CHEMICAL ANALYSIS AND BIOACTIVITIES ASSESSMENT OF *PINUS* NEEDLES LEAVES

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ABSTRACT

Pinus needles leaves have long history of its uses as food medicines and cosmetics in different parts of world . In current study *Pinus* needles leaves of different trees were evaluated for their proximate, total phenols, total flavonoids, tannins and total oil contents by using different methods as well as GC-MS and FT-IR techniques . Whereas various antioxidants, antibacterial ,anti mycobacterium tuberculosis and cytotoxicity assays were performed to determined bioactivities of different extracts of *pinus* needles leaves. Results indicates that pinusRoxburgiihave provided higher levels of moisture (18.5 ± 2.6 %), ash (2.4 ± 0.5 %) , dietary fiber (24.±2.8 %), crude lipid (8.5 ± 0.7 %), Carbohydrates (45.5 ± 2.5 %), total phenols (7.26±1.52) flavonoids (14.53±2.45), tannins (4.36±1.23) and total oils (1.92 ± 0.28) contents as compared to other extracts analyzed. GC-MS analysis exposed higher quantity of some saturated essential fatty acids in *Pinus*needles leaves, those were further confirmed by indication of their functional group by FT-IR analysis. According to results methanolic extracts of Pinusroxburgii has higher provided higher antioxidant values (DPPH, IC_{50} = 38.36±4.58 µg/ml) and higher zones of inhibition for various bacterial and anti mycobacterium strains. Whereas lower values of brine shrimp cytotoxicity assay depending on concentration of extracts was obtained for Pinusroxburgii as compared to other extracts analyzed. These results indicates that pinus needles leaves especially Pinusrox burgii has greater potential of antioxidants , antibacterial, anti mycobacterium activities as well as reliable cyto toxicity due to phenolic, flavonoids and essential oils presents in extracts.

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

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INTRODUCTION

A therapeutic plant has same properties as regular pharmaceutical medications. People have utilized them all through history to either cure or decrease side effects from an ailment. A pharmaceutical medication is a medication that is created in a research center to cure or help a disease. Names of some of the medicinal plants are: Amla, Ashok, Bael, BhumiAmla, Brahmi, Chiraita, Gudmar, long peeper *etc* The plant is utilized as a superior tonic in treatment of fever and for curing different skin infections (Alanis *et al*., 2003; Maryam *et al*., 2021).

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 (Ahmad *et al*., 2014). 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 (Al-Snafi, 2013)..Numerous medicinal plants contains awesome amount of antioxidant compounds instead of vitamins and carotenoids Therapeutic agents presents in plantspossessignificance 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 (Beech *et al*., 2017 : Gulfraz *et al*., 2008)

Extract from the leaves of Eremophila species (Myoporaceae) were the most dynamic, with Eremophiladuttonii displaying the best action (against Gram-positive microscopic organisms). The most dynamic antibacterial plants against both gram-positive and gram-negative microscopic organisms were Thymus vulgaris and Thymus origanium. The two extract from similar plants indicated diverse exercises; the organic extract demonstrated the same or more noteworthy activity than the watery extract . Out of the 14 plants examined, Fragariavirginiana Duchesne, Epilobiumangustifolium L. furthermore, Potentilla simplex Michx. shown solid antifungal potential.Fragariavirginiana had some level of action against the majority of the parasitic pathogens. AlnusviridisDC.,Betulaalleghaniensis Britt. what's more, SolidagogiganteaAit. additionally showed a huge level of action against a significant number of the yeast separates (Briskin, 2000).

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 (Burt, 2004; Cai *et al*., 2004).

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 (Cushine and Lamb, 2011; Essawi and Srour, 2000) .

From the tried raw extract, Inulagraveolens, Salvia dominica, Conyzacanadiensis and Achilleasantolinashowed powerful antiproliferative activity and the action dwelled in the chloroform/ethanolic separates. The most dynamic plant was I. graveolens with an IC50 of 3.83 µg/ml .

Three unique extracts (oil ether, ethylacetate, and methanol) from each plant species, were tried towards KB, HCT-15 COLADCAR and UISO-SQC-1 cell societies. The outcomes demonstrated that three plants Colubrinamacrocarpa (Cav.) Wear (Rhamnaceae), Acacia pennatula (Schltdl. furthermore, Cham.) Benth (Leguminosae) and Hemiangiumexcelsum (HBK.)Smith

(Hippocrateaceae), displayed essential cytotoxic action demonstrating a specific level of selectivity against the tried cells in culture (Graf *et al*., 2010; De Souza *et al*., 2007) .

Extracts taken from 10 South American therapeutic plants (Baccharistrinervis, Baccharisteindalensis, Eupatorium articulatum, Eupatorium glutinosum, Tagetespusilla, Neurolaenalobata, Conyza floribunda, Phytolaccabogotensis, Phytolaccarivinoides and Heisteriaacuminata) were selected for in vitro antiviral action against herpes simplex write I (HSV-1), vesicular stomatitis infection (VSV) and poliovirus compose 1. The most strong hindrance was seen with a fluid concentrate of B. trinervis, which restrained HSV-1 replication by 100% at 50–200 µg/mL, without indicating cytotoxic impacts. Great exercises were likewise found with the ethanol concentrate of H. cuminata and the fluid extract of E. articulatum, which showed antiviral impacts against both DNA and RNA infections (HSV-1 and VSV, individually) at 125– 250 µg/mL (Foster and Duke, 2000; Feng *et al*.,2011)

Five plants which have been utilized for the treatment of ailment, joint pain and edforgout in the conventional prescription of Saudi Arabia, were assessed for their anti inflammatory properties. Of these the ethanolic extract of Capparisdeciduas and the fluid concentrate of Capparisspinosa were found to have critical mitigating action against carrageenan actuated oedema in rats (De souza *et al*., 2007)

Plants are equipped with many chemical constituents. There are two types of plant constituents known as primary and secondary metabolites.Essential metabolites are associated with the essential digestion of plants. A portion of the essential metabolites are nucleic acids, starches, lipids, proteins and chlorophyll. Plants store the overabundance of essential metabolites that are discovered either in stem, leaves or roots, and are .Auxiliary metabolites incorporates synthetic constituents which are not being utilized in essential digestion. In the past they were viewed as excretory items or finished results. Presently their significance has been acknowledged by the solution. These constituents are presently viewed as therapeutically critical constituents. Optional metabolites assume a vital part of safeguard for the plants. They shield the plant from bugs and furthermore fend off the herbivores from them (Dynesius and Jansson, 2000).

The Pinaceae (pine family) are 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(Essawi and Sour, 2000; Hussain *et al*., 2008; Keeley, 2012).

Restorative Properties include: Germ-free, astringent, provocative, cell reinforcement, expectorant, high in Vitamin C for colds, influenza, hacks, clog, and even scurvy. Shikimic corrosive, the primary fixing in Tamiflu, is reaped from pine needles in Asia.

Pine Needle Tea: Pine needle drink 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 (Isman *et al*., 2011).

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 (Javanmardi *et al*., 2003; Ji et al., 2009). 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 antidiabetic agents.

MATERIAL AND METHODS

Collection and preparation of samples

The samples of needles leaves of three different pinus trees 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 Abbottabad regions. The samples were distinguished by a taxonomist at the Department of environmental Sciences, COMSATS University Islamabad (Abbottabad campus) and voucher specimen (No. 135) was deposited for future reference. Around 3 kg of plant material were moved to Biochemistry research laboratory, Division of Science, COMSATS University Islamabad,, Abbottabad. The needles leaves of the pinus species were used for the extraction of various phytochemicals and oils as well as determination of various bioactivities .

Plant materials were washed with demineralized water to evacuate undesirable materials including dust. Plants samples were shade and sun dried followed by oven dried for over night at 50 °C . The dried examples were ground with electric grinder, sieve 80mesh and saved in plastic bags at lower temperature till further uses.

Proximate analysis

The moisture contents of leaves were determined by weighing before after heating high temperature for overnight. Crude lipid was determined by using 5 gram of samples in 100 ml of ether through soxhlet apparatus , where as dietary fiber and ash contents of needles leaves were determined by using AOAC methods.

Determination of total phenols:

The concentration level of phenol in need leaves sample was determined in different solvents and amount was quantified by using method reported by various authors (Ji *et al*., 2009; Koehan and Carter, 2005). 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 (Monfalouti *et al*., 2010; Liang *et al*.,2008).

Determination of flavonoids

The extract of sample was prepared in five solvents . The flavonoid contents of extracts were quantified by using method reported by Husain et al.(2008). An aliquot (1 ml) of extract or a standard solution quercetin (4mg/ml) was added to 10 ml flask containing 4 ml distilled water and 0.3 ml 5% $NaNO_2$ was added. After 5 minutes, 0.3 ml of 10% $AlCl_3$ was added and after 6 minutes, 2 ml of 1M NaOH was further added and the total volume was made up to 10 ml with distilled water. Absorbance was read at 510nm with a spectrophotometer and the concentrations of flavonoid in the samples were expressed as mg quercetin equivalent /g of sample (Krymov, 2002).

Estimation of tannins:

The total concentration of tannins in different leaves extract was determined by Folin Denis method. The measurement of blue color formed by the reduction of phosphotungstomolybidic acid by tannins is used for colorometric determination of Tannins and 7.5 ml of distilled water was added to 1.0 ml of extract and standard solution of tannic acid. Then 1ml ofsodium carbonate was added following 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 (Muthu *et al*.,2006).

Analysis of flavonoids with High Performance Liquid Chromatography

The HPLC analyses were performed by using shimadzu system (Tokyo, Japan) C18 column (250 mm × 4.5 mm, 5 m) gradient pump, UV/Visible detector. The compounds were eluted with a gradient elution of mobile phases (Acetonitrile and 0.1% phosphoric acid ; 36:64), and the injection volume for all samples was 20µl. Flavonoids were monitored at 280 nm and 285 nm at a flow rate of 1 ml/min. The quercetin was used as standard and all determinations were performed in triplicates (Newman *et al.,* 2000).

Extraction of oils by Soxhlet methods

Steam refining is a partition procedure utilized by cleanse or separate temperature delicate materials,like normal sweet-smelling compounds. Steam or water is added to the refining contraption to bring down the breaking points of the mixes. The fundamental rule of steam refining is that its permits a compound or blend of mixes to be separated at a temperature impressively

underneath that of the breaking point of the individual constituent .basic oils contain substances with bringing down breaking points and within the sight of steam or bubbling water, these mixes are volatilized at a temperature of around 100°C (Masamgo, 2005).

The fundamental oil from crisp aeronautical parts of the plant was separated utilizing strategy effectively depicted (116) with a few adjustments. The ethereal parts of the chose plants were cut into littler pieces with the assistance of blade and scissors. The little bits of plant material could yield more noteworthy biomass that is effortlessly removed with high effectiveness .subsequent to changing over into little pieces, the heaviness of elevated parts of plant was controlled by utilizing an electric advanced adjust. The measured plant material was then raced into a spotless vessel of refining mechanical assembly. Roughly 2000ml refined water was added to the vessel and afterward put on warming mantle. The vessel was then associated with a condenser that was cooled by frosty faucet water. The stream of water through a condenser was begun and afterward the subsequent blend was bubbled for 3 hrs, the distillate containing unstable mixes was gathered in an isolating pipe in the wake of going through the water condenser. Layer of oil at the highest point of water in isolating cup obviously uncovered the nearness of basic oil. At that point, basic oil coasting on distillate was isolated and the rest of the distillate was utilized to isolate oil from it by fluid – fluid extraction (Newman *et al*., 2003).

The plug of isolating channel was opened for at some point to discharge weight. The shaking procedure was rehashed atleast three times and isolating channel was hanged in a stand holder. Following a couple of minutes a reasonable layer of hexane containing basic oil was framed over the water which was isolated in another flagon. A similar system was rehashed three times by including 70 mL of hexane in the leftover distillates. A few hints of water still present in hexane extricate was expelled by expansion of some measure of anhydrous magnesium sulfate to hexane separate. Hexane extricate containing magnesium sulfate was then sifted in a pre-weighed round base flagon (WHO, 2005; Palombo and Semple,2001).

Under reduced pressure most of the hexane was evaporated by using rotatory evaporator. On analytical balance oil free from hexane was weighed to find yield of oil by dividing the extracted oil mass by the mass of plant used for the extraction of oil . By means of specific glass adopter .round bottom flask having hexane extract was connected to rotary evaporator. To collect the hexane after evaporation collecting flask was connected to the condenser of rotatory evaporator.

At the beginning, the vaccum of rotatory evaporator was set at 200 mbe at 25℃. when the evaporation from flask get stable right after 3 mins the pressure was decreased to 100 or 80 nbr. The process was continued till all the hexane get evaporated from round bottom flask. Then the yield (%) of pure essential oil was calculated by dividing the mass of essential oil to the mass of fresh plant material used for the extraction of oil (Petrovska, 2012).

Briefly samples were waterlogged overnight and washed thoroughly to eliminate the pulp. The fruitssamples were air dried at room temperature crushed into powder from and used for the estimation of oil contents by using AOAC Official Method. Total 2 grams of sample by addition of appropriate amount of ether in Soxhlet apparatus was used for extraction of oil . The reaction continued for 16 hours and results were expressed as percentage of dry weight of fruit.

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 ℃. The stove temperature of GC was customized as: the underlying temperature of broiler was 40 ℃ and it was kept up for 2 min, the temperature was raised from 40 to 450 ℃ at a warming Rate of 4℃ 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

Bioactivity assessment of *Pinus* needles leaves

Antioxidant activity

The antioxidant activity of plant extracts was carried out according to method earlier reported (Javanmardi *et al*.,2003) Therefore, for determination of antioxidants, following bioassays were used.

DPPH scavenging bioassay

DPPH scavenging activity was done according to method 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

DPPH scavengingaction was carried out by technique with a few alterations. This strategy was depended on the capacity of cell reinforcement to rummage 1, 1-diphenyl-2-picryl hydrazyl (DPPH) activity radical. In a falcon tube, 100 µL of the example arrangement was taken and afterward 4mL of DPPH arrangement (0.1 mM) was included and blend was vortexed energetically. At that point the blend was brooded with Aluminum thwart to maintain a strategic distance from warm introduction oblivious for 30 min at room temperature. UV-Vis spectrophotometer was utilized to quantify the absorbance of the arrangement at 517 nm. Rate hindrance was ascertained by

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

ABTS scavenging bioassay

The ABTS radical scavenging bioassay was carried out by already reported method. 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 $0.7cm^{-1}$. Then the plant 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.

Scavenging of H_2O_2

Scavenging of H_2O_2 was determined by using method reported and 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 of H_2O_2 indicated strong free radical scavenging activity (Koehn and Carter, 2005).

Brine shrimp cytotoxicity bioassay

Hatching of shrimps

Brine shrimp eggs were hatched in a shallow rectangular dish (22 x 30 cm) filled with artificial sea water that was prepared with a commercial salt mixture and double distilled water. A plastic divider with 2 mm holes was clamped in the dish to make two unequal compartments, the eggs (50 mg) were sprinkled into larger compartment which was darkened while the smaller compartment was illuminated. After 48 hrs, the phototropic napulii were collected by pipette from the illuminated side, which was separated by the divider from their shells (Saeed *et al*.,2012).

Twenty shrimps were transferred to each sample vial using pipette and 5mL artificial sea water was added. The napulii can be counted in the stem of pipette against a lighted background. A drop of dry yeast suspension (3 mg in 6mL artificial sea water) was added as food to each vial (Solowey *et al*., 2014). The vials were maintained under illumination. Survivors were counted with the aid of 3 magnifying glass and after 24 hrs percent death at each dose and control were determined. In each case, where control deaths occurred, the data were corrected using Abbott's formula:

% Death = [Test – Control] / Control x 100

Antibacterial activity

Preparation of inoculum

Suspension of organisms was prepared as per McFarland's standard and 24 hours old culture was used for the preparation of bacterial suspension. Bacterial strains (colony) were picked in eppendorf tubes with the help of inoculation loops and placed in 37°C incubator for 30 minutes. Each bacterial colony collected was then mixed in 3mL distilled water and then shaked vigorously or vortexed and optical density was checked and made equal to 0.5 by using spectrophotometer at 600 nm wavelength (Sher, 2004).

Test organism: Bacterial strains

The plant extracts of three Pinus species were screened against five bacterial strains by following the method reported (Tsao and Liu, 2007). The strains of Gram +ve bacteria such as Staphylococcus aureus (KX262674) and Bacillus cereus (KX262674) and Gram –ve bacteria Escherichia coli (ATCC 10536), Salmonella typhi (ATCC 6539) and Pseudomonas aeruginosa (ATCC 9027) were included in this study. Each bacterial colony was mixed with 3 mL sterilized water, vortex ed and optical density was made equal to 0.5which means culture contained no. of colony forming units (CPU) in the range of $10^7 – 10^8$ per ml of suspension depending upon bacteria. These strains were grown in nutrient broth and then cultured on nutrient agar for their maintenance and were stored in refrigerator at low temperature for reculturing before use in experiment as reported earlier.

Antibacterial activity of Pinus essential oil

Antibacterial activity of essential oils extracted from was assessed using agar well diffusion method. Nutrient agar was prepared by pouring accurately weighed 13 g/L of nutrient broth and 14 g/L of agar technical in 1L of distilled water in reagent bottle and was mixed thoroughly that it may get mixed. Then the mixture was autoclaved with the temperature at 110°C for 60 mins. Then agar plates were poured by 30-35 mL of autoclaved nutrient media, covered and sealed with parafilm. The covered plates were allowed to rest for an hour so the agar can solidify and then petri plates were placed in an incubator at 37°C for overnight. 200 µL of each bacterial strain was

evenly spread on surface of petriplates using glass spreader. Then four uniform and equidistance wells were made with 6mm (diameter) cork borer in each plate. Each well was filled with 30µL of essential oil test solution and 2 replicates of each test petri plate were made. Hexane was used as negative control and streptomycin (1 mg/ mL) was selected for positive control as the reference for all bacterial strains. After half an hour, the plates were placed in incubator at 37°C for 24 hrs. The size of zone of inhibition of each well in a petri plate was individually measured in millimeters by using a scale at four different places around the inhibition zone circle (Shinwari, 2010).

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 by Cushine and Lamb(2011; Maryam *et al*., 2021).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 05 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 of (Gulfraz *et al*., 2008; Roy *et al*., 2994; Rios, 2010).

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.

RESULTS AND DISCUSSION

Proximate analysis

Results of proximate analysis indicates that pinus*Roxburghii* contained higher quantity of moisture (18.5 ± 2.6 %), dietary fiber (24.6 ± 2.8 %), ash (2.4 ± 0.5 %) ,crude lipid (5.6± 0.7 %) and carbohydrates (45.5 ± 2.5 %) as compared to other extracts (Table 1).

Table1. Proximate analysis (%) of pinus needles leaves

Extracts	**Moisture**	**Dietary fiber**	**Ash**	**Crude Lipid**	**Carbohydrates**
PinusRoxburghii	18.5±2.6	24. 6±2.8	2.4±0.5	5.6 ± 0.7	48.5 ±2.5
PinusWallichiana	14.6±1.5	23.5±1.6	2.2 ± 0.8	4.9 ± 0.5	54.5± 1.6
cedrus deodar	14.8±1.2	21.8±1.5	2.6±0. 7	3.5 ± 1.2	57.3 ±1.5

Mean ±SD (n=3)

Analysis of phytochemicals

Quantitative analysis of extracts shows that the amount of phenole, flavonoids and tannins (Table 1). Result s has shown that the extract from needles of *PinusRoxburghii*carries the higher amount of phenol (7.26 mg/g) followed by *Pinus Wallichiana*which contain 5.42 mg/g of phenol while *cedrus deodar* has 5.86 mg/g of phenol content in needles extracts.. The amount of flavonoids was maximum in plant extract of *PinusRoxburghii* (14.53 mg/g) whereas,*PinusWallichiana* and *cedrus* deodar contained 8.56 and 5.26 mg/g. Similarly the yield obtained from tannins was greater in *PinusRoxburghii(4.36* mg/g *)* followed by 3.46 mg/g in *PinusWallichiana* extract while 2.68 mg/g tannins were found in extract of cedrus deodar (Roy *et al*., 2004).

Table 2 Chemical analysis of *pinus* needle leavesof*PinusRoxburghii* (chir pine)

Constituents	**n-hexane**	**Ethanol**	**Methane**

Total phenol mg/g	2.06±0.27	4.28±0.72	7.26±1.52
Total flavonoids mg/g	4.13±0.16	5.15±1.38	14.53±2.45
Total tannins	1.33±0.25	2.87±0.54	4.36±1.23

Mean ±SD (n=3)

Table 3. Chemical analysis of *pinus* needle leaves of *PinusWallichiana*

Constituents	**N-hexane**	**Ethanol**	**Methane**
Total phenol mg/g	2.23±0.28	3.78±0.62	5.42±2.83
Total flavonoids mg/g	3.18±0.82	5.65±2.31	8.56±1.21
Total tannins	1.12±0.32	2.35±0.38	3.46±1.58

Mean ±SD (n=3)

Table 4. Chemical analysis of *pinus* needle leaves of *cedrus deodar*

Constituents	**N-hexane**	**Ethanol**	**Methane**
Total phenol mg/g	2.96±0.28	4.13±0.82	5.86±1.05
Total flavonoids mg/g	4.35±0.75	6.45±1.32	6.26±1.76
Total tannins	1.65±0.82	2.15±0.27	2.68±0.41

Mean ±SD (n=3)

Assesment of yield of essential oil

The extraction of essential oil was carried by hydro distillation from needles of *Pinus Roxburghii*, *Pinus Wallichiana* and *cedrusdeodara.* Needle extract of *PinusRoxburghii*and *PinusWallichiana* were rich in essential oil and yielded 1.92% and 1.68% essential oil respectively. *Cedrusdeodara*also showed good yield however, the amount was less as compared to other two *pinus*species (Table 2-4).

Table 4. Percentage yield of essential oils

Latin name	Family	% yield of essential oil
PinusRoxburghii	Pinaceae	3.92±0.28
PinusWallichiana	Pinaceae	3.68±0.52
cedrusdeodara	Pinaceae	2.25±0.05

Mean ± SD after triplicate analysis

HPLC analysis of flavonoids

Methanolic extracts of pinus needles was analyzed with HPLC and flavonoids were monitor at

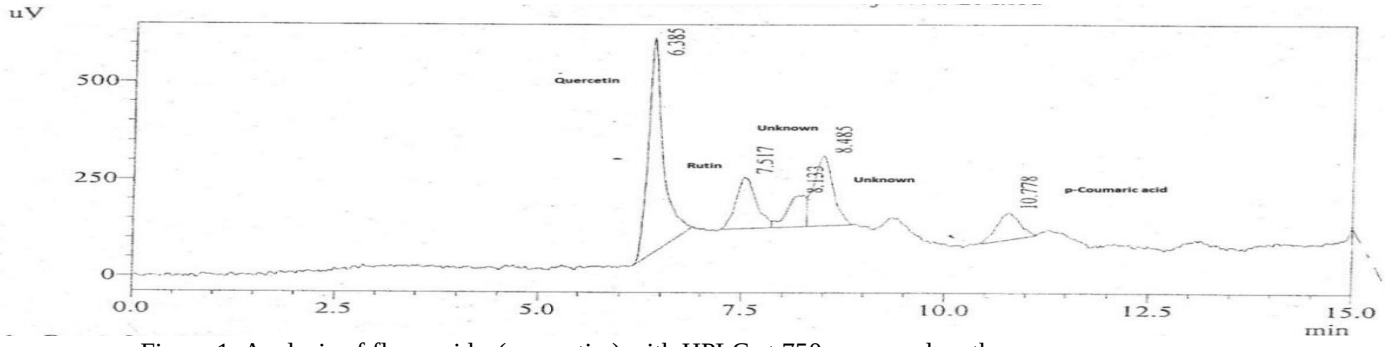

Figure 1. Analysis of flavonoids (quercetin) with HPLC at 750 nm wavelength

Analysis of oil by GC- MS

Oil extracted from various samples of pinus needles was analyzed by GC –MS .It was found many fatty acids were present (Table 5).

Table 5 Fatty acid contents of oil from cedrusdeodara needles leaves 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 cerdusDeodara by GC-MS . However, two compounds like tetrapentacontane (53.84 %) and Napthahalenone (41.5%) were present with higher concentration

Table 6 Fatty acid contents of oil from pinus Roxburgii 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

Figure 2. GC – MS analysis of pinus needl leaves

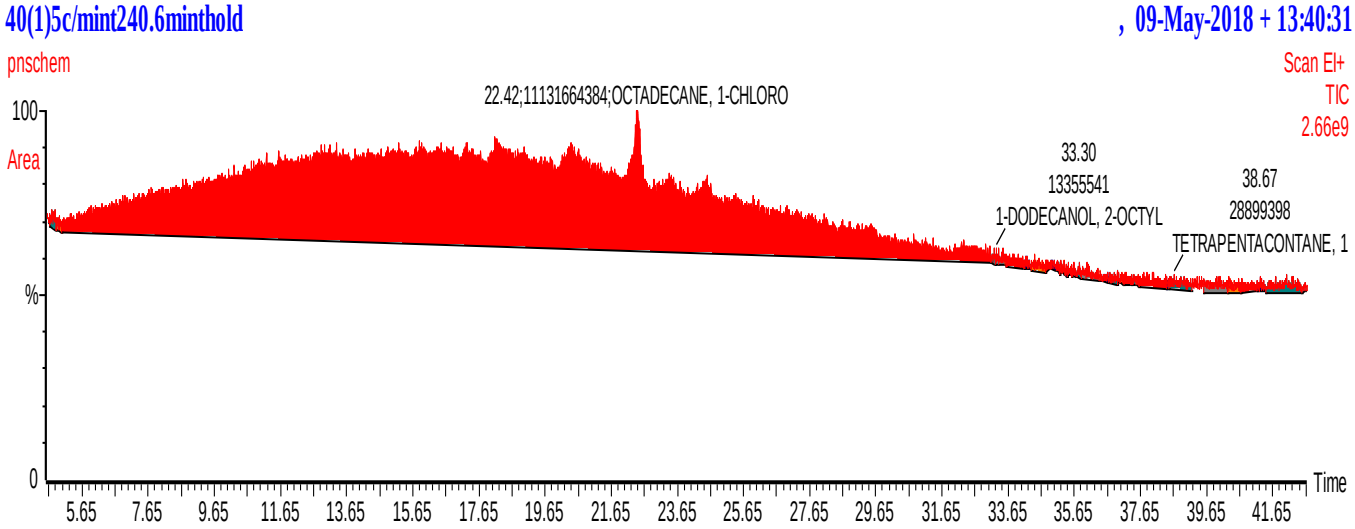

Table 7 Number of Carbon atoms of fatty acid found in pinus needles leaves

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

Antioxident 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

Table 8 . Antioxidant effects of leaves extracts of *PinusRoxburghii* (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 9. Antioxidant effects of leaves extracts of *Pinus Wallichiana (*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= $p<0.05$.

Table 10 .Antioxidant effects of fruit extracts of *Cedrusdeodara (*IC50 values µg/ml)

Extract 100 µg/ml	**DPPH**	**H_2O_2**	**ABTS**
Ethanol	47.16±3.64	53.65±2.11	48.15±2.13
Methanol	38.36±4.58	42.23±2.38	45.68±4.35
N hexane	54.14±5.64	48.37±3.61	48.29±1.26
Ascorbic acid	9.22±1.38	8.05 ±2.38	12.65±1.62
Gallic acid	8.16±1.32	7.53±1.26	9.16±1.36

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

Antibacterial activity of essential oil of*P. Roxburghii* , *P. Wallichiana and C.deodar was* tested againstthe strains of Gram+ bacteria such as *Staphylococcus aureus(KX262674)* and *Bacillus cereus(KX262674*) ;and Gram – bacteria *Escherichia coli (ATCC 10536)* and *Salmonella typhi (ATCC 6539)*. The results showsthat essential oils has inhibited growth of bacterial strain significantly. The zone of inhibition provided by *P. Roxburghii* for S. auresu (17.6 ± 0.6 mm), B. cereus (16.4 ± 0.9 mm), S trphi (21.5± 0.3 mm) and E. Coli (21.8 ± 0.4mm) were relevatively higher as compared to other methanolic plant extracts as well as standard antibiotic used (Table 11).

Table 11 Antibacterial activities of various methanolic pine needles extracts ; Zone of inhibition in mm

Extracts	*S. aureus*	*B. cereus*	*s.typhi*	*E.coli*
P. Roxburghii	19.6±0.6	17.4±0.9	23.5±0.3	21.8±0.4
P. Wallichiana	17.6±0.6	16.4±0.9	21.6±0.3	21.3±0.2
Cedrusdeodara	16.2 ±0.5	18.3±0.8	17.5±0.4	16.2±0.5
Cefixime (Antibiotic)	18.3±0.6	21.2±0.3	18.2±0.4	17.8±0.6
Negative control	0.0±0.0	0.2±0.0	0.3±0.0	0.2±0.0

Results mean± S D after triplicate analysis (n=3).

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 table 12.

Table 12.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

Antimycorbaterium activity of pinus needles leaves

Medicinal 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 (Table 13). Furthermore results obtained in our study showed higher percentage of inhibition results reported by other authors including Graf *et al*. (2010).

Table 13 .Anti-tuberculosis activities of *plant*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

Proximate parameters of any plant extracts give information regarding its suitability for used as feed or food, where as Phytochemicals present in the plants may be used as food and medicine. There is growing worldwide interest for characterization, isolation, the *in vivo* and *in vitro* 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, antibacterial and anti-tuberculosis

activities. In the present study, the plant extracts were assessed for the presence of flavonoids, total phenolics, saponin and tannin. The study indicates that the presence of these phytochemicals in all the three plants species contained the considerable amount of flavonoids, phenolics and tannins (Ullah and Khan, 2008). 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. In the current study, higher TPC was found to be possessed by the plant extracts. Highest quantity of phenolics was found in P. Roxburghii as compared to others extracts analyzed Tannins (flavonoids) are astringent polyphenolic biomolecules that binds to and precipitates proteins and various organic compounds including aminoacids and alkaloids. Tannins are considered to be antimutagenic and this mutagenicity of tannins is related to their anti oxidative property (Tsao and Liu, 2007). The reported studies showed that tannins are present in lower concentration as compared to other phytochemicals in majority of plants, but our study reveal that plant species do possess tannins in them in considerable amounts. The phytochemical analysis showed thatP. Roxburghii contained higher quantity of tannins

Phytochemical prospection indicated the presence of different secondary metabolites. So 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 (Shinwari, 2010).

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. The prominent peaks in the chromatogram of essential oil of indicates that pinus needles consist of some important fatty acids (Roy *et al*., 2004).

The variation in chemical constituents of essential oils might be due to the variation in the species of tree. 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 as mentioned above. The seasonal changes may account in the variation in chemical constituents of plants grown at different parts of the world .

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 (Saeed et al.,2012). 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. Among the three plants, the antioxidant potential of *P. Roxburghii* was higher as compared to other plant extracts analyzed

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 (Gulfraz et al., 2008).

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 whole plant extract 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 (Saeed *et al.*, 2012; Solowey *et al.*, 2014).

Antimicrobial activity of essential oil of *pinus needles* in methanol has been tested against Gram +ve and Gram –ve bacterial strains. The selected herbs have been used traditionally as a remedy for respiratory diseases like bronchitis, sinusitis, tuberculosis and common cold. These activities are mostly due to presence of oils like . The results indicate that essential oils and other phyochemicals present in plant extracts has exhibited significant antibacterial activity Results indicates that plant extracts has inhibited growth of bacterial strains So, it can be said that all the tested extracts are antibacterial in nature though the activity varies from extracts to extracts . The results obtained are in accordance with the reported findings (Ullah and Khan, 2008).

The antibacterial study indicates that the plant extractshave tremendous antibacterial activity at higher concentrations against various bacterial strains that are the major causative agents mainly of stomach problems. The finding of zone of inhibition was found to be higher than the results reported by other authors . 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 . Therefore 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 (Vaghasova *et al.*, 2011) . 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 (Tsao and Liu, 2007).

Cytotoxicity

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 forpinus needles leaves

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. P. Roxburghiishowed least toxicity as compared to other two *other*extracts during the bioassay. 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

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