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Identification and characterization of secondary metabolites isolated from the leaves of *Pinus roxburghii* (Sarg.)

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Abstract
In the present study, extracts prepared from the leaves of *Pinus roxburghii Sarg.* (Pinaceae) commonly known as "Chir pine", were evaluated for extraction and isolation of phytochemicals. *P. roxburghii* is traditionally used as a folk medicine as well as its other activities have been previously reported. The 50% ethanolic and aqueous extracts were tested for their phytochemicals screening and isolation of phytochemicals by column chromatography. The structure of isolated compounds was confirmed by spectral studies such as IR, NMR and MS spectroscopy and HPLC. The extracts showed the presences of secondary metabolites which are responsible for the therapeutic effects.

Keywords: HPLC, Phytochemicals, *Pinus roxburghii sarg,* Secondary metabolites

Introduction
Pinus roxburghii Sarg. (Pinaceae) locally known as "Chir pine", named after William Roxburgh, a pine inhabitant extending through the wholesome Himalayan areas of Kashmir, Sikkim, Uttarakhand, North India, Punjab and Himachal Pradesh in India, is like a jewel specimen and having many different medicated values. It is also found in nearby countries of India like Nepal, Bhutan and Pakistan [1]. Pinus is known to be a rich source of terpenoids [2], flavonoids, tannins [3], xanthones, saponins, phenolic compounds [1], triterpenes and steroids [4] from all the parts of plant. The foremost phytochemicals secluded from the leaves of *P. roxburghii* were like caryophyllene, 3-carene, α -humulne, α-pinene, α-terpeniol [5] and longifolene [6]. Leaves oil contains alpha-limonene, alpha-phellandrene, bomeol, longifolene and alpha-cadinene [7].
Integrated portions of pine tree gives different medicated purposes namely; haemostatic, anthelmintic, digestive, liver tonic, diaphoretic, diuretic, antiseptic, rubefacient, expectorant and febrifuge [8]. It is good to use in eye, ear, and pharynx diseases, foul ulcers, haemorroids, haemoptysis, worn infections, flatulence, fatty liver, bronchitis, TB, inflammations, skin diseases, pruritus, giddiness [9], snake bites, scorpion stings, skin diseases and hair remover [10]. It is utilized for various therapeutic purposes earlier including intestinal antiseptic, antidyslipidemic, spasmolytic, antioxidant [11], antidiabetic [12], analgesic, anti-inflammatory [13], anticonvulsant [14], antiasthmatic, hepatoprotective [4], lipoxygenase [15], α-amylase inhibitory [16], antibacterial and antifungal [5], mosquito repellent and larvicidal [17], allopathic, cytotoxicity[18] and contact dermatitis[19].
In this research paper, we report the isolation and characterization of gallic acid and catechin from the leaves of *P. roxburghii*.

Material and Methods
Neighboring local data in addition to reporting on the subject of the medicinal properties helped in the choice of this plant. In the month of February 2015, the leaves of *P. roxburghii* were collected from Kumaun district of Nainital. It was authenticated as well as the herbarium was submitted at Botanical Survey of India (BSI), Allahabad under voucher accession number 100230.

Extraction of plant material (50% ethanolic and aqueous extracts)
AR grade solvents as well as purified water were used in all process of extraction and isolation which were purchased from S.D. Fine chemicals, Mumbai, India. The leaves were washed with distilled water in order to eliminate dirt and impurities, dried away from the sunlight. Air and shed dehydrated leaves from *P. roxburghii* was grinded and overwrought in the route of 30 meshes (0.5 mm).

In the preparation of 50% ethanolic extract, thinly crushed plant material (100 gm) was stimulated in a percolator in addition to treated with ethanol: water (1:1 v/v; 500 ml) at 26-30°C for the night. The marc was extracted three times by cold percolation (1:1 v/v; 500 ml×3) and the pooled percolate (1000 ml) was evaporated at 45-50°C underneath vacuum evaporator to get 5-7% dry extract. The finely grinded leaves (100 gm) were treated by 500 ml MilliQ water at 55-70°C for 6-8 h. This procedure was continual further for three times. The warm water extract was filtered by Whatman filter paper number 1. The total filtrate (1300 ml) was distilled at 50-55°C beneath vacuum pressure to get concentrate 300 ml extract. The extract was followed by lyophilized at -20 to -40°C to afford 6-8% dehydrated extract. Both the extracts were stored at -20 to - 40º C or further analysis [20].

Phytochemical studies

The Qualitative phytochemical studies of both extracts from the leaves of *P. roxburghii* crude powder were examine to recognize the component like alkaloids, flavonoids, glycosides, proteins and amino acid, phenols, saponins, steroids, tannins and terpenoids [21].

Isolation of compounds by column chromatography

The isolation of phytochemicals was passed away by 50% ethanolic extract. The 50% ethanolic extract (10g) prepared from leaves of *P. roxburghii* was loaded on silica gel 230-400 mesh size belonging to Rankem Laboratory reagent. And it was eluted by using following solvents hexane, chloroform, ethyl acetate and ethanol.

A glass column, calculating 5cm by 60cm, was filled with silica gel of 230-400 mesh size. The filtrate occurred from the chloroform fraction was ground fit by a minute size of silica gel and loaded on to the top of the column that was eluted with solvents by means of escalating polarity *via* hexane, ethyl acetate, ethanol etc. Fractions of 120 ml were collected every time and the homogeneity was tested on TLC using silica gel 60-120 mesh size through appropriate solvents method. Approximately 150 fractions were accumulated. Distinct fractions like 1-4, 5-12, 13-21, 22-35, 36-48, 49-62, 63-80, 81-92, 93-107, 108-120, 121-132 and 133-150 were eluted. TLC examine were brought out with many solvent system as mobile phase. Two compounds were isolated from this plant. Fractions in between the 49-62 exhibited single spot using TLC solvent system toluene: ethyl acetate : formic acid system 3:3.5:0.5 with Rf value 0.9 which showed yellow spot under UV light at 272 and 221 nm and anisaldehyde-sulphuric acid used as spraying agent for compound (A). In this series, fractions in between 93-107 showed single spot using TLC solvent system ethyl acetate: toluene: formic acid (5:5:1, v/v/v) with Rf value 0.33 which showed brownish spot under UV light at 275 nm and 5% FeCl3 solution and vanillin sulfuric acid (VSR) were used as derivatising reagent for compound (B). Fractions with similar spots were merged collectively and concentrated at reduced pressure and temperature. For compound (A), the concentrated fraction after evaporation revealed pure pale white powder (72 mg) and for compound (B), it was found to be pure brown powder (55 mg) designated as isolated compounds. Both the phytochemicals were recognized through direct evaluation with the spectroscopic data (UV, IR, NMR and MS) and HPLC reported in the journalism.

Characterization of compounds

The structure of isolated compound was recognized on the basis of IR, 1H-NMR and mass spectral analysis.

The instrument used in the analysis of IR spectra was PerkinElmer Spectrum Version 10.03.06. Potassium bromide (KBR) pellets of compounds were used for recording. FT-IR examination was made at 4000-4501/cm.

The instrument used in the analysis of NMR spectra was model no - BRUKER AVANCE 400 (FT NMR) MHz FT NMR with, 5 mm multi- nuclear Broad band inverse probe, VT facility and also with HRMAS accessory, spectrophotometer for proton NMR spectral study. Deuterated Dimethyl sulfoxide-d6 (DMSO) was used as the solvent for recording.

The instrument used in the study of DART-MS spectra was JEOL-Accu TOF JMS-T100LC Mass spectrometer having a DART (Direct Analysis in Real Time) source. The specified samples were subjected as such in front of DART basis. Dry Helium was used with 4 LPM flow rate for ionization at 350° C. The orifice 1 was set at 28 V and collected the averaged spectra of 6-8 scan.

Qualitative and quantitative analysis for separation of the compounds were performed by HPLC with a SYSTRONICS LC-100 PLUS HPLC system with UV detector and injection regulator with 20-µL sample loop. The separation of compounds was on a 4.6 mm × 250 mm, i.d., 5-µm pore size welchrom RP-C18 column.

Results and Discussion

Description of phytochemicals

The Phytochemical studies showed that 50% ethanolic extract contain alkaloids, tannins, saponins, steroids, phenols, terpenoids, flavonoids, and glycosides. In contrast, aqueous extract contained alkaloids, tannins, saponins, phenols, terpenoids, flavonoids, glycosides but not steroids. At the same time protein and amino acid was absent in both extract (Table-I).

Table 1: Phytochemical analysis of leaves extract of *P. roxburghii*

S No	Compounds	50% Ethanolic Extract	Aqueous Extract
1.	Alkaloids	+	+
2.	Flavonoids	+	+
3.	Glycosides	+	+
4.	Protein and Amino Acid	-	-
5.	Phenols	+	+
6.	Saponins	+	+
7.	Steroids	+	-
8.	Tannins	+	+
9.	Terpenoids	+	+

(+): detected; (-): not detected

Structure elucidation of compounds

Compound (A): was obtained as pale white powder with molecular formula C7H6O5, Melting Point- 259.2°C, soluble in alcohol, acetone and water.

UV-vis (Ethanol) λmax = 272, 221 nm; ESI-MS m/z: 168.9 [M-H] (Fig-1-a), IR cm-1(KBr): 3369.3 (O-H str.), 3020.92 (Ar C-H str.),

2401.19 (O-H str.), 1613.42 (C=O str.), 1429.39 (Ar C=C str.) (Fig-2-a). 1H-NMR δ (DMSO, 300 MHz) δ: 9.158 (S, 1H, COOH), 6.912 (S, 2H, Ar-H), 3.335 (4H, OH) (Fig-3-a). 1H-NMR information was relevant with those reported in the literature for the compound [22]. Compound A was identified as gallic acid. The chromatographic separation was detained away using a mobile phase by phosphoric acid: water 0.05% as solvent A and methanol as solvent B at a flow rate of 1

ml/min. The gradient plan was as follows: 90-10% B (10 min), 70-30% B (3 min), 40-60% B (5 min), 60-40% B (3 min), 80-20% B (3 min) and 90-10% B (6 min). The peak was identified at 272 nm and the retention time was 8.4 min [23] (Fig-4-a).

Compound (B): was obtained as light brown powder with molecular formula C15H14O6, Melting Point- 176°C, soluble in ethanol or ethanol-water and DMSO. UV-vis (Ethanol) λmax = 275 nm; ESI-MS m/z: 291[M-H] (Fig-1-b),
IR cm-1(KBr): 3395.72 (O-H str.), 3021.23 (Ar C-H str.), 2401.28 (O-H str.), 1439.22 (Ar C=C str.), 1215.42 (C-O str.) (Fig-2-b).
1H-NMR δ (DMSO, 300 MHz) δ: 9.156 (S, 1H, 4-OH), 6.719 (S, 1H, H- 6, 8), 6.694 (S, 1H, H-21, 51), 5.686 (S, 1H, H-5, 7), 3.467 (S, 1H, H-3) (Fig-3-b-i, ii, iii). 1H-NMR data were compatible with those described in the literature for the compound [24]. Compound B was identified as catechin.
The chromatographic separation was passed out *via* a mobile phase with solvents compositions (A) water: acetonitrile: formic acid (94.7:4.3:1 v/v) and (B) water: acetonitrile: formic acid (49.5:49.5:1 v/v). The gradient program was as follows: 90% solvent A and 10% solvent B, being increased linearly to 30% solvent B in 10 min, along with a linear increase of solvent B to 80% in 5 min, the final conditions being bore for further 5 min. The peaks were detected at 275 nm and the retention time was 11.8 min [25] (Fig-4-b).
Thus, above results confirm the presence of gallic acid and catechin from the leaves of *P. roxburghii*. Gallic acid is a polyphenol, possesses the antioxidant capacity and neuroprotective agents. In environment, it is found in nearly every part of plant. Catechin is also a natural phenol and has antioxidant property. So, in this way this plant contains highly medicinal properties.

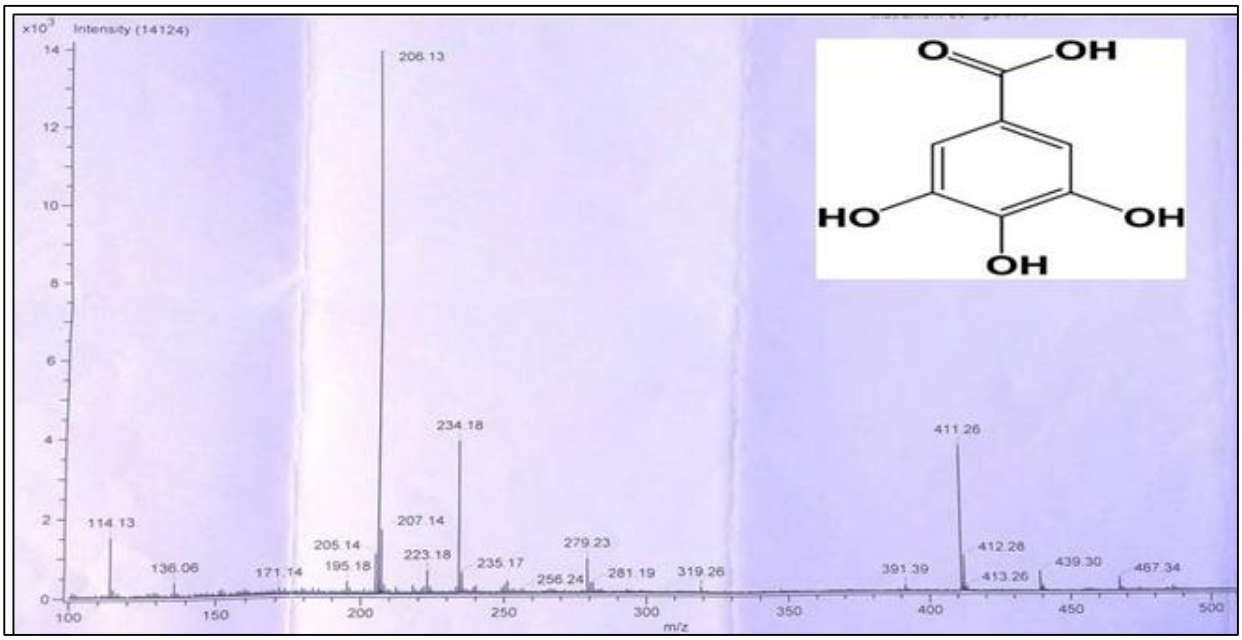

Fig 1(a): Mass spectrum study of isolated compound A of *P. roxburghii.*

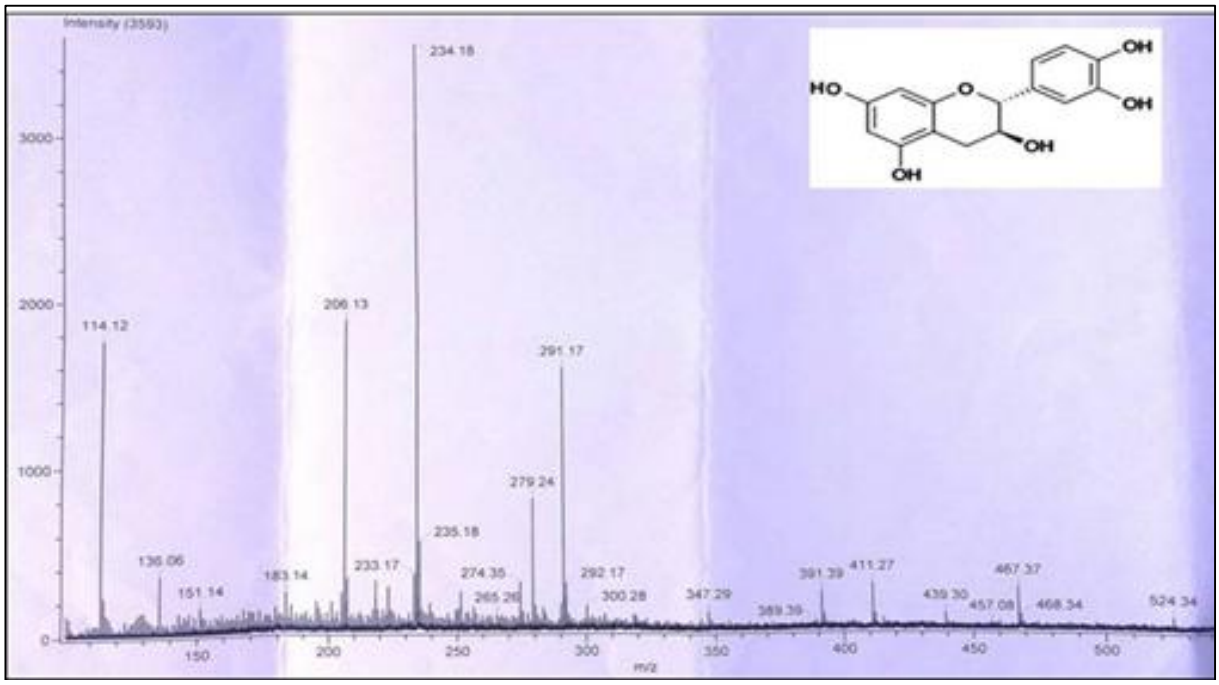

Fig 1 (b): Mass spectrum study of isolated compound B of *P. roxburghii.*

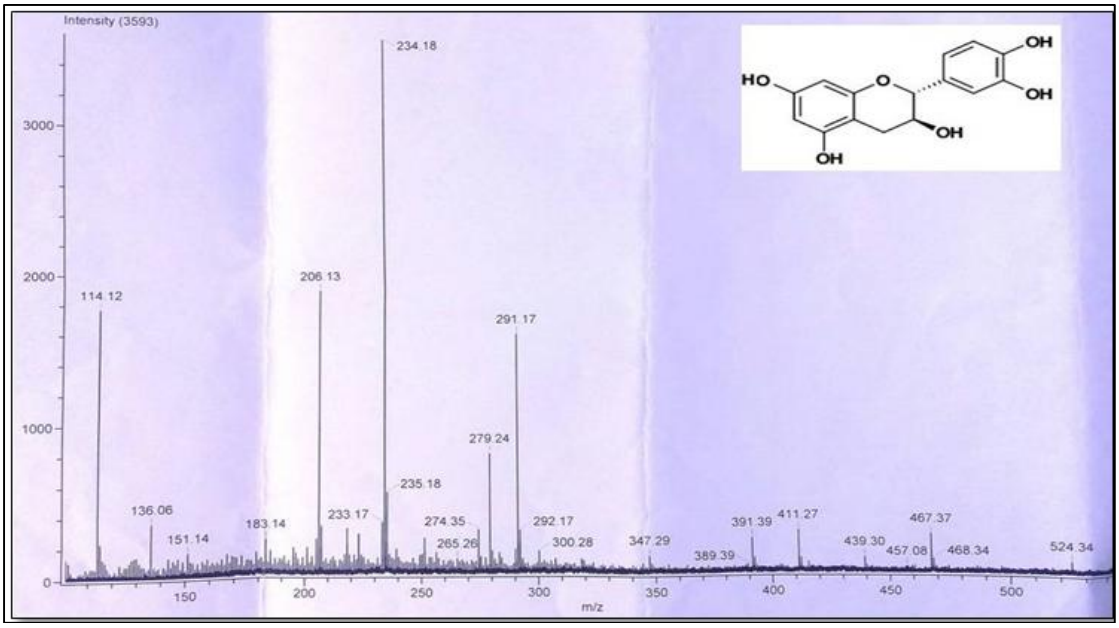

Fig 2: (a) FT-IR Spectral data of isolated compound A of *P. roxburghii.*

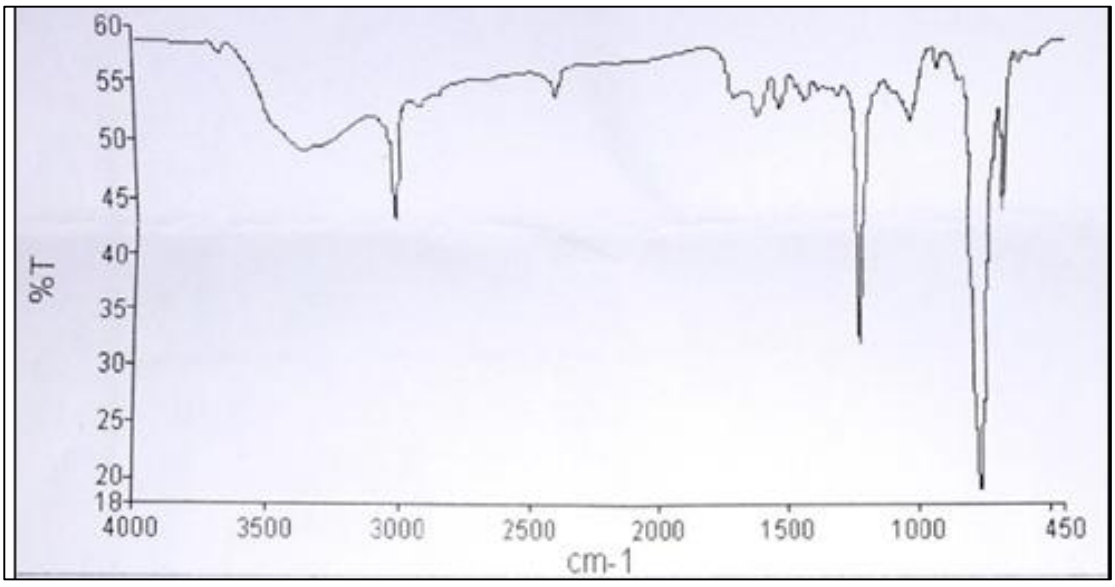

Fig 1(b): Mass spectrum study of isolated compound B of *P. roxburghii.*

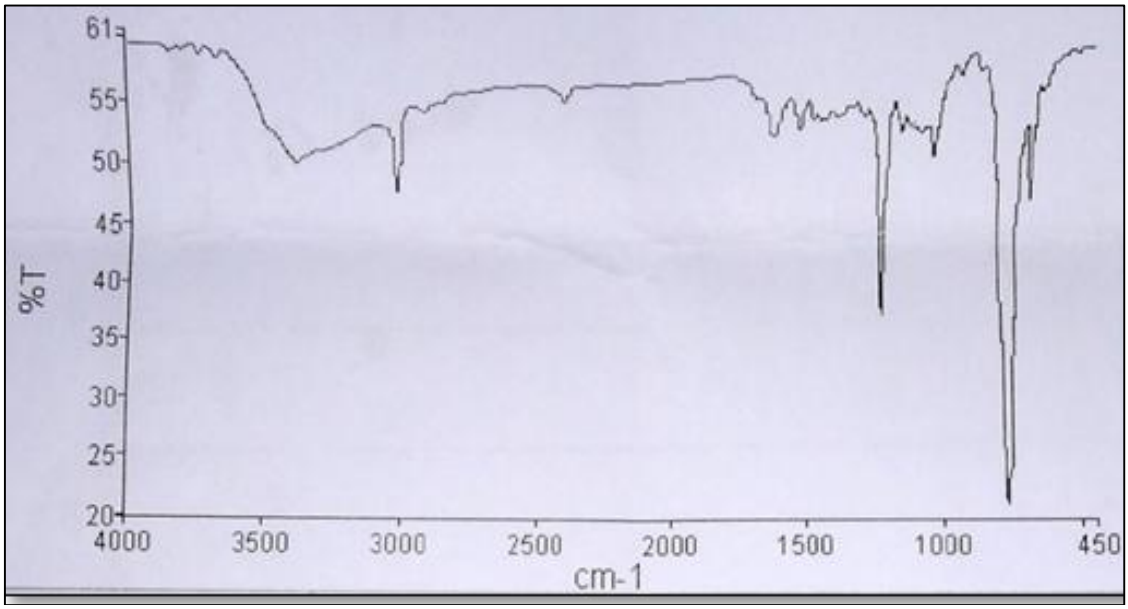

Fig 2 (b): FT-IR Spectral data of isolated compound B of *P. roxburghii.*

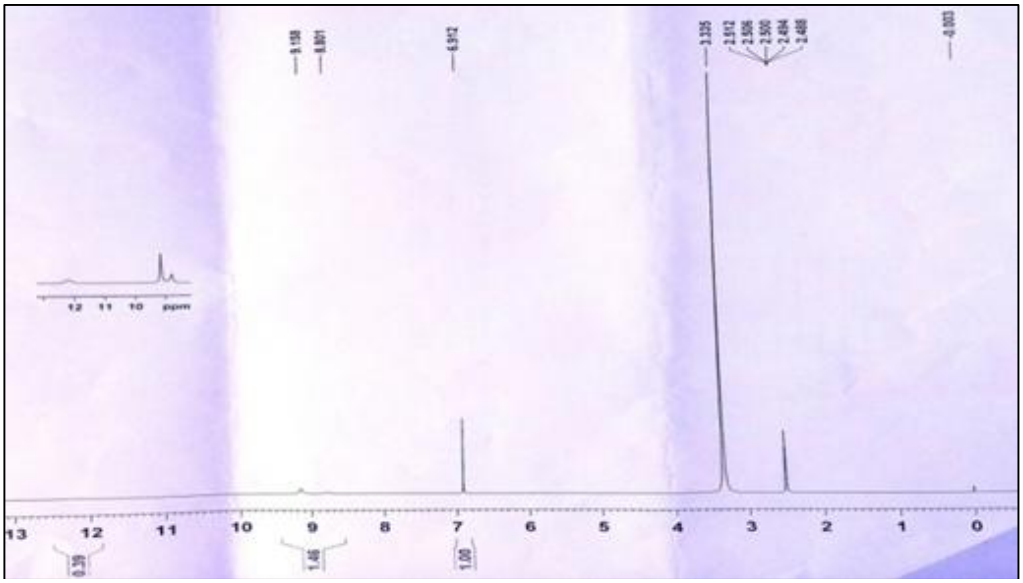

Fig 3(a): [1]H-NMR Spectral data of isolated compound A of *P. roxburghii.*

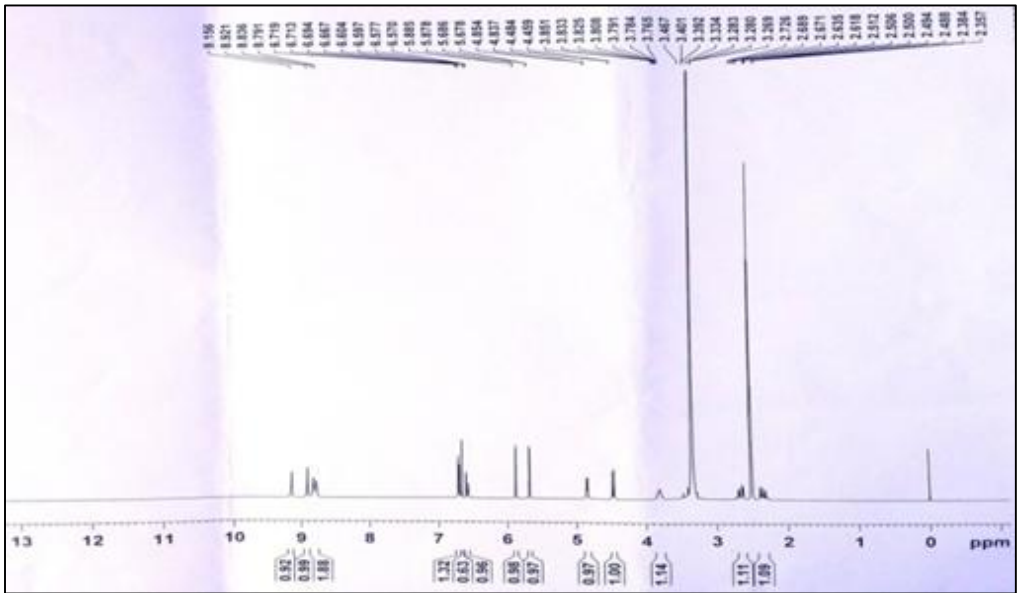

Fig 3 (b-i): [1]H-NMR Spectral data of isolated compound B of *P. roxburghii*

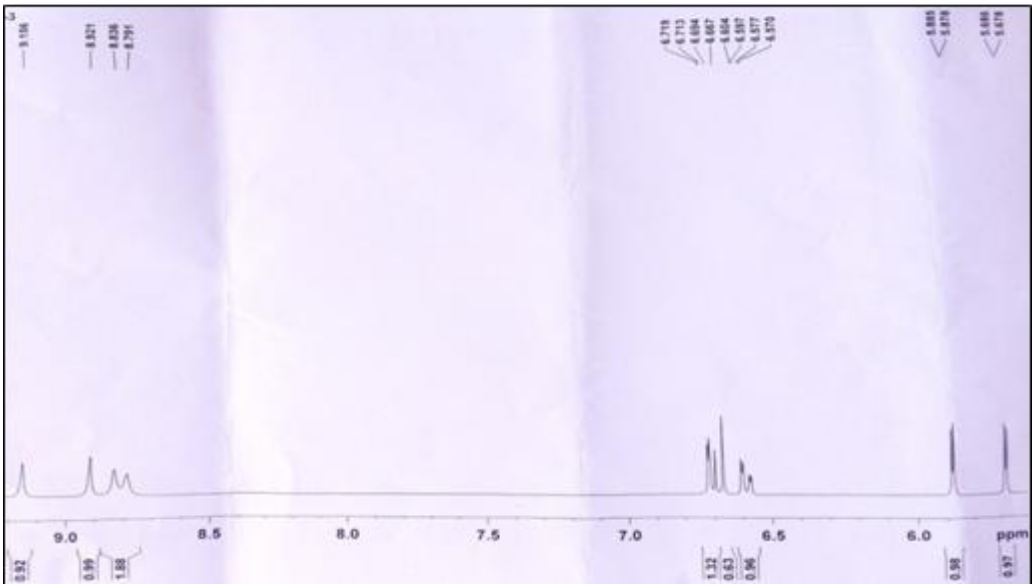

Fig 3 (b-ii): [1]H-NMR Spectral data of isolated compound B of P. roxburghii.

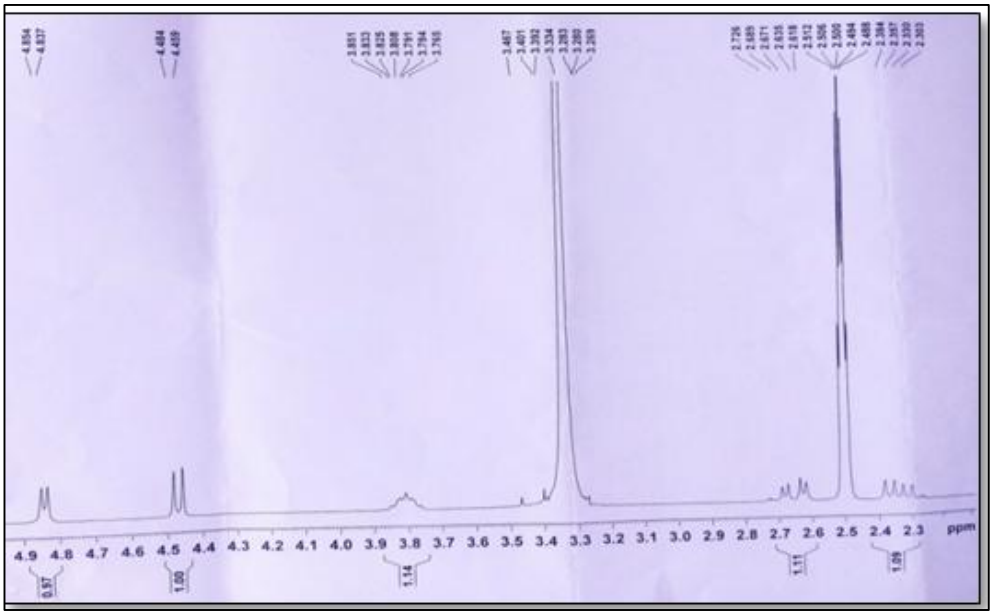

Fig 3(b-iii): [1]H-NMR Spectral data of isolated compound B of P. roxburghii.

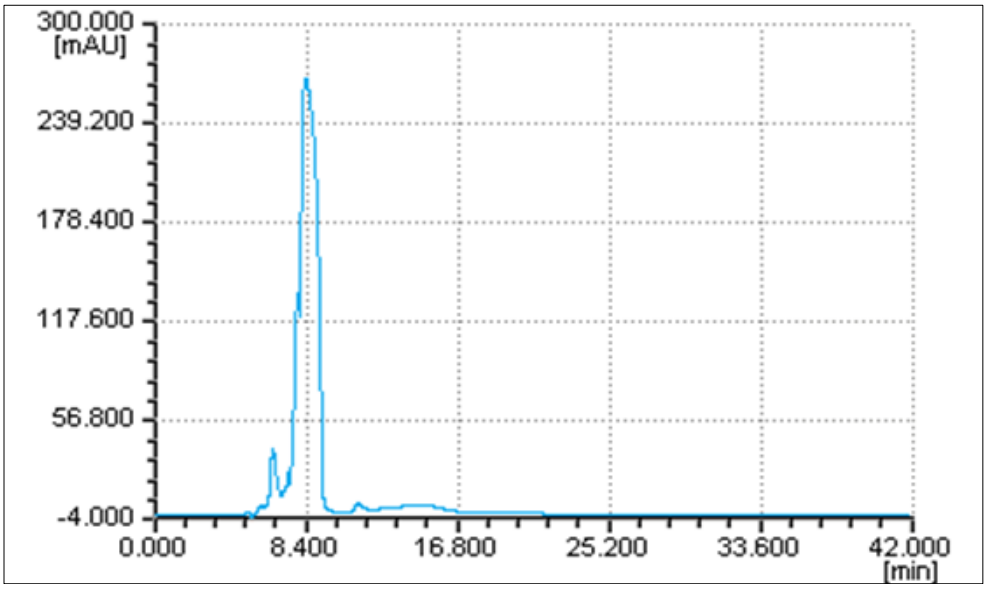

Fig 4(a): HPLC of isolated compound A of *P. roxburghii.*

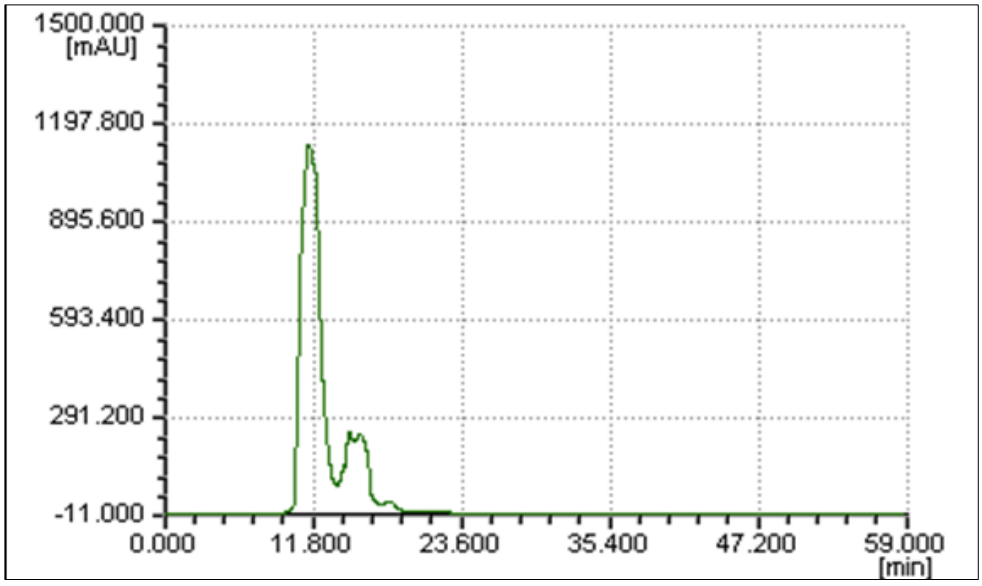

Fig 4(b): HPLC of isolated compound B of *P. roxburghii.*

Conclusions

The isolation and identification of gallic acid and catechin from the leaves of *P. roxburghii* was reported from this plant. The effort was carried out by using many kinds of chromatographic separation techniques and spectroscopic studies.

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Conflicts of interest

The authors declare no conflicts of interests.

References

1. Kaushik P, Kaushik D, Khokra SL. Ethnobotany and Phytopharmacology of *Pinus roxburghii* Sarg: a plant review. J of Integrative Med. 2013; 11:371-376.
2. Inayatullah, S, Prenzler PD, Obied HK, Rehman AU, Mirza B. Bioprospecting traditional Pakistani medicinal plants for potent antioxidants. Food Chem. 2012; 132: 222-229.
3. Ahmad M, Nazli S, Anwar MM. Studies on tannins from barks of *Pinus roxburghii.* J Chem Soc Pak. 1989; 11:213-217.
4. Khan I, Singh V, Chaudhary AK. Hepatoprotective activity of *Pinus roxburghii Sarg.* Wood oil against carbon tetrachloride and ethanol induced hepatotoxicity. Bangladesh J Pharmacol. 2012; 7:94-99.
5. Hassan A, Amjid I. Gas chromatography-mass spectrometric studies of essential oil of *Pinus roxburghii* stems and their antibacterial and antifungal activities. JMPR. 2009; 3(9):670-673.
6. Kunwar RM, Uprety Y, Burla K, Chowdhary CL, Bussmann RW. Indigeneous use and ethnopharmacology of medicinal plants in west Nepal. J Plants People Appl Res. 2009; 7:5-28.
7. Shuaib M, Ali M, Ahamad J, Naquvi KJ, Ahamad MI. Pharmacognosy of *Pinus roxburghii*: a review. J of Pharmaco and Phytochem. 2013; 2:262-268.
8. Abbasi AM, Khan MA, Ahmad M, Zafar M, Jahan S, Sultana S. Ethnopharmacological application of medicinal plants to cure skin diseases and in folk cosmetics among the tribal communities of North-West Frontier Province Pakistan. J Ethnopharm. 2010; 128:322-335.
9. Chopra RN, Nayar SL, Chopra IC. Glossary of Indian Medicinal Plant, CSIR, New Delhi, 1986.
10. Murad W, Ahmad A, Gilani SA, Khan MA. Indigenous knowledge and folk use of medicinal plants by the tribal communities of Hazar Nao Forest, Malak and District, North Pakistan. J Med Plant Res. 2011; 5:1072-1086.
11. Puri A, Srivastava AK, Singhal B, Mishra SK, Srivastava S, Lakshmi S. Antidyslipidemic and antioxidant activity of *Pinus roxburghii* needles. Med Chem Res. 2010; 20:1589-1593.
12. Marles RJ, Farnsworth NR. Antidiabetic plants and their active constituents. Phytomed. 1995; 2:137-139.
13. Kaushik D, Kumar A, Kaushik P, Rana AC. Analgesic and anti-inflammatory activity of *Pinus roxburghii sarg.* Advances in Pharmacological Sci. 2012; 2012:1-6.
14. Kaushik D, Kumar A, Kaushik P, Rana AC. Anticonvulsant activity of alcoholic extract of bark of *Pinus roxburghii Sarg*. J of Chinese Integrative Med. 2012; 10:1056-1060.
15. Kuchimanchi SN, Murthy BGK, Sampath Kumaran PS. Isomerization of abietic acid to generate medicinally important diterpene building blocks. American Chemical Society, Washington, D.C., Conference held at Boston, United States; 2002:18-22.
16. Kaushik P, Singh G, Khokra SL, Kaushik D. Bioassay guided fractionation and α-amylase inhibitory activity of flavanoid isolated from *Pinus roxburghii Sarg.*. Nat Prod Chem Res. 2015; 3:1-3.
17. Ansari MA, Mittal PK, Razdan RK, Sreehari U. Larvicidal and mosquito repellent activities of pine (*Pinus longifolia*, family: Pinaceae) oil. J Vector Borne Dis. 2005; 42:95-99.
18. Islam WT. Composition and bioactivities of the essential oils of twigs of four Pinus species cultivated in Egypt. Egypt J Biomed Sci. 2004; 15:452-464.
19. Mahajan VK, Sharma NL. Occupational airborne contact dermatitis caused by *Pinus roxburghii sawdust*. Contact Dermat. 2011; 64:110-11.
20. Kulshrestha S, Khan SH. Identification and characterization of secondary metabolite isolated from the stem bark of *Prosopis cineraria (Linn.)* Druce and their *in vitro* anticancer activity against A549 human lung cancer cell line. JPR. 2018; 12:611-618.
21. Harborne JB. Phytochemical Methods-A Guide to Modern Techniques of Plant Analysis. Edn 3, Chapman and Hall, London, 1998, 203-42.
22. Abri A, Maleki M. Isolation and identification of Gallic acid from the *Elaeagnus angustifolia* leaves and determination of total phenolic, flavonoids contents and investigation of antioxidant activity. Iran Chem Commun. 2016; 4:146-154.
23. Fernandesa FHA, Batista b Ray S. de A, Medeirosa Franc D. de, Santosb FS, Medeiros Ana CD. Development of a rapid and simple HPLC-UV method for determination of gallic acid in Schinopsis brasiliensis. Revis Brasil de Farmacognosia. 2015; 25:208–211.
24. Desail S, Tatke P, Gabhe SY. Isolation of catechin from stem bark of *Albizia Lebbeck*. IJAPBS. 2014; 3:31-35.
25. Fernandez PL, Martín MJ, González AG, Pablos F. HPLC determination of catechins and caffeine in tea. Differentiation of green, black and instant teas. Analyst. 2000; 125:421-425.