Isolation and Identification of Some Antimicrobial, Insecticidal and Antidiabetic Phytochemicals from the Medicinal Plants

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DECLARATION

I, Aditi Gupta, student of Ph. D (Regular) under the Department of Chemistry of Lovely Professional University, Punjab, hereby declare that the thesis entitled "**Isolation and Identification of Some Antimicrobial, Insecticidal and Antidiabetic Phytochemicals from the Medicinal Plants"** has been prepared by me under the guidance of Dr. Monika Gupta, Assistant Professor of Department of Chemistry, Lovely Professional University. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Aditi Gupta has prepared her thesis entitled, **"Isolation and Identification of Some Antimicrobial, Insecticidal and Antidiabetic Phytochemicals from the Medicinal Plants"** for the award of Ph. D degree of the Lovely Professional University under my guidance. She has carried out the work at the Department of Chemistry, Lovely Professional University.

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ABSTRACT

Isolation and Identification of Some Antimicrobial, Insecticidal and Antidiabetic Phytochemicals from the Medicinal Plants

Plants are the elixir of life. Throughout the ages, humans have relied on nature for their basic needs - food, shelter, clothing, fertilizers and medicines. There are ample evidences from the past such as written documents, preserved monuments and original plant medicines that shows the connection between man and his search for drugs. Rigveda, the most previous book, provides enormous information on this matter. Awareness of medicinal plants usage is a result of the many years of struggles against illnesses due to which man learned to pursue drug in bark, seed, fruit bodies and other parts of the plants. The Tang Herbals and The Chinese Materia Medica are the documented records of the use of natural products.

Turmeric has been used in Indian Ayurveda as medicine as early as 1900 B.C. Many other herbs and minerals used in Ayurveda were later described by ancient Indian herbalists such as Charaka and Sushruta during the first millennium. 64 preparations from mineral sources and 57 preparations based on animal sources are reported in „*Sushruta Samhita'*

Ayurveda has been derived from the Indian words, "Ayar" stands for life and "Vedas" for knowledge. Treatment of plants for various human ailments has been described in Ayurveda and other Indian literature. Phytotherapists, the modern practitioners tried to explain the action of various herbs in terms of their chemical constituents. It is assumed that specific combination of various secondary metabolites present in plants enhances efficacy and removes adverse effects. Pharmacists prefer the use of single isolated phytochemical as medicine where as herbalists believe that various phytochemicals present in plant extracts interact to increase the therapeutic effect and decrease toxicity. The first active principles isolated from the plants were morphine, atropine and colchicine. Morphine is the first commercial pure natural product isolated by E. Merck in 1826.

In recent years, plant derived substances has obtained greater attention to cure and prevent human ailments as they are considered more bio-friendly. Plant produces these secondary metabolites to protect itself but researchers have described the use of these phytoconstituents into curing of various diseases. Knowledge of the chemical constituents of plants is a subject of curiosity because such information will be of value for the

synthesis of complex chemical substances. Replacement of synthetic insecticides by plant based insecticides can be a feasible plant pest management method as botanicals produce fewer hazards to the environment or to non-targeted organisms and results in better human health.

Plants produce secondary metabolites having healing potential and antimicrobial principles. The active principles obtained from various plant extracts have potential against Diabetes mellitus, a major global health and economic problem characterized by high level of blood glucose due to lack of insulin action, its production or both. Diabetes is affecting 6% of the world‟s population and 7% of the U.S.A population. According to the World Health Organization (WHO), there are approximately 160,000 diabetics worldwide and is expected to double in the year 2025 and the global cost of treating diabetes reach 1 trillion US dollar annually.

The present research work is focused on the chemistry of four plants namely *Piper nigrum, Psoralea corylifolia, Solanum nigrum* and *Eclipta alba* with an emphasis on the search for new natural products having antimicrobial/ insecticidal/ antidiabetic activity. These plants are investigated with the help of various spectroscopic analysis such as UV, IR, ^{1}H-NMR, ^{13}C-NMR, MS, LC-MS, GC-MS and X-Ray Analysis.

Piper nigrum (Family: Piperaceae) is a perennial climbing herb and acts as CNS stimulant, analgesic, antipyretic, antifungal, antidepressant, antioxidant, anti-inflammatory and anticonvulsant etc. Cold extraction of piper seeds was done with pet. ether, toluene, chloroform, ethyl acetate and methanol. The pet. ether and toluene crude extracts were kept as such for around two months, shiny yellow crystals are settled at the bottom of beaker which were identified by spectroscopic studies as Pn 1 and Pn 2 respectively. Hot extraction of piper seeds was done via soxhlet apparatus with various solvents on the basis of polarity gradient such as pet. ether, toluene, dichloromethane, chloroform, ethyl acetate and methanol. The essential oils isolated from various hot extracts were subjected to GC-MS analysis for the identification of chemical constituents. GC-MS analysis of hot pet. ether extract reports methyl benzene as major and δ-elemene as minor component; DCM extract reports c-murrolene as major and 2-methyl naphthalene as the minor component; Chloroform extract reports 2, 4-di-t-butyl phenol as the major and c-murrolene as the minor component; Ethyl acetate extract reveals caryophyllene as the major and piperine as the minor component. Hot pet. ether, toluene, DCM, chloroform, ethyl acetate extracts and crystals Pn 2 are subjected to antimicrobial activity against the bacterias such

as *E. coli, Bacillus subtilis, Pseudomonas aeruginosa* and *Salmonella typhimurium* and fungus *Aspergillus niger* and *Penicillium chrysogenum.* Hot pet. ether, methanol extracts and crystals Pn 2 are checked for insecticidal activity against the agricultural crop insect

Coccinella septumpunctata and stored grain insect *Sitophilus oryzae.* Hot pet. ether, methanol extracts and crystals Pn 2 are also checked for diabetic activity using 18 h fasted rat model on Wistar rats.

Psoralea corylifolia (Family: Fabaceae) is an annual herb used as antihelminthic, diuretic, deobstruent and against skin diseases. The seeds of the plant were extracted with various solvents on the basis of polarity gradient such as pet. ether, chloroform, ethyl acetate and methanol. The crude methanol extract was chromatographed and the white crystals (Pc 1) obtained from the solvent ratio (7:3) toluene: chloroform, keeping it for around 10 days were identified as crystals of psoralen by different spectroscopic analysis. The essential oil obtained through column chromatography of methanol extract of *Psoralea corylifolia* using (1:1) petroleum ether: toluene solvent ratio was subjected to GC-MS analysis and reported the presence of many phytoconstituents such as α-pinene, β-pinene, terpinolene and thumbergene etc. The above said extracts and crystal Pc 1 are tested for *in vitro* antimicrobial activity against the bacterias such as *E. coli, Bacillus subtilis, Pseudomonas aeruginosa* and *Salmonella typhimurium* and fungus *Aspergillus niger* and

Penicillium chrysogenum. Pet. ether and methanol extracts and crystals Pc 1 are checked for insecticidal activity against the agricultural crop insect *Coccinella septumpunctata* and stored grain insect *Sitophilus oryzae.* Pet. ether and methanol extracts and crystals Pc 1 are also checked for diabetic activity using 18 h fasted rat model on Wistar rats.

Solanum nigrum (Family: Solanaceae) is a wild herb and extensively used in traditional medicine in India and other parts of world to cure liver disorders, cough, wounds, asthma, ulcers, hemorrhoids, leprosy, dropsy and chronic skin ailments (psoriasis and ringworm). The aerial parts of *Solanum nigrum* were extracted with petroleum ether, toluene, dichloromethane, chloroform and methanol via soxhlate apparatus and crude extracts were collected. DCM extract was subjected to column chromatography and the fraction obtained from the toluene: dichloromethane (5:3) ratio was settled in the form of brown powder (Sn1) and subjected to LC-MS analysis. Methanol extract was subjected to basic hydrolysis and its aqueous layer reports the presence of white sugar like crystals Sn 2. The above said extracts and crystal Sn 2 are tested for *in vitro* antimicrobial activity against the bacterias such as *E. coli, Bacillus subtilis, Pseudomonas aeruginosa* and *Salmonella typhimurium*

and fungus *Aspergillus niger* and *Penicillium chrysogenum.* Crude pet. ether and methanol extracts are checked for insecticidal activity against the agricultural crop insect

Coccinella septumpunctata and stored grain insect *Sitophilus oryzae.* Pet. ether and methanol extracts and crystals Sn 2 are further checked for diabetic activity using 18 h fasted rat model on Wistar rats.

Eclipta alba (Family: Compositae) is a trailing annual weed acts as tonic and has hepatoprotective activity and used for treatment of jaundice and other ailments of the liver and gall bladder and viral hepatitis. The pet. ether extracts of *Eclipta alba* is subjected to LC-MS analysis. Basic hydrolysis of pet. ether extracts results in separation of white sugar like crystals (Ea 2) which are subjected to various spectroscopic analysis for characterization. Crude pet. ether, toluene, chloroform, ethyl acetate, methanol extracts and crystals Ea 2 are tested for *in vitro* antimicrobial activity against the bacterias such as

E. coli, Bacillus subtilis, Pseudomonas aeruginosa and *Salmonella typhimurium;* fungus

Aspergillus niger and *Penicillium chrysogenum.* Pet. ether and methanol extracts are checked for insecticidal activity against the agricultural crop insect

Coccinella septumpunctata and stored grain insect *Sitophilus oryzae.* Pet. ether extracts are also verified for diabetic activity using 18 h fasted rat model on Wistar rats.

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Glossary of Abbreviations

S.No.	Abbreviation	Description
1.	WHO	World Health Organization
2.	US	United State
3.	°C	Degree Celsius
4.	AIDS	Acquired immunodeficiency syndrome
5.	NADH	Nicotinamide adenine dinucleotide
6.	HIV	Human immunodeficiency virus
7.	*E. coli*	*Escherichia coli*
8.	CNS	Central Nervous System
9.	IUPAC	International Union of Pure and Applied Chemistry
10.	UV	Ultra violet
11.	IR	Infra Red
12.	^{1}H-NMR	Proton Nuclear Magnetic Resonance
13.	^{13}C-NMR	Carbon Nuclear Magnetic Resonance
14.	MS	Mass spectrometry
15.	LC-MS	Liquid Chromatography-Mass Spectrometry
16.	GC-MS	Gas Chromatography-Mass Spectrometry
17.	TLC	Thin Layer Chromatography
18.	CC	Column Chromatography
19.	nm	Nanometre
20.	EtOH	Ethanol
21.	OH	Hydroxy
22.	ppm	Parts per million
23.	EI	Electron Ionization
24.	CI	Chemical Ionization

25.	FD	Field Desorption
26.	Conc.	Concentrated
27.	ml	Milli-litre
28.	N	Normal
29.	cm	Centi-metre
30.	m	Metre
31.	BHT	Butylated hydroxytoluene
32.	BHA	Butyrated hydroxyanisole
33.	MeOD	Deuterated Methanol
34.	DCM	Dichloromethane
35.	Pet. ether	Petroleum ether
36.	NCIM	National Collection of Industrial Microorganisms
37.	gm	Gram
38.	Lt	Litre
39.	µl	Micro-litre
40.	h	Hour
41.	IIIM	Indian Institute of Integrative Medicine
42.	m.p.	Melting Point
43.	Rf	Retention Factor
44.	HRMS	High Resolution Mass Spectrum
45.	DEPT	Distortion less Enhancement by Polarization Transfer
46.	LPU	Lovely Professional University
47.	R.	Time Retention Time
48.	R.H.	Relative Humidity
49.	J&K	Jammu and Kashmir
50.	ha	Hectare

Achievements

Publications with full details

1. Monika Gupta, Aditi Gupta and Sudhakar Gupta; Phytochemical Analysis of Methanol Extracts of *Psoralea corylifolia;* International J. of Indigenous Medicinal Plants, 2013. Vol-46, issue-2, pp 1196-1199.

2. Aditi Gupta, Monika Gupta and Sudhakar Gupta; Phytochemical Analysis of hot Petroleum ether Extracts of *Piper nigrum,* Current World Environment. 2013. Vol-8, issue-1, pp 151-152.

3. Monika Gupta, Aditi Gupta and Sudhakar Gupta; Chemical composition and Insecticidal activity of Essential oil obtained from DCM extracts of *Psoralea corylifolia* against Agricultural pests, Current World Environment. 2013. Vol-8, issue-2, pp 309-312.

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1.1: Introduction

Since ancient times, humans were dependent on nature for their fundamental requirements such as food, shelter, clothing, fertilizers and medicines. Plants have been the backbone of Ayurvedic medicinal system since ages. As diseases, death and degradation are parallel to life so the knowledge about the diseases and their ailments are also associated with the birth of human intelligence. Rigveda, the historic book in library of man, provides enormous information on the matter.[1] Relieve with the help of therapeutic agents is as old as humanity itself. The curious nature of man has always encouraged him for the new discoveries of drugs. The evidences are present in the form of written reports, preserved gravestones and original plant parts that can be used as medicines. Since the Stone Age man has been fighting for illness and searching sources of remedies. That results the use of bark (*Cinchona*), stem (*Eucalyptus*), leaves (*Ocimum*, *Azadirachta indica*), seed (pepper), flower (*Angelica*), roots (*Dahlia*), oil (*Brassica, Cocos nucifera*).[2] The importance of plants as remedies of various human ailments has been given in Ayurveda and the other literature across the world. About 45000 plant species are being reported in India, among them several thousands are possessing medicinal properties.[3] In Ayurveda about 8000 herbal treatments are given. A description of 67 medicinal plants is given in Rigveda, 81 species in Yjurveda and 290 species in Atharvaveda. Charak Samhita and Sushruta Samhita had given a detail of medicinal importance of 110 and 1270 species respectively in drug synthesis and as a part of some drugs.[4]

Since ancient times, plants have been used by men against common infectious diseases and some of these plant parts are habitually used by humans against various ailments.[5]

The earliest evidences of use of natural compounds were pictured on clay tablets in cuneiform reported from Mesopotamia which informed about oils from *Cupressus sempervirens* (Cypress) and *Commiphora* species (myrrh). It has been used against inflammation, cough and cold. The Chinese Materia Medica and the Tang Herbal are written evidences of the applications of naturally existing chemical compounds. The Greek physician, Dioscorides documented the assemblage, storage and the utility of medicinal herbs. The religious organizations in France, Germany, Ireland and England maintained this Western knowledge where as the Arabs saved the Greco-Roman knowledge and elaborated the applications of their own resources along with Chinese and Indian herbs unknown to the Greco-Roman world. Arabs were the first to open their own

private pharmacies. A Persian pharmacist, physician, philosopher and poet i.e. Avicenna, contributed to the field of pharmaceutical and medicinal works.[6-7]

The modern medicinal system in most of the parts of the world is termed as medication as Yoga and Ayurveda are contemporary to each other. In 2005, WHO defined the importance of medication in terms of expenses and applications in both developed and developing countries and surveyed that 80% of the Africans and 42% of US people are turned to common domestic methods of relieve. The significance of traditional medicine in the developing countries and its emerging popularity in industrialized countries arouse interest in the mind of scientists.[8]

During the development of mankind, the biologically active phytoconstituents can act as medicine to combat pain and diseases.[1]

The remarkable medical advances in the last two decades have brought us face to face with a peculiar dilemma by decreasing death rate and increasing life expectancy. On the contrary the uncontrolled population explosion has resulted in the escalation of our food, energy and material requirements at a rate that natural resources would hardly be able to sustain for long.

This reminds us of the most unsettling predicaments of our times, that creativity and inventiveness of man, from his reproductive capabilities up to the most sophisticated accomplishment of applied knowledge, appears to be potentially self destructive. Now man either ought to find ways and means of self restraints for self preservation or face the gruesome prospect of losing initiative to nature once again.[9]

The various preparations such as herbal teas, decoctions, tinctures, extracts and infusions etc. are formulated from medicinal plants

1. **Herbal teas-Herbal remedies**: The mixture of herbal extracts in hot water of grounded/ungrounded materials to which drug extracts/therapeutic agents can be added. The mixture should be as possible as homogenous.

2. **Drug extracts**: The drugs extracted by the plants may be either by cold/hot extraction agents called “Menstrua”. After removal of liquid part, the residue of drug is called “Miscella” or fluid extract which is further processed for next step at the earliest.

3. **Aqueous drug extracts**: Drugs in required amount can be prepared from the various extracts of plant parts such as root, bark, leaves, flowers, fruits, seeds and herbs.

3.1. **Decoctions**: The drug in the required amount is added into water at temperature more than 90°C and kept under water bath for 30 minutes at the same temperature, with continuous stirring. It is then filtered out while hot.

3.2. **Infusions**: Small amount of drug in required quantity is being crushed for several times in a mortar with some amount of water and allowed to keep for 15 minutes. The remaining boiling water is then added on to the mixture which is stringed in a container in a water bath and again kept as such for 5 minutes with continuous stirring at more than 90°C temperature. The mixture is covered and kept untouched till cool.

3.3. **Macerates**: The adequate amount of water has been added to the drug at room temperature and then left to stand for thirty minutes with occasional stirring. The extract is then filtered and made up to the required amount with rinsing.

3.4. **Tinctures**: It is usually an alcoholic extract of flora or fauna of a low volatility substance (such as iodine and mercurochrome). In herbal medicine, alcoholic tinctures are prepared from different concentrations of ethanol. Among them the sample of 25% ethanol is most common. The other solvents used in herbal tinctures except ethanol are glycerol, propylene glycol, vinegar and ether. All of them cannot be used for oral intake.

3.5. **Fluid extracts:** These are also the liquid preparations but more concentrated than tinctures.

3.6. **Dry extracts:** They are hygroscopic so prepared under extremely dry conditions with no moisture. Intermediates and end product must be kept in dry conditions.[4]

A. Status of Phytochemical Investigations

No doubt modern medicine has registered a polymorphic growth yet a cure for many vulnerable diseases such as malignancy, epilepsy, meningitis and diabetes. Parkinson's diseases and now AIDS is still illusory. Most of the prescribed diseases, including the multi-drug regimens are associated with severe and many times irreversible side effects. Our failures force us to profess the smart theory of rejuvenating our immune system without a sound scientific know-how. While population explosion continues to be a matter of grave concern particularly to the developing countries, the premature ageing and various diseases like cardiovascular dysfunctions, diabetes, Alzheimer's diseases and osteoporosis are increasingly inflicting young generations. The treatments in the current use, especially psychosomatic drugs, analgesics and purely steroidal preparations suppress the conditions only and their prolonged administration proves counter-productive.[10]

The wide therapeutic properties associated with the natural products, which show insignificant contraindications within a specified LD_{50} dose, has made the scientific community, all over the world, to retrieve and search for the new biological active natural products, especially of plant origin.

As the knowledge about the constitution, therapeutic and other biochemical properties of new complex yet fascinating natural products emerges, the genius of synthetic chemists confronts new challenges and the demand for the natural product based preparations grows exponentially.[10]

Natural product chemistry began with the work of Serturner who first isolated morphine from *Papaver somniferum*.[1] Apomorphine derivatives are proved to regulate blood pressure, manage Parkinson's diseases and cure psychosomatic ageing in males. The triterpenoid saponins and sapogenins of *Phytolacca acilosa* are the most powerful non-steroidal drugs prescribed for the treatment of arthritis. Likewise boswellic acid derived from *Boswellia* species has become the comparable drug for the same purpose.

Stereochemistry plays an astounding role in the therapeutics of natural products. Thus a detailed structural study of a natural product, in a potent plant is very important. Besides techniques need to be developed for the rapid analysis and drug standardization of phytochemicals. Realising the need for such indepth studies, profound attention is being paid to the interdisciplinary studies in the field of phytochemistry. No doubt then, phytochemistry continues to be fertile field of fruitful research.

As a consequence of huge studies on phytotherapeutics, promising results in the management of some diseases like carcinoma, meningitis, encephalitis and AIDS, have been recorded in the recent past although a perfect cure is far from the way.

To cite some examples sanguidimerine from *Corydalis* species and vincristine from *Vinca rosea* are widely used against different types of cancers. Acetogenins inhibit NADH-oxidase in plasma membranes of tumour cells hence show anti-tumor cytotoxic activity. These molecules also exhibit antibacterial and antiparasitic activity. Estratab-a plant derived harmone is a safe drug for sparing females from post menopausal osteoporosis and the risk of endometrial hyperplasia- a precursor of endometrial carcinoma. Kusumoto et. al., have reported that limonoids are active against HIV. Though it is too early to suggest a possible cure for AIDS, hopes to discover a perfect cure are high because some plants have shown a strong tendency to combat this virus.[10]

Arctostaphylos uva-ursi (Bearberry) and *Vaccinium macrocarp (*Cranberry juice) are used against urinary tract infections as given in different manuals of phytotherapy, *Melissa officinalis* (Lemon balm), *Allium sativum* (garlic) and *Melaleuca alternifolia* (tee tree) can act as broad-spectrum antimicrobial agents.[5]

Recently iridoids have been reported to possess a marked immune modulating activity on animals. Furanoditerpenoids of clerodane type have shown high activity against peptic ulcers. Rutin, a flavanone, acts as an antioxidant towards adrenaline and ascorbic acid. It behaves as a general enzyme inhibitor and acts as an antiageing substance. The flavanone is readily absorbed by skin and its prolonged use reduces the dermal lining texture. Likewise isoflavones, like genistein and Diadzein, have oestrogenic activity. Coumasterol is a highly potent oestrogen, being thirty times more active than oestradiol and has no toxicity against male and female humans.

The chemistry of natural products leads to developments in the fields of pharmaceutical chemistry and synthetic organic chemistry. Even today, more than twenty five percent of the prescribed drugs, excluding the thirteen percent of the microbial preparations, are derived from plants and these earns a multi-million dollars profit to the industry, in the international market.

During the last three decades the multiprolonged strategy adopted in the phytochemical investigations, coupled with the rapid technologies have elevated the status of natural product based pharmaceutical industry. The recognition of vast pharmacognostical and

agricultural potential of natural products arouse curiosity in interdisciplinary scientists all over the world.

There is a long list of phytochemicals with proved biological activity and many of them are in commercial use and few are under further scrutiny. However much remains to be done before phytopharmaceutical industry is put on sound footing. For this reason phytochemical investigation with an emphasis on the research for new pharmacodynamic products, alternative sources for known drugs and their efficacious utilization will have a priority in pharmaceutical research.

With its varied geographical conditions at different places, India is a vast emporium of aromatic and medicinal plants. No doubt then, India has been a major contributor to the knowledge on oriental medicine, which culminated ultimately into the Ayurvedic System of Medicine. A large number of natural products have been added to the literature from time to time. India has offered a large number of new and potential natural products to pharmaceutical industry which helped in regaining once lost position in marketing some potential galenical preparations, medicinal plants and their products.[10]

B. List of plants which are used as medicines

1. ***Aloe barbadensis*** **(Aloe vera):** Aloe vera also known as medicinal aloe belongs to family liliaceae and cultivated in almost all parts of India, grows wildly on the coasts of Gujarat, Maharashtra and some parts of South India. It acts as emmenagogue, purgative, anti-inflammatory, antimicrobial (used for wound healing, sun burn), in sporadic constipations, pelvic congestion, intestinal diseases, uterine disorders and appendicitis. The *Ayurvedic Pharmapoecia of India* prescribes the usage of dry leaves in dysmenorrhoea and diseases of liver. The phytoconstituents aloin (Anthraquinone glycoside), acts as a stimulant in digestion in small quantity and behaves as a laxative as well as enhance colonic secretions and peristaltic contractions at high doses. The pulp is used in menstrual suppressions.[11]

2. ***Allium sativum*** **(Garlic):** *Allium sativum* is usually called as garlic and belongs to family liliaceae. It is native to Central India and cultivated all over India. It is commonly used as antibiotic, bacteriostatic, fungicide, antihelminthic, hypotensive and hypocholesterolaemic. According to *British Herbal Pharmacopoeia* it has antimicrobial properties. *The Ayurvedic Pharmacopoeia of India* suggests the use of the garlic bulb as a brain stimulant in epilepsy and mental disorders. Cloves of garlic are rich in sulphur containing amino acids called as alliin. The antibiotic effect is due to the presence of allicin; hypoglycemic effect is due to presence of allicin and allylprophyldisulphide.[11]

3. ***Azadirachta indica*** **(Neem):** *Melia azadirachta* belongs to family Meliaceae. It is indigenous to Burma and found all over India. Leaf and bark are used as antimicrobial, antifungal, insecticidal, antiviral, antipyretic, antiperiodic, antimalarial, antihelmintic, mosquito larvicidal, antifertility, spermicidal, anti-inflammatory and hypoglycemic, in infection of gums, sores, gingivitis and periodonitis. It is prescribed for various ailments such as enlargement of spleen, malarial fever, fever during child birth, measles, smallpox, head scald and cutaneous infections. The oil obtained from the plant is used as contraceptive and as well as mosquito repellant. The bark's methanol extract exhibits antimalarial activity against *Plasmodium falciparum*. The aqueous extract of leaves showed anti-inflammatory and antiulcer activity. The water-soluble part of alcoholic extract of leaves decreases blood glucose level in glucose-fed and adrenaline induced hyperglycemic rats. Nimbidin obtained from *Azadirachta indica* has antiulcer properties.[4, 11]

4. ***Ocimum sanctum* (Tulsi):** *Ocimum sanctum* also known as tulsi belongs to family lamiaceae, used as a medicine since ancient times. The leaves possess antihelminthic, expectorant, diuretic and stimulant effects. The decoction of roots is used against urino-genital disorders and malaria. It is also found to possess chemo preventive, anti-stress, anti-convulsant, anti diabetic, analgesic, anti-oxidant, anticancer, immunomodulatory and anti- inflammatory activities.[12]

5. ***Cinchona ledgeriana* (Quina):** Cinchona, also known as crown bark tree, is indigenous to tropical South America. It grows in Costa Rica, Bolivia, Peru, Indonesia, Columbia, Sri Lanka and Tanzania. It is best known for the antimalarial drug '*quinine*' derived from the plant's bark. Out of 35 alkaloids obtained from the plant, the most valuable are quinidine, quinine, cinchonidine and cinchonine. The above said alkaloids are present in the form of salts such as quinic acid, quinovic acid and cinchotannic acids. The cultivated bark contains major portion (7-10%) of the alkaloid found in the plant, among them 70% is quinine. Out of all alkaloids, 60% of the root bark are quinine. Quinine is derived in the form of quinine sulphate. It is recommended against malarial fever, cold, pneumonia, typhoid, influenza, whooping coughs, septicaemia, pin worms, sciatica, amoebic dysentery, lumbago, bronchial neuritis, intercostals neuralgia and internal hemorrhoids. They are also used as contraceptive and anesthetic. [4]

6. ***Papaver somniferum* (Opium poppy):** Poppy is indigenous plant of western Mediterranean region. It was introduced into India in the early sixteenth century, now grown in Punjab, Rajasthan, Uttar Pradesh and Madhya Pradesh. There are 25 poppy alkaloids out of which morphine, theanine, codeine, narcotine and papaverine are important. Morphine acts as pain killer and codeine as anticough agent and Opium acts as sedative, narcotic, hypnotic, sudorific, anodyne, analgesic and antispasmodic. Poppy seeds are used as emollient, nutritive demulcent, spasmolytic, lack of narcotic properties. The nutrients found in the seeds are thiamine, riboflavin, folic acid, pantothenic acid and niacin. The seed oil contains gamma-tocopherol, alpha-tocopherol and beta-tocopherol. The components of fatty oil obtained from seeds are stearic acid, palmitic acid, linoleic acid, oleic acid and linolenic acids. The seed extracts shows highly significant antidiarrhoeal activity against *E. coli.* The triglycerides present in the seeds exhibit anti-tumour activity against *Ehrlich's ascites* in mice.[5-6]

7. ***Acacia catechu*** **(Black Kutch):** The plant is moderate sized, prickly, caducous, can achieve upto 13m in height. The gummy myrrh of the wood is called katha or cutch. The various phytoconstituents of the plant are epecatechin, catechin, epicatechin gallate, epigallocatechin, phloroglucin, protocatechuic acid, quarcetin, lupenone, poriferasterol glucosides, procyanidin and kaemferol. The sugars reported from the plant are D-galactose, L-arabinose and D-rhamnose. Aldobiuronic acid, mineral, afzelchin gum and taxifolin were also reported. The concentrated aqueous extract of Heartwood is called Cutch and Katha.

   The cutch and Katha is commonly used in Ayurveda and have styptic, chilling and digestive properties. It is also recommended for ulcers, cold, cough, uterine haemorrhages, bleeding piles, atonic dyspepsia and chronic bronchitis etc.[13]

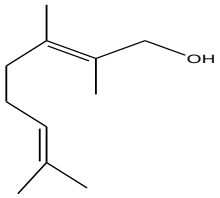
1.2: Important Classes of Active Compounds

Plant products or secondary metabolites are naturally occurring chemical constituents obtained from living organisms including plants. The study of these natural compounds includes the separation of compounds in pure form, elucidation of their structure, uses and purpose in the organisms. These secondary metabolites function as defense against predators and pathogens. They can be classified as terpenoids, alkaloids, flavonoids, steroids, saponins, lignans, amino acids, fatty acids and hydrocarbons.

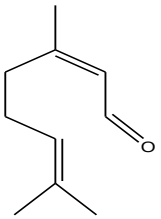
1.2a Terpenoids: The predominant group of phytoconstituents belongs to terpenoids. It is a group of natural products structurally depends upon the number of isoprene (2- methyl-1, 3-diene) units. The first conceptual framework for a common terpenoids was formulated by Wallach in 1887. According to his theory the terpenoids can be viewed as made up of one or more isoprene units attached together in a head to tail manner. The terpenoids are classified on the basis of number of isoprene units present in them. Thus the basic carbon framework of terpenoids is build up by the union of two or more C-5 isoprene units.

Monoterpenoids containing two isoprene units are C-10 compounds, responsible for characteristic aroma. The essential oil present in the plants belonging to different families (e.g., Asteraceae, Lauraceae, Myrtaceae and Liliaceae etc.) are the important sources of monoterpenes. The examples of acyclic monoterpenes are geraniol **(I),** citral **(II)** and monocyclic monoterpenes are camphor **(III)** and Carvacrol **(IV)**.

Acyclic monoterpenes

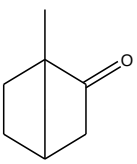


(I)



(II)

Monocyclic monoterpenes



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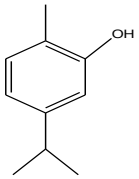
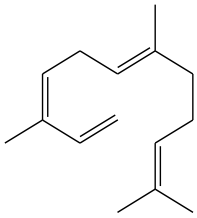


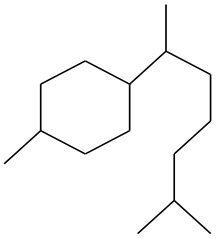
(IV)

Sesquiterpenes are the other group of terpenoids series containing three isoprene units. These are present in plants, liverworts, mosses, fungi and algae. Farnesene **(V)** is a acyclic sesquiterpene and plant growth inhibitor substance, abscicin and bisabolene **(VI)** are the examples of monocyclic sesquiterpenes. Cadinene is bicyclic and cedral is tricyclic terpene.

Acyclic sesquiterpene Monocyclic sesquiterpene

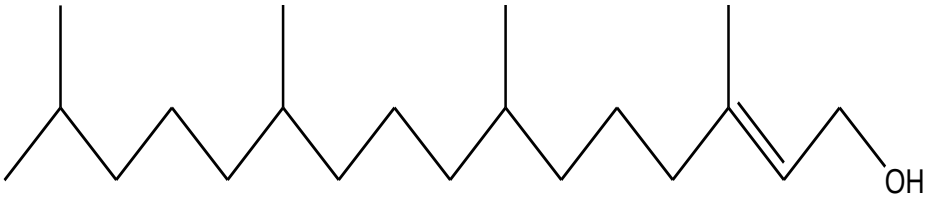


(V)



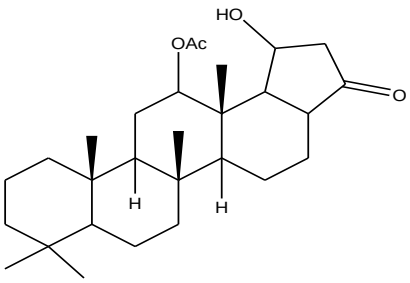
(VI)

The diterpenes are C-20 compounds of four isoprene units derived from geranyl phosphate. At present around 180 frame works of diterpenes are known. These terpenes may be acyclic (e.g. phytol **VII)**, monocyclic (e.g. cembrene), bicyclic (e.g., labdanolic acid), tricyclic (e.g., abetic acid) or tetracyclic (e.g., gibberellins) which possess various functional groups and unsaturation at different places.



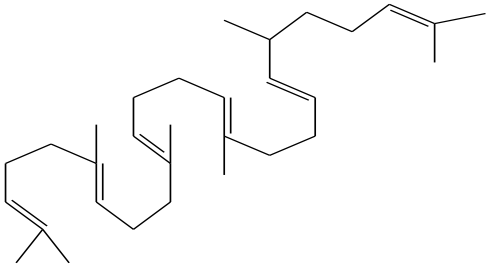
(VII)

The sesterterpenoids are the small group of pentaprenyl terpenoid derivative possessing five isoprene units. The terrestrial lichens, fungi, waxes, plant resins and various marine organisms are the major sources of these terpenoids. The scalarene **(VIII)** sestertriterpenoid have been reported in the sponges, found to have ecological role in preventing predation.



(VIII)

The triterpenoids are ubiquitous, non steroidal secondary metabolites and have a carbon skeleton based on six isoprene units, derived from squalene **(IX)**. These are cyclic or acyclic, colourless crystalline structures with high optical activity. These compounds may have monocyclic, bicyclic, tricyclic, tetracyclic or pentacyclic carbon skeleton.



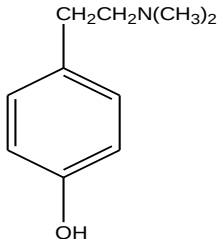
(IX)

The triterpenoid saponins are naturally occurring sugar conjugate. They form stable froth on shaking with water. The sugar part probably oligosaccharide joined to a hydroxyl group or carbonyl group or both. The saponins possess the oleanane ring system or rarely ursane, lupane, lanostane or serratene system. The carbohydrate group attached to the terpenic skeleton is glucose, galactose, rhamnose, arabinose, fucose and xylose. All triterpenes exhibit strong to weak haemolytic activity.[1]

1.2b Alkaloids: The name alkaloids was given to all organic bases (alkali like) obtained from the plants. In 1880, Konigs defines alkaloids as naturally occurring organic bases which contain a pyridine ring. Later on Ladenburg modified the definition of alkaloids as natural phytoconstituents that possess basic character and have atleast single nitrogen in a heterocyclic ring. Alkaloids are usually toxic but can be used in traces in medicines. Alkaloids are classified as[14]

1. **Phenyl ethylamine group:** These are monoamine alkaloids and functions as neuromodulator/neurotransmitter in the mammalian CNS. The outstanding physiological role is to increase the blood pressure so referred to as the pressor drugs.

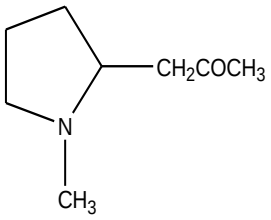
   Example: Hordenine:



(X)

2. **Pyrrolidine group:** It is a cyclic secondary amine and classified as saturated heterocycle.

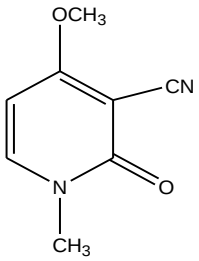
   Example: Hygrine



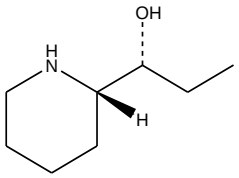
(XI)

3. **Pyridine and Piperidine group:** Pyridine alkaloids have unsaturated heterocyclic, nitrogen containing ring while piperidine alkaloids have saturated nitrogenous ring.

Example: Ricinine **(XII)** and Conhydrine **(XIII)**



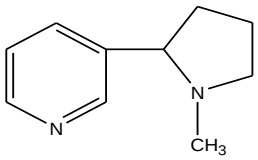
(XII)



(XIII)

4. **Pyrrolidine-Pyridine group:** Pyrrolidine alkaloids develop from solitary nitrogen containing five membered ring and pyridine alkaloids develop from aromatic 6 membered ring system.

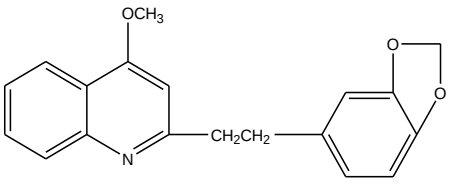
Example: Tobacco alkaloid Nicotine **(XIV)**



(XIV)

5. **Quinoline group:** Quinoline is a benzopyridine that is composed of a benzene ring fused to a pyridine ring.

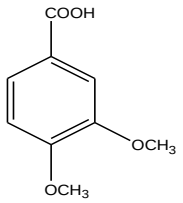
Example: Angostura bark alkaloid Cusparine **(XV)**



(XV)

6. **Isoquinoline group:** Isoquinoline is a structural isomer of Quinoline.

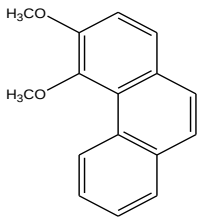
Example: Veratric acid **(XVI)**



(XVI)

7. **Phenanthrene group:** These alkaloids are derived from Isoquinoline nucleus.

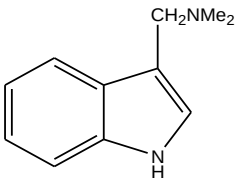
Example: 3, 4-dimethoxy Phenanthrene **(XVII)**



(XVI)

8. **Indole group:** Indole group of alkaloids is the largest class of alkaloids. It is containing indole nucleus as a structural moiety.

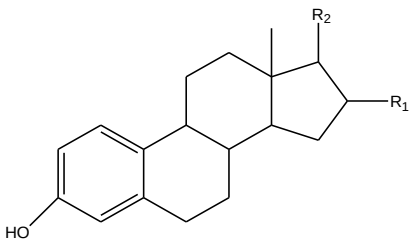
Example: Gramine **(XVIII)**



(XVII)

1.2c Steroids and Saponins: The steroids constitute a large group of naturally occurring chemical constituents possessing a perhydro-1, 2-cyclopenteno phenanthrene ring skeleton. These contain three cyclohexane rings A, B, C and the 4th cyclopentane ring D. These are generally crystalline compounds and are largely present in plants and animals. A steroid can be defined as any compound that gives Diel's hydrocarbon on distillation with Se at 360°C. The steroids possessing alcoholic group **(XIX)** may be saturated (plant sterol) or unsaturated (animal and plant).[1]

Historically, a number of plants have been used as sex hormones in the natural medicine. The pomegranate was a symbol of fertility, immortality and its legendary powers date back to Greek and Judeo Christian mythology. The pollen grains of the *Phoenix dectylifera var* have been reported to induce fertility in women in Egypt. A number of sterols have since been isolated and evaluated as estrogens.



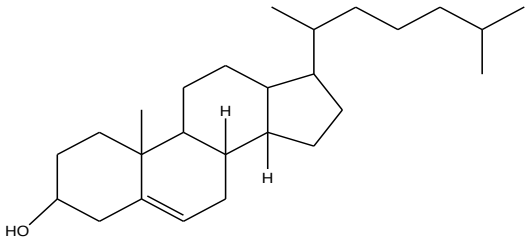
(XIX)

Depending upon the source of isolation they may be classified as:

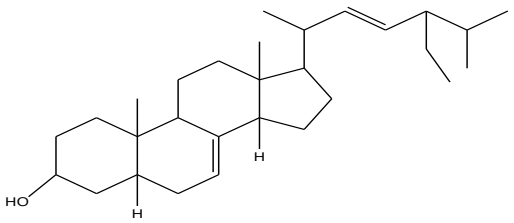
(i) Zoosterol- Isolated from animals (e.g. cholesterol **(XX).**

(ii) Phytosterol- Obtained from plant sources (e.g. spinosterol **(XXI)**, campesterol and stigmasterol.

(iii) Mycosterol isolated from yeast and fungi (e.g. zymosterol).



(XX)

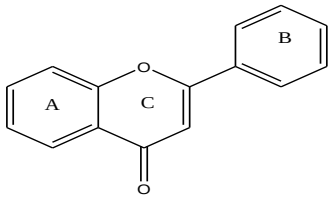


(XXI)

Saponins: Saponins are the plant glycosides which form colloidal soapy solution in water. These plant products have the C_{27} framework of cholesterol rather than that of C_{29} sitosterol. The sapogenin side chain differs in having two oxide bridge linked at C_{22} in a ketospiroacetal grouping which is transformed into many grouping.[14]

1.2e Flavonoids: The fascinating colours of flowers and fruits are due to the presence of important class of natural compounds called flavonoids. Flavonoids are found everywhere in photosynthesizing cells and thus occurs almost in the entire plant kingdom. These are present in various parts of the plants such as stem, fruits, seeds, vegetables, nuts and flowers as well as in wine, tea and honey. These constitute a very important part of human diet.

In flowers, the role of flavonoids is to impart colours that attract plant pollinators. Flavonoids protect the leaves from fungal pathogens and UV-B radiations hence promoting physiological survival of the plant. Flavonoids also act as photo sensitizers, energy transfer agents, plant's growth harmone regulators, involved in respiration control, photosynthesis, morphogenesis and determination of sex. The fundamental structural unit of flavonoids is the flavane nucleus **(XXII)**, which comprises of two benzene rings (A and B) linked through a heterocyclic pyrane ring (C) as shown below:

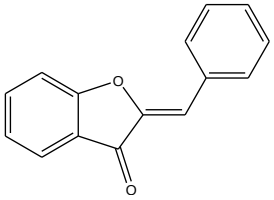


(XXII)

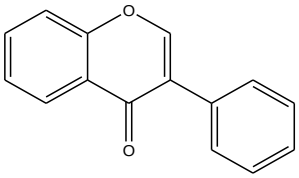
Fig.1.1. The skeleton structure of the flavones (a class of flavonoids), with rings named and positions numbered.

Flavonoids can be classified on the basis of biosynthetic origin. Some classes of flavonoids contains compounds that acts as both intermediates in biosynthesis as well as end products and can be stored in plant tissues for example flavanones, chalcones, flavan-3-ols and flavan-3, 4-diols. The classes of flavonoids that contain only the end products of biosynthesis are proanthocyanidins, anthocyanidins, flavonols and flavones. The flavonoids in which the 2-phenyl side chain isomerizes to 3rd position are called isoflavones and those in which it isomerizes to the 4th position are called neoflavonoids. Trivial names are widely used and sometimes also show its class or plant source. Finally the flavonoids are called by IUPAC names such as 3,4-dihydro-2-phenyl-2H-1-benzopyran for flavan.[15]

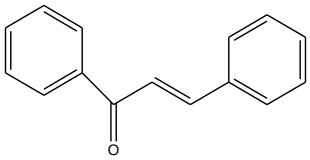
The skeleton structure of main classes of flavonoids is shown below:



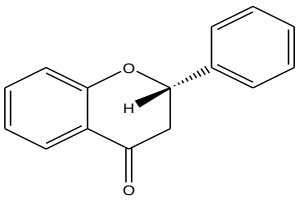
(XXIII) Aurone



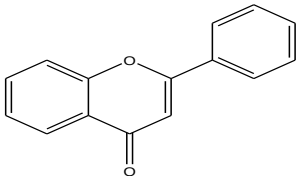
(XXIV) Isoflavone



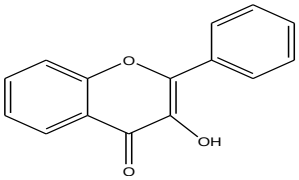
(XXV) Chalcone



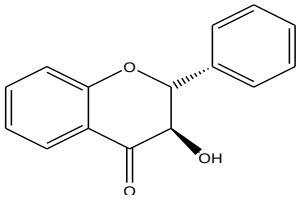
(XXVI) Flavanone



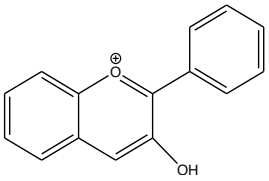
(XXVII) Flavone



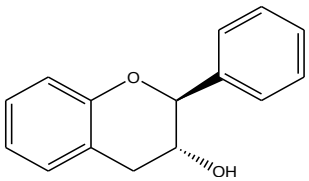
(XXVIII) Flavonol



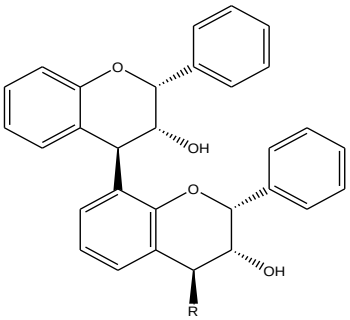
(XXIX) Flavon-3-ol



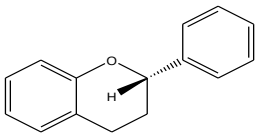
(XXX) Anthocyanidin



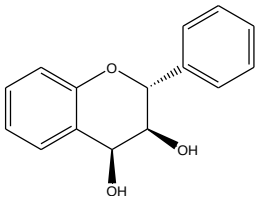
(XXXI) Flavan-3-ol



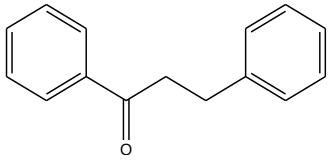
(XXXII) Proanthocyanidin



(XXXIII) Flavan



(XXXIV) Flavan-3, 4-diol



(XXXV) Dihydrochalcone

1.3: Techniques for the Purification and Identification of Compounds

There are many methods which have been used to obtain and identify the substances in high state of purity. Besides chemical methods many physical methods like fractional precipitation, distillation and crystallization have been effectively used for separation and purification of the chemical compounds. Likewise in the fields of chemistry and biosciences many substances are obtained, where the classical methods for their identification and purification don't work satisfactorily e.g. protein is a complex mixture of several amino acids which are very much alike to each other, in such cases chromatography plays a very important and significant role in solving all such problems. The physico chemical methods which are generally employed in the identification and structure elucidation of compound isolated from plants are Chromatography, UV, IR, NMR (^{1}H and ^{13}C), Mass spectroscopy, Liquid chromatography Mass Spectroscopy (LC-MS), Gas Chromatography Mass Spectroscopy (GC-MS), and X-Ray Spectroscopy.

1.3.1 Chromatography: Chromatography includes a number of laboratory techniques used to separate mixture into individual components. In Greek, Chroma means "color" and graphein means "to write". The IUPAC has defined "Chromatography as a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction".[16]

1.3.1a Thin Layer Chromatography (TLC): Thin layer chromatography (TLC) is a chromatographic technique used to separate mixtures into its components. For TLC, a sheet of glass/plastic/aluminium foil is used which is coated with a thin layer of adsorbents such as silica gel, aluminium oxide or cellulose. The adsorbent layer is called as the stationary phase. When the adsorbent get dry, a spot of the sample has been applied near the base on the plate. The plate is then kept in a solvent or solvent mixture (known as the mobile phase) that moves upwards due to capillary action. As various analytes are having different polarities so these move upwards on the TLC plate at different rates and hence results in separation.[17]

The TLC plates were made by decanting the silica gel G slurry on the glass plates. The plates were then allowed to dry for 30 minutes in air and in an oven at a temperature of 110°C for another 30 minutes. A sample spot was placed near the bottom of the plate and it is kept at an inclination of 45° in the development chamber containing solvent up to 1 cm.

The plate was finally allowed to dry after the solvent front was marked. The coloured analytes are visible on the chromatogram while colourless components are visualized with the help of iodine chamber. As different analytes are having different Rf values thus these migrates to different extent hence leads to qualitative evaluation.[17]

1.3.1b Column Chromatography (CC): The column was prepared by pouring slurry of TLC silica gel 60 GF_{254} (Merck) mixed with a column volume of solvent into a glass tube with a cotton cloth attached at the end to retain the stationary phase. The silica was allowed to settle and the solvent run off until there was just sufficient to cover the support. Sample adsorbed onto a small amount of silica gel was added and elution performed until non-retained material reached the end of the column. At this stage, the cotton cloth was removed and the contents of the column extruded with a plunger. The components were detected under UV light and the column silica as required.[23]

1.3.2 UV Spectroscopy: Ultraviolet absorption spectroscopy deals with the measurement of energy absorbed when electrons are excited to higher energy levels. The application of UV Spectroscopy lies in detection of double bonds in conjugation, aromatic conjugation within various molecules. Isolated double bonds do not give strong band, but when conjugated systems are present; the bands are usually strong and are of longer wavelength.[14, 18]

Alkanes such as methane, propane, cyclohexane shows λ_{Max} for σ-σ^* transition below 140 nm. All non conjugated alkenes have an intense absorption due to $\pi - \pi^*$ transition below 200 nm example ethylene absorbs in the vapour phase at 165 nm. An isolated ethylenic linkage exhibits a high intensity band near 170 nm and isolated carbonyl group displays a low intensity band near 290 nm. However in α-β-unsaturated carbonyl compounds, both the bands undergo bathochromic shifts to the regions 210-230 and 310-330 nm respectively. Carboxylic acids and esters exhibit a weak intensity band at near 200 nm, lactones exhibit absorption in the region 200-240 nm, α-β-unsaturated lactams exhibit an additional band near 250 nm.[18]

UV spectra of flavone and flavonols generally exhibit high intensified absorption in the 300- 380 nm and the 240- 270 nm and alkaloids shows λ_{Max} (EtOH) 212, 235 and 285 nm (log E, 4.51, 4.10 and 3.94 respectively).[1]

1.3.3 IR Spectroscopy: The usefulness of IR spectroscopy has been overshadowed in recent years by NMR spectroscopy. As many of the structural features brought out by the

IR spectrum are more clearly discernible in the ^{1}H-NMR and ^{13}C-NMR spectra, in spite of this the IR spectra offers the first clue to the nature of compounds giving an idea about the nature of the different functional groups in a compound under investigations. The IR spectrum of terpenoids is helpful in identification of different categories of terpenoid compounds. It also designate the groups present in them.

The IR spectrum of monoterpenes shows absorption bands in the regions 3100-2800 cm^{-1}(C-H stretching), 1470 – 1420 cm^{-1}(C-H asymmetrical), 1385-1305 cm^{-1}(C-H symmetrical) and 825-720 cm^{-1}(C-H bending). Unsaturated monoterpenes shows a band in the region 1670-1600 $cm^{-1,}$ due to C=C stretching vibrations. The free hydroxyl group absorbs near 3630-3520 cm^{-1} as a sharp band. This band is shifted to 3330 cm^{-1} in compounds possessing intermolecular hydrogen bonding. The carbonyl groups of ketones, aldehydes, carboxylic acids and esters are detected near 1755-1670 cm^{-1}.

IR bands were observed very clearly in case of sesquiterpenoids. The presence of functional groups such as hydroxyl (3600-3200 cm^{-1}), furan (3140-3050, 1590, 1540, 815 and 755 cm^{-1}), lactones (1750-1650 cm^{-1}), carbonyl (1870-1660 cm^{-1}), unsaturation (~ 1600 cm^{-1}) and gem dimethyl/ isopropyl (1390, 1380, 1308 cm^{-1}) groups are readily detected by inspection of IR spectrum. The IR absorption bands near 1760 and 1660 cm^{-1} reveals the presence of gamma-lactone.

The diterpenoids are also recognized by their peculiar IR spectrum. The IR bands in the region 3610-3200 cm^{-1} reveals the presence of hydroxyl group in the molecule. A chelated hydroxyl function absorbs at lower frequency (nearly 3360 cm^{-1}). The phenolic hydroxyls also appear in the same range. A carboxyl group may appear in the range 1850-1650 cm^{-1} and the location of a particular ketonic group is established as δ-lactone (1778 cm^{-1}), aldehyde (1740-1720 cm^{-1}), five membered carbonyl (1745 cm^{-1}), six membered carbonyl (1720 cm^{-1}), α-β-unsaturated carbonyl (3000 br, 1665 cm^{-1}) and methoxy carbonylic (1720, 1250 cm^{-1}). A strong band at 1774 cm^{-1}, is due to aromatic acetate group and a weaker one at 1729 cm^{-1} ascribed to the acetate group bonded to a saturated carbon atom. Also the bands in region 1660-1590 cm^{-1} reveals the presence of unsaturated bonds.

The IR spectra of triterpenes resemble with the spectra of steroids. But for identical positions in C-3 oxo steroid, the C-2 and C-4 methylene groups absorbs near 1420 cm^{-1} while in the corresponding 3-oxo triterpenes, the C-2 methylene absorbs near 1430 cm^{-1}. A C-11 methylene in 12-oxo steroid absorbs at 1434 cm^{-1}, whereas the same group in

12-oxo triterpenes absorbs close to 1420 cm^{-1}. On the basis of IR spectroscopic studies tertiary equatorial (3613 cm^{-1}) and axial (3617 cm^{-1}) hydroxyl groups can be distinguished.

Compounds containing α-β-unsaturated carbonyl group show IR absorption band in the region 1665-1600 cm^{-1}, saturated CO group at 1700 cm^{-1} and α-β-unsaturated β lactone at 1760 cm^{-1}, δ-lactone at 1720 and 1250 cm^{-1}, ester carbonyl group at 1725 and 1080 cm^{-1}, aliphatic C-H near 2800 cm^{-1}, COOH group at 3420-2500 cm^{-1}, CO at 1150-1000 cm^{-1}, trisubstituted double bond (1670, 830 cm^{-1}). Band close to 1792 cm^{-1} is due to the presence of carbonyl group of gamma lactone, with an ether linkage α- to oxygen. Significant absorption at 1043 cm^{-1} and 1000 cm^{-1} are due to COOH stretching vibrations of an A/B trans triterpenes.[1] The IR frequencies of various functional groups are given in table 1.1.

Table 1.I: Vibrational Wave numbers for functional groups

S. No.	Functionality	IR range in cm^{-1}	Intensity
1	C-H (s)	3000-2850	Strong
2	C-H (b)	1470-1430	Weak
3	$-CH_3$ (sb)	1390-1370	Medium
4	$-CH_2-$ (rock)	700-750	Weak
5	-C≡ C-H (C-H)	3300	Strong
6	C=C	1680-1620	Strong
7	-O-H (s)	3650-3590	Strong
8	-O-H (b)	1410-1260	Strong
9	C-OH (s)	1150-1050	Strong
10	N-H (ss/as)	3500-3300	Medium
11	C=O (s)	1780-1680	Very Strong
12	Hydrogen bond	3570-3200	Strong
13	-C≡C-H (s)	3300	Strong

s= Stretch; b = Bend; sb = Symmetric bend; ss = Symmetric stretch; as = Asymmetric stretch

1.3.4 NMR Spectroscopy: Nuclear magnetic resonance (NMR) spectrometry is a type of absorption spectroscopy which is based on absorption of electromagnetic radiations in the radiofrequency region at a particular frequency that is characteristics of the given compound. A **NMR spectrum** consists of a plot between frequencies of absorption versus peak intensity.[19] It involves excitation of a spin active nucleus from one spin state to another when placed in magnetic field. The applications of 2D-NMR lie in determining the structures of complex compounds.[18]

1.3.4.a Proton NMR (^{1}H-NMR): The application of NMR spectroscopy has proven most powerful tool in the structure determination of terpenoids, alkaloids, steroids, flavonoids and hydrocarbons. By the use of silyl derivative, double irradiation techniques, solvent induced shift studies and recently introduced lanthanides induced shift studies; one can come to the structure of the chemical constituent occurring even in minor quantities without tedious and time consuming chemical degradation and synthesis.

The ^{1}H-NMR spectra of monoterpenes gives information about the pattern of attachment of hydrogen atoms to their respective carbons and their spatial arrangements. The exocyclic methylene protons appear as two doublets in the range δ 5.3-4.7. The sharp signal in the upfield range at δ 2.3-0.7 is due to methyl functionalities attached to different carbons.

Most of the sesquiterpenoids possess an isopropenyl group which exhibits a characteristics singlet for the olefinic methyl functional at δ 1.7-1.9 and the terminal olefinic protons as two broad singlets at δ 4.6-4.5. The presence of isopropyl group at sp^3 carbon is detected by the appearance of two doublets at δ 0.72 (J= 6.5 Hz, Me) and δ 0.88 (J= 6.5 Hz, Me) and a multiplet for methine function at δ 1.70. Additional methyl absorptions δ 0.9-1.6 are present in each case. Methyl signals in the range δ 1.5- 2.3 indicate the attachment to unsaturated carbon.

The ^{1}H-NMR spectra of diterpenoids compounds reveal many facts about the attachment of protons. The methyl groups appears in the range of δ 0.65-2.20 and their coupling constants and absorption fields determine their attachment to primary (δ 5.06, t) secondary (δ 0.94 d, J = 6.5 Hz), tertiary (δ 1.34-0.74, s), allylic or olefinic (δ 1.60-1.95, s) carbons, acetyl function (δ 2.20-1.95, s) and isopropyl methyls as two doublets at δ 0.09 and 0.96. An isopropyl group attached to the benzoquinone ring appear at δ 1.25 as a doublet (6H, J=7Hz) and δ 3.35 (1H, sept, J=7Hz). Most of the cyclic ring diterpenoids possess tertiary C-methyl groups, the number of which is determined from the characteristic three proton singlet signals in the ^{1}H-NMR spectrum at δ 1.0 ppm. Two doublets near δ 4.76-3.73 and δ 4.14-3.70 (J = 11 Hz) are assigned to a methylene group bearing an oxygen substituent (OH). A carbonyl group attached to a double bond appears in the downfield range δ 4.28 and 4.10. An acetoxy methylene function resonates at δ 5.26- 4.76 as two signals. A pair of doublet at δ 3.47 and 2.49 is assigned to methylene group adjacent to a keto group. The existence of methylene group of an oxirane ring is determined by the location of two doublets near δ 2.60-2.25 and δ 3.63- 3.17 (J = 5.4 Hz).

The ^{1}H-NMR spectra of triterpenoid containing carbomethoxyl function, the chemical shift of the highly shielded C-methyl group partially indicates the position of the carbomethoxyl group. Methyl group attached to an unsaturated carbon atom containing a carboxyl group CH_2(COOH)-C=C-, also resonate at δ 2.05-1.85. The peak due to presence of acetoxy group appears between δ 2.07-1.82 as sharp signals. In pentacyclic triterpenes methyl ester, angular methyls and acetoxy groups give sharp signal. These triterpenes contain a number of quaternary methyl groups whose signals are oftenly formed overlapped. Normal methyl group signals appear in the range δ 0.62- 1.55. A trisubstituted double bond proton in ursane and oleanane compounds absorbs in the range δ 5.50 - 4.90. If the double bond is in conjugation with a carbonyl group at C-11 (e.g. 11 –keto-α-boswellic acid methyl ester), the vinylic proton is observed shielded at δ 5.55 as sharp signal. The vinylic protons absorb at higher field near δ 4.30–5.87 as two proton signal in compounds containing terminal double bonds.

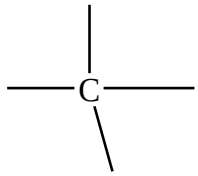
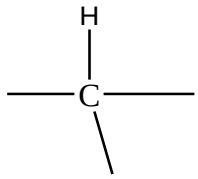
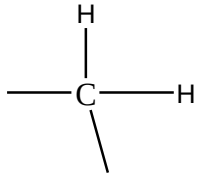
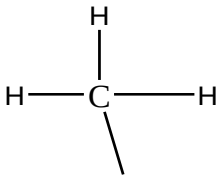
In case of flavonoids the chemical shift of the protons of ring A and B are independent of each other but are affected by the nature of the ring C. The other peaks in the aromatic region will reveal the pattern of oxygen substitution in rings A and B and confirm the nature of ring C. The proton of 5-OH group adjacent to a 4 carbonyl group in a flavonoid shows a sharp signal at very low field (δ 3.00). Also the 3-OH group which reduces the hydrogen bonding causes an up field shift of this peak and methylation of a hydroxyl group causes deshielding of the ring protons. The ^{1}H-NMR spectrum of a common sterol indicated the chemical shift for six methyl groups at δ 0.7 -1.07 assigned to methyl protons, 18, 19, 21 and 26, 27 and 29.[1]

1.3.4.b ^{13}C NMR Spectroscopy: Like proton NMR, ^{13}C- NMR is a plot of signals given by various types of carbons depends upon their environment. As the carbon skeletons of rings and chains are central to organic chemistry, most of the chemists would have selected carbon nucleus over the hydrogen nucleus for immediate investigation. The ^{12}C nucleus is magnetically inactive but the ^{13}C nucleus is magnetically active and has spin number of ½. However, since the natural abundance of ^{13}C is only 1.1 % than that of ^{12}C, its sensitivity is only about 1.6 % that of ^{1}H, the overall sensitivity of ^{13}C compared with ^{1}H is about 1/5700.[19]

Thus the studies of the carbon nuclei had greater utility over the equivalent proton studies as it gives direct information of carbon skeleton, signals are spread over a chemical range of 200 ppm, compared to 20 ppm for proton spectra.[18]

Alkanes generally absorb from -2 to 55 ppm, carbonyl carbon absorbs far downfield at 200 ppm, carbons of alkenes and benzene ring absorb in the same region which can be distinguished by ^{1}H NMR. Various kind of carbons or sets of equivalent carbons present in a molecule are shown by the number of signals in proton decoupled ^{13}C NMR spectrum. The number of H-atoms attached to a given carbon is given by the splitting of a signal in ^{13}C proton off resonance decoupled NMR spectrum. The signals for various kinds of carbons are given in table 1.2.

Table 1.II: Signals for various carbons

1.		SINGLET
2.		DOUBLET
3.		TRIPLET
4.		QUARTET

The signal for sp^3 hybridized carbons occur upfield in the range of -2 to 55 ppm where as sp^2 hybridized carbons, the signals appear over 100 ppm downfield from them i.e 110-170 ppm. The triply bonded hybridized carbons in acetylenes absorb in the region between sp^3 and sp^2 hybridized carbons. The alkyl halide or amine shows signal at 10-65 ppm, alcohols or ethers at 50-90 ppm, alkynes at 65-90 ppm, alkenes at 110-170 ppm, nitriles at 130-150 ppm, amides, carboxylic acids and esters at 150-185 ppm, aldehydes or ketones at 180-220 ppm.[18]

The details of ^{13}C NMR are helpful in assigning the structure i.e basic skeleton of any compound. It also helps in designation of functional groups present in the compound.

1.3.5 Mass Spectroscopy: Mass spectrometry is a micro analytical technique requiring only a few nanomoles of the substance to obtain the information regarding the structure and molecular mass of the compound. It is unlike the other forms of spectroscopy, in that it is not concerned with nondestructive interactions between molecules and electromagnetic radiation. In mass spectrometry the ionized parent molecules are produced, separated and degraded into ionic fragments and then the relative abundance of the different ions produced is measured. As the sample is consumed during analysis so it is called a destructive technique.

Mostly the nascent molecular ion of the analyte produces fragment ions by cleavage of the bonds and the resulting fragmentation pattern obtained constitutes the mass spectrum. Thus each chemical compound is having a unique mass spectrum that is characteristic of the sample, also called "chemical fingerprint".

A mass spectrum is the plot of relative abundance of ions against their mass/charge ratio. Various kinds of mass spectrometers and different analyte introduction techniques help in analyzing large number of samples.[18]

Detection of the molecular ion peaks of amino acids can be difficult. The EI spectra of amino acids or their esters give weak or nonexistent molecular ion peak but CI and FD give either molecular ion peak or quasimolecular ion peak as the amino acids easily lose their carboxyl group and the esters easily lose their carboalkoxyl group upon electron impact. Poly hydroxyl steroids give EI spectra that show weak or nonexistent molecular ion peak. Triglycerides give rise to characteristic [M- O_2CR] ions arising from positive charge stabilization by neighbouring oxygen.[19]

1.3.6 Gas Chromatography-Mass Spectrometry: In this spectroscopic technique a mixture of compounds is to be analyzed, shot into the GC where the mixture is evaporated in a heated chamber (injector). The mixture in vaporized form travels through a GC column containing a carrier gas where the compounds get separated as they interact with the stationary phase of the column. These isolated compounds then immediately enter into the mass spectrometry that provides the mass spectrum of the single component.[1]

Thus GC-MS is one of the best techniques for scientists involved in lipid analysis. GC-MS arrangements such as the Finnigan MAT "ion trap" detector and the Hewlett Packard "mass selective detector" have become cheap, authentic and easy to use. For fatty acid identification especially, basic system can provide answers to most of the questions.

GC-MS provides two types of informations of a given compound such as its mass spectra as well as its GC retention time.

The methyl ester derivatives of long-chain saturated fatty acids are easily identified by EI-MS and their spectra are characterized by a prominent molecular ion (M^+) peak and other important peaks are present at *m/z* = M−31 due to the elimination of methanol and M−43 due to elimination of C2, C3 and C4 as a result of a complex rearrangement, together with a series of ions of general formula - $[CH_3COO(CH_2)_n]^+$, often with intensity maxima at *m/z* = 87, 143 and 199. The base ion peak at *m/z* = 74 appears due to "McLafferty rearrangement ion" and is formed when parent compound breaks at a position beta to the carboxyl group. Pyrrolidine derivatives of saturated fatty acids have eminent molecular ion peaks and a base peak at *m/z* = 113 due to the McLafferty ion in their mass spectrum.

EI-MS of unsaturated fatty acids are different from their saturated analogues. The peak observed during release of methanol is at ($[M-32]^+$. The base peak with some instruments is also observed. During the elimination of methoxyl radical $[M-31]^+$, $[M-74]^+$ and $[M-116]^+$ peaks are observed. In the mass spectra of dienes and trienes, the molecular ion peaks are eminent where as the peaks representing losses of 32, 74 and 116 amu are less eminent.[20]

1.3.7 Liquid Chromatography-Mass Spectrometry: Liquid chromatography–mass spectrometry (LC–MS) is a combined technique that includes both separation based on liquid chromatography and molecular mass determination using mass spectrometry. This technique is largely used for fingerprinting analysis of isoflavonoids, carotenoids pigments and toxins in marine algae.[21]

1.3.8 X-Ray Analysis: X-rays are electromagnetic radiation with wavelengths of the order 10^{10} m. X-ray diffraction method is widely used physical procedure for the complete molecular structure determination provided the material should be crystalline in nature. X-ray analysis could be successfully employed for obtaining the complete electron distribution in the molecule, information about the intermolecular interactions in the molecule, a detailed picture of the thermal vibrations of each atom in the crystal.[22]

In Laue Photographic method, a single, small crystal is placed in the path of a narrow beam of X-rays from a tungsten anticathode and the resulted diffracted beam is allowed to fall on a photographic plate. When the photographic plate is developed, a characteristic pattern,

known as Laue pattern of spots is seen. From the positions of the spots and the distance of the photographic plate from the crystal, θ is calculated and the relative spacing between the planes is estimated. Laue pattern can be used to orient crystals for solid state experiments and to determine the symmetry of single crystal.

In Bragg's method, the X-rays are also allowed to fall on the crystal surface. On applying Bragg's equation ($n \lambda = 2 d \sin \theta$), ratio of lattice spacing for various groups of planes can be obtained. This ratio has been found to be different for different crystals.[22]

Thus X-ray is very useful technique to find out the crystal structure of isolated natural products.

1.4: Tests for Identification of Functional Groups

1.4.1 Test for the presence of alkaloids:

Mayer's test:

Alkaloids on treatment with Mayer's reagent [Potassium mercuric iodide solution] give cream coloured precipitates.

Dragendorff's test:

Alkaloids on treatment with Dragendorff's reagent [Potassium bismuth iodide solution] give reddish brown precipitates.

Hager's test:

Alkaloids on treatment with Hager's reagent [saturated solution of Picric acid] give yellow color precipitates.

1.4.2 Test for the presence of glycosides:

Raymond's test:

On treatment with dinitro- benzene in hot methanolic alkali, glycosides give violet color.

1.4.3 Test for the presence of tannins and phenolic compounds:

Ferric chloride test:

On treatment with ferric chloride solution, phenolic compounds give reddish brown colour.

1.4.4 Test for the presence of flavonoids:

Shinoda test (Magnesium Hydrochloride reduction test):

On addition of few fragments of Magnesium ribbon and concentrated hydrochloric acid drop wise, flavonoids give pink scarlet, crimson red or occasionally green to blue colour after few minutes.

Zinc Hydrochloride reduction test:

On adding Zinc dust and conc. hydrochloric acid, flavonoids give red colour after few minutes.

Alkaline reagent test:

On adding few drops of sodium hydroxide solution, flavonoid gives intense yellow coloration, which turns to colourless on further addition of few drops of dil. acid.

1.4.5 Test for the presence of sterols and triterpenoids:

Libermann- Buchard test:

Test solution is treated with few drops of acetic anhydride, boil and cool. Sulfuric acid is added from the sides of the test tube, a brown ring appears at the junction of two layers. If the upper layer turns green indicates the presence of steroids and formation of deep red color reveals the presence of triterpenoids.

Salkowski test:

Test solution is treated with chloroform and few drops of conc. Sulfuric acid. Shake well and kept it untouched for some time. Appearance of red coloured lower layer indicates the presence of steroids and yellow coloured lower layer indicates the presence of triterpenoids.

1.4.6 Test for the presence of carbohydrates:

Molisch's test:

To the test solution, add few drops of alcoholic alpha napthol and 0.2 ml of concentrated Sulfuric acid slowly through the sides of the test tube, a purple to violet color ring appears at the junction indicates the presence of carbohydrates.

Fehling's test:

To the test solution, add equal volume of Fehling's A (Copper sulfate in distilled water) and Fehling's B (Potassium tartarate and Sodium hydroxide in distilled water) and boil. Brick red precipitate of cuprous oxide appears if reducing sugars are present.

1.4.7 Test for the presence of fats and fixed oils:

Saponification test:

To the test solution, add a few drops of 0.5N alcoholic potassium hydroxide along with a drop of phenolphthalein and heat on a water bath for 1-2 hrs. The formation of soap or partial neutralization of alkali shows the presence of fixed oils and fats.[17]

1.5: Aims and Objectives of Study

Aim: Analysis of insect killing and antidiabetic phytochemicals from some herbs also their effective role on microorganism**.**

Objective of Present Study:

1. To isolate the phytochemicals from different medicinal plants in different solvent system and their identification through different spectroscopic techniques.
2. To identify the phytochemicals extract/ constituents for its antimicrobial activity.
3. To analyse the phytoconstituents against different crop insects.
4. To check the antidiabetic activity of some phytochemicals.

Novelty:

To identify a novel antimicrobial, insecticidal and antidiabetic plant extracts/ constituent from the reported medicinal plants.

Secondary Metabolites obtained from medicinal plants either in their pure form or in the form of plant extracts, provide a vast opportunity to obtain new drug leads because of wide chemical diversity. Thus seeking therapeutic agents from natural products particularly from the edible plants is a matter of interest for the scientists throughout the world. Various types of bioactive compounds are contained in herbal formulations. Thus there is an opportunity to get various pharmaceutical agents due to wide aromatic and medicinal flora of India.

The present investigation concerned with the study on the chemistry of four plants namely *Piper nigrum, Psoralea corylifolia, Solanum nigrum, Eclipta alba* with an emphasis on the search for new natural products. The results of this investigation are discussed in the following chapters.

2.1: Introduction

Piper nigrum, commonly called as black pepper, is a member of family piperaceae. The genus piper includes 700 species distributed in northern and southern hemispheres. It is also known as "The King of spices" and has major contribution in Indian Spice Export Scenario. The fruit of *Piper nigrum*, also called as pepper is an ancient and famous spice throughout the world. *Piper nigrum* is a native of Malabar seashore of India and has its utility as a redolent stimulant in cholera, flatulence, arthritis disease, dyspepsia and anti-periodic in malarial fever. *Piper nigrum* is familiar species of genus piper because of its high economic, commercial and medicinal values. The genus piper is having a rich phytochemistry and researchers showed the ample presence of alkaloids, amides and terpenoids. The various pharmacological activities of *Piper nigrum* are radical scavenging, antioxidant, anti-insecticidal, allelopathy, anticonvulsant, anti-inflammatory, anti-tubercular, antibacterial, antipyretic, exterofective and antioxidant.[24-25]

In the recent years, phytoconstituents arouse curiosity in the mind of researchers as they are more biofriendly to cure and prevent human ailments. About 6000 plants are used in folk, herbal and traditional medicine in India and contribute about 75% of medicinal needs of the world. Phytoconstituents are nonnutritive compounds synthesized by plants and have protective or disease preventive activities. Plant synthesizes these compounds to defend itself but research reveals that many phytoconstituents can also protect humans against various ailments. Knowledge of the phytochemicals is required because such information will be valuable for the researchers to synthesize new complex chemical substances.[26]

Botanical Description: *Piper nigrum* is a perennial climbing shrub.[27] Branches are hard, dragging and have roots at the nodes. Leaves are intact, 12.5 – 17.5 cm by 5.0 – 12.5 cm and have variable breadth. Fruiting spikes are having different lengths. Flowers are narrow at spikes and mostly dioecious. Fruits are spherical and lustrous red when ripen and belong to category drupe. Seeds are almost spherical.

Geographical Distribution: The plant is indigenous to India and is a costly spice since ancient times.[28] The plant is extensively found in Western Ghat forests. The plant adapts itself to huge altitudinal diversity up to an elevation of 1300 m almost from sea level and to a large range of soils and climatic conditions, thus the genus has a vast interspecies diversity.[29] It is distributed all over the tropics and subtropics of the world.[30] It grows in hot and moist places. Since biblical times, the history of the pepper is well explored and

demonstrates the importance of the spice. Black pepper originates in Kerala in India. The other antediluvian countries growing the plant are Malagasy Republic, Sri Lanka, Brazil, Malaysia, South Pacific Islands, many South East Asian countries, Latin America and some African countries. In terms of tonnage, India is the largest producer (average 191,000 tonnes per annum). The country having highest productivity in terms of kg per ha is Thailand (3595). As per the report of the International Trade Centre (Geneva), pepper has calculated the current trade in spices to be 400,000-450,000 metric tonnes valued at US dollars 1.5-2.0 billion per annum. It contributes 34% of the total trade in spices.[32]

Pharmacognosy:

Macroscopic: The entire fruit is nearly spherical, brown-black in colour, 4-6.5 mm diameter; the surface is scratchy and aromatic with a pungent taste.

Pharmacology: It is used as nervine tonic and against stultification, itchiness and flatulency.[30] It is familiar for large number of therapeutic properties such as analgesic, antifeedant activities and antipyretic[31], antiplatelet, antifungal, antidepressant and anxiolytic[33], anti-tumor, anti-bacterial, anti-inflammatory, anticonvulsant, antioxidant and hepatoprotective activities.[34]

Phytochemistry: The research in the field of Chemistry reveals that the genus piper has a great phytochemistry including long and short chain esters, alkaloids, flavonoids, terpenes, steroids, unsaturated amides, lignans, aristolactams and propenyl phenols.[35]

The alkaloid piperine is the biochemical component that attributes pungency to black pepper while the components responsible for its aroma and flavour are myrcene, sabinene, a- and b-pinenes, camphene, b-caryophyllene and limonene, etc.

Many components of essential oil and its variability in various black pepper cultivars are found by investigators. Few researchers described the significant components of essential oil of *Piper nigrum* as limonene, b-pinene, b-caryophyllene and sabinene. Elemol was present in ample quantities in *P. nigrum* leaf oil. Limonene, Germacrene-D, a-pinene, b-pinene and b-ocimine are present in major amounts in black pepper. Major components such as b-pinene, a-phellandrene and a-humulene as well as minor constituents such as elemicin, d-carene, b-phellandrene, bulnesol, T-muurolol and cubenol are significant for the characteristics odour of pepper. The major phytoconstituent contributes pungency to pepper oleoresin is piperine. The various phytoconstituents reported from the acetone extract of pepper are guineensine (3.23%), piper amide (3.4%), piperolein b (13.7%) and

piperine (33.5%). Studies on essential oil of *Piper nigrum* leaves collected from Western Ghats of Kerala and Karnataka shows large diversity in nerolidol and b-caryophyllene. Diversity in chlorophyll and carotene percentage in the leaves of *Piper nigrum* over a narrow geographical range shows spatial influence in its biochemical activities. The phenolic compounds present in leaves range from 0.3 to 0.8 mg/g. Many authors have reported that different black pepper cultivars have different amount of b-caryophyllene in berry oil. However research showing correlation between leaf and fruit metabolites in black pepper cultivars is not available[36]

Various species of genus piper have enjoyed considerable importance in Ayurvedic medicine numerous ailments, which has attracted the attention of research workers throughout the world. Attention is directed towards the evaluation of pharmacological and chemical properties and an extensive study of the different constituents of various species has been made in the last two decades. A galaxy of chemically active constituents have been isolated and characterized by research workers. These include hydrocarbons, steroids, pyrones and chalcones like kawain and flavokawain, o-allyl compounds like chavicol, chavibetol and lignans such as cubenin, sesamin, eudesmin, diaeudesmin, clusin, epieudesmin, piperine, piperettine, cyclohexane epoxides like pipoxide, croteepoxide and isobutyl amides. The various *Piper* species and the phytochemicals obtained from them are shown in table 2.1. The leaves and seeds of *Piper nigrum* are given in figure 2.I and 2.II respectively.

Table 2.I: Various piper species and phytochemicals isolated from them are as follows[37]

S. No.	Name of the species	Compounds isolated and identified
1	*P. aduncum*	1-allyl-2,3-(methylenedioxy)-4, 5-dimethoxybenzene, 4-methoxy-3,5-bis(3'methyl-2'-butenyl) benzoic acid.
2	*P. amalgo*	5'-methoxy-3',4'-methylene dioxy-cinnamic acid pyrrolidide, 2-methoxy-4,5-methylenedioxy trans-cinnamoyl piperidide, 2-methoxy-4,5-methylenedioxy-trans cinnamoyl pyrolidide.
3	*P. cubeba*	(-)cubebinone, (-)isoyatein, (-)yatein, (-)thujaplicatin trimethyl ether, (-)cubebininolide, (2R, 3R)-2-(3", 4"-methylene-dioxybenzyl)-3-(3',4'-dimethoxybenzyl) butyrolactone.
4	*P. futokadsura*	Kadsurenone, Kadsurin A, Kadsurin B.
5	*P. hancei*	Hancinone, Kadsurenone, denudatin B.
6	*P. hispidum*	1-allyl-2,3(methylenedioxy)-4,5-dimethoxy benzene, 4-methoxy-3,5-bis(3'-methyl-2' butenyl) benzoic acid.
7	*P. lenticellusom*	Elemicin, isosafrol, o-methyl eugenol sarisan, 3,5-dimethoxytoluene, 2-methoxy-4,5-methylene dioxy benzaldehyde, Trans-2-methoxy-4,5-methylenedioxy benzaldehyde.
8	*P. retrofractum*	Retrofractamide A, retrofractamide B, retrofractamide C, retrofractamide D, sesamin.

Fig. 2.I Leaves of *Piper nigrum*

Fig. 2.II Seeds of *Piper nigrum*

Seeds of plant are of great importance in terms of chemical composition. So far almost all the compounds isolated from plants have been isolated from either fresh fruits or seeds. The seeds have always been an interesting subject of investigation for researchers. Various chemical components have been reported from the seeds of *Piper nigrum.*

Volatile oil: Volatile oils include sabinene, limonene and a- and b-pinenes as the major components. Caryophyllene is sesquiterpene hydrocarbon obtained from the pepper oil in majority while eugenol, myristicin and safrole are also present.[33]

Alkaloids and Amides: The pungent nature of *Piper* has been considered due to the presence of alkaloids and amides. These are isopiperolein B[31], Pellitorine[39], Retrofractamide A, pipercide, piperchabamide D and dehydropipernonaline[40], Dipiperamides- A, B and C.[41] The other phytochemicals isolated are sesamin, pipataline, guineensine, brachystamide B, pellitorine, 5-hydroxy-7, 4-dimethoxyflavone, diaeudesmin, 5-hydroxy-7, 3, 4-trimethoxyflavone, dihydropiperlonguminine and 7, 4-dihydroxy-5-methoxyflavone.[38]

Medicinal Uses of Pepper*:*

Although many plants have been reported for their specific medicinal property but *Piper nigrum* is one of them.

Pepper is identified as a medicine that helps in digestion, increases appetency, acts as a remedy for cough, cold, throat diseases, colic, dyspnoea, intermittent fever, dysentery, worms and piles. It is not only used as a spice but also have a broad spectrum antimicrobial property. Piperine proves as an active component possessing antipyretic, analgesic and anti-inflammatory actions. Pepper and piperine protects liver enzymes and create no toxicity in liver. Peppers have significant mutagenic and carcinogenic properties based on the Ames test. Pepper phenolic amides are better antioxidants as compared to the synthetic compounds BHT and BHA.[32]

2.2: Materials and Methods:

2.2.1 Plant Material: Seeds of *Piper nigrum* were bought from an authentic seed shop of Kathua district of Jammu and Kashmir and classified systematically by Dr. Gurdev Singh of the Botany Department at Lovely professional university.

2.2.2 Experimental:

Analytical tools: The solvent apparatus (JSGW) was used for the extraction of plant material. The melting point was found out on Lab fit melting point apparatus. A UV spectrum was obtained on SHIMADZU UV-1800 UV spectrophotometer and the solvent used is ethanol. An IR spectrum was recorded on SHIMADZU FTIR-8400S (Fourier Transforms infrared spectrophotometer). ^{1}H-NMR (400MHz) and ^{13}C-NMR were recorded in MeOD on Bruker, Avance 400 MHz NMR Spectrometer. Chemical shifts are given as δ with TMS as internal standard. A HR-MS was obtained on Agilent, 6540, Q-TOF (HR-MS) mass spectrometer. The essential oil was examined by using Varian 4000 GC-MS. The instrument works on the following conditions: fitted with fused silica 30 m (CP-Sil-8,Varian), the internal diameter and film thickness of capillary column are 0.25 mm and 0.25 µm, the Helium carrier gas is introduced at a rate of 1 ml/min, a capillary injector operating at 280°C in the split mode (1:150), flame ionization detector (FID) running at temperature 300°C, the column oven temperature programming was 50°C for 5 min and then enhanced from 50 to 250°C at the rate of 3°C/min and hold for 7 min. X-Ray diffraction analysis was performed using X-Ray diffractometer (Rigaku Co. Ltd. Japan) with Mo Kα radiation with wavelength 0.7103 A^{o} in the range of Bragg's angle $3.59<\theta<26.00^{o}$ at room temperature.

Cold Extraction: The seeds (one kg) of *Piper nigrum* were washed, dried and crushed in different solvents on the basis of polarity such as petroleum ether, toluene, dichloromethane, chloroform, ethyl acetate, ethanol and water for 120 hours to prepare crude extracts.

Hot Extraction: The washed, dried and mashed *Piper nigrum* seeds (one kg) were soxhlated in ethanol with different solvents according to their increasing polarity gradient such as petroleum ether, toluene, dichloromethane, chloroform, ethyl acetate, ethanol and water. The various crude extracts are then distilled to remove excess of solvent. Petroleum ether, DCM, chloroform, ethyl acetate extracts separated into oils which were further investigated for the presence of phytoconstituents and results are given ahead.

A. Analysis of antimicrobial activity of all the hot extracts of *Piper nigrum*:

Bacterial strains: The biological activities of different hot extracts were tested against bacteria *E. coli* (NCIM No.- 2563), *Bacillius subtilis* (NCBI No.- PS3 KJ489411.1) *Pseudomonas aeruginosa* (NCBI No.- jogii JX276925.1) and fungus *Aspergillus niger* (NCIM No.- 619). These bacterial cultures are ordered from NCIM Pune and maintained at 4°C on nutrient broth in Biotechnology labs of LPU. Potato dextrose broth is used for fungal culture.

Chemicals: Nutrient broth and nutrient agar for bacterial growth; potato dextrose agar, potato dextrose broth for fungal growth and standard antibiotic like gentamicin were purchased from Hi Media Laboratories Pvt. Ltd., Mumbai.

Composition of nutrient broth:

1. Peptic digest animal tissue-5 gm/lt
2. Sodium chloride powder-5 gm/lt
3. Beef extract-1.50 gm/lt
4. Yeast extract-1.50 gm/lt
5. Distilled water- 1 lt
6. P^H- 7.4 ± 0.2 at 25°C

Composition of nutrient Agar:

1. Agar- 15 gm/lt
2. Peptic digest animal tissue-5 gm/lt
3. Sodium chloride powder- 5 gm/lt
4. Beef extract-1.50 gm/lt
5. Yeast extract- 1.50 gm/lt
6. Distiller water- 1 lt
7. P^H- 7.4 ± 0.2 at 25°C

Composition of potato dextrose broth

1. Potato peeled and diced into small pieces- 200 gm/lt
2. Glucose- 20 gm/lt
3. Distilled water- 1000 ml

Composition of potato dextrose agar

1. Agar- 15gm/lt
2. Potato peeled and diced into small pieces- 200 gm/lt
3. Glucose- 20 gm/lt
4. Distilled water- 1000 ml

Preparation of nutrient agar medium: Dissolve 28 grams of nutrient agar in 1000 ml distilled water. Gently heat to dissolve the components completely. The nutrient agar medium was then sterilized in an autoclave at 15 psi (121°C) for 15 minutes and then cooled at room temperature in a laminar. After cooling it was decanted in the sterilized petriplates and placed in laminar till solidification.

Antimicrobial activity of plant extracts: One gram of the extracts was dissolved in same solvent in such a way that the final concentration of each extract would be 1gm/ml of respective solvent.

Disc diffusion method: The *in vitro* antimicrobial activity of various extracts of pepper was checked by disc diffusion method. Bacterial culture was inoculated in nutrient agar and plated. The 5 µl of various extracts were poured on to different discs prepared from whatman No: 1 filter paper. The 2 or 3 discs were then placed on the petriplates containing cultures and incubated bacterias for 24 hours at 37°C. The diameter of zone of inhibition was measured.[25]

B. Insecticidal activity of hot petroleum ether and ethanol extracts of *Piper nigrum* seeds

The insecticidal activity of nonpolar petroleum ether and polar ethanol extracts of *Piper nigrum* L. (Piperaceae) was investigated against agricultural insect "*Coccinella septumpunctata"* and rice weevil "*Sitophilus oryzae"* in a laboratory. Insecticidal activity was find out at a temperature of 25 ± 4°C and 68 ± 5% relative humidity in dark conditions. The mortality rate of both the insects was checked against 1%, 5% and 10% concentration of petroleum ether and ethanol extracts. Finding insecticidal activity is of great importance as using plant extracts as insecticides are biodegrable and do not produce toxicity hence are ecofriendly.

Insecticidal Testing: The petroleum ether and ethanol extracts were prepared at three different concentrations (1 %v/v, 5%v/v, 10%v/v).

(i). Insect Assayed: Test insects: The Epilachna insect *Coccinella septumpunctata* were collected from the fields and rice weevil *Sitophilus oryzae* were collected from the rice mill and identified by Entomologist Dr. Sudhakar Gupta of Lovely Professional University.

(ii). Determination of Insecticidal Activity by Contact toxicity assay: The Wheat leaves (for Epilachna insect) and rice grains (for rice weevil) are taken from the field and houses, washed and dried and then insects were allowed to feed on them under controlled conditions of temperature and humidity. These washed and dried wheat leaves and rice grains are then dipped in the respective percentage of plant extracts (i.e. 1%, 5%, 10% v/v) for two hours and then insects are allowed to feed on these leaves in each jar and mortality rate of the insects was checked. Results were recorded by counting the number of survivals in each jar. Control dishes with petroleum ether, ethanol, distilled water and without solvent were performed separately up to 72 hours. Mortality was assessed after 3, 4, 7 and 12 h of the treatment. The calculation of mortality rate was corrected for control mortality according to Abbott's formula[45]:

$$Mc = (Mo-Mc/100-Me) * 100$$

Where, Mo = Observed mortality rate of treated adults (%), Me = mortality rate of control (%), and Mc = corrected mortality rate (%).

C. Antidiabetic activity of hot petroleum ether and ethanol extracts of *Piper nigrum* seeds

Diabetes mellitus is a most common cause of death illness, major health and economic trouble worldwide characterized by increase in blood glucose level due to abnormality in action or production or both of insulin hormone. It is affecting 6% of total population in the world and 7% of the U.S.A population. Diabetes type 2 is the most common and involves 90-95% of total diabetics. The 60-90 % of the people is obese but not all when the disease is diagnosed. Worldwide projection suggests that the global population of diabetic patients reach 300 million in the year 2025 and it costs around one trillion US dollars annually to treat diabetes and complications associated with it. The disease is characterized by chronic hyperglycemia due to deficiency of insulin production or its action on the target tissue.[48] WHO reports about one lakh sixty thousand diabetics globally and the number would become double in the last few years and again expected to get double by the year 2025.[49]

Diabetes mellitus is a common metabolic disease around the world. The disease is induced by stressful life style, fast food eating, lack of exercise and genetic makeup. Diabetes and its related complications are closely related with the oxidative stress of the body. Diabetes is closely interlinked with cardiovascular and renal disorder at advanced stage and creates fatal disease syndrome.[47]

The search for novel, bioactive therapeutic agents to treat various diseases is still going on. This makes the use of experimental models for the disease imperative.

Antidiabetic activity of nonpolar petroleum ether and polar ethanol extracts of pepper was determined in CSIR lab, IIIM Jammu on wistar rats by 18h Fasted rats model.

***In vivo* models**: **18h Fasted rats model**

Four groups of six Wistar rats (male/female) were selected randomly and fasted overnight. First group was given Normal Vehicle Control, second and third groups were given petroleum ether and ethanol treated diet and fourth group was treated with reference (glibenclamide) treated group. Blood glucose level of all the rats was found out prior to any treatment (0h) and post-drug administration (3h).[46]

2.3: Results and Discussion

2.3.1 Cold Extraction: Cold extraction is performed as shown in the figure 2.III given below:

Fig. 2.III Cold extraction of *Piper nigrum* seeds

The various extracts separated after cold extraction of *Piper nigrum* seeds are:

A. Petroleum ether extract

B. Toluene extract

C. Dichloromethane extract

D. Methanol extract

A. Investigation of cold petroleum ether extracts of *Piper nigrum*

The petroleum ether extract kept for around two months, shows two different layers the upper oily layer [(A) fraction] and lower thick portion as a precipitate [(B) fraction]. The oily fraction on GC-MS analysis shown to be mixture of around fifteen components and the precipitate portion on the repeated process of crystallization gave shiny, pale yellow crystals (pn 1) of melting point 132°C.

1. Spectroscopic analysis of yellow crystals obtained from petroleum ether extracts [pn 1 from fraction B] : The shiny pale yellow crystals (m. p. 132°C) obtained from petroleum ether extract of *Piper nigrum* seeds was found to be an alkaloid on performing Dragendroff test.[42] The alkaloid framework was also supported by UV and IR spectroscopy. TLC of the crystals which were recrystallised using ethanol showed Rf value equals to 0.23 that is similar to Rf value of piperine observed in literature was 0.25[34] thus crystals obtained may be of piperine. TLC plate is shown in figure 2.IV.

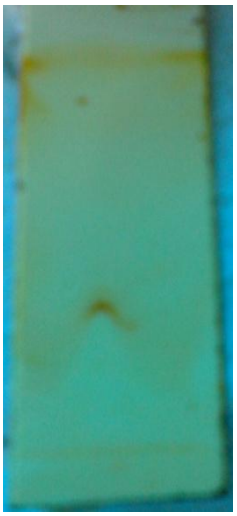


Fig. 2.IV TLC of the crystals in 1:4 petroleum ether: chloroform as solvent system

The molecular formula was established as $C_{17}H_{19}O_3N$ by Agilent, 6540 Q-TOF (HRMS) mass spectrometer.

1. **UV spectrum of pn 1:** The presence of an alkaloid framework was suggested by the UV spectrum (Fig. 2.V), showing absorption at 342 nm.[43] The peak given is taken from the UV spectra.

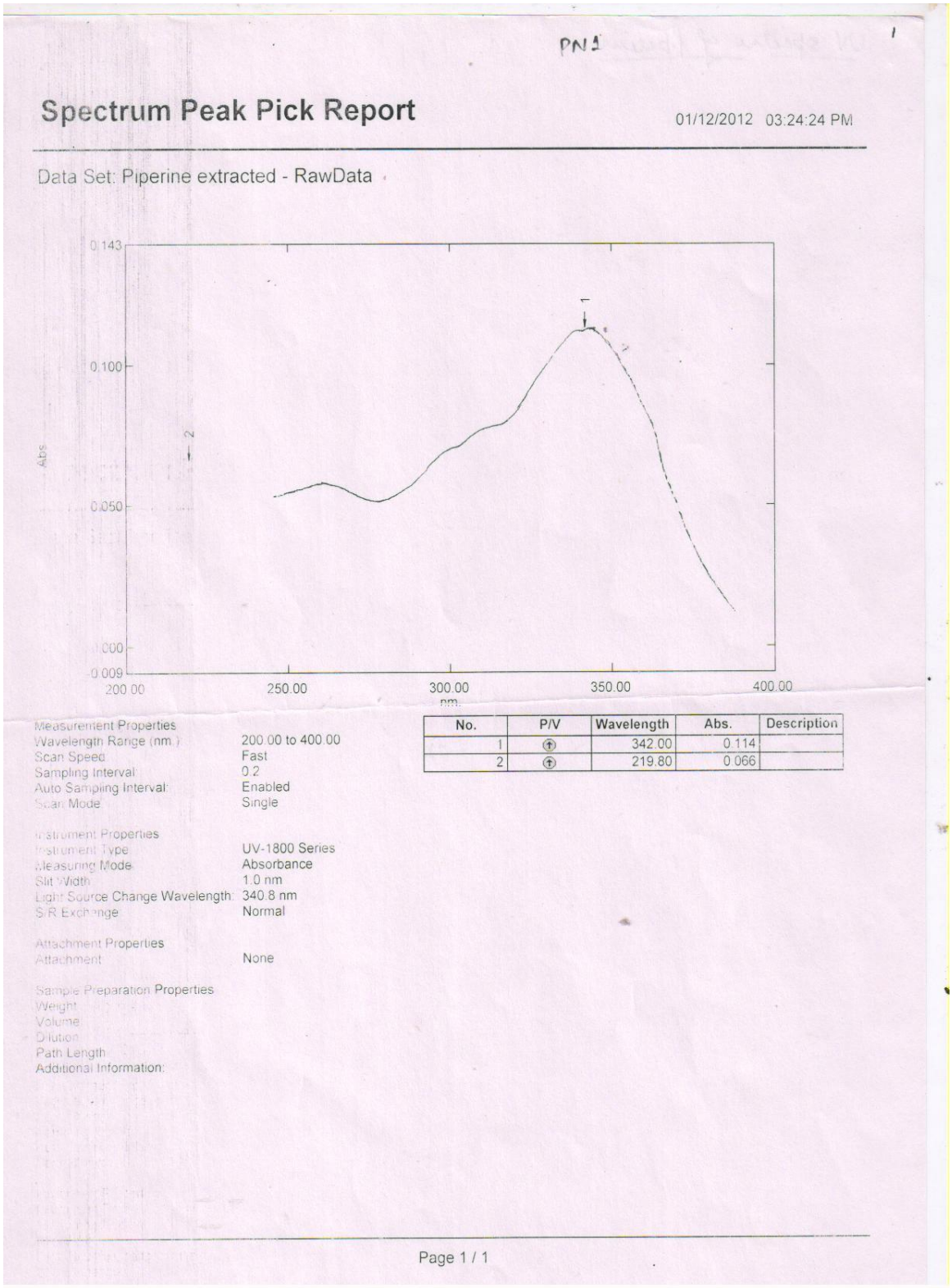
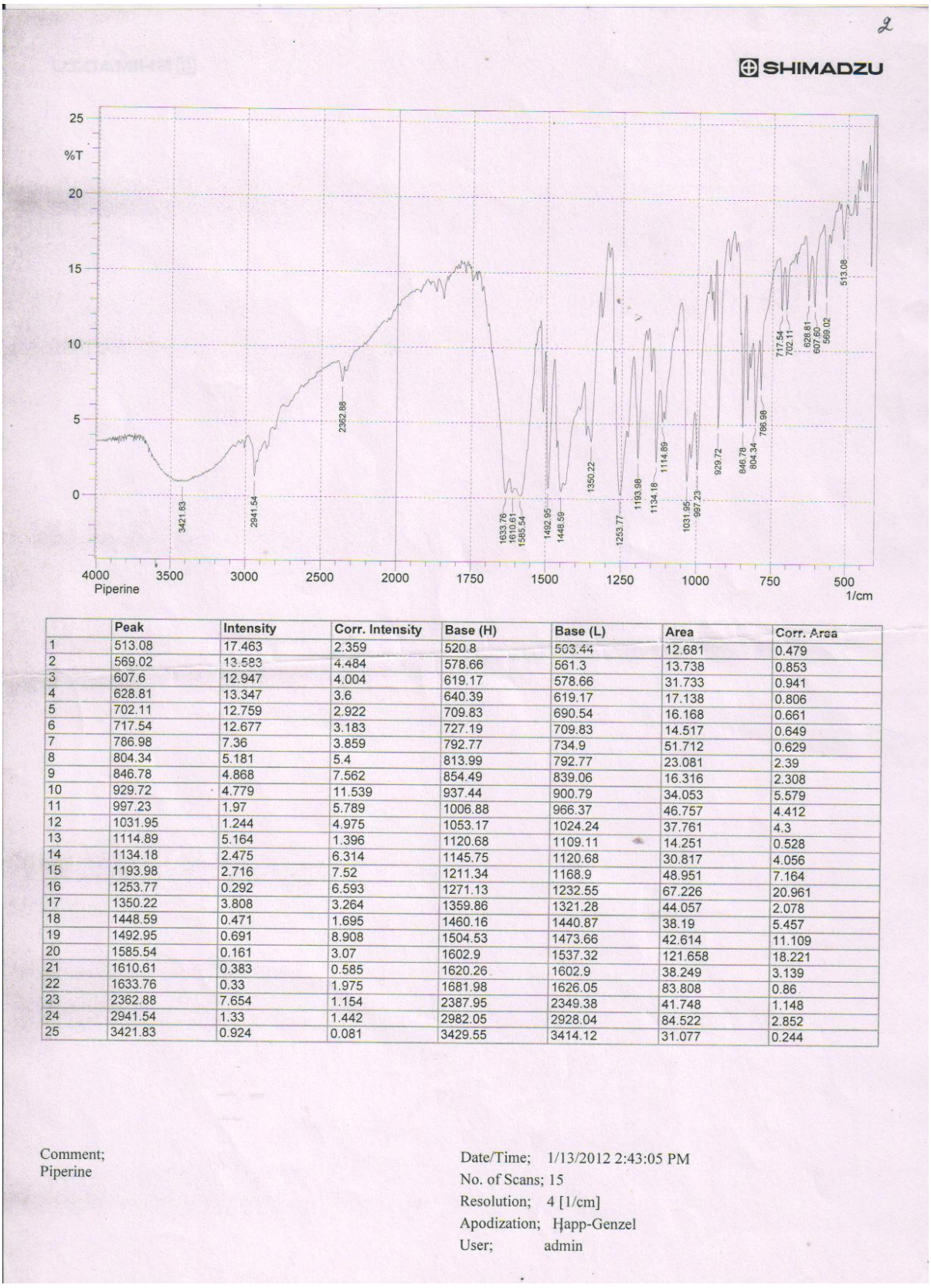


Fig. 2.V UV spectrum of yellow crystals from cold petroleum ether extracts

2. **IR spectral detail of pn 1:** The IR spectrum (Fig. 2.VI) showed the presence of absorption bands at 3421 cm^{-1} (intermolecular H-bonding), 3000cm^{-1} (aromatic C-H stretch), 1633 and 1610 cm^{-1} (symmetric and asymmetric stretching of c=c of dienes), 1610,1585,1492 cm^{-1} (aromatic stretching of c=c of phenyl ring), 1633 cm^{-1} (stretching of -CO-N group), 2941 and 2859 cm^{-1} (CH_2 asymmetric and symmetric stretching), 1448 cm^{-1} (CH_2 bending), 1253 and 1193 cm^{-1} (asymmetric stretching of =C-O-C), 929 cm^{-1} (C-O stretching), 1134 cm^{-1} (in plane bending of phenyl CH), 997 cm^{-1} (CH bending for trans –CH=CH-), 848, 830 and 804 cm^{-1} (out of plane C-H bending). It resembles to the IR spectra of piperine given in literature.[43]



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	513.08	17.463	2.359	520.8	503.44	12.681	0.479
2	569.02	13.583	4.484	578.66	561.3	13.738	0.853
3	607.6	12.947	4.004	619.17	578.66	31.733	0.941
4	628.81	13.347	3.6	640.39	619.17	17.138	0.806
5	702.11	12.759	2.922	709.83	690.54	16.168	0.661
6	717.54	12.677	3.183	727.19	709.83	14.517	0.649
7	786.98	7.36	3.859	792.77	734.9	51.712	0.629
8	804.34	5.181	5.4	813.99	792.77	23.081	2.39
9	846.78	4.868	7.562	854.49	839.06	16.316	2.308
10	929.72	4.779	11.539	937.44	900.79	34.053	5.579
11	997.23	1.97	5.789	1006.88	966.37	46.757	4.412
12	1031.95	1.244	4.975	1053.17	1024.24	37.761	4.3
13	1114.89	5.164	1.396	1120.68	1109.11	14.251	0.528
14	1134.18	2.475	6.314	1145.75	1120.68	30.817	4.056
15	1193.98	2.716	7.52	1211.34	1168.9	48.951	7.164
16	1253.77	0.292	6.593	1271.13	1232.55	67.226	20.961
17	1350.22	3.808	3.264	1359.86	1321.28	44.057	2.078
18	1448.59	0.471	1.695	1460.16	1440.87	38.19	5.457
19	1492.95	0.691	8.908	1504.53	1473.66	42.614	11.109
20	1585.54	0.161	3.07	1602.9	1537.32	121.658	18.221
21	1610.61	0.383	0.585	1620.26	1602.9	38.249	3.139
22	1633.76	0.33	1.975	1681.98	1626.05	83.808	0.86
23	2362.88	7.654	1.154	2387.95	2349.38	41.748	1.148
24	2941.54	1.33	1.442	2982.05	2928.04	84.522	2.852
25	3421.83	0.924	0.081	3429.55	3414.12	31.077	0.244

Comment;
Piperine

Date/Time; 1/13/2012 2:43:05 PM
No. of Scans; 15
Resolution; 4 [1/cm]
Apodization; Happ-Genzel
User; admin

Fig. 2.VI IR spectrum of yellow crystals obtained from petroleum ether extracts

3. **^{1}H-NMR spectrum of pn 1:** The 400 MHz ^{1}H NMR (Fig. 2.VII and 2.VIII) spectrum showed methylene dioxy signal at δ 5.9 (2H, s, O-CH_2-O) and other hydrogen atoms signals at δ 7.3 (1H, ddd, J=10.5, 4.12, 10.53 Hz, H-3), δ 6.63 (1H, d J=15 Hz, H-2), δ 3.3-3.6 (4H,m, H-c), δ 1.6- 1.7 (5H,m,H-a,b).

Fig. 2.VII ^{1}H-NMR spectrum of yellow crystals obtained from pet. ether extracts

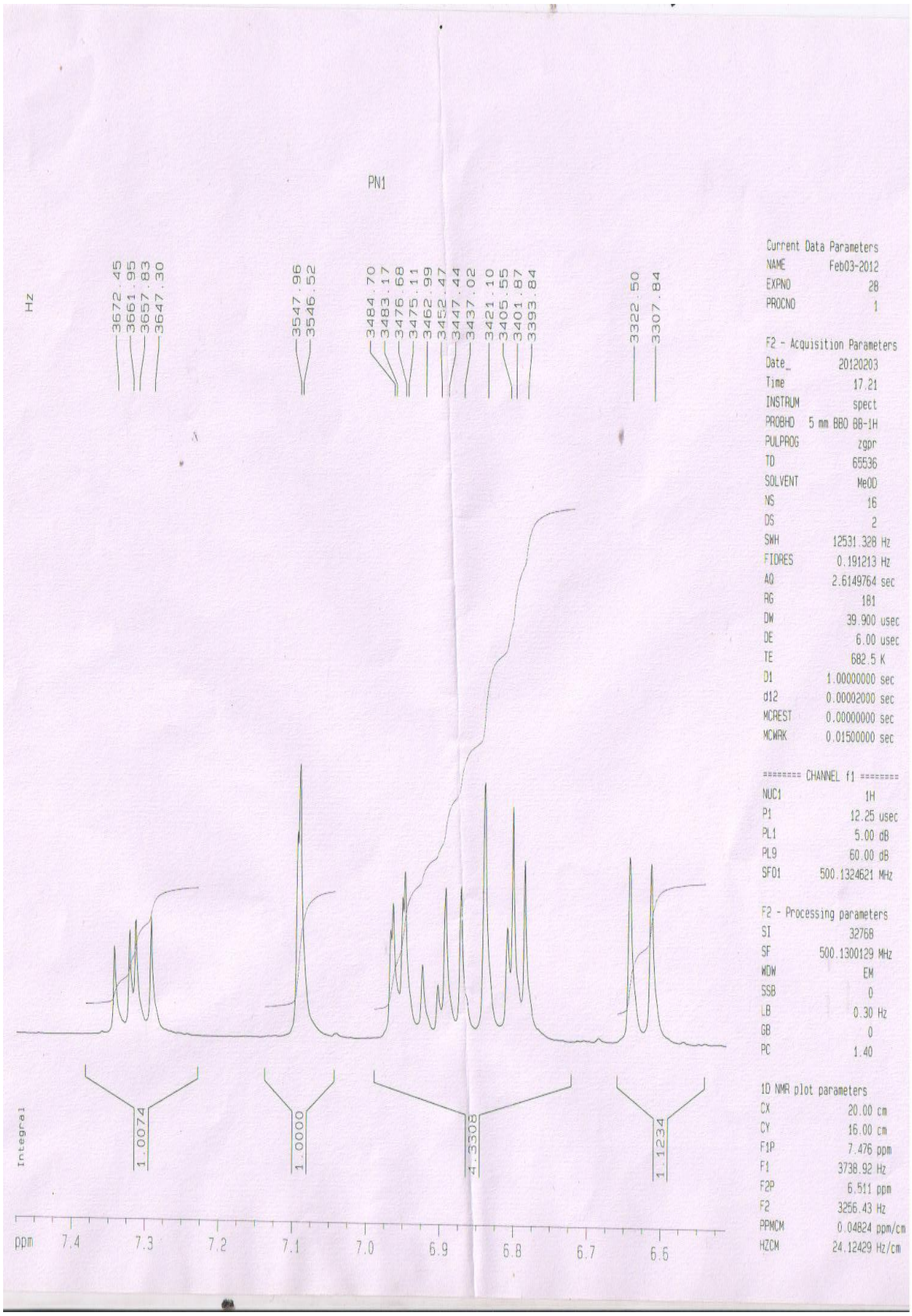


Fig. 2.VIII Magnified ^{1}H-NMR spectrum of pn 1

4. **^{13}C-NMR spectrum of pn 1:** The ^{13}C NMR spectrum (Fig. 2.IX) showed the peaks at 167.7 for C-1(C=O), δ 120.6, δ 140.2 for C-2 and C-3(C=C), δ 123.9, 132.4 for C-4 and C-5(C=C), δ 106.7 (C-2'), δ 149.7-149.8 for C-3 and C-4'(C=C), δ 109.4, δ 207 for C-5'and C-6'(C=C), δ 102.7 (C-7') giving the information of C=O, C=C and other carbon atoms. The DEPT spectrum showing primary, secondary or tertiary carbons of pn 1 is given in figure 2.X.

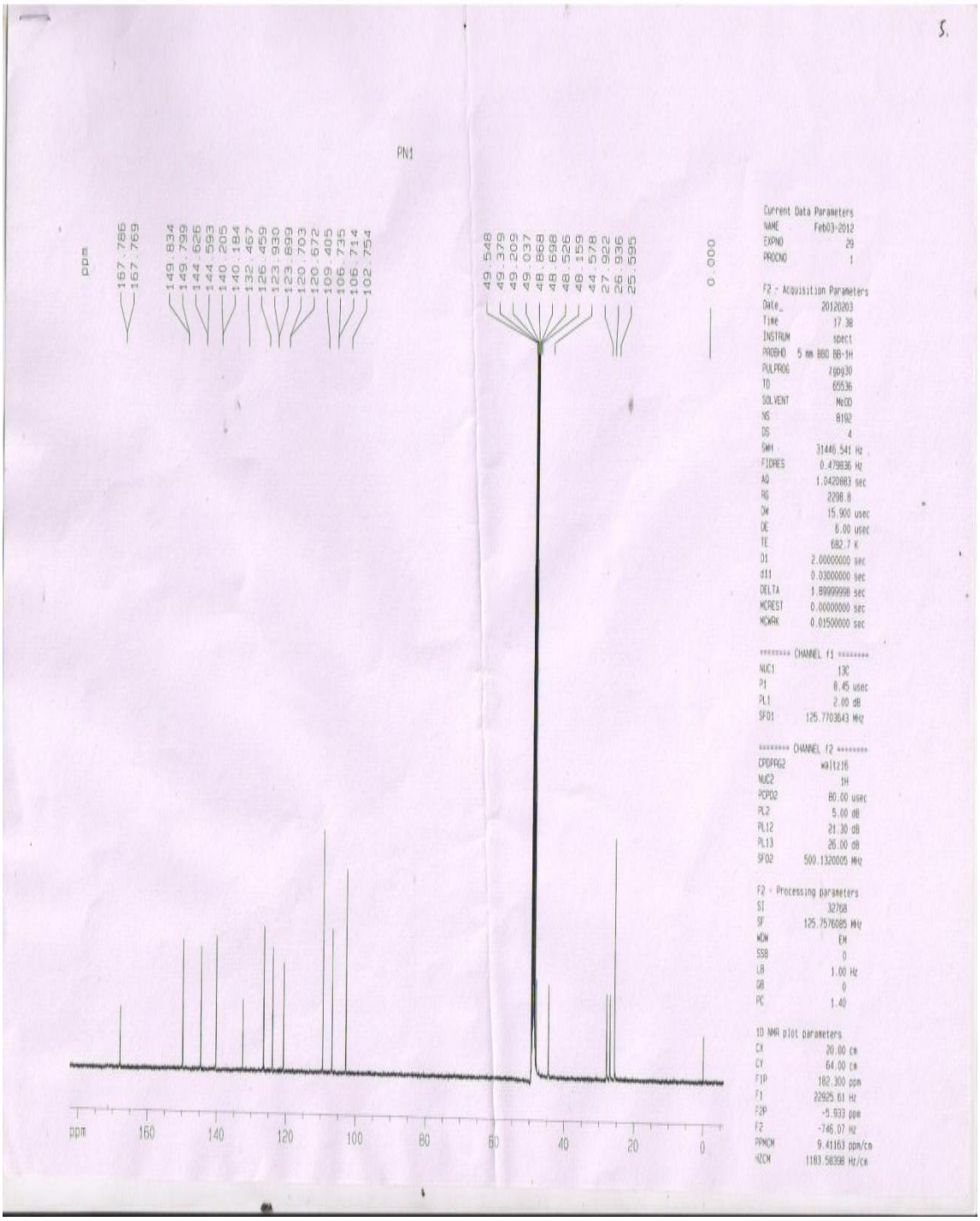


Fig. 2.IX ^{13}C-NMR spectrum of pn 1

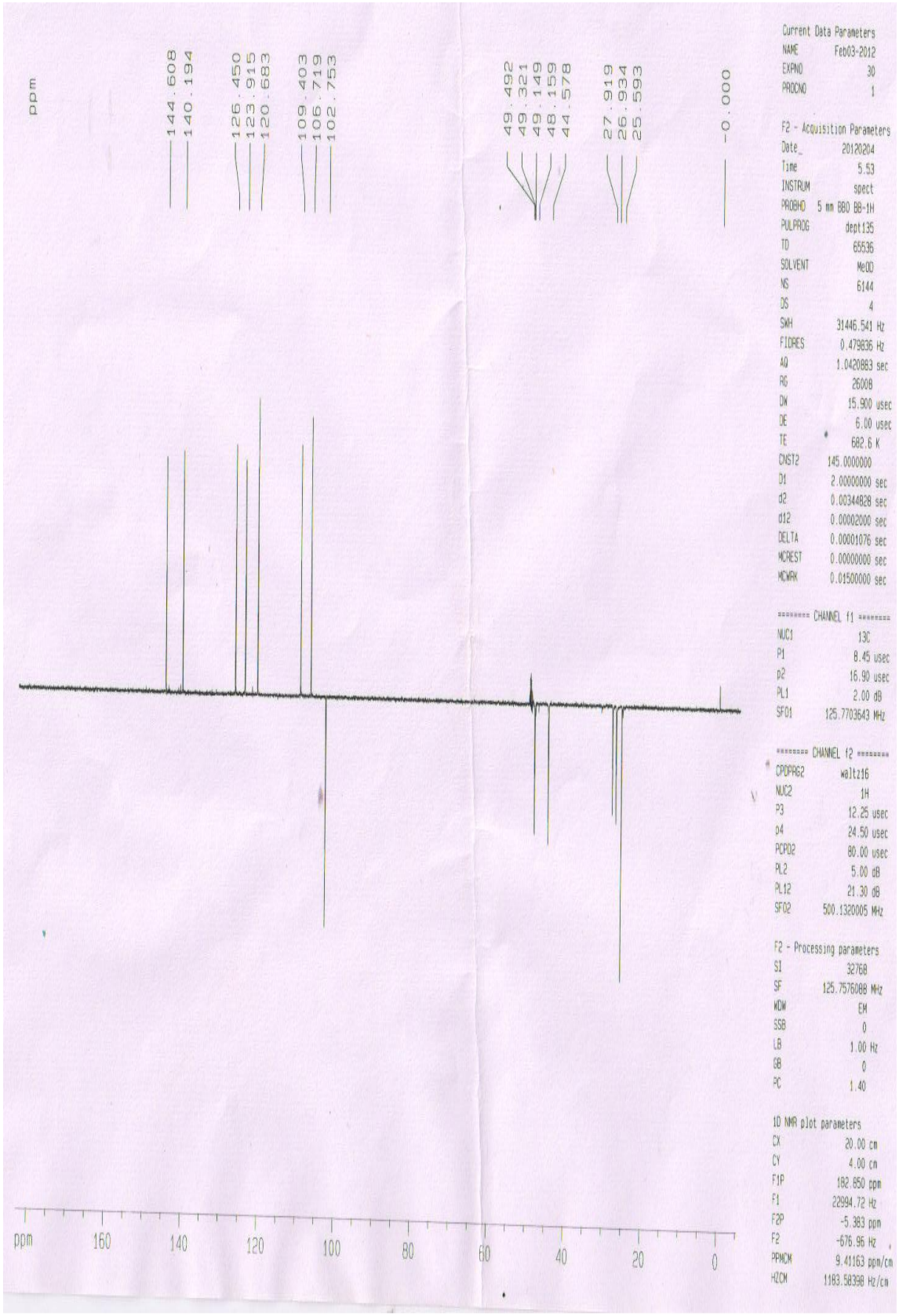


Fig. 2.X DEPT spectrum of yellow crystals obtained from petroleum ether extracts

5. **Mass spectrum of pn 1:** The HR-mass spectrum (Fig. 2.XI) showed a $[M+H]^+$ peak at 286.14,$[2M+H]^+$ peak at 571.2 and $[2M+Na]^+$ peak at 593.2.

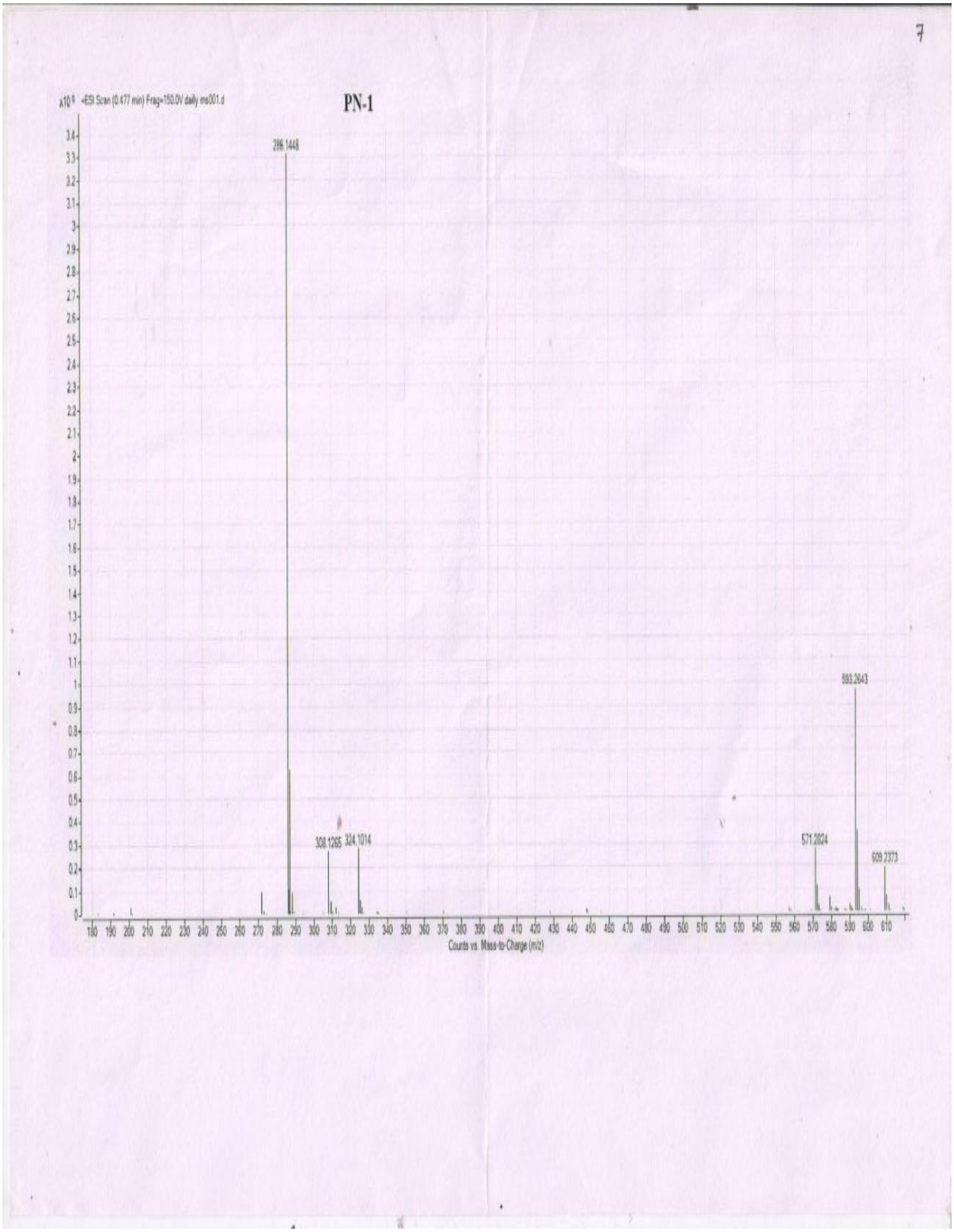


Fig. 2.XI Mass spectrum of pn 1

On the basis of above spectral studies and chemical analysis it is expected that the isolated shiny yellow shiny rod shaped crystals may be of piperine. Picture of isolated crystals and structure is given in figure 2.XII and 2.XIII respectively.

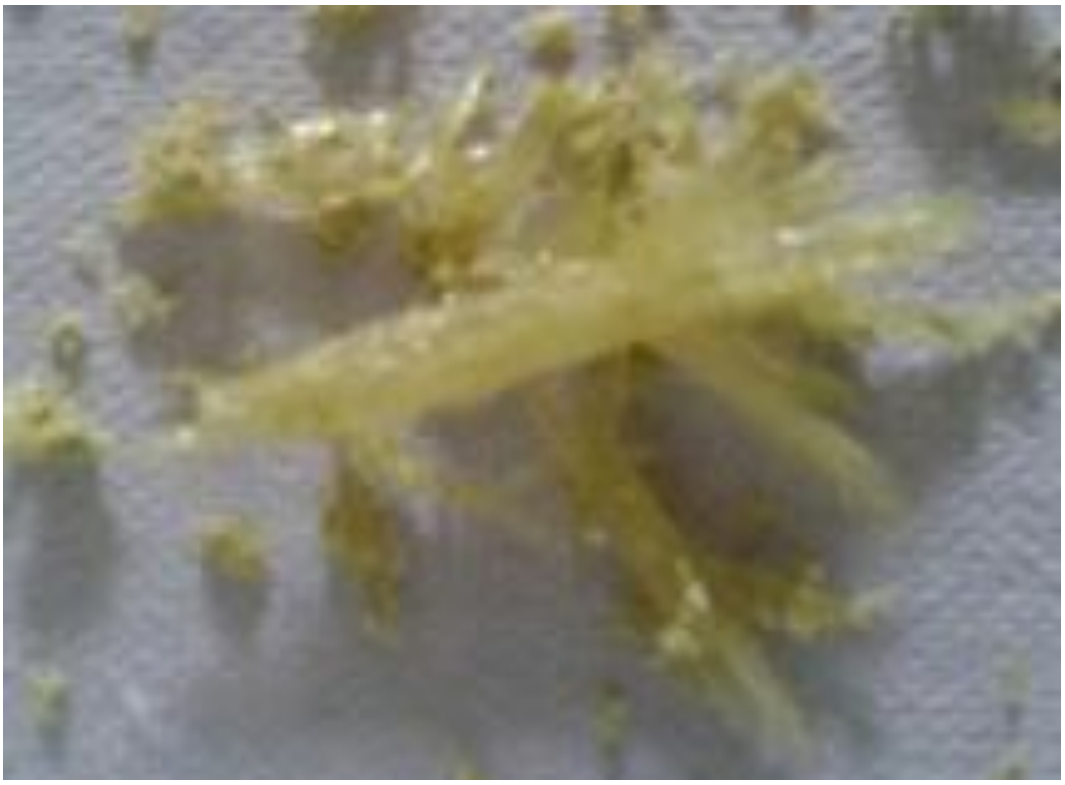


Fig. 2.XII Piperine crystals

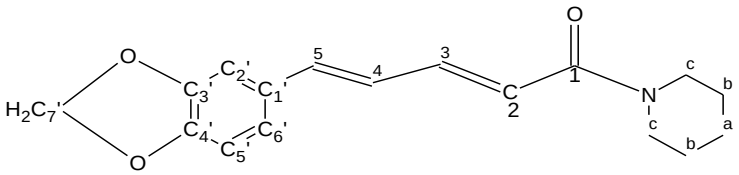
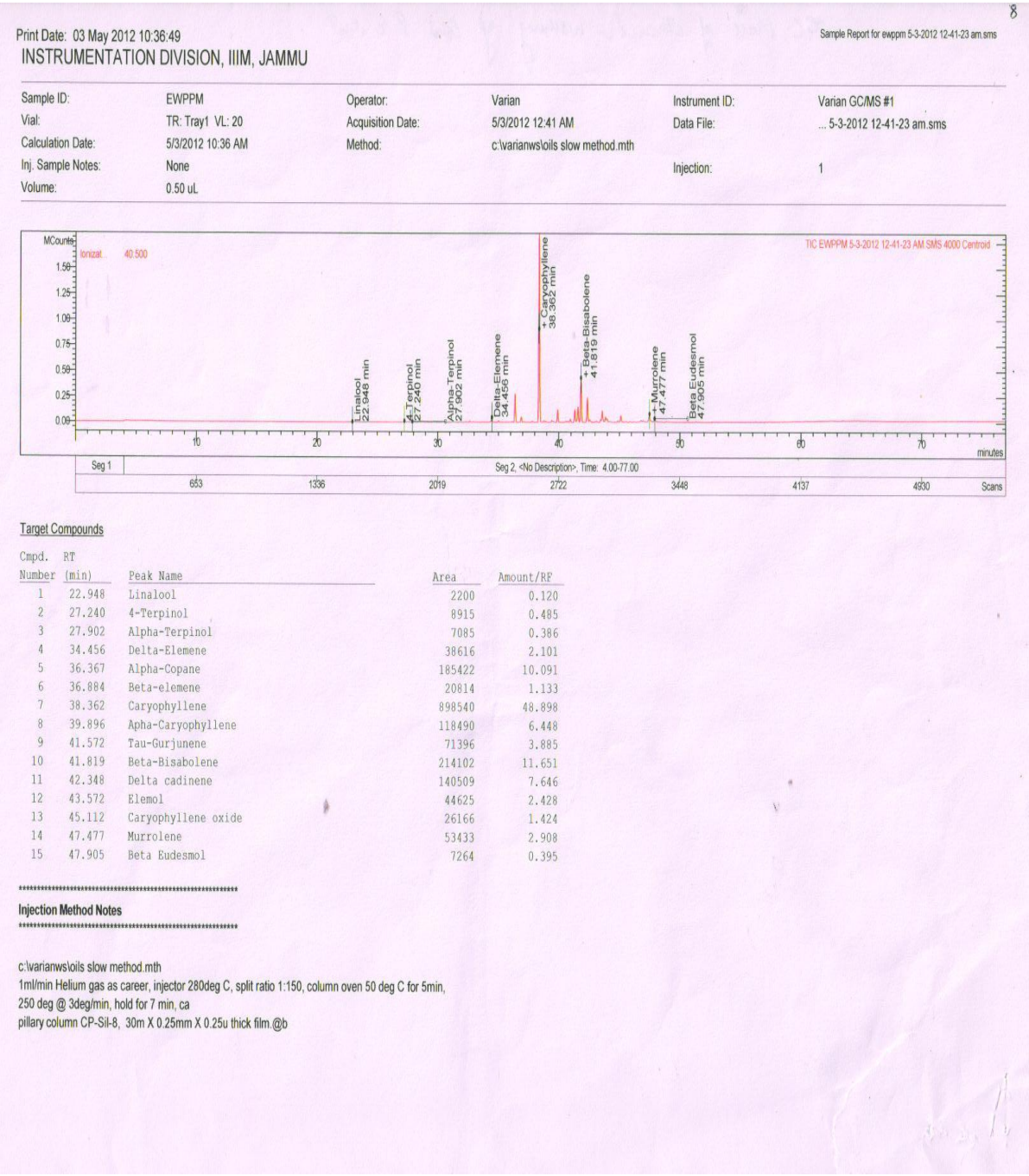


Fig. 2.XIII Piperine structure

2. GC-MS analysis of oily fraction separated from petroleum ether extract [Fraction A]: Compounds were identified by their GC retention time relative to known compounds and by comparison of their mass spectra with those present in IIIM library**.** The GC-MS spectrum (Fig. 2.XIV) of the oily fraction of petroleum ether extracts of *Piper nigrum* unveiled the presence of following components.



Target Compounds

Cmpd. Number	RT (min)	Peak Name	Area	Amount/RF
1	22.948	Linalool	2200	0.120
2	27.240	4-Terpinol	8915	0.485
3	27.902	Alpha-Terpinol	7085	0.386
4	34.456	Delta-Elemene	38616	2.101
5	36.367	Alpha-Copane	185422	10.091
6	36.884	Beta-elemene	20814	1.133
7	38.362	Caryophyllene	898540	48.898
8	39.896	Apha-Caryophyllene	118490	6.448
9	41.572	Tau-Gurjunene	71396	3.885
10	41.819	Beta-Bisabolene	214102	11.651
11	42.348	Delta cadinene	140509	7.646
12	43.572	Elemol	44625	2.428
13	45.112	Caryophyllene oxide	26166	1.424
14	47.477	Murrolene	53433	2.908
15	47.905	Beta Eudesmol	7264	0.395

****
Injection Method Notes
****

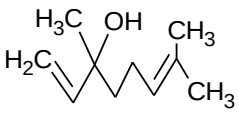
c:\varianws\oils slow method.mth
1ml/min Helium gas as career, injector 280deg C, split ratio 1:150, column oven 50 deg C for 5min,
250 deg @ 3deg/min, hold for 7 min, ca
pillary column CP-Sil-8, 30m X 0.25mm X 0.25u thick film.@b

Fig. 2.XIV GC-MS analysis of oil obtained from petroleum ether extracts of *Piper nigrum*

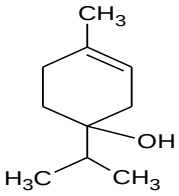
The compounds identified are (linalool, 4-terpinol, alpha terpinol, delta elemene, alpha copane, beta elemene, caryophyllene, alpha caryophyllene, tau gurjunene, beta bisabolene, delta cadinene, elemol, caryophyllene oxide, murrolene, beta eudesmol) displayed in table 2.II and Fig. 2.XV.

Table 2.II Various components obtained from GC-MS analysis of petroleum ether extracts

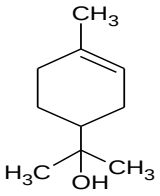
S. No.	RT (min)	Peak Name	Area	Amount/RF
1.	22.948	Linalool	2200	0.120
2.	27.240	4-terpinol	8915	0.485
3.	27.902	α-terpinol	7085	0.386
4.	34.456	δ-elemene	38616	2.101
5.	34.367	α-copane	185422	10.091
6.	36.884	β-elemene	20814	1.133
7.	38.362	Caryophyllene	898540	48.8998
8.	39.896	α-caryophyllene	118490	6.448
9.	41.819	β-bisabolene	214102	11.651
10.	42.348	δ-cadinene	140509	7.646
11.	43.572	Elemol	44625	2.428
12.	45.112	Caryophyllene oxide	26166	1.424
13.	47.477	Murrolene	53433	2.908
14.	47.905	β-eudesmol	7264	0.395
15.	41.572	τ-gurjunene	71396	3.885



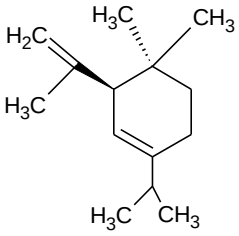
(1) linalool



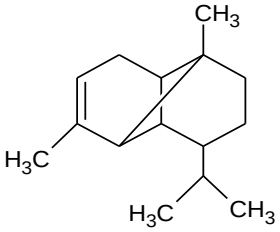
(2) 4-terpinol



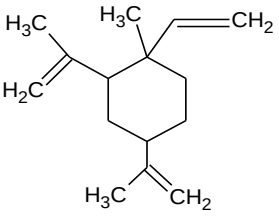
(3) alpha terpinol



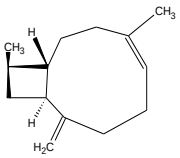
(4) delta elemene



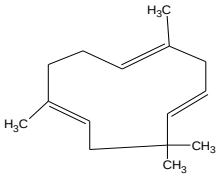
(5) alpha copane



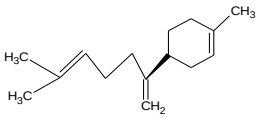
(6) beta elemene



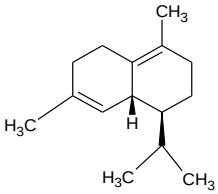
(7) caryophyllene



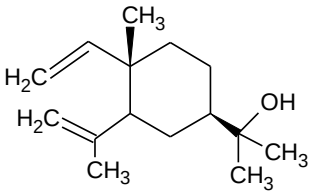
(8) alpha caryophyllene



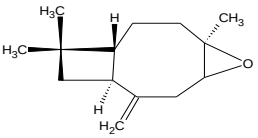
(9) beta bisabolene



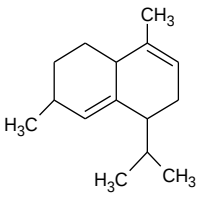
(10) delta cadinene



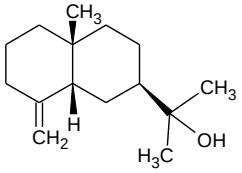
(11) elemol



(12) caryophyllene oxide



(13) Murrolene



(14) beta eudesmol

Fig. 2.XV Structures of various components obtained by GC-MS analysis of oil of petroleum ether extracts

B. Investigation of cold toluene extracts of *Piper nigrum*

Toluene extract kept for around three and half months, separated into upper brown coloured oily layer [fraction (A)] and rod shaped shiny pale yellow crystals (Fig. 2.XVI) settled at the bottom of the beaker [fraction (B)]. The crystals obtained were further identified on the basis of their spectral studies. The oil obtained was subjected to GC-MS analysis for identification of phytoconstituents.

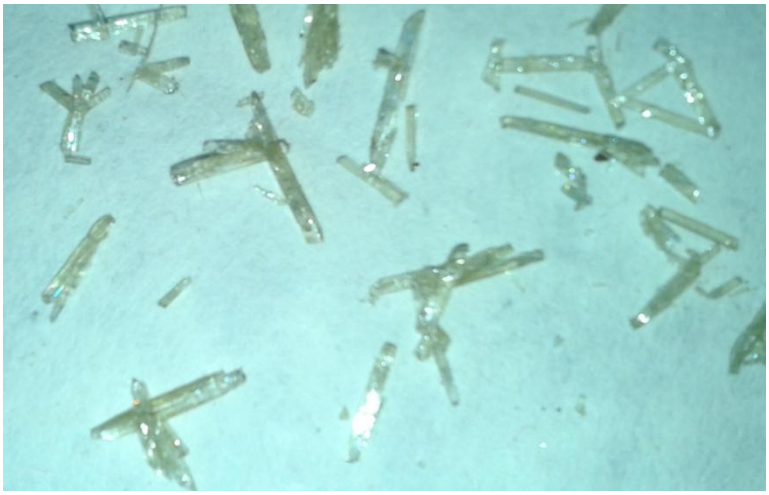
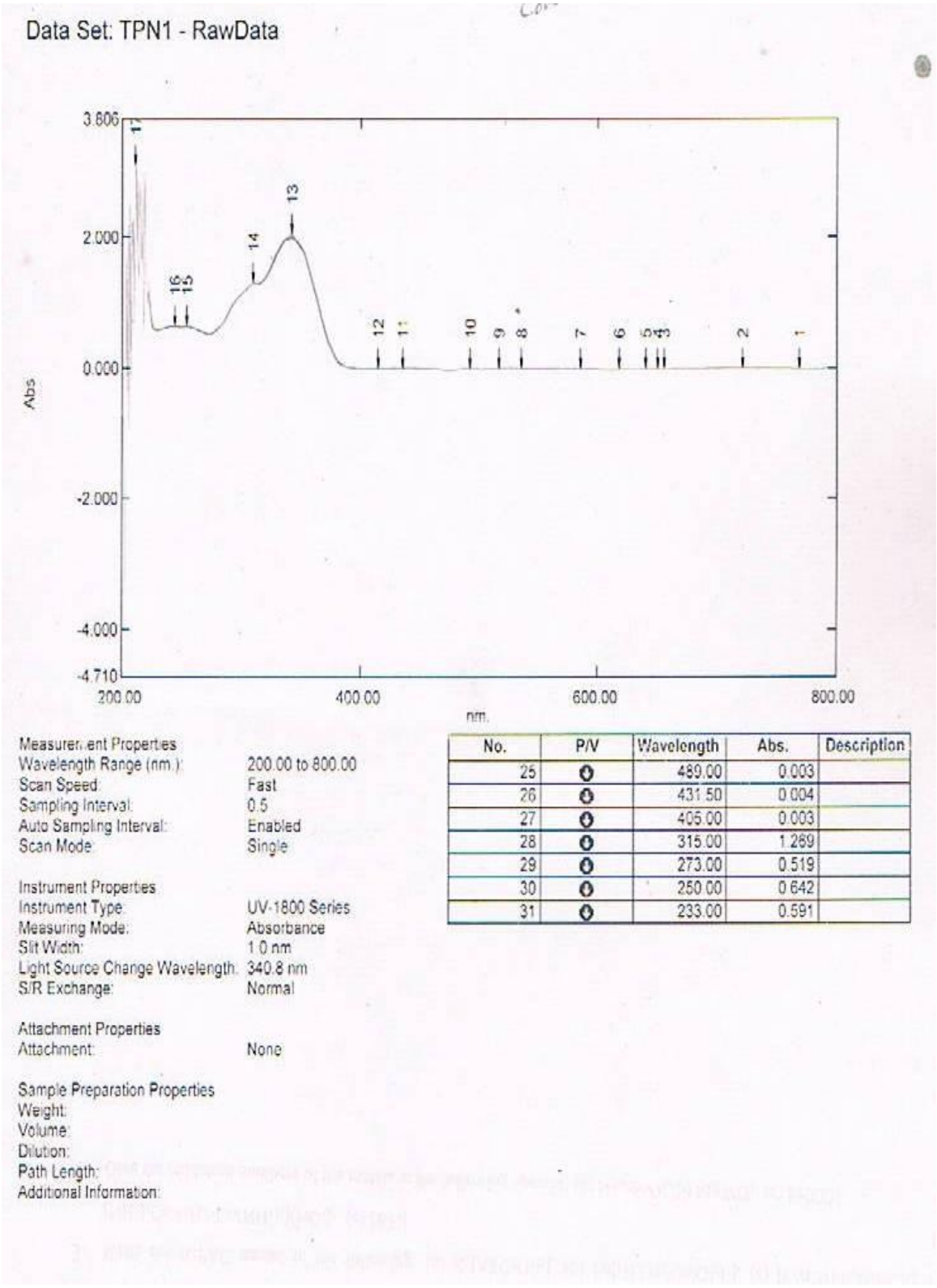


Fig. 2.XVI Yellow crystals from toluene extracts of *Piper nigrum*

1. Fraction B: The shiny pale yellow crystals (m. p. 132°C, pn 2) obtained from toluene extract of *Piper nigrum* seeds is found to be an alkaloid on performing Mayer's reagent test.[42] The molecular formula was established as $C_{17}H_{19}O_3N$ by Agilent, 6540 Q-TOF (HRMS) mass spectrometer and the TLC of the pure crystals showed Rf value 0.24 which is very close to standard Rf value of piperine, reported in literature that is 0.25[34], The spectral studies of these crystals are exactly similar to the crystals obtained from petroleum ether fraction (B) thus the crystals obtained may be of piperine.

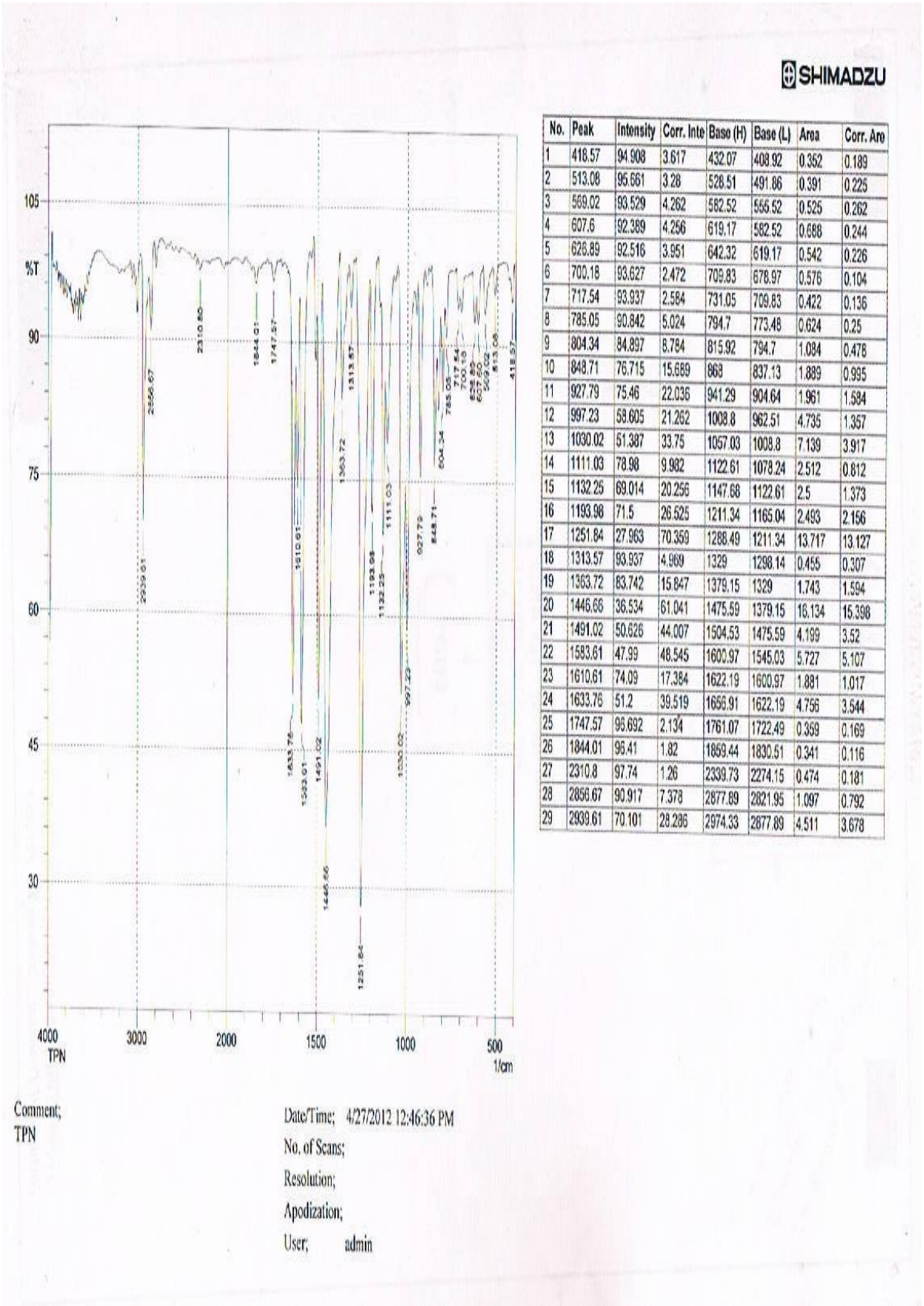
1. **UV spectrum of pn 2:** The UV spectrum showing alkaloid framework is given in figure 2.XVII.



No.	P/V	Wavelength	Abs.	Description
25	⬇	489.00	0.003	
26	⬇	431.50	0.004	
27	⬇	405.00	0.003	
28	⬇	315.00	1.269	
29	⬇	273.00	0.519	
30	⬇	250.00	0.642	
31	⬇	233.00	0.591	

Fig. 2.XVII UV spectrum of crystals from cold toluene extracts of *Piper nigrum*

2. **IR spectrum of pn 2:** IR spectrum (Fig. 2.XVIII) is compared with the original spectrum of piperine[32] that showed absorption bands at 3000 cm^{-1} corresponds to aromatic C-H stretch; at 1633 and 1610 cm^{-1} due to symmetric and asymmetric stretch of C=C (diene); 1610,1583 and 1491 cm^{-1} corresponds to aromatic stretching of C=C phenyl ring ; 1633 cm^{-1} corresponds to stretching of -CO-N stretch, 2939, 2856 cm^{-1} due to CH_2 asymmetric and symmetric stretching, 1446 cm^{-1} corresponds to CH_2 bending; 1251,1193 cm^{-1} due to asymmetric stretch of =C-O-C; 1030 cm^{-1} due to symmetric stretch of =C-O-C; 927 cm^{-1} due to C-O stretching, 1132 cm^{-1} corresponds to in plane bending of phenyl CH ; 997cm^{-1} corresponds to C-H bending for trans –CH=CH- ;848, 830 and 804 cm^{-1} corresponds to out of plane C-H bending.



No.	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	418.57	94.908	3.617	432.07	408.92	0.352	0.189
2	513.08	95.661	3.28	526.51	491.86	0.391	0.225
3	569.02	93.529	4.262	582.52	555.52	0.525	0.262
4	607.6	92.389	4.256	619.17	582.52	0.688	0.244
5	626.89	92.516	3.951	642.32	619.17	0.542	0.226
6	700.18	93.627	2.472	709.83	678.97	0.576	0.104
7	717.54	93.937	2.584	731.05	709.83	0.422	0.136
8	785.05	90.842	5.024	794.7	773.48	0.624	0.25
9	804.34	84.897	8.784	815.92	794.7	1.084	0.476
10	848.71	76.715	15.689	868	837.13	1.889	0.995
11	927.79	75.46	22.036	941.29	904.64	1.961	1.584
12	997.23	58.605	21.262	1008.8	962.51	4.735	1.357
13	1030.02	51.387	33.75	1057.03	1008.8	7.139	3.917
14	1111.03	78.98	9.982	1122.61	1078.24	2.512	0.812
15	1132.25	69.014	20.256	1147.68	1122.61	2.5	1.373
16	1193.98	71.5	26.525	1211.34	1165.04	2.493	2.156
17	1251.84	27.963	70.359	1288.49	1211.34	13.717	13.127
18	1313.57	93.937	4.969	1329	1298.14	0.455	0.307
19	1363.72	83.742	15.847	1379.15	1329	1.743	1.594
20	1446.66	36.534	61.041	1475.59	1379.15	16.134	15.398
21	1491.02	50.626	44.007	1504.53	1475.59	4.199	3.52
22	1583.61	47.99	48.545	1600.97	1545.03	5.727	5.107
23	1610.61	74.09	17.384	1622.19	1600.97	1.881	1.017
24	1633.76	51.2	39.519	1656.91	1622.19	4.756	3.544
25	1747.57	96.692	2.134	1761.07	1722.49	0.369	0.169
26	1844.01	96.41	1.82	1859.44	1830.51	0.341	0.116
27	2310.8	97.74	1.26	2339.73	2274.15	0.474	0.181
28	2856.67	90.917	7.378	2877.89	2821.95	1.097	0.792
29	2939.61	70.101	28.286	2974.33	2877.89	4.511	3.678

Fig. 2.XVIII IR spectrum of crystals obtained from toluene extracts

3. **^{1}H-NMR spectrum of pn 2:** The 400 MHz ^{1}H-NMR spectrum (Fig. 2.XIX and 2.XX) showed the peaks at δ 5.976(2H, 7'), δ 7.39 (1H, 3), δ 6.43 (1H, 2), δ 3.529-3.633 (4H,C) and δ 1.562-1.688 (5H, b, a).

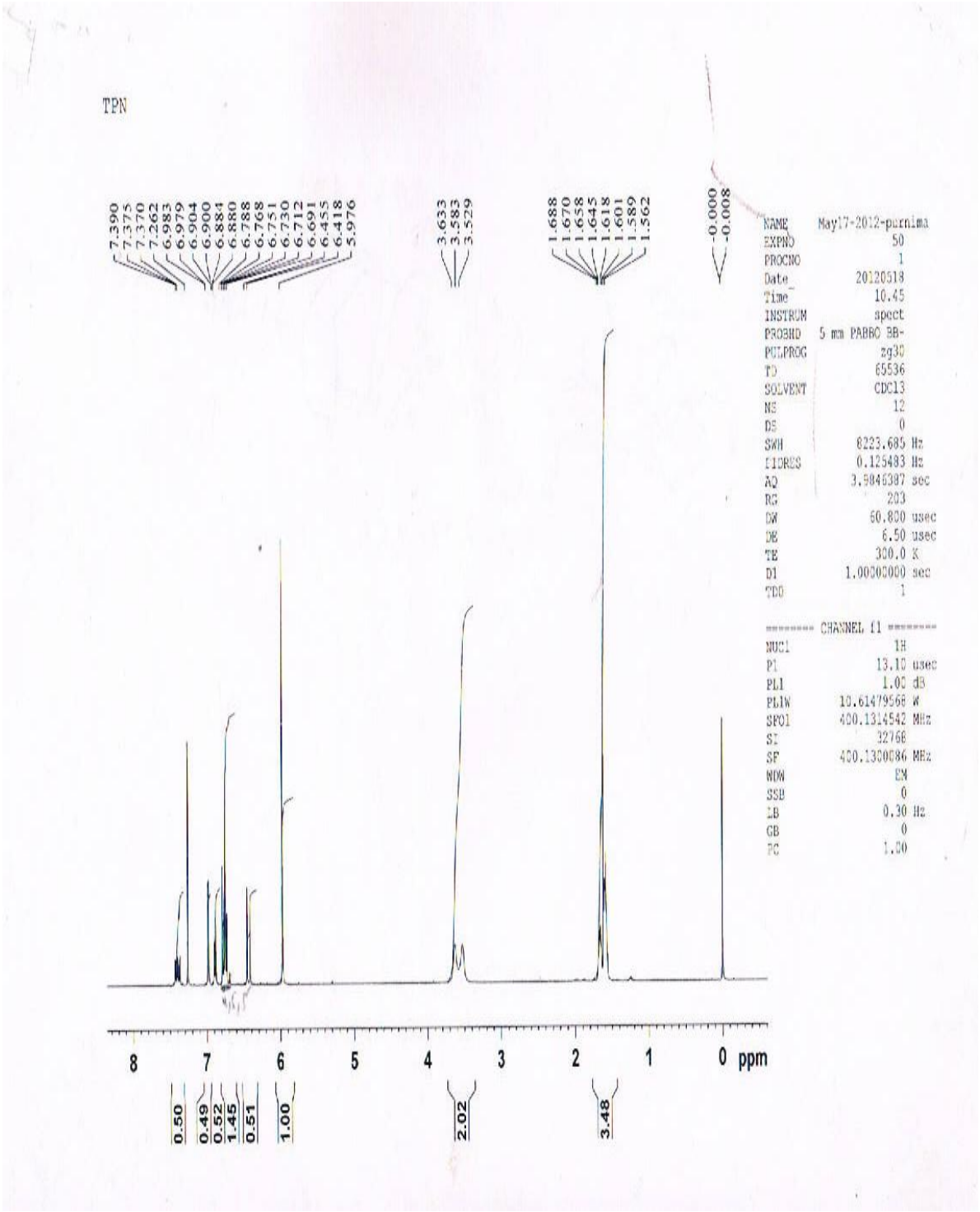


Fig. 2.XIX ^{1}H-NMR spectrum of crystals obtained from toluene extracts

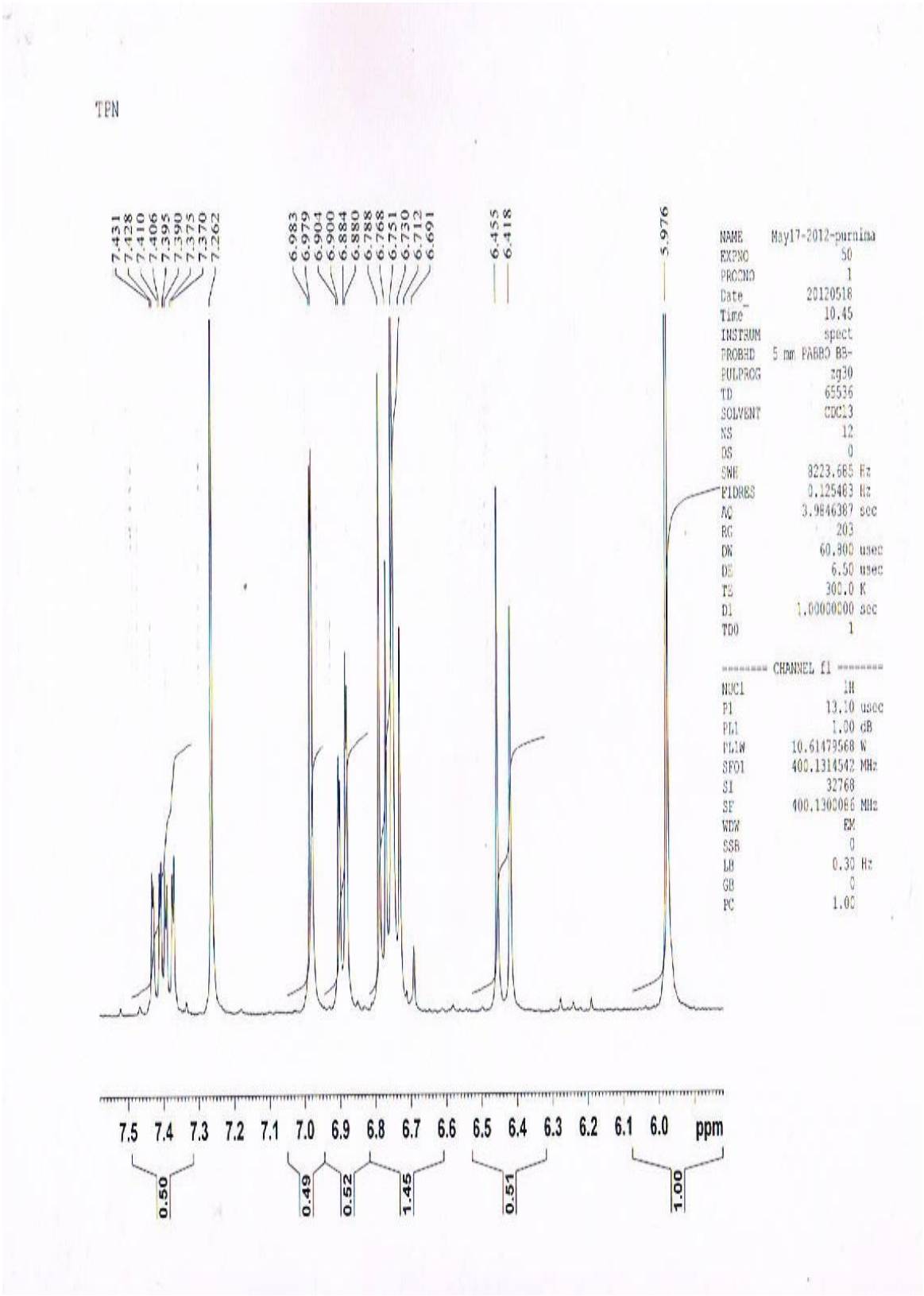


Fig. 2.XX Magnified ^{1}H-NMR spectrum of crystals obtained from toluene extracts

4. **^{13}C-NMR spectrum of pn 2:** The ^{13}C-NMR spectrum revealed the peaks at δ 167.7 (C-1), δ 120.6 (C-2), δ 140.2 (C-3), δ 123.9 (C-4), δ 132.4(C-1'), δ 106.7 (C-2'), δ 149.7-149.8 (C-3',C-4'), δ 109.4 (C-5'), δ 207 (C-6') and δ 102.7 (C-7').

5. **Mass spectrum of pn 2:** The HR-MS (Fig. 2.XXI) showed a molecular ion peak at $[M+H]^+$ 286.14, $[2M+H]^+$ peak at 571.27 and $[2M+Na]^+$ peak at 593.25.

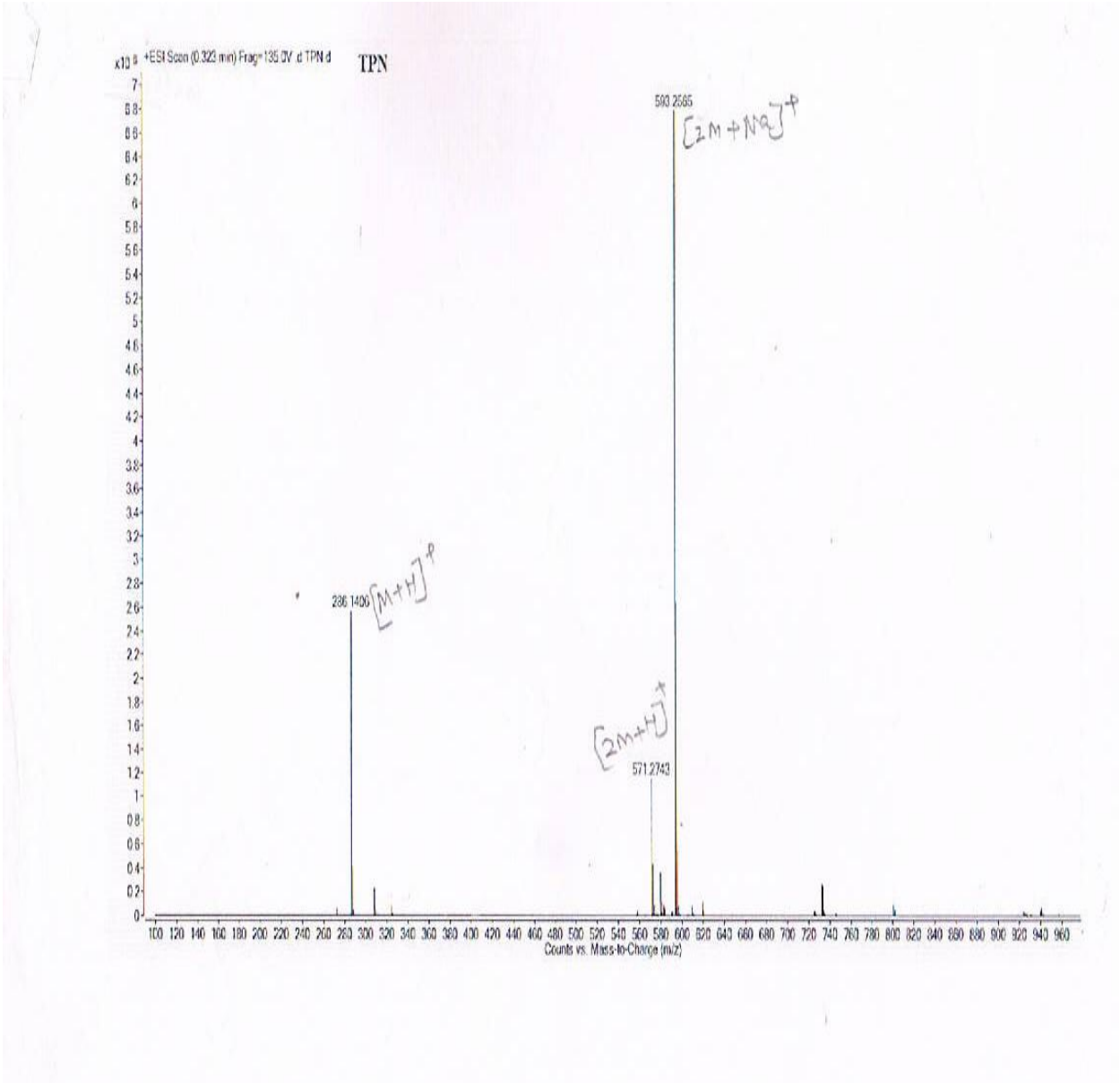


Fig. 2.XXI Mass spectrum of crystals obtained from toluene extract

6. **X-Ray analysis of the crystals shows the presence of following structure:** The structure obtained as a result of X-ray analysis is shown in figure 2.XXII. Bond lengths and bond angles between various bonds are given in table 2.III.

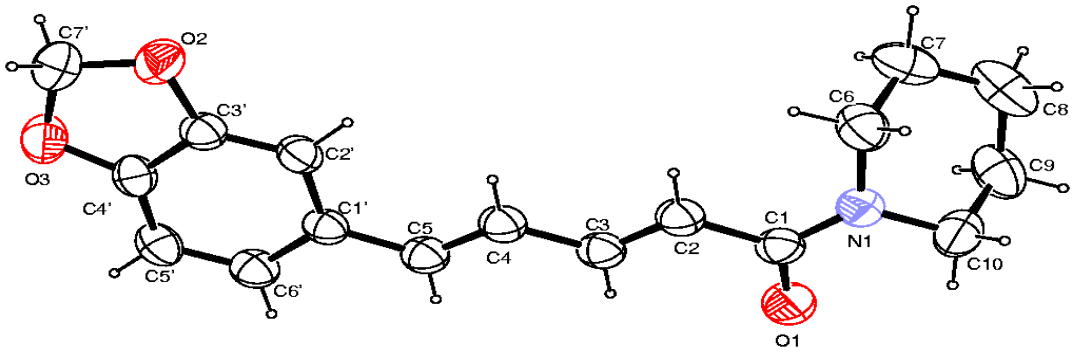


Fig. 2.XXII ORTEP View of the molecule showing the atom-labeling scheme. Displacement ellipsoids are drawn at 50% probability level and H- atoms are shown as small spheres of arbitrary radii.

Table 2.III The X-Ray analysis is given in the form of table below

S. No	Bonds	Bondlengths and angles
1	C(5)-C(4)	1.323(2)
2	C(5)-C(1')	1.457(2)
3	C(5)-H(5)	0.9300
4	C(2')-C(3')	1.357(2)
5	C(2')-C(1')	1.408(2)
6	C(2')-H(2')	0.9300
7	C(1')-C(6')	1.385(2)
8	O(3)-C(4')	1.373(2)
9	O(3)-C(7')	1.427(2)
10	C(3)-C(2)	1.317(3)
11	C(3)-C(4)	1.434(2)
12	C(3)-H(3)	0.9300
13	C(4')-C(5')	1.362(3)
14	C(4')-C(3')	1.373(2)
15	N(1)-C(1)	1.348(2)
16	N(1)-C(10)	1.453(2)
17	N(1)-C(6)	1.461(3)
18	C(3')-O(2)	1.372(2)
19	O(1)-C(1)	1.234(2)
20	C(4)-H(4)	0.9300
21	C(2)-C(1)	1.474(3)
22	C(2)-H(2)	0.9300
23	C(5')-C(6')	1.389(3)
24	C(5')-H(5')	0.9300
25	C(6')-H(6')	0.9300
26	O(2)-C(7')	1.423(2)
27	C(10)-C(9)	1.500(3)
28	C(10)-(10A)	0.9700
29	C(10)-H(10B)	0.9700
30	C(6)-C(7)	.1.500(3)
31	C(6)-H(6A)	0.9700
32	C(6)-H(6B)	0.9700
33	C(7)-C(8)	1.507(3)
34	C(7)-H(7A)	0.9700
35	C(7)-H(7B)	0.9700
36	C(7')-H(7'1)	0.9700
37	C(7')-H(7'2)	0.9700
38	C(8)-C(9)	1.504(3)
39	C(8)-H(8A)	0.9700
40	C(8)-H(8B)	0.9700
41	C(9)-H(9A)	0.9700
42	C(9)-H(9B)	0.9700

S. No	Bonds	Bondlengths and angles
43	C(4)-C(5)-C(1')	127.05(18)
44	C(4)-C(5)-H(5)	116.5
45	C(1')-C(5)-H(5)	116.5
46	C(3')-C(2')-C(1')	117.99(17)
47	C(3')-C(2')-H(2')	121.0
48	C(1')-C(2')-H(2')	121.0
49	C(6')-C(1')-C(2')	118.56(17)
50	C(6')-C(1')-C(5)	120.00(17)
51	C(2')-C(1')-C(5)	121.44(16)
52	C(4')-O(3)-C(7')	105.74(14)
53	C(2)-C(3)-C(4)	125.15(18)
54	C(2)-C(3)-H(3)	117.4
55	C(4)-C(3)-H(3)	117.4
56	C(5')-C(4')-O(3)	128.41(18)
57	C(5')-C(4')-C(3')	121.49(18)
58	O(3)-C(4')-C(3')	110.10(17)
59	C(1)-N(1)-C(10)	119.31(17)
60	C(1)-N(1)-C(6)	126.84(16)
61	C(10)-N(1)-C(6)	112.53(17)
62	C(2')-C(3')-O(2)	127.82(17)
63	C(2')-C(3')-C(4')	122.36(18)
64	O(2)-C(3')-C(4')	109.82(16)
65	C(5)-C(4)-C(3)	126.33(18)
66	C(5)-C(4)-H(4)	116.8
67	C(3)-C(4)-H(4)	116.8
68	C(3)-C(2)-C(1)	122.54(18)
69	C(3)-C(2)-H(2)	118.7
70	C(1)-C(2)-H(2)	118.7
71	O(1)-C(1)-N(1)	121.32(17)
72	O(1)-C(1)-C(2)	119.84(18)
73	N(1)-C(1)-C(2)	118.84(17)
74	C(4')-C(5')-C(6')	116.83(18)
75	C(4')-C(5')-H(5')	121.6
76	C(6')-C(5')-H(5')	121.6
77	C(1')-C(6')-C(5')	122.77(18)
78	C(1')-C(6')-H(6')	118.6
79	C(5')-C(6')-H(6')	106.04(15)
80	C(3')-O(2)-C(7')	110.02(18)
81	N(1)-C(10)-C(9)	109.7
82	N(1)-C(10)-H(10A)	109.7
83	C(9)-C(10)-H(10A)	109.7
84	N(1)-C(10)-H(10B)	109.7

S. No	Bonds	Bondlengths and angles
85	C(9)-C(10)-H(10B)	108.2
86	H(10A)-C(10)-H(10B)	110.04(17)
87	N(1)-C(6)-C(7)	109.7
88	N(1)-C(6)-H(6A)	109.7
89	C(7)-C(6)-H(6A)	109.7
90	N(1)-C(6)-H(6B)	109.7
91	C(7)-C(6)-H(6B)	108.2
92	H(6A)-C(6)-H(6B)	112.2(2)
93	C(6)-C(7)-C(8	109.2
94	C(6)-C(7)-H(7A)	109.2
95	C(8)-C(7)-H(7A)	109.2
96	C(6)-C(7)-H(7B)	109.2
97	C(8)-C(7)-H(7B)	109.2
98	H(7A)-C(7)-H(7B)	107.9
99	O(2)-C(7')-O(3)	108.29(16)
100	O(2)-C(7')-H(7'1)	110.0

S. No	Bonds	Bondlengths and angles
101	O(3)-C(7')-H(7'1)	110.0
102	O(2)-C(7')-H(7'2)	110.0
103	O(3)-C(7')-H(7'2)	110.0
104	H(7'1)-C(7')-H(7'2)	108.4
105	C(9)-C(8)-C(7)	111.0(2)
106	C(9)-C(8)-H(8A)	109.4
107	C(7)-C(8)-H(8A)	109.4
108	C(9)-C(8)-H(8B)	109.4
109	C(7)-C(8)-H(8B)	109.4
110	H(8A)-C(8)-H(8B)	108.0
111	C(10)-C(9)-C(8)	111.2(2)
112	C(10)-C(9)-H(9A)	109.4
113	C(8)-C(9)-H(9A)	109.4
114	C(10)-C(9)-H(9B)	109.4
115	C(8)-C(9)-H(9B)	109.4
116	H(9A)-C(9)-H(9B)	108.0

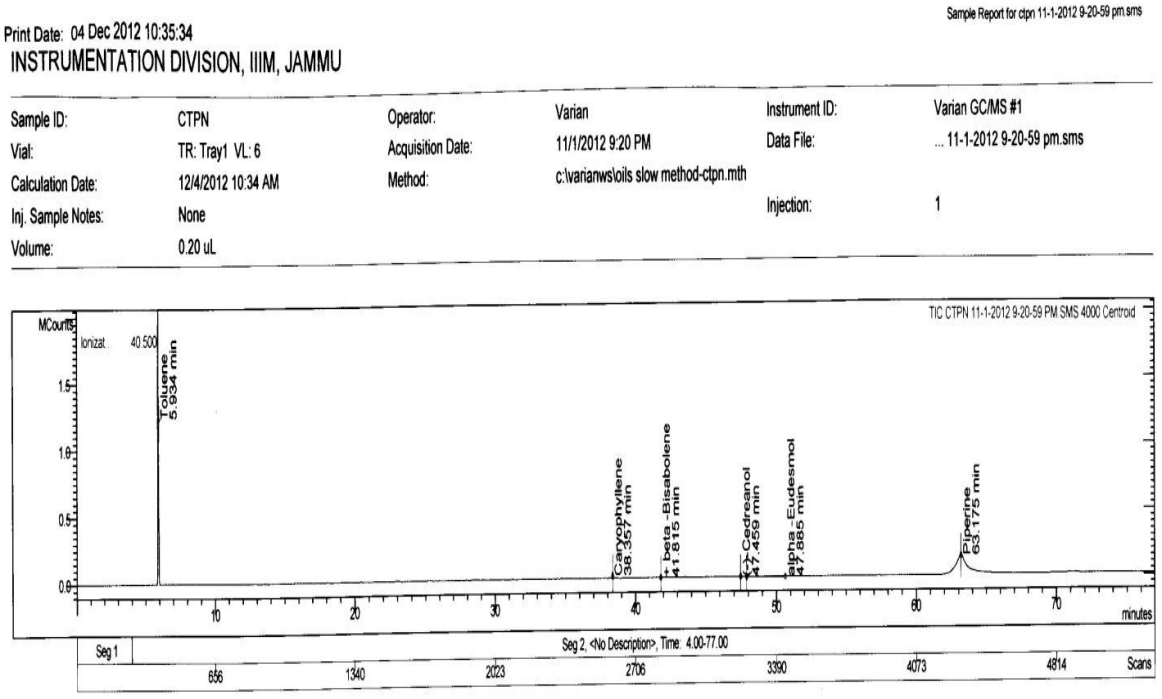
X-Ray analysis depicts the following:

1. Crystal size — 0.30 x 0.20 x 0.15 mm
2. Empirical formula — $C_{17}H_{10}NO_3$
3. Formula weight — 285.33
4. Radiation, Wavelength — Mo *K*α, 0.71073 Å
5. Unit cell dimensions — a = 8.7284(4), b = 13.6455(6), c = 13.1589(6) Å; β = 108.744(5)^{O}
6. Crystal system, Space group — monoclinic, $P2_1/n$
7. Unit cell volume — 1484.15(12) $Å^3$
8. No. of molecules per unit cell, Z — 4
9. Absorption coefficient — 0.087 mm^{-1}
10. F(000) — 608
11. θ range for entire data collection — $3.59 < \theta < 26.00^{o}$
12. Reflections collected / unique — 21086 / 2914
13. Reflections observed (I>2σ(I)) — 1848

14. No. of parameters refined	190
15. Final R-factor	0.0482
16. $wR(F^2)$	0.1138
17. Goodness-of-fit	1.024
18. $(\Delta/\sigma)_{max}$	0.001
19. Final residual electron density	$-0.157<\Delta\rho<0.123$ eÅ^{-3}

On the basis of above spectroscopic analysis the crystals (pn 2) obtained are identified as that of piperine. It is for the very first time piperine has been obtained from the toluene extracts of *Piper nigrum.*

2. GC-MS analysis of oily fraction (A) obtained from toluene extracts: Compounds were identified by their GC retention time relative to known compounds and by comparison of their mass spectra with those present in IIIM library**.** The GC-MS spectra (Fig. 2.XXIII) of the oily fraction of toluene extracts of *Piper nigrum* unveiled the presence of many components, Rf value and structures of isolated components are given in table 2.IV and figure 2.XXIV:



Target Compounds

Cmpd. Number	RT (min)	Peak Name	Area	Amount/RF
1	5.934	Toluene	3.929e+6	99.216
2	38.357	Caryophyllene	10878	0.275
4	41.815	beta -Bisabolene	2341	0.059
5	42.325	delta -Cadinene	1828	0.046
6	43.562	Elemol	2101	0.053
10	47.459	(-)-Cedreanol	3755	0.095
11	47.885	alpha -Eudesmol	905	0.023
12	63.175	Piperine	9249	0.234

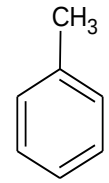
Method Notes

Injection Method: c:\varianws\oils slow method.mth
1ml/min Helium gas as career, injector 280deg C, split ratio 1:150, column oven 50 deg C for 5min,
250 deg @ 3deg/min, hold for 7 min, ca
pillary column CP-Sil-8, 30m X 0.32mm X 0.25u thick film.77.00
Recalc Method: c:\varianws\oils slow method-ctpn.mth
1ml/min Helium gas as career, injector 280deg C, split ratio 1:150, column oven 50 deg C for 5min,
250 deg @ 3deg/min, hold for 7 min, ca
pillary column CP-Sil-8, 30m X 0.32mm X 0.25u thick film.77.00

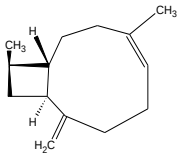
Fig. 2.XXIII GC-MS analysis of oily fraction (A) obtained from toluene extract

Table 2.IV Various components obtained from GC-MS analysis of toluene extracts:

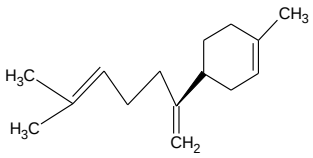
S. No.	RT(min)	Peak name	Area	Amount/Rf
1	5.934	Toluene	3.929e+6	99.216
2	38.357	Caryophyllene	10878	0.275
3	41.815	β–bisabolene	2341	0.059
4	42.325	δ –Cadinene	1828	0.046
5	43.562	Elemol	2101	0.053
6	47.459	(-)- Cedreanol	3755	0.095
7	47.885	α- Eudesmol	905	0.023
8	63.175	Piperine	9249	0.234



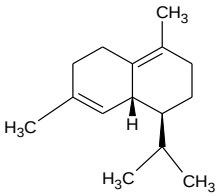
(1)Toluene



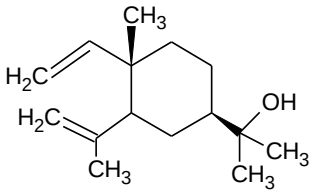
(2) Caryophyllene



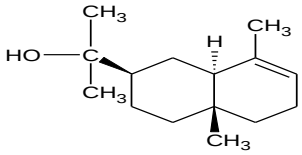
(3) β–bisabolene



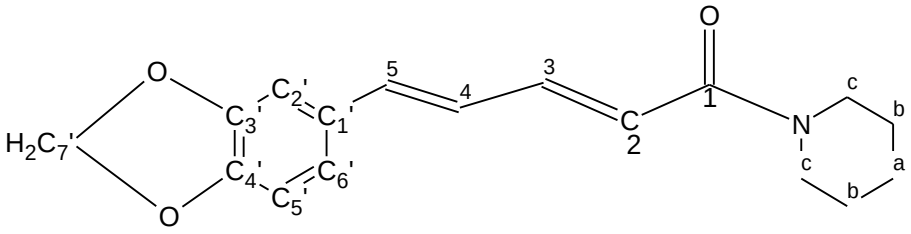
(4) δ –Cadinene



(5) Elemol



(6) α- Eudesmol



(7) Piperine

Fig. 2.XXIV Structures of various components present in toluene extracts

C. Investigation of cold dichloromethane extracts of *Piper nigrum*

DCM extracts of *Piper nigrum* seeds kept for around one weak and repeatedly purified for crystallization. But colourless oil is found after the process from the DCM extract which was subjected to GC-MS analysis for separation of various phytochemicals. The spectra is attached as Fig. 2.XXV, amount and structure of the isolated components are given in table 2.V and figure 2.XXVI.

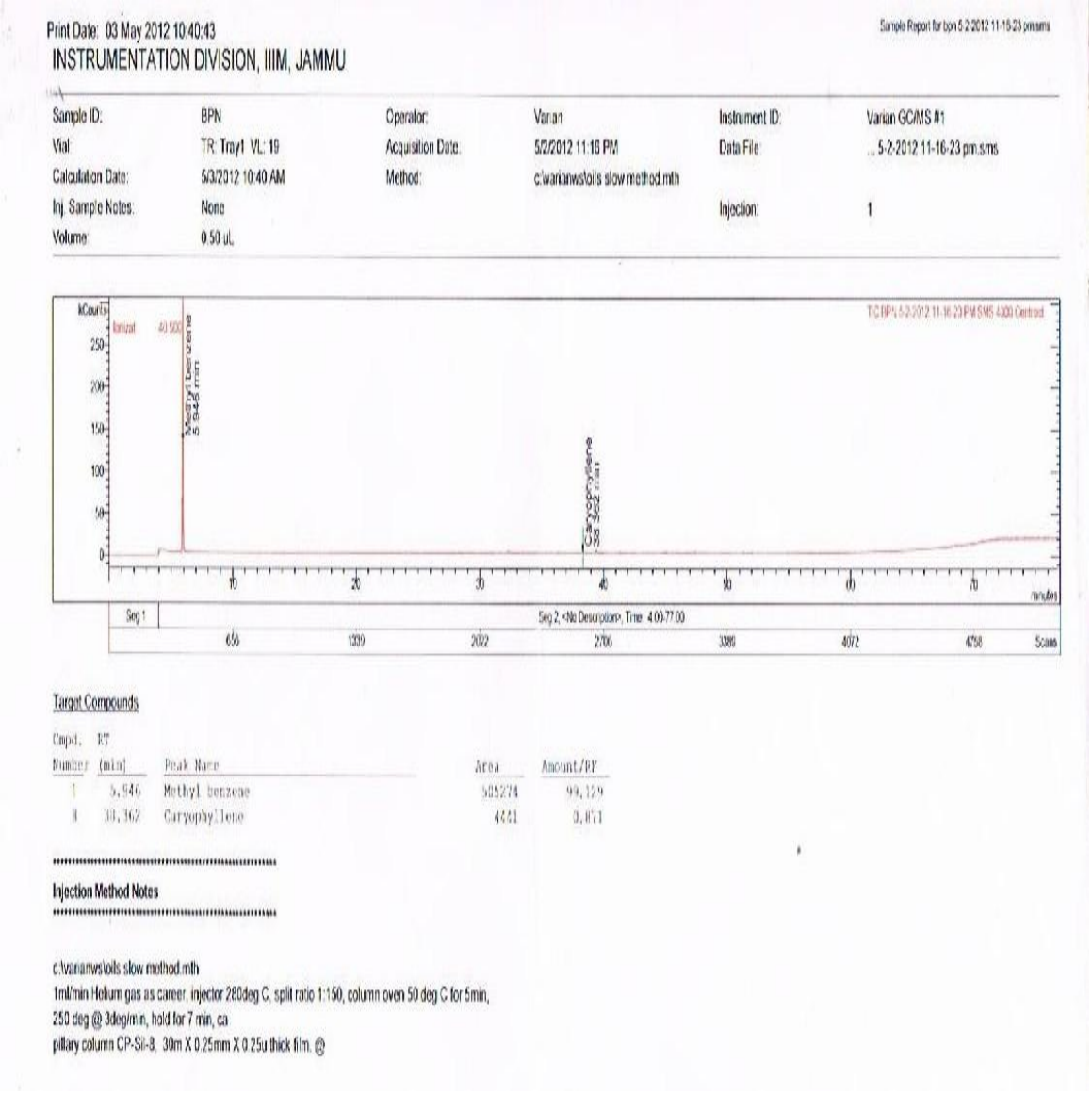
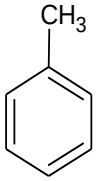


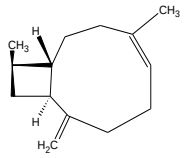
Fig. 2.XXV Various components obtained from cold dichloroform extracts of *Piper nigrum*

Table 2.V The phytochemicals identified by GC-MS of dichloromethane extracts

S. No.	RT(min)	Peak name	Area	Amount/Rf
1	5.934	Toluene	3.929e+6	99.216
2	38.357	Caryophyllene	10878	0.275



(1) Toluene



(2) Caryophyllene

Fig. 2.XXVI Structure of components from cold dichloromethane extracts of *Piper nigrum*

D. Investigation of cold methanol extracts of *Piper nigrum*

The very less quantity of methanol extract was separated. The amount was so less that could not separated into oil or crystals. Only qualitative analysis of this extract is done where it shows the presence and absence of few secondary metabolites.[42, 44]The presence or absence of secondary metabolites are given in table 2.VI.

Table2.VI Presence or absence of various secondary metabolites

S. No.	Name of secondary metabolite	Test performed	Present(+)/ Absent(-)
1	Alkaloids	Wagner's reagent test	(+)
2	Carbohydrates	Molisch reagent test	(+)
3	Flavonoids	Shinoda test	(-)
4	Terpenoids	Liebermann Burchard test	(+)
5	Steroids	Liebermann Burchard test	(-)
6	Tannins	Ferric chloride test	(-)
7	Glycosides	Borntrager's test	(-)
8	Fats or oils	Saponification test	(+)

2.3.2 Hot Extraction: Hot extraction of *Piper nigrum* seeds was soxhlated with methanol. The crude extract was further separate into different solvent extracts. The extraction is performed as shown in figure 2.XXVII.

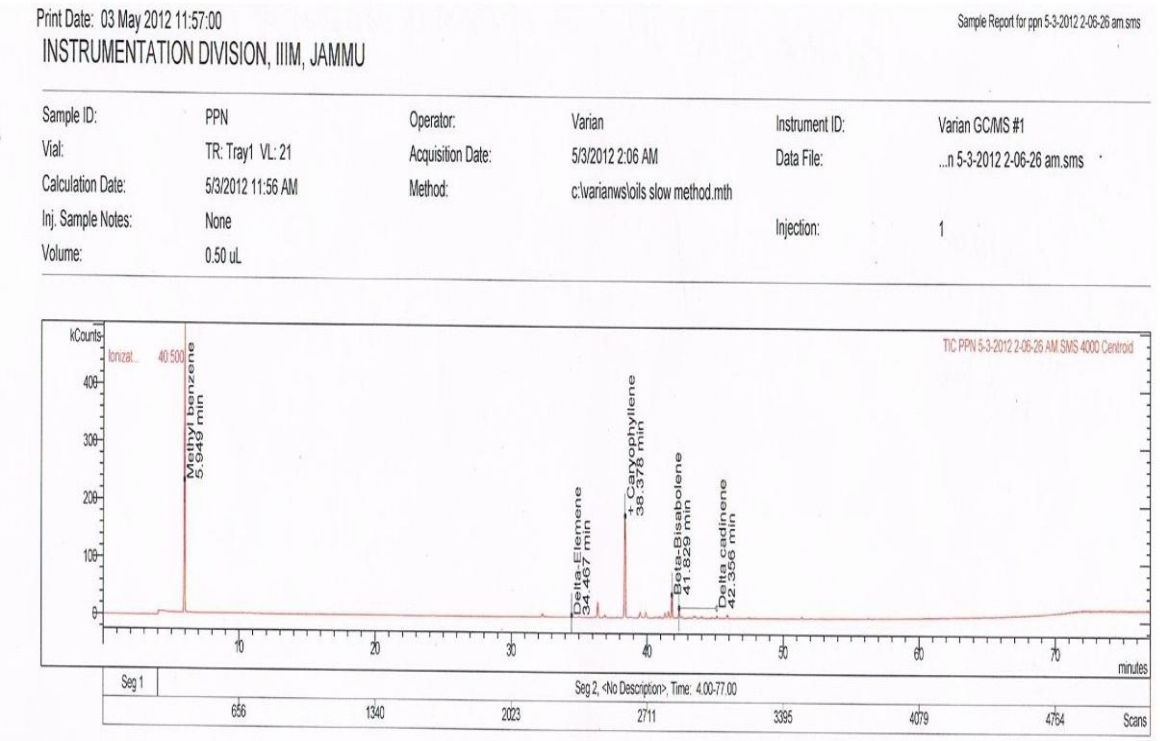
Fig. 2.XXVII Hot extraction of pepper

The various extracts separated are:

A. Petroleum ether extract
B. Dichloromethane extract
C. Chloroform extract
D. Ethyl acetate extract
E. Methanol extract

A. Investigation of hot petroleum ether extracts of *Piper nigrum* seeds

The GC-MS (Fig. 2.XXVIII) of oily fraction of hot petroleum ether extracts of *Piper nigrum* recorded from Varian 4000 GC-MS/MS from IIIM Jammu, unveiled the presence of following components. The amount and structure of various phytochemicals are given in table 2.VII and figure 2.XXIX.



Target Compounds

Cmpd. Number	RT (min)	Peak Name	Area	Amount/RF
1	5.949	Methyl benzene	833309	84.671
5	34.467	Delta-Elemene	2804	0.285
6	36.378	Alpha-Copane	18083	1.837
8	38.378	Caryophyllene	91584	9.306
9	39.904	Apha-Caryophyllene	9096	0.924
11	41.829	Beta-Bisabolene	19815	2.013
12	42.356	Delta cadinene	9482	0.963

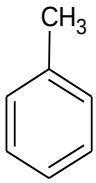
Injection Method Notes

c:\varianws\oils slow method.mth
1ml/min Helium gas as career, injector 280deg C, split ratio 1:150, column oven 50 deg C for 5min,
250 deg @ 3deg/min, hold for 7 min, ca
pillary column CP-Sil-8, 30m X 0.25mm X 0.25u thick film.

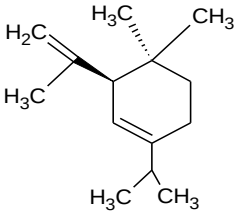
Fig. 2.XXVIII Various components obtained from hot petroleum ether extracts of *Piper nigrum*

Table 2.VII Phytochemicals identified by GC-MS of hot petroleum ether extracts are:

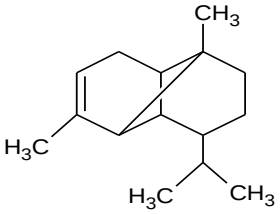
S. No.	RT(min)	Peak name	Area	Amount/Rf
1	5.949	Methyl benzene	833309	84.671
2	34.467	δ – elemene	2804	0.285
3	36.378	α – copane	18083	1.837
4	38.378	Caryophyllene	91584	9.306
5	39.904	α- caryophyllene	9096	0.924
6	41.829	β–bisabolene	19815	2.013
7	42.356	δ –cadinene	9482	0.963



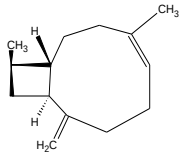
(1) Methyl benzene



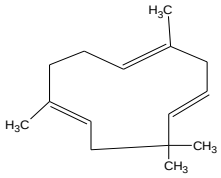
(2) δ – elemene



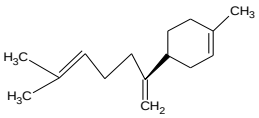
(3) α – copane



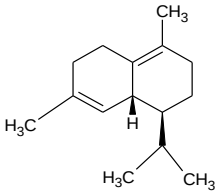
(4) Caryophyllene



(5) α- caryophyllene



(6) β–bisabolene



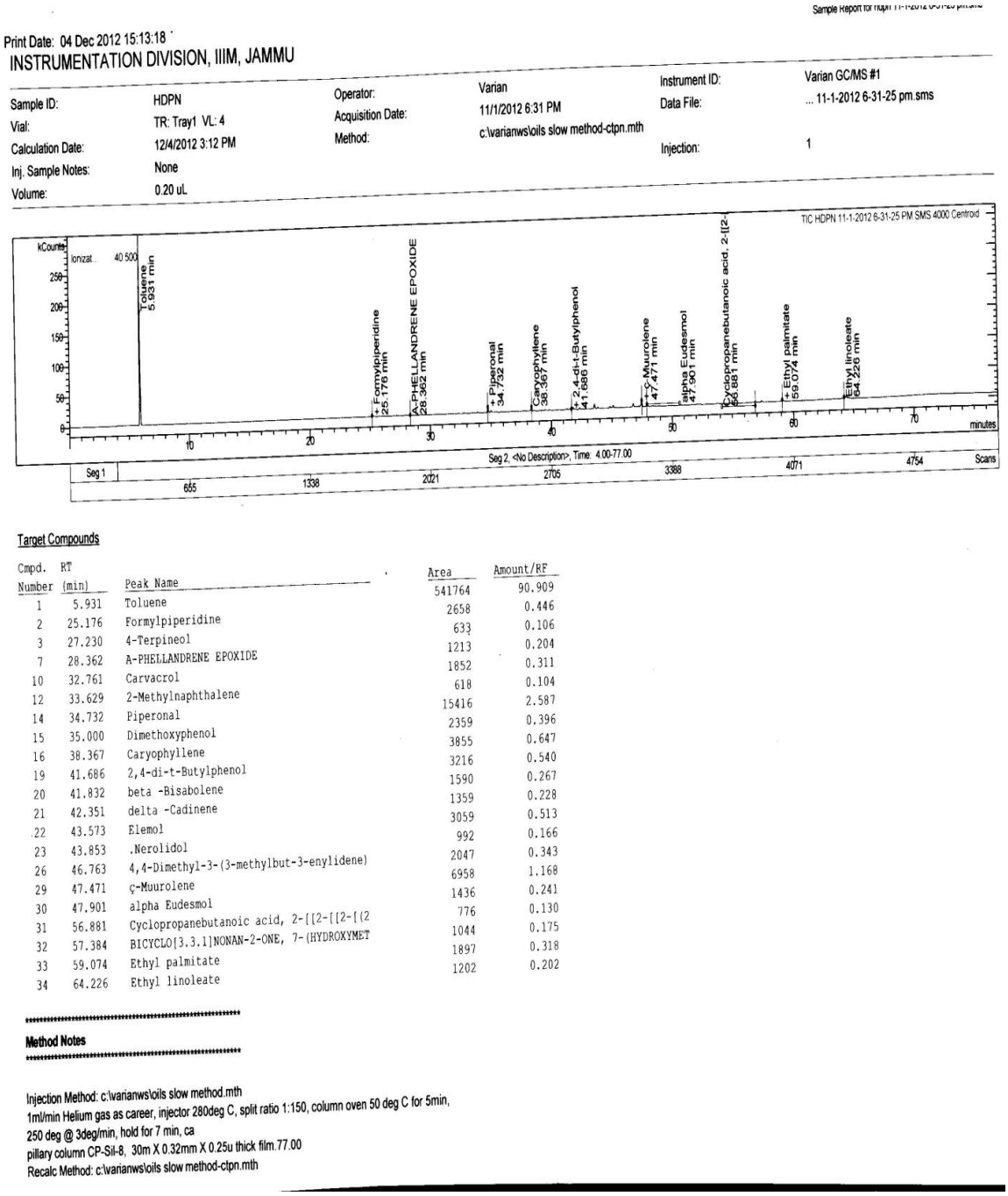
(7) δ –cadinene

Fig. 2.XXIX Various components from hot petroleum ether extracts of *Piper nigrum*

Among these chemical constituents methyl benzene is present as major component and δ – elemene as minor component.

B. Investigation of hot dichloromethane extracts of *Piper nigrum*

The GC-MS spectrum (Fig. 2.XXX) of oily fraction of hot dichloromethane extracts of *Piper nigrum* recorded from Varian 4000 GC-MS/MS from IIIM Jammu, unveiled the presence of following components. The amount and structure of various phytochemicals are given in table 2.VIII and figure 2.XXX.



Target Compounds

Cmpd. Number	RT (min)	Peak Name	Area	Amount/RF
1	5.931	Toluene	541764	90.909
2	25.176	Formylpiperidine	2658	0.446
3	27.230	4-Terpineol	633	0.106
7	28.362	A-PHELLANDRENE EPOXIDE	1213	0.204
10	32.761	Carvacrol	1852	0.311
12	33.629	2-Methylnaphthalene	618	0.104
14	34.732	Piperonal	15416	2.587
15	35.000	Dimethoxyphenol	2359	0.396
16	38.367	Caryophyllene	3855	0.647
19	41.686	2,4-di-t-Butylphenol	3216	0.540
20	41.832	beta -Bisabolene	1590	0.267
21	42.351	delta -Cadinene	1359	0.228
22	43.573	Elemol	3059	0.513
23	43.853	.Nerolidol	992	0.166
26	46.763	4,4-Dimethyl-3-(3-methylbut-3-enylidene)	2047	0.343
29	47.471	ç-Muurolene	6958	1.168
30	47.901	alpha Eudesmol	1436	0.241
31	56.881	Cyclopropanebutanoic acid, 2-[[2-[[2-[(2	776	0.130
32	57.384	BICYCLO[3.3.1]NONAN-2-ONE, 7-(HYDROXYMET	1044	0.175
33	59.074	Ethyl palmitate	1897	0.318
34	64.226	Ethyl linoleate	1202	0.202

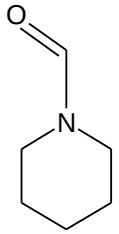
Method Notes

Injection Method: c:\varianws\oils slow method.mth
1ml/min Helium gas as career, injector 280deg C, split ratio 1:150, column oven 50 deg C for 5min,
250 deg @ 3deg/min, hold for 7 min, ca
pillary column CP-Sil-8, 30m X 0.32mm X 0.25u thick film.77.00
Recalc Method: c:\varianws\oils slow method-ctpn.mth

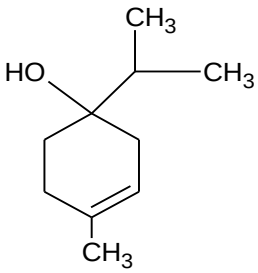
Fig. 2.XXX Various components from hot dichloromethane extracts of *Piper nigrum*

Table 2.VIII Secondary metabolites from dichloromethane extracts

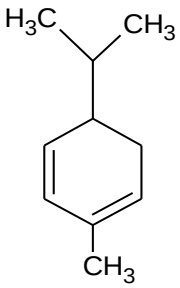
S. No.	RT (min)	Peak Name	Area	Amount/Rf
1	25.176	Formylpiperidine	2658	0.446
2	27.230	4-Terpeneol	633	0.106
3	28.362	A-phellandrene epoxide	1213	0.204
4	32.761	Carvacrol	1852	0.311
5	33.629	2-Methyl naphthalene	618	0.104
6	34.732	Piperonal	15416	2.587
7	35.000	Dimethoxyphenol	2359	0.396
8	38.367	Caryophyllene	3855	0.647
9	41.686	2,4-di-t-butylphenol	3216	0.540
10	41.832	β–bisabolene	1590	0.267
11	42.351	δ –Cadinene	1359	0.228
12	43.573	Elemol	3059	0.513
13	43.853	Nerolidol	992	0.166
14	46.763	4,4-dimethyl-3-(3-methylbut-3-enylidene)	2047	0.343
15	47.471	c-murrolene	6958	1.168
16	47.901	α- Eudesmol	1436	0.241
17	56.881	Cyclopropanebutanoic acid	776	0.130
18	57.384	Bicyclo(3.3.1) Nonan-2-one	1044	0.175
19	59.074	Ethyl palmitate	1897	0.318
20	64.226	Ethyl linoleate	1202	0.202



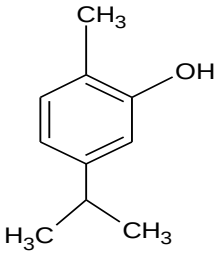
(1) Formylpiperidine



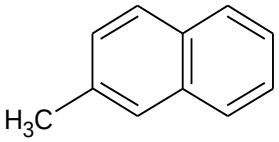
(2) 4- terpinol



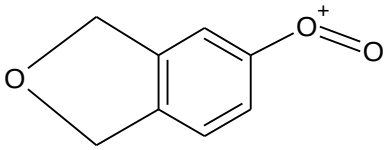
(3) A-phellandrene epoxide



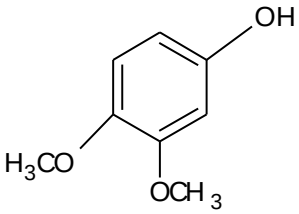
(4) Carvacrol



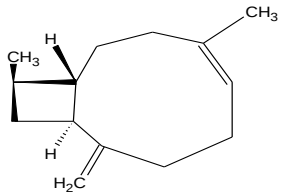
(5) 2-Methyl naphthalene



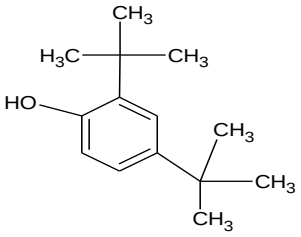
(6) Piperonal



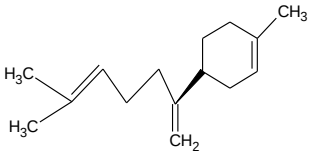
(7) Dimethoxyphenol



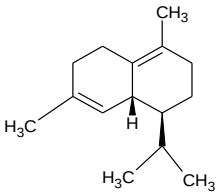
(8) Caryophyllene



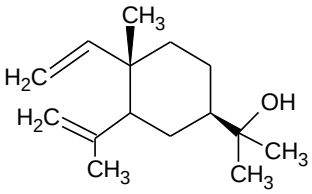
(9) 2, 4-di-t-butylphenol



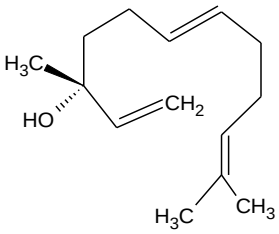
(10) beta bisabolene



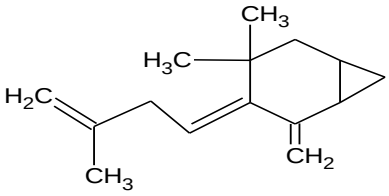
(11) delta cadinene



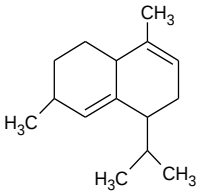
(12) Elemol



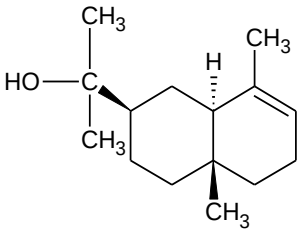
(13) Nerolidol



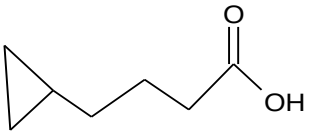
(14) 4,4-dimethyl-3-(3-methylbut-3-enylidene) methylenebicyclo [4.1.0] heptanes



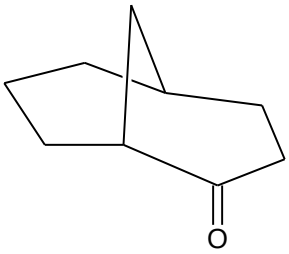
(15) c-murrolene



(16) α- Eudesmol

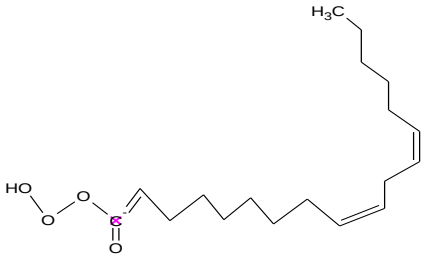


(17) Cyclopropanebutanoic acid



(18) Bicyclo (3.3.1) Nonan-2-one

(19) Ethyl palmitate



(20) Ethyl linoleate

Fig. 2.XXXI Structures of various components present in dichloromethane extracts

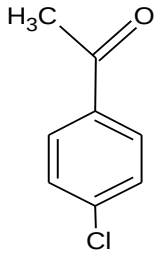
The DCM extracts reveals c-murrolene as major and 2-methyl naphthalene as minor components.

C. Investigation of hot chloroform extracts of *Piper nigrum*

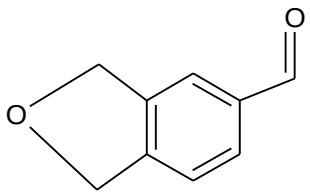
Chloroform extract of *Piper nigrum* seeds after keeping undisturbed for fifteen days separated into colourless oil which is subjected to GC-MS analysis for identification of phytoconstituents. 2,4-di-t-butyl phenol is found to be as major component where as c-murrolene is appeared as minor component. The amount and structure of the isolated components are given in the table 2.IX and figure 2.XXXII.

Table 2.IX Secondary metabolites from chloroform extracts

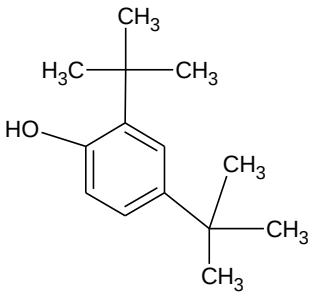
S. No.	RT (min)	Peak name	Area	Amount/RF
1	21.336	Chloroacetophenone	2355	0.395
2	34.754	Piperonyl aldehyde	2720	0.456
3	41.674	2,4-di-t-butyl phenol	4725	0.793
4	47.452	c-murrolene	1394	0.234



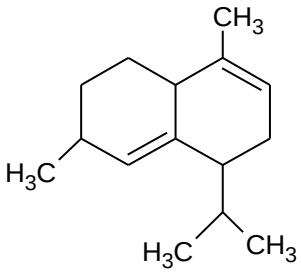
(1) Chloroacetophenone



(2) Piperonyl aldehyde



(3) 2,4-di-t-butyl phenol



(4) c-murrolene

Fig. 2.XXXII Structures of various components isolated from chloroform extracts of *Piper nigrum.*

D. Investigation of ethyl acetate extracts of *Piper nigrum* seeds

The oil obtained from ethyl acetate extracts was analyzed by GC-MS in IIIM Jammu. The spectra, amount and structures of identified compounds are given below in figure 2.XXXIII, table 2.IX and figure 2.XXXIV.

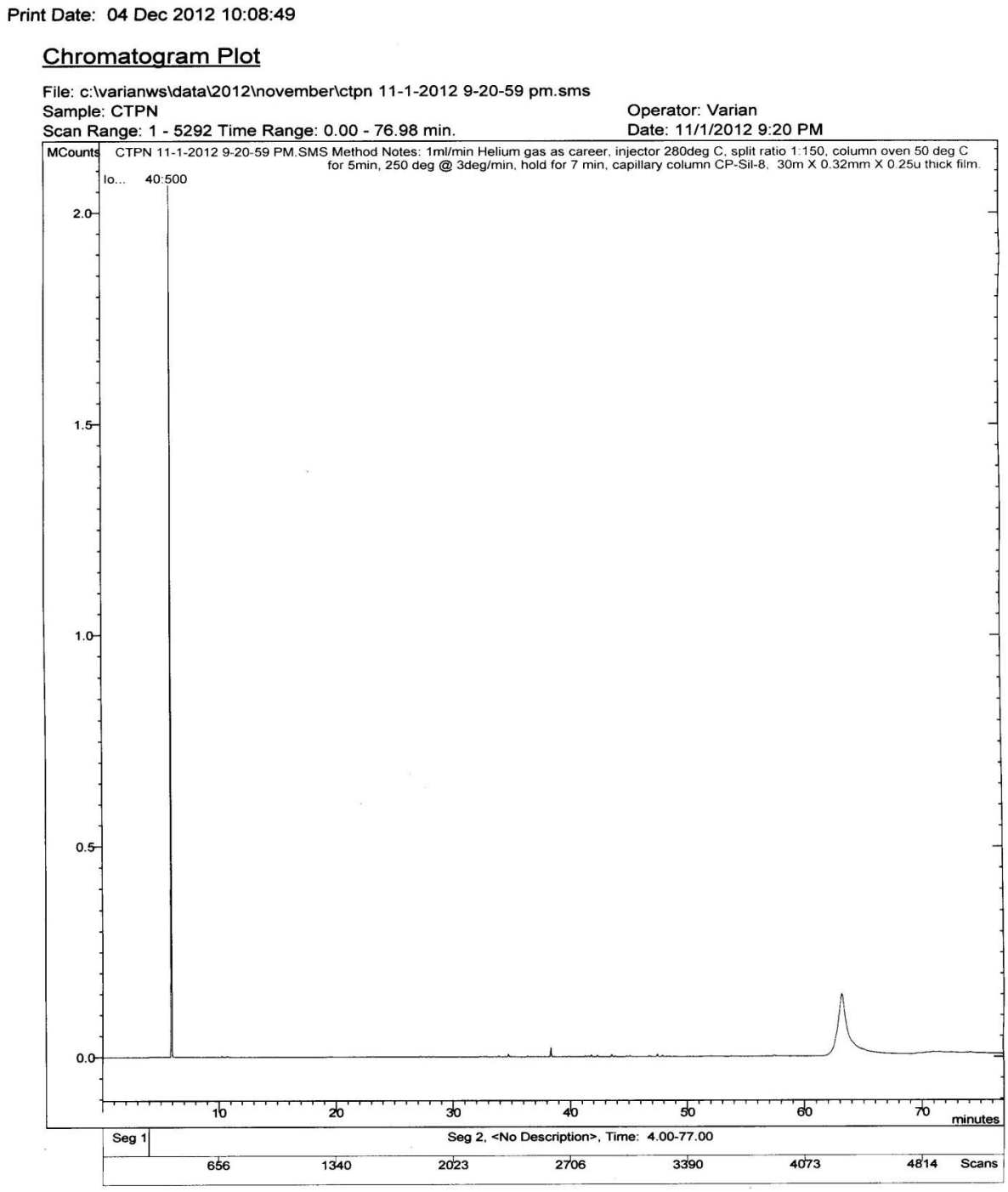
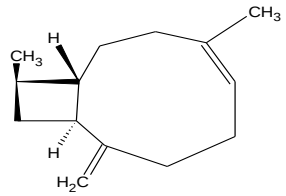


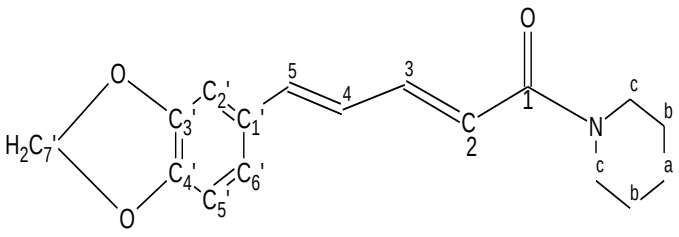
Fig. 2.XXXIII Gas chromatography-mass spectrum of ethyl acetate extracts of *Piper nigrum*

Table 2.X Secondary metabolites from ethyl acetate extracts

S. No.	RT (min)	Peak name	Area	Amount/RF
1	38.378	Caryophyllene	91584	9.306
2	63.170	Piperine	27747	0.702



(1) Caryophyllene



(2) Piperine

Fig. 2.XXXIV Structures of various components present in ethyl acetate extracts

The analysis of the spectrum shows that caryophyllene is major component where as piperine is minor component in the oil.

E. Investigation of hot ethanol extracts of *Piper nigrum*

Ethanol extract of *Piper nigrum* is investigated for the presence of unknown natural metabolites such as alkaloids, carbohydrates, flavonoids, terpenoids, steroids, tannins, glycosides, fats or oils[42, 44] and results are given in the table below in table 2.XI:

Table 2.XI Presence or absence of various secondary metabolites

S. No.	Name of secondary metabolite	Test performed	Present(+)/ Absent(-)
1	Alkaloids	Wagner's reagent test	(+)
2	Carbohydrates	Molisch reagent test	(+)
3	Flavonoids	Shinoda test	(-)
4	Terpenoids	Liebermann Burchard test	(+)
5	Steroids	Liebermann Burchard test	(-)
6	Tannins	Ferric chloride test	(-)
7	Glycosides	Borntrager's test	(-)
8	Fats or oils	Saponification test	(-)

2.4: Antimicrobial activity of various hot extracts of *Piper nigrum* against the bacterias and fungus

A. Antimicrobial activity of petroleum ether extracts against various microbes

The average measurement of diameter of zone of inhibition against various microbes is given in the table. *In vitro* antimicrobial activity of petroleum ether extracts are tabulated in table 2.XII and figure 2.XXXV.

Table 2.XII *In vitro* antimicrobial activity of petroleum ether extracts

S.No.	Stain	Microbes	Diameter in (mm)
1	Gram (+)	Bacteria *Bacillus subtilis*	(-)
2	Gram (-)	Bacteria *Escherichia coli*	19 mm
3	Gram (-)	Bacteria *Pseudomonas aeruginosa*	(-)
4		Fungus *Aspergillus niger*	(-)

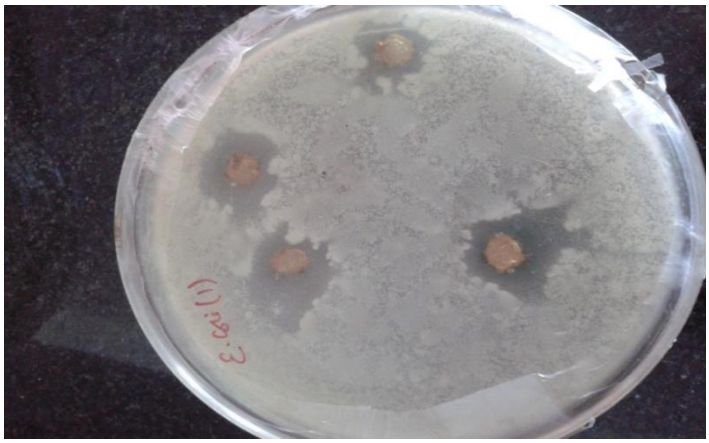


Fig. 2.XXXV Antibacterial activity of petroleum ether extracts against *E. coli*

B. Antimicrobial activity of toluene extracts: *In vitro* antimicrobial activity of toluene extract is given in table 2.XIII

Table 2.XIII *Invitro* antimicrobial activity of toluene extracts

S.No.	Stain	Microbes	Diameter in (mm)
1	Gram (+)	Bacteria *Bacillus subtilis*	(-)
2	Gram (-)	Bacteria *Escherichia coli*	(-)
3	Gram (-)	Bacteria *Pseudomonas aeruginosa*	(-)
4		Fungus *Aspergillus niger*	(-)

C. Antimicrobial activity of dichloromethane extracts The average measurement of diameter of zone of inhibition is given in the form of table 2.XIV and activity is shown in figure 2.XXXVI

Table 2.XIV *In vitro* antimicrobial activity of dichloromethane extracts

S.No.	Stain	Microbes	Diameter in (mm)
1	Gram (+)	Bacteria *Bacillus subtilis*	(-)
2	Gram (-)	Bacteria *Escherichia coli*	(-)
3	Gram(-)	Bacteria *Pseudomonas aeruginosa*	(-)
4		*Aspergillus niger*	16 mm

The dichloromethane extract of *Piper nigrum* seeds is active against fungus *Aspergillus niger***.**

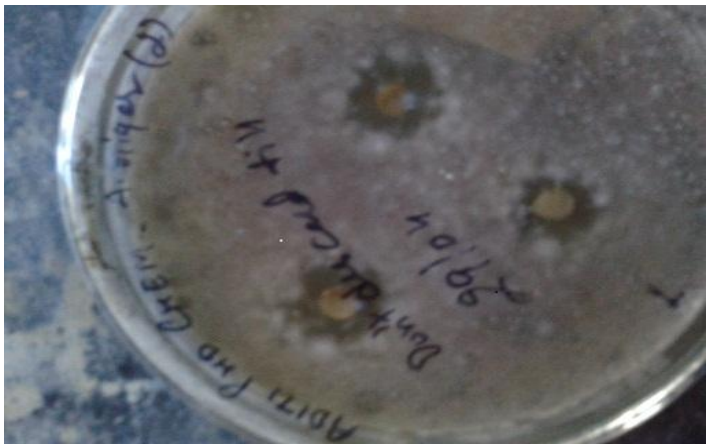


Fig. 2.XXXVI Antimicrobial activity against fungus *Aspergillus niger*

D. Antimicrobial activity of chloroform extracts: *In vitro* antimicrobial activity of chloroform extracts are given in table 2.XV and figures 2.XXXVII and 2.XXXVIII.

Table 2.XV *In vitro* antimicrobial activity of chloroform extracts

S. No.	Stain	Microbes	Diameter in (mm)
1	Gram (+)	Bacteria *Bacillus subtilis*	(-)
2	Gram (-)	Bacteria *Escherichia coli*	12 mm
3	Gram (-)	Bacteria *Pseudomonas aeruginosa*	(-)
4		*Aspergillus niger*	12 mm

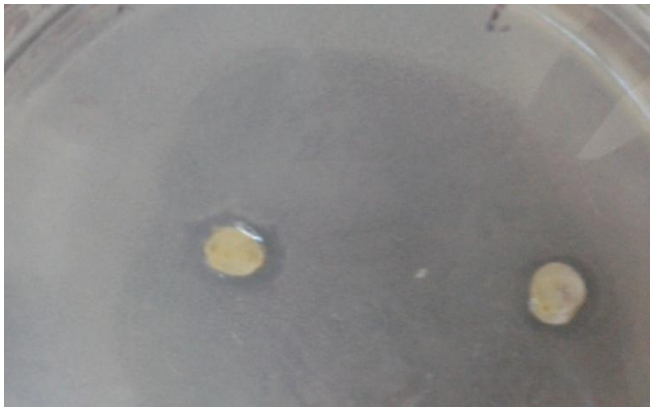


Fig. 2.XXXVII Antimicrobial activity against bacteria *E.coli*

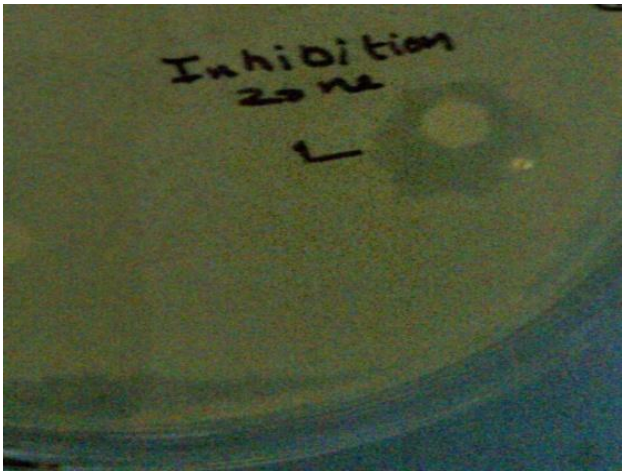


Fig. 2.XXXVIII Antimicrobial activity against fungus *Aspergillus niger*

E. Antimicrobial activity of ethyl acetate extracts: *In vitro* antimicrobial activity of ethyl acetate extracts of *Piper nigrum* are given in table 2.XVI and figure 2.XXXIX.

Table 2.XVI *In vitro* antimicrobial activity of ethyl acetate extracts

S. No.	Stain	Microbes	Diameter in (mm)
1	Gram (+)	Bacteria *Bacillus subtilis*	(-)
2	Gram (-)	Bacteria *Escherichia coli*	13 mm
3	Gram (-)	Bacteria *Pseudomonas aeruginosa*	(-)
4		*Aspergillus niger*	(-)

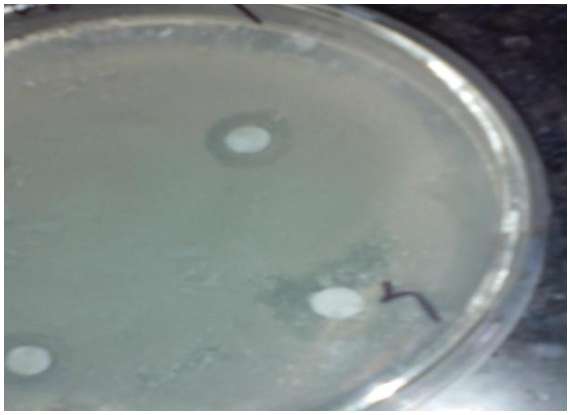


Fig. 2.XXXIX Antimicrobial activity of ethyl acetate extracts of *Piper nigrum* seeds against *E.coli*

2.5: Insecticidal activity of hot petroleum ether and methanol extracts of *Piper nigrum* against agricultural pest (*Coccinella septumpunctata)* and stored grain pest (*Sitophilus oryzae*): Insecticidal activity was performed in the laboratory under controlled conditions. The photographs of experimental work are attached herewith in figure 2.XXXX and table 2.XVII.

Fig. 2.XXXX Insecticidal activity against rice weevil and epilachna insect

The results are mentioned below in the table:

Table 2.XVII The mortality effect of *Piper nigrum* plant extracts on *Coccinella septumpunctata* and *Sitophilus oryzae*

S.No.	Extract of Plant	Insect	% of Extract (v/v)	No. of Insects used	No. of insects Dead (hours)				Total No. of insects dead	% of average mortality	% Corrected Mortality
					3h	4h	7h	12h			
1.	*P. nigrum* (Pet. ether extracts)	*Coccinella septumpunctata*	1 %	10	10	-	-	-	10	100	100
			5 %	10	10	-	-	-	10	100	100
			10 %	10	10	-	-	-	10	100	100
2.	*P. nigrum* (Pet. ether extracts)	*Sitophilus oryzae*	1 %	10	5	3	2	-	10	100	100
			5 %	10	10	-	-	-	10	100	100
			10 %	10	10	-	-	-	10	100	100
3.	*P. nigrum* (Methanol extracts)	*Coccinella septumpunctata*	1 %	10	1	-	-	-	1	10	10
			5 %	10	-	-	-	-	-	-	-
			10 %	10	2	-	-	-	2	20	20
4.	*P. nigrum* (Methanol extracts)	*Sitophilus oryzae*	1 %	10	-	-	-	-	-	-	-
			5 %	10	-	-	-	-	-	-	-
			10 %	10	1	1	1	1	1	10	10

Conclusion: The results of above experimental work shows that petroleum ether extracts showed good mortality effect rather than ethanol extracts.

2.6: Study of Antidiabetic action of hot petroleum ether and ethanol extracts of *Piper nigrum* seeds against Wistar rat model (Fig.2.XXXXI): This activity is performed at IIIM Jammu under controlled conditions and results are given in table 2.XVII and graph 2.I.

Fig. 2.XXXXI Wistar Rat

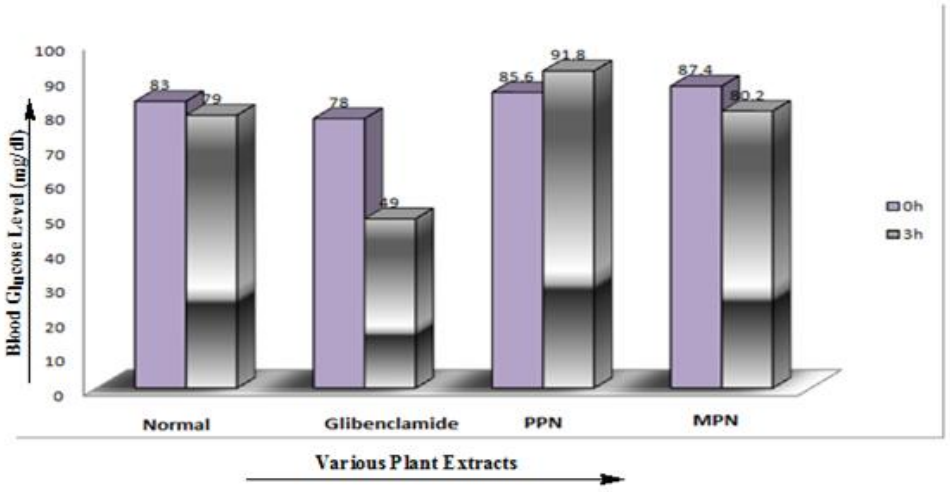
Table 2.XVII Hyperglycemic and hypoglycemic action of petroleum ether extracts and methanol extracts

S. No.	Treatment group (mg/kg p.o.)	Blood glucose level (mg/dl) Mean±S.E	
		0h	3h
1	Normal Control	83±4.65	79±4.09
2	Glibenclamide (0.5)	78±4.02	49±2.56***
3	Petroleum ether extracts of pepper (250)	85.6±3.32	91.8±1.98
4	Methanol extracts of pepper (250	87.4±5.39	80.2±4.31

n = (Number of animals): 5

$p < 0.001$ compared to 0h blood glucose level

Petroleum ether extracts of pepper is showing hyperglycemic activity while its methanol extracts is showing hypoglycemic activity.



Where NC = Normal control, PPN = Petroleum ether extracts of *Piper nigrum*, MPN = Methanol extracts of *Piper nigrum*

Graph 2.I Graphical representation of hyperglycemic and hypoglycemic action of petroleum ether extracts and methanol extracts

3.1: Introduction

Psoralea corylifolia belongs to family Fabaceae. It is mentioned as a valuable medicinal herb in Indian and Chinese Pharmacopeia.[50] It is commonly occurring in tropics and subtropics worldwide.[51-52] It is a leguminous plant and commonly called as Buguzhi in China. The dry fruits of the plant are popularly and conventionally used as a medicine in China and formally given in Chinese Pharmacopeia.[53] It is used as a tonic or an aphrodisiacal. The ethanol extracts of the seeds acts as a medicine against bone fracture, osteomalacia and osteoporosis.[54]

Psoralea corylifolia is an endangered species and its therapeutic importance is described in Chinese, British and American Pharmacopeias; Indian pharmaceutical codex and also in various conventional medicinal systems such as Ayurveda, Unani and Siddha.[55]

Botanical Description: It is erect, small, annual herb and can achieve up to 60-120 cm in height. It can grow in sandy and loamy soils throughout the plains of East and Central India.

Stems are gland-dotted and well grooved.

Leaves are rounded, simple, mucronate and broadly elliptic and both the sides are covered with white hairs, have many black dots, from the base five nerves are coming out.

Flowers are axillary, dense with yellowish or bluish purple corolla and fluorescence is raceme with 10-30 flowers. Usually flowering season is from the month of August to December.

Fruits are sub globular, small, indehiscent, 5 mm long, one-seeded pod adhered to pericarp, slightly compressed, pitted black, beaked without hairs.[56]

Seeds are 2 to 4mm in length, 2 to 3 mm in breadth and 1 to 1.5 mm in thickness, kidney shaped, oblong, hard, smooth, brown-black in colour, flattened, redolent odour, exalbuminous with straw-colored hard testa and acrid taste.[56] Due to the hard seed coat of this medicinally important plant, seed germination percentage is very low i.e. 5-7% and thus its need of the hour to cultivate this endangered and therapeutically important plant species.[55]

Pods are 3.5-4.5 mm × 2.0-3.0 mm, small, oval, mucronate, oblong, somewhat compressed, chocolate to black in colour.[55]

The factors that don't favour the commercial cultivation of the plant are long gestation period, low germination percentage and viability of the seeds and delicate field-handling.[55]

Geographical Distribution: It is distributed throughout the tropics and subtropics worldwide especially in South Africa and China. In India, this herb is distributed throughout the plains, especially in Rajasthan, Eastern Punjab and nearby areas of U.P, Himalayas, Bundelkhand, Oudh, Bengal, Dehradun, Karnataka, Bihar, Deccan and Bombay.[56]

Parts Used: The important parts are roots, leaves, seeds and seed oil.[56] Roots are used in dental aids. Leaves are used against diarrhoea. Fruits are used as aphrodisiacal, laxative and are used against psoriasis, leucoderma, leprosy and inflammatory problems of the skin.[57]

Pharmacognosy: Microscopic: T.S of the fruit reveals the presence of outer pericarp with prominent ridges and depressions with collapsed parenchyma and secretory glands containing oleo-resinous material; testa, an outer layer of palisade epidermis, layer of bearer cells and 2-3 layers of parenchyma; cotyledons of polyhedral parenchyma and 3 layers of palisade cells on the adaxial side.[56]

Pharmacology: All blooms such as roots, stems, leaves and seeds have utility to treat various diseases of skin like leucoderma, skin rashes and infections. It is also called by the name "Kushtanashini" i.e., Leprosy destroyer. Since antiquity it is used as a medicine for leucoderma and extensively used by the Indian Medicine Practitioners and Western system followers. Psoralens are the furanocoumarins that helps in enhancing pigmentation.[56]

The plant has its utility in both ways i.e., internal and external. The seed oil can be applied externally against many skin disorders. It is also used in disease characterized by hypopigmented lesions such as leucoderma and psoriasis both via local application and oral therapy. The paste prepared by mixing seed powder with Haratala Bhasma (Yellow arsenic) in the ratio of 4:1 in cow' urine can be used to treat the patches of leucoderma.[55]

It has been mentioned in Vedas that the seed powder can be given orally for leucoderma and leprosy and applied externally in the form of paste and ointments. The oil obtained from the plant is used against the skin *Streptococci*. It is also used against the disorder vitiligo which is characterized by the appearance of patches on the skin due to less pigmentation. It is beneficial against dermatitis, inflammatory disorders, mucomembranous disorders and edematous problems of the skin. It helps in relieving boils and eruptions of

the skin. It also acts as blood purifier. The plant plays an important role to treat itching, eruptions, ringworm, extensive eczema with thickened dermis, itching red papules, dermatosis with fissures and scabies, rough and discolored dermatosis. It is found to enhance the colour of nails, skin and hairs. Seeds are useful against bilious disorders and are also given in scorpion-sting and snake bite. Extracts of *Psoralea corylifolia* are known to possess antidepressant, antihyperglycemic, antioxidant and antitumour activities. Its aqueous extract exhibits antibacterial activities. The powder of seeds is used as an antihelminthic, laxative, diuretic, stomachic, stimulant, aphrodisiac, diaphoretic and for healing wounds. It helps in the treatment of many types of diseases like nephritis, cough and asthma. Psoralen and isopsoralen are present in major amount and are known to exhibit antitumour, antiviral and antibacterial properties. It acts as a beneficial hair tonic and useful in disease alopecia areata and hair loss.[56]

It is an efficient invigorant againt uterine hemorrhage, impotence and menstrual disorders. It acts as a cure for gynecological bleedings. It also has its utility against premature ejaculation and spermatorrhea. It exhibits coronary vasodilatory actions. The seeds are an important deobstruent, cure blood disorders, heals ulcer, heart troubles and elephantiasis.

The drug in crude form has its utility in the treatment of enuresis, pollakiuria, low back pains, debility, pain of cold in the knees and waist and weak kidney and other troubles associated with kidney inefficiency like frequent urination, febrile disorder, bed wetting and incontinence.

The roots are useful to treat dental caries. *P. corylifolia* has its utility in enhancing bone calcification, in the treatment of bone fractures and osteoporosis. Leaves are useful in relieving diarrhoea. Fruit cures difficulty in micturition, prevent vomiting, in treatment of piles, bronchitis, anemia and enhance complexion. The phytoconstituents psoralen, corylifolinin and bavachinin inhibit the multiplication of lung cancer cells, malignant ascites, osteosarcoma, leukemia and fibrosarcoma and also have hepatoprotective actions.

It is used against the diseases tuberculosis and lumbago. The essential oil obtained from the plant acts as a tonic and aphrodisiacal. Seeds are acrid, astringent and bitter. Seeds play an important role in imparting vitality and vigor; enhances receptive power of mind and digestive power. Seeds act as alexiteric and antipyretic. In vata diseases, the plant acts as a famous nervine tonic. The plant plays an important role in the treatment of intestinal amebiasis. The herb is antirepellant, cytotoxic and antimutagenic.

Other uses: Seeds have utility to prepare perfume oils. In Japan, seed's ethanolic extract is used as a preservative in some processed foods or pickles. Due to high percentage of minerals and nitrogen in seed cake, it is used as manure or cattle feed.[56]

Phytochemistry: Phytochemical analysis of *Psoralea corylifolia* showed the presence of β-sitosterol, terpenoids, phenolic compounds, saponins, glycosides, tannins[58], chalcones derivatives, coumestans, coumarins[59], monoterpenes, benzofuran glycosides.[60] The seeds contain nonvolatile terpenoid oil, 13.2% of extractive matter, a dark brown resin (8.6%), sugar, ash (7.4%), an essential oil (0.05%), albumin and traces of alkaloidal substance and manganese in traces. Seeds also contain unsaponifiable oil having the formula $C_{17}H_{24}O$, with boiling point 180-190^{0}C. A yellowish acidic substance $C_{40}H_{45}O_{10}$ and a methyl glycoside with 4 OH groups and melting point 105-107^{0}C are also obtained from the alcoholic extracts of seeds.[56]

The components of essential oil are linalool, α-elemene, 4-terpineol, γ-elemene, limonene, β-caryophylenoxide, geranylacetate, active component psoralen (identical with ficusin; $C_{11}H_6O_3$, m.p. 161-162°C). Petroleum ether extract of the seeds are known to possess resin acids (21.5%) and glycerides of linoleic acid, myristolic acid, oleic acid, stearic acid, myristic acid, linolenic acid and palmitic acid.[56]

The various phytochemicals reported are psoralen, psoralidin, corylin, bakuchiol, isopsoralen and corylifolin[61], 4-methoxy flavone[51], Monoterpenoids- bakuchiol A and B and (S)-Bakuchiol, bavachin, bavachinin, bavachalcone, corylifol A, B and C, neobavaisochalcone, isoneobavachalcone, isobavachalcone, 8-prenyl Diadzein, brosimacutin, bakuchalcone and erythrinin.[62]

The various other phytochemicals isolated are 12, 13-dihydro-12, 13-epoxy bakuchiol, psoracorylifol A-E.[63-64] Angelicin and cyclobakuchiols A and B have been reported from dichloromethane extracts of *Psoralea corylifolia.*[65] Bavachin, bavachinin, 6-prenyl naringenin, 3-hydroxy bakuchiol, γ-cadinene, diadzein, genistein, psoralester, psorachromene, 7-methoxybavachin, chromenoflavone, 4-hydroxylonchocarpin, bavachalcone, bavachin, corylifolinin, bavachinin are some other phytoconstituents documented from *Psoralea corylifolia.*[51-52, 58, 70]

Leaves and flowers are given in figure 3.I and seeds in figure 3.II.

Fig. 3.I Leaves and flowers of *Psoralea corylifolia*

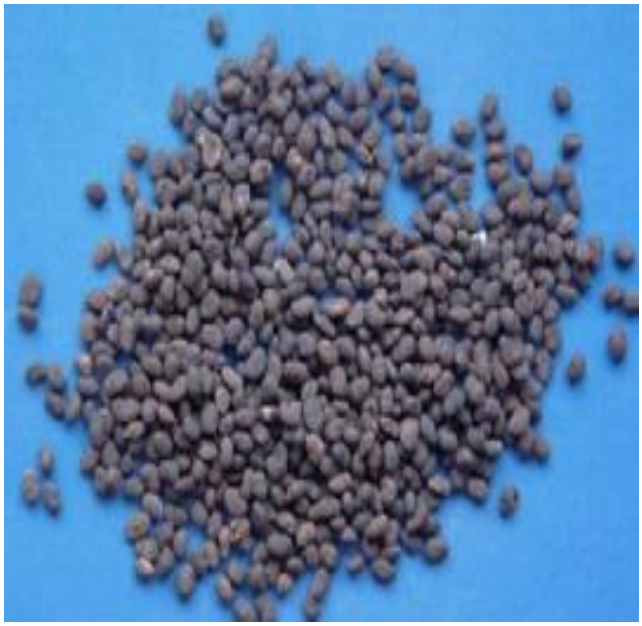


Fig. 3.II Seeds of *Psoralea corylifolia*

3.2: Materials and Methods

Plant Materials: *Psoralea corylifolia* seeds (1Kg) were bought from an authentic seed shop of Kathua district of Jammu and Kashmir and classified systematically by Dr. Gurdev Singh of Botany Department of LPU.

Hot Extraction: The seeds were dried, crushed and soxhlated in methanol for 120 hours. The crude methanol extract was successively distilled with different solvents according to their increasing polarity gradient such as petroleum ether, dichloromethane, ethyl acetate and methanol to make the respective fractions.

Experimental

Analytical tools: The solvent apparatus (JSGW) was used for the extraction of plant material. Lab fit melting point apparatus was used to determine the melting point. A UV spectrum was recorded on SHIMADZU UV-1800 UV spectrophotometer using ethanol as solvent. IR spectra were obtained on Shimadzu FTIR-8400S (Fourier Transforms Infrared Spectrophotometer). ^{1}H-NMR (400MHz) and ^{13}C-NMR were recorded in MeOD on Bruker, Avance 400 MHz NMR spectrometer. Chemical shifts are given as δ with TMS as internal standard. A HR-mass spectrum was recorded on Agilent, 6540, Q-TOF (HR-MS) mass spectrometer. The oil was analyzed by using Varian 4000 GC-MS. The instrument works on the following conditions: fitted with fused silica 30 m (CP-Sil-8,Varian), the internal diameter and film thickness of capillary column are 0.25 mm and 0.25 µm, the Helium carrier gas is introduced at a rate of 1 ml/min, a capillary injector operating at 280°C in the split mode (1:150), flame ionization detector (FID) running at temperature 300°C, the column oven temperature programming was 50°C for 5 min and then enhanced from 50 to 250°C at the rate of 3°C/min and hold for 7 min.

Apparatus and Equipments: The equipments laminar air flow, incubator and oven were of Yorko Industries where as autoclave of JSWG. Glassware and heating mantle were from Perfit India.

3.3: Results and Discussion

A. Investigation of hot methanol extracts

The crude methanol extracts was subjected to column chromatography. The fraction obtained from the column of solvent ratio (7:3) toluene **:** chloroform was settled at the bottom of the beaker in the form of white rod shaped shiny needle like crystals (Fraction 1 i.e., pc 1). A solvent ratio of petroleum ether **:** dichloromethane collected from the column after being kept for 10-12 days shows oil (pc 2) which is then stored in refrigerator in airtight glassware and further subjected to GC-MS analysis.

Fraction 1: Spectroscopic analysis of white crystals (pc 1): The melting point of the crystals determined by Labfit apparatus was found out to be 166 ^{0}C which is close to 169 ^{0}C given in the literature.[70]. The white crystals were repeatedly purified with ethanol and TLC showed Rf value of 0.30 which is very similar to that of psoralen as given in literature. The molecular formula was established as $C_{11}H_6O_3$ by Agilent, 6540 Q-TOF (HRMS) mass spectrometer.[71]

1. UV spectrum of pc 1: The presence of coumarin derivative was indicated by the presence of UV absorption at 260, 290, 330 nm which is in accordance with that given in literature.[71] The UV spectral peaks resemble exactly to the spectral peaks of UV spectrum of psoralen as reported in literature.[45] The UV spectrum is attached in the form of figure 3.III.

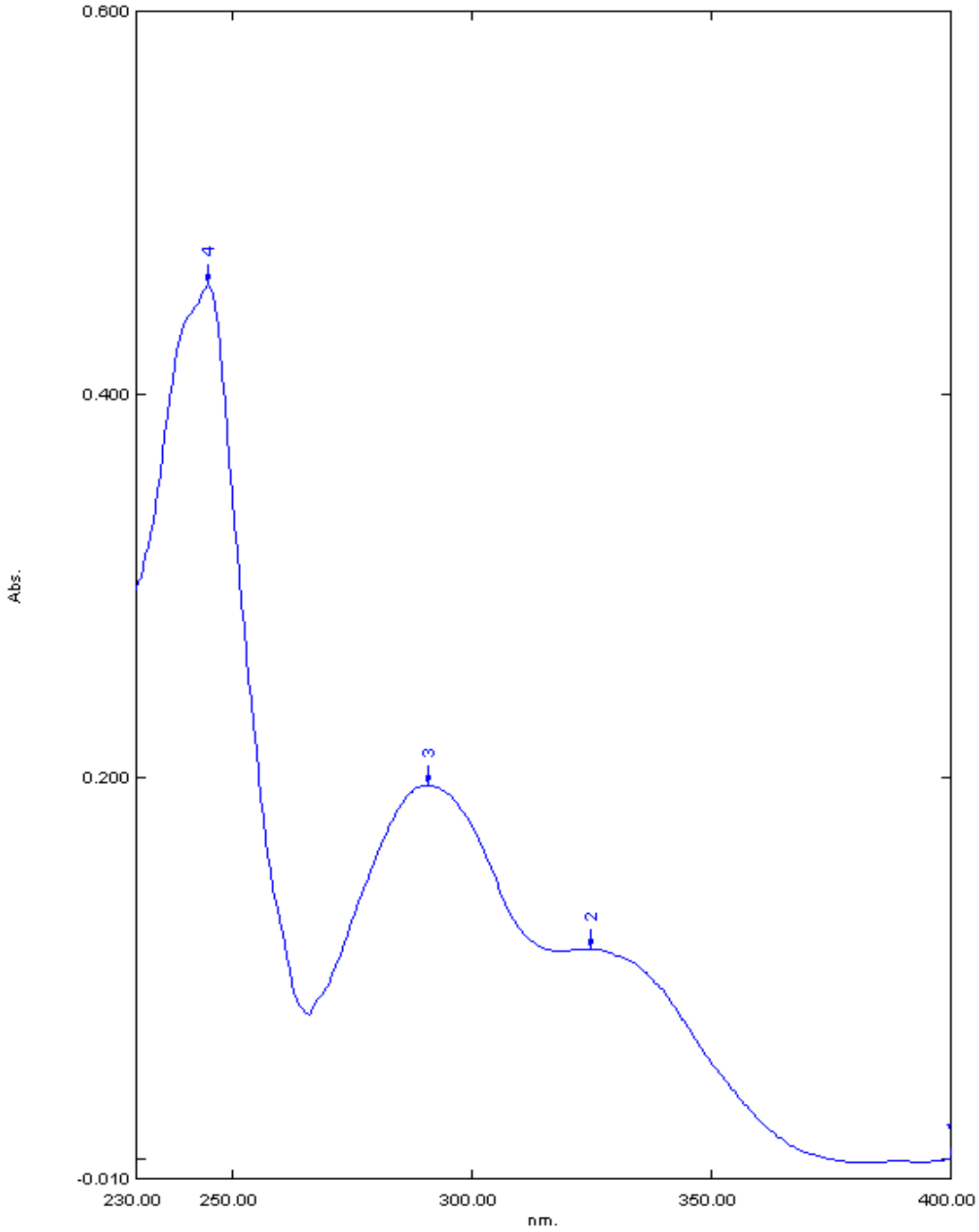
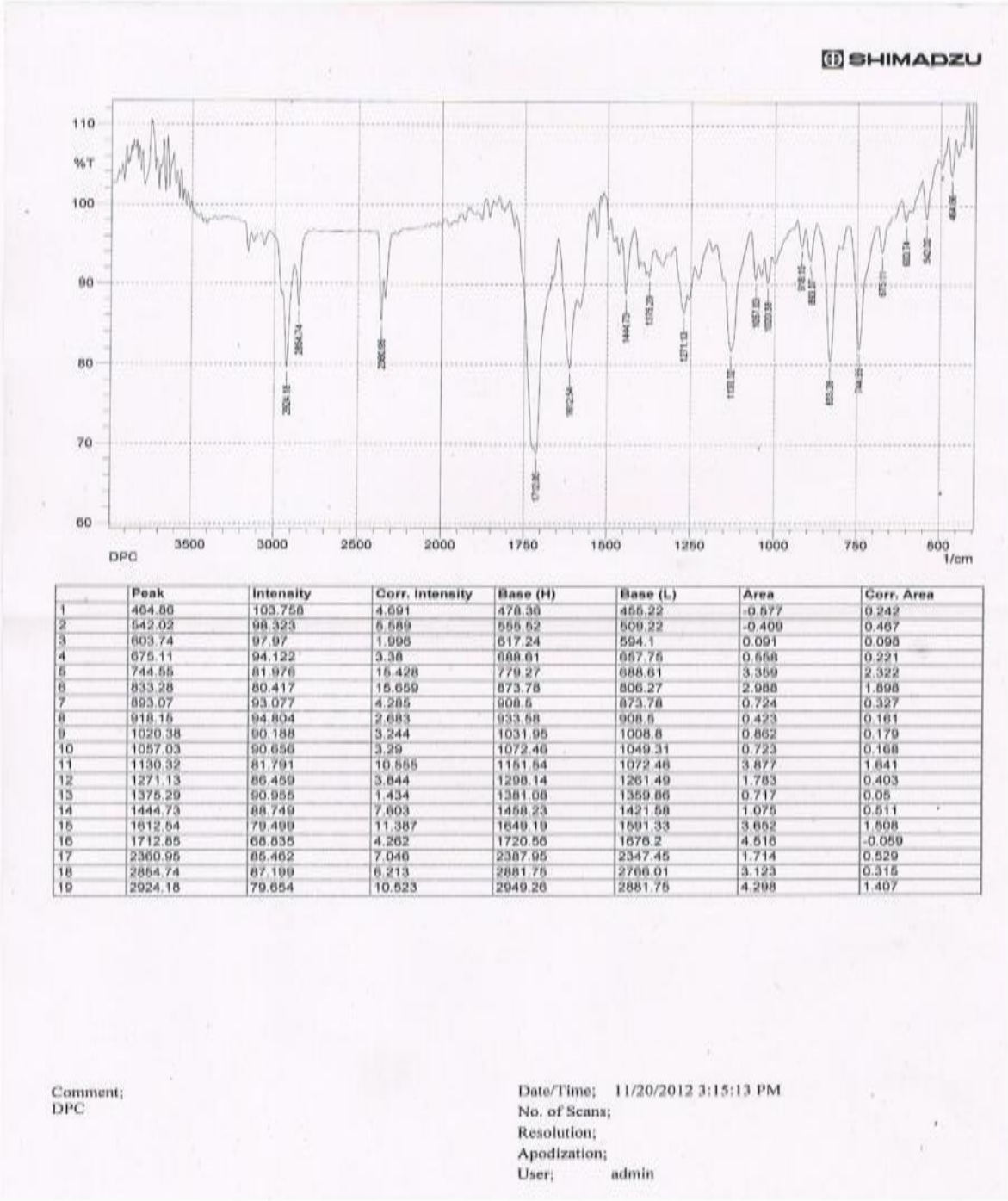


Fig. 3.III UV spectrum of white crystals

2. IR spectrum of pc 1: The IR spectral peak at 1712 cm^{-1} confirms the presence of carbonyl group in the structure, 1020.38, 1057.03, 1130.32 cm^{-1} confirms the group C-O-C in the structure, 2924 and 2854 cm^{-1} confirms aromatic and vinylic hydrocarbon group (C-H) in the structure, 1612 cm^{-1} confirms C=C group in the structure, 1444 cm^{-1} confirms the presence of aromatic ring in the structure. The spectrum (figure 3.IV) is very similar to IR spectrum of psoralen as reported in the literature.[73]



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	464.86	103.758	4.691	478.36	455.22	-0.577	0.242
2	542.02	98.323	6.589	555.52	509.22	-0.409	0.467
3	603.74	97.97	1.996	617.24	594.1	0.091	0.098
4	675.11	94.122	3.38	688.61	657.75	0.658	0.221
5	744.55	81.976	15.428	779.27	688.61	3.359	2.322
6	833.28	80.417	15.659	873.78	806.27	2.988	1.898
7	893.07	93.077	4.285	908.5	873.78	0.724	0.327
8	918.16	94.804	2.683	933.58	908.5	0.423	0.161
9	1020.38	90.188	3.244	1031.95	1008.8	0.862	0.179
10	1057.03	90.656	3.29	1072.46	1049.31	0.723	0.168
11	1130.32	81.791	10.555	1151.64	1072.46	3.877	1.641
12	1271.13	86.459	3.844	1298.14	1261.49	1.783	0.403
13	1375.29	90.955	1.434	1381.08	1359.86	0.717	0.05
14	1444.73	88.749	7.603	1458.23	1421.58	1.075	0.511
15	1612.64	79.499	11.387	1649.19	1591.33	3.652	1.508
16	1712.85	66.835	4.262	1720.56	1676.2	4.516	-0.059
17	2360.95	85.462	7.046	2387.95	2347.45	1.714	0.529
18	2854.74	87.199	6.213	2881.75	2766.01	3.123	0.315
19	2924.18	79.654	10.523	2949.26	2881.75	4.298	1.407

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Fig. 3.IV IR spectrum of white crystals

3. ^{1}H-NMR spectrum of pc 1: The ^{1}H-NMR spectrum (Fig. 3.V) shows the peak at δ 7.80 (d, 1H, J=1.00, C_4-H), δ 7.70 (d, 1H, J = 1.08, C_{12}-H), δ 7.69 (1H, s, C_5-H), δ 7.49 (1H, s, C_8-H), δ 6.84 (1H, d, 1H, J=0.16, C_{11}-H), δ 6.39 (1H, D, J=0.96, C_3-H).[61, 72]

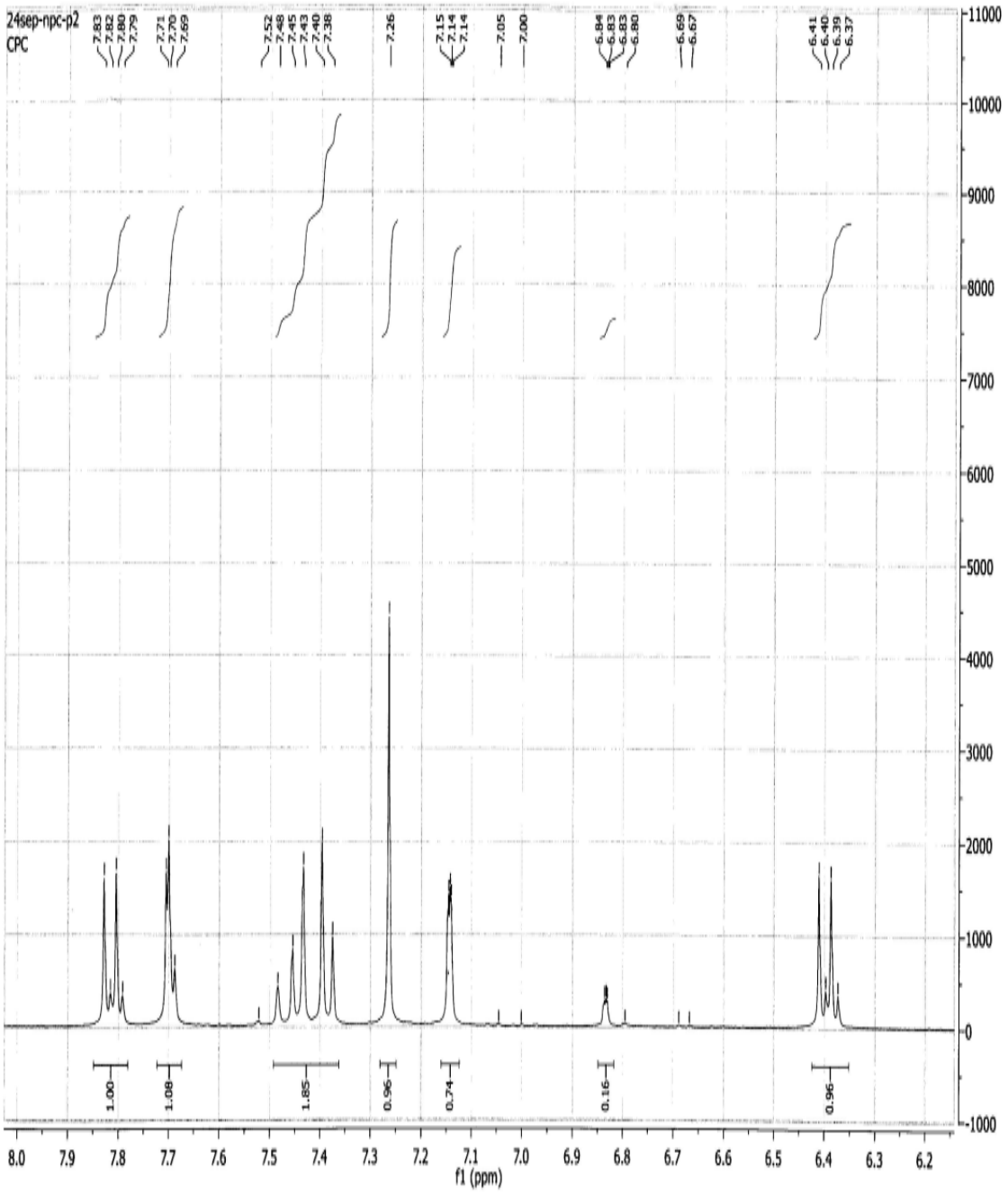


Fig. 3.V ^{1}H-NMR spectrum of white crystals

4. ^{13}C-NMR spectrum of pc 1: The ^{13}C-NMR spectrum (Fig. 3.VI) reveals the presence of peaks at δ-158 (C-2), δ-157 (C-7), δ-146.92 (C-9), δ-145.89 (C-12), δ 144.53 (C-4), δ 123.83 (C-6), δ 119.84 (C-5), δ 114.70 (C-10), δ 114.14 (C-3), δ 106.39 (C-11), δ 99.92 (C-8) that resembles to that of psoralen as given in literature.[61, 72]

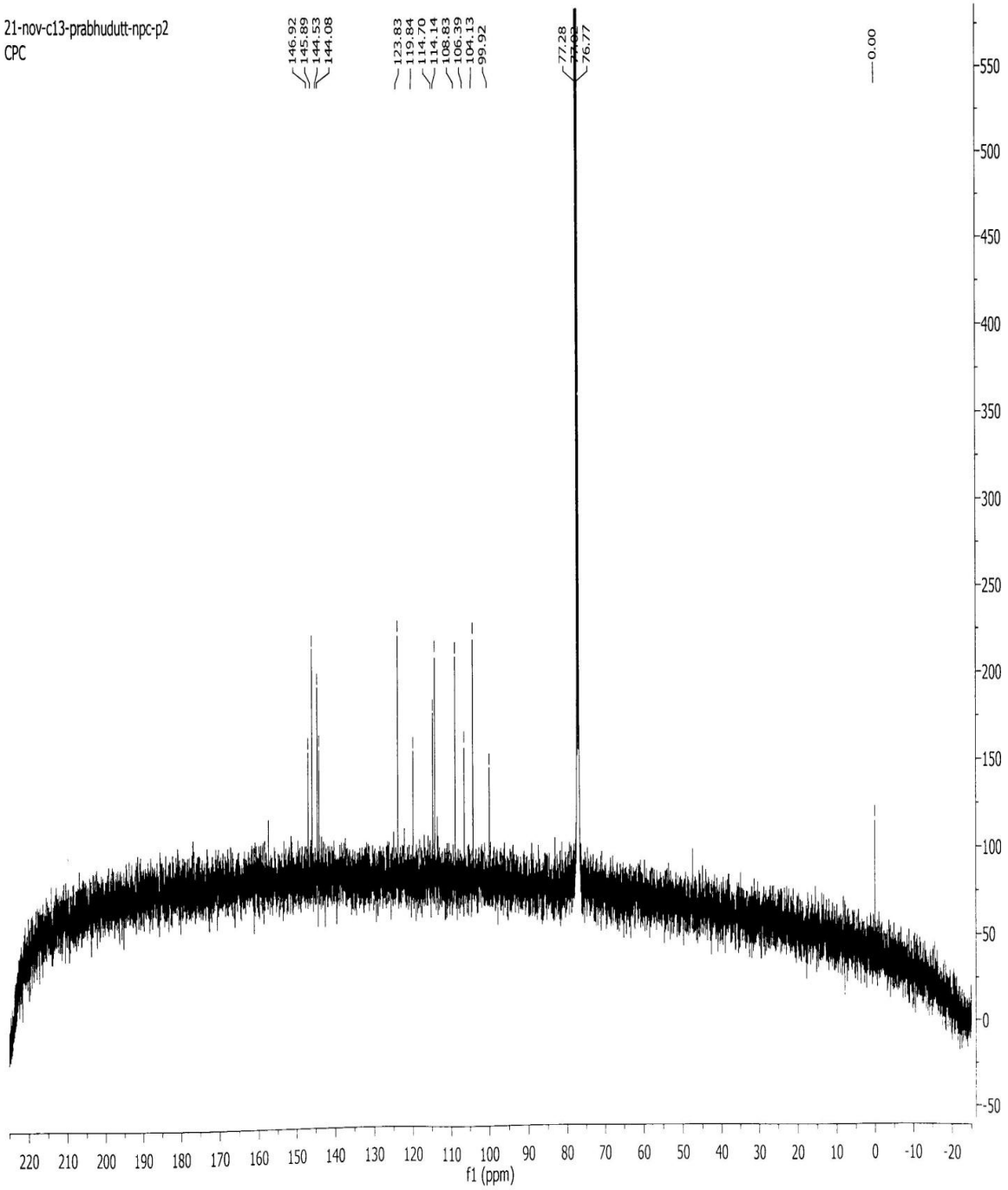


Fig. 3.VI ^{13}C-NMR spectrum of pc 1

5. Mass spectrum of pc 1: The HR-MS (Fig. 3.VII) showed following peaks which are explained in the table 3.I below:

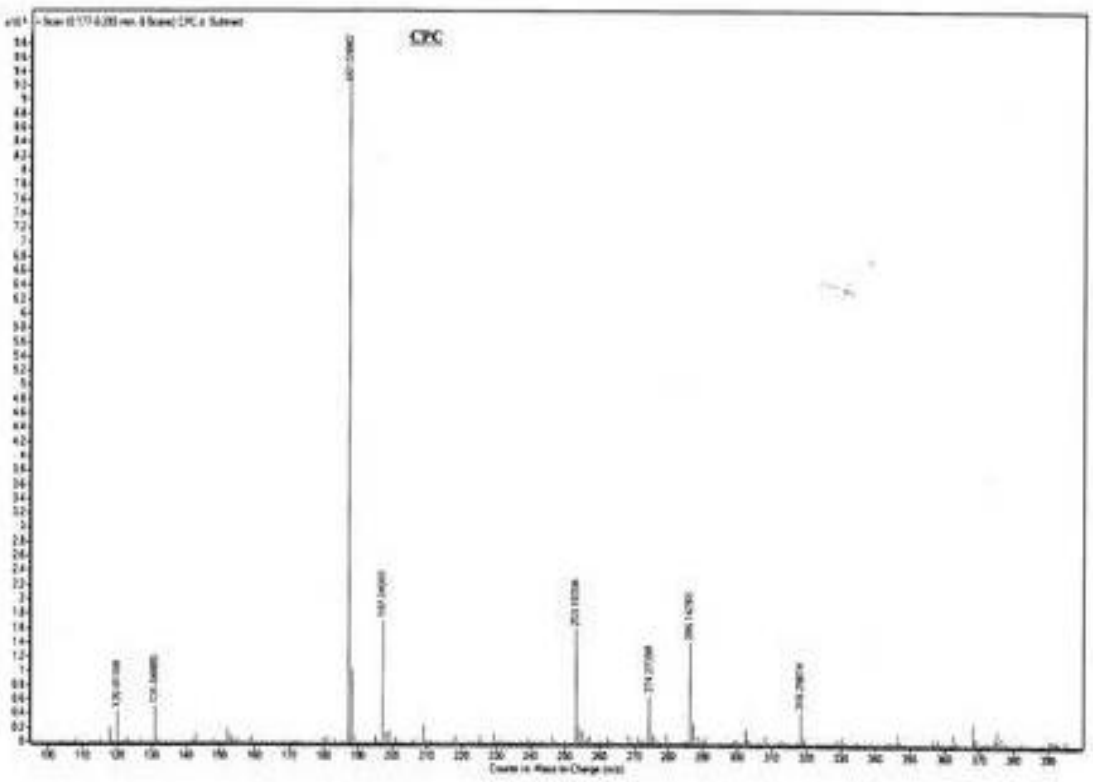


Fig. 3.VII Mass spectrum of white crystals

Table 3.I : Peaks obtained in MS of white crystals

S.No.	**Peak**	**Corresponds to**
1	187	$[M + H]^+$
2	121	$[M - C_4H_40]^+$
3	253	$[M + 2H + C_4H_40]^+$
4	130	$[M - C_4H_7]^+$
5	208	$[M + Na]^+$
6	318	$[2M – 2 CO + 3H]^+$

On the basis of above spectral and chemical analysis the crystals obtained are assigned as psoralen (Fig.3.VIII and 3.IX).

Fig. 3.VIII Psoralen crystals

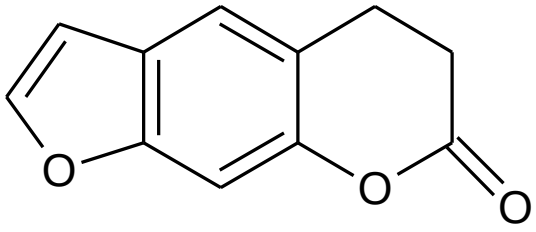


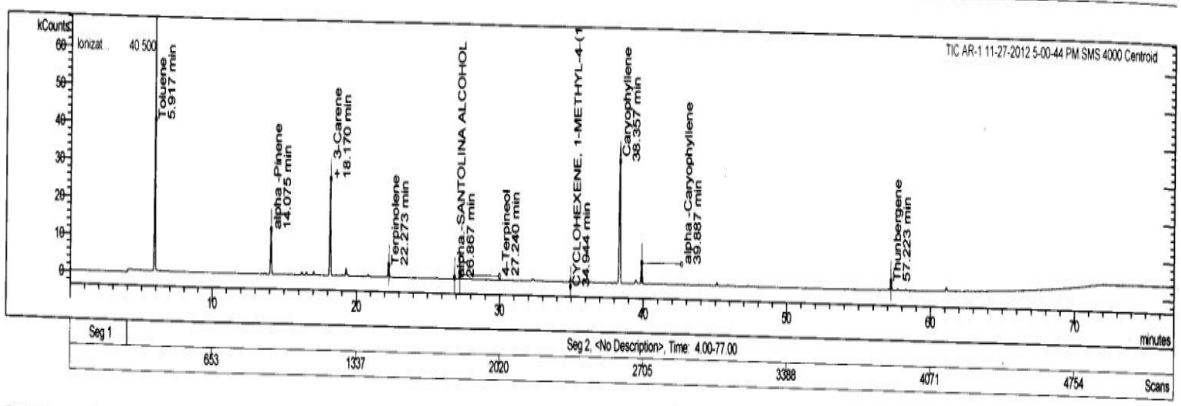
Fig. 3.IX Psoralen structure

Pc 2: GC-MS analysis of oil: Compounds were identified by their GC retention time relative to known compounds and by comparison of their mass spectra with those present in IIIM library**.** The spectrum is given in figure 3.X. The amount and structures of isolated phytoconstituents are given in table 3.II and figure 3.XI.

Print Date: 03 Dec 2012 14:55:08
INSTRUMENTATION DIVISION, IIIM, JAMMU

Sample Report for ar-1 11-27-2012 5-00-44 pm.sms

Sample ID:	AR-1	Operator:	Varian	Instrument ID:	Varian GC/MS #1
Vial:	TR: Tray1 VL: 2	Acquisition Date:	11/27/2012 5:00 PM	Data File:	...11-27-2012 5-00-44 pm.sms
Calculation Date:	12/3/2012 2:53 PM	Method:	c:\varianws\oils slow methodar-1.mth		
Inj. Sample Notes:	None			Injection:	1
Volume:	0.20 uL				



Target Compounds

Cmpd. Number	RT (min)	Peak Name	Area	Amount/RF
1	5.917	Toluene	119838	62.255
2	14.075	alpha -Pinene	12947	6.726
3	16.530	L-beta -pinene	951	0.494
4	17.035	beta -Pinene	951	0.494
5	18.170	3-Carene	24278	12.612
6	19.282	Limonene	1823	0.947
7	20.824	Gamma terpinene	547	0.284
8	22.273	Terpinolene	2727	1.417
9	26.867	alpha.-SANTOLINA ALCOHOL	1182	0.614
10	27.240	4-Terpineol	910	0.473
13	34.944	CYCLOHEXENE, 1-METHYL-4-(1-METHYLETHENYL	706	0.367
14	38.357	Caryophyllene	19037	9.890
16	39.887	alpha -Caryophyllene	5702	2.962
18	57.223	Thunbergene	897	0.466

****

Method Notes

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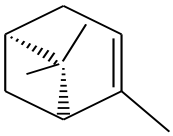
Injection Method: c:\varianws\oils slow method.mth
1ml/min Helium gas as career, injector 280deg C, split ratio 1:150, column oven 50 deg C for 5min,
250 deg @ 3deg/min, hold for 7 min, ca
pillary column CP-Sil-8, 30m X 0.32mm X 0.25u thick film.
Recalc Method: c:\varianws\oils slow methodar-1.mth
1ml/min Helium gas as career, injector 280deg C, split ratio 1:150, column oven 50 deg C for 5min,
250 deg @ 3deg/min, hold for 7 min, ca
pillary column CP-Sil-8, 30m X 0.32mm X 0.25u thick film.T Rol

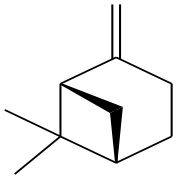
Fig. 3.X GC-MS of fraction 2

Table 3.II: The list of phytochemicals identified and their peak value, retention time by comparing with entries in IIIM library

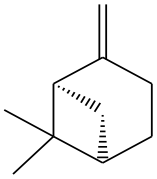
Peak	R. time	Area	Name of compound
1	62.255	119838	Toluene
2	6.726	12947	α-pinene
3	0.494	951	L-β-pinene
4	0.494	951	β-pinene
5	24278	18.170	3- Carene
6	19.282	1823	Limonene
7	0.284	547	γ-terpinene
8	1.417	2727	Terpinolene
9	0.614	1182	α- santolina alcohol
10	0.473	910	4- terpineol
11	38.357	19037	Caryophyllene
12	39.887	5702	α-Caryophyllene
13	0.466	897	Thumbergene



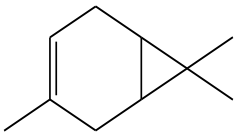
(1) α-pinene



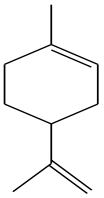
(2) L-β-pinene



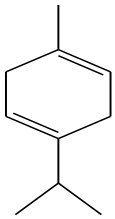
(3) β-pinene



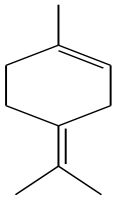
(4) 3- Carene



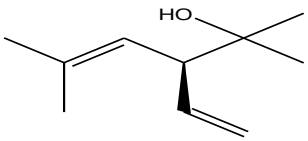
(5) Limonene



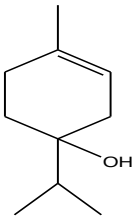
(6) γ-terpinene



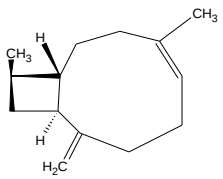
(7) Terpinolene



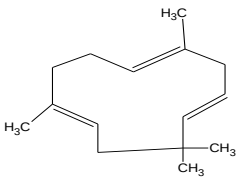
(8) α-santolina alcohol



(9) 4- terpineol



(10) Caryophyllene



(11) α-Caryophyllene

Fig. 3.XI Various components obtained by GC-MS of oil (fraction 2)

3.4: Antimicrobial activity of various hot extracts of *Psoralea corylifolia* against the bacterias and fungus

Bacterial strains: The biological activities of different hot extracts were tested against bacteria *E. coli* (NCIM No.- 2563), *Bacillius subtilis* (NCBI No.- PS3 KJ489411.1), *Pseudomonas aeruginosa* (NCBI No.- jogii JX276925.1) and fungus *Aspergillus niger* (NCIM No.- 619). These bacterial cultures were ordered from NCIM Pune and maintained at 4^0C on nutrient broth in Biotechnology labs of LPU. Potato dextrose broth was used for fungal culture.

Chemicals: Nutrient agar and nutrient broth for bacterial cultivation; potato dextrose agar, potato dextrose broth and standard antibiotic like gentamicin were purchased from Hi Media Laboratories Pvt. Ltd., Mumbai.

Preparation of nutrient agar medium: Dissolve 28 grams of nutrient agar in 1000 ml distilled water. Gently heat to dissolve the components completely. The nutrient agar medium was then sterilized in an autoclave at 15 psi (121^0C) for 15 minutes and then cooled at room temperature in a laminar. After cooling it was decanted in the sterilized petriplates and placed in laminar till solidification.

Antimicrobial activity of plant extracts: One gram of the extracts was dissolved in same solvent in such a way that the final concentration of each extract would be 1gm/ml of respective solvent.

Disc diffusion method: The *in vitro* antimicrobial activity of various extracts of *Psoralea corylifolia* seeds was checked by disc diffusion method. Bacterial culture was inoculated in nutrient agar and plated. The 5 µl of various extracts were poured on to different discs prepared from whatman No: 1 filter paper. The 2 or 3 discs were then placed on the petriplates containing cultures and incubated bacterias for 24 hours at 37^0C. The diameter of zone of inhibition was measured. All the experiments were triplicated.[25]

A. Antimicrobial activity of various extracts: The average measurement of zone of inhibition against various microbes is given in the table. The *in vitro* antimicrobial activity of petroleum ether extract is given in table 3.III and figure 3.XII. The *in vitro* antimicrobial activity of DCM extract is given in table 3.IV, *in vitro* antimicrobial activity of ethyl acetate extract is given in table 3.V and figure 3.XIII, *in vitro* antimicrobial activity of methanol extract is given in table 3.VI and figure 3.XIV.

Table 3.III: *In vitro* antimicrobial activity of petroleum ether extracts

S. No.	Stain	Microbes	Diameter in (cm)
1	Gram (+)	Bacteria *Bacillus subtilis*	(-)
2	Gram (-)	Bacteria *E. coli*	22 mm
3	Gram (-)	Bacteria *Pseudomonas aeruginosa*	(-)
4		*Trichoderma ressi*	(-)

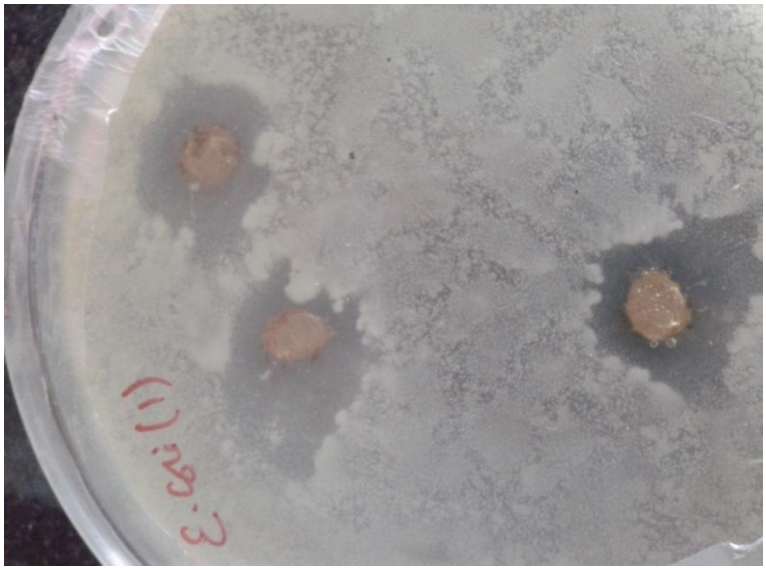


Fig. 3.XII Antibacterial activity of pet. ether extracts against *E.coli*

Table 3.IV *In vitro* antimicrobial activity of dichloromethane extracts

S.No.	Stain	Microbes	Diameter in (mm)
1	Gram (+)	Bacteria *Bacillus subtilis*	(-)
2	Gram (-)	Bacteria *E. coli*	(-)
3	Gram (-)	Bacteria *Pseudomonas aeruginosa*	(-)
4		*Trichoderma ressi*	(-)

Table 3.V *In vitro* antimicrobial activity of ethyl acetate extracts

S. No.	Stain	Microbes	Diameter in (mm)
1	Gram (+)	Bacteria *Bacillus subtilis*	(-)
2	Gram (-)	Bacteria *E.coli*	14 mm
3	Gram (-)	Bacteria *Pseudomonas aeruginosa*	(-)
4		*Trichoderma ressi*	(-)

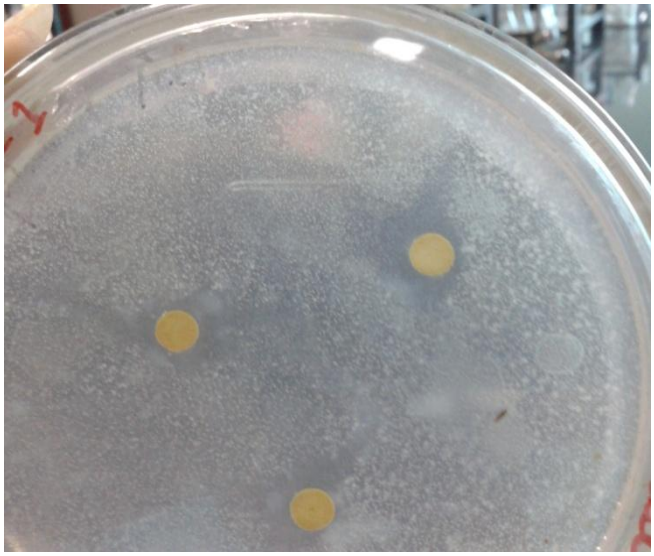


Fig. 3.XIII Antibacterial activity of ethyl acetate extracts against *E.coli*

Table 3.VI *In vitro* antimicrobial activity of methanol extracts

S. No.	Stain	Microbes	Diameter in (mm)
1	Gram (+)	Bacteria *Bacillus subtilis*	(-)
2	Gram (-)	Bacteria *E. coli*	20 m
3	Gram (-)	Bacteria *Pseudomonas aeruginosa*	(-)
4		*Trichoderma ressi*	(-)

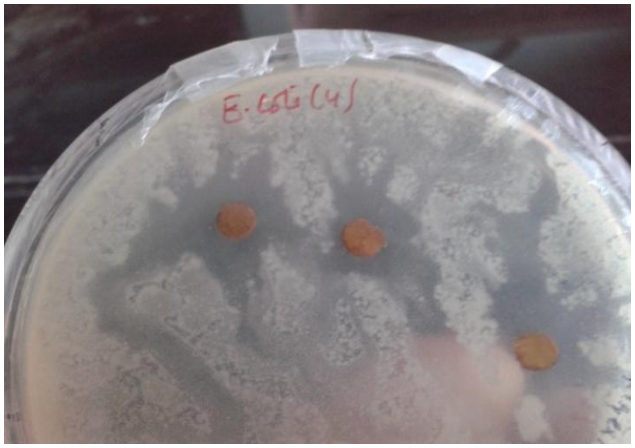


Fig. 3.XIV Antimicrobial activity of methanol extracts against *E. coli*

3.5: Insecticidal activity of hot petroleum ether and methanol extracts of *Psoralea corylifolia* seeds

The insecticidal activity of petroleum ether and methanol extracts were investigated against agricultural epilachna insect *Coccinella septumpunctata* and rice weevil *Sitophilus oryzae* in a laboratory. Insecticidal activity was found out at a temperature of 25 ± 4^0C and 70 ± 5% relative humidity in dark conditions. The mortality rate of both the insects was checked against 1%, 5% and 10% concentration of petroleum ether and ethanol extracts. Finding insecticidal activity is of great importance as using plant extracts as insecticides are biodegrable and do not produce toxicity hence are ecofriendly.

Insecticidal Testing: The petroleum ether and methanol extracts were prepared at three different concentrations (1%v/v, 5%v/v, 10%v/v).

i. **Insect Assayed: Test insects:** The epilachna insect *Coccinella septumpunctata* were collected from the fields and rice weevil *Sitophilus oryzae* were collected from the rice mill and identified by Entomologist Dr. Sudhakar Gupta of Lovely Professional University.

ii. **Determination of Insecticidal activity by Contact toxicity assay:** The Wheat leaves (for Epilachna insect) (Fig. 3.XV) and rice grains (for rice weevil) (Fig. 3.XVI) are taken from the field and house, washed and dried and then insects were allowed to feed on them under controlled conditions of temperature and humidity. These washed and dried wheat leaves and rice grains are then dipped in the respective percentage of plant extracts (i.e. 1%, 5%, 10% v/v) for two hours and then insects are allowed to feed on these leaves in each jar and mortality rate of the insects was checked. Results were recorded by counting the number of survivals in each jar. Control dishes with petroleum ether, methanol, distilled water and without solvent were performed separately up to 72 hours. Mortality was assessed after 3, 4, 7 and 12 h of the treatment. The calculation of mortality rate was corrected for control mortality according to Abbott's formula[45]:

$$Mc = (Mo-Mc/100-Me) * 100$$

Where Mo = observed mortality rate of treated adults (%), Me = mortality rate of control (%) and Mc = corrected mortality rate (%)

Results are given in table 3.VII.

Fig.3.XV Insecticidal activity against *Coccinella septumpunctata*

Fig.3.XVI Insecticidal activity against *Sitophilus oryzae*

Table 3.VII: Toxicity of *Psoralea corylifolia* plant extracts on agricultural insects and stored grain insect

S.No.	Extract of Plant	Insect	% of Extract (v/v)	No. of Insects used	No. of insects Dead (hours)				Total No. of insects dead	% of average mortality	% Corrected Mortality
					3h	4h	7h	12h			
1.	*Psoralea corylifolia* (Pet. ether extracts)	*Coccinella septumpunctata*	1 %	10	10	-	-	-	10	100	100
			5 %	10	10	-	-	-	10	10	100
			10 %	10	10	-	-	-	10	10	100
2.	*Psoralea corylifolia* (Pet.ether extracts)	*Sitophilus oryzae*	1 %	10	7	-	3	-	10	100	100
			5 %	10	10	-	-	-	10	100	100
			10 %	10	9	1	-	-	10	100	100
3.	*Psoralea corylifolia* (Methanol extracts)	*Coccinella septumpunctata*	1 %	10	1	-	-	-	1	10	10
			5 %	10	10	-	-	-	-	100	100
			10 %	10	10	-	-	-	10	100	100
4.	*Psoralea corylifolia* (Methanol extracts)	*Sitophilus oryzae*	1 %	10	10	-	-	-	10	100	100
			5 %	10	10	-	-	-	10	100	100
			10 %	10	10	-	-	-	10	100	100

Results and Discussion: Petroleum ether extract of the plant is showing 100 % mortality against the agricultural pest *Coccinella septumpunctata* and stored grain insect *Sitophilus oryzae* where as methanol extract is showing only 10 % mortality against *Sitophilus oryzae* and less active against *Coccinella septumpunctata.*

Conclusion: Petroleum ether extract is active against both the insects while methanol extract is more active against epilachna insect.

3.6: Antidiabetic activity of hot petroleum ether and methanol extracts of *Psoralea corylifolia* seeds

Antidiabetic activity of petroleum ether and methanol extracts of *Psoralea corylifolia* seeds were determined in CSIR lab, IIIM Jammu on wistar rats by 18h Fasted rats model.

Experimental

***In vivo* models**: **18h Fasted rats model**

Four groups of six Wistar rats (male/female) were selected randomly and fasted overnight. First group was given Normal Vehicle Control, second and third groups were given petroleum ether and methanol extracts treated diet and fourth group was given glibenclamide treated diet. Blood glucose level of all the rats was found out prior to any treatment (0 hour) and post-drug administration (3 hour).[46] Results are tabulated in table 3.VIII and Graph 3.I.

Table 3.VIII: Diabetic activity of petroleum ether and methanol extracts of *Psoralea corylifolia*

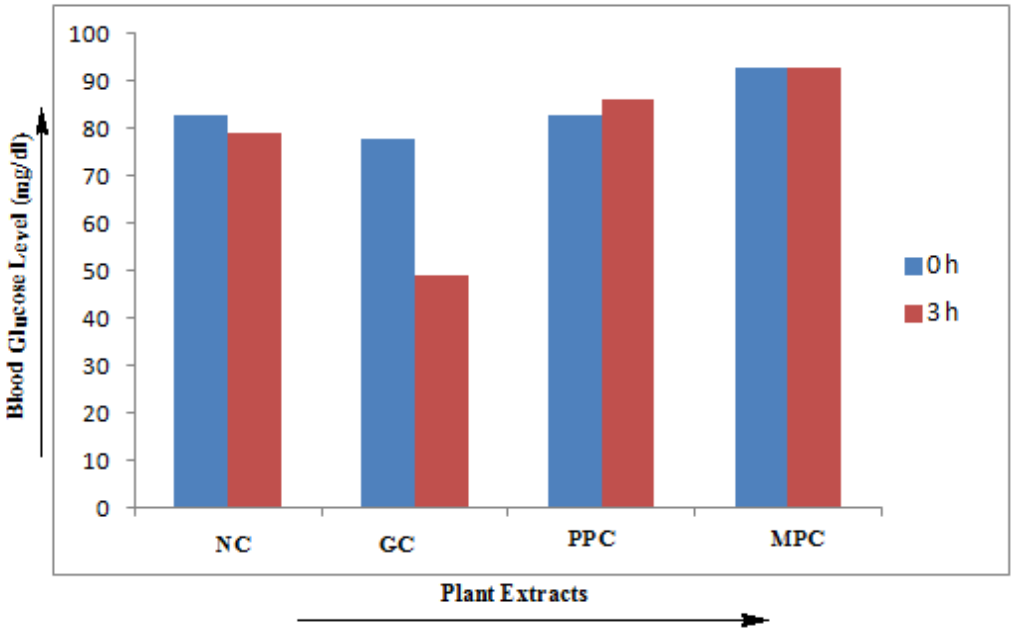
S.No.	Treatment group (mg/kg p.o.)	Blood glucose level (mg/dl) Mean±S.E	
		0h	3h
1	Normal Control	83±4.65	79±4.09
2	Glibenclamide (0.5)	78±4.02	49±2.56***
3	Pet. ether extracts of *Psoralea corylifolia* seeds (250)	83.0±3.82	86±6.09
4	Methanol extracts of *Psoralea corylifolia* seeds (250)	93.6±2.51	93.2±2.53

n (Number of animals): 5

$p < 0.001$ compared to 0h blood glucose level

Results and Discussion: Petroleum ether extracts increases the blood glucose level of the rat from 83mg/dl to 86 mg/dl thus hyperglycemic while methanol extract do not show any significant increase or decrease in blood glucose level.

Conclusion: The above results show petroleum ether extracts as hyperglycemic where as methanol extract is neutral for the diabetic activity.



Where NC = Normal control, GC = Glibenclamide, PPC = Petroleum ether extracts of *Psoralea corylifolia*, MPC = Methanol extracts of *Psoralea corylifolia*

Graph 3.I : Graph representing antidiabetic activity of nonpolar and polar extracts of *Psoralea corylifolia*

4.1: Introduction

Solanum nigrum Linn. belongs to family Solanaceae and also known by the name "Black Nightshade". In India and many parts of the world, it is widely used in conventional medicine and acts as a remedy against bilious disorders, inflammatory disorders, chronic skin problems (psoriasis and ringworm), fevers, diarrhoea, hydrophobia, cough, dropsy, painful sensations and eye diseases etc.[74-76] The genus *Solanum* constitutes wide number of around 1400 species occurring throughout the tropics and temperate zones of the world.[77] It is an African pediatric plant utilized for several ailments that are responsible for to infant mortality especially feverish convulsions.[78] It occurs mostly in old fields, ditches, waste land and roadsides as well as on cultivated land.[78]

Black nightshade is highly nutritious and has a rich phytochemistry which is capable to supply many proteins, vitamins, minerals and harmones.[79] It is commonly known as 'hierba mora', and its fruit is useful as a nervous tonic in conventional medicines in Mexico. Its fruit has been used as a cure for nervous conditions by local communities of Hidalgo. A clear yellow liquid is prepared by boiling its fruit in water and a small cup is taken daily as medicine by the people of Hidalgo.[80]

Solanum nigrum is a herb that has its utility in traditional folk medicines due to diuretic and antipyretic effects.

Since centuries, it is used to cure edema, hepatic cancer, inflammation and mastitis.[81] It is a traditional Chinese medicinal herb bestowed with hepatoprotective effects and a major ingredient of folk anticancerous drugs.[82] The phytochemical studies revealed that the plant contains glycoalkaloids, steroidal glycosides, steroidal saponin, steroidal genin, tannin, alkaloids and polyphenolic compounds.[83] It is also reported to have antitumour activity.[84]

Botanical Description: It is an annual weed (Fig. 4.I), can achieve up to 60 cm in height, erect, branched and occurs wildly in crop fields and wastelands.[74] In Ethiopia, the Welayta people don't remove this weed that appears in their gardens as they like to cook and eat the leaves.[85]

Leaves are juicy, ovate/lanceolate, dull dark green, toothless or slightly toothed on the margins.

Flowers are white, short, small pedicellate and five widely spread petals which surround yellow prominent bright anthers.

Berries are 6-8 mm in diameter, purple or dull black in colour. The ripen berries are plucked and eaten by the children normally while during the periods of famines, berries acts as a food source and taken by affected people in Ethiopia.[85]

Geographical Distribution: It is found throughout the tropical and temperate regions of the world.[77]

Pharmacology: The various pharmacological activities reported are anticancer, immunomodulatory effects, antimicrobial, nematicidal, molluscicidal, antioxidant, hepatoprotective, anticonvulsant, antiulcerogenic, antiinflammatory, hypolideamic, anti-hyperglycemic and hypotensive potentials.[78]

Phytochemistry: Citric, acetic, tartaric and malic acids are the major organic acids present in *Solanum nigrum.* A glycoalkaloid called solanine is present in major amounts in many plant parts and highest levels are found in unripe berries. The various other components reported are steroidal alkaloids such as solanine, solasodine, steroidal glycosides such as beta 2-solamargine, solamargine and degalactotigonin, steroidal saponins collectively called solanigrosides and nigrumnin 1 and 2, non-saponins namely p-hydroxybenzoic acid, 3,4-dihydroxhbenzoic acid, pinoresinol-4-O-beta-D-glucopyranoside, 6-methoyhydroxycoumarin, syringaresinol-4-O-beta-D-glucopyranoside and 3- methoxy-4-hydroxyienzoic acid.[78]

Fig. 4.I *Solanum nigrum*

4.2: Materials and Methods:

Plant Material: *Solanum nigrum* whole plants except roots were plucked from Ravi river banks of Kathua district of J&K and identified by the taxonomist Dr. Rajesh Manhas of Botany Department of University of Jammu.

Preparation of Plant Samples: The live plants collected were cleaned and washed properly first with tap water and then with distilled water and then kept under shade to dry without any contamination for 3-4 weeks. The plant materials are then crushed and soxhlated with various solvents in soxhlet apparatus to prepare various extracts according to their polarity gradient such as petroleum ether, toluene, dichloromethane, chloroform, ethyl acetate and methanol. The DCM extracts were than subjected to column chromatography and the brown solid fraction (Sn 1) obtained from 5**:**3 petroleum ether **:** dichloromethane was further subjected to LC-MS for the identification of various chemical constituents.

The methanol extracts of *Solanum nigrum* was subjected to basic hydrolysis In the process of basic hydrolysis to methanol extracts added equal amount of 10% KOH, heated on water bath for two hours, cooled and add chloroform to it. Separate both the organic and aqueous layers with the help of separating funnel. Aqueous layer after keeping for three months results in the formation of white sugar like crystals (Sn 2) that were subjected to various spectroscopic analysis for identification.

Experimental

Analytical tools: The hot extraction of plant material was done with JSGW soxhlate apparatus. Labfit melting point apparatus was used to determine melting point. SHIMADZU UV-1800 UV Spectrophotometer was used for recording UV spectrum and ethanol was used as a solvent. SHIMADZU FTIR-8400 S (Fourier Transform Infrared spectrophotometer) was used to obtain IR spectrum. Bruker Avance 400 MHz NMR spectrophotometer was used to record ^{1}H-NMR and ^{13}C-NMR spectrophotometer and solvent used was CH_3OD. Chemical shifts were given in δ using tetramethylsilane as internal standard. Agilent 6540, Q-TOF (HR-MS) was used to record mass spectrum. X-Ray diffraction analysis was performed using X-Ray diffractometer (Rigaku Co. Ltd. Japan) with Mo Kα radiation and wavelength 0.7103 A° at room temperature.

LC-MS Spectrometry: The various components present in the plant were analyzed using LC-MS spectroscopy from IIIM Jammu. Agilent 1100 LC coupled with Bruker MS

(Model Esquire 3000) was used for LC-MS analysis. The various instrumental parts of liquid chromatography are binary gradient pump with online degasser, column oven, autosampler with capacity of 100 samples and PDA detector. Sample inlet system, liquid chromatographic system and syringe pump inlets are the components of mass spectroscopy. Electron Spray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI) are the ionization sources and connected with ion trap analyzer with MS_n facility. The LC-MS instrument helps in determination of molecular mass of the components of a mixture and number of components present in a given mixture. It has utility in both qualitative and quantitative analysis.

Apparatus and Equipments: The equipments laminar air flow, incubator and oven were of Yorko Industries where as autoclave of JSWG. Glassware and heating mantle were from Perfit India.

4.3: Results and Discussion:

LC-MS analysis of Sn 1 (Fig. 4.11) reveals the presence of following components

Evaluation of various peaks

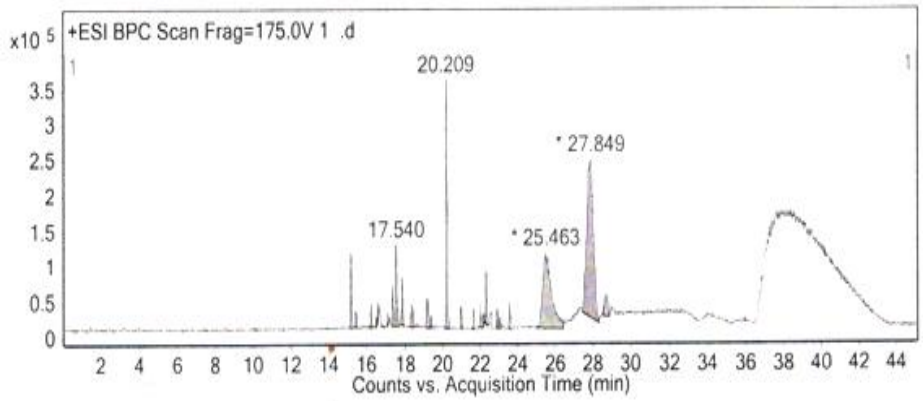
Qualitative Analysis Report

Data Filename	1 .d	**Sample Name**	1
Sample Type	Sample	**Position**	Vial 2
Instrument Name	Instrument 1	**User Name**	
Acq Method	khajuria sir.m	**Acquired Time**	22-02-2013 AM 11:01:13
IRM Calibration Status	Success	**DA Method**	SamplePurity-Default.m
Comment			

Sample Group	**Info.**
Acquisition SW Version	6200 series TOF/6500 series Q-TOF B.05.01 (B5125)

User Chromatograms

Fragmentor Voltage 175 **Collision Energy** 0 **Ionization Mode** ESI

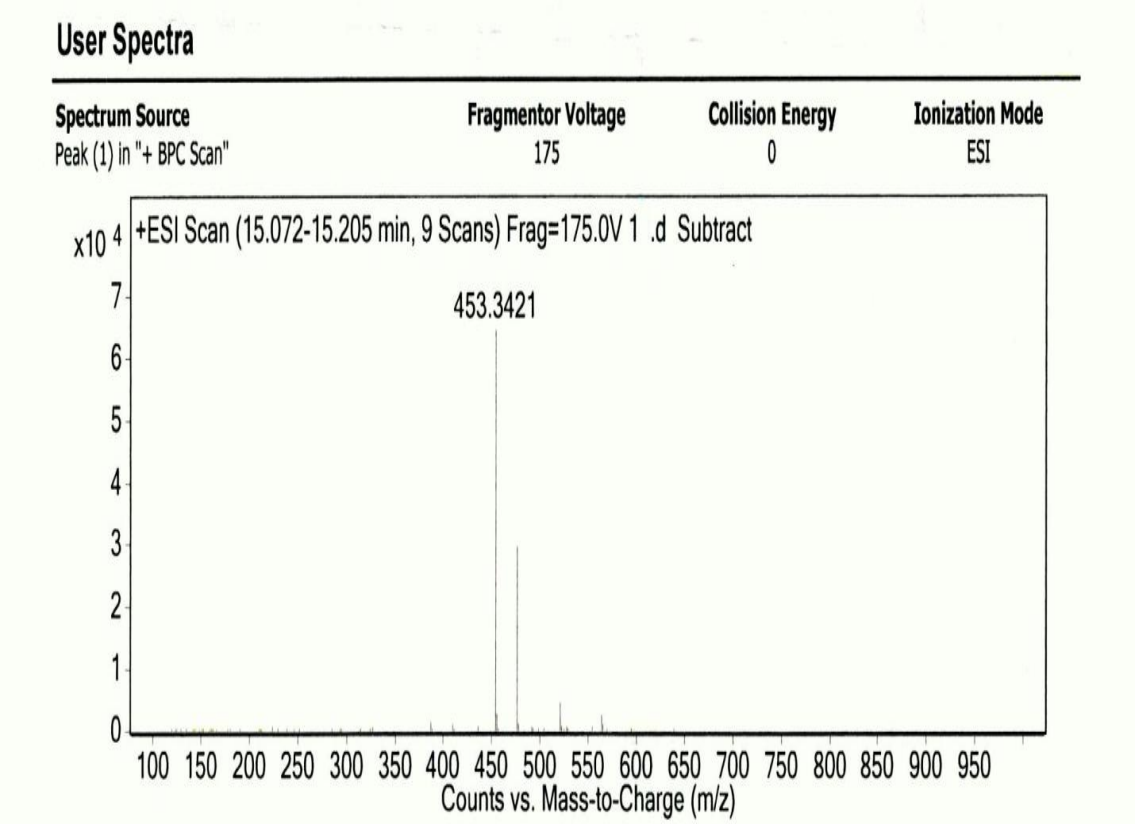


Integration Peak List

Start	RT	End	Height	Area	Area %
15.055	15.122	15.255	104473	486985	8.89
17.29	17.357	17.44	61380	255876	4.67
17.457	17.54	17.707	114900	638650	11.66
17.774	17.857	17.957	66495	294328	5.38
19.092	19.175	19.292	42337	267855	4.89
20.109	20.209	20.393	352340	2039582	37.25
22.227	22.311	22.394	73326	357357	6.53
23.478	23.545	23.629	37193	170270	3.11
24.996	25.463	26.397	106029	3391819	61.95
27.398	27.849	28.282	222463	5475067	100

Fig 4.II LC-MS analysis

1. **Peak 1.** Corresponds to 2-hydroxymethylene-3-methyl pentane dioic acid 1-methyl ester 5-(3, 4, 5- trihydroxy-6-phenethyl oxy tetrahydropyran-2-yl-methyl ester). **a.** Peak 453 – $[M+H]^+$, **b.** 475 – $[M+Na]^+$, **c.** 476 – $[M+Na+H]^+$.[86] The spectrum is given in figure 4.III.

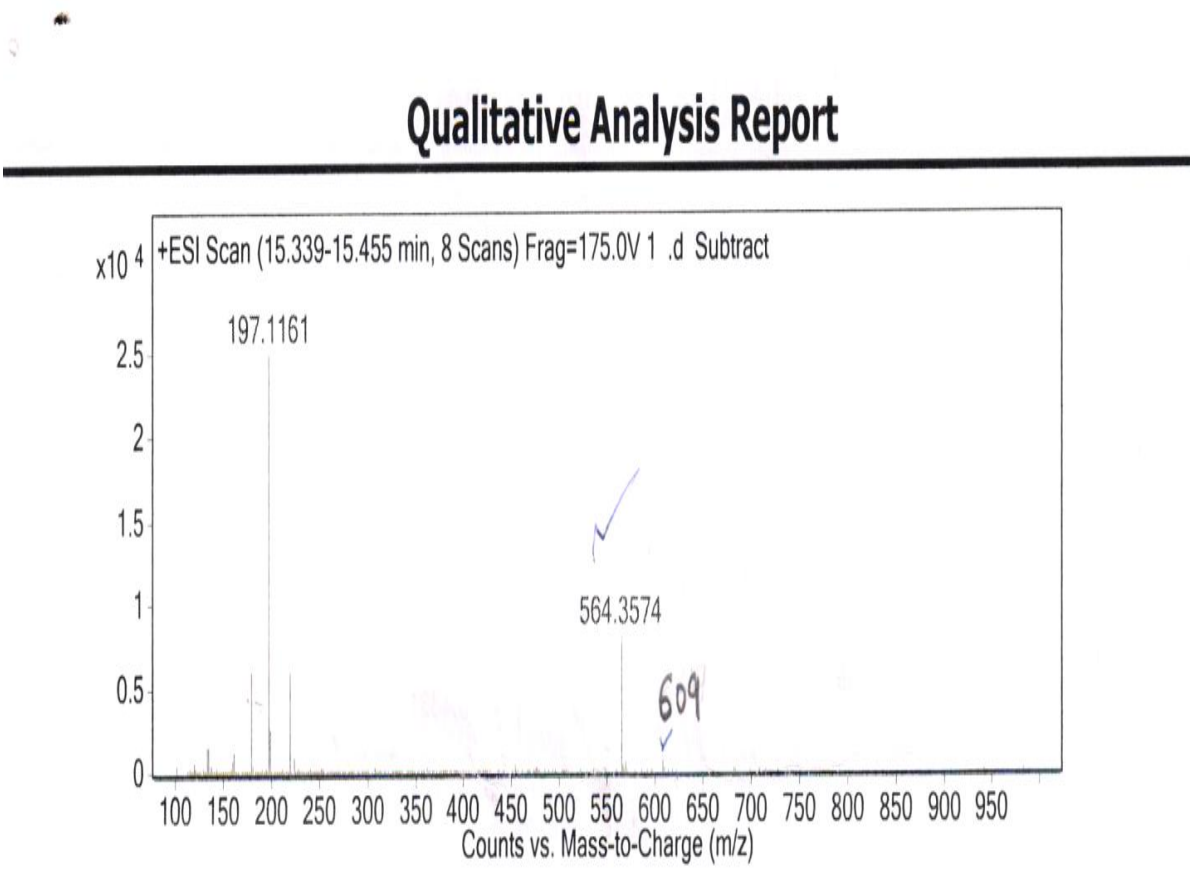


Peak List

m/z	z	Abund
453.3421	1	64680.41
454.3451	1	18034.93
475.3239	1	29969.21
476.3214	1	6368.29
520.3316		4683.45

Fig 4.III Peak 1

2. **Peak 2.** Corresponds to 2-methyl-4,6-dinitro phenol. **a.** Peak 197 – $[M]^+$, **b.** 609 – $[3M+H_2O+H]^+$, **c.** 564 – $[3M-CO]^+$. The spectrum is given in figure 4.IV.



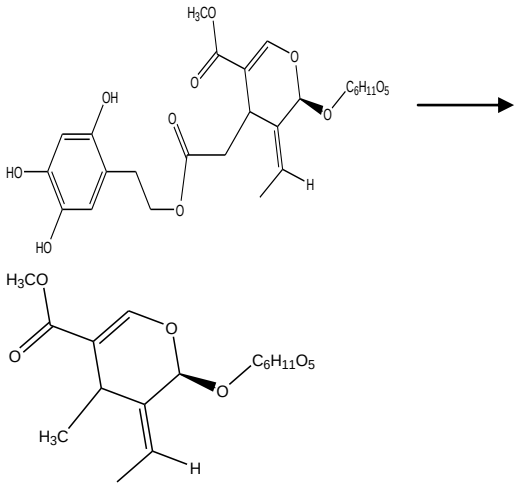
Peak List

m/z	z	Abund
133.0996		1526.25
135.1159		1501.96
179.107		5982.53
197.1161	1	25035.01
198.12	1	2616.48
219.0981		5993.4
564.3574	1	8133.17
565.3612	1	2242.44

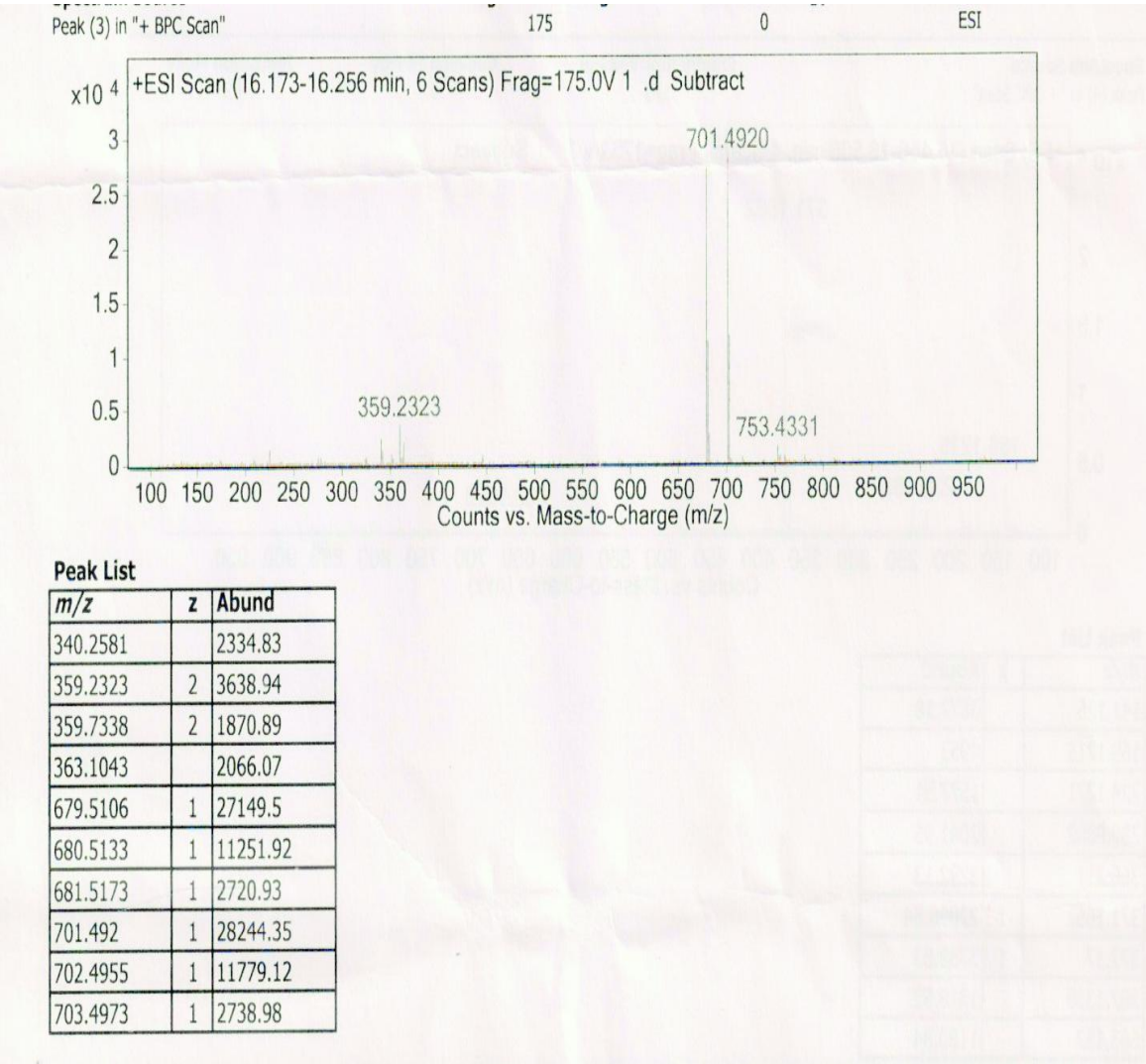
Fig 4.IV Peak 2

3. **Peak 3.** Corresponds to Oleuropein glucoside. **a.** Peak 701 – $[M]^+$, **b.** 702 – $[M+H]^+$, **c.** 753 – $[M+2H_2O+OH]^+$, **d.** 359 – $[M-C_{16}H_{24}O_9]^+$.[86] The spectrum is given in figure 4.V.

Fragmentation pattern:



$C_{25}H_{32}O_{14}$ $C_{16}H_{24}O_9$



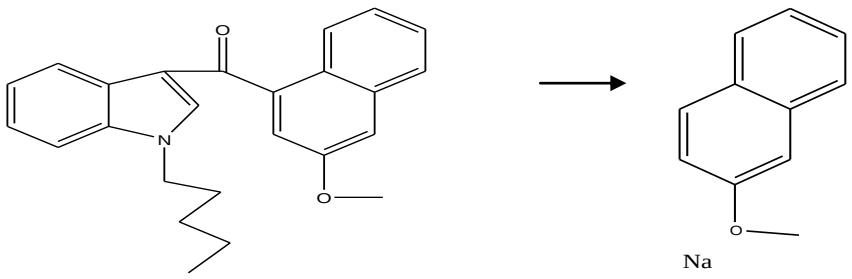
Peak List

m/z	z	Abund
340.2581		2334.83
359.2323	2	3638.94
359.7338	2	1870.89
363.1043		2066.07
679.5106	1	27149.5
680.5133	1	11251.92
681.5173	1	2720.93
701.492	1	28244.35
702.4955	1	11779.12
703.4973	1	2738.98

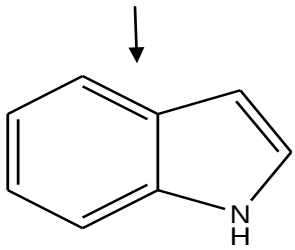
Fig. 4.V Peak 3

4. **Peak 4.** Corresponds to (3-Methoxy- naphthalene-1-yl)-(1-pentyl-1H-indol-3 yl)-methanone. **a.** Peak 371 – $[M+H]^+$, **b.** 169 – $[C_{10}H_9ONa]$, **c.**141 $[C_8H_7N + 2Mg]$.[87] The spectrum is given in figure 4.VI.

Structure and Fragmentation Pattern

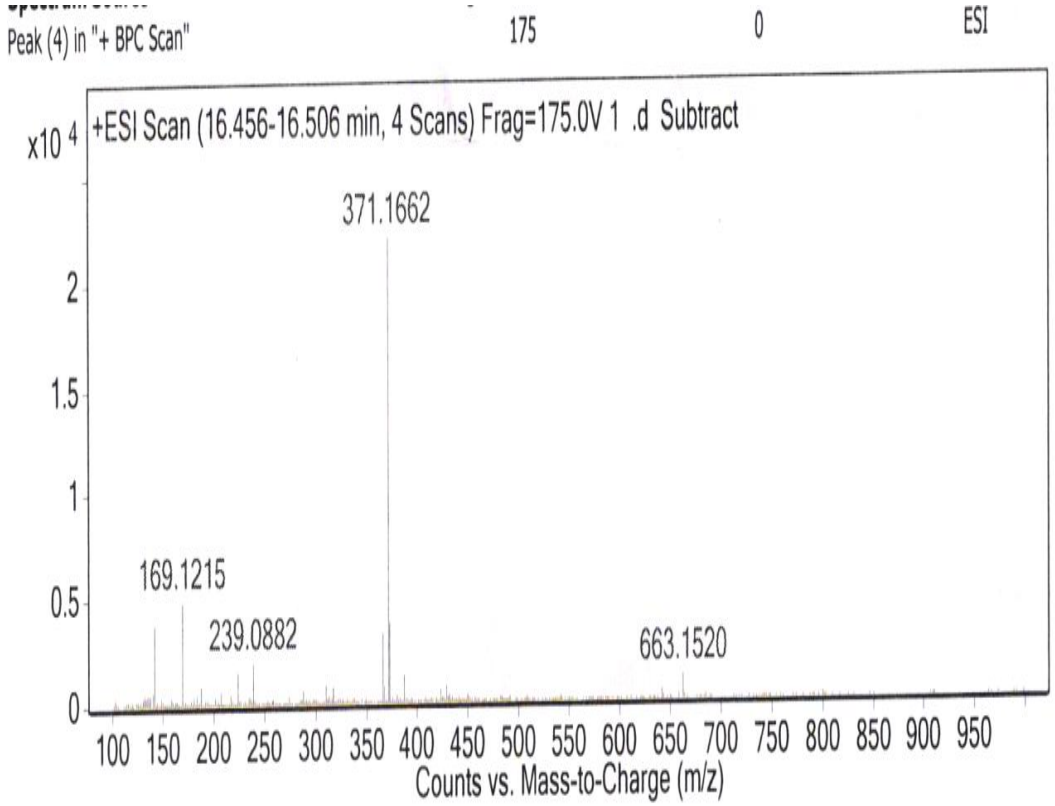


a. b.



2Mg

c.

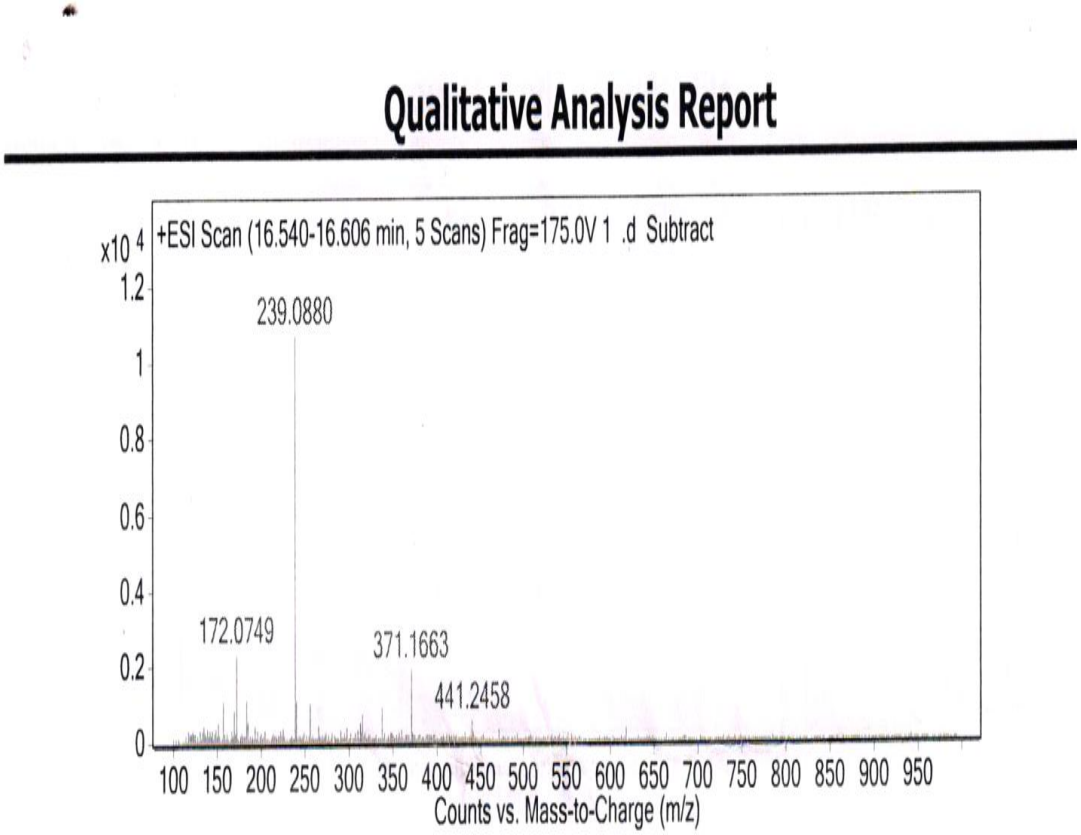


Peak List

m/z	z	Abund
141.125		3872.18
169.1215		4953
224.1271		1577.51
239.0882		2041.95
366.211		3292.13
371.1662	1	22098.84
372.17	1	3788.83
387.1358		1318.52
663.152		1180.84

Fig 4.VI Peak 4

5. **Peak 5.** Corresponds to 1-(3-Methoxy-naphthalene-1-yl)-pent-2-en-1-one. **a.** Peak 239 – [M^+], **b.** 371 – corresponds to its fragment (3-methoxy-naphthalene-1-yl)-(1-pentyl-1H-indol-3-yl)-methanone, **c.** 172 belongs to 3-hydroxy-Naphthalene-1-carbaldehyde. The spectrum is given in figure 4.VII.

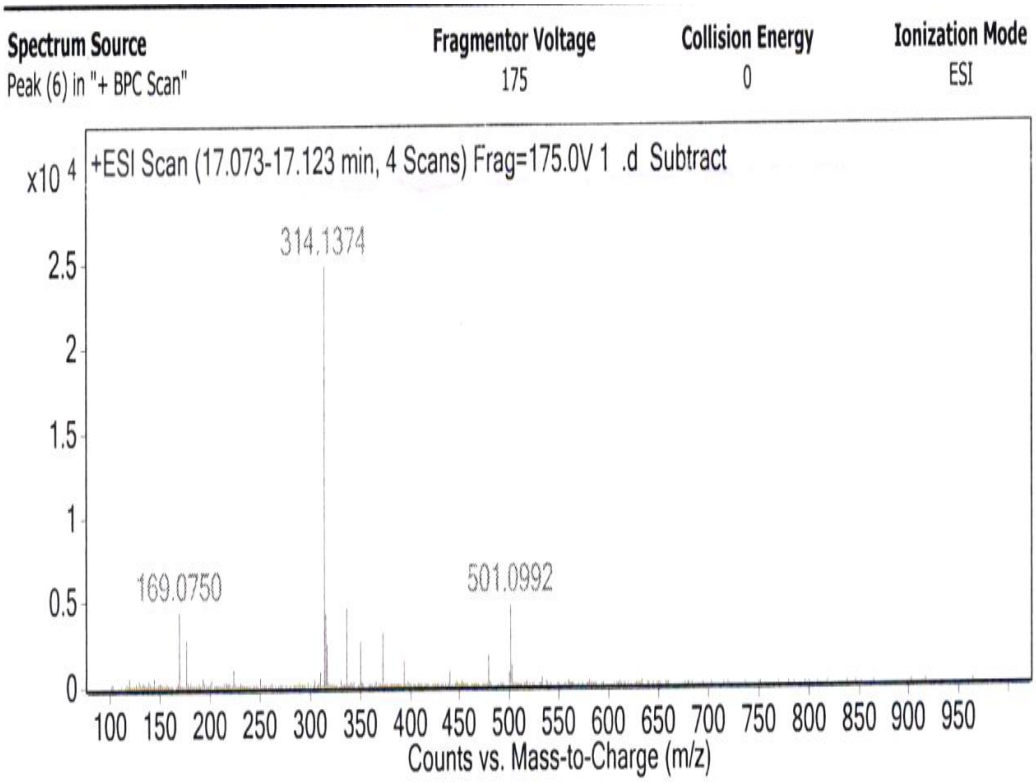


Peak List

m/z	z	Abund
157.0709		1074.81
169.1212		832.04
172.0749		2324.21
183.0318		1092.4
239.088	1	10734.08
240.0915	1	1079.96
255.053		1034.11
316.1732		679.78
338.1562		924.67
371.1663		1909.06

Fig. 4.VII Peak 5

6. **Peak 6.** Corresponds to coumarin compound 6-O-demethyl salutaridine [$C_{18}H_{19}NO_4$]. **a.** Peak 314 – $[M+2H]^+$, **b.** 315 – $[M+3H]^+$, **c.** 169 – [M- $C_8H_{10}O_4$ i.e. M- mass of 2-oxo-5, 6-dihydro-2H-pyran-3-carboxylic acid ethyl ester], **d.** 501 – $[2M-C_6H_8O_3]^-$ i.e. 2M- 2-Formyl acrylic acid ethyl ester.[88] The spectrum is given in figure 4.VIII.



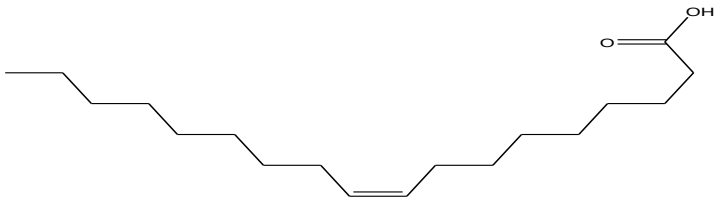
Peak List

m/z	z	Abund
169.075		4429.4
177.0531		2753.02
314.1374	1	24850.15
315.1408	1	4247.98
317.0646		2498.97
336.1194		4592.86
350.159		2651.65
372.14		3134.19
479.1168		1718.74
501.0992	1	4782.07

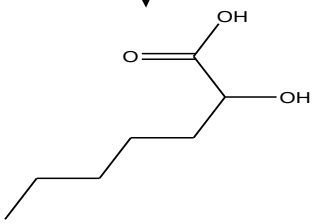
Fig. 4.VIII Peak 6

7. **Peak 7.** Corresponds to Oleic acid. **a.** Peak 284 – $[M+H]^-$, 285 – due to isotope effect, **b.** Peak 147 – corresponds to fragment $C_7H_{14}O_3$ (2-hydroxy heptanoic acid). The spectrum is given in figure 4. IX.

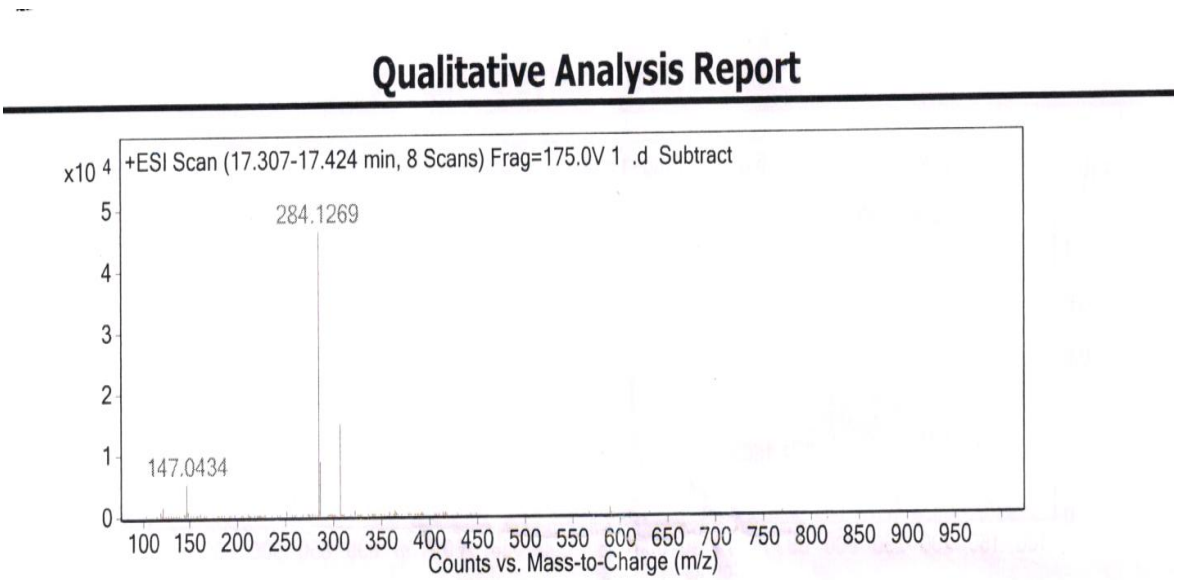
Fragmentation Pattern:



a.



b.

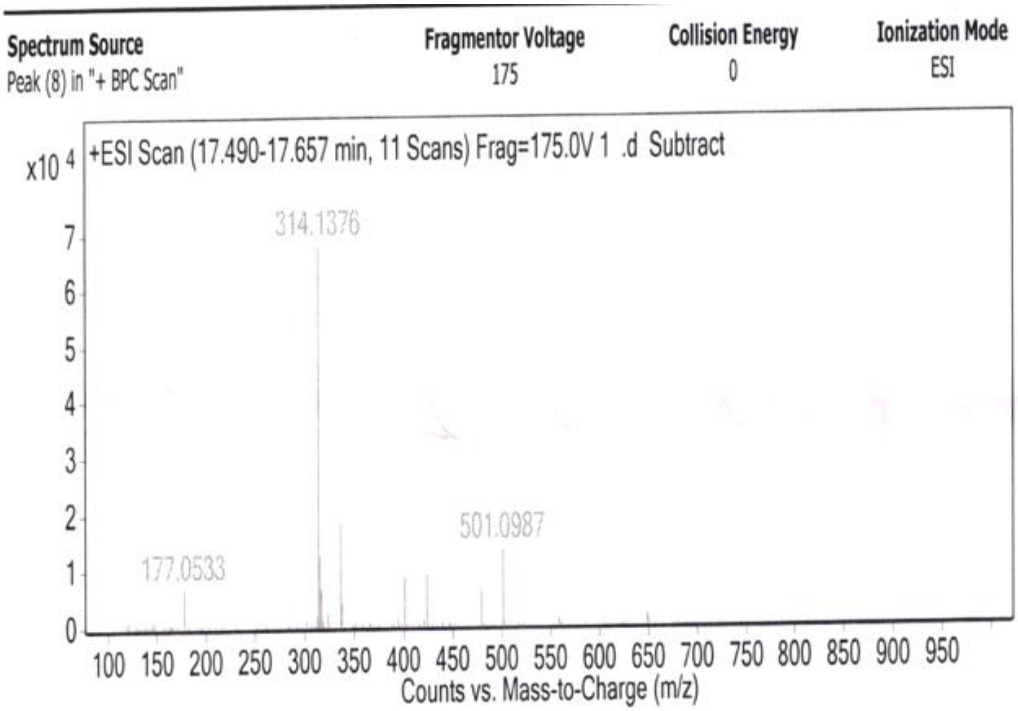


Peak List

m/z	z	Abund
147.0434		5365.87
284.1269	1	46453.91
285.1301	1	8968.63
306.1088	1	15124.11
307.1125	1	2722.14

Fig. 4.IX Peak 7

8. **Peak 8. a.** M/Z at 314 corresponds to 10-oxo-2, 3, 5, 6-tetrahydro-1H, 4H, 10H-11oxa-3a-azobenzo [de] anthracene-9-carboxylic acid ethyl ester **b.** Peak at 315 is due to isotopic effect **c.** Peak at 501 corresponds to [2M-$C_6H_8O_3$] i.e 2M- Mass of 2-formyl acrylic acid ethyl ester. The spectrum is given in figure 4.X.



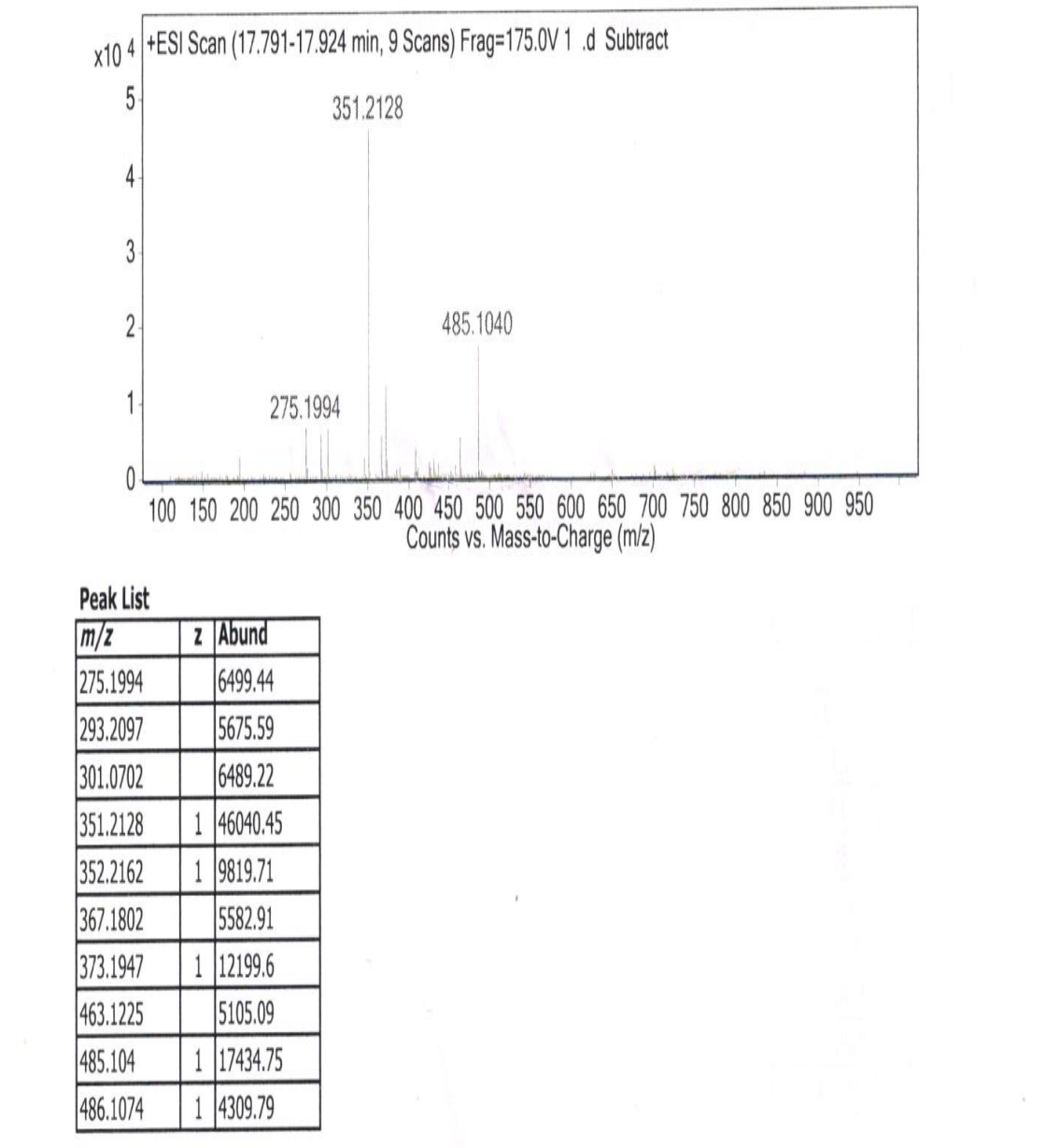
Peak List

m/z	z	Abund
177.0533		6740.13
314.1376	1	67838.1
315.1406	1	12519.92
317.0641		6794.97
336.1194	1	18318.45
337.1226	1	3916.12
401.2632		8499.74
423.2449		9300.25
479.1168		6206.01
501.0987		13400.13

Fig. 4.X Peak 8

9. **Peak 9. a.** M/Z at 351 corresponds to retrorsine.[89] **b.** M/Z at 352 is due to isotopic effect.

The spectrum is given in figure 4.XI.

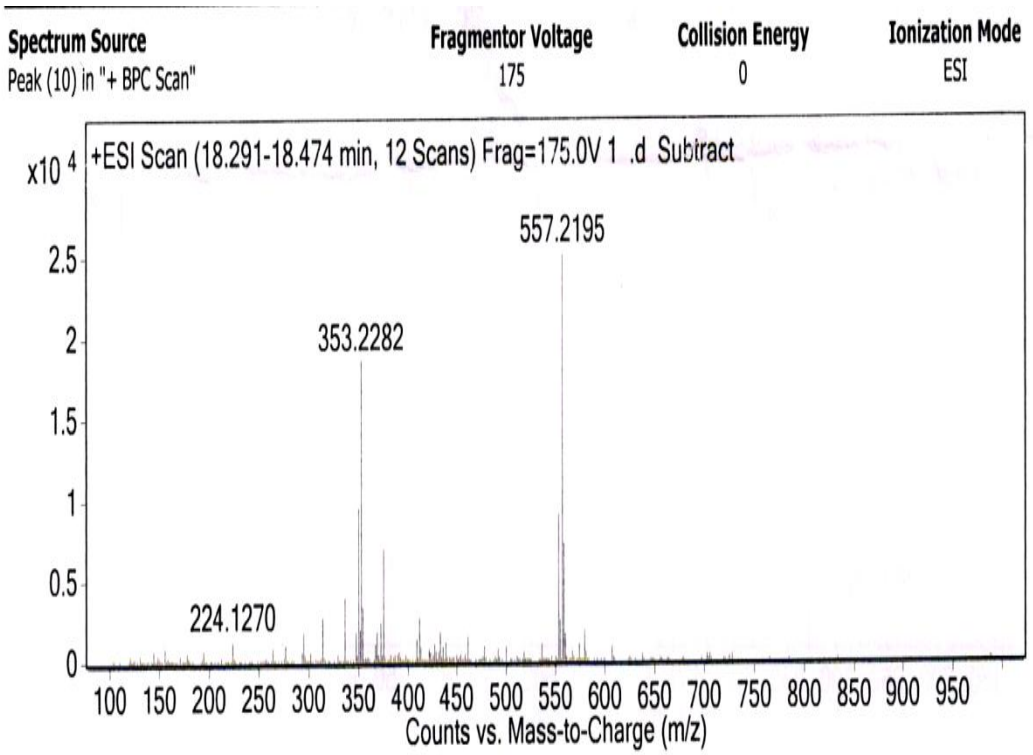


Peak List

m/z	z	Abund
275.1994		6499.44
293.2097		5675.59
301.0702		6489.22
351.2128	1	46040.45
352.2162	1	9819.71
367.1802		5582.91
373.1947	1	12199.6
463.1225		5105.09
485.104	1	17434.75
486.1074	1	4309.79

Fig. 4.XI Peak 9

10. **Peak 10. a.** M/Z at 557 corresponds to 2’-chloro-4’-(2-(2,4-di-tert-pentyl phenoxy) butyryl amino)-5’-hydroxy benzanilide.[90] The spectrum is given in figure 4.XII.



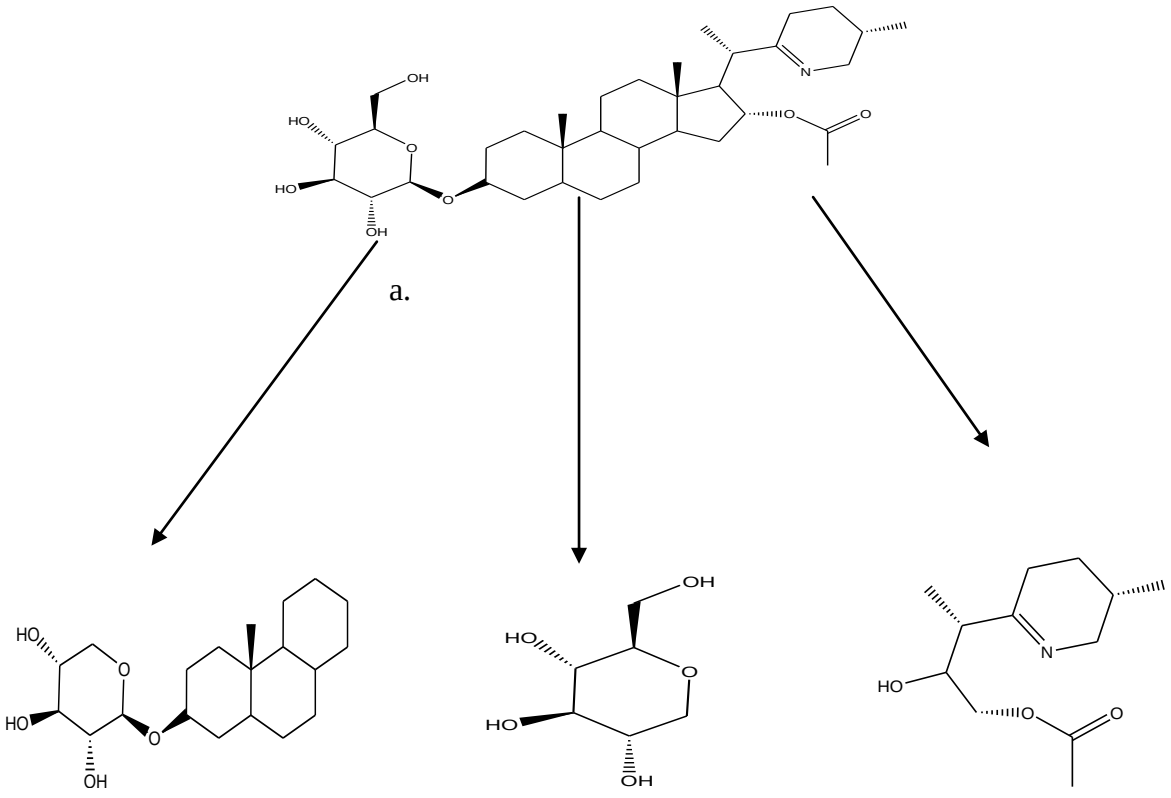
Peak List

m/z	z	Abund
314.1587		2739.75
336.1403		3873.68
351.2127	1	9516.96
353.2282	1	18591.73
354.2312	1	3239.41
375.2105	1	6955.33
411.1845		2754.25
552.2638	1	9049.46
557.2195	1	25161.29
558.2224	1	7200.57

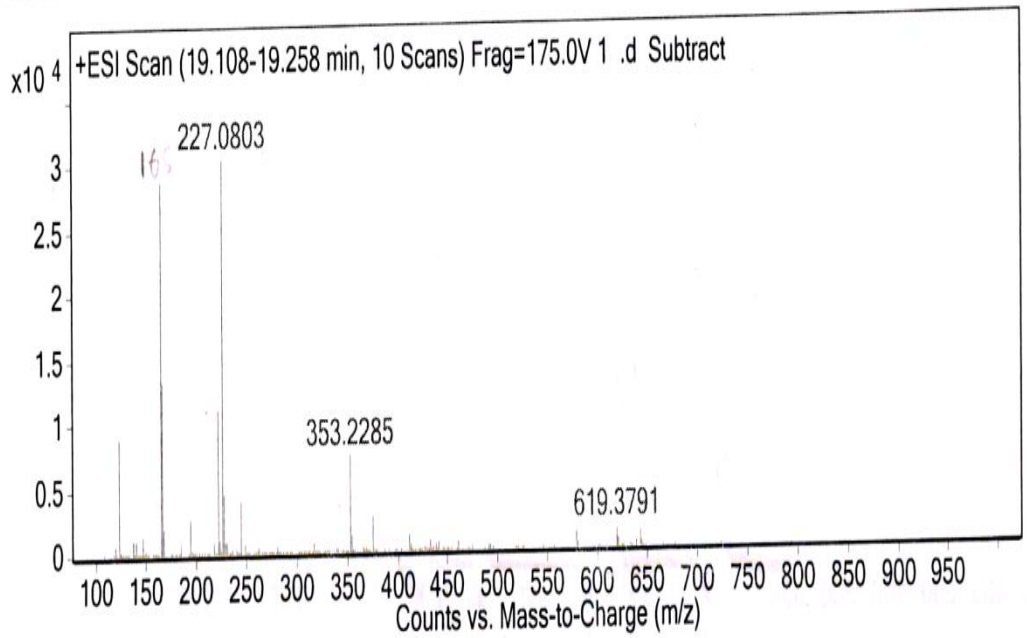
Fig. 4.XII Peak 10

11. **Peak 11.** Corresponds to steroidal alkaloid havanine ($C_{35}H_{57}NO_8$).[91] **a**. Peak at 619 corresponds to $[M+H]^+$**,** **b**. Peak at 353 corresponds to $[M-C_{15}H_{11}O_3]$ i.e. it corresponds to the fragment [2-(4a-Methyl tetradeca hydro-phenanthrene-2-yl oxy) tetrahydropyran-3, 4, 5 triol **c.** Peak at 165- $[M- C_{29}H_{45}O_3 +2H]^+$ i.e. belongs to 2-hydroxy methyl –tetrahydropyran-3,4,5-triol, **d.** Peak at 227 goes to $[M-C_{23}H_{34}O_5+H]^+$ i.e. belongs to Acetic acid 2-hydroxy-3-(5-methyl-3,4,5,6-tetrahydropyridin-2-yl)-butyl ester. The spectrum is given in figure 4.XIII.

Structure and Fragmentation Pattern



Qualitative Analysis Report

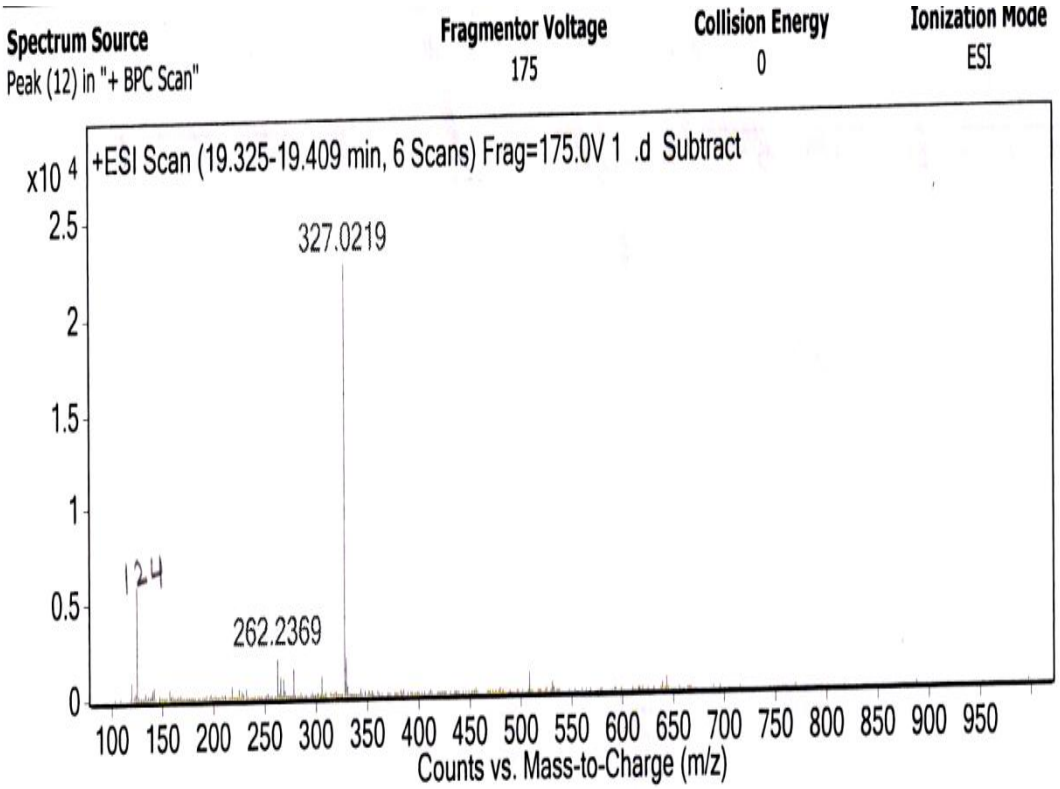


Peak List

m/z	z	Abund
123.0451		8967.55
165.09	1	28704.52
167.0595	1	13328.75
195.0548		2783.87
222.1114		11088.08
227.0803	1	30436.59
228.0839	1	4486.41
244.0926		4094.69
353.2285		7388.66
375.2107		2772.44

Fig. 4.XIII Peak 11

12. **Peak 12.** Corresponds to O-(3, 5-dichloro-2,4-dihydroxy benzoyl) benzoic acid. **a.** 327 – $[M+H]^+$, **b.** 328 – $[M+2H]^+$, **c.** 329 – $[M+3H]^+$ (M+2 Peaks of halogen isotopes), **d.** Peak at 124 is due to $[M-C_7H_2Cl_2O_3 + 2H]$. The spectrum is given in figure 4.XIV.

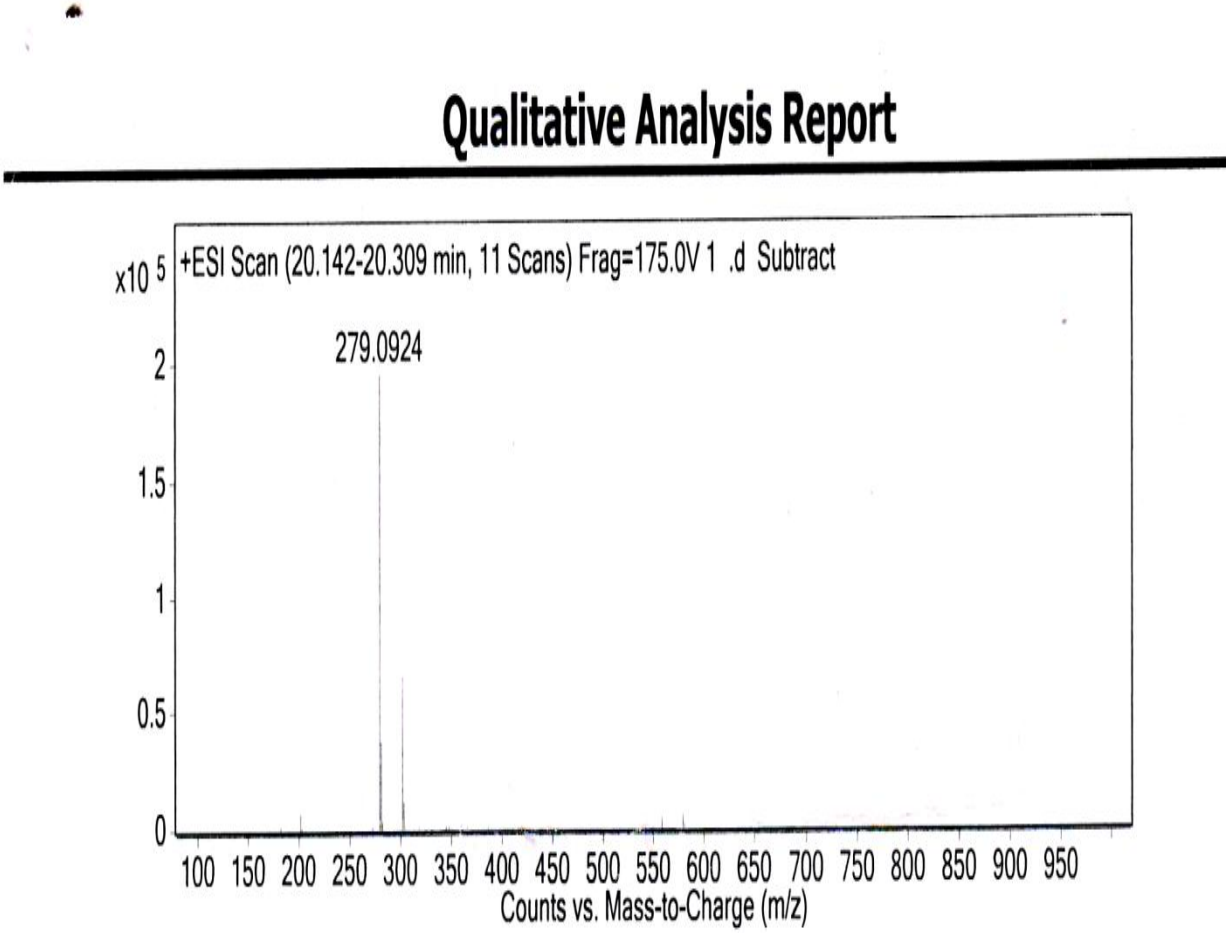


Peak List

m/z	z	Abund
124.0208		5957.7
262.2369		2091.23
277.046		1439.14
327.0219	1	22736.28
328.0247	1	3752.44
329.0203	1	2011.4

Fig. 4.XIV Peak 12

13. **Peak 13.** Corresponds to linoleic acid. **a.** 280 – $[M-H]^-$, **b.** 279 – $[M]^+$, **c.** 302 – $[M+Na]^+$, **d.** 301 – $[M-H+Na]^+$. The spectrum is given in figure 4.XV.

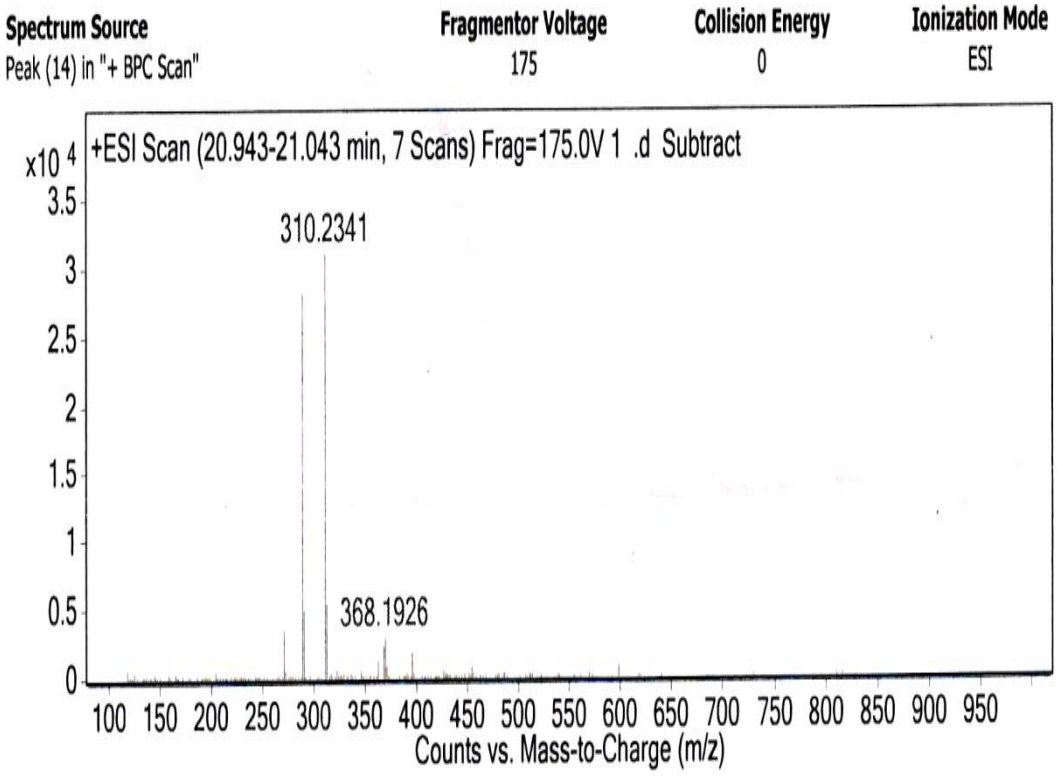


Peak List

m/z	z	Abund
279.0924	1	195984.25
280.0956	1	37222.24
301.0741	1	65570.63
302.0774	1	12198.35

Fig. 4.XV Peak 13

14. **Peak 14.** Corresponds to tetracosanoic acid ($C_{24}H_{48}O_2$). **a.** 368 – [ESI-MS], **b.** 311 – $[M\text{-}C_4H_8]^+$ i.e. icosanoic acid, **c.** 310 – $[M\text{-}C_4H_8\text{+}H]^+$, **d.** 288 – $[C_{19}H_{40}\text{+}H_3O\text{+}OH]^-$, **e.** 289 – $[C_{19}H_{40}\text{+}H_3O\text{+}2H]^-$.[92] The spectrum is given in figure 4.XVI.



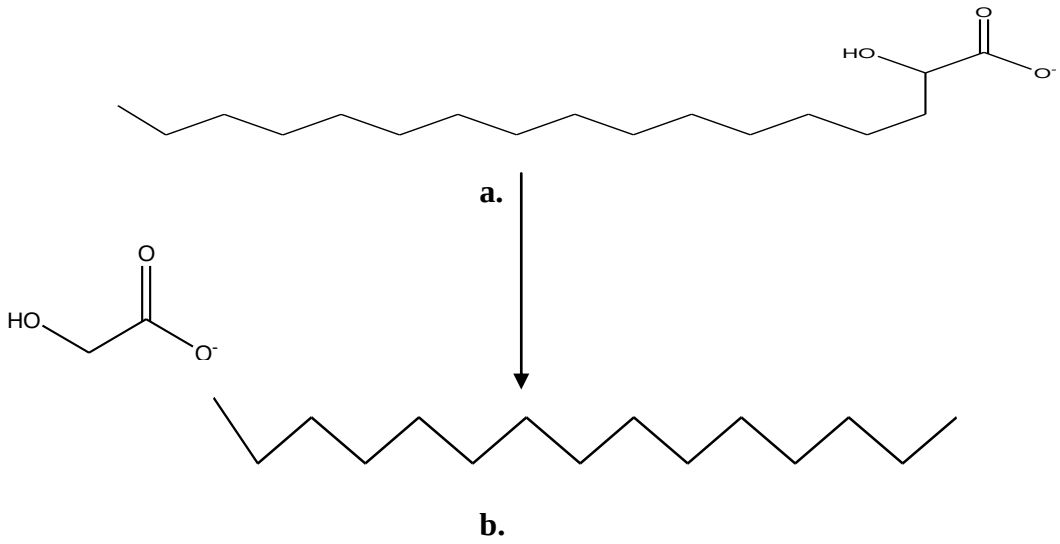
Peak List

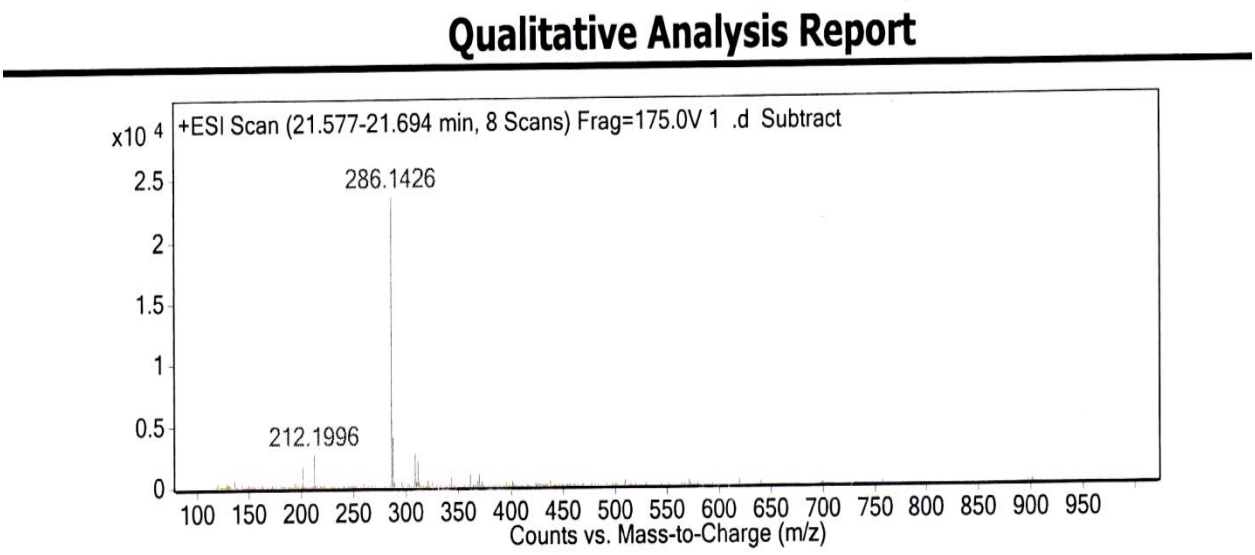
m/z	z	Abund
270.2417		3528.99
288.2523	1	28320.41
289.2556	1	4982.37
310.2341	1	31112.77
311.2373	1	5391.74
367.2447		2418
368.1926		2945.79
395.2121		1838.68

Fig. 4.XVI Peak 14

15. **Peak 15.** Corresponds to 2-hydroxy hexadecanoate. **a.** Peak at 286 – $[M+H]^+$, **b.** 212 – $[M- C_2H_3O_3^- + H^+]$.[93] The spectrum is given in figure 4.XVII

Structure and Fragmentation Pattern:





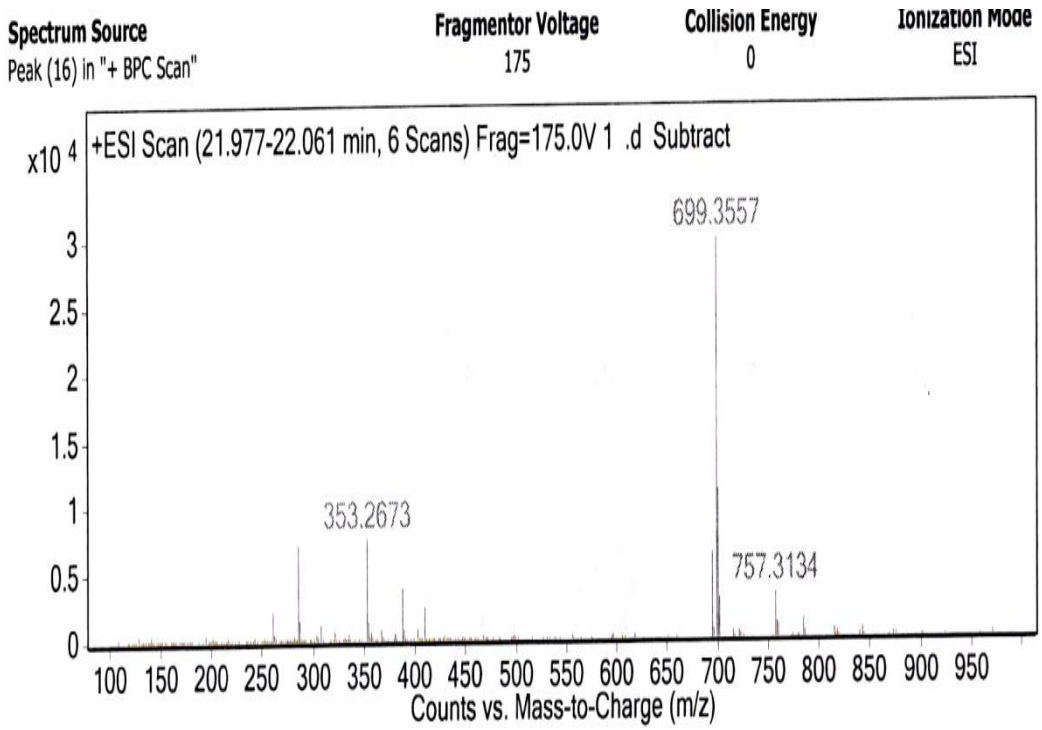
Peak List

m/z	z	Abund
201.0541		1668.68
212.1996		2743.85
286.1426	1	23668.46
287.1462	1	4133.37
308.1248		2719.37
311.1257		2174.99

Fig. 4.XVII Peak 15

16. Peak 16. M/Z 757 corresponds to Delphinidin-3-cis-coumaroyl rutinoside-5-glucoside.[94]

The spectrum is given in figure 4.XVIII.

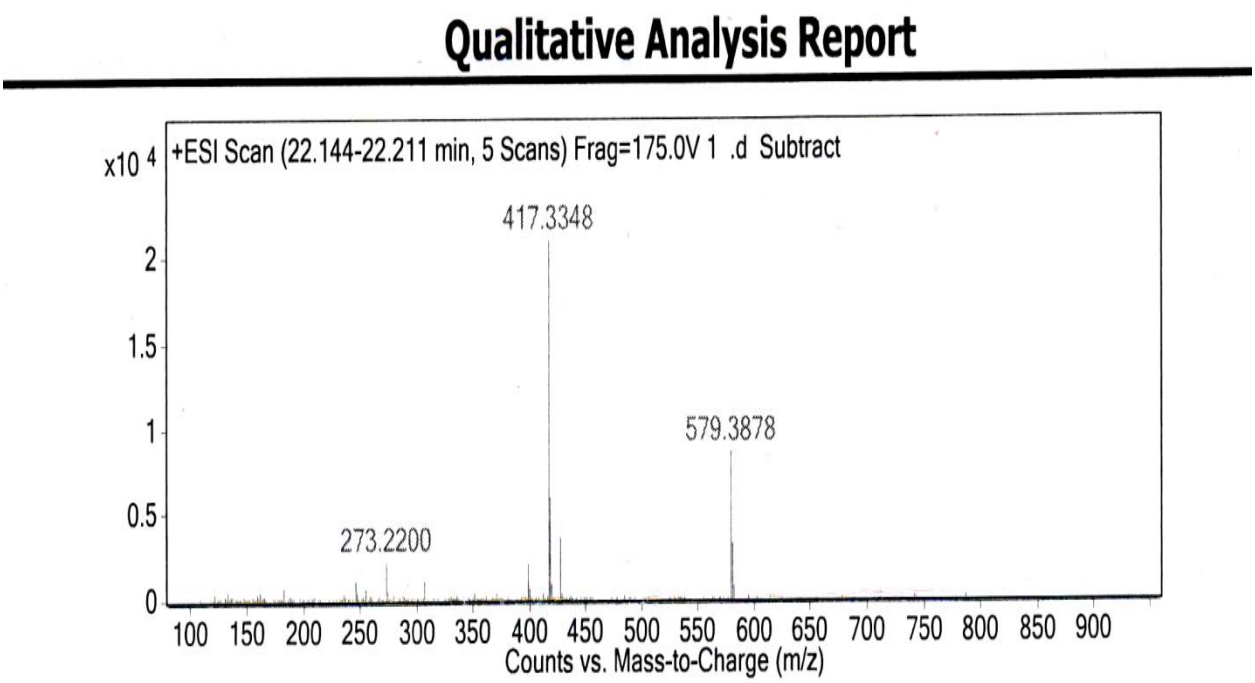


Peak List

m/z	z	Abund
286.1426	1	7113.1
353.2673		7648.25
387.1921		3982.67
409.1746		2495.12
694.4	1	6502.85
695.4031	1	2498.16
699.3557	1	30030.83
700.3586	1	11068.21
701.361	1	3053.06
757.3134		3349.85

Fig. 4.XVIII Peak 16

17. **Peak 17.** M/Z 417 corresponds to alkaloid veremivirine. **a.** Peak 417 is due to $[M+H]^+$, **b.** Peak 579 – [M-H+ 3-Hydroxy-3-methyl glutaric acid]$^+$. The spectrum is given in figure 4.XIX.

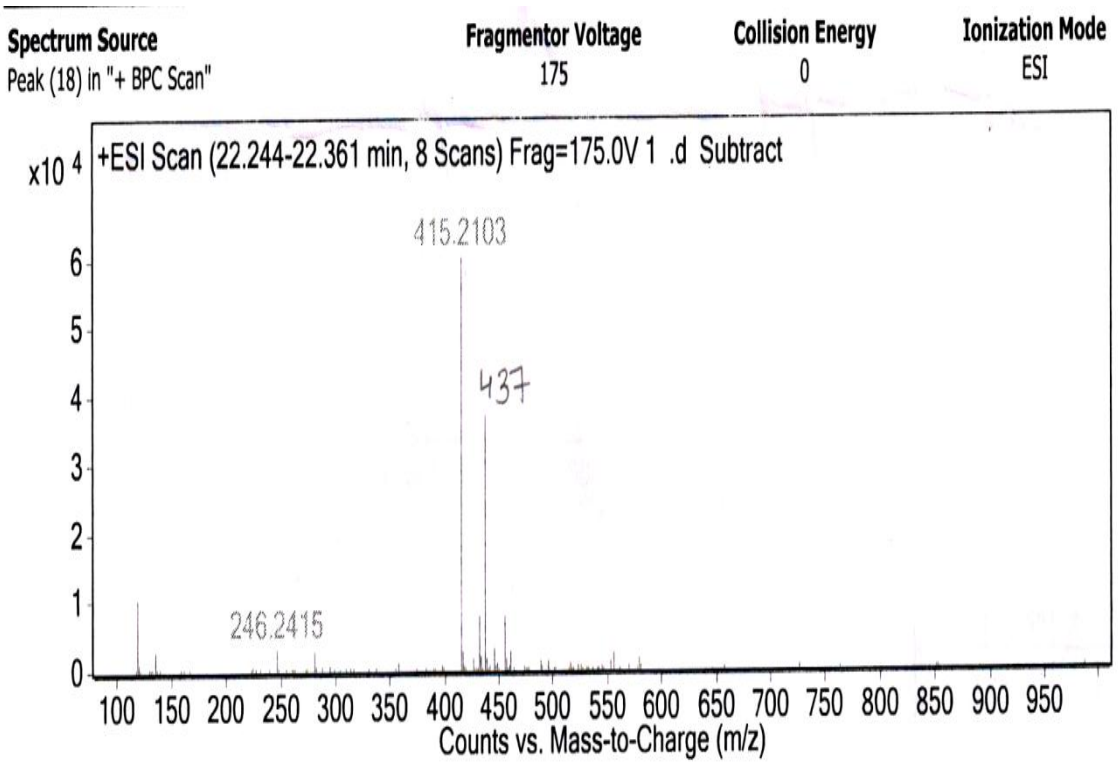


Peak List

m/z	z	Abund
246.2412		1126.79
273.22		2261.77
307.1295		1133.6
399.3238		2158.71
m/z	z	Abund
417.3348	1	21073.39
418.338	1	6111.74
419.3398	1	1059.11
427.1484		3595.6
579.3878	1	8705.54
580.3909	1	3269.79

Fig. 4.XIX Peak 17

18. **Peak 18.** M/Z 415 corresponds to phenolic compound 1-acetoxy pinoresinol. **a.** 415 – $[M+H]^+$, **b.** 416 – $[M]^+$ **c.** 437 – $[M+Na+H]^+$.[95] The spectrum is given in figure 4.XX.

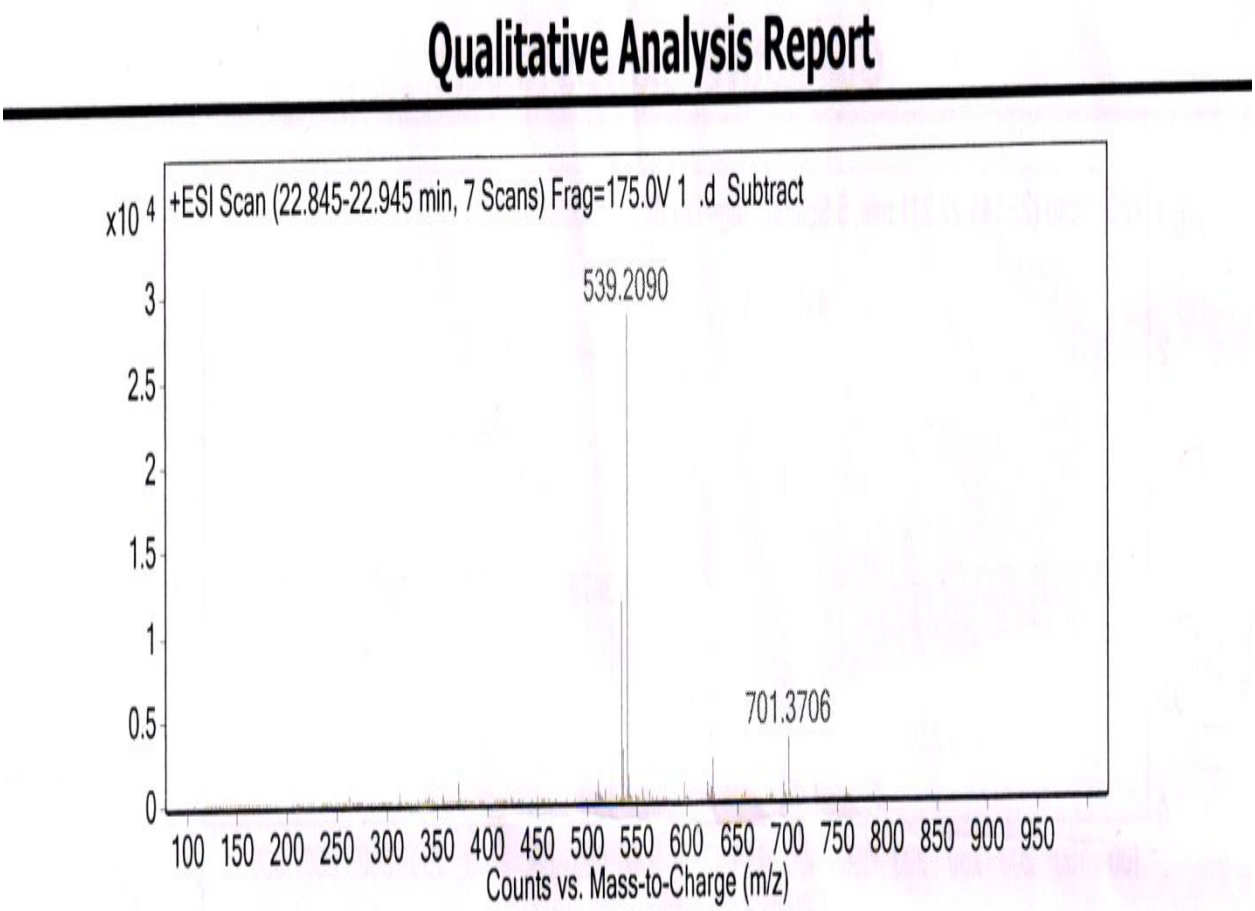


Peak List

m/z	z	Abund
119.0847		10618.46
246.2415		3155.18
415.2103	1	60313.72
416.2136	1	16514.54
432.2374		7964.06
437.1923	1	37330.69
438.1955	1	9490.45
446.2522		3297.9
455.3832		7983.14

Fig. 4.XX Peak 18

19. Peak 19. a. M/Z 539 Corresponds to 7-Methoxy-Salcolin B [22]. **b**. Peak 701 – [2M-$C_{18}H_{21}O_7$ + CO].[96] The spectrum is given in figure 4.XXI.

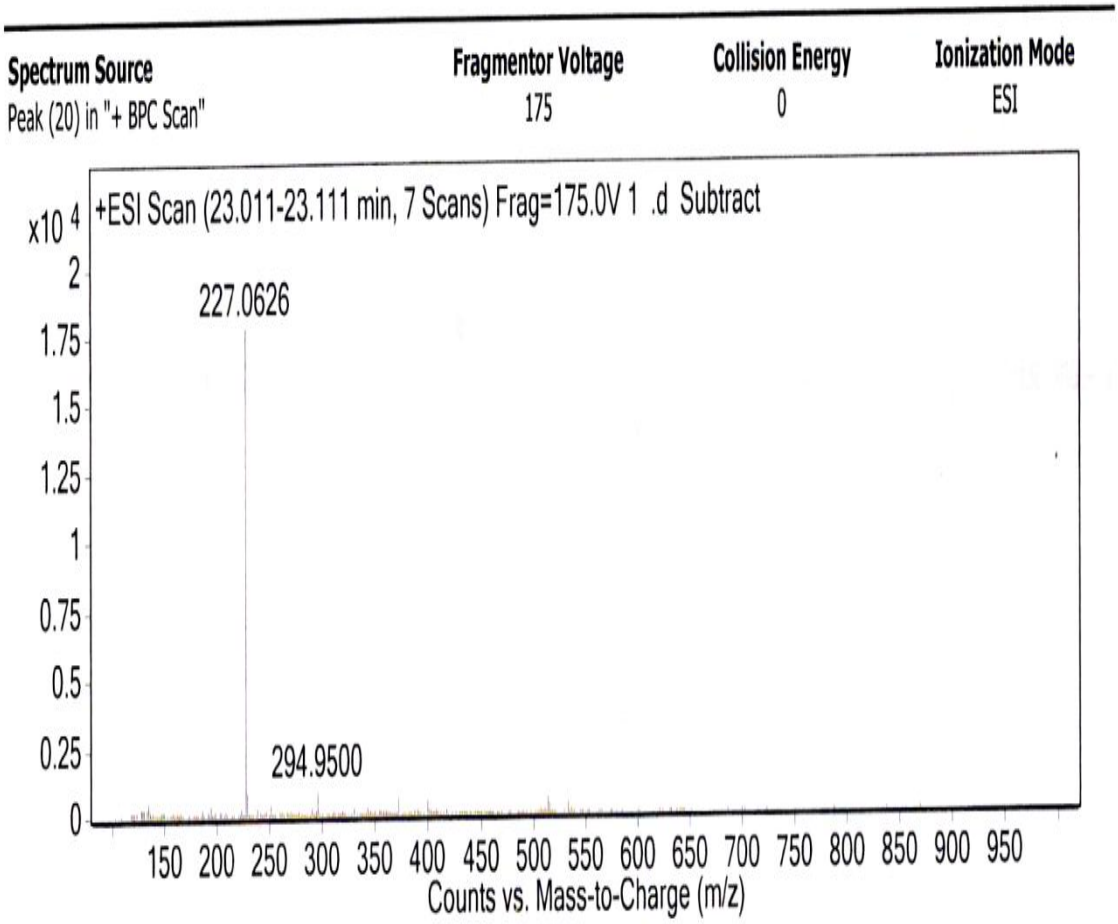


Peak List

m/z	z	Abund
534.2532	1	11807.62
535.2583	1	3053.44
539.209	1	28600.96
540.2124	1	7311.59
541.2145	1	1582.31
625.318		2433.21
701.3706		3559.81

Fig. 4.XXI Peak 19

20. **Peak 20. a.** M/Z 227 corresponds to Myristic acid $[M]^+$, **b.** Peak 228- $[M+H]^+$.[97] The spectrum is given in figure 4.XXII.

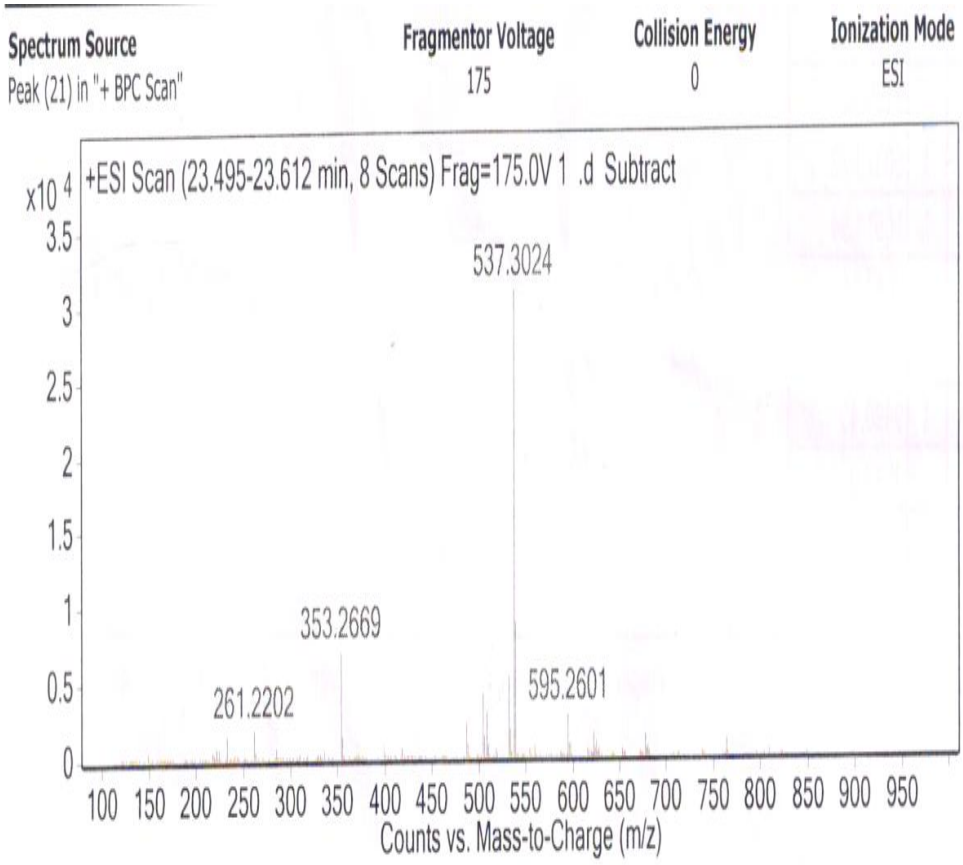


Peak List

m/z	z	Abund
227.0626	1	17844.05
228.0658	1	2741.12
294.95		963.29

Fig. 4.XXII Peak 20

21. **Peak 21. a.** M/Z 537 corresponds to trimethylacetyl delcosine $[M+H]^+$, **b.** 538 – $[M+2H]^+$, **c.** 509 corresponds to delcosine, **d.** 504 – $[M-OCH_3+3H]^+$, **e.** 595 – $[M+2C_2H_5]$.[98] The spectrum is given in figure 4.XXIII.

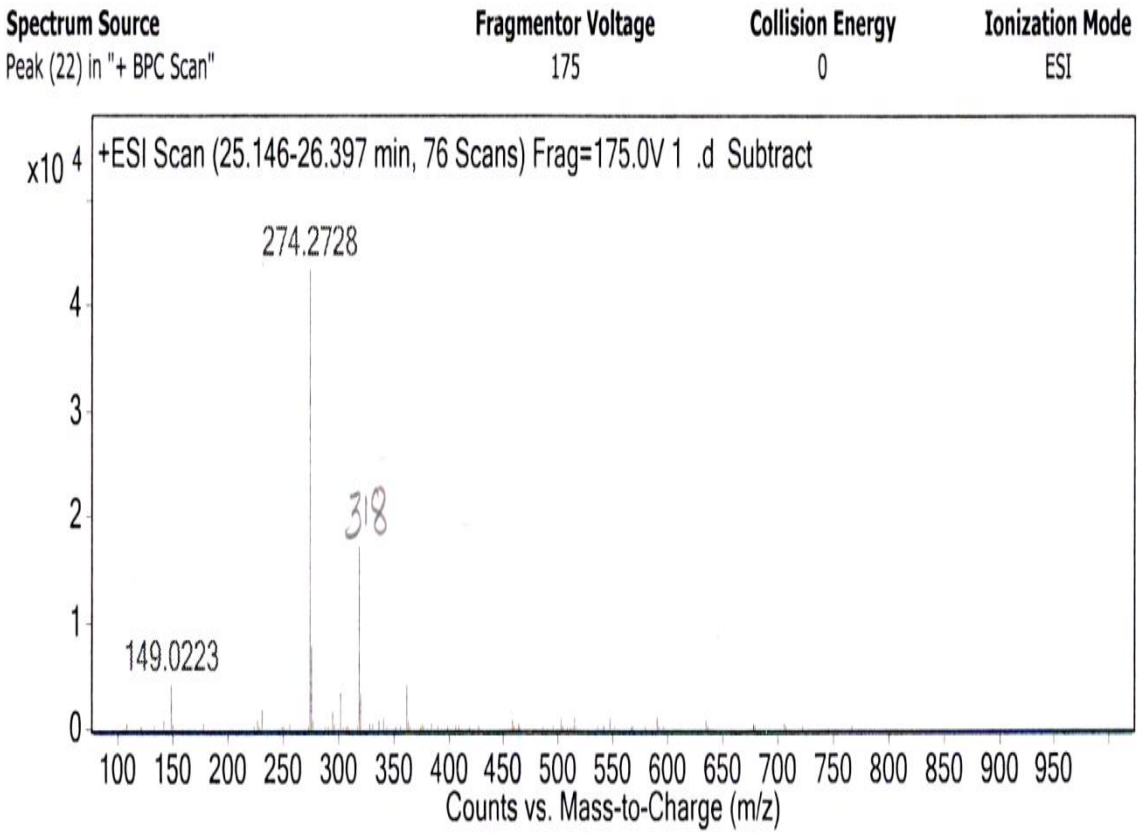


Peak List

m/z	z	Abund
261.2202		1981.78
353.2669	1	7134.73
487.296		2476.48
504.3223	1	4226.82
509.2783		2973.17
531.2597		1993.09
532.3477	1	5476.4
537.3024	1	31142.54
538.306	1	9003.6
595.2601		2876.78

Fig. 4.XXIII Peak 21

22. **Peak 22. a.** M/Z 274 corresponds to 10, 13-dimethyl-hexadecahydrocyclopenta [a] phenanthrene$[M+H]^+$ **b.** 275 – $[M+2H]^+$ **c.** 318 – $[M+COOH]^+$ **d.** 319 – $[M+COOH+H]^+$ **e.** 301 – $[M+CO^+]$.[99] The spectrum is given in figure 4.XXIV.

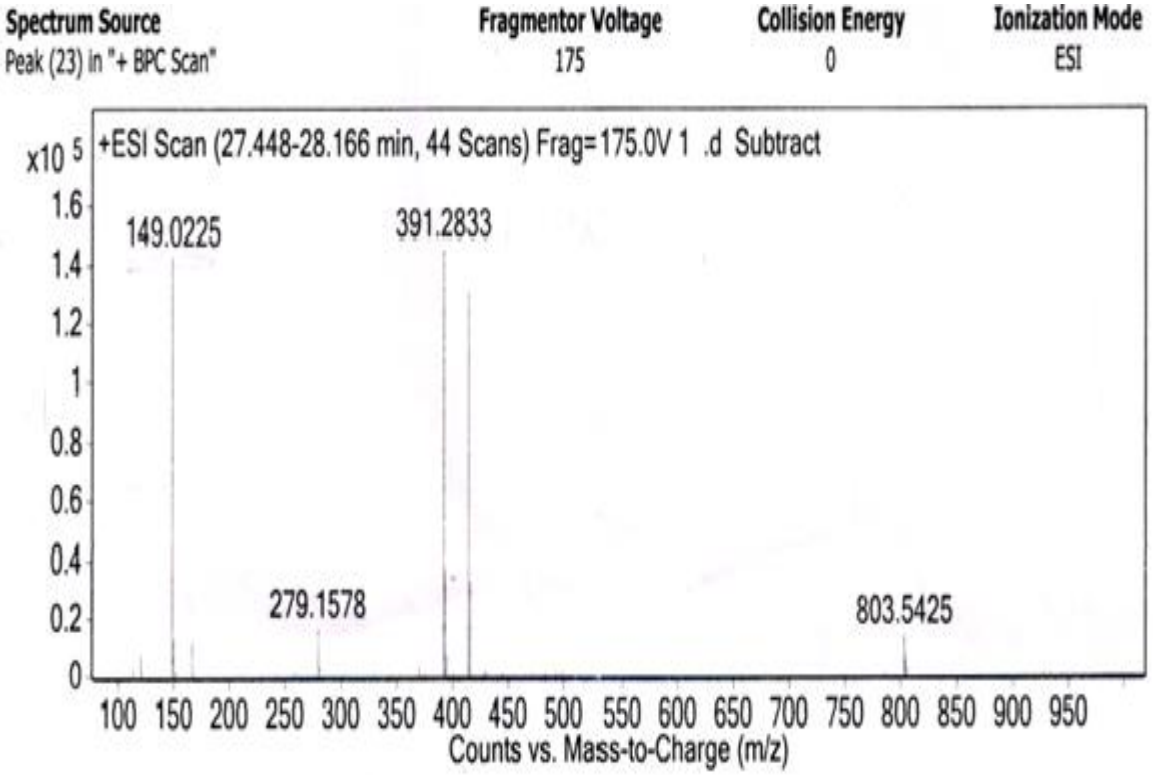


Peak List

m/z	z	Abund
149.0223		4219.13
274.2728	1	43549.95
275.2761	1	7803.12
301.1395		3512.32
318.2989	1	17195.21
319.3011	1	3260.1
362.3258		4269.59

Fig. 4.XXIV Peak 22

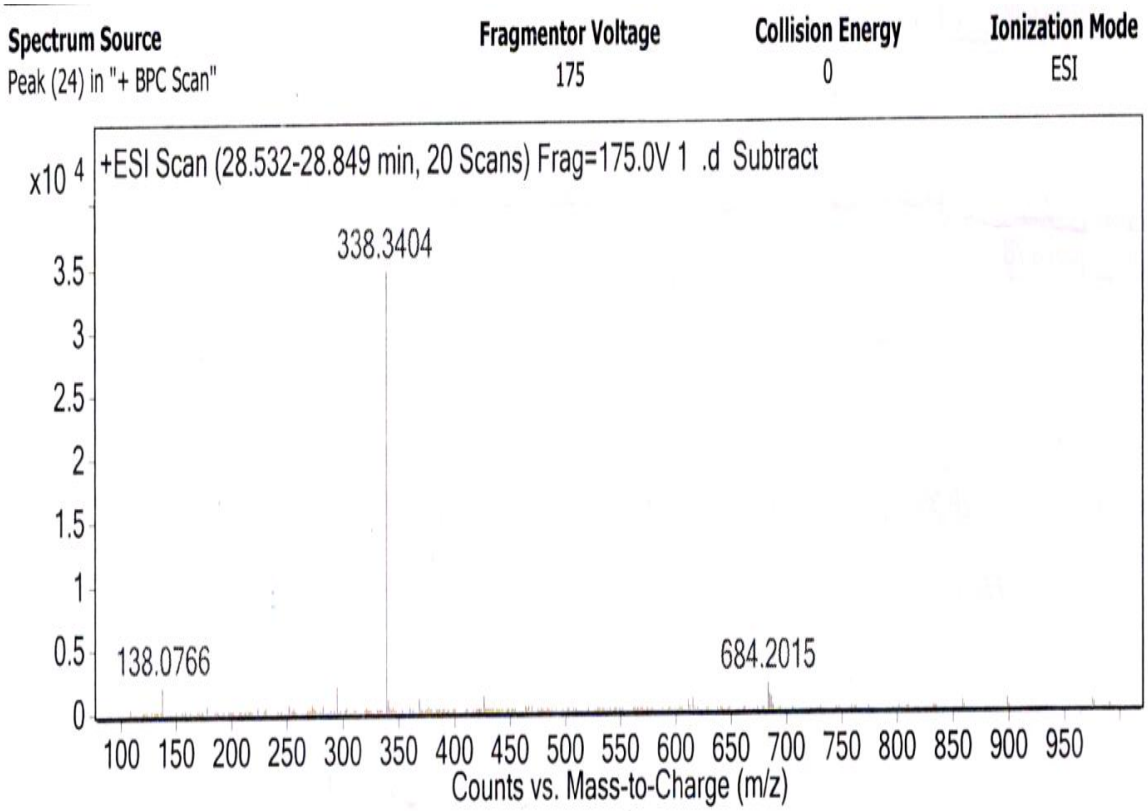
23. Peak 23. a. M/Z 149 corresponds to Trimethylsilyl glycolic acid $[M+2H]^+$ **b.** 150 – $[M+3H]^+$ **c.** 167 – [M+OH] **d.** 391 – $[2M+H+C_7H_7^+]$ **e.** 392- $[2M+2H+C_7H_7^+]$. The spectrum is given in figure 4.XXV.



m/z	z	Abund
121.0287		8099.85
149.0225	1	142808.38
150.0258	1	12857.35
167.0327		11983.29
279.1578		16464.83
391.2833	1	145486.73
392.2865	1	37304.67
413.2652	1	130523.41
414.2683	1	33302.92
803.5425	1	13667.76

Fig. 4.XXV Peak 23

24. Peak 24. a. M/Z 338 corresponds to Trans-13-docasenoic acid ($C_{22}H_{42}O_2$) [M^+] **b.** M/Z 339 – $[M+H]^+$ **c.** 138 – Corresponds to fragment Sodium heptanoate.[98] The spectrum is given in figure 4.XXVI.



Peak List

m/z	z	Abund
138.0766		2139.18
294.9498		2113.22
338.3404	1	34733.52
339.3437	1	8117.14
684.2015		2160.73

Fig. 4.XXVI Peak 24

4.3.2 Spectroscopic analysis of white sugar like crystals obtained from methanol extracts (Sn 2)

Solubility: The white sugar like crystals (Fig. 4.XXVII) obtained were soluble in water and toluene.

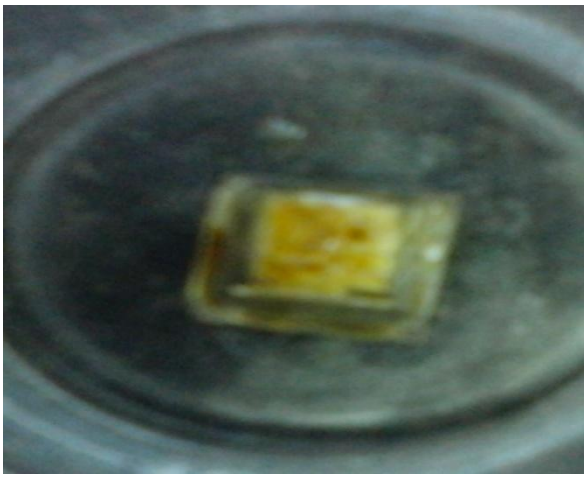


Fig. 4.XXVII Sn 2 Crystal

1. UV spectrum of white crystals: The UV spectrum (Fig. 4.XXVIII) of the crystals were obtained in water as solvent.

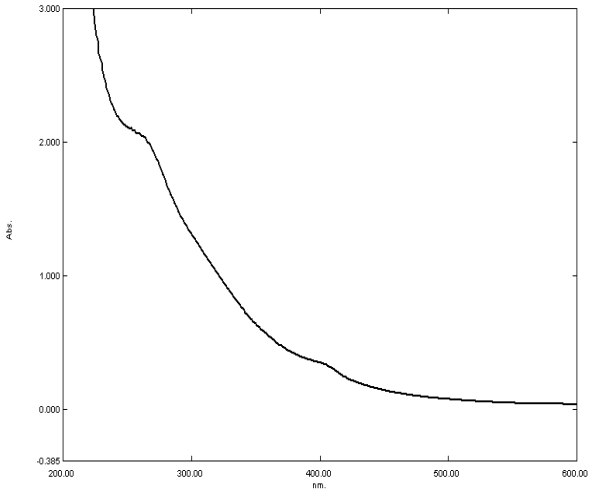
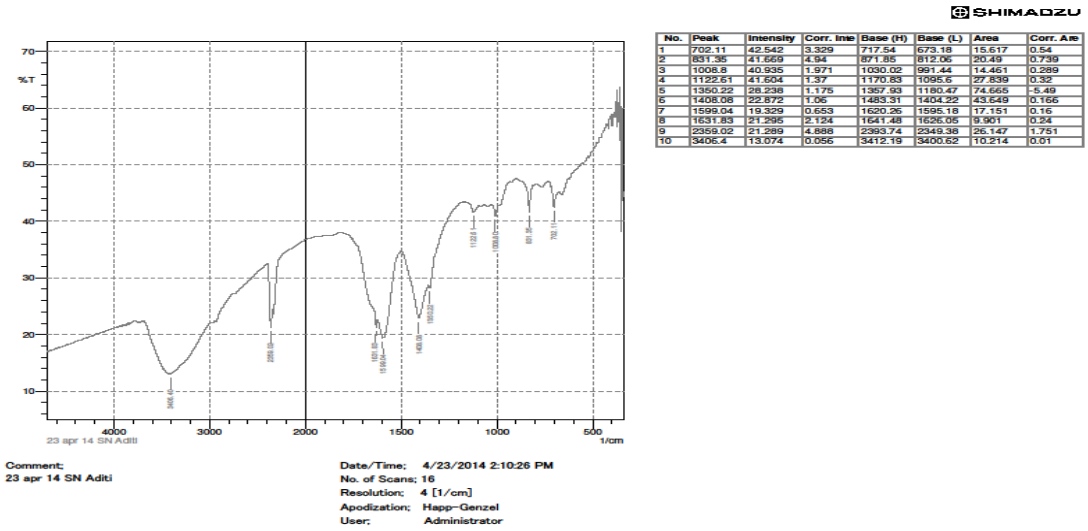


Fig. 4.XXVIII UV spectrum of white crystals

2. IR spectrum of the crystals: IR spectrum of Sn 2 (Fig. 4.XXIX) is given below



No.	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	702.11	42.542	3.329	717.54	673.18	15.617	0.54
2	831.35	41.669	4.94	871.85	812.06	20.49	0.739
3	1008.8	40.935	1.971	1030.02	991.44	14.461	0.289
4	1122.61	41.604	1.37	1170.83	1095.6	27.839	0.32
5	1350.22	28.238	1.175	1357.93	1180.47	74.665	-5.49
6	1408.08	22.872	1.06	1483.31	1404.22	43.649	0.166
7	1599.04	19.329	0.653	1620.26	1595.18	17.151	0.16
8	1631.83	21.295	2.124	1641.48	1626.05	9.901	0.24
9	2359.02	21.289	4.888	2393.74	2349.38	26.147	1.751
10	3405.4	13.074	0.056	3412.19	3400.62	10.214	0.01

Fig. 4.XXIX IR spectrum of Sn 2

3. ^{1}H-NMR spectrum (Fig. 4.XXX) of Sn 2 is given below

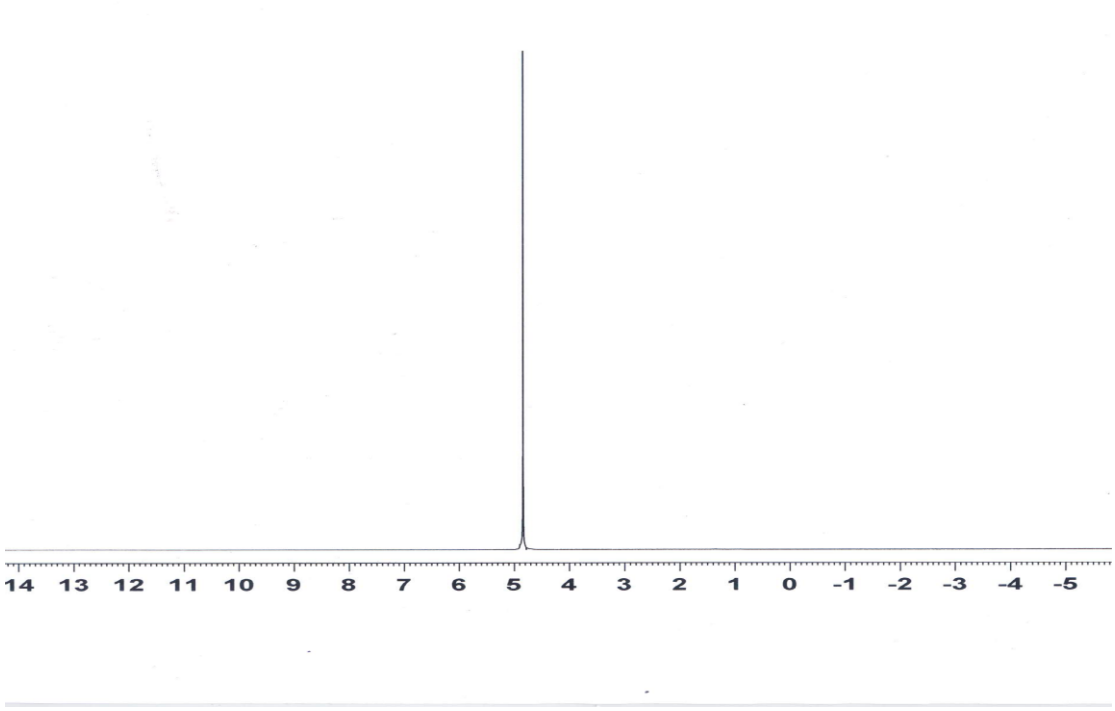


Fig. 4.XXX ^{1}H-NMR spectrum of Sn 2

The ^{1}H-NMR spectrum in solvent D_2O gives only a single peak

4. X-RAY analysis of Sn 2 : It reveals the following

1. Cell length a = 3.1463(5)
2. Cell length b = 3.1463(5)
3. Cell length c = 3.1463(5)
4. Cell angle α = 90 ºC
5. Cell angle β = 90 ºC
6. Cell angle γ = 90 ºC
7. Cell Volume = 31.100(12)
8. Empirical Formula = $C_{28}H_{28}N_5O_1Na_1Cl_1$
9. Radiation, Wavelength = Mo Kα, 0.7107 Aº
10. Crystal System = Cubic

4.4: Antimicrobial activity of various hot extracts of *Solanum nigrum* against the bacterias and fungus

Bacterial strains: The biological activities of different hot extracts were tested against bacteria *E. coli* (NCIM No.- 2563), *Bacillius subtilis* (PS3 KJ489411.1), *Pseudomonas aeruginosa* (jogii JX276925.1) and fungus *Aspergillus niger* (NCIM No.- 619). These microbial cultures were ordered from NCIM Pune and maintained at 4 °C on nutrient broth in Biotechnology labs of LPU. Potato dextrose broth was used for fungal culture.

Chemicals: For bacterial cultivation, nutrient agar and nutrient broth were used. Potato dextrose agar and potato dextrose broth were used for fungal cultivation. Antibiotic gentamicin is used as control and bought from HI-Media Laboratories, Mumbai.

Preparation of nutrient agar medium: Dissolve 28 grams of nutrient agar in 1000 ml distilled water. Gently heat to dissolve the components completely. This nutrient agar was then disinfected in an autoclave at 15 psi (121 °C) for 15 minutes, cooled at room temperature. After cooling it was poured in the sterilized petriplates and placed in laminar till solidification.

Antimicrobial activity of plant extracts: One gm of the various plant extracts was dissolved in 1ml of respective solvent to make the final concentration of 1gm/ml.

Disc diffusion method: Disc diffusion method was used to check *in vitro* antimicrobial activity of various extracts of *Solanum nigrum.* Bacterial culture in was allowed to inoculate on the petriplates containing nutrient agar. Discs were prepared from Whatmann No.-1 filter paper and poured in 5 µl of various extracts. Then two or three discs were placed on petriplates containing inoculated bacterias and kept for 24 hours at 37°C. Experiments were triplicated and average of diameter of zone of inhibition was measured.[25]

A. Antimicrobial activity of various extracts against various microbes

The average diameter of zone of inhibition against various microbes is given in the table. The *in vitro* antimicrobial activity of pet. ether, toluene, DCM, chloroform and ethyl acetate extracts are given in table 4.I, 4.II, 4.III, 4.IV and 4.V respectively.

Table 4.I: *In vitro* antimicrobial activity of petroleum ether extracts

S. No.	Microbes		Diameter in (mm)
1	Gram (+)	Bacteria *Bacillus subtilis*	(-)
2	Gram (-)	Bacteria *Escherichia coli*	(-)
3	Gram (-)	Bacteria *Pseudomonas aeruginosa*	(-)
4		*Aspergillus niger*	(-)

Table 4.II: *In vitro* antimicrobial activity of toluene extracts

S. No.	Microbes		Diameter in (mm)
1	Gram (+)	Bacteria *Bacillus subtilis*	(-)
2	Gram (-)	Bacteria *Escherichia coli*	(-)
3	Gram (-)	Bacteria *Pseudomonas aeruginosa*	(-)
4		*Aspergillus niger*	(-)

Table 4.III: *In vitro* antimicrobial activity of dichloromethane extracts

S. No.	Microbes		Diameter in (mm)
1	Gram (+)	Bacteria *Bacillus subtilis*	(-)
2	Gram (-)	Bacteria *Escherichia coli*	(-)
3	Gram (-)	Bacteria *Pseudomonas aeruginosa*	(-)
4		*Aspergillus niger*	(-)

Table 4.IV: *In vitro* antimicrobial activity of chloroform extracts

S. No.	Microbes		Diameter in (mm)
1	Gram (+)	Bacteria *Bacillus subtilis*	(-)
2	Gram (-)	Bacteria *Escherichia coli*	(-)
3	Gram (-)	Bacteria *Pseudomonas aeruginosa*	(-)
4		*Aspergillus niger*	(-)

Table 4.V: *In vitro* antimicrobial activity of ethyl acetate extracts

S. No.	Microbes		Diameter in (mm)
1	Gram (+)	Bacteria *Bacillus subtilis*	(-)
2	Gram (-)	Bacteria *Escherichia coli*	(-)
3	Gram (-)	Bacteria *Pseudomonas aeruginosa*	(-)
4		*Aspergillus niger*	(-)

Results and Discussion: None of the extract is found active against the bacterias *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and fungi *Aspergillus niger*

4.5: Insecticidal activity of hot petroleum ether and methanol extracts of *Solanum nigrum*

The insecticidal activity of petroleum ether and methanol extracts of *Solanum nigrum* are investigated against agricultural epilachna insect *Coccinella septumpunctata* and rice weevil *Sitophilus oryzae* in a laboratory. Insecticidal activity was found out at a temperature of 25±4 ºC and 70 ± 5 % relative humidity in dark conditions. The mortality rate of both the insects was checked against 1 %, 5 % and 10 % concentration of petroleum ether and ethanol extracts. Finding insecticidal activity is of great importance as using plant extracts as insecticides biodegrable and do not produce toxicity hence ecofriendly.

Insecticidal Testing: The petroleum ether and methanol extracts were prepared at three different concentrations (1 %v/v, 5 % v/v, 10 % v/v).

(i). Insect Assayed: Test insects: The epilachna insect *Coccinella septumpunctata* (Fig. 4.XXXI) were collected from the fields and rice weevil *Sitophilus oryzae* (Fig. 4.XXXII) were collected from the rice mill and identified by Entomologist Dr. Sudhakar Gupta of Lovely Professional University.

(ii). Determination of Insecticidal activity by Contact toxicity assay: The Wheat leaves (for Epilachna insect) and rice grains (for rice weevil) are taken from the field and house, washed and dried and then insects were allowed to feed on them under controlled conditions of temperature and humidity. These washed and dried wheat leaves and rice grains are then dipped in the respective percentage of plant extracts (i.e. 1%, 5%, 10% v/v) for two hours and then insects are allowed to feed on these leaves in each jar and mortality rate of the insects was checked. Results were recorded by counting the number of survivals in each jar. Control experiments with petroleum ether, methanol, distilled water and without solvent were performed separately up to 72 hours. Mortality was assessed after 3, 4, 7 and 12 h of the treatment. The calculation of mortality rate was corrected for control mortality according to Abbott's formula[45]:

$$Mc = (Mo-Mc/100-Me) * 100$$

Where, Mo = Observed mortality rate of treated adults (%), Me = mortality rate of control (%), and Mc = corrected mortality rate (%).

Results are tabulated in the form of table 4.VI and 4.VII.

Fig. 4.XXXI Insecticidal activity against *Coccinella septumpunctata*

Fig. 4.XXXII Insecticidal activity against *Sitophilus oryzae*

Table 4.VI: Insecticidal activity of pet. ether extracts against *Coccinella septempunctata* and *Sitophilus oryzae*

S.No.	Plant Name	Insect	% of Extract	No. of Insects used	No. of Insects died (hr)				Total No. of insects Died	% Average Mortality	% Corrected Mortality
					3	4	7	12			
1	*Solanum nigrum*	*Coccinella septempunctata*	1 %	10	8	2	-	-	10	100	100
2	*Solanum nigrum*	*Coccinella septempunctata*	5 %	10	9	1	-	-	10	100	100
3	*Solanum nigrum*	*Coccinella septempunctata*	10 %	10	8	2	-	-	10	100	100
4	*Solanum nigrum*	*Sitophilus oryzae*	1 %	10	10	-	-	-	10	100	100
5	*Solanum nigrum*	*Sitophilus oryzae*	5 %	10	9	-	-	-	10	100	100
6	*Solanum nigrum*	*Sitophilus oryzae*	10 %	10	10	-	-	-	10	100	100

Table 4.VII: Insecticidal activity of methanol extracts against *Coccinella septempunctata* and *Sitophilus oryzae*

S. No.	Plant Name	Insect	% of Extract	No. of Insects used	No. of Insects died				Total No. of insects Died	%Average Mortality	% Corrected Mortality
					3	4	7	12			
1	*Solanum nigrum*	*Coccinella septempunctata*	1 %	10	-	3	-	2	5	50	50
2	*Solanum nigrum*	*Coccinella septempunctata*	5 %	10	5	5	-	-	10	100	100
3	*Solanum nigrum*	*Coccinella septempunctata*	10 %	10	10	-	-	-	10	100	100
4	*Solanum nigrum*	*Sitophilus oryzae*	1 %	10	10	-	-	-	-	100	100
5	*Solanum nigrum*	*Sitophilus oryzae*	5 %	10	9	1	-	-	10	100	100
6	*Solanum nigrum*	*Sitophilus oryzae*	10 %	10	10	-	-	-	10	100	100

Results and Discussion: Petroleum ether extract of the plant is showing 100 % mortality against the agricultural pest *Coccinella septumpunctata* and stored grain insect *Sitophilus oryzae* where as methanol extract (1 %) is less active towards *Coccinella septumpunctata* but 5 % and 10 % extracts are showing 100 % mortality against *Coccinella septumpunctata.* Methanol extracts at 1 %, 5 % and 10 % is very well active and showing 100 % mortality against the stored grain insect *Sitophilus oryzae.*

Conclusion: Both the extracts of *Solanum nigrum* shows strong toxic effects against the agricultural pest *Coccinella septempunctata* and stored grain pest *Sitophilus oryzae.*

4.6 Antidiabetic activity of hot petroleum ether and methanol extracts of *Solanum nigrum* plant

Antidiabetic activity of petroleum ether and methanol extracts of *Solanum nigrum* is determined in CSIR lab, IIIM Jammu on wistar rats by 18h Fasted rats model.

Experimental

***In vivo* models**: **18h Fasted rats model**

Four groups of six Wistar rats (male/female) were selected randomly and fasted overnight. First group was given Normal Vehicle Control, second and third groups were given petroleum ether and methanol extracts treated diet and fourth group was given glibenclamide treated diet. Blood glucose level of all the rats was found out prior to any treatment (0 hour) and post-drug administration (3 hour).[46] Results are given in table 4.VIII and graph 4.I

Table 4.VIII: Diabetic activity of petroleum ether and methanol extracts of *Solanum nigrum*

S.No.	Treatment group (mg/kg p.o.)	Blood glucose level (mg/dl) Mean±S.E.	
		0h	3h
1	Normal Control	83±4.65	79±4.09
2	Glibenclamide (0.5)	78±4.02	49±2.56***
3	Pet. ether extracts of *Solanum nigrum* (250)	94.2±3.19	92.0±4.46
4	Methanol extracts of *Solanum nigrum* (250)	96±7.22	90±5.43

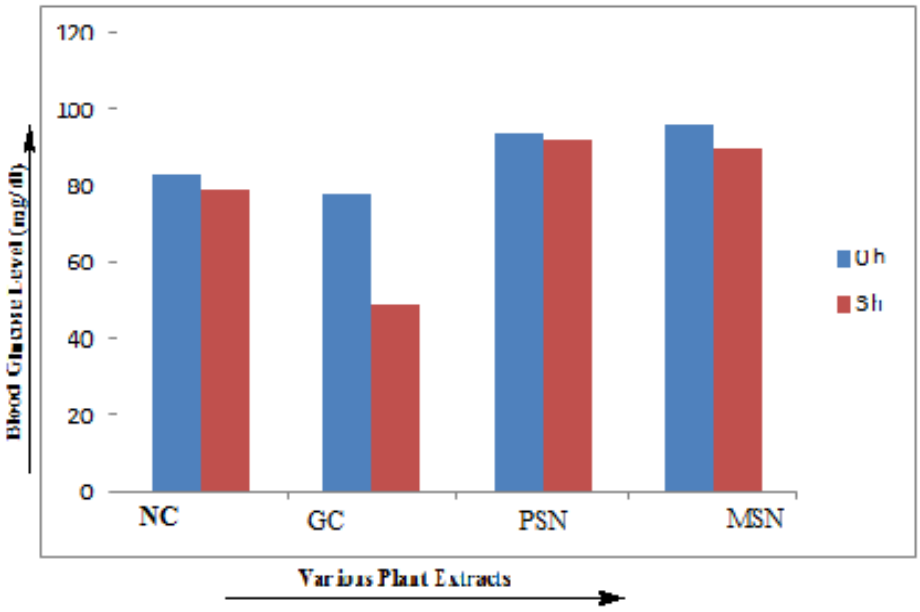
n (Number of animals) : 5

*** $p < 0.001$ compared to 0 h blood glucose level.

Results and Discussion: Petroleum ether extracts decreases the blood glucose level of the rat from 94 mg/dl to 92 mg/dl while methanol extract show significant decrease in blood glucose level from 96 mg/dl to 90 mg/dl thus both showing hypoglycemic actions.

Conclusion: Pet. Ether and methanol extracts of *Solanum nigrum* are showing hypoglycemic activity.

Graphical representation of hypoglycemic activity of both extracts is given below:



Where NC = Normal control, GC = Glibenclamide, PPN = Petroleum ether extracts of *Solanum nigrum* MPN = Methanol extracts of *Solanum nigrum*

Graph 4.I Graphical representation of hyperglycemic action of petroleum ether extracts and methanol extracts

5.1: Introduction:

Eclipta alba (Fig. 5.1) is an annual herb belongs to family Asteraceae and widely distributed in Tropical countries.[100] It is commonly called as 'Bhringraj' in Ayurveda and is useful in the treatment of Kapha and Vata disorders. It's a primary liver herb also called as 'Kesharaja' or 'Kesharanjana' in Ayurveda; Kadim-il-bint in Arab; Vellai Karisilankani in Tamil.[101-102] The herb is found in moist atmosphere throughout India up to an altitude of 600 feet on the hills and has characteristics white flower heads. In Ayurveda and Unani medicinal system, it is regarded as a herbal medicine and reported as a major component of Indian antihepatotoxic phytopharmaceutical formulations.[103] The whole plant is dried, powdered and taken in liver disorders especially jaundice in conventional medicinal system.[104] The aerial part of *E. alba* is one of important traditional Chinese medicines known as Mohanlian in Chinese and mainly used as a tonic for enriching the blood.[105]

Botanical Description: The plant has a thin woody stem, dark green leaves and white small flowers and can achieve up to two feet in height.[102]

Stem is dark green in colour, cylindrical, 2 to 5 cm in diameter with longitudinal ridges.

Leaves are 2 to 6 mm in diameter, opposite, sessile, lanceolate, usually oblong with entire or dentate margins.[106]

Geographical Distribution: In India, the plant springs up itself as a weed ascending up to 1800 m in Himalayas, Upper Gangetic Plains and pasture lands, in moist and cool places of Punjab, Orissa, Bihar, West and South India.[106]

Pharmacognosy:

Macroscopic: The plant is branched, prostate or erect with characteristics white flowers. Its stem is profusely branched, pubescent, round or flat. Leaves are 3 to 5 cm in length and blackish green in colour[107-108], Inflorescence is capitulum, 2 to 6 mm in diameter and white in colour. Achenes (dry fruit) are flattened, elliptical, 2 to 3 mm in length, brown to pale colour, with slight odour and taste.[106]

Pharmacology:

Hepatoprotective activity: Wedeolactone and demethylwedeolactone are the coumestans attributing antihepatotoxic activities to the plant in phalloidin, CCl_4, gallactosamine induced liver damage in rats.

CNS Activity: 300 mg/Kg of aqueous extract of the plant shows nootropic activity in rats.

Antimicrobial activity: Antibacterial activity is shown by its shoot extracts against the bacterias E. *coli* and *Staphylococcus aureus.*

Antinociceptive activity: Due to the presence of coumarin derivatives in hydro-alcoholic extracts, this extract of the plant at a concentration of 200 mg/Kg shows antinociceptive activities.

Bronchodilator and Antiinflammatory activity: Coumarin phytoconstituents attributes bronchodilator and antiinflammatory activities.

Immunomodulatory activity: Immunomodulatory effects are shown by methanolic extracts. The phytoconstituents demethylwedeolactone and wedeolactone shows *in vitro* trypsin inhibition.

Antiviral activity: Antiviral activity against Ranikhet disease is shown by its alcoholic extracts.

Analgesic activity: Total alkaloids produced in ethanolic extracts are responsible for good analgesic activity in Albino mice by tail flick and tail clip methods.

Antioxidant activity: Free radical scavenging activity for hydroxyl radical and DPPH are reported by methanolic extracts of aerial parts of the plant.

Hair Growth activity: In anagenic phase, 5% petroleum ether extracts of the plant produces greater number of hair follicles. It is reported that petroleum ether extracts (2 % and 5%) were showing more activity than 2 % minoxidil treatment.

Wound Healing Activity: Ethanol extracts of leaves at a concentration of 150 and 300 mg/Kg is reported to heal wounds in anaesthetized wistar rats using excision, incision and dead space wound model.

Antifungal activity: Whole plant shows antifungal activity against *Candida albicans, Candida tropicalis, Rhodotorula glutinis*.[106]

Phytochemistry: The various coumestans derivatives reported in the dried leaves of the plant are ecliptine, nicotine, stigmasterol, wedelic acid, apigenin, luteolin and their glucosides, demethylwedelolactone, wedelolactone, desmethylwedeolactone-7-glucoside, α-terthienymethanol, 25β-hydroxyverazine.[106]

The phytoconstituents of roots contains thiophene acetylenes such as hentriacontanol, ecliptal, stigmasterol, 5I-tigloyloxymethylene-2-(isovaleryloxybut-3-ynyl)-dithiophene, 5I-senecioyloxymethylene-2-(4-isovalery oxybut-3-ynyl)-dithiophene and 14-heptacosanol.

The phytoconstituents reported in stem are wedelic acid, luteolin, wedeolactone, L-terthienyl methanol and apigenin.

Sterols are the secondary metabolite reported in seeds. The phytoconstituents apigenin, β-amyrin, cinnaroside, luteolin-7-O-glucoside, Phytosterol and sulphur compounds are present in aerial parts of the herb. Whole plants of *Eclipta alba* is reported to possess triterpene saponin, oleanolic acid, resin, ecliptine, nicotine, reducing sugar, stigmasterol, ursolic acid, eclalbatin and α-amyrin.[106, 109]

Types of secondary metabolites:

Terpenoids:

Eclalbasaponins are the taraxastane terpene glycoside reported along with oleanane glycosides. The structures of eclalbasaponins were characterized as 3β, 16β, 20β, and 3β, 20β, 28β trihydroxytaraxastane glycosides and their sulphated saponins. A ubiquitous steroid stigmasterol along with two oleanane type glycosides eclalbasaponin I and eclalbasaponin II were reported from the n-hexane extract of *Eclipta prostrata'* s stem bark. Six triterpene glycosides characterized as echinocystic acid glycosides and sulphated saponins were reported from the entire plant of *Eclipta alba Hassk.*

Alkaloids:

The presence of alkaloid ecliptine in the plant is confirmed by contemporary clinical tests. (20S)(25S)-22,26-imino-cholesta-5,22(A)-dien-3P-ol (verazine) was the major component of alkaloids whereas 4P-hydroxyverazine, 25β-hydroxyverazine, ecliptalbine [(20R)-20-pyridyl-cholesta-5-ene-3P,23-diol], (20R)-4P-hydroxyverazine, (20R)-25P-hydroxyverazine and 20-epi-3-dehydroxy-3-oxo-5,6-dihydro-4,5-dehydroverazine were identified as new alkaloids. Ecliptalbine, in which the 22, 26-imino ring of verazine was replaced by a 3-hydroxypyridine moiety, had comparable bioactivity to verazine.

Volatile oils:

Aerial parts of the herb were subjected to hydrodistillation and analysed by GC-MS to obtain various volatile components**.** A sum of 55 phytoconstituents constitutes the major portion i.e., 91.7% of the volatiles were identified by comparing their mass spectra with the mass spectrum library (NIST 05.L). The various volatile constituents are pentadecane, heptadecane, w-hexadecanoic acid, 1,2-benzenedicarboxylic acid diisooctyl ester, (Z)-7,11-dimethyl-3- methylene-1,6,10-dodecatriene,(Z,Z)-9.12-octadecadienoic acid,

(Z,Z,Z)-1,5,9,9-tetramethyl-1,4,7-cycloundecatriene, eudesma-4(14),11-diene, 6,10,14-trimethyl-2-pentadecanone, octadec-9-enoic acid and phytol.

Saponins:

A triterpenes saponin called Eclalbatin and ursolic acid, oleanolic acid and alpha-amyrin were obtained from *Eclipta alba* entire plant. On the basis of spectrochemical analysis, 3-O-β-D-glucopyranosyl-3-β-hydroxy-olean-12-en-28oicacid,28-O-β-D-arabinopyranoside is assigned as the structure of eclalbatin. A phytochemical Dasyscyphin C, isolated from *Eclipta prostrata,* is studied for anticancer activity on the HeLa cells.[110]

Fig.5.I *Eclipta alba*

5.2: Materials and Methods:

Plant Material: The whole plant of *Eclipta alba* except roots were plucked from Ravi river banks and Billawar Hills of Kathua district of J&K and classified systematically by the taxonomist Dr. Rajesh Manhas of Botany Department of University of Jammu.

Hot Extraction: The live plants collected were washed properly under tap water, then rinsed in distilled water and shade dried without any contamination for 3-4 weeks. The plant materials were then crushed and soxhlated with various solvents in soxhlet apparatus to prepare various extracts according to their polarity gradient such as petroleum ether, toluene, chloroform, ethyl acetate and methanol. The petroleum ether extract was subjected to column chromatography and the brown-black fraction (Ea 1) obtained from 7:3 pet. ether **:** DCM was further subjected to LC- MS for the identification of various chemical constituents.

Basic Hydrolysis of Petroleum ether extracts: To the petroleum ether extracts (20 ml), added 20 ml of 10 % KOH, heated on water bath for 3 hours and cooled. Added equal qty of chloroform and again heated for on water bath for 2 hours at a temp of 20°C. Poured the contents in a separating funnel to separate organic and aqueous layer. Lower layer results in the formation of sugar like crystals (Ea 2), soluble in water, that were subjected to various spectroscopic analysis for identification.

Experimental

Analytical tools: The hot extraction of plant material was done with JSGW soxhlate apparatus. Labfit melting point apparatus was used to determine melting point. SHIMADZU UV-1800 UV Spectrophotometer was used for recording UV spectrum and ethanol was used as a solvent. SHIMADZU FTIR-8400 S (Fourier Transform Infrared spectrophotometer) was used to obtain IR spectrum. Bruker Avance 400 MHz NMR spectrophotometer was used to record ^{1}H-NMR and ^{13}C-NMR spectrophotometer and solvent used was CH_3OD. Chemical shifts were given in δ using tetramethylsilane as internal standard. Agilent 6540, Q-TOF (HR-MS) was used to record mass spectrum. X-Ray diffraction analysis was performed using X-Ray diffractometer (Rigaku Co. Ltd. Japan) with Mo Kα radiation and wavelength 0.7103 A° at room temperature.

LC-MS: The various components present in the plant were analyzed using LC-MS from IIIM Jammu. Agilent 1100 LC coupled with Bruker MS (Model Esquire 3000) was used for LC-MS analysis. The various instrumental parts of liquid chromatography are binary

gradient pump with online degasser, column oven, autosampler with capacity of 100 samples and PDA detector. Sample inlet system, liquid chromatographic system and syringe pump inlets are the components of Mass spectroscopy. Electron Spray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI) are the ionization sources and connected with ion trap analyzer with MS_n facility. The LC-MS instrument helps in determination of molecular mass of the components of a mixture and number of components present in a given mixture. It has utility in both qualitative and quantitative analysis.

Apparatus and Equipments: The equipments laminar air flow, incubator and oven were of Yorko Industries where as autoclave of JSWG. Glassware and heating mantle were from Perfit India.

5.3: Results and Discussion:

A. LC-MS analysis of (Ea 1)(Fig. 5.II) is given below:

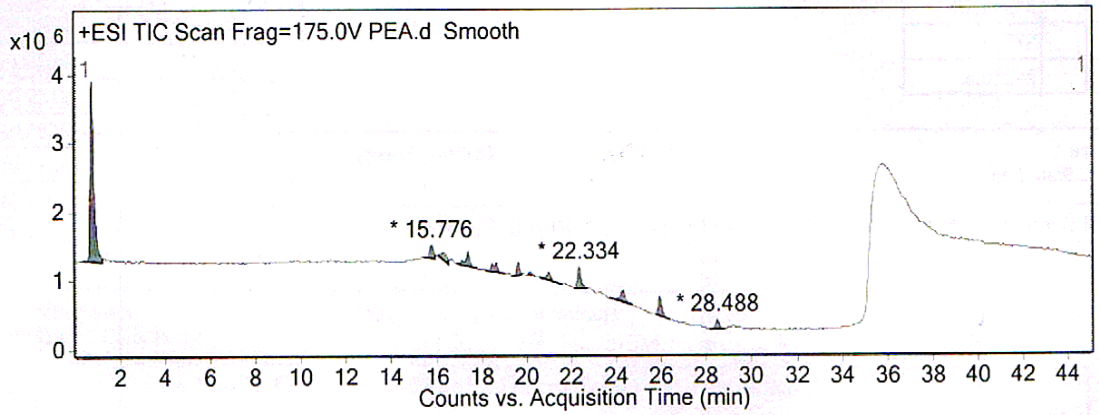
Qualitative Analysis Report

Data Filename	PEA.d	**Sample Name**	PEA
Sample Type	Sample	**Position**	Vial 41
Instrument Name	Instrument 1	**User Name**	vishal
Acq Method	khazuria sir.m	**Acquired Time**	04-05-2013 PM 12:45:51
IRM Calibration Status	Success	**DA Method**	daily_report.m
Comment			

Sample Group	**Info.**
Acquisition SW Version	6200 series TOF/6500 series Q-TOF B.05.01 (B5125)

User Chromatograms

Fragmentor Voltage 175 **Collision Energy** 0 **Ionization Mode** ESI



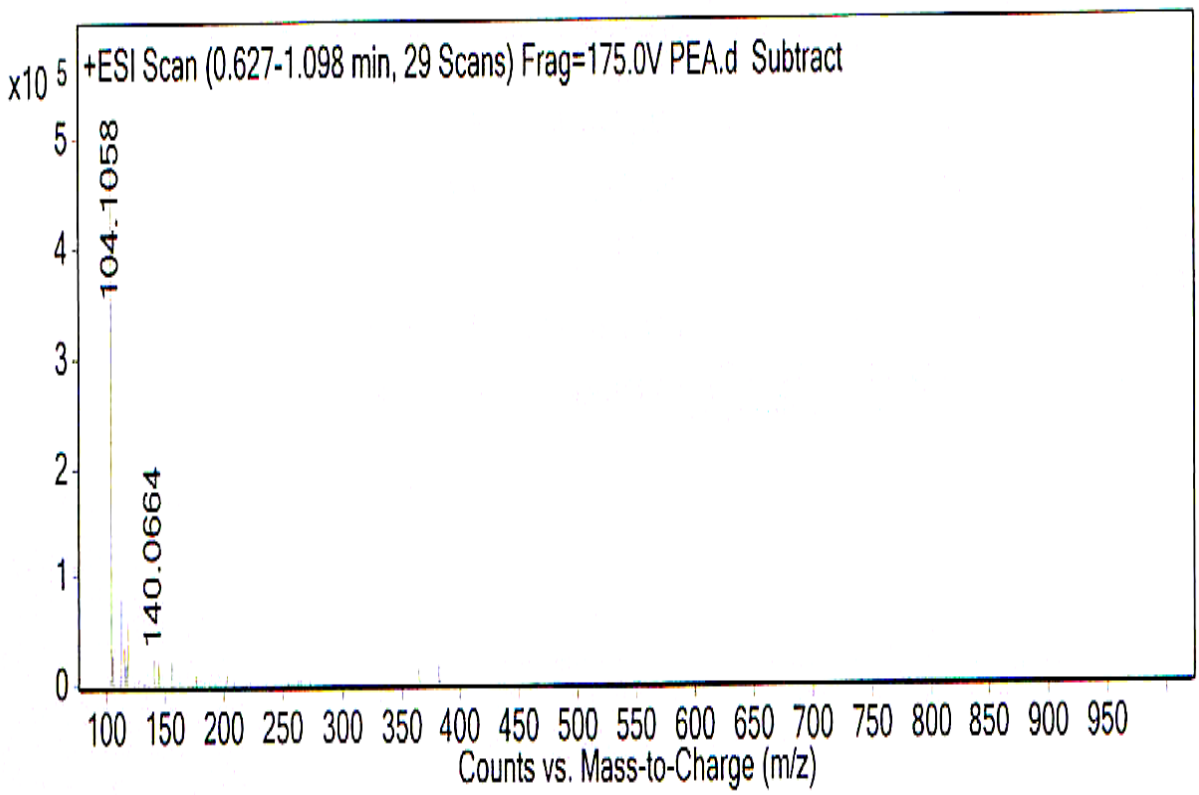
Integration Peak List

Start	RT	End	Height	Area	Area %
0.358	0.745	1.232	2625909	32439004	100
15.44	15.776	15.928	190279	2341706	7.22
16.92	17.407	17.828	215349	3070551	9.47
18.181	18.652	19.021	139652	2092597	6.45
19.341	19.627	19.812	196665	1893778	5.84
20.585	21.006	21.56	121979	1144439	3.53
22.132	22.334	22.737	315975	3662251	11.29
23.679	24.301	24.738	164399	2636105	8.13
25.73	25.915	26.386	281845	3433391	10.58
28.151	28.488	28.824	147056	1650512	5.09

Fig. 5.II LC-MS analysis of Ea 1

Evaluation of various peaks

1. **Peak 1.** Corresponds to styrene. **a.** Peak 104 – [M], **b.** 105 – [M+H], **c.** 112 – Chlorobenzene $C_6H_5Cl^{35}$, **d.** 114 - $C_6H_5Cl^{37}$, **e.** 140 – (chloro-vinyl)-benzene.[111] The spectrum is attached in fig. 5.III.

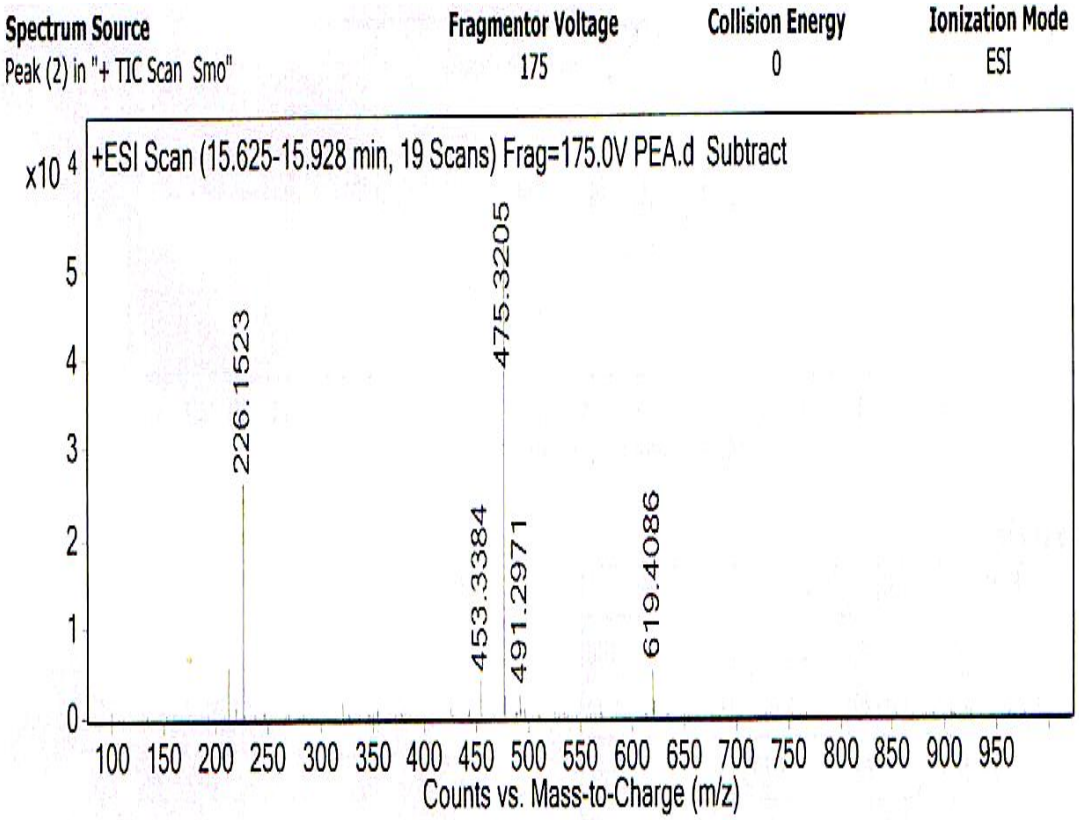


Peak List

m/z	z	Abund
104.1058	1	481265.78
105.1092	1	25971.77
112.8942		78753.2
114.8915		34244.58
118.0848		57505.2
140.0664		24220.5

Fig. 5.III Peak 1

2. Peak 2. The fragment ion at *m/z* 475 corresponds to 20(*S*)-protopanaxatriol aglycon moiety. **a.** Peak 619 – Rha-Glc-H_2O where Rha = α-L-rhamnopyranose and Glc = α-D glucopyranose.[112] The spectrum is attached in fig. 5.IV.

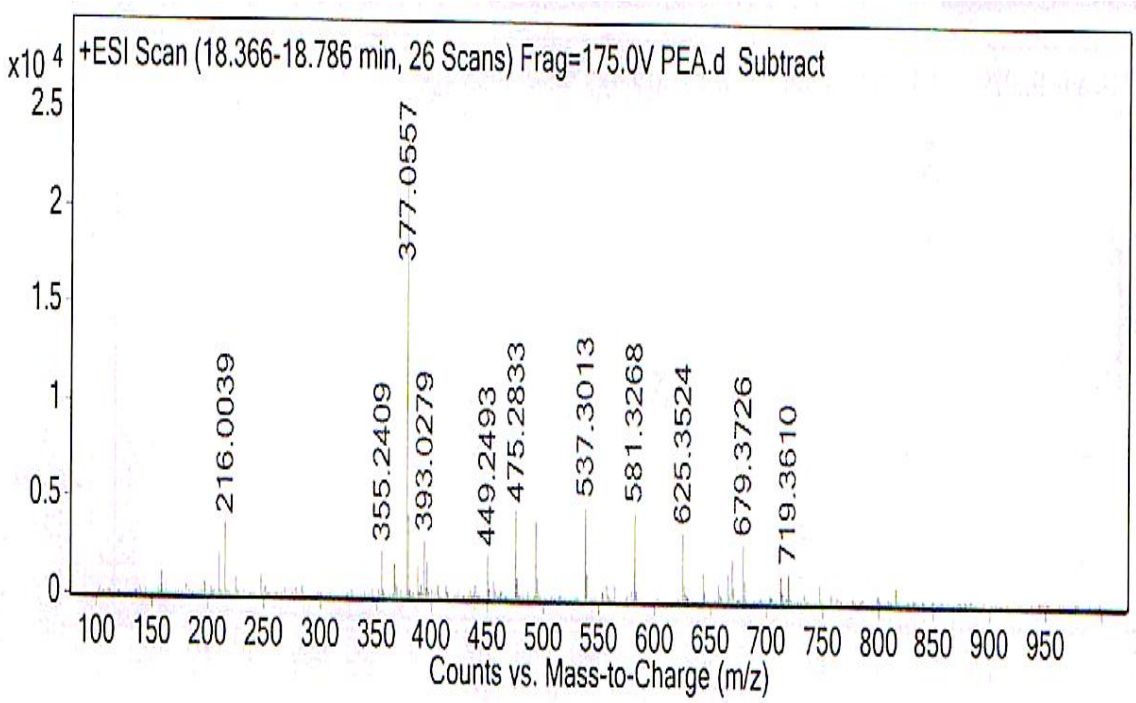


Peak List

m/z	z	Abund
212.1733		5592.38
226.1523	1	26162.66
227.1574	1	3027.41
453.3384		4084.1
475.3205	1	53309.34
476.3235	1	14265.19
491.2971		2731.96
619.4086		5326.05

Fig. 5.IV Peak 2

3. **Peak 5. a.** The mass spectra (parent ion m⁄ z = 719, corresponds to loroxanthin derivative $[M+H-18]^+$, **b.** 581 – $[M-3H^+]$, **c.** m/z 377 corresponds to fragment 4-(3-Hydroxy methyl -7,12-dimethyl tetradeca-1, 3, 5, 7, 9, 11, 13-heptaenyl)-3, 5, 5-trimethyl cyclohex-3-enol.[113] The spectrum is attached in fig. 5.V.

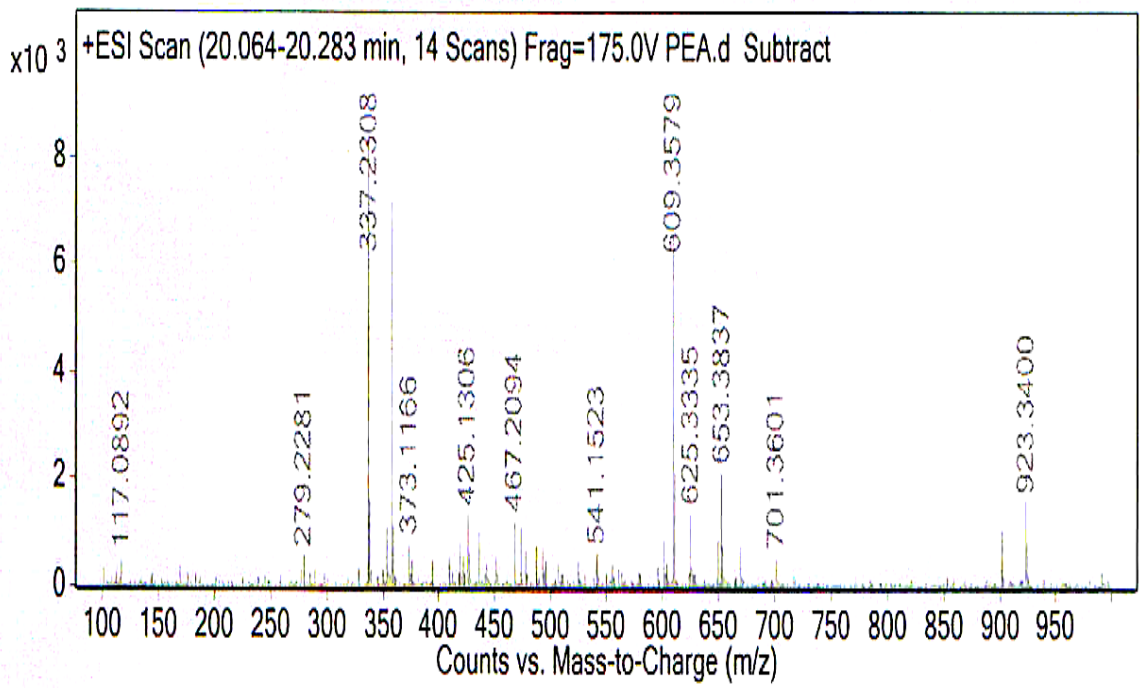


Peak List

m/z	z	Abund
216.0039		3669.67
377.0557	1	23497.99
378.0584	1	4316.69
393.0279		2911.45
475.2833		4511.31
493.2749		4031.36
537.3013	1	4844.38
581.3268	1	4559.25
625.3524		3519.53
679.3726		2955.05

Fig. 5.V Peak 5

4. Peak 7. a. Peak 609 – Quercetin-3-rutinoside $[M-H]^-$, **b.** 625 – $[M+OH+2H]^+$, **c.** 653 – $[M+COO^-]^+$.[114] The spectrum is attached in fig. 5.VI.



Peak List

m/z	z	Abund
337.2308	1	8511.3
338.2338	1	1596.85
357.1429	1	7175.51
358.1463	1	1501.06
425.1306		1344.45
609.3579	1	6612.11
610.3595	1	2111.62
625.3335		1363.87
653.3837	1	2136.57
923.34	1	1585

Fig. 5.VI Peak 7

5. Peak 11. a. Peak 388 – 2-[6-Amino-1-(2-hydroxy-1-phenyl- ethyl)-1, 4, 5, 6-tetrahydropurin-9-yl]-5-hydroxy methyl-tetrahydrofuran-3, 4-diol [M-3H$^+$], **b.** 389 – [M-2H$^+$].[115] The spectrum is attached in fig. 5.VII.

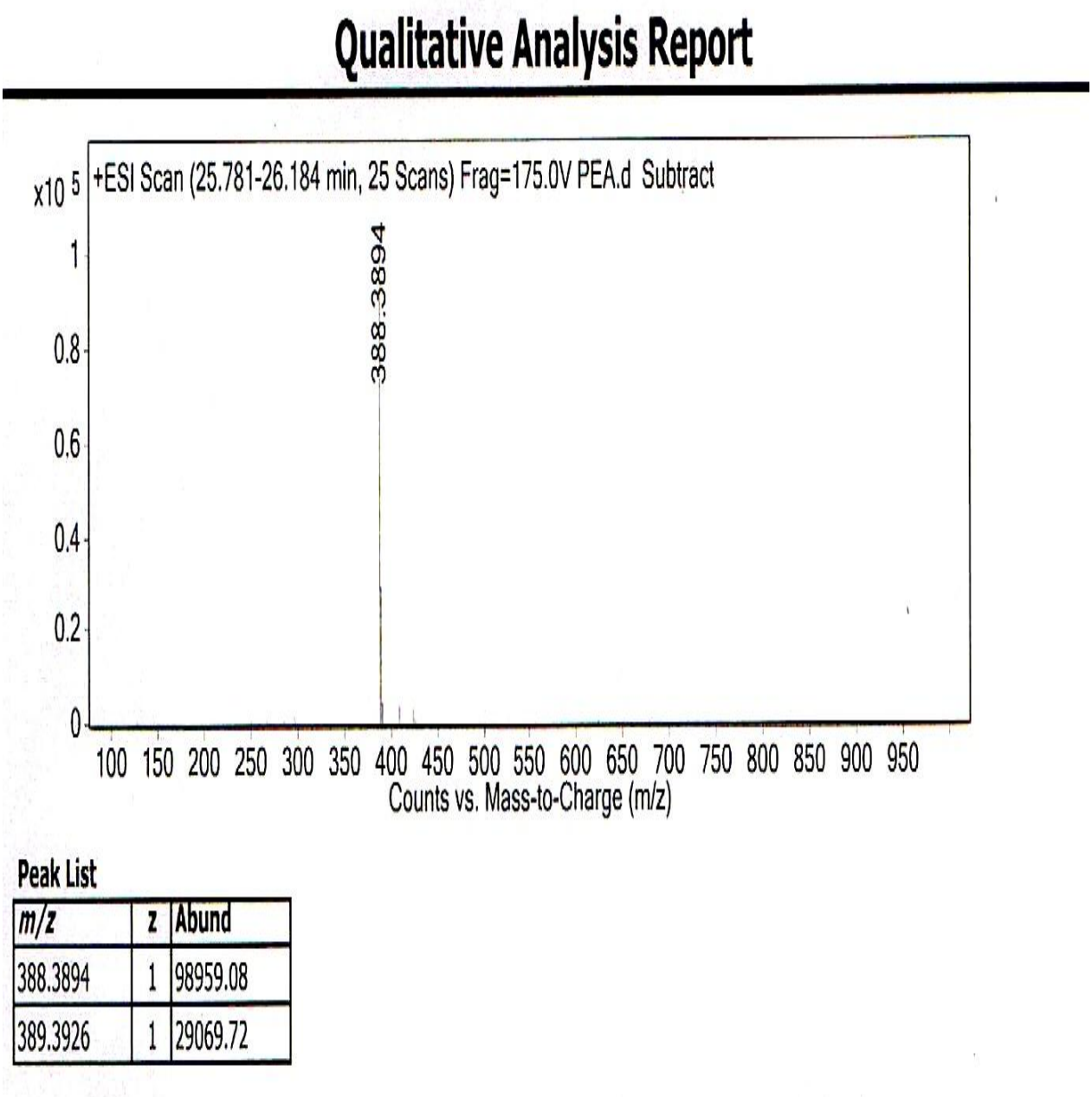
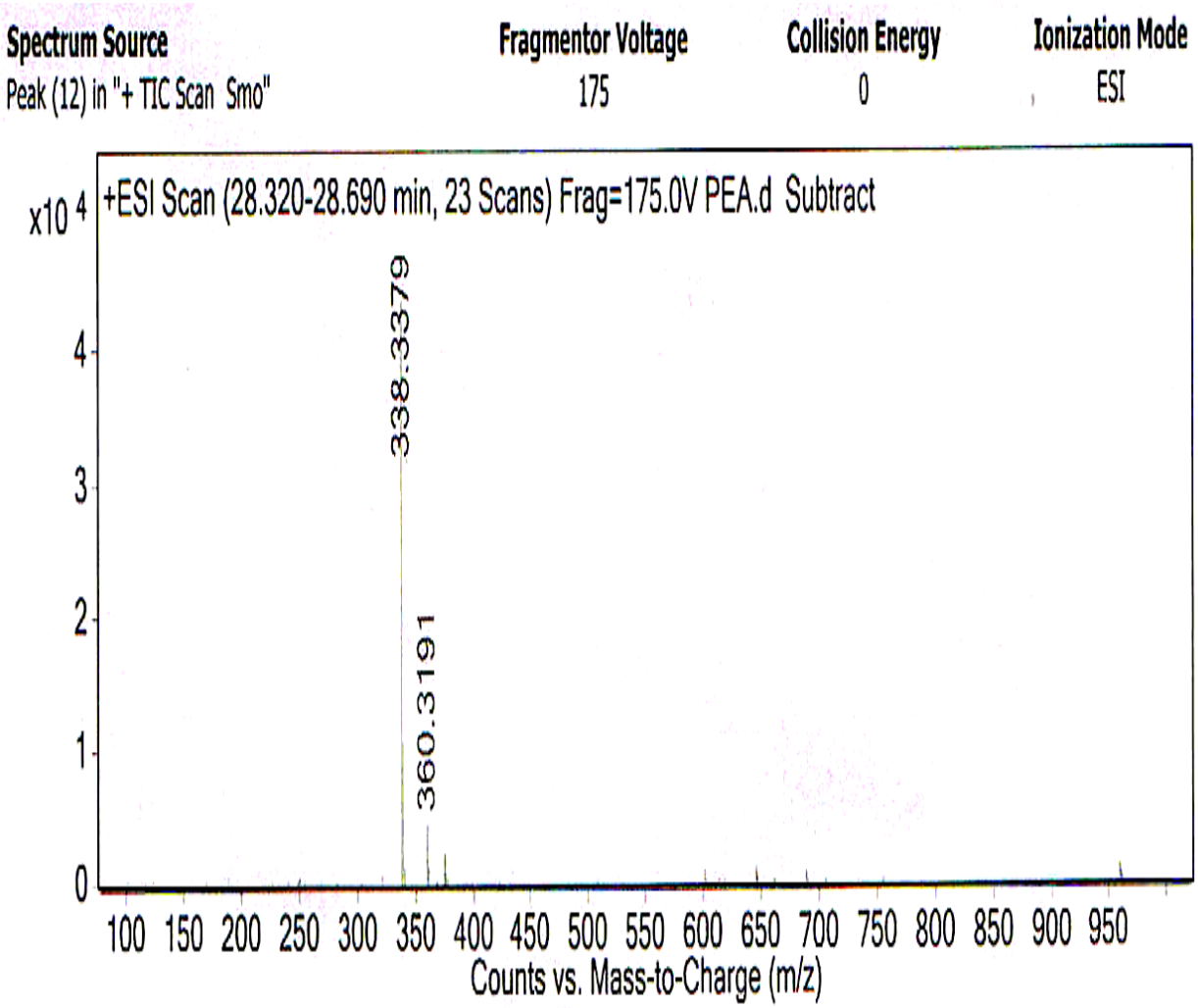


Fig. 5.VII Peak 11

6. Peak 12. a. Peak 338 – 2, 4-dichloro-8,9 dihydroxy-6-H-benzofuro [3,2-c]-[1] benzopyran-6-one $[M+H]^+$, **b.** 339 – $[M+2H]^+$, **c.** 360 – $[M+Na]^+$.[116] The spectrum is attached in fig. 5.VIII.



Peak List

m/z	z	Abund
338.3379	1	43678.09
339.3411	1	10652.99
360.3191		4558.11
376.2933		2259.45

Fig. 5.VIII Peak 12

B. Spectroscopic analysis of white sugar like crystals (Ea 2) obtained from basic hydrolysis of petroleum ether extracts (Fig. 5.IX)

The white sugar like crystals obtained was soluble in water.

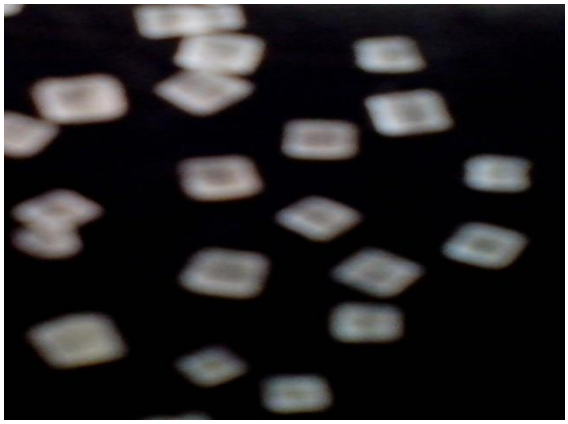


Fig. 5.IX Ea 2 Crystals

1. UV spectrum of (Ea 2): The UV spectrum of the crystals (Fig. 5.X) were obtained in water as solvent.

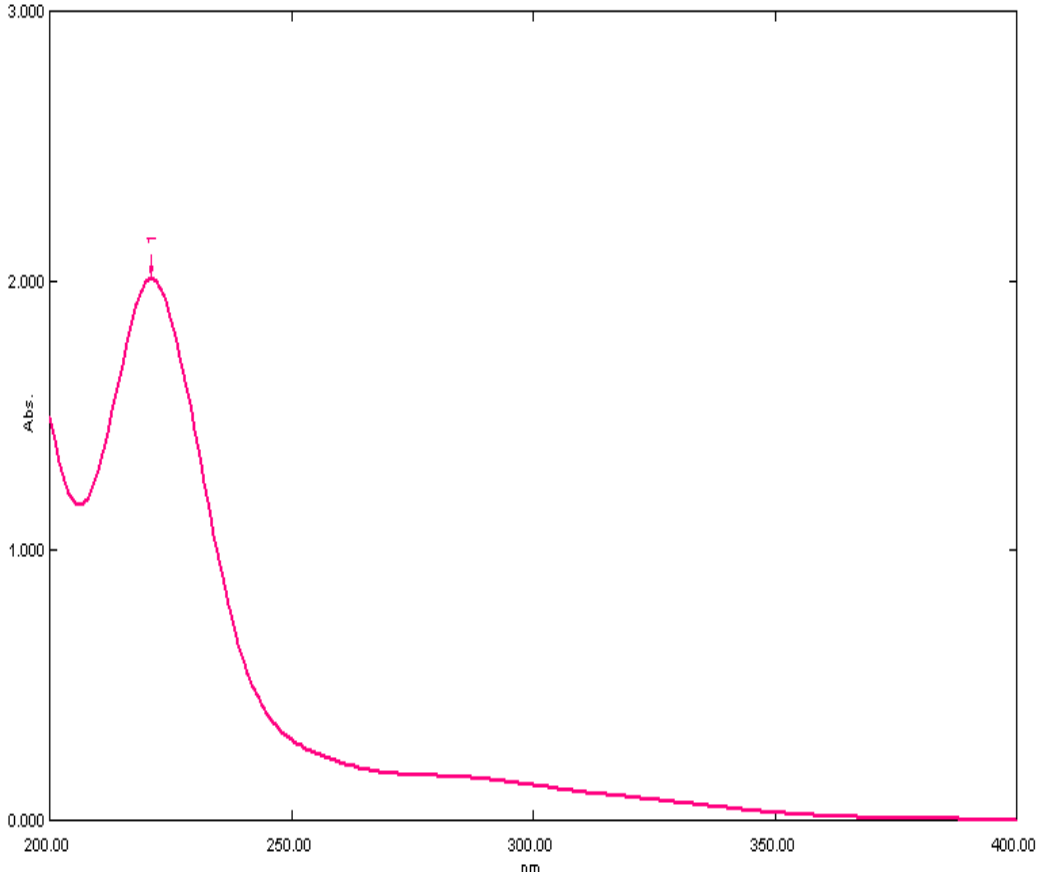
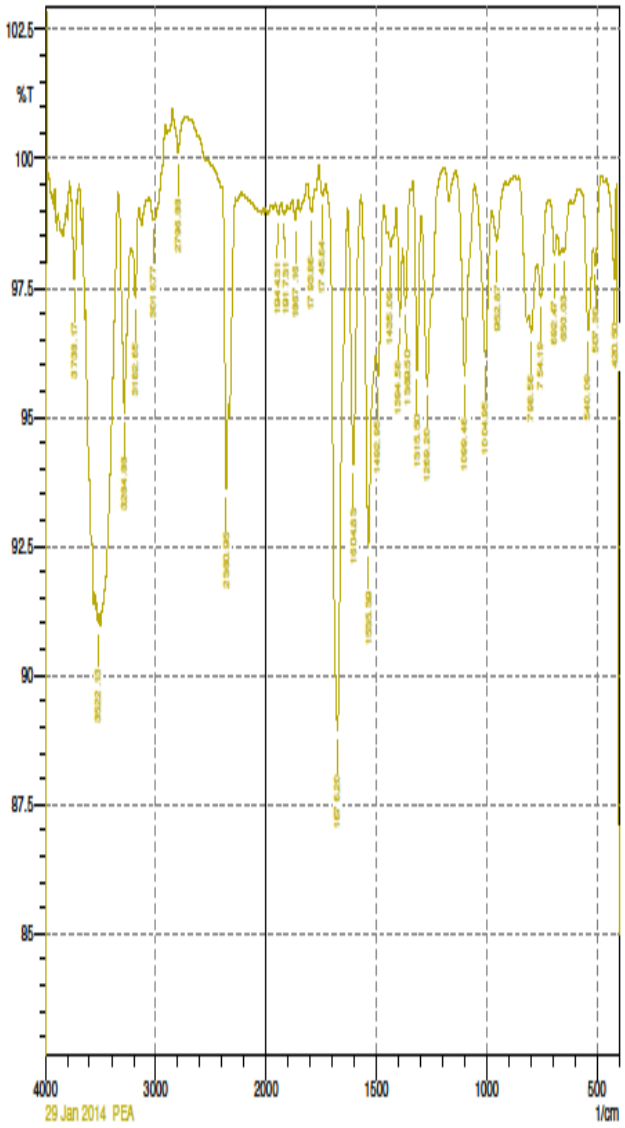


Fig. 5.X UV spectrum of white crystals

2. IR Spectrum of (Ea 2): IR spectrum is given in figure 5.XI.



	Peak	Intensity	Corr. Int	Base (H)	Base (L)	Area	Corr. Are
1	420.5	97.685	1.855	459.07	412.78	0.239	0.153
2	507.3	97.946	0.651	516.94	478.36	0.211	0.036
3	540.09	96.672	1.977	582.52	516.94	0.516	0.178
4	650.03	98.21	0.245	657.75	619.17	0.226	0.012
5	692.47	98.137	0.603	719.47	680.89	0.224	0.034
6	754.19	97.312	1.072	771.55	719.47	0.409	0.088
7	798.56	96.661	0.609	810.13	771.55	0.477	0.047
8	952.87	98.438	0.763	977.94	914.29	0.291	0.085
9	1004.95	96.17	2.994	1055.1	977.94	0.682	0.426
10	1099.46	95.83	3.806	1141.9	1055.1	0.716	0.577
11	1269.2	95.626	3.521	1298.14	1190.12	0.865	0.566
12	1315.5	95.924	3.302	1332.86	1298.14	0.359	0.242
13	1369.5	97.163	1.393	1381.08	1332.86	0.314	0.082
14	1394.58	96.938	1.67	1410.01	1381.08	0.272	0.099
15	1435.09	98.293	0.434	1448.59	1410.01	0.25	0.047
16	1492.95	95.788	1.149	1502.6	1465.95	0.463	0.078
17	1535.39	92.508	5.174	1570.11	1502.6	1.287	0.609
18	1604.83	94.09	5.03	1629.9	1570.11	0.804	0.58
19	1676.2	88.934	10.311	1730.21	1629.9	2.211	1.889
20	1745.64	99.291	0.396	1761.07	1730.21	0.072	0.03
21	1793.86	98.976	0.471	1813.15	1778.43	0.117	0.034
22	1857.16	98.812	0.395	1880.66	1855.58	0.109	0.022
23	1917.31	98.924	0.195	1930.81	1903.8	0.116	0.012
24	1944.31	98.923	0.209	1957.81	1930.81	0.114	0.011
25	2360.95	93.628	3.02	2395.67	2343.59	0.891	0.278
26	2796.88	100.105	0.781	2847.03	2737.08	-0.261	0.157
27	3016.77	98.816	0.862	3061.13	2914.54	0.431	0.401
28	3182.65	97.292	1.312	3211.59	3151.79	0.503	0.141
29	3284.88	95.093	3.709	3338.89	3228.95	1.387	0.806
30	3522.13	91.006	0.263	3535.64	3512.49	0.931	0.014
31	3738.17	97.689	1.615	3782.53	3715.02	0.399	0.212

Fig. 5.XI IR spectrum of the crystals

3. ^{1}H-NMR spectrum (Ea 2): ^{1}H-NMR spectrum of Ea 2 is given in figure 5.XII

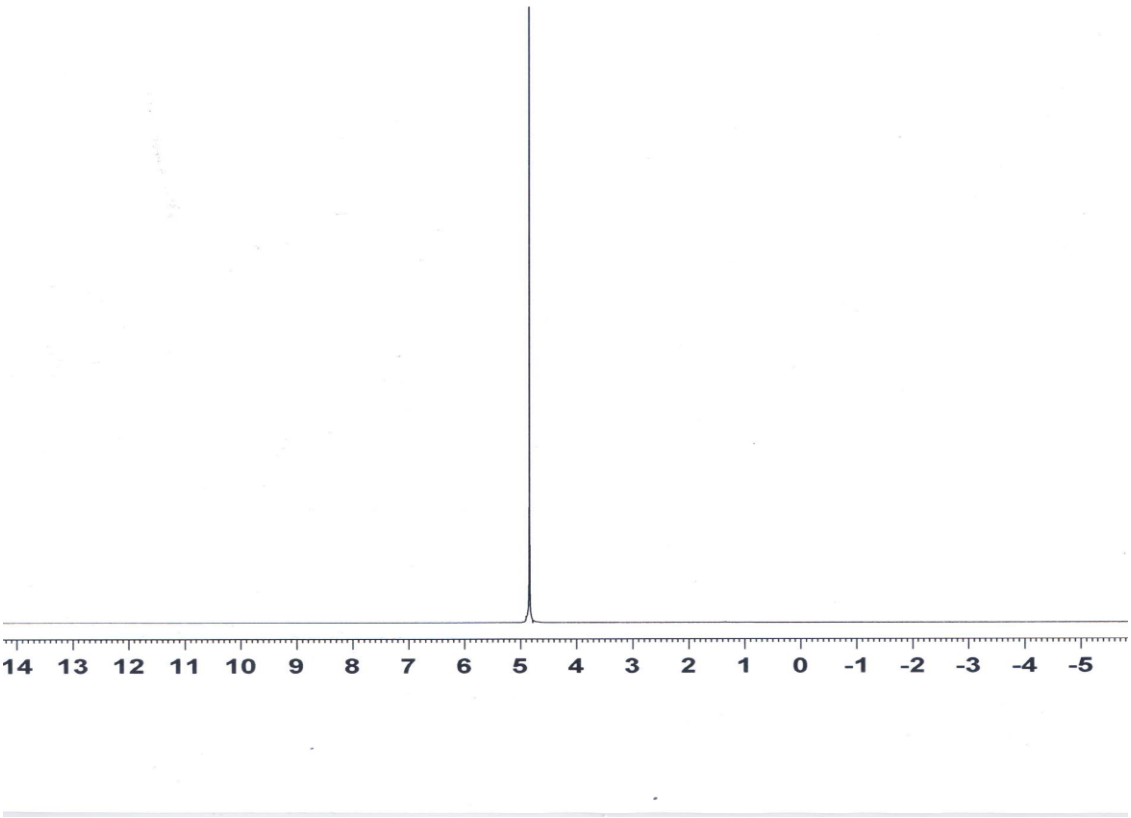


Fig. 5.XII ^{1}H-NMR spectrum of white crystals

The ^{1}H-NMR spectrum in solvent D_2O gives only a single peak.

4. X-RAY analysis of Ea 2 : Reveals the following data

1. Cell length a = 3.1463(5)
2. Cell length b = 3.1463(5)
3. Cell length c = 3.1463(5)
4. Cell angle α = 90 °C
5. Cell angle β = 90 °C
6. Cell angle γ = 90 °C
7. Cell Volume = 31.100(12)
8. Empirical Formula = $C_{28}H_{28}N_5O_1Na_1Cl_1$
9. Radiation, Wavelength = Mo Kα, 0.7107 A°
10. Crystal System = Cubic

5.4: Antimicrobial activity of various hot extracts of *Eclipta alba* against the bacterias and fungi

Bacterial strains: The biological activities of different hot extracts were tested against bacteria *E. coli* (NCIM No.-2563), *Salmonella typhimurium* (NCIM No.-2501) and fungus *Aspergillus fumigatus* (NCIM No.- 902) and *Penicillium chrysogenum* (NCIM No.- 738). These microbial cultures were ordered from NCIM Pune and maintained at 4 ºC on nutrient broth in Biotechnology labs of LPU. Potato dextrose broth was used for fungal culture.

Chemicals: For bacterial cultivation, nutrient agar and nutrient broth were used. Potato dextrose agar and potato dextrose broth were used for fungal cultivation. Antibiotic gentamicin is used as control and bought from HI-Media Laboratories, Mumbai.

Preparation of Nutrient agar medium: Dissolve 28 grams of nutrient agar in 1000 ml distilled water. Gently heat to dissolve the components completely. This nutrient agar was then disinfected in an autoclave at 15 psi (121 ºC) for 15 minutes, cooled at room temperature. After cooling it was poured in the sterilized petriplates and placed in laminar till solidification.

Antimicrobial activity of plant extracts: One gm of the various plant extracts was dissolved in 1ml of respective solvent to make the final concentration of 1gm/ml.

Disc diffusion method: Disc diffusion method was used to check *in vitro* antimicrobial activity of various extracts of *Eclipta alba.* Bacterial culture in was allowed to inoculate on the petriplates containing nutrient agar. Discs were prepared from Whatmann No.-1 filter paper and poured in 5 µl of various extracts. Then two or three discs were kept on petriplates containing inoculated bacterias and kept at 37ºC for 24 hours. Experiments were triplicated and average of diameter of zone of inhibition was measured.[25]

A. Antimicrobial activity of various extracts against various microbes

The average diameter of zone of inhibition against various microbes is given in the table. *In vitro* antimicrobial activity of pet. ether, toluene, chloroform, ethyl acetate and methanol extracts are given in tables 5.I, 5.II, 5.III, 5.IV and 5.V and figures 5.XIII, 5.XIV and 5.XV.

Table 5.I: *In vitro* antimicrobial activity of petroleum ether extracts

S. No.	**Microbes**		**Diameter in (mm)**
1	Gram (-)	Bacteria *Salmonella typhimurium*	(-)
2	Gram (-)	Bacteria *Escherichia coli*	(-)
3		*Penicillium chrysogenum*	(-)
4		*Aspergillus fumigatus*	(-)

Table 5.II: *In vitro* antimicrobial activity of toluene extracts

S. No.	**Microbes**		**Diameter in (mm)**
1	Gram (-)	Bacteria *Salmonella typhimurium*	(-)
2	Gram (-)	Bacteria *Escherichia coli*	(-)
3		*Penicillium chrysogenum*	(-)
4		*Aspergillus fumigatus*	(-)

Table 5.III: *In vitro* antimicrobial activity of chloroform extracts

S. No.	**Microbes**		**Diameter in (mm)**
1	Gram (-)	Bacteria *Salmonella typhimurium*	(-)
2	Gram (-)	Bacteria *Escherichia coli*	(-)
3		*Penicillium chrysogenum*	(-)
4		*Aspergillus fumigatus*	(-)

Table 5.IV: *In vitro* antimicrobial activity of ethyl acetate extracts

S. No.	Microbes		Diameter in (mm)
1	Gram (-)	Bacteria *Salmonella typhimurium*	(-)
2	Gram (-)	Bacteria *Escherichia coli*	(22 mm)
3		*Penicillium chrysogenum*	(-)
4		*Aspergillus fumigatus*	(-)

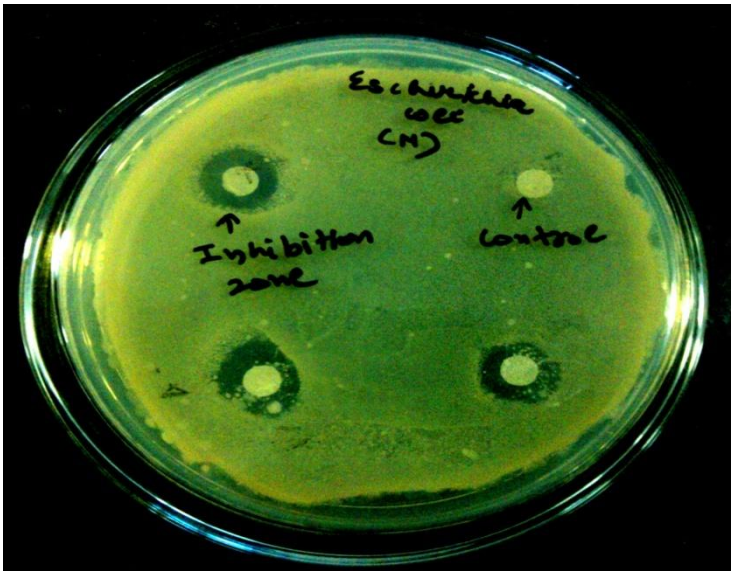


Fig. 5.XIII Antimicrobial activity of ethyl acetate extracts against *E coli*

Table 5.V: *In vitro* antimicrobial activity of methanol extracts

S. No.	Microbes		Diameter in (mm)
1	Gram (-)	Bacteria *Salmonella typhimurium*	(24 mm)
2	Gram (-)	Bacteria *Escherichia coli*	(20 mm)
3		*Penicillium chrysogenum*	(-)
4		*Aspergillus fumigatus*	(-)

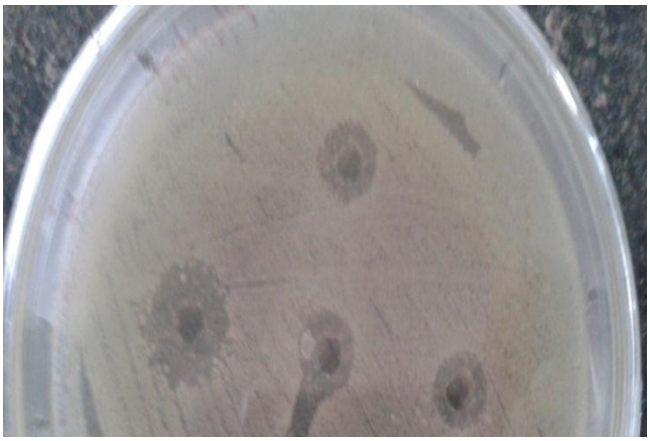


Fig. 5.XIV Antimicrobial activity of methanol extracts against *E. coli*

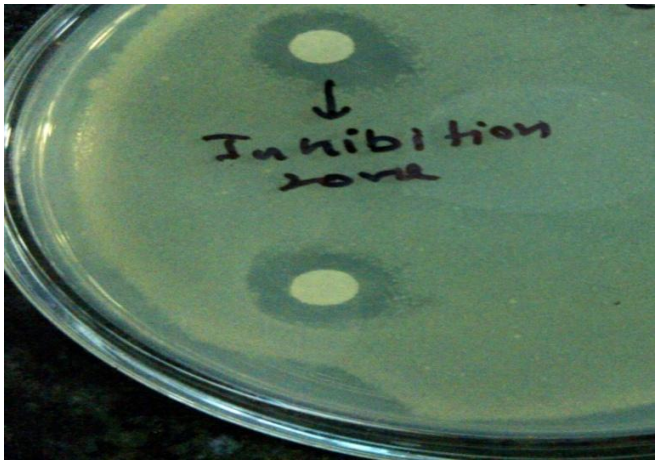


Fig. 5.XV Antimicrobial activity of methanol extracts against *Salmonella typhimurium*

5.5: Insecticidal activity of hot petroleum ether and methanol extracts of *Eclipta alba*

The insecticidal activity of pet. ether and methanol extracts of *Eclipta alba* are investigated against agricultural epilachna insect *Coccinella septumpunctata* and rice weevil *Sitophilus oryzae* in a laboratory. Insecticidal activity was found out at a temperature of 25±4 °C and 70 ± 5 % relative humidity in dark conditions. The mortality rate of both the insects was checked against 1 %, 5 % and 10 % concentration of petroleum ether and ethanol extracts. Finding insecticidal activity is of great importance as using plant extracts as insecticides are biodegrable and do not produce toxicity hence are ecofriendly.

Insecticidal Testing: The petroleum ether and methanol extracts were prepared at three different concentrations (1 % v/v, 5 % v/v, 10 % v/v).

(i). Insect Assayed: Test insects: The epilachna insect *Coccinella septumpunctata* (Fig. 5.XVI) were collected from the fields and rice weevil *Sitophilus oryzae* (Fig. 5.XVII) were collected from the rice mill and identified by Entomologist Dr. Sudhakar Gupta of Lovely Professional University.

(ii). Determination of Insecticidal activity by Contact toxicity assay: The Wheat leaves (for epilachna insect) and rice grains (for rice weevil) are taken from the field and house, washed and dried and then insects were allowed to feed on them under controlled conditions of temperature and humidity. These washed and dried wheat leaves and rice grains are then dipped in the respective percentage of plant extracts (i.e. 1%, 5%, 10% v/v) for two hours and then insects are allowed to feed on these leaves in each jar and mortality rate of the insects was checked. Results were recorded by counting the number of survivals in each jar. Control dishes with petroleum ether, methanol, distilled water and without solvent were performed separately up to 72 hours. Mortality was assessed after 3, 4, 7 and 12 h of the treatment. The calculation of mortality rate was corrected for control mortality according to Abbott's formula[45]:

$$Mc = (Mo-Mc/100-Me) * 100$$

Where, Mo = Observed mortality rate of treated adults (%), Me = mortality rate of control (%), and Mc = corrected mortality rate (%).

Results were tabulated in tables 5.VI and 5.VII.

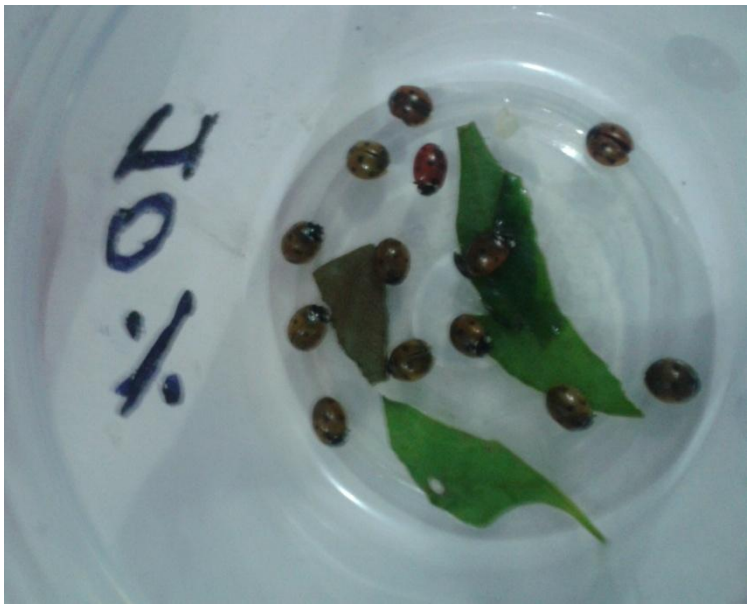


Fig. 5.XVI Insecticidal activity against *Coccinella septumpunctata*

Fig. 5.XVII Insecticidal activity against *Sitophilus oryzae*

Table 5.V1: Insecticidal activity of petroleum ether extracts against *Coccinella septempunctata* and *Sitophilus oryzae*

S.No.	Plant Name	Insect	% of Extract	No. of Insects used	No. of Insects died (hr)					Total No. of insects Died	% Average Mortality	% Corrected Mortality
					3	4	7	12	72			
1	*Eclipta alba*	*Coccinella septempunctata*	1 %	10	10	-	-	-	-	10	100	100
2	*Eclipta alba*	*Coccinella septempunctata*	5 %	10	6	-	4	-	-	10	100	100
3	*Eclipta alba*	*Coccinella septempunctata*	10 %	10	10	-	-	-	-	10	100	100
4	*Eclipta alba*	*Sitophilus oryzae*	1 %	10	10	-	-	-	-	10	100	100
5	*Eclipta alba*	*Sitophilus oryzae*	5 %	10	10	-	-	-	-	10	100	100
6	*Eclipta alba*	*Sitophilus oryzae*	10 %	10	10	-	-	-	-	10	100	100

Table 5.VII: Insecticidal activity of methanol extracts against *Coccinella septempunctata* and *Sitophilus oryzae*

S.No.	Plant Name	Insect	% of Extract	No. of Insects used	No. of Insects died					Total No. of insects Died	% Average Mortality	% Corrected Mortality
					3	4	7	12	72			
1	*Eclipta alba*	*Coccinella septempunctata*	1 %	10	-	1	-	2	-	3	30	30
2	*Eclipta alba*	*Coccinella septempunctata*	5 %	10	8	2	-	-	-	10	100	100
3	*Eclipta alba*	*Coccinella septempunctata*	10 %	10	8	2	-	-	-	10	100	100
4	*Eclipta alba*	*Sitophilus oryzae*	1 %	10	8	2	-	-	-	10	100	100
5	*Eclipta alba*	*Sitophilus oryzae*	5 %	10	10	-	-	-	-	10	100	100
6	*Eclipta alba*	*Sitophilus oryzae*	10 %	10	8	2	-	-	-	10	100	100

Results and Discussion: Petroleum ether extract of the plant at all concentrations is showing 100 % mortality against the agricultural pest *Coccinella septumpunctata* and stored grain insect *Sitophilus oryzae* where as methanol extract (1 %) is less active towards *Coccinella septumpunctata* but 5 % and 10 % of methanol extracts are showing 100 % mortality against *Coccinella septumpunctata* and *Sitophilus oryzae.*

Conclusion: The *Eclipta alba* extract shows strong toxic effects against the agricultural pest *Coccinella septempunctata* but 1% methanol extract is less effective against *Coccinella septumpunctata.*

5.6: Antidiabetic activity of hot petroleum ether and methanol extracts of *Eclipta alba:*

Antidiabetic activity of petroleum ether and methanol extracts of *Eclipta alba* is determined in CSIR lab, IIIM Jammu on wistar rats by 18h Fasted rats model.

Experimental

^{1}H-NMR models: **18h Fasted rats model**

Four groups of six Wistar rats (male/female) were selected randomly and fasted overnight. First group was given Normal Vehicle Control, second and third groups were given petroleum ether and methanol extracts treated diet and fourth group was given glibenclamide treated diet. Blood glucose level of all the rats was found out prior to any treatment (0 hour) and post-drug administration (3 hour).[46] Results are given in table 5.VIII.

Table 5.VIII: Diabetic activity of petroleum ether and methanol extracts of *Eclipta alba*

S.No.	Treatment group (mg/kg p.o.)	Blood glucose level (mg/dl) Mean±S.E.	
		0h	3h
1	Normal Control	83±4.65	79±4.09
2	Glibenclamide (0.5)	78±4.02	49±2.56***
3	Pet. ether extracts of *Eclipta alba* (250)	98.00±4.88	98.00±7.46

n (Number of animals) : 5

*** $p < 0.001$ compared to 0h blood glucose level.

Results and Discussion: Thus petroleum ether extracts of *Eclipta alba* is not showing increase or decrease in blood glucose level thus neither hypoglycemic nor hyperglycemic activity.

Comparative analysis of the different extracts of *Piper nigrum, Psoralea corylifolia, Solanum nigrum, Eclipta alba* and various phytochemicals

After the investigation of various extracts of above said plants, the isolated phytochemicals, antimicrobial, insecticidal and diabetic activity (Hyperglycemic or Hypoglycemic) were compared.

6.1: Comparative analysis of presence of secondary metabolites: The various extracts of all the four plants were also investigated for the presence of general categories of secondary metabolites such as alkaloids, flavonoids, terpenoids, steroids, tannins, carbohydrates and phenolic compounds etc. and results were mentioned in tables 6.I, 6.II, 6.III and 6.IV.

Table 6.I: The presence of various secondary metabolites in different extracts of *Piper nigrum* is tabulated

Secondary metabolites	Pet. ether extract	DCM extract	Chloroform extract	Ethyl acetate extract	Methanol extract
Alkaloids	(+)	(+)	(+)	(+)	(+)
Carbohydrates	(+)	(+)	(-)	(-)	(+)
Flavonoids	(-)	(-)	(-)	(-)	(-)
Terpenoids	(+)	(+)	(+)	(+)	(+)
Steroids	(-)	(-)	(-)	(-)	(-)
Tannins and Phenolic compounds	(+)	(+)	(+)	(-)	(-)
Glycosides	(-)	(-)	(-)	(-)	(-)
Fats/oil	(+)	(+)	(-)	(-)	(+)

Table 6.II: The presence of various secondary metabolites in different extracts of *Psoralea corylifolia* is mentioned below:

Secondary metabolites	Pet. ether extract	DCM extract	Ethyl acetate extract	Methanol extract
Alkaloids	(-)	(+)	(+)	(+)
Carbohydrates	(-)	(-)	(+)	(+)
Flavonoids	(+)	(+)	(+)	(+)
Terpenoids	(+)	(-)	(-)	(+)
Steroids	(-)	(+)	(+)	(+)
Tannins and Phenolic compounds	(-)	(-)	(-)	(-)
Glycosides	(-)	(-)	(-)	(-)
Fats/oil	(-)	(+)	(-)	(-)

Table 6.III: The presence of various secondary metabolites in different extracts of *Solanum nigrum* is tabulated:

Secondary metabolites	Pet. ether extract	Toluene extract	DCM extract	Chloroform extract	Methanol extract
Alkaloids	(+)	(+)	(+)	(+)	(-)
Carbohydrates	(-)	(-)	(-)	(-)	(+)
Flavonoids	(+)	(+)	(+)	(-)	(+)
Terpenoids	(+)	(+)	(+)	(+)	(+)
Steroids	(+)	(+)	(-)	(+)	(+)
Tannins and Phenolic compounds	(-)	(-)	(-)	(-)	(+)
Glycosides	(-)	(-)	(-)	(-)	(-)
Fats/oil	(+)	(+)	(+)	(+)	(+)

Table 6.IV: The presence of various secondary metabolites in different extracts of *Eclipta alba* is tabulated:

Secondary metabolites	Pet. ether extract	Toluene extract	Chloroform extract	Ethyl acetate extract	Methanol extract
Alkaloids	(+)	(+)	(+)	(+)	(+)
Carbohydrates	(-)	(-)	(-)	(-)	(+)
Flavonoids	(+)	(+)	(+)	(-)	(+)
Terpenoids	(-)	(-)	(+)	(+)	(-)
Steroids	(+)	(+)	(-)	(-)	(-)
Tannins and Phenolic compounds	(+)	(+)	(+)	(+)	(-)
Glycosides	(-)	(-)	(+)	(-)	(-)
Fats/oil	(+)	(+)	(+)	(-)	(+)

6.2: Comparative antimicrobial activity of *Piper nigrum, Psoralea corylifolia, Solanum nigrum and Eclipta alba*

Antimicrobial activity of various extracts of all the four plants was investigated against various bacterias and fungi by disc diffusion method. A comparative analysis of antimicrobial activity of various plants against various microbes is given in the form of table 6.V below.

Table 6.V Comparative analysis of antimicrobial activity of various plant's extracts

S. No.	**Microbes**	**Plant's Name, Extract and Diameter of Zone of Inhibition (mm)**																			
		Piper nigrum					**Psoralea corylifolia**				**Solanum nigrum**					**Eclipta alba**				
		P	T	D	C	E	P	D	E	M	P	T	D	C	M	P	T	C	E	M
1.	*Bacillus subtilis*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	N	N	N
2.	*Escherichia coli*	19	-	-	12	3	12	-	14	24	-	-	-	-	-	-	-	-	22	18
3.	*Pseudomonas aeruginosa*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	N	N	N	N
4.	*Aspergillus niger*	-	-	16	12	-	N	N	N	N	-	-	-	-	-	N	N	N	N	N
5.	*Trichoderma ressi*	N	N	N	N	N	-	-	-	-	N	N	N	N	N	N	N	N	N	N
6.	*Salmonella typhimurium*	N	N	N	N	N	N	N	N	N	N	N	N	N	N	-	-	-	-	14
7.	*Penicillium chrysogenum*	N	N	N	N	N	N	N	N	N	N	N	N	N	N	-	-	-	-	-
8.	*Aspergillus fumigatus*	N	N	N	N	N	N	N	N	N	N	N	N	N	N	-	-	-	-	-

Where P = Petroleum ether extracts, T = Toluene extracts, D = Dichloromethane extracts, C = Chloroform extracts, E = Ethyl acetate extracts, M = Methanol extracts

N stands for "Not found" and – for negative appearance of Zone of inhibition.

6.3: Comparative chart of insecticidal activity of *Piper nigrum, Psoralea corylifolia, Solanum nigrum and Eclipta alba*:

Insecticidal activity of these plants was checked with least polar petroleum ether and highly polar methanol extracts against stored grain insect *Sitophilus oryzae* and agricultural insect *Coccinella septumpunctata.* A comparative chart of insecticidal activity of these plants is tabulated in table 6.VI.

Table 6.VI : Comparative insecticidal activity of various plant extracts

S.No.	Extract of plant	Insect	% of Extract (v/v)	No. of Insects used	No. of insects Dead (hours)				Total No. of insects dead	% of average mortality	% Corrected Mortality
					3h	4h	7h	12h			
1.	*P. nigrum* (Pet. ether extracts)	*Coccinella septumpunctata*	1 %	10	10	-	-	-	10	100	100
			5 %	10	10	-	-	-	10	100	100
			10 %	10	10	-	-	-	10	100	100
2.	*P. nigrum* (Pet. ether extracts)	*Sitophilus oryzae*	1 %	10	5	3	2	-	10	100	100
			5 %	10	10	-	-	-	10	100	100
			10 %	10	10	-	-	-	10	100	100
3.	*P. nigrum* (Methanol extracts)	*Coccinella septumpunctata*	1 %	10	1	-	-	-	1	10	10
			5 %	10	-	-	-	-	-	-	-
			10 %	10	2	-	-	-	2	20	20
4.	*P. nigrum* (Methanol extracts)	*Sitophilus oryzae*	1 %	10	-	-	-	-	-	-	-
			5 %	10	-	-	-	-	-	-	-
			10 %	10	1	1	1	1	1	10	10
5.	*P. corylifolia* (Pet. ether extracts)	*Coccinella septumpunctata*	1 %	10	10	-	-	-	10	100	100
			5 %	10	10	-	-	-	10	10	100
			10 %	10	10	-	-	-	10	10	100

6.	*P. corylifolia* (Petroleum ether extracts)	*Sitophilus oryzae*	1 %	10	7	-	3	-	10	100	100
			5 %	10	10	-	-	-	10	100	100
			10 %	10	9	1	-	-	10	100	100
7.	*P. corylifolia* (Methanol extracts)	*Coccinella septumpunctata*	1 %	10	1	-	-	-	1	10	10
			5 %	10	10	-	-	-	-	100	100
			10 %	10	10	-	-	-	10	100	100
8.	*P. corylifolia* (Methanol extracts)	*Sitophilus oryzae*	1 %	10	10	-	-	-	10	100	100
			5 %	10	10	-	-	-	10	100	100
			10 %	10	10	-	-	-	10	100	100
9.	*S. nigrum* (Pet. ether extracts)	*Coccinella septumpunctata*	1 %	10	10	-	-	-	10	100	100
			5 %	10	10	-	-	-	10	100	100
			10 %	10	10	-	-	-	10	100	100
10.	*S. nigrum* (Pet. ether extracts)	*Sitophilus oryzae*	1 %	10	10	-	-	-	10	100	100
			5 %	10	9	-	1	-	10	100	100
			10 %	10	10	-	-		10	100	100
11.	*S. nigrum* (Methanol extracts)	*Coccinella septumpunctata*	1 %	10	3	-	2		5	50	50
			5 %	10	10	-	-		10	100	100
			10 %	10	10	-	-		10	100	100
12.	*S. nigrum* (Methanol extracts)	*Sitophilus oryzae*	1 %	10	10	-	-		-	100	100
			5 %	10	10	-	-		10	100	100
			10 %	10	10	-	-		10	100	100
13.	*E. alba* (Pet. ether extracts)	*Coccinella septumpunctata*	1 %	10	10	-	-	-	10	100	100
			5 %	10	6	4	-	-	10	100	100
			10 %	10	10	-	-	-	10	100	100

14.	*E. alba* (Pet. ether extract)	*Sitophilus oryzae*	1 %	10	10	-	-	-	10	100	100
			5 %	10	10	-	-	-	10	100	100
			10 %	10	10	-	-	-	10	100	100
15.	*Eclipta alba* (Methanol extracts)	*Coccinella septumpunctata*	1 %	10	1	-	-	2	3	30	30
			5 %	10	10	-	-	-	10	100	100
			10 %	10	10	-	-	-	10	100	100
16.	*Eclipta alba* (Methanol extracts)	*Sitophilus oryzae*	1 %	10	10	-	-	-	10	100	100
			5 %	10	10	-	-	-	10	100	100
			10 %	10	10	-	-	-	10	100	100

6.4. Comparative analysis of diabetic activity of *Piper nigrum, Psoralea corylifolia, Solanum nigrum* and *Eclipta alba*

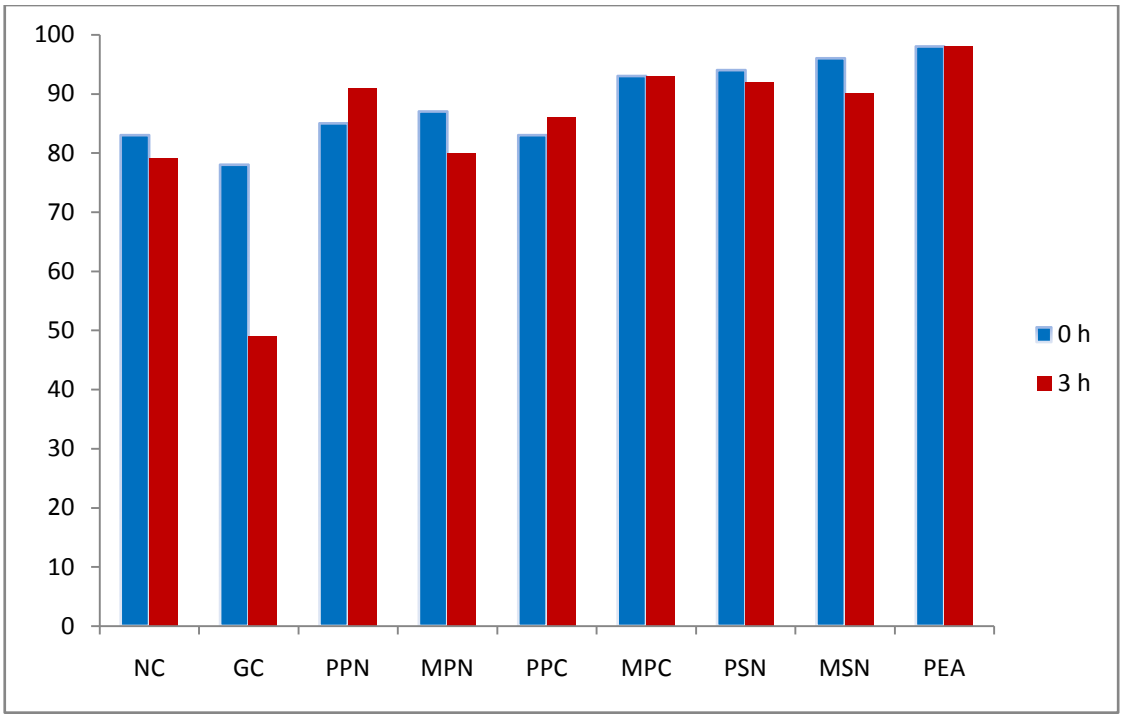
Petroleum ether and methanol extracts of *Piper nigrum, Psoralea corylifolia, Solanum nigrum* and *Eclipta alba* were investigated for hypoglycemic and hyperglycemic effects in normal rats using 18 hr fasted rat model. Petroleum ether extracts of *Piper nigrum* is showing hyperglycemic activity while its methanol extracts is showing hypoglycemic activity. Pet. ether extracts of *Psoralea corylifolia* is showing minor hyperglycemic activity while its methanol extract is not showing much effect on serum glucose level. Pet. ether and methanol extracts of *Solanum nigrum* are showing hypoglycemic activity. Pet. ether extract of *Eclipta alba* is not showing effect on glucose level of Wistar rats. The results of hyperglycemic or hypoglycemic activity of various plant extracts are given in the table 6.VII and graph 6.I.

Table 6.VII Comparative diabetic activity of various plant extracts

S.No.	Treatment group (mg/kg p.o.)	Blood glucose level (mg/dl) Mean±S.E.	
		0h	3h
1	Normal Control	83±4.65	79±4.09
2	Glibenclamide (0.5)	78±4.02	49±2.56***
3	Pet ether extract of pepper (250)	85.6±3.32	91.8±1.98
4	Methanol extract of pepper (250)	87.4±5.39	80.2±4.31
5	Pet. ether extract of *Psoralea corylifolia* (250)	83±3.82	86±6.09
6	Methanol extract of *Psoralea corylifolia* (250)	93.6±2.51	93.2±2.53
7	Pet. ether extract of *Solanum nigru*m(250)	94.2±3.19	92.0±4.46
8	Methanol extract of *Solanum nigrum* (250)	96±7.22	90±5.43
9	Pet. ether extract of *Eclipta alba* (250)	98.00±4.88	98.00±7.46

n = Number of animals: 6

*** $p < 0.001$ compared to 0h blood glucose level



Where NC = Normal control, GC = Glibenclamide, PPN = Petroleum ether extract of *Piper nigrum*, MPN = Methanol extracts of *Piper nigrum*, PPC = Petroleum ether extract of *Psoralea corylifolia*, MPC = Methanol extract of *Psoralea corylifolia*, PSN = Petroleum ether extract of *Solanum nigrum*, MSN = Methanol extract of *Solanum nigrum*, PEA = Petroleum ether extract of *Eclipta alba.*

Graph 6.I Graphical representation of hyperglycemic and hypoglycemic activity of various extracts

6.5: Comparative analysis of isolated crystals from *Piper nigrum, Psoralea corylifolia, Solanum nigrum* and *Eclipta alba* : The various crystals isolated from various plants (Fig. 6.I) were further investigated for antimicrobial, insecticidal and diabetic activity.

Pn 2 from *Piper nigrum*

Pc 1 from *Psoralea corylifolia*

Sn 2 from *Solanum nigrum*

Ea 2 from *Eclipta alba*

Fig. 6.I Crystals from various plants

6.5.1 Antimicrobial activity of Pn 2, Pc 1, Sn 2 and Ea 2 crystals : 0.01 gm of the crystals of various plants were dissolved in 10 ml of solvent in which they are soluble (Pn 2 in EtOH, Pc 1 in EtOH, Sn 2 and Ea 2 in water) to make 10 ml of 1000 ppm of the solution. Took 5 ml of it and added 5 ml of respective solvent again to make 500 ppm of solution. Took 5 ml of 500 ppm solution and added 5 ml of solvent again to make 250 ppm of the solution. These crystals were checked against various microbes and comparative results are given below in the form of table 6.VIII.

Table 6.VIII Comparative analysis of antimicrobial activity of various crystals

S. No.	Crystals	Microbes	Control	250 ppm	500 ppm	1000 ppm
1	***Pn 2***	*Aspergillus fumigatus*	(-)	(-)	(-)	(-)
		Penicillium chrysogenum	(-)	15 mm	20 mm	25 mm
		E.coli	(-)	(-)	(-)	(-)
		Salmonella typhimurium	(-)	30 mm	33 mm	36 mm
2.	***Pc 1***	*Aspergillus fumigatus*	(-)	(-)	(-)	(-)
		Penicillium chrysogenum	(-)	(-)	(-)	(-)
		E.coli	(-)	(-)	(-)	(-)
		Salmonella typhimurium	(-)	25 mm	30 mm	42 mm
3.	***Sn 2***	*Aspergillus fumigatus*	(-)	(-)	(-)	(-)
		Penicillium chrysogenum	(-)	(-)	(-)	(-)
		E.coli	(-)	(-)	(-)	(-)
		Salmonella typhimurium	(-)	(-)	(-)	(-)
4.	***Ea 2***	*Aspergillus fumigatus*	(-)	(-)	(-)	(-)
		Penicillium chrysogenum	(-)	(-)	(-)	(-)
		E.coli	(-)	(-)	(-)	(-)
		Salmonella typhimurium	(-)	(-)	(-)	(-)

6.5.2 Comparative analysis of insecticidal activity of Pn 2 and Pc 1: 1 mg of both Pn 2 and Pc 1 were dissolved in 1ml of EtOH to make 1mg/ml of standard solution. 1 %, 5 % and 10 % of the above solutions were taken and tested against *Coccinella septumpunctata* and *Sitophilus oryzae*. The results are given below in the form of table 6.IX.

Table 6.IX Comparative insecticidal activity of crystals

S.No.	Crystals	Insect	% of Extract (v/v)	No. of Insects used	No. of insects Dead (hours)				Total No. of insects dead	% of average mortality	% Corrected Mortality
					3h	4h	7h	12h			
1.	***Pn 2***	*Coccinella septumpunctata*	1 %	10	5	1	-	-	6	60	60
			5 %	10	6	4	-	-	10	100	100
			10 %	10	10	-	-	-	10	100	100
2.	***Pn 2***	*Sitophilus oryzae*	1 %	10	5	3	2	-	10	100	100
			5 %	10	10	-	-	-	10	100	100
			10 %	10	10	-	-	-	10	100	100
3.	***Pc 1***	*Coccinella septumpunctata*	1 %	10	6	-	-	-	6	60	60
			5 %	10	-	-	-	-	-	NM	NM
			10 %	10	7	-	1	-	8	80	80
4.	***Pc 1***	*Sitophilus oryzae*	1 %	10	10	-	-	-	10	100	100
			5 %	10	10	-	-	-	10	100	100
			10 %	10	10	-	-	-	10	100	100

Where NM = no mortality.

Both the crystals are showing 100 % mortality against *Sitophilus oryzae* while 1 % solution of piperine crystals are less active against *Coccinella septumpunctata.*

6.5.3 Comparative analysis of diabetic activity of Pn 2, Pc 1 and Sn 2:

Study 1 : Hypoglycemic effect

Experimental model : Normal rats

Experimental animals: Adult male/female Wistar rats (8 weeks), weighing 180–200 g bred in the Animal House, Regional Research Laboratory (CSIR), Jammu, were used. All animal experiments were approved by the Institutional Animal Ethic Committee (IAEC), Regional Research Laboratory (CSIR), Jammu. The animals were housed in polycarbonate cages in a room with a 12h day-night cycle, temperature of 22 ± 2°C, humidity of 45–64%. During the whole experimental period, animals were fed with a balanced commercial pellet diet (Ashirwad Industries, Chandigarh, India) and water ad libitum.

18h Fasted rats model : Wistar rats (male/female), 6 animals in each group were fasted overnight. The animals were divided into Normal Vehicle Control, test (test extract/fraction) treated and reference (glibenclamide) treated group. Blood glucose determination was done at 0h (prior to any treatment), 3h (Post-drug administration)**.**

Hypoglycemic effect of Pn 2, Pc 1 and Sn 2 in 18h fasted rats : The hypoglycemic effect of the AD-1, AD-2 and AD-3 was studied in normal 18h fasted rats in which the blood glucose level was monitored after the test drug administration. In the 18h fasted normal rats, as compared to the normal untreated control with glibenclamide as standard, Pn 2 and Sn 2 showed significant hypoglycemic effect. Pn 2 and Sn 2 showed fall of 32 and 46% respectively whereas glibenclamide exhibited 43% in blood sugar at 3h post drug administration (Table 6.X below).

Table 6.X Comparative % fall in blood sugar level by various crystals

S.No.	Treatment group (mg/kg p.o.)	Blood glucose level(mg/dl) Mean±S.E.		% Fall in Blood Sugar
		0h	3h	
1	Pn 2	96± 3.91	65±3.36	32
2	Pc 1	86±4.04	83±3.98	3.4
3	Sn 2	100±5.06	54±5.36	46
4	Glibenclamide (1)	104±3.46	59±5.72	43
5	Control	100±3.65	90±4.48	10

Additional Study 2: Antiinflammatory activity

Effect on production of TNF-alpha: Isolation of hPBMC: Blood was obtained by venipuncture from healthy volunteers. PBMCs were separated on Ficoll-Paque (Sigma) density gradients, washed twice with calcium- and magnesium-free PBS, and resuspended in RPMI 1640 with l-glutamine containing 10% fetal bovine serum and 25 mM HEPES at a density of 10^6 cells/ml. The cells were exposed to 100 ng of LPS per ml.

ELISA for cytokine release

Cells were seeded into 24-well plates at a density of 10^6 cells/well and were incubated for 6 h in the presence of LPS in presence and absence of test compounds. The cell-free supernatant was collected by centrifugation and stored at −70°C. The concentrations of tumor necrosis factor alpha (TNF-α was assayed with enzyme-linked immunosorbent assay (ELISA) kit according to the protocol of the manufacturer.

The effect of Pn 2, Pc 1 and Sn 2 on the production of proinflammatory cytokines from human peripheral blood mononuclear cells (PBMCs) was evaluated and tabulated in table 6.XI. Pn 2 and Sn 2 inhibited the production of tumor necrosis factor alpha (TNF-α) by PBMCs stimulated with lipopolysaccharide (LPS), without cytotoxic effects.

Table 6.XI: Comparative % Inhibition by various crystals

S.No.	Treatment group (mg/kg p.o.)	TNF-alpha (pg/ml)	% inhibition
1	Pn 2	451.78	44
2	Pc 1	873.58	7
3	Sn 2	736.02	21.6
4	LPS Control	812.16	0.0
5	Dexamethasone Control	597.97	26

Both Pn 2 and Sn 2 shows good anti-inflammatory activity.

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