UTILIZATION OF WILD PLANTS SPECIES FOR INNOVATIVE BIO BASED INDUSTRIAL COMPOUNDS

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ABSTRACT

Pinus Roxburghii (chir pine) and similar others species of this family are very important and dominated trees of forest of Murree and Kotli sattian areas (Pakistan). The needles leaves, stem, resins and cones of these trees are important due to their domestic and commercial uses like food , cosmetics and fertilizers . In the current study *Pinus* needles leaves and resins were chemically analyzed to assess levels of phenols, flavonoids, tannins and total oil contents by using different analytical methods as well as GC-MS and FT-IR techniques. Antioxidants, Anti mycobacterium tuberculosis (MT) and cytotoxicity assays were performed to determined bioactivities of different extracts of leaves. Where as total oil contents of resins were determined by using Soxhlet method and oil was converted into different products like glycerin and biodiesel by using trans etherification process. Results indicates that *pinus* leaves contains total phenols (7.26±1.52) flavonoids (14.53±2.45) and tannins (4.36±1.23). Where as total oils was found in *pinus* needles leaves (1.92 ± 0.28) as compared to pinus resins . GC-MS analysis exposed higher quantity of organic compounds (fatty acids) , those were further confirmed by indication of their functional group by FT-IR analysis. According to results methanolic extracts of Pinus roxburgii has provided higher antioxidant values (DPPH, IC_{50} = 38.36±4.58 µg/ml) and higher zones of inhibition for MT strains as compared to lower value of brine shrimp cytotoxicity assay and these values are probably due to presence of total phenols , flavonoids and essential oils contents. Where as *pinus* resins results indicates presence of glycerin and fuel oil those have higher economical values especially for developing country like Pakistan

Key words: *Pinus* needles leaves, Resins, Chemical anlaysis, Organic acids, Bioactivities

1. Introduction

Agro forestry is a useful low-cost income applying to many forms of integrated land and to contributes to a green economy by promoting long-term, sustainable, and renewable forest management, especially for small-scale producers (1).

The Pinaceae (pine family) are main tress of forest located on northern east part of Punjab provinces as well as other parts of Pakistan. *Pinus Roxburghii* commonly known as chir pine

A very important tree as its leaves, stem, resins and cone all have commercial uses since ancient time (2).

Pinus belongs to Pinaceae as a result of having shoot dimorphism, which includes short shoots (fascicles) that have one to eight narrow needles surrounded by bud scales at the base. Strong woody cone scales with the apical structure exposed after the first growing season (bump) and in the mature cone are also typical of the genus Pinus. Currently, Pinus is treated as a monophyletic taxon [3]. The subgenus Pinus (pines) has two fibrovascular bundles per needle, diverging pulvini at cataphyll bases ,which usually have persistent sheaths. There are two to eight needles per fascicle and the position of the resin canals is polymorphic (septa; internal, medial external); the seed wings are articulated or oppressed [4].

Pinus trees or bushes, including a considerable number of the outstanding conifers of business significance, for example, cedars, firs, hemlocks, larches, pines and spruces.Pine Bark Band: The inward bark can be used as a germ-free band for cuts and scraps. It is applied to wounds with channel tape, handkerchief, or cordage (3)

Needle leaf tree (coniferous evergreen) have longer , thin leaves that resemble needles. The leaves remain on the tree year-round and are replaced slowly and continuously rather than all at once. The smaller, tighter needles are more waterproof and wind tight than the larger, wider leaves found on broadleaf or deciduous trees (2, 3,4) . Pine needles can be found on evergreen trees throughout the world and can be found on roughly 114 species in the Pinus genus. Most common pines are predominantly from two subgenera of the Pinaceae family, Pinus and Strobus (5).

Pinus needles has being use for animals caring, ceiling of houses and as fertilizers uses since ancient time. Leaves has also being used as feed and food as well as source of some cosmetic and similar other chemicals (6,7).

Rosin is a solid resin derived from the oleo-resin (crude turpentine) discharged by various species of pine tree. Rosin is extracted by tipping the stem of tree . Natural resins are typically fusible and flammable organic substances that are transparent or translucent and are yellowish to brown in colour (8).

It may also extracted by heating the oleo-resin to vaporize the essential oils (spirit of turpentine), or through a naphtha solvent process (9).. Rosin is used in the manufacture of varnishes and it is often combined with other resins (8,9).

Conifer cone is a seed bearing organ on gymnosperm plants. It is a type of fruit usually wood, avoid to globular including scales and bracts arranged around a central axis. The seeds of cone are delicious and contains important nutrients especially essential oil (10).

Wood of *Pinus Roxburghii* are very important and use domestically as fuel , house hold items and for preparation of furniture etc., Pine needle tea is taken to remove the valuable stuff when one feel influenza like manifestations in body . Essential oils have been widely used since long for bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, medicinal and cosmetic applications. Nowadays they are widely used in pharmaceutical, sanitary, cosmetic, agricultural and food industries (10, 11).

Around 80% of world population depends on home grown solutions for treatment of different human afflictions. Therapeutic plants have the fundamental part in allopathic medications, extensive number of current medications, for example, dioxin, morphine, codeine, ibuprofen, vinblastine, cocaine, emetine, ephedrine, vinocristine, pilocarpine and so forth., have been disengaged from plants (12). After greater progression of manufactured science during the later half of twentieth century over common items, again the enthusiasm of pharmaceutical industry in restorative plants stir and they took a gander at wellspring of natural plants as synthetic platforms for amalgamation of medications. The normal compounds from therapeutic plants are more secure and could be found to supplant the engineered drugs which constitutes around 70% of our

medications (13, 14).Numerous medicinal plants contains awesome amount of antioxidant compounds instead of vitamins and carotenoids Therapeutic agents presents in plants posses significance and extraordinary biological activities. Therapeutic herbs displayed more antioxidant activity and contained fundamentally larger amounts of phenolics and other secondary metabolites than regularly known vegetables and fruits those are considered as rich sources of common dietary antioxidants (15,16)

A basic oil portion from Canella winterana was additionally tried. The antimycobacterial movement of these substances was tried against Mycobacterium tuberculosis, M.aviumand M. kansasaii utilizing the Middlebrook 7H11 agar medium, the Bactec 460-TB radiometric system, and assurance of bacterial suitable tallies. Three mixes, to be specific ibogaine, voacangine and texalin, demonstrated antimycobacterium action (17).

The extracts were taken from Urticamembranacea (Urticaceae), Artemesiamonosperma (Asteraceae), and Origanumdayi post (Labiatae). Each of the three plant extracts showed dosage and time-subordinate killing abilities in different human inferred tumor cell lines and essential societies set up from patients' biopsies. The executing action was particular toward tumor cells, as the plant extracts had no impact on essential societies of solid human cells (18,19)

Various strategies are utilized so far for the extraction of fundamental oil from plant material, for example, steam refining, dissolvable extraction and so forth. The fundamental oil extraction strategy can be isolated into two noteworthy classes in light of temperature utilized for extraction i.e extraction at low or high temperature and extraction at room temperature. The strategies are headspace gathering of volatiles, hydro distillation, steam distillation , soxhletextraction and water and steam refining (19). The possible role and mode of action of these natural products is discussed with regard to the prevention and treatment of cancer, cardiovascular diseases including atherosclerosis and thrombosis, as well as their bioactivity as antibacterial, antiviral, antioxidants and anti diabetic agents (20).

2. Material and Methods

2.1. Collection and preparation of samples

The leaves samples of *pinus Roxburghii* (chir pine) and resins were collected in fine plastic bags duly labeled with date and areas of collection of samples. The collection of samples based on ethnobotanical uses by inhabitants of rural areas of Kotli sattian (Rawalpindi) . The resin was extracted from bark of *pinus* tree in fine plastic bags and treated separately. The samples were identified by a taxonomist by expert from Department of Botany PMAS Arid Agriculture University Rawalpindi and voucher specimen (No. 142) was deposited for future reference. Around 3 kg of plant material were moved to UIBB ,PMAS Arid Agriculture University Rawalpindi. for further process (13).

Figure 1. ***Pinus wallichaina***

The samples of *pinus* needles leaves and *pinus* resins were used for the extraction of various phytochemicals as well as oils and their bioactivities were determined (21). .

Plant materials were washed with demineralized water to evacuate undesirable materials including dust. The leaves samples were shade and sun dried followed by oven dried for over night at 50 °C. The dried samples were ground with electric grinder, sieve 80mesh and saved in plastic bags at lower temperature till further uses. After washing and sun drying , *pinus* resins samples were used for oil extraction (21,22).

2.2.Extraction of *pinus* resins from bark of *pinus* tree

Resin is usually collected by causing minor damage to the tree by making a hole far enough into the trunk to puncture the vacuoles, to let sap exit the tree, known as tapping, and then letting the tree repair its damage by filling the wound with resin within few days (22,23). Natural resins are typically fusible and flammable organic substances that are transparent or translucent and are yellowish to brown in colour. The most generalized technique can be the extraction of the drug with alcoholic solvents and then subsequent precipitation of resin by adding concentrated alcoholic extract to a large proportion of water. The method of distillation or hydrodistillation can be used for the separation of volatile oils from resin (23).

2.3.Determination of total phenols:

The concentration level of phenol in leaves and resins was determined in different solvents and amount was quantified by using method reported by various authors (21). Briefly 100 µl of extract was diluted with 3 ml of distilled water and 0.5 ml of Folin-Ciocalteu reagent was added. After 3 min, 2 ml of 20% sodium carbonate was added and the contents were mixed thoroughly. The color was developed and absorbance was measured at 650 nm in spectrophotometer (Shimadzu UV-1800). Gallic acid was used as standard and different concentrations of Gallic acid were used to draw standard curve ($R^2 = 0.9926$). The amounts of total phenolics were expressed as gallic acid equivalents (GAE) mg/100g of dry matter (19).

2.4.Determination of flavonoids

The flavonoid contents of extracts were determined by using method reported by Husain et al.(2008). Briefly an aliquot (1 ml) of extract or a standard solution of quercetin (4mg/ml) was added to 10 ml flask containing 4 ml distilled water and 0.3

ml 5% $NaNO_2$. After 5 minutes, 0.3 ml of 10% $AlCl_3$ was added followed by addition of 2 ml of 1M NaOH after 6 minutes and total volume was made up to 10 ml with distilled water. Absorbance was measured at 510 nm with a spectrophotometer and the concentrations of flavonoid in the samples were expressed as mg quercetin equivalent /g of sample (21,22,24).

2.5.Estimation of Tannins:

The total concentration of tannins in leaves extract was determined by Folin Denis method . It is measurement of blue color formed by the reduction of phosphotungstomolybidic acid by tannins. According to method, the colorometric determination of tannins was carried out by adding 7.5 ml of distilled water to 1.0 ml of extract and standard solution of tannic acid. Then 1ml of sodium carbonate was added following by addition of 0.5 ml of Folin Denis reagent. The solution was diluted with distilled water up to 10 ml and absorbance was measured at 700 nm . The total tannic acid concentration was expressed as mg of tannic acid equivalent per gram of extract (25,26).

2.6.Extraction of oils by Soxhlet method

Oil was extracted from *pinus* needles leaves and *pinus* resins by using Soxhlet apparatus as reported (27). Total 15 grams of a powdered form of a sample was taken in the clean thimble. Then fix the thimble in the loading chamber and connect the loading chamber with the condenser . Added methanol (300 mL) in the flask and connect it to a continuous supply of water. Fix the whole assembly in the heating mental and heat the flask up to 50 ºC. High temperature caused the vaporization of the solvent and vapors moved upward by the tubes and hit the condenser and turned to hot liquid (28).

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2.7.Conversion of oil into Fuel (Biodiesel) and glycerin

Crude oil contains many impurities such as free fatty acids, phospholipids and sterols whereas refined oil contains the very small amount of free fatty acids and other impurities. These impurities in the form of FFA and water have significant effects on the reaction, glycerides.

Alcoholysis is the chemical reaction in which the oil or fat reacted to the methanol followed by alkali (sodium hydroxide) catalyst to produce ester and glycerol. For getting the high yields of ester in transesterification reaction, use of an extra quantity of methanol is preferred (23).

2.8.Trans esterification process

In the base catalyzed reaction the methanol as a solvent and alkali (sodium hydroxide) was used as a catalyst. Different catalyst concentrations of NaOH such as 0.5 %, 1 %, or 1.5 % (w/w) may be used along with the methanol. The homogeneous solution of sodium hydroxide and methanol was prepared. Oil has firstly heated to 60 ºC and then cooled to room temperature. Then add the catalyst, and methanol mixture in the oil flask, set the whole in the reaction assembly apparatus, the mixture was stirred at 800 rpm for 2 h. After the complete reaction time, the whole mixture was taken in separating funnel with the passage of time, two layers were separated. The upper layer was methylated ester, and the lower layer was glycerin. The upper layer consists of different impurities such as unreacted methanol, catalyst, water, and glycerin . Further more neutralization and washing process were carried out for refinement of Biodiesel (29).

2.9.Physical and chemical analysis of Fuel (Biodiesel)

Different Physical and Chemical analysis were carried out for assessment of quality of Biodiesel like pH, Iodine , acid and saponification values by following AOAC method (28.29).

2.10.Analysis of oil for fatty acids with GC-MS

Fatty acid methyl esters: *pinus* needles leaves oil in n-heptane (0.20 g per 2 mL) was transmethylated using a cold solution of KOH (2 mol L−1) (200 μL) and methyl esters (FAME) was analyzed. The composition of extracted oil was examined by utilizing Gas Chromatography-Mass Spectrometry. The hawlett Packard framework was utilized to analyze the sample. The 6890N was outfitted with DB-5 section (30 m length, 0.25mm inner distance across and 0.25 µm stationary stage film thickness) and combined with a HP 5973 Mass spectrometer (MS; Agilent Advances Inc. USA). The injector was worked at 235 °C. The stove temperature of GC was customized as: the underlying temperature of broiler was 40 °C and it was kept up for 2 min, the temperature was raised from 40 to 450 °C at a warming Rate of 4°C and stayed at higher temperature for 8 mins. Exceptionally unadulterated Helium gas was utilized as portable stage with a steady stream of 1mL/min.The weakened arrangement of fundamental oil was infused

with volume of 1 µL in split less mode. Mass spectra was performed by an electron ionization framework worked at the ionization vitality of 70 eV. The particle source temperature of mass spectrometer was set at 180°C and the deferral for dissolvable was 5 mins. The mass spectra filter extend was 30-400amu. GC top territories were utilized to register the rate structure of an example without utilizing remedy factors. The distinguishing proof of fundamental oil constituents was at first completed by contrasting mass spectra of a compound and NIST-2008 MS library. As a second step, the maintenance lists of isolated mixes were resolved in respect to the maintenance times of standard C9 to C24 n-alkanes at a similar GC-MS parameters utilized for the basic oils. The figured maintenance lists of mixes were contrasted and the distributed information for the assurance of elution request and distinguishing proof of mixes. At last, the distinguishing proof of compound was accomplished by co-infusion

2.11. Bioactivity assessment of *Pinus* needles leaves

2.11.1.Antioxidant activity

The antioxidant activity of plant extracts was carried out according to method earlier reported (30) Therefore, for determination of antioxidants, following bioassays were used.

2.12.2. DPPH scavenging bioassay

DPPH scavenging activity was done according to method reported by (30) with some modifications. This method was based on the ability of antioxidant to scavenge 1, 1-diphenyl-2-picryl hydrazyl (DPPH) action radical. In a falcon tube, 100 µL of the sample solution was taken and then 4mL of DPPH solution (0.1 mM) was added and mixture was vortexed vigorously. Then the mixture was incubated with Aluminium foil to avoid heat exposure in the dark for 30 min at room temperature. UV-Vis spectrophotometer was used to measure the absorbance of the solution at 517 nm. Percentage inhibition was calculated by following formulae

$$\% \text{ Inhibition} = A_{blank} - A_{sample} \times 100 / A_{blank}$$

2.11.3. ABTS scavenging bioassay

The ABTS radical scavenging bioassay was carried out by already reported method (29,30). The ABTS radical cation was prepared by mixing 5 mL of a 7 mM aqueous ABTS solution to an about 88µL of 140 mM potassium per sulphate by making the final concentration of solution to be $0.7cm^{-1}$. The leaves extracts were prepared in ethanol to a volume equal to 25 µL. 10µL of sample solution was added to the reaction mixture and final percentage reduction in absorbance was measured at 730nm. The expected final absorbance was assumed to be 20-80% decreased as compared to the initial absorbance of reaction mixture.

2.11.4.Scavenging of H_2O_2

Scavenging of H_2O_2 was determined by using method reported (31). Briefly about 4 mM of H_2O_2 (0.6 mL) solution was added to 4 mL of extract and incubated for 10 min. The absorbance of solution was measured at 230 nm against a blank solution via spectrophotometer method. Free radical scavenging potency as determined from %age of H_2O_2. Lower value of H_2O_2 indicated strong free radical scavenging activity of leaves extracts (30,31).

2.12. Anti mycobacterium activity

The two drug resistant strains of *Mycobacterium tuberculosis* ,bg 206 and bg 1972 along with a sensitive strain H37Rv were used in this experiment. Inoculum of all strains were prepared by using method described (9).From dilution (10^{-2}) of each strains of *Mycobacterium tuberculosis* suspensions, about 60µl was streaked on the LJ slants using loop with 3mm external diameter. The crude extract at various concentrations of 5 mg/ml, 10 mg/mL and 50 mg/mL were separately incorporated in the medium and this process was performed for all extracts. Antimycobacterium activity was done according to the methods (9, 30).

2.13.Brine shrimp cytotoxicity bioassay

2.13.1.Hatching of shrimps

In a shallow rectangular dish measuring 22 by 30 cm, brine shrimp eggs were incubated. The fake seawater was created by mixing commercial salt combination with double-distilled water. Two uneven sections were created in the dish by clamping a plastic separator with 2 mm holes. The bigger compartment was filled with 50 mg of eggs and was darkened, while the smaller

compartment was left lit. The phototropic napulii were isolated from their shells by the separator and collected by pipette from the lit side after 48 hours. Using a pipette, twenty shrimp were placed into each sample vial, and then 5 milliliters of fake seawater were added. Against a bright background, the napulii in the pipette stem may be counted. Each vial was fed with a drop of dry yeast solution (3 mg in 6 mL of artificial sea water). The vials were kept under lighted conditions. Using three magnifying glasses, the survivors were tallied, and after a 24-hour period, the percentage of deaths at each dose and control was established. The statistics were adjusted in each instance when control deaths transpired by applying Abbott's methodology:

$$\% \text{ Death} = [\text{ Test} - \text{Control}] / \text{Control} \times 100$$

2.14. Statistical analysis

Data obtained were analyzed statistically by using one way ANOVA and results were expressed in form of mean, standard deviation and percentage values.

3. Results and Discussion

Samples of *pinus* needles leaves and *pinus* resins were chemically analyzed for various parameters and results were mentioned in following sections

Assessment of oil contents of needles leaves and resins

The extraction of oil was carried by distillation method by using Soxhlet apparatus and oils was extract from needles of *Pinus Roxburghii,* and *pinus* resins and results were mentioned [Tables 1-2].

Table 1. Extraction of oils (%) from Needle leaves samples of *pinus* roxburgii

Samples	Methanol	Ethanol	Acetone	Hexane
Needle leaves 1	45.5 ± 1.2	41.5 ± 1.4	11.6 ± 0.9	8.6 ± 0.6
Needle leaves 2	42.4 ± 1.5	37.6 ± 1.2	3.2 ± 2.1	4.7 ± 0.8
Needle leaves 3	33.5 ± 2.3	31.4 ± 0.7	1.6 ± 1.4	1.91 ± 0.7

Mean ± S.D (n=3).

Table 2. Extraction of oils (%) from resins samples obtained from stems of *pinus* roxburgii

Samples	Methanol	Ethanol	Acetone	Hexane
Resins 1	51.5 ± 1.5	48.3 ± 1.8	14.5 ± 0.8	12.7 ± 0.7
Resins 2	45.6 ± 1.1	41.5 ± 1.1	6.5 ± 2.3	3.9 ± 0.9
Resins 3	36.4 ± 2.1	31.0 ± 0.9	2.7 ± 1.1	1.90 ± 0.6

Mean ± S.D (n=3).

Table 3. Extraction of Biodiesel and glycerin after trans esterification process

Samples	Biodiesel (%)	Glycerin (%)
Methanol	10.5 ± 1.2	15.6 ± 3.2
Ethanol	8.2 ± 1.6	11.3 ± 1.5
Acetone	6.3 ± 1.1	7.2 ± 1.5
Hexane	7.4 ± 1.6	9.3± 1.6

Mean ± S.D (n=3).

Conversion of oil into Biodiesel and glycerin

Results regarding biodiesel and glycerin production, after trans esterification process of crude oil are given into table3. Higher quantity of Biodiesel was produced when transesterfifction was carried out by using methanol and sodium hydroxide, 10.5 ± 1.2 % / 100 ml of crude oil . Where as 15.6 ± 3.2 % of glycerin was produced when 100 ml of crude oil was used for transesterifiction process .

Pure glycerin / glycerol has various uses in food, pharmaceutical , medical and personal care industries as reported by different research workers [31,32].

Physical and chemical analysis of Biodiesel

Conformity tests of Biodiesel were carried out and results of pH, acidity, iodine values and saponification values are given in table 4

Table 4 . Various parameters of Biodiesel

Parameter	Results
Color	Pale yellow
pH	6.5
Acid value (mg of NaOH/g of oil	1.3 ± 0.01
Iodine (mg of I2/g of oil)	81.5± 0.2
Saponification value (mg of KOH/g of fat	175± 1.18
Refractive index	1.46± 0.01

Mean ± SD

Analysis of Phyto chemicals

Quantitative analysis of needle leaves and resins are given in table 3. According to results higher quantity of flavonoids fallowed by total phenols and tannins were present in needles leaves. Where as lower quantity was obtained from pinus resins [9].

Table 5. Analysis of methanolic extracts of *pinus* needle leaves and resins of *Pinus Roxburghii* (chir pine) for different phytochemicals

Constituents	Resins	Needles leaves
Total phenol mg/g	3.28±0.72	7.26±1.52
Total flavonoids mg/g	4.15±1.38	14.53±2.45
Total tannins	2.87±0.54	4.36±1.23

Mean ±SD (n=3)

Analysis of oil by GC- MS

Oil extracted from various samples of pinus needles leaves and resins was analyzed by GC – MS .It was found many fatty acids were present [Table 5].

Table 6 Fatty acid contents of oil from *pinus* resins analyzed by GC-MS

Name	RT	%compounds
1-DODECANOL, 2-OCTYL-	8.801	0.008304
TETRAPENTACONTANE, 1,54-DIBROM	11.042	53.84524
1(2H)-NAPHTHALENONE, 6-(1,1-DI	21.181	41.54379
1-DODECANOL, 2-HEXYL-	32.356	0.013741

There were four compounds of fatty acids detected from oil of by GC-MS . However, two compounds like tetrapentacontane (53.84 %) and Napthahalenone (41.5%) were present with higher concentration [Table 6, 7 , Fig.2].

Table 7 Fatty acid contents of oil from needles leaves analyzed by GC-MS

Name	RT	Area	%composition
OCTADECANE, 1-CHLORO-	22.422	11131664384	96.51861
1-DODECANOL, 2-OCTYL-	33.801	25381554	0.220074
1-DODECANOL, 2-HEXYL-	34.807	6787248.5	0.05885
HEPTACOSANE, 1-CHLORO-	36.943	10392788	0.090112
17-PENTATRIACONTENE	37.098	3289870.25	0.028525
1-PENTACONTANOL	37.538	744749.688	0.006457
TETRAPENTACONTANE, 1,54-DIBROM	42.08	53473556	0.46365

There were seven compounds of fatty acids detected from oil of pinusroxburgii by GC-MS . However, compound like OCTADECANE, 1-CHLORO- (96.51) is present with higher concentration [Tables 6-8, Fig.2]

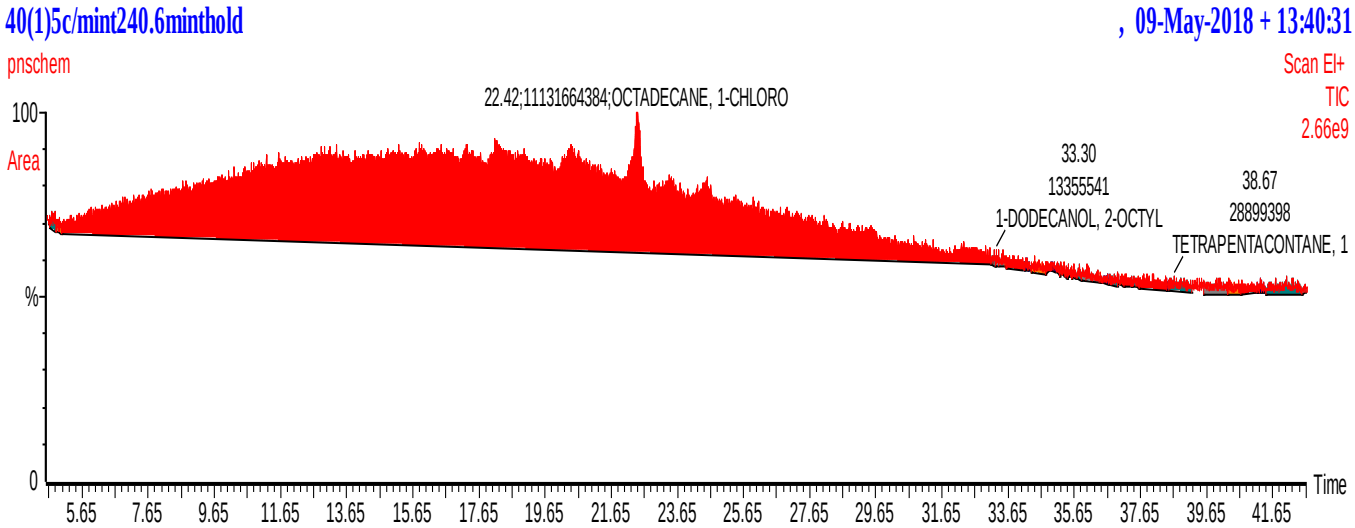

Figure 2. GC – MS analysis of pinus needl leaves

Table 8. Number of Carbon atoms of fatty acid found in organic compounds

Fatty acid	P. R (%)	C
C18 :O	96.5	0.08
C 15: 0	0.64	
C 20 :0	0.22	53.84
C 27:0	0.31	0.13
C35: 0	0.62	-
C 54: 0	0.46	-
C8 :0	-	41.54

Bioactivities of *pinus* needles leaves

Antioxidant activities of leaves extract

In order to find out antioxidant activities, the extracts from needles of Pinus were employed for the DPPH, ABTS and H_2O_2scavenging bioassays. The scavenging activity of the needles

extracts was much greater in H_2O_2 scavenging assay as compared to ABTS and DPPH scavenging assay [Tables 9-10]

Table 9 . Antioxidant activity of leaves extracts of *Pinus Roxburghii* (chir pine) (IC50 values µg/ml)

Extract 100 µg/ml	**DPPH**	**H_2O_2**	**ABTS**
Ethanol	45.17±3.26	58.54±5.26	45.32±2.81
Methanol	25.38±4.15	42.56±3.15	39.46±2.28
N hexane	52.18±1.36	61.52±4.85	48.24±1.35
Ascorbic acid	9 .65±2.52	7.62 ±1.36	16.25±2.38
Gallic acid	6.34±1.32	5.65±1.25	8.26±1.36

Means ± SD, (n = 3).

Table 10. Antioxidant activity of *Pinus resins* extracts (IC50 values µg/ml)

Extract 100 µg/ml	**DPPH**	**H_2O_2**	**ABTS**
Ethanol	36.17±1.25	45.69±4.15	39.45±2.16
Methanol	27.28±1.48	38.21±3.17	32.89±4.36
N hexane	45.16±1.51	48.93±6.53	53.27±3.25
Ascorbic acid	12.29±1.43	9.12 ±2.36	12.35±1.25
Gallic acid	8.35±1.36	7.68±1.25	8.24±1.32

Means ± SD, (n = 3), whereas $^{\alpha}$ = $p<0.01$,Þ= $p<0.05$.

Antimycorbaterium activity of pinus needles leaves

Medicial plants offer a hope for developing alternate medicines for the treatment of TB. The present study was carried out to evaluate in vitro anti-tubercular activity of different extracts of pinus needles leaves extracts against different strains of mycobacterium tuberculosis (Tables 11- 12). Furthermore results obtained in our study showed higher percentage of inhibition results reported by other authors including [13]

Table 11 .Anti-tuberculosis activities of *pinus needles leaves* extracts on LJ Media

Extracts	Isolates	Mean CFU on media				Percentage Inhibition		
		Control	5mg/ml	10mg/ml	50mg/ml	5mg/ml	10mg/ml	50mg/ml
Methanolic leaves extracts	H37Rv	140	36	2	0	70	99	92
	bg 206	150	49	23	0	65	85	82
	bg 1972	130	74	51	0	40	61	80
Methanolic leaves extract	H37Rv	140	30	0	0	75	100	85
	bg 206	150	45	20	0	72	87	82
	bg 1972	130	65	50	0	52	62	80
Methanolic leaves extracts	H37Rv	140	80	76	20	45	46	65
	bg 206	150	90	60	35	35	60	70
	bg 1972	130	86	64	40	30	51	50

Table 12 .Anti-tuberculosis activities of *resins* extracts on LJ Media

Extracts	Isolates	Mean CFU on media				Percentage Inhibition		
		Control	5mg/ml	10mg/ml	50mg/ml	5mg/ml	10mg/ml	50mg/ml
Methanolic leaves extracts	H37Rv	140	36	2	0	74	99	100
	bg 206	150	49	23	0	67	85	100
	bg 1972	130	74	51	0	43	61	100

Methanolic leaves extract	H37Rv	140	30	0	0	79	100	100
	bg 206	150	45	20	0	70	87	100
	bg 1972	130	65	50	0	50	62	100
Methanolic leaves extracts	H37Rv	140	80	76	20	43	46	86
	bg 206	150	90	60	35	40	60	77
	bg 1972	130	86	64	40	34	51	69

Cytotoxiciy assessment of pinus needles leaves

Assessment of cytotoxic behavior of medicines pants used for drugs development are important which indicates that plant extracts is how much toxic to any cell. Results of brine shrimp cytotoxicity assay is given in tables [13-14]

Table 13.Cytotoxicity screening of methanolic*P. Roxburghi* pine needles extracts (µg/ml)

Concentration (ug/ml)	**Total nupuli**	**Live after 24 hours**	**Death after 24 hours**	**% of death**
10	18	17	1	5.56
100	20	14	6	30.0
300	20	12	8	40.0
600	19	10	9	47.37

Values are Mean±SD ,(n=3) and significantly different ($P<0.05$); positive control are saline sea salt

Table 14.Cytotoxicity screening of methanolic pine resins extracts (µg/ml)

Concentration (ug/ml)	**Total nupuli**	**Live after 24 hours**	**Death after 24 hours**	**% of death**
10	18	15	3	16.66
100	20	12	8	60

300	20	8	12	40.0
600	19	6	13	31.50

Values are Mean±SD ,(n=3) and significantly different ($P<0.05$); positive control are saline sea salt

People around the world use herbal supplements and medicines due to their beneficial effects on human health [8]. Bark, needles, pollen and other parts of numerous pine species have been used for many years and proven to constitute excellent raw materials in the production of goods [4].

Conifer shoots are virtually unused as a food ingredient, despite their common availability in many parts of the world. The addition of Pinus extracts increases the antioxidant potential of juices and dairy products as regard to juices, which indicates that it may also serve as an ingredient providing flavour and aroma [15]. In the case of the addition of pine extract to bread and meat, the substance acted as a shelf life extender by inhibiting the growth of bacteria and oxidisation of fats [6].

The seeds of pinus have higher energy value due to a high fat content [21]. The seeds also generally have the highest content of the tested nutrients, excluding vitamin C, which is higher in the conifer needles. The seeds of pinus can be a good source of Mg, P and especially Zn [24].

Macronutrients affect biochemical processes, physiological responses and yield quantity [17,25]. When it comes to macronutrients, their role in plant organisms includes many life processes that determine plant functioning [24,26]. Therefore, it is very difficult to clearly indicate a specific role of elements because they act in a complex way. The role of micronutrients, on the other hand, is more specific, as it is related to specific, well-defined life processes in the plant and to plant growth [27,28]. Nutrient deficiency results in various disorders in terms of the normal growth and development of the plant

It was reported earlier that Pine essential oils contain more than 50 ingredients (19). Their concentrations vary depending on the plant variety, crop, distillation method and part of the plant (Tables 5-7). Studies have shown that these phytochemicals exhibit diverse biological activity, which contributes to their various uses and applications. They can be used as fungicides, flavours and fragrances, as well as antiviral and antimicrobial agents . The uses of α- and β-pinene go beyond therapeutic and nutritional applications. They are versatile compounds that are used in polymer synthesis [20].

Polyphenols are essential secondary metabolites that allow plants to grow and develop. They also protect plants from insects and other factors [13]. Polyphenols found in plants are involved in functions related to sensory properties such as colour, bitterness and sourness [13,14]. Simple phenols and flavonoids correspond to most natural phenolic substances. Moreover, flavonoids belong to the most common group of these compounds. The demand for phenolic acids is very high in many industries because they are used as precursors to other important bioactive molecules that are regularly needed for therapeutic and cosmetic purposes, as well as for food industry. Phenolic acids are also commercially available as dietary supplements [15]. Various parts of a pine (needles, seeds, bark and cones) and different solvents can be used to extract polyphenols. The pine bark is the best-examined part.

mechanism of action of polyphenols is strongly associated with their antioxidant activity and reduction of reactive oxygen species in the human body [14,15]. Furthermore, the health-promoting properties of plant polyphenols include anti-inflammatory, anti-allergic, anti-atherosclerotic, anticoagulant and antimutagenic effects [16]. The daily intake of polyphenols among the general population ranges from 0.1 to 1.0 g per day. Fruit, vegetables, herbs, spices, coffee, tea and wine are the main source of polyphenols [17].

The assessment of biological activities of the phytochemicals to have beneficial therapeutic capacity for curing human from various illness. Therefore, variety of chemical compounds obtained by the tested plants impart significant anti proliferative, cytotoxic, and anti-tuberculosis activities. In the present study, the plant extracts were assessed for the presence of flavonoids, total phenolics and tannin. The study indicates that *pinus* needles leaves and *pinus* resins. According to results pinus needles tree and resins are comprised significant amount of total phenols and tannins [tables 4-5]. Phenols constitute the largest group of secondary metabolites, varying in size from a simple structure with aromatic ring to complex ones. Phenolic compounds, ubiquitous in plants are an essential part of human diet, and are of considerable interest due to their antioxidant properties. The presence of significant amount of these important phyto-constituents bestow the plant with high medicinal activities like free radical scavenging and antimicrobial activities. The variation in quantity of phytochemicals may be dependant upon both the chemical structure and the amount of individual compounds in plant material [7].

By comparison of chromatogram of GC-MS, the area of peaks of compounds was calculated and other required information was obtained by NIST library data base [Tables 6-8. The prominent peaks in the chromatogram of essential oil of indicates that *pinus* needles and resins consist of

some important fatty acids [9]. It was observed that the variation in chemical constituents of essential oils might be due to the variation in contents of leaves and resins because physical structures of needles leaves are different from resins [17]. The composition of oil directly affects the effectiveness of biological activities which have displaces difference in its constituents depending on the growing area whereas chemistry of oil is complex and variable The seasonal changes may account in the variation in chemical constituents of plants grown at different parts of the world [24] .

Chemical constituents with antioxidant activity present in plants determine the role of plants in prevention of many degenerative diseases. The human food supplements including herbs, contain higher amount of compounds that are capable of deactivating free radicals [13] . The plant extracts were assessed for their antioxidant potential by three methods that includes DPPH scavenging, ABTS scavenging and H_2O_2 scavenging bioassays. The methanolic extracts of the plants showed significant scavenging of free radicals. By comparing DPPH and ABTS bioassays results. It was assumed that the antioxidant potential of the three plant extracts is much increased in case of DPPH bioassay as compared to ABTS bioassay. The antioxidant potential of *P. Roxburghii* was higher as compared to resins extracts analyzed [Tables 9-10]

DPPH and other scavenging bioassay revealed that free radical scavenging potential was present in plant extracts as been reported in literature by many authors might be significant antioxidant agent due to its excellent antioxidant activity for Reactive oxygen species (ROS). ROS and reactive nitrogen species (RNS) are some forms of activated oxygen and nitrogen respectively, which include free radicals such as superoxide ions, hydroxyl and nitric oxide radicals as well as non-free radical species such as hydrogen peroxide (H_2O_2), and nitrous acid (HNO_2). ROS and RNS have been the cause of more than 100 diseases which includes malaria, AIDS, heart diseases, stroke, diabetes and carcinogenity. It was reported thatpinus needles leaves have antioxidant as well as antimicrobial activity which may be attributed to the presence of various active secondary metabolites [27].

Similarly in the current research work, has shown a considerable amount of antioxidant potential. However the studies conducted in the other parts of world revealed the good antioxidant potential and anti-inflammatory activity of *pinus needles leaves* .

Scavenging of H_2O_2 displayed by extracts of plants was dose dependent. The total radical scavenging activity (superoxide and hydroxyl radical) of pinus needles was found to be quite significant and showed effective scavenging activity in dose dependent manner and suppressed the production of H_2O_2 at the dose concentration 300 µg/mL which is a characteristic of chain-breaking antioxidants, and has been observed in oxidation of linoleic acid emulsion with extract . Flavonoids, phenolic acids and phenolic diterpenes are the examples of phenolic components with antioxidant properties. Scavenging of extracts may be characterized to phenolic content which is capable of donation of electron to H_2O_2, thus nullifying to water. Although reactivity o H_2O_2 is not much, even then it can periodically be cytotoxic by ascending hydroxyl radicals in the cells. Expulsion of H_2O_2 by food stuffs is too much necessary [20].

The selected herbs have been used traditionally as a remedy for respiratory diseases like bronchitis, sinusitis, tuberculosis and common cold [21] . These activities are mostly due to presence of essential oils . The results indicates that essential oils and other phyochemicals present in plant extracts has exhibited significant antibacterial activity [Tables 11-12]. Therefore it is assumed that due to presence of essential oils, phenols and flavonoids . Extracts of *Pinus* needles leaves as well as *pinus* reins might be good remedy against infection causes by mycobacterium tuberculoses . All the tested extracts are antibacterial in nature and results obtained are in accordance with the reported findings [25]. Due to multidrug resistant strains of various bacterial strains new medicines are required to overcome tuberculosis in human population. Various plants have some active secondary metabolites those have wide range of application against such human disorders . There is need of continuous development of new and efficient methods to determine the susceptibility of isolates of mycobacterium tuberculosis in search for new novel antimycobacterium agents from natural products of plants sources. Therefore in present study various solvents extracts of *pinus* needles leaves have provide remarkable anti mycobacterium activities indicating its usefulness control aliments of tuberculosis in human population [26] . Tuberculosis (TB) is an infectious disease mostly caused by mycobacterium tuberculosis mostly affects on lung but some cases also affects other

parts of body . According to literature about one- third of world population is suffering TB , that is increasing at rate of 1 % per years [19].

Practice for uses of pinus needles leaves as antimicrobial agent is taking since many decades . It was reported by many research workers that smell of pinus needles are important for respirator tract system [20]. According to results of anti mycobacterium activity of pinus needles were proposing . The increasing incidence of infectious diseases, severe side effects related to the intake of many antibiotics and the development of antibiotic resistance substantiate the growing interest in the identification of new antimicrobial compounds, both natural and synthetic agents [24].

Plant resin has been applied to treat diseases in folk medicine for thousands of years. It was also used in the pharmaceutical industry before the introduction of modern antibiotics. The antimicrobial activity of extracts, oils and resins from trees of the Pinus genus may be related to various organic Plants

The discovery of biological effects of the chemical compounds available Pinus suggests that they may be applied in the creation of environmentally friendly and biocompatible pharmaceuticals [32].

Cytotoxicity refers to the ability of certain chemicals to destroy the living cells in the body. By a cytotoxic compound, healthy living cells either induce necrosis (accidental cell death) or apoptosis (programmed cell death). Brine shrimp lethality bioassay used for *pinus* needles leaves and resins indicates that all the plant extracts are very less toxic and are suitable to be used in folk medicine, which also indicates importance of *these* extracts for its application in pharmaceutical industry for development of drugs.. The toxicity of extracts was assessed which revealed that all of the plant extracts were less toxic towards shrimp's napulii at higher concentration (600 µg/mL) which confirmed their efficiency to be used in preparation of future drugs [31,32]

4.Conclusion

Various parts of *pinus* tree contains different bioactive compounds. Green and dried Needles leaves , resins and cones are important sources of food materials as well as material required

domestically. The pine bark extracts are commercially available, there is no universal method of extraction that is suitable for all phenols. Depending on the ultimate goal of extraction, an individual examination should be performed to ensure the most appropriate extraction procedure. Regardless of the solvent, method, pine species and plant part used, all pine extracts contain a high number of polyphenols. Nevertheless, individual compounds are characterized by different concentrations, types and levels of their bioactivity. There are few studies on the identification and even fewer studies presenting the quantitative determination of individual polyphenols contained in pine extracts. Pine tree extracts exhibit several described biological activities that may be beneficial to human health. The available examples of the application of pine elements in food are promising. Pine tree extracts, syrups and other intermediates may be components that impart functional properties, extend the shelf life and assign desirable qualities to food products. Pine extracts and oils exhibit great potential as formulation ingredients for food, cosmetic and pharmaceutical industries. *Pinus* resins is natural fuel that can be converted into other fuels like biodiesel etc to meet future requirements of energy .The reuse of residual pine elements is still limited compared to its potential. In this case, it is necessary to conduct more research to find and develop new products and applications of pine residues and by-products.

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