Comparative analysis of phytochemical constituents of *Pinus roxburghii* male cones taken at different time intervals and their Anti-bacterial screening

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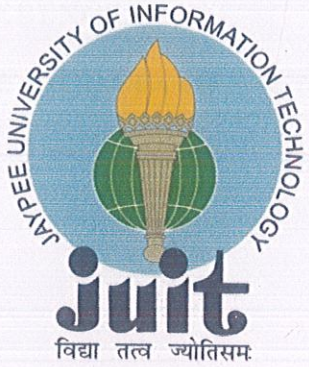

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CERTIFICATE

This is to certify that the work titled **"Comparative analysis of phytochemical constituents of Pinus roxburghii male cones taken at different time intervals and their antibacterial screnning"** submitted by "**Reetika Mahajan** and **Ishaan Verma**"in partial fulfillment for the award of degree of **B.Pharm.** of Jaypee University of Information and Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of supervisor .....

Name of supervisor ...Dr. Gokul Singh Bisht.

Designation .....

Date .....28/05/12.....

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It is with a sense of pride and pleasure when I look back to acknowledge those who have been a source of encouragement in my entire endeavour. It is my great pleasure to express my deep sense of gratitude and sincere thanks to my respected magnanimous guide **Dr. Gopal Singh Bisht**, Senior Lecturer, Department of Pharmacy, Jaypee University of Information and Technology, Waknaghat for his unparalleled and excellent guidance, profound interest, meticulous care, support, creative criticism and encouragement. He cared me more than as a guide, and I am grateful to him, whatever knowledge I have gained from him. Whenever I felt low and nervous during last days of my project work, he encouraged me with great affection. He was with me and helped till last moment of the project. Throughout the project work, he always kept my spirit high and I learnt a lot by working under him.

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SUMMARY

In the recent years, research on medicinal plants has attracted a lot of attentions globally. Large body of evidence has accumulated to demonstrate the promising potential of Medicinal Plants used in various traditional, complementary and alternate systems of treatment of human diseases. Natural products have been the single most productive source of leads for the development of drugs. Studies have shown that antibacterial screening of leaves using four solvents (petroleum ether, chloroform, ethanol and water), petroleum ether extracts exhibited the best antibacterial activity and *Pinus roxburghii* was found to be the most effective species [30]. The main objective of this project is to analyze the change in the phytoconstituents as the pine cone grows by collected them at an interval of two months each and to carry out qualitative test for the various phytoconstituents.

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Chapter - 1

Introduction

Natural products remain a prolific source for the discovery of new drugs and drug leads even from Vedic period. Recent data suggests that 80% drug molecules are natural products or natural compound inspired [1].Studies on sources of new drugs from 1981 to 2007 reveal that almost half of the drugs approved since 1994 are based on natural products .Indian natural products, particularly those from traditional medicinal plants which are reported in the classic texts like Ayurveda and Charak Samhita, have contributed towards this 'boom' in drug discovery[30].The traditional Indian system of medicines has a very long term history of usage in number of diseases and disorders.

Monoterpenes, sesquiterpenes, and their oxygenated derivatives such as alcohols, aldehydes, esters, ethers, ketones, and phenols, are the main components present in essential oils, which may be involved in its physiological and biological activities. However, their actual compositions and biological activities are quite different among the plants, even in the same species, depending on their environmental and genetic variations. All the more, the chemical and biological properties are quite different from the essential oils depending on the part of the same plant, which makes it difficult to understand systematically the effectiveness of essential oils.[2,3,4,5]

Essential oils from medicinal as well as other edible plants have been recognized as safe food flavoring agents and aromatic disinfectants with antimicrobial and antioxidizing activities. Essential oils are mixtures of volatile oils from various parts of plants, and are considered as important antimicrobial, antifungal, and insecticidal or insect-repelling agents present in plants and some have been reported to endow antioxidant, anti-inflammatory, antitumor, antiaging, antimutation, and sedative effects. [6,7]

In this project we are mainly concerned with the male cones and needles (leaves) of *Pinus roxburghii.* We collected the samples of needles and male cones for this pinus species in and around Solan. The sample for the male cones were collected in a gap of 2 months (17^{th} Aug, 17^{th} Oct, 17^{th} Dec 2011, 17^{th} Feb 2012) because the main objective is to analyze whether there is any change in the phytochemical constituents at different interval of its growth period and to carry out the phytochemical test on the different extracts of leaves and male cones.
We carried out this work because our main area of interest is in natural products. There is a "paradigm shift" in drug discovery now days, marked by the isolation of pure bioactive entities from medicinal plants and ***Pinus roxburghii*** is reported to have medicinal values as it

is used as an Antiseptic, Diuretic, Rubifacient, Vermifuge, its valuable remedy internally is used in the treatment of kidney and bladder complaints[8,9]. It is used in steam bath for treating rheumatic infections but we were concerned in its Anti-bacterial Activity which can prove to be beneficial and not much work has been carried on this species for this area. This species is available easily in this place so the samples can be collected on the required interval.

The pine needles of this species has been previously reported to have anti-bacterial activity of certain extracts and here our main focus is on screening the compound responsible for the Anti-bacterial Activity from the Pet. Ether and Methanol extract of Pine leaves as these extracts shows maximum Anti-bacterial Activity as compared to other. We also observed the Anti-bacterial Activity of different extracts, collected at different interval, for male cones on a particular bacterial culture (*E. coli*).

Chapter-2

Review of Literature

World is endowed with a rich wealth of medicinal plants and man cannot survive on this earth for long healthy life without the plant kingdom because the plant products and their active constituents play an important role in maintaining perfect health. The use of medicinal plant to treat human diseases has its roots in pre – historic times. Medicinal plants are used by 80% of the world population as the only available medicines, especially in developing countries .A wide range of medicinal plants parts is used for extract as raw drugs and they posses varied medicinal properties. The different parts include leaves, roots, stem, flowers, fruit, twigs exudates and modified plant organs. Medicinal and aromatic plants and their essence are rich in Antibacterial compounds which could be an alternate way to combat bacterial diseases even against so bacteria which are becoming resistant to certain synthetic medicines.[8]

Pinus roxburghii.*, belonging to family Pinaceae, is commonly known as Chir Pine.** The Chir Pine, *Pinus roxburghii*, named after William Roxburgh, is a pine native to the Himalaya. The range extends from northern Pakistan (North-West Frontier Province, Azad Kashmir), across northern India (Jammu and Kashmir, Punjab, Himachal Pradesh, Uttarakhand, Sikkim) and Nepal to Bhutan. It generally occurs at lower altitudes than other pines in the Himalaya, from 500-2000 m, occasionally up to 2,300 metres (7,500 ft). The other Himalayan pines are Blue Pine, Bhutan White Pine, Chinese White Pine, Chilgoza Pine and Sikang Pine. ***Pinus roxburghii is a large tree reaching 30-50 m with a trunk diameter of up to 2 m, exceptionally 3 m. The leaves are needle-like, in fascicles of three, very slender, 20-35 cm long, and distinctly yellowish green. The cones are ovoid conic, 12-24 cm long and 5-8 cm broad at the base when closed, green at first, ripening glossy chestnut-brown when 24 months old. They open slowly over the next year or so, or after being heated by a forest fire, to release the seeds, opening to 9-18 cm broad. The seeds are 8-9 mm long, with a 40 mm wing, and are wind-dispersed. Chir pine is widely planted for timber in its native area, being one of the most important trees in forestry in northern Pakistan, India and Nepal. For local building purposes, the wood of this tree is the least preferred, as it is the weakest and most prone to decay when compared with other conifers. However, in most low altitude regions, there is no other choice, except for the fact that these being tropical latitudes there are other trees at lower altitudes.

Old trees which die from fire or drought undergo some metamorphosis in their wood due to the crystallization of the resin inside the heart wood. This makes the wood become brightly colored (various shades from translucent yellow to dark red) and very aromatic with a brittle, glassy feel.

This form of wood known as *Jhukti* by the locals is very easy to ignite (It never gets wet or waterlogged). They use it for starting fires and even for lighting, as a small piece of this burn for a long time (owing to the high resin content). Of all the conifer species in the area, only this one seems to be ideal for that purpose. Every autumn, the dried needle of this tree forms a dense carpet on the forest floor, which the locals gather in large bundles to serve as bedding for their cattle, for the year round. The green needles are also used to make tiny hand brooms. The locals of the Jhaunsar region of Uttarakhand have several uses for this tree which is known in the local dialect as *Salli*. It is also occasionally used as an ornamental tree, planted in parks and gardens in hot dry areas, where its heat and drought tolerance is valued.

It is believed the pine plant originated in Austria and Russia and spread in different parts of the world. Because the pine tree can withstand both cold and relatively dry conditions, it grows easily and well in many different places. Native Americans used pine trees in several ways. They chewed the needles to ward off scurvy, and stuffed the oil-rich needles in their mattresses to discourage lice and fleas. The needles contain quite a lot of vitamin C, so it was a useful remedy for hunters if they were short of the vitamin. Hippocrates used pine to treat pulmonary conditions (lung-related medical conditions) and Pliny recommended pine oil for respiratory problems. Pine oil has antibacterial, energizing, antiseptic, and aromatic properties. It's been used as a tool in the medical armoury for thousands of years.

Medicinal Uses:-

It is also tapped commercially for resin. On distillation, the resin yields an essential oil, commonly known as turpentine, and non-volatile rosin. The proportion of rosin and turpentine oil in Chir Pine is 75% and 22% respectively with 3% losses, etc.The turpentine obtained from the resin of all pine trees act as an **antiseptic**, **diuretic**, **rubifacient** and **vermifuge**. It is a valuable remedy used internally in the treatment of kidney and bladder complaints. It is used in steam bath for the treatment of rheumatic infection.[10]

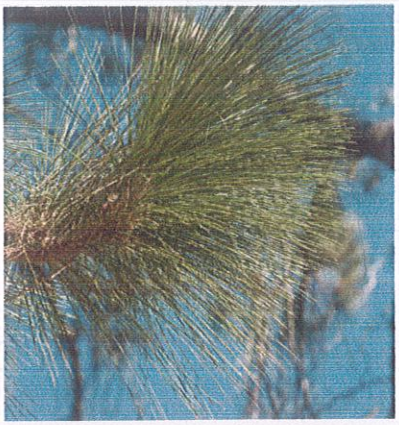

Fig.1: Pinus roxburghii Needles

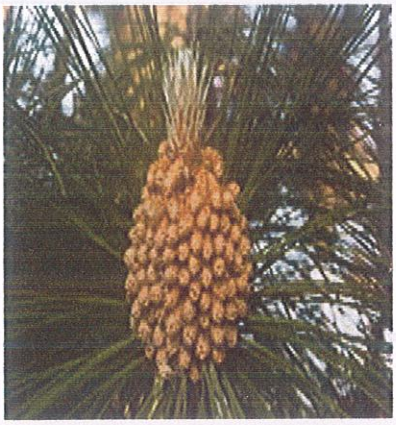

Fig.2: Male Cones (Matured)

Source – www.wikipedia.com

The turpentine is chiefly used as a solvent in pharmaceutical preparations, perfume industry, in manufacture of synthetic pine oil, disinfectants, insecticides and denaturants. It is one of the most important basic raw materials for the synthesis of terpene chemicals which are used in a wide variety of industries such as adhesives, paper and rubber, etc. Chir Pine rosin is principally used in paper, soap, cosmetics, paint, varnish, rubber and polish industries. Esides these, other uses include manufacture of linoleum, explosives, insecticides and disinfectants, as a flux in soldering, in brewing and in mineral beneficiation as a frothing agent.

In the present study, phytochemical analysis of male cones of *Pinus roxburghii* will be carried out at time intervals (2 months) and will be analyzed the change in phytochemical constituents with cone growth. *Pinus roxburghii* needles of the same tree will be studied for isolation and screening of the bioactive compounds.

The most common commercially available pine bark extract is Pycnogenol, a 14avender14zed extract of *Pinus 14avender*, which has been reported to have cardiovascular benefits and enhance microcirculation. The study conducted by Ozlem Yesil-Celiktas et al in 2008 was conducted to determine the chemical composition of four pine bark extracts, assess their biological activities and to compare the results with Pycnogenol. The *Pinus* species were analysed by LC and LC-MS; extracts of *P. brutia* and *P. nigra* showed higher levels of phenolic constituents compared to *P. sylvestris* and *P. pinea*. In particular, *P. brutia*

contained extremely high concentrations of taxifolin (18.5%). The highest radical scavenging activities were attained with *P.pinea* (88.6%), *P. nigra* (87.2%) and *P. brutia* (86.4%) bark extracts. Additionally, anticarcinogenic effects of the extracts and their kinetics were determined in four cell lines including human prostate (PC-3, DU 145, LNCaP) and breast adenocarcinoma (MCF7) by the MTT assay. Cell viability was reduced to 40% by extracts of *P. pinea,* and *P. sylvestris* in PC-3 cells showing a similar effect like the positive control, CPT-11. *Pinus* species other than *P. 15avender* definitively possess high biological activities, and therefore present a huge potential to be 15avender in the food and the pharmaceutical industries.[11]

Other uses of pine-

- **Skin Care:** The most widely known use of pine essential oil is in treating skin problems. Dermatologists often prescribe the oil in treating the following conditions: psoriasis, itching, eczema, scabies, sores, and fleas.[1,11]
- **Cosmetics:** Pine essential oil has a distinctive essence and gives a sweet aroma to the cosmetics it is used in. Pine plant oil is widely used as massage oil and in perfumes and other scents.
- **Stress Relief:** The health benefits of pine oil include some emotional benefits. It gives an energizing, invigorating feeling. Pine plant oil is also useful for people suffering from loss of concentration and loss of memory. Regular massage with pine essential oil lends mental clarity.[11]
- **Injuries:** Pine essential oil is antiseptic and can be used in treating cuts, sores, and fungal infections.[11]
- **Aromatherapy:** Oil from pine trees is one of the most important essential oils used in aromatherapy. Pine essential oil blends well with cedarwood, rosemary, 15avender, sage, and juniper oils. The "clean" smell means that people having an aromatherapy massage find pine oil invigorating and cleansing.[11]

Pine resin is a natural resin obtained from coniferous trees and is commercially produced from *Pinus massoniana*, *Pinus tabulaeformi* and other species of Pinus in many countries, and has countless applications at home and at work. Traditionally, pine resin has been used to treat inflammation, to relieve cough symptoms, and to alleviate pain. In Chinese medicine, pine resin is used for the treatment of skin diseases, burn and scald wounds, trachitis, pulmonary tuberculosis and as a good antiseptic (Hu et al., 1998).Modern studies have

indicated that pine resin mainly consists of the chemical constituents of abietane and pimarane diterpene acids, which were reported to have many biological and pharmacological functions, including antitumor, anti-inflammatory, pesticidal, and antibacterial properties, as well as lowering cholesterol and inhibiting ATPase activities. Different solvent extracts of P. massoniana resin were actively screened, and its petroleum ether extract showed moderate cytotoxicities against A431 and A549 cells. So the petroleum ether extract was chemically investigated, and a series of diterpenoids, including abietane, pimarane and labdane diterpenes and podocarpane norditerpenes, were isolated. In this work, we deal with isolation, structural elucidation and cytotoxicity against human epithelial carcinoma A431 and human lung cancer A549 cells of our new diterpenes, one new norditerpene, 12 known diterpenes and two known norditerpenes from P. massoniana resin.[12]

Pine oil contains phenols, which are acidic chemicals that have germ-killing properties. Pine oil disinfectants are effective against yeast spores, E.coli, and other household germs. Mildew and mould cannot withstand the chemicals in pine oil.[8] Studies have shown that antibacterial screening of four solvents (petroleum eher, chloroform, ethanol and water), petroleum ether extracts exhibited the best antibacterial activity and pinus roxburghi was found to be the most effective species [13].

Chapter-3

Material and Methods

Materials:-

Plant material was collected in and around Solan (H.P) Chemicals pet. Ether (LOBA), Ethyl acetate (LOBA), Hexane (LOBA), methanol (LOBA), Ethanol (LOBA), Acetic acid (MERCK), carbon tetrachloride (LOBA), α-Napthol (LOBA), ferric chloride (SRL.CHEM), Lead Ethinoite (MERCK), Fehlings solution (FISCHER'S SCIENTIFIC), Benedicts Reagent (MERCK), Sodium Hydroxide (MERCK), Chloroform (LOBA), nutrient media (HIMEDIA), Sulphuric acid (LOBA), Hydrochloric acid (LOBA)

EQUIPMENTS

Rota evaporator (EQUITRON, HEIDOLPH), Lypholizer (ALIED FROST), Autoclave(EQUITRON), Laminer Air Flow Chamber(S.M INERNATIONAL), Heating Mantle(JAIN SCIENTIFIC), Soxhlet Apparatus(JSGW), Incubator (ORBITEK), Oven (MACFLOW ENGINEERING), HPLC(waters 2424)

METHODS:-

Collection of plant material leaves **and** male cones of *Pinus roxburghii* **were** collected on from in and around Solan. Collected plant material was dried for 20 days in the Laboratory.

Extracts Preparation

Dried needles (27g) and crushed male cones (27g) was packed and extracted in a soxhlet apparatus using 300 ml of different solvents (Ethyl acetate, petroleum ether, methanol and water). After soxhlet extraction solvent was concentrated in Rota Evaporator till dryness. The methanol solvent was evaporated and residue obtained was weighed.. For bulk extraction of needles, 120g of powdered needles were macerated in 2L of methanol solvent in 5L of conical flask and filtered. The compounds obtained were subjected to various phytochemical tests.

Phytochemical Tests:

Th[illegible]st for Carbohydrates (Molisch Test)

Few drops of α-napthol was added to each portion dissolved in distilled water, this was then followed by the addition of 1 ml of conc. H_2SO_4 by the side of the test tube. The mixture was then allowed to stand for 2 min. Formation of a red or dull color at the interphase of the two layers was a posi[illegible]e test.

***The test for Tannins*:**

About 0.01g of each extract was stirred with 10 ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2ml of the filtrate occurrence of blue-black, green or blue –green precipitate indicates the presence of tannins.

The test for Terpenoids (Liebermann-Burchard test):

To 0.02g of each, 2ml of acetic acid was added; the solution was cooled well in ice followed by the addition of conc. H_2SO_4 carefully. Color development from violet to blue or bluish-green indicated the presence of terpenoids.

The test for Saponins:

Some portion of each extract was boiled with 5ml of distilled water, filtered. To the filtrate, about 3ml of distilled water was further added and shaken vigorously for 5 min. Frothing which persisted on warming was taken as an may be the presence of saponins.

***The test for Flavonoids*:**

Few quantity of the each portion was dissolved in water and filtered. To 5ml of each of the filtrate, 3ml of Lead ethanoate solution was then added. Appearance of buff-colored ppt indicates the presence of flavonoids.

The test for Alkaloids:

Few quantity of the each portion was stirred with 5ml of 1% aq. HCl on water bath and then filtered. Of the filtrate, 1ml was taken individually into 2 test tubes. To 1ml, **Mayer's Reagent** was added and appearance of buff-colored ppt will be an indication for the presence of alkaloids.

Thin Layer Chromatography of Methanolic extract:

The Thin Layer Chromatography was carried out for the different extract in various solvent system-

- Carbon tetrachloride : methane (9:1)
- Chloroform : methane (9:1)
- Chloroform : ethyl acetate (9:1)
- Hexane : ethyl acetate (8:2)
- Hexane : ethyl acetate (7:3)
- Butanol :acetic acid :water (3:2:1)

Column Chromatography: The column was first filled with dry stationary phase powder (silica), followed by the addition of mobile phase, which is flushed through the column until it is completely wet, and from this point is never allowed to run dry. Care must be taken to avoid air bubbles . The individual components are retained by the stationary phase differently and separate from each other while they are running at different speeds through the column with the eluent as shown in Fig.3. At the end of the column they elute one at a time. During the entire chromatography process the eluent is collected in a series of fractions [14] as shown in Fig:4 respectively.

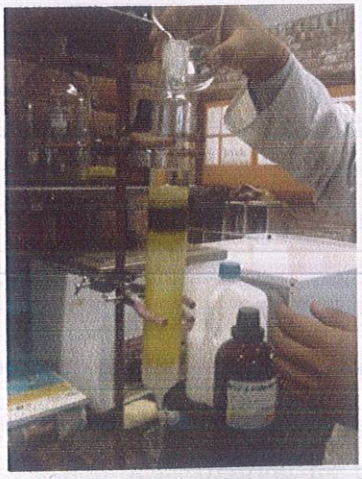

Fig.3: Column Chromatography

Fig.4: Different Fractions Collected in test tubes.

After, the TLC was observed for various fractions as shown in Fig:5. The solvent was evaporated from the isolated fractions which were containing the compound.

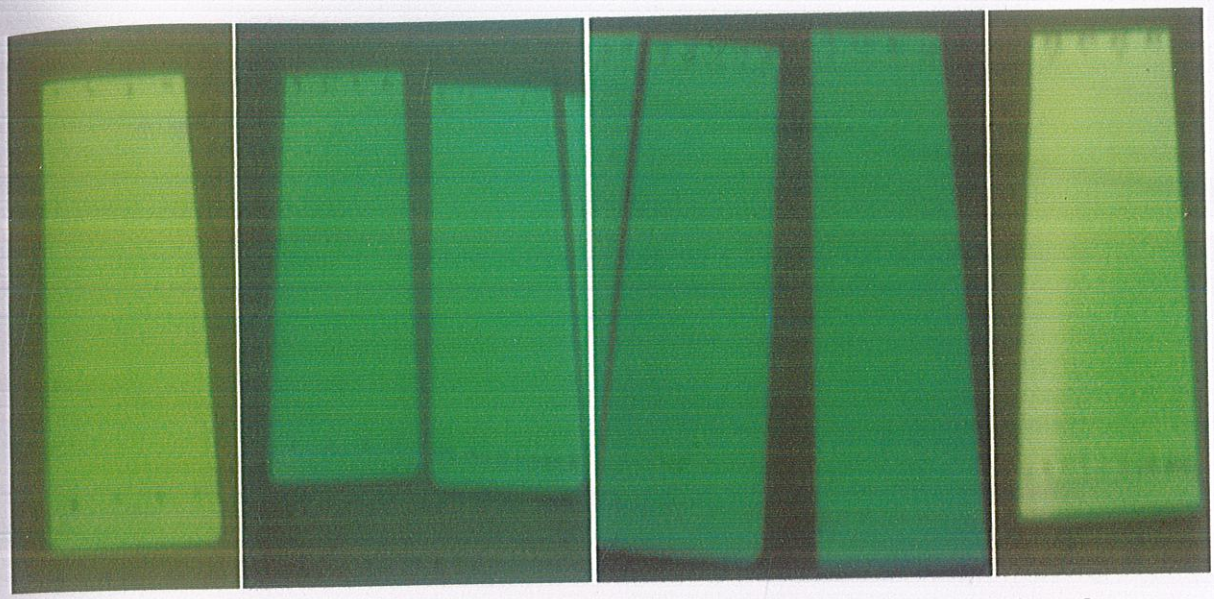

Fig.5: Thin Layer Chromatography of different Fractions collected.

Lyophilization:-

Freeze-drying is a dehydration process typically used to preserve a perishable material or make the material more convenient for transport. Freeze-drying works by freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublimate directly from the solid phase to the gas phase.[14] The purpose of freeze-drying is to remove a solvent (usually water) from dissolved or dispersed solids. Freeze-drying is method for preserving materials, which are unstable in solution. In addition, freeze-drying can be used to separate and recover volatile substances, and to purify materials. The fundamental process steps are:

1. Freezing: The product is frozen. This provides a necessary condition for low temperature drying.
2. Vacuum: After freezing, the product is placed under vacuum. This enables the frozen solvent in the product to vaporize without passing through the liquid phase, a process known as sublimation.
3. Heat: Heat is applied to the frozen product to accelerate sublimation.

4. Condensation: Low-temperature condenser plates remove the vaporized solvent from the vacuum chamber by converting it back to a solid. This completes the seperation process.

Autoclaving:-

An **autoclave** is an instrument used to sterilize equipment and supplies by subjecting them to high pressure saturated steam at 121 °C for around 15–20 minutes depending on the size of the load and the contents. Autoclaves are widely used in microbiology, medicine, veterinary science etc. They vary in size and function depending on the media to be sterilized. Once a medium is sterilized, it will remain sterile until contaminated by microorganisms (i.e., they do not appear by spontaneous generation.).

Heating is one of the most convenient methods for sterilization. Simple boiling at 100°C, however, is not completely effective for sterilization because many spores can survive this temperature. However, the boiling point of water goes up when exposed to increased pressures. In a pressure cooker where the pressure is 15 pounds/sq inch (1 atmosphere) above standard pressure (760 mm Hg), water boils at 121°C. This temperature kills all life forms in 15 minutes or less. These conditions have become the standard for sterilizing apparatus or small volumes of liquid: i.e., treat over boiling water at 15 pounds pressure for 15 minutes (commonly called "15 pounds for 15 minutes"). An autoclave is a piece of laboratory apparatus which acts as an automated pressure cooker. The conditions inside are thermostatically controlled so that heat is applied until 121C is achieved, at which time the timer starts, and the temperature is maintained for the selected time.

Laminar Air Flow Chamber:-

A **laminar flow cabinet** or **laminar flow closet** or **tissue culture hood** is a carefully enclosed bench designed to prevent contamination of semiconductor wafers, biological samples, or any particle sensitive device. Air is drawn through a HEPA filter and blown in a very smooth, laminar flow towards the user. The cabinet is usually made of stainless steel with no gaps or joints where spores might collect.Such hoods exist in both horizontal and vertical configurations, and there are many different types of cabinets with a variety of airflow patterns and acceptable uses.

Laminar flow cabinets may have a UV-C germicidal lamp to sterilize the shell and contents when not in use. (It is important to switch this light off during use, as it will quickly give any exposed skin sunburn and may cause cataracts).

Rotatory Evaporater:-

Rotatory evaporators are distillation units that incorporate an efficient condenser with a rotator flask system. As the flask containing the solvent is rotated it continually transfers a thin layer of liquid over the entire surface. This gives a very large surface area for evaporation that is effected by heating from the accessory waterbath. They are the ideal tools for many everyday laboratory applications including:-

- Concentration of solutions
- Reclamation of solvents
- Vacuum drying of wet solids
- Degassing liquids

The rotating system is fitted with a special seal that allows the apparatus to be placed under vacuum. This reduces the boiling point of the solvents and removes the vapour phase making the process much more efficient. Each unit is also provided with an easy to use vacuum release and a continuous feed system, which allows more solvent to be drawn into the rotating Florentine flask without the need to stop the operation.

Fig.6: Rota – Evaporator

Source- www.wikipedia.com

Vacuum evaporators as a class function because lowering the pressure above a bulk liquid lowers the boiling point of the component liquids in it. Generally, the component liquids of interest in applications of rotary evaporation are research solvents that one desires to remove from a sample after an extraction, for instance, following a natural product isolation or a step in an organic synthesis. Use of a "rotavap" therefore allows liquid solvents to be removed without excessive heating of what are often complex and sensitive solvent-solute combinations. Rotary evaporation is most often and conveniently applied to separate "low boiling" solvents such a n-hexane or ethyl acetate from compounds which are solid at room temperature and pressure. However, careful application also allows removal of a solvent from a sample containing a liquid compound if there is minimal co-evaporation (azeotropic behavior), and a sufficient difference in boiling points at the chosen temperature and reduced pressure.

The main components of a rotary evaporator are:

1. A motor unit that rotates the evaporation flask or vial containing the user's sample.
2. A vapor duct that is the axis for sample rotation, and is a vacuum-tight conduit for the vapor being drawn off of the sample.
3. A vacuum system, to substantially reduce the pressure within the evaporator system.
4. A heated fluid bath (generally water) to heat the sample.
5. A condenser with either a coil passing coolant, or a "cold finger" into which coolant mixtures such as dry ice and acetone are placed.
6. A condensate-collecting flask at the bottom of the condenser, to catch the distilling solvent after it re-condenses.
7. A mechanical or motorized mechanism to quickly lift the evaporation flask from the heating bath.

High-Performance Liquid Chromatography:- (sometimes referred to as **High-Pressure Liquid Chromatography**)

HPLC, is a chromatographic technique used to separate a mixture of compounds in analytical chemistry and biochemistry with the purpose of identifying, quantifying and purifying the individual components of the mixture. HPLC typically utilizes different types of stationary phases (i.e. sorbents) contained in columns, a pump that moves the mobile phase and sample components through the column, and a detector capable of providing characteristic retention times for the sample components and area counts reflecting the amount of each analyte passing through the detector.[14]

The detector may also provide additional information related to the analyte, (i.e. UV/Vis spectroscopic data, if so equipped). Analyte retention time varies depending on the strength of its interactions with the stationary phase, the composition and flow rate of mobile phase used, and on the column dimensions. HPLC is a form of liquid chromatography that utilizes small size columns (typically 250 mm or shorter and 4.6 mm i.d. or smaller; packed with smaller particles), and higher mobile phase pressures compared to ordinary liquid chromatography.

High performance liquid chromatography is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it

is forced through under high pressures of up to 400 atmospheres. That makes it much faster. It also allows you to use a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture.

The other major improvement over column chromatography concerns the detection methods which can be used. These methods are highly automated and extremely sensitive.[14]

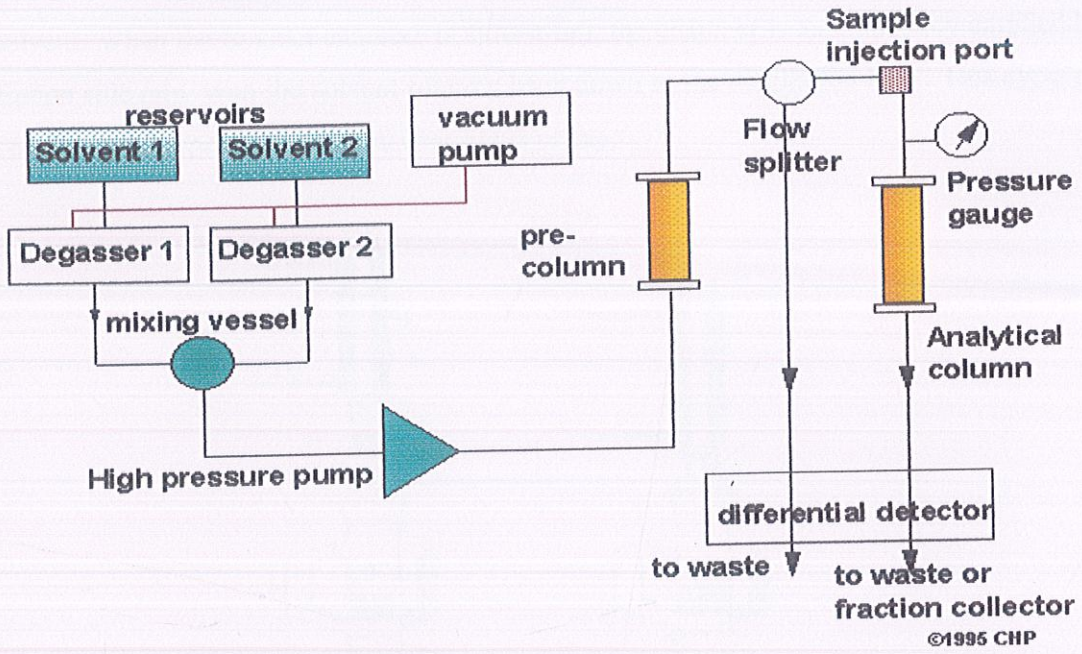

Fig.7: Outline of HPLC

Source - www.files.chem.vt.edu

Soxhlet Apparatus:-

A Soxhlet extractor is not limited to the extraction of lipids. Typically, a Soxhlet extraction is only required where the desired compound has a *limited* solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a significant solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance.Fruit extraction in progress. The sample is placed in the thimble. [8]

Normally a solid material containing some of the desired compound is placed inside a thimble made from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor is placed onto a flask containing the extraction solvent. The Soxhlet is then equipped with a condenser.The solvent is heated to reflux.

The solvent vapour travels up a distillation arm, and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the solid material.The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle may be allowed to repeat many times, over hours or days.

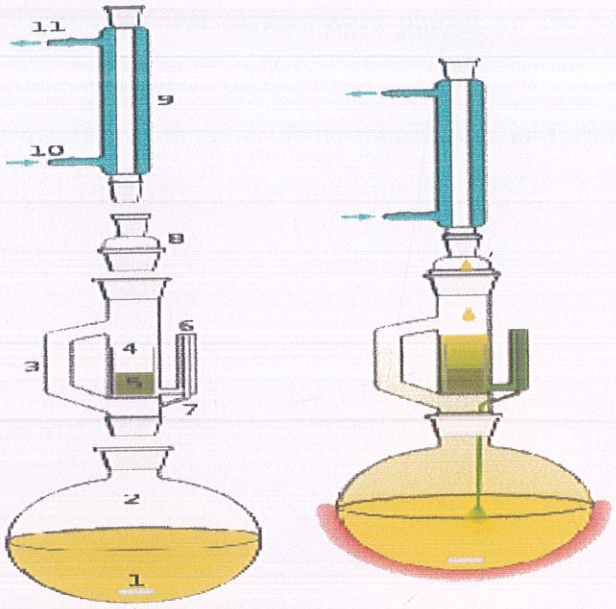

Fig.8: Soxhlet Apparatus Source- www.wikipedia.com

During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled.

After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound. The non-soluble portion of the extracted solid remains in the thimble, and is usually discarded.

Antimicrobial susceptibility test:-

All the extracts were screened against bacterial strains. the organism was *Escherichia coli* (MTCC-0723). The disk diffusion method was used to test the Anti-bacterial activity of the plant extracts. Sterlised nutrient agar medium (20ml) were poured into the sterlised petridish. The plates were allowed to solidify for 5 min and ----µl of bacterial suspension was swabbed uniformly. The entire agar surface of each plate was inoculated with this swab, first in the horizontal direction and then in the vertical direction, which ensure the even distribution of organism over the agar surface. The filter paper disc (0.4cms in radius) loaded with the desired concentration of wet extract were placed on the surface of the bacterial seeded agar plates and the compound was allowed to diffuse for 5 min and then the plates were incubated at 37°c for 24h. at the end of the incubation, inhibition zone formed around the disc were measured with transparent ruler in centimeters. [8]

Chapter- 4

Results and Discussions

Extract Preparation and their Phytochemical Test:-

Dried needles (27g) were packed and extracted in a soxhlet apparatus using 300ml of different solvents, moving from Pet. Ether(non – polar) to Water (polar) as shown in Table 1. The extractive values are different for different solvents and the extractive values of non-polar and polar extract is somewhat similar as shown in Table 1.

Table 1:-Soxhlet extraction of leaves of *Pinus Roxburghii (collected on 8th August)* in different solvents.

Solvent	Weight of dried leaves (g)	Vol. of Solvent (ml)	Duration of 1st cycle (min.)	No. of cycles	Extractive values (w/w)%
Petroleum ether	27	300	21	28	3.37
Ethyl Acetate	27	300	28	28	3.03
Methanol	27	300	23	28	3.0
Water	27	300	45	28	3.55

After the extracts were obtained the phytochemical test were carried out on different extracts like Pet. Ether, Ethyl Acetate, Methanol and Water extracts gives positive result for Saponins , while Pet. Ether and Ethyl Acetate extract gives positive results for Terpinoids whereas Methanol and Water extract gives positive results for Tannins. Only Water extract gives positive result for Carbohydrates as shown in Table 2.

Table 2 :- Different phytochemical test done on the Extracts of leaves collected on 8th August

Extract	Test for Carbohydra	Test for Alkaloid	Test for Saponins	Test for Tannins	Test for Flavanoids	Test for Terpinoid
Pet. ether	Negative	Negative	Positive	Negative	Negative	Positive
Ethyl Acetate	Negative	Negative	Positive	Negative	Negative	Positive
Methanol	Negative	Negative	Positive	Positive	Negative	Negative
Water	Positive	Negative	Positive	Positive	Negative	Negative

Crushed male cones (27g) were packed and extracted in a soxhlet apparatus using 300ml of different solvent, moving from Pet. Ether (non – polar) to Water (polar) as solvents for extraction. From the Table.3 we can say that the non – polar compounds in the Male cones , collected on 17th Aug. , is in more quantity as compared to polar compounds. So we can conclude from Table.3 that in the initial stage of the growth of Male cones the quantity of non – polar compounds are more

Table 3:- Soxhlet extraction of Male Cones of *Pinus Roxburghii* in different solvents collected on 17th August, 2011.

Solvent	Weight of dried cones (g)	Vol. of Solvent (ml)	Duration of 1st cycle (min.)	No. of cycles	Extractive value (w/w)%
Petroleum ether	27	300	22	28	10.7
Ethyl Acetate	27	300	26	28	8.9
Methanol	27	300	29	28	5.4
Water	27	300	38	28	4.8

Crushed male cones (27g) were packed and extracted in a soxhlet apparatus using 300ml of different solvent, moving from Pet. Ether (non – polar) to Water (polar) as solvents for extraction. From the Table.4 we can say that the non – polar compounds in the Male cones , collected on 17th Oct. is in more quantity as compared to polar compounds but the extractive values decreases as compared to the previous extraction of Male cones (Table.1). So we can conclude that in the 2nd stage of the growth of Male cones the quantity of non – polar compounds remains more in comparision but extractive values decreases.

Table 4:- Soxhlet extraction of Male Cones of *Pinus Roxburghii* in different solvents collected on 17th October, 2011.

Solvent	Weight of dried cones (g)	Vol. of Solvent (ml)	Duration of 1st cycle (min.)	No. of cycles	Extractive value (w/w)%
Petroleum ether	27	300	22	28	4.40
Ethyl Acetate	27	300	26	28	3.90
Methanol	27	300	29	28	3.62
Water	27	300	38	28	2.45

From the Table.5 we can say that the extractive value for polar compounds in the Male cones , collected on 17th Dec. increases as compared to non - polar compounds. So we can conclude from Table.5 that in the 3rd stage of the growth of Male cones the quantity of polar compounds increases as compared to the previous data.

Table 5:- Soxhlet extraction of Male Cones of *Pinus Roxburghii* in different solvents collected on 17th December, 2011.

Solvent	Weight of dried cones (g)	Vol. of Solvent (ml)	Duration of 1st cycle (min.)	No. of cycles	Extractive value (w/w)%
Petroleum ether	27	300	22	28	7.11
Ethyl Acetate	27	300	26	28	3.02
Methanol	27	300	29	28	8.0
Water	27	300	38	28	4.5

From Table.6 we can say that the extractive values of polar and non – polar compounds in the Male cones , collected on 17th Feb. , are similar. So we can conclude from Table.6 that in the Final stage of the growth of Male cones the quantity of polar and non - polar compounds is somewhat similar.

Table 6:- Soxhlet extraction of Male Cones of *Pinus Roxburghii* in different solvents collected on 17th Feburary, 2012

Solvent	Weight of dried cones (g)	Vol. of Solvent (ml)	Duration of 1st cycle (min.)	No. of cycles	Extractive value (w/w)%
Petroleum ether	27	300	22	28	6.2
Ethyl Acetate	27	300	26	28	6.0
Methanol	27	300	29	28	8.1
Water	27	300	38	28	3.2

We carried out the phytochemical test on the different extracts of the Male cones and the thing we observed that all the Male cones collected on different interval gave similar phytochemical results. So we can conclude that there is not much change in the phytochemical constituents of Male cones at different stages of its growth. The only change we observed is in the morphology and in the extractive values as the growth progresses.

Table 7: - Different phytochemical tests done on the extracts of Males cones collected on 17th August 2011,October 2011,December 2011 and Feburary 2012.

Extract	Test for Carbohydrate	Test for Alkaloid	Test for Saponins	Test for Tannins	Test for Flavanoids	Test for Terpinoids
Pet. ether	Negative	Negative	Positive	Negative	Negative	Positive
Ethyl Acetate	Negative	Negative	Positive	Negative	Negative	Positive
Methanol	Negative	Negative	Positive	Positive	Negative	Negative
Water	Negative	Negative	Positive	Positive	Negative	Negative

In comparison of the extractive values for different solvent ,for the Male cones collected on different time interval (17th Aug, Oct, Dec. 2011, 17th Feb. 2012) , in observation of the above data we can conclude that with growth of Male cones the quantity of non – polar compound in the initial stage is increases and in the later stages the quantity of polar compound increases somewhat.

Table 8 :- Comparison of the Extractive values for different extracts of Male cone collected at different intervals

Solvent	Extractive values(w/w)% of Cones (collected on 17th Aug,11)	Extractive values(w/w)% of Cones(collected on 17th Oct,11)	Extractive values(w/w)% of Cones(collected on 17th Dec,11)	Extractive values(w/w)% of Cones(collected on 17th Feb,12)
Pet. Ether	10.7	4.40	7.11	6.2
Ethyl Acetate	8.9	3.90	3.02	6.0
Methanol	5.4	3.62	8.0	8.2
Water	4.8	2.75	4.5	3.1

ZONE OF INHIBITIONS OF CONES COLLECTED

Radius of the disk =0.2cm

No Zone of inhibition was observed in Hexane and Water when they were used blank. A clear zone of inhibition was observed in Ethyl Acetate and Methanol indicating the Anti - bacterial Activity of these extracts of Male cones as shown in Table 9.

Table 9:- The comparison of the Anti – bacterial Activity for different extracts of Male cones.

Date on which cones were collected	Zone of inhibition Hexane(cm)	Zone of inhibition Ethyl Acetate (cm)	Zone of inhibition Methanol (cm)	Zone of inhibition water (cm)
17.8.11	—	0.9	0.3	—
17.10.11	0.7	0.4	0.5	—
17.11.11	—	0.7	0.7	—
17.02.11	—	0.8	0.6	—

Anti-bacterial Activity of the Ethyl Acetate extract of Male cones collected at different time intervals.

After, the comparison of the Anti-bacterial Activity of different extracts we found that Ethyl Acetate and Methanol extracts for different intervals of Male cones, gave zone of inhibition. Then we observed the Anti – bacterial Activity of Ethyl Acetate and Methanol extracts ,of Male Cone collected at different intervals, for the concentration as 1mg/ml, 5mg/ml, 10mg/ml, as shown below.

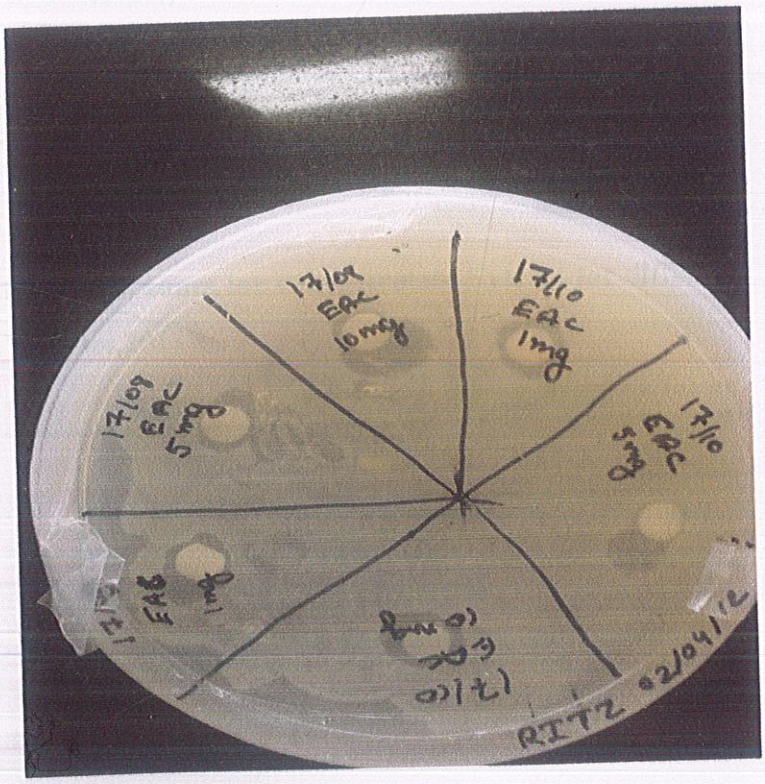

Fig.9: The Inhibition zone for the Ethyl Acetate extracts(17th Aug,Oct. 2011) for different concentrations.

Table 10:- The inhibition zones for Ethyl Acetate Extract for different concentrations(17th Aug,2011).

Dated	Conct (mg/ml)	extract	Zone of inhibition(cm)
17-08-11	1mg/ml	ethylacetate	0.6
17-08-11	5mg/ml	ethylacetate	0.7
17-08-11	10mg/ml	ethylacetate	0.9

The Anti-bacterial Activity increases as we increased the concentration so we can conclude from Table 10. that the activity is concentration dependent that is as the concentration increases the activity increases.

Table 11:- The inhibition zones for Ethyl Acetate Extract for different concentrations(17th Oct,2011).

Dated	Conc (mg/ml)	extract	Zone of inhibition (cm)
17.10.11	1mg/ml	ethylacetate	0.6
17.10.11	5mg/ml	ethylacetate	0.9
17.10.11	10mg/ml	Ethylacetate	0.6

The Anti-bacterial Activity should increase as we increased the concentration but we found that for the Male colonies dated 17th Oct.2011 that upto 5mg/ml it increased but as we moved to 10mg/ml it decreased as shown in Table 11.

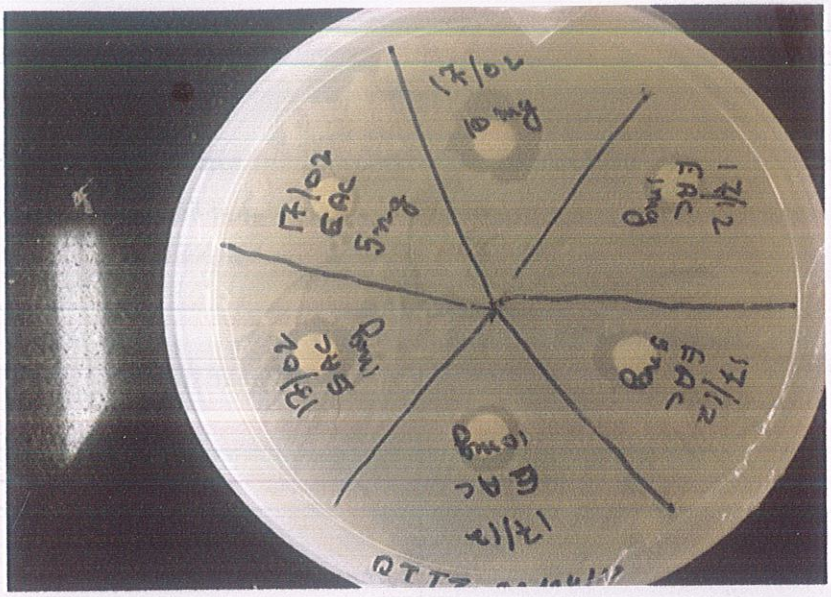

Fig.10: The Inhibition zone for the Ethyl Acetate extracts(17th Dec. 2011, Feb.2012) for different concentrations.

Table 12:- The inhibition zones for Ethyl Acetate Extract for differentconcentrations(17th Dec,2011).

Dated	Conc. (mg/ml)	Extract	Zone of inhibitions (cm)
17.12.11	1mg/ml	ethylacetate	0.5
17.12.11	5mg/ml	ethylacetate	0.6
17.12.11	10mg/ml	ethylacetate	0.5

The Anti-bacterial Activity should increase as we increased the concentration but we found that for the Male cones dated 17th Dec. 2011. that upto 5mg/ml it increased but as we moved to 10mg/ml it decreased as shown Table 12.

Table 13:- The inhibition zones for Ethyl Acetate Extract for differentconcentrations(17th Feb,2012).

Dated	Conc.(mg/ml)	Extract	Zone of Inhibition (cm)
17.2.12	1mg/ml	ethylacetate	0.7
17.2.12	5mg/ml	ethylacetate	0.5
17.2.12	10mg/ml	ethylacetate	0.8

The Anti-bacterial Activity should increase as we increased the concentration but we found that for the Male cones dated 17th Feb.2012. that as the conc. increases from 1mg/ml to 5mg/ml the Activity decreases but moving further to 10mg/ml the Activity increases.

Anti-bacterial Activity of Methanolic Extract of Male cones collected at different intervals.

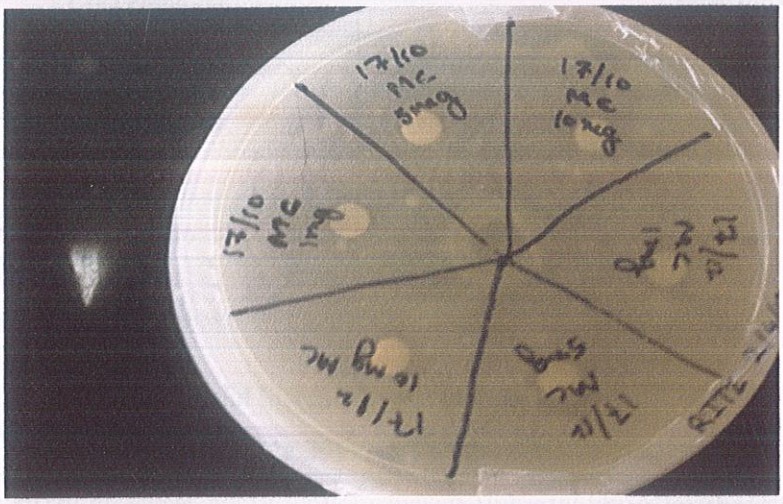

Fig.11: The Inhibition zone for Methanolic extracts(17th Oct, Dec 2011) for different concentrations.

Table 14:- The inhibition zones for Methanolic Extract for different concentrations(17thOct,2011).

Dated	Concentration(mg/ml)	Extract	Zone of inhibition(cms)
17.10.11	1mg/ml	methanol	0.5
17.10.11	5mg/ml	methanol	—
17.10.11	10mg/ml	methanol	0.4

The Anti-bacterial Activity should increase as we increased the concentration but we found that for the Male cones dated 17th Oct.2011. that as the conc. increases from 1mg/ml to 5mg/ml the Activity decreases but moving further to 10mg/ml the Activity increases somehow but not more then the inhibition zone of of 1mg/ml as shown in Table 14.

Table 15:- The inhibition zones for Methanolic Extract for different concentrations(17th Dec,2011).

Dated	Concentration(mg/ml)	Extract	Zone of inhibition(cms)
17.12.11	1mg/ml	methanolic	0.5
17.12.11	5mg/ml	methanolic	0.8
17.12.11	10mg/ml	methanolic	0.6

The Anti-bacterial Activity should increase as we increased the concentration but we found that for the Male cones dated 17th Dec.2011. that as the conc. increases from 1mg/ml to 5mg/ml the Activity increases but moving further to 10mg/ml the Activity decreases as shown in Table 15.

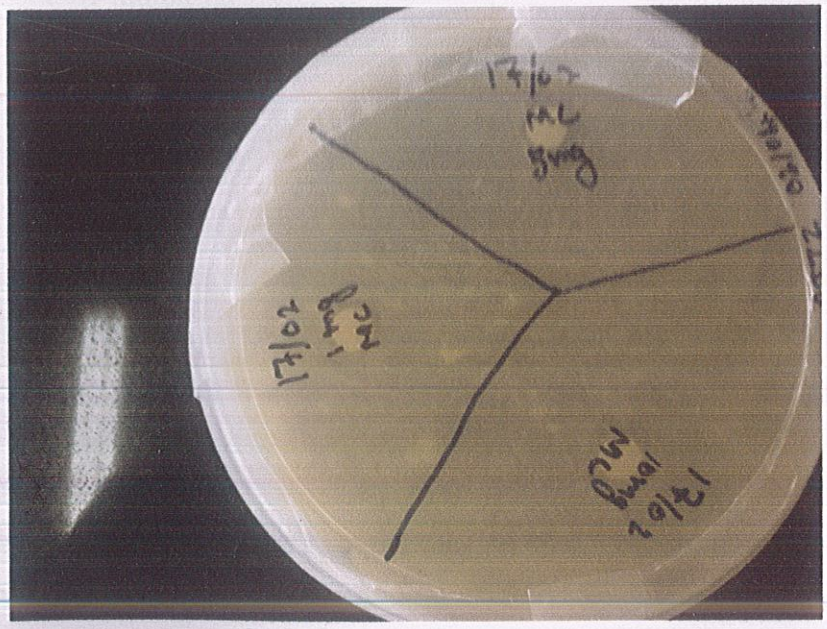

Fig.12: The Inhibition zone for Methanolic extracts(17th Feb, 2012) for different concentrations.

Table 16:- The inhibition zones for Methanolic Extract for different concentrations(17th Feb,2011).

Dated	Concentration(mg/ml)	extract	Zone of inhibition
17.2.12	1	methanolic	0.5
17.2.12	5	methanolic	0.5
17.2.12	10	methanolic	0.6

The zone of inhibition should increase as we increased the concentration but we found that for the Male cones dated 17th Dec.2011., with increase in concentration from 1mg/ml to 5mg/ml the the zone of inhibtion does not increases it remain the same but further moving to 10mg/ml the zone of inhibition increases a bit. So, there is a steady increase as shown in Table 16.

CONCLUSIONS & FUTURE WORK

The Soxhlet extraction of pine needles were carried out for different solvents (Pet. Ether, Ethyl Acetate, Methanol and Water) the non-polar component dominated the polar components, after the extracts were obtained the phytochemical test were carried out on different extracts. Pet. Ether, Ethyl Acetate, Methanol and Water extracts gives positive result for Saponins while Pet. Ether and Ethyl Acetate extract gives negative results for Tannins whereas Methanol and Water extract gives positive results for Tannins. Only Water extract gives positive result for Carbohydrates.

Then the Soxhlet extraction were carried on for the male cones collected at different time intervals, for different solvents (Pet. Ether, Ethyl Acetate, Methanol and Water). The extractive values were obtained and it was found that in the initial stage of the growth of the male cones the non-polar content is more and as it matures to the final stage the polar content increases as non-polar content decreases (Table.8) and also phytochemical test for the male cones collected at different interval of its growth, the different dated extracts give similar test.

The Anti-bacterial Activity of the male cones for different extract was obtained and it was found that different extracts of Ethyl Acetate and Methanol for different time interval give better Activity results as compared to other extracts. So, in order to observe the potency of the Ethyl Acetate and Methanol extracts for different dated extract of male cones, the Anti.bacterial Activity for different concentrations were observed and the Activity is concentration dependent in some of the cases.

In the future we will like to isolate the compound from the Ethyl acetate and Methanol extract of Male cones which is responsible for the Anti-bacterial Activity.

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