for applying chromatography fingerprint to assess quality of Chinese herbal medicine," *Traditional Chinese Drug Research & Clinical Pharmacology*, 2001; 12 (3): 141–169.

Fig 1: ***Pinus gerardiana*** **(Leaves)**

Table 1: Collection of leaves part of *Pinus gerardiana*

S.NO.	Plant	Part	Place	Altitude	Time of collection
1.	*Pinus geradiana*	Leaves	ReckongPeo, Kinnaur, H.P	2290 m	20-05-2017
2.			Lug village, Killar, Chamba, H.P	2600 m	26-07-2018

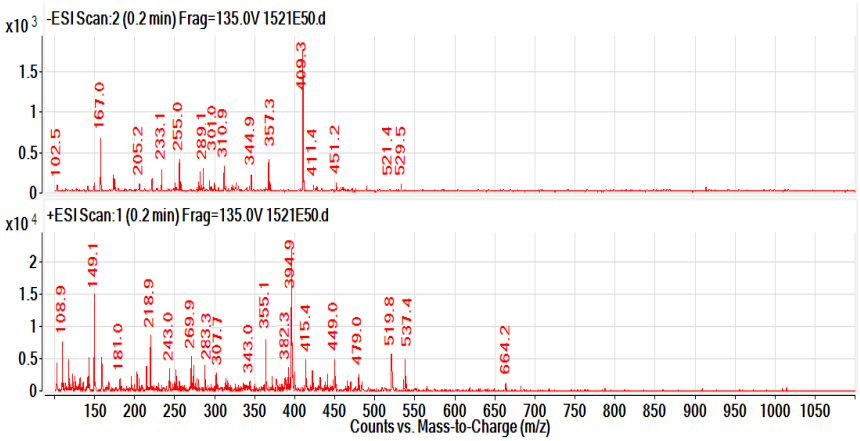


Fig 1: LC-MS spectrum of Alcohol:DCM/ 1:1 extract of *P. gerardiana*showing the major identified compounds in both +/- mode.

Table:2 Identified compounds and their expected and observed masses.

Compound As In Spectra	Expected Mass (g/mol)	Observed Mass (g/mol)
Caffeic acid+ H^+	181.0	181.0
-sitosterol+ H^+	415.4	415.4
Catechin- H^+	289.1	289.1
Quercetin- H^+	301.0	301.0
Lupeol+ H^+	382.3	382.3
Gallocatechin + H^+	307.7	307.7
p-Coumaric acid+ H^+	165.0	165.0
Chlorogenic acid+ H^+	355.0	355.0
Lutein- H^+	529.5	529.5
Lycopene - H^+	521.4	521.4
Tocopherol+ H^+	415.4	415.4
Campesterol- H^+	357.3	357.3
Stigmasterol- H^+	411.4	411.4
Ellagic acid- H^+	301.0	301.0
Vannilic acid - H^+	167.0	167.0
Gallic acid- H^+	167.0	167.0
-Carotene- H^+	521.4	521.4
-Carotene + H^+	537.4	537.4
Oleic acid + H^+	283.3	283.3
-Pinene+ H^+	149.1	149.1
-Pinene+ H^+	149.1	149.1

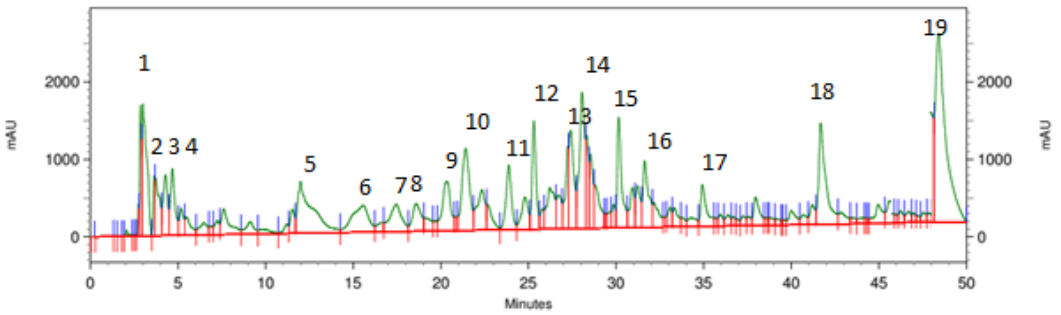


Fig2: HPLC chromatogram of Alcohol:DCM/ 1:1 extract of *P. gerardiana*showing the major identified 19 compounds

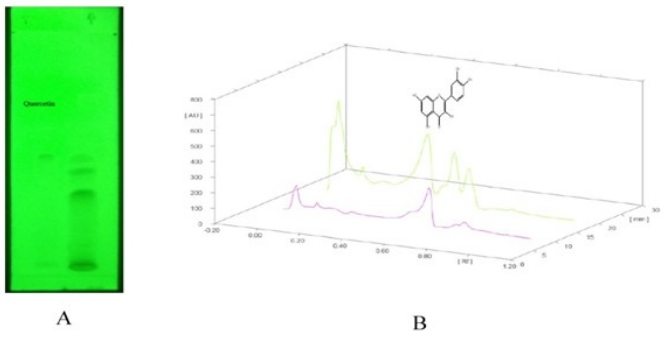


Fig 3: TLC and HPTLC estimation of quercetin in the extract. (A) TLC plate of extract with Quercetin marker (B) HPTLC chromatogram of extract and quercetin in 3D mode.

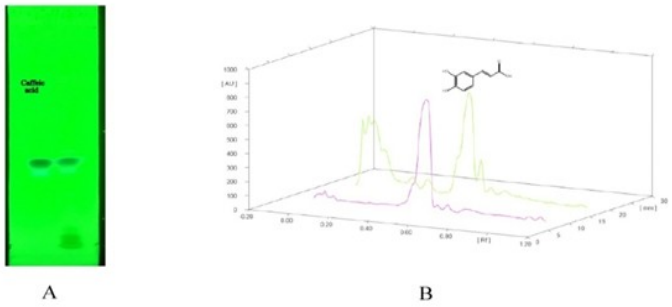


Fig 4: TLC and HPTLC estimation of Caffeic acid in extract. (A) TLC plate of extract with Caffeic acid marker (B) HPTLC chromatogram of extract and Caffeic acid in 3D mode.

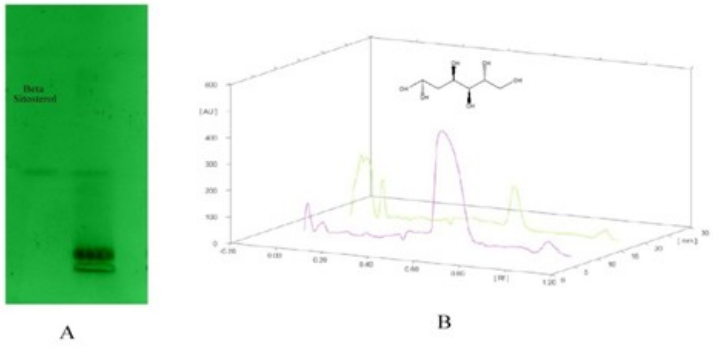


Fig 5: TLC and HPTLC estimation of β-Sitosterol in extract. (A) TLC plate of extract with β-Sitosterolmarker (B) HPTLC chromatogram of extract and β-Sitosterol in 3D mode.

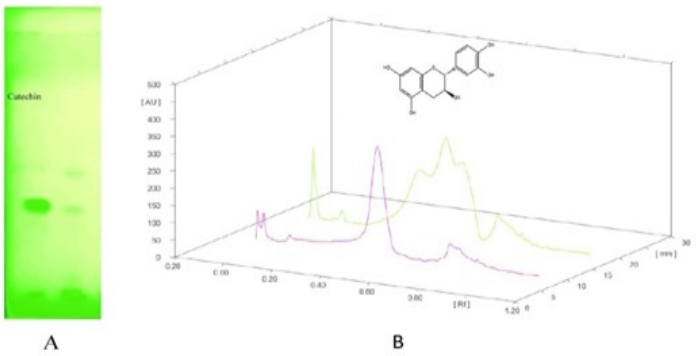


Fig 6: TLC and HPTLC estimation of Catechin in extract. (A) TLC plate of extract with Catechin (B) HPTLC chromatogram of Catechin and extract in 3D model.

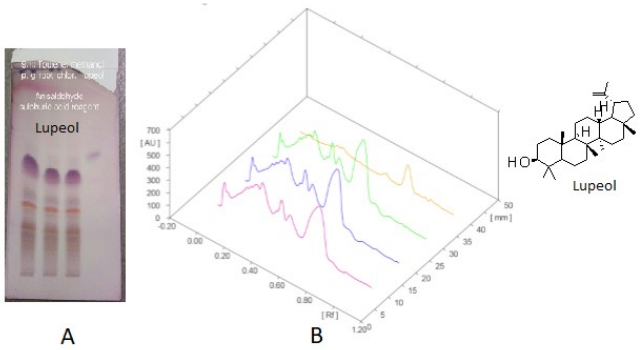


Fig 7: TLC and HPTLC estimation of Lupeol in extract. (A) TLC plate of extract with Lupeol (B) HPTLC chromatogram of extract and Lupeol in 3D model.

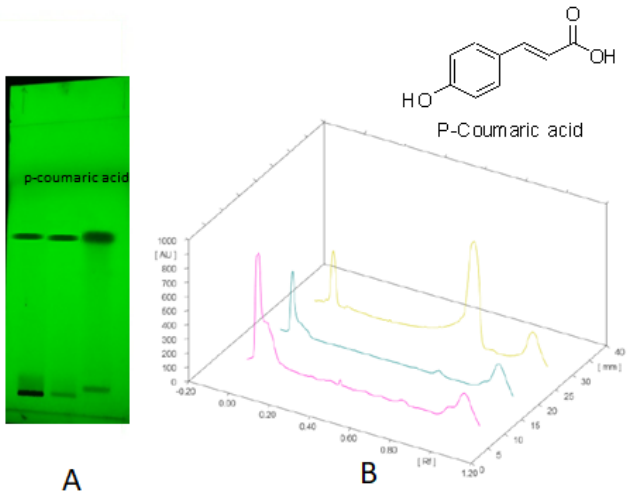


Fig 8: TLC and HPTLC estimation of *p*-Coumaric acid in DCM/Alcoholic extract. (A) TLC plate of extract with *p*-coumaric acid (B) HPTLC chromatogram of extract and *p*-coumaric acid in 3D model

Table 3: Antimicrobial activity of different eight different extracts of *Pinus gerardiana* leaves

Extracts	zone of inhibition				
	S.aureus	***S.mutans***	***E.coli***	***K.pneumonia***	***P.areuginosa***
1.	11.66 ± 0.43	10.66 ± 0.47	13.66± 0.65	13.33 ± 0.41	11.66 ± 0.65
2.	10.33 ± 0.37	9.33 ± 0.28	14.30 ± 0.32	15.33 ± 0.43	16.33 ± 0.38
3.	12.03 ± 0.05	13.66± 0.30	12.66 ± 049	12.66 ± 0.56	13.33 ± 0.44
4.	9.60 ± 0.17	8.66 ± 0.20	13.66 ± 0.57	14.66 ± 0.26	15.66 ± 0.05
5.	11.33 ± 0.27	12.66 ± 0.23	16.33 ± 0.42	17.35 ± 0.58	15.35 ± 0.15
6.	12.66 ± 0.40	16.01 ± 1.81	16.04 ± 0.61	13.6 ± 0.25	14.01 ± 0.06
7.	13.33 ± 0.41	13.66 ± 0.36	10.33 ± 0.46	11.66 ± 0.32	12.06 ± 0.43
8.	14.33 ± 0.25	14.66 ± 0.47	13.60 ± 0.43	11.33 ± 0.15	14.30 ± 0.47
Positive	24.33 ± 0.47	22.36 ± 0.40	23.66 ± 0.47	23.33 ± 0.49	24.66 ± 0.46

N.B. Antimicrobial activity expressed as diameter of zone of inhibition in mm. Value (mm) ±S.D in triplicates.

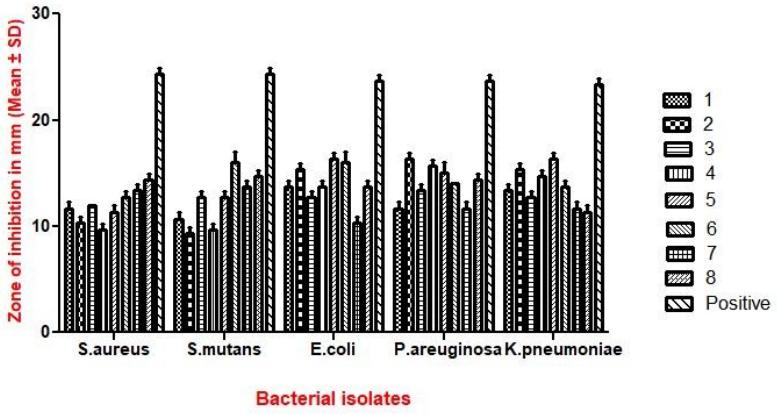


Fig 9: Graphical representation of plant extracts showing a varied degree of sensitivity against test bacteria *S.aureus*, *S.mutans*, *E.coli*, *P.areuginosa* and *K.pneumonia*where 1,2,3,4,5,6,7,8 and positive are Aqueous, n-hexane, Methanolic, Choroform, Ethyl acetate, Acetone, *n*-butanol, Hydroalcoholic, chloramphenicol (standard drug) , respectively.

Fig 10: Antimicrobial activity of eight extract of *Pinus gerardiana* against the test bacteria *Stapylococcus aureus*

Fig 11: Antimicrobial activity of eight extract of *Pinus gerardiana* against the test bacteria *Stapylococcusmutans*

Fig 12: Antimicrobial activity of eight extract of *Pinus gerardiana* against the test bacteria *Escherichia coli*

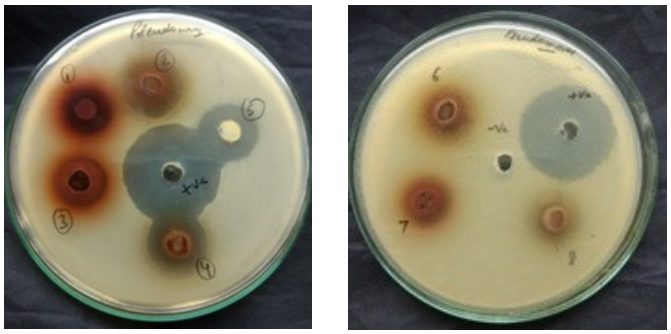


Fig 13: Antimicrobial activity of eight extract of *Pinus gerardiana* against the test bacteria *Pseudomonas areuginosa*

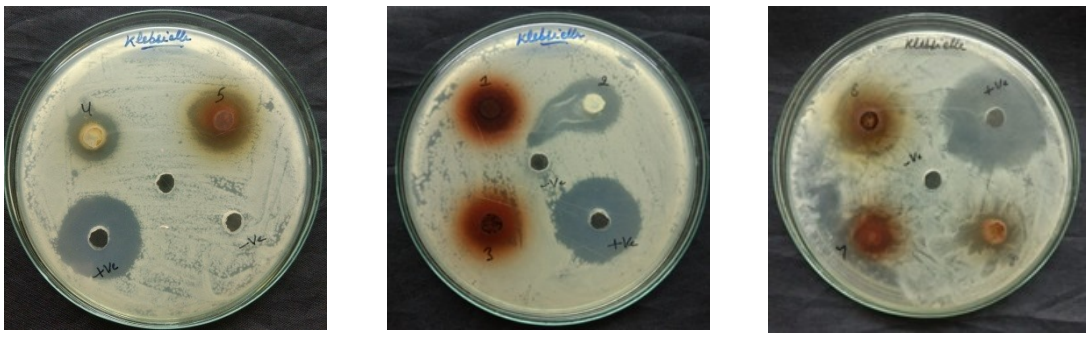


Fig 14: Antimicrobial activity of eight extract of *Pinus gerardiana* against the test bacteria *Klebsiella pneumonia*

In the petri plate, 1,2,3,4,5,6,7,8 are Aqueous,n-hexane, Methanol, Chloroform, Ethyl acetate, Acetone, n-butanol, Hydroalcholic are extracts respectively

Table 4: Scavenging activity IC_{50} Value of Pinus gerardiana of different extracts compared to Ascorbic acid as standard

S.No.	Extract	DPPH method µg/ml
1.	Hexane	189.5±1.3
2.	Chloroform	134.2±3.4
3.	Ethyl acetate	56.2±1.4
4.	Acetone	59.3±3.6
5.	n-butanol	58.2±4.3
6.	Methanol	51.3±3.1
7.	Hydroalcholic	63.2±2.8
8.	Aqueous	159.5±1.6
9.	Ascorbic acid (positive control)	17.9±2.1

N.B. Antioxidant activity expressed as IC_{50}Value in µg/ml±S.D of three replicates.

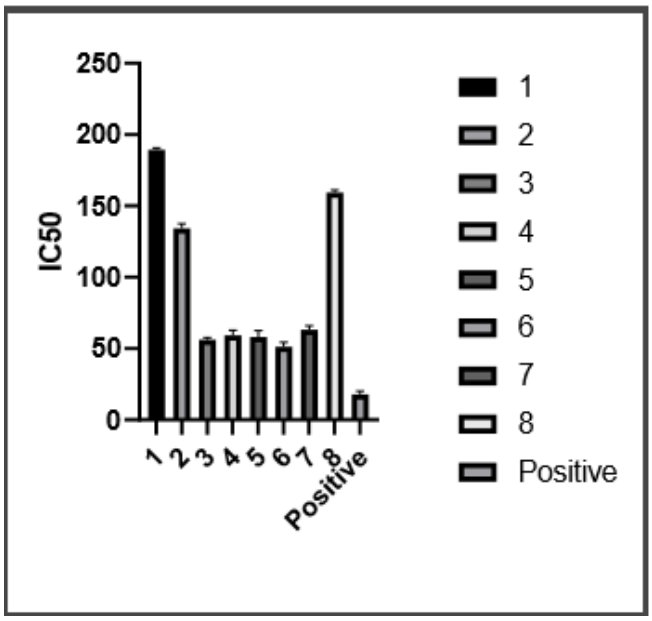
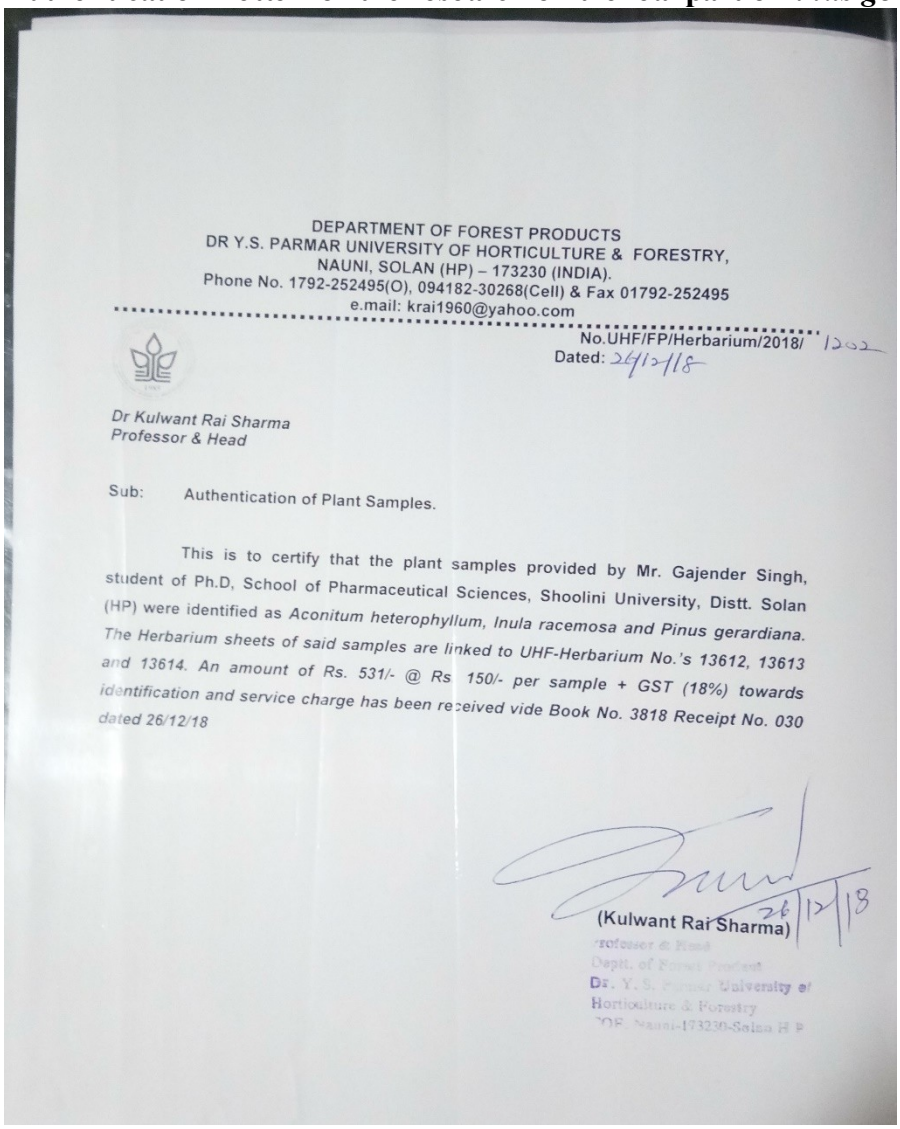


Fig 15: Graphical representation of *in-vitro*antioxidant acitivity of eight plant extracts and ascorbic acid (positive control) against DPPH inducing oxidation. Here 1,2,3,4,5,6,7,8 and positive are n-hexane, Choroform, Ethyl acetate, Acetone, n-butanol, Methanolic, Hydroalcoholic, Aqueous, Ascorbic acid (standard drug) , respectively.

Supporting Information

Authentication Letter for the research on the leaf part of *Pinus gerardiana*



DEPARTMENT OF FOREST PRODUCTS
DR Y.S. PARMAR UNIVERSITY OF HORTICULTURE & FORESTRY,
NAUNI, SOLAN (HP) – 173230 (INDIA).
Phone No. 1792-252495(O), 094182-30268(Cell) & Fax 01792-252495
e.mail: krai1960@yahoo.com

No.UHF/FP/Herbarium/2018/ 1202
Dated: 26/12/18

Dr Kulwant Rai Sharma
Professor & Head

Sub: Authentication of Plant Samples.

This is to certify that the plant samples provided by Mr. Gajender Singh, student of Ph.D, School of Pharmaceutical Sciences, Shoolini University, Distt. Solan (HP) were identified as *Aconitum heterophyllum, Inula racemosa and Pinus gerardiana.* The Herbarium sheets of said samples are linked to UHF-Herbarium No.'s 13612, 13613 and 13614. An amount of Rs. 531/- @ Rs 150/- per sample + GST (18%) towards identification and service charge has been received vide Book No. 3818 Receipt No. 030 dated 26/12/18

(Kulwant Rai Sharma) 26/12/18
Professor & Head
Deptt. of Forest Products
Dr. Y. S. Parmar University of
Horticulture & Forestry
(HP), Nauni-173230-Solan H P

Table :2 HPLC retention time and peak area indicating the presence of 19 major compounds.

Sr. No.	Retention Time	Area	Percentage of total area
1.	2.847	692471	0.33
2.	4.307	10037352	4.76
3.	4.680	10928558	5.19
4.	5.500	3996661	1.90
5.	12.913	6776387	3.22
6.	16.613	2669874	1.27
7.	18.527	4031058	1.91
8.	19.187	2378890	1.13
9.	21.427	1393913	0.66
10.	22.380	2868623	1.36
11.	24.860	2883783	1.37
12	26.013	3507383	1.66
13	28.533	2134708	1.01
14	29.860	1445043	0.69
15	31.260	2060673	0.98
16	32.867	921402	0.44
17	36.440	2210942	1.05
18	42.973	4061402	1.93
19	49.647	664093	0.32

3. HPTLC

Source of variation	Degrees of Freedom	Sum of Squares	Mean square
Column Factor	8.0	1609	201.2
Row Factor	4.0	70.22	17.56
Interaction	32.0	307.4	9.606
Residual (error)	90.0	32.67	0.3630
Total	134.0	2019	