Studies Directed Towards The Chemical Investigation Of Some Medicinal And Aromatic Plants And Transformation Of Their Major Constituents Into Pharmacodynamic Compounds

DISSERTATION

Submitted in partial fulfilment of the requirements provided for the award of the degree of

Masters of Philosophy

In

Chemistry

By

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Under the supervision of

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Certificate from the supervisor

This is to certify that *Ms Shaista Nazir* has carried out the research work presented in this dissertation entitled ***"Studies directed towards the chemical investigation of some medicinal and aromatic plants and transformation of their major constituents into Pharmacodynamic compounds",*** under my supervision for the award of Pre-Doctoral degree in Chemistry and fulfils all the requirements for the degree from University of Kashmir, Srinagar. This work is original and has not been submitted for any degree or diploma to this or any other University or Institution.

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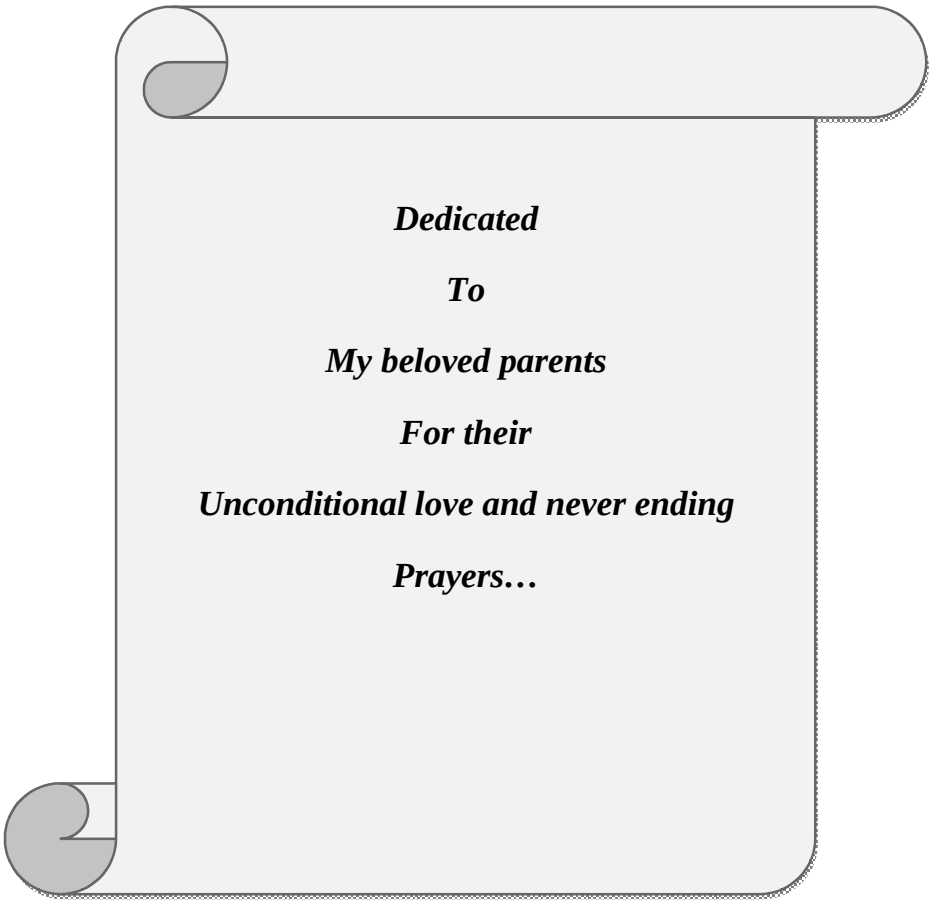
Declaration

I hereby declare that the dissertation entitled "***Studies Directed towards the Chemical Investigation of Some Medicinal and Aromatic Plants and Transformation of Their Major Constituents into Pharmacodynamic Compounds"*** submitted for M. Phil degree to University Of Kashmir has been carried out at University Of Kashmir under the supervision of *Prof. Mushtaq Ahmad Qurshi* (Department Of Chemistry, University Of Kashmir). The work embodied in this dissertation is original and has not been submitted in part or full for any degree or diploma to this or any other University or Institution.

Dated *Shaista Nazir*


Dedicated
To
My beloved parents
For their
Unconditional love and never ending
Prayers…

Acknowledgements

I bow my head before Almighty Allah the most beneficent and most merciful. It is the blessing of Almighty Allah and His Prophet Hazrat Muhammad (PBUH) which enabled me to achieve this goal. I am indebted to owe my gratitude to Him for providing me an opportunity to explore texture of His beauties.

I would like to take this opportunity to convey my cordial gratitude and appreciation to my worthy, reverently and ingenious supervisor Prof. M. A Qurishi (Former Dean Physical and Material sciences, Director IQA and HOD Chemistry) Department of Chemistry, University of Kashmir, for his valuable assistance, encouragement, vigilant guidance, technical suggestions and kind help during the course of my M. Phil programme. I wish to express my sincere thanks and greatest depth of gratitude to Prof. G.M. Peerzada, Head, and K.U. Zaman, former Head Department of Chemistry, University Of Kashmir for material and moral support.

I would like to pay my heartfelt thanks to all the teachers of the Department for their valuable suggestions during this endeavour.

I would like to pay my humble thanks to Dr A. S Shawl, Former Head, Indian Institute Of Integrative Medicine (CSIR) Srinagar, for providing me an opportunity to carry some part of my work in the respective institute.

I wish to express my deep sense of appreciation and gratitude to my senior colleague Mr Oyais Ahmad Chat, Senior Research fellow, Department Of Chemistry and Ms Yasrib Qureshi, formerly (Research Scholar, IIIM Jammu) currently RA, IISC Bangalore for their cooperation, which helped me to take my work to its logical end.

I sincerely thank all my colleague and friends both inside and outside the Department for their camaraderie and constant support. My special thanks to my lab mates Ms Kuratull- Ain, Ms Saima, Ms Soubiya, Mr Yosuf, Mr Mubashir, Mr Showkat and also other colleagues Mr Shakeel-ur-Rehman, Mr Syed Raashid Maqsood, Mr Hafeez Dar, Ms.

Roheena Jan, Ms. Shabnum and Ms Sumyra for their assistance and good company, marvellous behaviour and friendly attitude. I would like to express my heartfelt gratitude respect and deepest appreciation to my parents for their support, guidance, endless love and encouragement during the course of my work. I acknowledge with regards the unshakable desire of my father especially, which inspired me to develop the research attitude. I would also like to acknowledge my sisters Bushra and Rumaisa for their unconditional love, invaluable encouragement and support throughout my research endeavour.

Sense of gratitude would be imperfect without mentioning the name of Dr. Akhtar Malik Department Of Plant Taxonomy, Kashmir University for identification of plants.

Shaista Nazir

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Chapter 1

Introduction

Introduction

1.1 General overview of medicinal plants

Since prehistoric times, alleviation of diseases has been one of the primary concerns of mankind. Local practitioners have used indigenous plants and herbs for centuries all over the world in a variety of ailments showing definite pharmacological activities and even as poisons. Those plants which provide toxic effects were being used in hunting or warfare, while plant products like opium and hashish has long been used as hallucinating agents.

Traditional medicines are developed over the millennia through the acquired experience and accumulated knowledge of man for the beneficial or harmful effects of plant materials against various human sufferings, culminating in self-contained theories. Thus man succeeded in discovering cure of ailments with herbal, animal and mineral medicinal products. Much indigenous medicine began as myth, transferred to new generations as folk medicine and developed with times as the complex modern science.

The history of medicine is an account of man's effort to deal with human illness and disease from primitive attempts of preliterate man to present complex array of specialities in treatments. The Egyptian civilization is supposed to have delineated the use of many medicinal plants for the treatment of human ailments. With the advent of Islam, Arabs acquired, collected, translated and documented the knowledge of use of herbal medicine, from almost all the famous civilizations. It is well recognised that the modern medicinal science has its roots in the Islamic civilization of middle ages and resulted in the flowering of medical technology in Europe during the days of Renaissance.[1] Although the plants have served through the ages as the mainstay for the treatment of various ailments, it is astonishing that from

the plant kingdom which comprises approximately 600,000 plant species, only 2% of these plant species have been subjected to pharmaco-chemical studies. [2]

Plants are used medicinally in different countries and are a source of potent and powerful drugs. A wide range of medicinal plants are used as extracts for raw drugs and they possess varied medicinal properties. Some of them are collected in smaller quantities by local communities and folk healers for local use, many other raw drugs are collected in large quantities and traded in the market as the raw material by many herbal industries.[3] The drugs are derived from the whole plant or from different parts of the plant like leaves, stem, bark, roots, flower, seed, etc. Some drugs are extracted from excretory plant product such as gum, resin, and latex. Not only that plant derived drug offers a stable market worldwide, but plants also continue to be an important source of new drugs. Although encountering drastic challenges from chemical synthesis, combinatorial chemistry, computer aimed molecular modelling and other drug discovery approaches, drug discovery from natural products, especially medicinal plants have continued to provide new drugs and drug leads against various pharmacological targets such as tumours, viruses, fungi etc. For thousands of years various plant families and mostly herbaceous plants have been used as therapeutic agents against wide range of diseases and these were known as herbal medicines. [4]

The use of natural products with therapeutic properties is as ancient as human civilization and for a long time, mineral, plant, and animal products were the main source of drugs.[5] The industrial revolution and development of organic chemistry resulted in a preference for synthetic product for pharmacological treatment. The reason for this was that pure compounds were easily obtained, structural modifications to produce potentially more active and safer drugs could be easily

performed and the economic power of the pharmaceutical companies was increasing. About 25% of the drugs prescribed worldwide come from plants.

1.2 Role of natural product chemistry in drug discovery

For thousands of years, medicine and natural products have been closely related through the use of traditional medicine and natural poisons.[6-7] The discovery of antibacterial filtrate "penicillin" by Fleming in 1928, re-isolation and clinical studies by Chain Florey and co- workers in early 1940's and commercialization of synthetic penicillin revolutionized drug discovery research.[8-10] Following the success of penicillin, drug companies and research groups soon assembled large micro-organism culture collection in order to discover new drugs "antibiotics". The output of early years of antibiotic research was prolific and included examples such as streptomycin,[8] chloramphenicol,[9] chlortetracycline,[10] cephalosporin C, erythromycin and vancomycin.[11] All these compounds and their derivatives are even used today.

1.3 Current scenario of natural products in drug discovery

Despite competition from other drug discovery methods, natural products are still providing their fair share of new clinical candidates and drugs. This was demonstrated recently by New man, Cragg and Snoder, who analysed the number of natural product derived drugs launched from 1981-2002.[12-13] They concluded that natural products are still a significant source of new drugs, especially in anticancer and anti-hypertensive therapeutic areas. Natural product derived drugs are well represented in the top 35 world selling ethical drug sales of 2000, 2001 and 2002. The percentage of natural product derived drugs was 40% in 2000 and remained approx. constant at 24% in 2001 and 2002. Therefore in addition to being a proven and important source of drug lead, natural product derived drugs also contribute to the profitability of many

companies. In 1998, “shu” published a review on natural products in drug development from an industrial perspective listing most compounds that were then in clinical trials. [14] In that review drugs were well represented in the anticancer, anti-infectives, immune suppression and neurological disease and some of these compounds have since progressed further into clinical trials or into market. [15]

Given that natural products (NP’s) have historically provided many drug leads, one would assume that NP’s would still play a pivotal role in drug discovery strategy of big pharma companies. However most big pharma companies have terminated or scaled down their natural product operations in the last 10 years.[16-17] The advent of combinatorial chemistry about 15 years ago created huge excitement in the pharmaceutical industry, and most big pharma companies quickly changed their drug discovery strategy to include a significant proportion of combinatorial chemistry.[18-19] The basic premises was that combinatorial chemistry would generate libraries consisting of millions of compounds, which would be screened by HTS and produce drug leads by sheer weight of numbers. In addition, most synthetic compound libraries have no IP issues that are involved with natural products.[20-21] The lead would be developed in quicker time and in greater numbers for all therapeutic areas compared to traditional drug discovery method and as a consequence it was not surprising that NP research was often assigned a lower priority. However results from early combinatorial libraries were often disappointing and by the mid 1990’s there were series of doubts about usefulness and value of large libraries generated till that time.[22,18] The reason for the lack of lead compounds from the synthetic libraries in some therapeutic areas such as anti-infectives, immune suppression, oncology and metabolic diseases may be due to different chemical space occupied by NP’s and synthetic compounds.[22] This different chemical space makes NP’s an attractive

alternative to the synthetic libraries, especially in therapeutic areas, that have a dearth of lead compounds. In an interesting development, some groups of companies have begun to synthesise more complex structures to match chemical space occupied by natural products.[23]

CONCLUSION

A common misperception has been that NP research has not kept pace with other drug discovery methods and as a consequence become uncompetitive for lead discovery. However improvement in instrumentation, robotics and bioassay technologies have increased the speed of bioassay guided isolation and structure elucidation of NP's considerably and these improvements have allowed NP research to become more competitive with synthetic compound screening.

1.4 Plants - A herbal wealth, as a potential source of ISM (Indian System Of Medicine) drugs

ISM medicines mainly based on plants, enjoy a respectable position today, especially in the developing countries, where modern health services are limited. Herbal medicines have been the main source of primary health care all over the world. About 80% of the world population still depends on traditional medicine. Herbal medicines are finished, labelled medicinal products that contain active ingredients from plants. Medicines containing that plant material combined with active substances, including chemically modified isolated constituents of plants are not considered to be herbal medicines.[24] Herbal medicine continues to be major market in U.S pharmaceuticals and constitute a multi-billion dollar business. About 1500 botanicals are sold as dietary supplements. Formulations are subjected to food and drug administration (FDA) clinical toxicity testing, to assure their safety and efficacy. [25] ISM remains an important system of medicine and drug therapy in India. Plant alkaloids are primary

active ingredients of ISM drugs. Today the pharmacologically active ingredients of ISM drugs are being identified and are being used in drug therapy.[26] In India, around 15000 medicinal plants have been recorded, however traditional communities are using only 7000-7500 plants for curing different disease.[27] The valuable medicinal properties of different plants are due to presence of several constituents e.g. saponins, alkenyl phenols, glycolic alkaloids, flavonoids, sesquiterpene lactones and terpeniods. Among them some act as synergistic and enhance the bioactivity of other compounds.[28] Artemisinin, produced by *Artemisia annua* plant is very effective against *plasmodium falciparum, plasmodium vivax* and also drug resistant parasites. The main active constituent in *Artemisia annua* is a sesquiterpenoids lactone endoperonides, named artemisinic acid.

For more than a century, quinine, an alkaloid obtained from the bark of many species of cinchona tree has been used in the treatment of malaria and interestingly was the first agent used for the treatment of amoebic dysentery. Reserpine, isolated from raw plant extract *Rouvolfia serpentine*, is used as tranquilizer and in control of high blood pressure. Although synthetic drugs are often used in treatment of certain diseases but a remarkable interest and confidence on plant medicine is also found. [29]

1.5 Drug Development based on natural products

The search for new pharmacologically active agents obtained by screening natural sources such as microbial fermentation and plant extracts has led to the discovery of many clinically useful drugs that play a major role in treatment of human diseases. A recent review pointed out that approx. 60% of the anti-tumour and anti-infective agents that are commercially available or in late stages of clinical trials today, are of

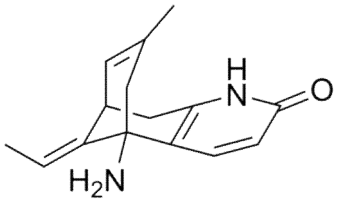
natural origin.[30] A large number of natural products especially plant derived drugs continue to be discovered on the basis of traditional or empirical local medical practices.[31] Role of natural product in the drug discovery can be summarized briefly Viz-a-Viz five major disease areas with some representative examples of interesting natural product molecules provided for each.

1.5.1 Infectious diseases

Important development of antibacterial agents over recent years has been related to some well-known natural product classes such as β-lactam structures e.g. (cephalosporin's), tetracycline structures e.g. (glycylcyclines), macrolides e.g. (erthromycin and rifamycin analogues), spectenomycin e.g. (vancomycins and teicoplanin analogues). In the antifungal areas, many chemistry and formulation studies have also been undertaken on known natural product classes such as polyenes e.g. amphotericin B and mysstatin analogues and nucleosides e.g. nikkomycin Z.

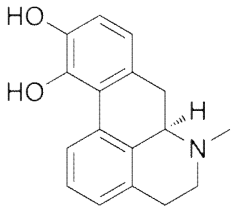
1.5.2 Neurological disease

The dramatic effect of ergot (claviceps purpurae) and its constituent indole alkaloids when ingested by humans has been recognized for nearly two centuries. To varying degrees, these agents act as partial agonists or antagonists at α- adrenergic serotoninergic and dopaminergic receptors. The impact of their action limits the therapeutic use of natural ergot alkaloids, but clinical application of their analogues exist for the treatment of migraine, Parkinson's disease and post-partum haemorrhage.[32] For several years/centuries, elderly people in some parts of Main Land China have obtained tea from the leaves of the club moss (Huperzia serrata) for improvement of their memory. In early 1980's Chinese scientists isolated Huperzine A from this traditional medicine as a potent reversible, selective and inhibitor of acetyl choline esterase.



Huperzine A

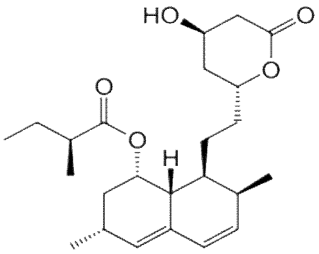
Apomorphine, a semisynthetic opium alkaloid, has long been known for its erectile activity at the effective dose of 2-6mg. As a dopamine D_2 agonist, Apomorphine represents a significant advance in the treatment of well-developed motor fluctuations in Parkinson's disease, through subcutaneous injections or infusions. [33]



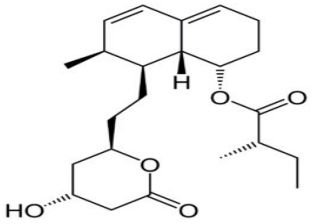
Apomorphine

1.5.3 Cardiovascular and metabolic disease

An earlier example of mechanism based high volume screen, is the use of HMG Co-A reductase, as a biological target. The extensive screening and isolation chemistry studies by Endo, co-workers and Sankyo, let the discovery of *Mevastatin* (compactin), with inhibitory activity against the enzymes in 1976.[34] In November 1978, Lovastatin was discovered to be responsible for its activity against HMG Co-A reductase after testing in various biochemistry assay.



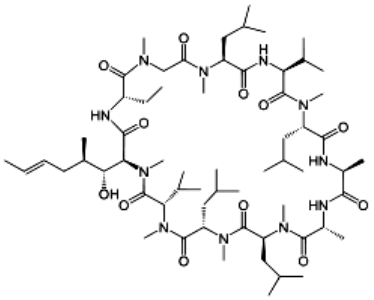
Lovastatin



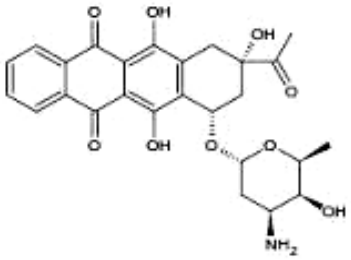
Mevastatin

1.5.4 Immunological, Inflammatory and related disease

It is wisely claimed that the recent increase of successful organ transplantations is largely due to the effective use of cyclosporine A (sandimmune). Since the introduction of cyclosporine A, as many as 10 new non-peptide drugs have been brought into various stages of clinical and pre-clinical development. Natural products have thus become a rich source of immunosuppressive agents for organ transplantation.With the approval of tacrolimus and other new agents, the outlook for successful organ transplantation is even more promising.[35] Cyclosporine, a fungal cyclic oligopeptide discovered at Sandoz and Sirolimus (rapamycin) a macrolide, antibiotic discovered at Wyeth-Ayerst, were originally recognised as antifungal agents in 1970's. In 1980's their remarkable immune suppressant activities were recognized and vigorously pursued.[36]



Cyclosporine



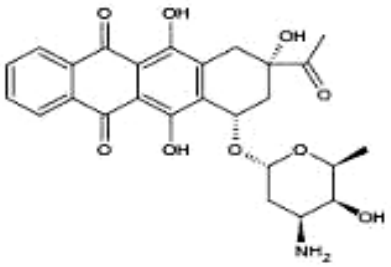
Sirolimus

Similarly mycophenolic acid, a phenolic fungal metabolite with known antitumor, anti-viral and immune suppressive effects is an inhibitor of two enzymes, inosine monophosphate dehydrogenase and guanylate synthetase.[37]

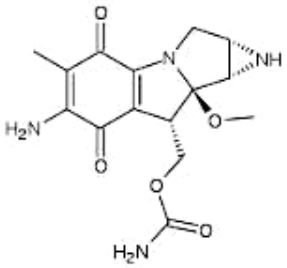
1.5.5 Oncological disease

Historically natural products have been used invaluably as agents to regulate cell cycle, leading to fruitful achievements in the antitumor areas in particular. With a

better under-standing of many molecular details of cycle, NP's are likely to offer continued promise for the future. Recent drug development efforts have produced new generations and improved versions of historically well-known antitumour agents such as vinca alkaloids, anthracyclines, mytomycin analogues, bleomycin analogues and others.[38]



Anthracycline



Mytomycin

The major achievements of NP research of past 10-15 years has been summarized so far, which clearly demonstrated that NP represents an unparallel source of molecular diversity to drug discovery and development and are indeed complimentary to those emerging molecular sources, such as combinatorial libraries.

Chapter 2

Phytochemical investigation of genus Nymphaea

2.1 An introduction to genus *Nymphaea*

Nymphaea is a genus of perennial, rhizomatous, aquatic herbs, widely distributed in temperate and tropical regions. Five species occur in India and a few are grown in gardens as ornamental plants.

Known as water lilies, many species of *Nymphaea* bear showy flowers and are highly prized in aquatic horticulture. Numerous local varieties and cultivated hybrids are known. Some of them are day bloomers, opening with the rising of sun, some bloom only in the night and opens after sun sets. A few are fragrant. Natural hybridization occurs wherever two or more species are grown in the same pond and the hybrid may be fertile or sterile. Important among the horticulture types grown in India are “Marliac” and “Laydekeri" hybrids derived from *Nymphaea alba* linn., *Nymphaea mexicana* Zucc., *Nymphaea odorata* A.t., *Nymphaea tetragona* Georgi and others. They are handy and require 60-90 cm of water for growth.[39]

I ***Nymphaea Alba*: (**European white water lily. Kashmir- Brimposh, Nilofar, Kanud**)**

A perennial aquatic herb, found in the lakes of Kashmir, at altitudes below 1800 m. Leaves are round, cordate, entire rhizome black ; flowers solitary about 10-13 cm across, floating fruits are spongy berries, ripening under water ; seeds are minute striate, punctate, buried in pulp. The starchy rhizomes and seeds of the plants are eaten in times of scarcity. Rhizomes are boiled before they are consumed and seeds are parched. Rhizomes contain starch 46%, crude fibre 10%, crude proteins 6.4% and ash 10.8% ; an alkaloid **nymphaeine** ($C_{14}H_{23}O_2N$), M. p 76-77 ^{O}C with a pyrole ring, glycoside and tannins are also present. Seeds contain 47% starch; fatty oil of seeds contain, di, tri and tetraenoic acid.[40] The alkaloid **nymphaeine** is present in all parts

of the plant except the seeds. It is toxic to frog and produces tetanus like symptoms. Alcoholic extracts of rhizomes containing alkaloids have a mild sedative and spasmolytic action. They do not significantly depress the heart in large doses and have a paralysing action on medulla. [41] The leaves of plants contain a flavone glycoside, *myricitin*. A glycoside nymphalin M.p 40 ^{0}C with digitalis like action has been identified in the flowers. Various parts of the plants contain ascorbic acid, the value recorded for nodules and foliage is 235 mg and 170 mg /100 g respectively. [42]

II ***Nymphaea nouchali:*** (Indian Red Water lily, Hindi- Kanwal)

Aquatic herb with short, erect, roundish, tuberous rhizomes, found in jheels, tanks, ponds and ditches, throughout the warmer parts of India. Leaves are peltate 15-25 cm, deem, orbicular or Reni form, sagittate when young, sharply sinute toothed, pubescent below; flowers solitary, variable in colour from deep red to pure white; fruit a spongy berry, 3cm in diameter, ripening under water; seeds minute, broadly ellipsoid, rough, buried in pulp. All parts of the plants are eaten in times of scarcity. The starchy rhizomes are eaten raw or boiled or sometimes baked. Analysis of rhizomes (from Philippines) gave the following values, moisture 53.95 %, crude proteins 5.87 % , fat 1.06 % , starch 27.37% , fibre 1.55 %, other carbohydrates 9.07 % and ash 1.13 %. Its seeds are edible and can be eaten or after parching they may also be grounded into flour and made into a kind of bread or cooked into kanji with water. They produce toxic effects when consumed in excessive quantities. The rhizomes are considered demulcent and used for dysentery and dyspepsia. Flowers are astringent and cardiotonic. some preparations like “gholland” etc. are reported to be prepared from the flowers. [43]

III ***Nymphaea stellate:*** (Indian blue water lily)

A large perennial aquatic herb with ovoid, acute root stock, found in ponds and ditches throughout the warmer parts of India. Various parts of the plant are edible. The pyriform egg sized rhizomes, tender leaves and flowers, peduncle are used as vegetables. The Powdered rhizomes are given in dyspepsia, diarrhoea and piles. An infusion of rhizomes and stem is considered emollient and diuretic. It is used against the diseases of urinary tract. Macerated leaves are used as a lotion in eruptive fevers. A decoction of flowers is considered narcotic and seeds are stomach restorative.

IV ***Nymphaea Candida:***

Nymphaea Candida is a species of aquatic perennial herbaceous plants of the genus *Nymphaea*, native to quite fresh water habitat in Eurasia. It is in flower from July to August. It is sometimes treated as subspecies of *Nymphaea alba*. A decoction of the roots may be used in the treatment of diarrhoea or to treat bronchial catarrh, kidney pain and can be taken as the gargle for sore throat. It is also used as a poultice to treat boils. The flowers have generally calming and sedative effects on the nervous system making them useful in the treatment of insomnia, anxiety and similar disorders.[(44)]

2.2 Review of literature

Previous phytochemical work on genus *Nymphaea* has resulted in the isolation of various classes of compounds which is present in the form of table 2.1

Table 2.1 Phytoconstituents of various species of *Nymphaea.*

S. No	Species	Class of compounds isolated	References
1.	***Nymphaea coerula***	Flavonoids – delphidin-3′-gallaylgagalactoside, seed lipids.	Fossen, Torgills., Andrers, Oyvind M. **50** (7), 1185 -88**.** (1999)
2.	***Nymphaea alba***	Tocopherol esters, phenolic content, luteolin glucopyranoside, ascorbic acid, tannic acid, gallic acid, sterols, flavnoids, hydrolysableTannins.	*Studies in Natural product*. **35**, Brazil Elzeviers. 5.(2008)
		alkaloids.	Chromatographic analysis of alkaloids from *N. alba* and *N. candida*. A*cta. Pharm*.**21** (2). 129-34
3.	***Nymphaea Stellate***	B- sitosterol, Cocluarine	Mukherjee, K.S., Buche, A and Gulacar, F.O. *Phytochemistry*, **36**, 813-14, (1994)
4.	***Nymphaea odorata***	Sterols(β-Sitosterol)	Segal, A., The component of N. odorata, P.hd dissertation, New York University (1965)
		tri-terpenes(α and β amyrin lupeol and taxasterol), lignans, Nymphaeoside A and icariside E	Hooper, S. N and Chandler, R. F., *J. Ethanopharmocol*, **10,** 181-194
5.	***Nymphaea mexicana***	28-29 C, Alkanes of surface waxes	*Aquatic. Bot*. **36** (3), 281-86. (1990)

6.	***Nymphaea marliacea***	The novel flavonol, Myricetin-3-o-α-rhamnopyranosyl	*Phytochemistry*. **49**(7), 1999 – 2002. (1998)
7.	***Nymphaea tetragona***	Geranin- a hydrolysable tannin.	B*iotechanol, Biochem*, **57** (9), 1570- 71.(1993)
		Aminopropylhemosp-ermidine	Hamana, K., Matsozaki, S., Niitsu, M and Samejima, K., *Can. j. Bot.* **72**. 1114-1120, (1994)
8.	***Nymphaea odorata***	Phenolic compounds	*Journal Of Natural Products.* ***66,*** *548-50. (2003).*

2.3 Results

2.3.1 Extraction. The shade dried plant material was subjected to sequential extraction in organic solvents e.g. Pet-ether, Chloroform, Ethyl-Acetate, Methanol and Water. Maximum yield was found in Methanolic extract of *Nymphaea alba* and chloroform extract of *Nymphaea mexicana*. The extract values of both the species have been tabulated in table 2.2.

Table 2.2 Extract values of *Nymphaea alba* and *Nymphaea mexicana*

S. No	Extracts	Extractive values % (W/W)	
		Nymphaea alba	***Nymphaea mexicana***
1.	Petroleum ether	2.6	1.48
2.	Chloroform	1.5	**2.03**
3.	Ethyl- acetate	1.23	1.5
4.	Methanol	**8**	1.87
5.	Water	4.4	1.3

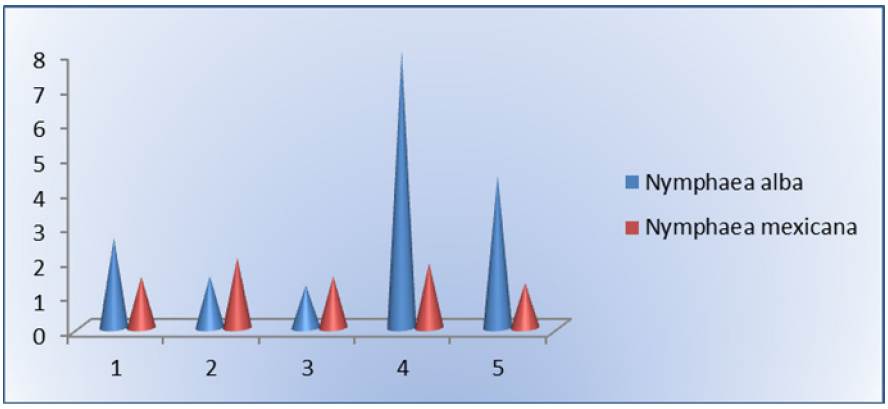


Fig.1 *Extract values of Nymphaea alba* and *Nymphaea mexicana*, a graphical representation

2.3.2 Qualitative chemical analysis of extracts.

Qualitative chemical tests were performed using standard procedures in literature (Farnsworth N.R. Biological and phytochemical screening of plants.*J .Pharm. Sci*. 55: 225-276. (1966). The tests revealed the presence of phenols, alkaloids, flavonoids, terpenoids, glycosides etc in the successive extracts of *Nymphaea alba* and *Nymphaea mexicana*.

Table 2.3 Phytochemical screening of various extracts

S. No	Constituents	Observations	
		Nymphaea alba	***Nymphaea mexicana***
1.	Terpenoids	Absent	Present
2.	Flavonoid**s**	Present	Present
3.	Steroids	Present	Absent
4.	Anthraquinones	Absent	Absent
5	Glycosides	Present	Present
6	Alkaloids	Present	Absent
7	Phenols	Present	Present

Since phenols, alkaloids, flavonoids and terpenoids are responsible for broad pharmacological profile including antioxidant, antibacterial, anti-allergic activity, therefore these extracts were preliminary screened for some of these possible activities.

2.3.3 Biological activities

2.3.3.1 Antioxidant activity

Extracts of both the plant species were evaluated for antioxidant activity by free radical scavenging activity using 1,1-diphenyl-2-picryl hydrazyl ether (DPPH) free radical method. The assay is based on the measurement of the scavenging ability of antioxidant test substance towards the stable radical. The method is based on spectrophotometry, in which the absorbance of the solutions is measured at 517nm. The decrease in absorbance indicates an increase in DPPH radical activity. Quercetin (sigma) served as the positive control.

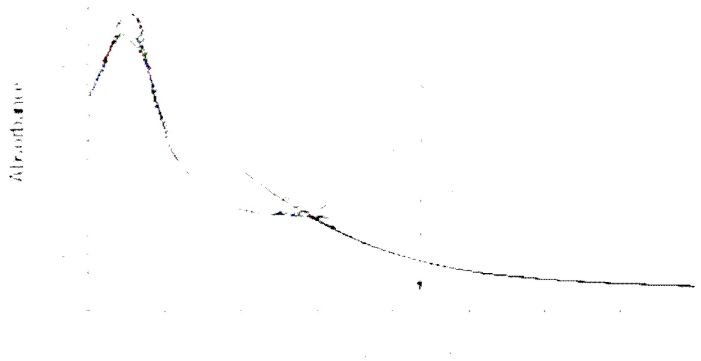


Fig 2: Prototype graph, showing the effect of increasing concentration of the aqueous extract (*N. mexicana)* on the DPPH radical scavenging activity.

The percentage scavenging activity of Petroleum Ether (PEE), Chloroform (CE), Ethyl Acetate (EA), Methanol (ME) and aqueous extract (AE) of the flowers of *Nymphaea alba* is reported in table 2.4

Table 2.4: Antioxidant activity (%) of *Nymphaea alba* as compared to the standard quercetin using stable DPPH radical

Aqueous		Methanol		Ethyl acetate		Chloroform		Pet ether	
Conc. µg/ml	Activity	Conc. µg/ml	Activity	Conc. µg/ml	Activity	Conc. µg/ml	Activity	Conc. µg/ml	Activity
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4.0	7.529	6.00	26.557	8.0	17.571	30.00	4.304	80.00	2.712
8.0	13.109	8.00	39.927	12.00	27.241	40.00	6.227	100.00	2.594
10.0	17.803	10.00	48.077	16.00	36.439	50.00	7.601	120.00	3.184
12.0	26.041	12.00	55.586	20.00	56.014	60.00	8.883	160.00	5.071
14.0	26.216	16.00	71.429	25.00	63.529	80.00	12.179	200.00	7.783
16.o	29.672	24.00	84.432	32.00	73.585	120.00	17.308	280.00	9.906
20.0	35.341	32.00	84.707	40.00	85.967	160.00	22.619	400.00	15.684
24.0	40.655	48.00	84.432	48.00	89.976	280.00	33.059		
32.0	57.307	64.00	84.158	56.00	90.802	400.00	42.216		
40.0	69.885			80.00	91.509	500.00	48.260		
60.0	70.236								

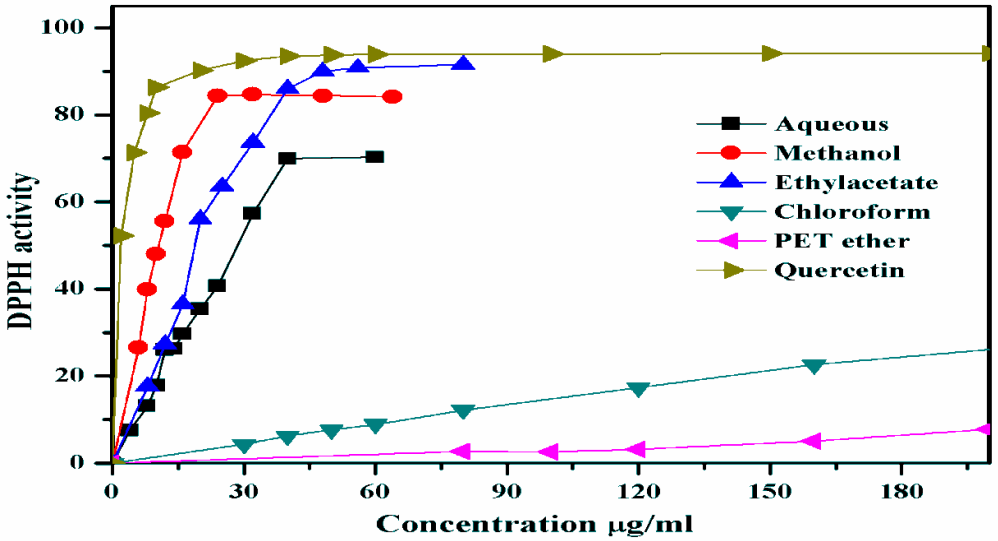


Fig 2.1: Radical scavenging activity of *Nymphaea alba* and standard quercetin using stable DPPH Radical.

The percentage scavenging activity of petroleum ether (PEE), chloroform (CE), ethyl acetate (EA), methanol (ME) and aqueous extract (AE) of the flowers of *Nymphaea mexicana* is reported as in table 2.5

Table 2.5: Antioxidant activity (%) of *Nymphaea mexicana* as compared to the standard quercitin, using stable DPPH radical

Aqueous		Methanol		Ethyl acetate		Chloroform		Pet ether	
Conc. µg/ml	Activity	Conc µg /ml	Activity	Conc µg /ml	Activity	Conc µg /ml	Activity	Conc µg /ml	Activity
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4.00	63.153	10.00	40.921	10.00	15.323	10.00	32.241	80.0	4.960
8.00	83.260	20.00	65.810	20.00	35.341	20.00	34.987	100.0	6.112
10.00	88.751	25.00	76.971	25.00	43.933	25.00	37.201	120.0	6.643
12.00	89.194	30.00	89.283	30.00	55.979	30.00	40.124	160.0	6.997
14.00	86.714	35.00	91.320	35.00	65.456	35.00	41.187	200.0	10.275
15.00	90.257	40.00	94.154	40.00	75.456	40.00	43.047	240.0	11.780
20.00	93.180	50.00	94.597	50.00	90.168	50.00	47.653	280.0	12.400
24.00	91.763	60.00	94.686	60.00	93.800	60.00	51.107	320.0	14.526
32.00	91.940	80.00	95.040	80.00	94.243	80.00	55.624	400.0	17.095
40.00	91.763	100	95.040	100	94.508	100	62.356		

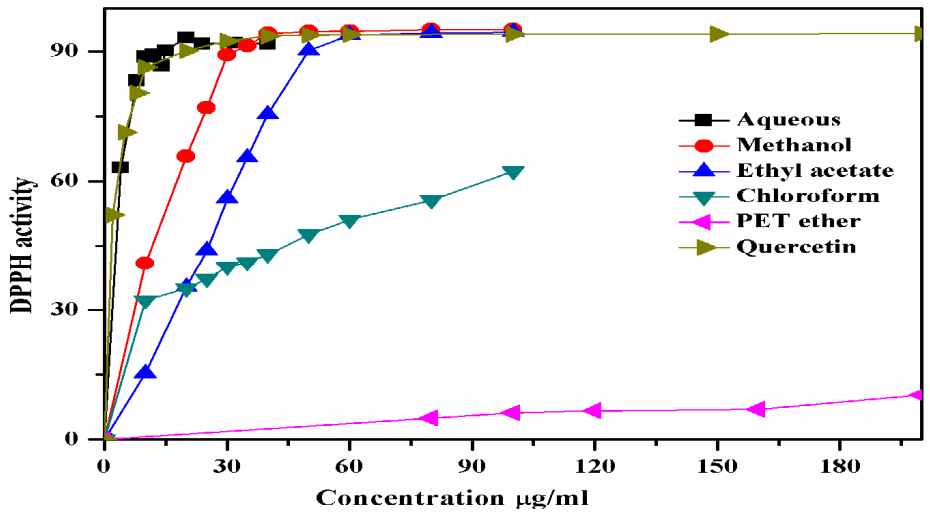


Fig 2.2: Radical scavenging activity of *Nymphaea mexicana* and standard quercetin using stable DPPH Radical.

Observation/Assessment

The results of DPPH free radical scavenging assay suggest that methanol, ethyl acetate and aqueous extract of both species show most promising antioxidant activity while Pet ether extract in both species show least activity. A comparative study of IC_{50} values of active extracts with the standard Quercetin further suggests that

Aqueous extract of *N. mexicana* is the most active extract with IC_{50} value of 3µg/ml as compared to Quercetin with IC $_{50}$ value of 1.66µg/ml.

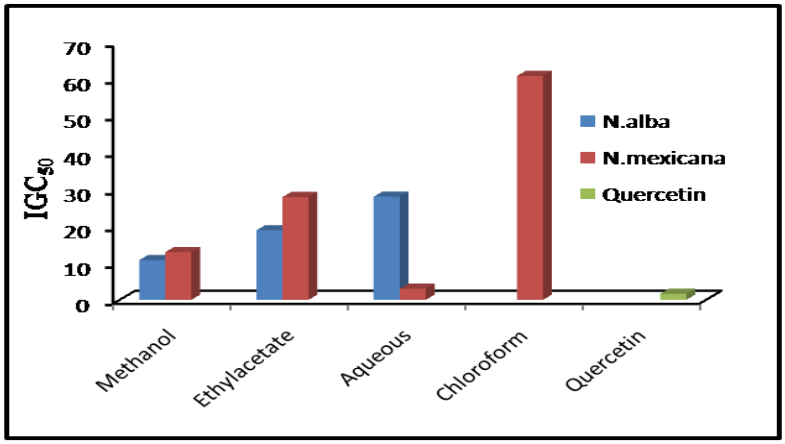


Fig. 2.3: Comparative study of IC_{50} values of the active extracts and the standard quercetin

The active extracts especially aqueous extract of *Nymphaea mexicana* and methanol extracts of both species possess strong free radical scavenging activity which could exert a beneficial action against liver damage and many other ailments induced by different exogenous and endogenous sources. The present study further suggests that the antioxidant potential of these extracts may be mainly due to presence of phenolic compounds like gallic acid and ellagic acid that could provide a good source of antioxidants which could offer potential protective effects against lipid oxidation. It can be concluded that free radical scavenging effects of these species is highly promising (especially aqueous extract of *Nymphaea mexicana and* methanol extracts of both species) and they can be used as an accessible source of natural antioxidants with consequent health benefits.

2.3.3.2 Anticancer activity

All the extracts of *Nymphaea alba* and *Nymphaea Mexicana* were screened for invitro anticancer activity. In this activity Sulpharhodamine-B assay was performed (Skehan et al. 1990) against human cancer cell lines, namely THP-1 (leukaemia), A-549 (lung), colon (HCT-15) and prostrate (PC- 3), which revealed increase in growth of inhibition during 48 hour incubation at concentrations of 100µg/ml and 50µg/ml of the sample. DMSO control was set up separately to cancel out the cell death occurred by DMSO, which was used as the solvent for dissolving sample homogenously. 5-Fluoro Uracil was used as the positive control. The data pertaining to results of cytotoxic potential of the extracts of *Nymphaea mexicana* and *Nymphaea alba* is presented in the form of table. 2.6 and 2.7 respectively

Table 2.6 In vitro cytotoxic activity of various extracts of *Nymphaea mexicana*.

Tissue Type			Leukemia	Prostate	Colon	Lung
Cell Line Type			THP-1	PC-3	HCT-15	A549
S.No.	Extract	Conc (µg/ml)	% GROWTH INHIBITION			
1.	Ethyl acetate	100	**91**	**79**	**69**	**71**
		50	**61**	45	39	38
2.	Pet ether	100	49	35	29	17
		50	28	12	9	1
3.	Methanol	100	56	**59**	**61**	**72**
		50	23	21	32	29
4.	Chloroform	100	24	28	46	41
		50	11	13	26	21
5.	5-Fluoro uracil	10^{-5} M	92	43	90	62

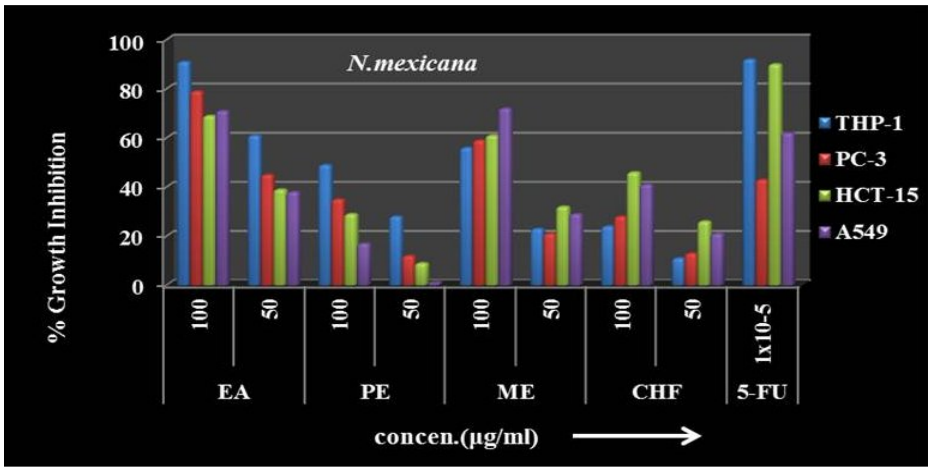


Fig.2.4: % age growth inhibition of *Nymphaea mexicana* against different human cancer cells in comparison to the control 5-Fluoro Uracil

Table 2.7 In vitro cytotoxic activity of various extracts of *Nymphaea alba*

Tissue type			Leukemia	Prostate	Colon	Lung
Cell Line Type			THP-1	PC-3	HCT-15	A-549
S.No.	Extract	Conc(µg/ml)	% GROWTH INHIBITION			
1.	Aqueous	100	**76**	**63**	**71**	**58**
		50	**43**	28	32	25
2.	Ethyl acetate	100	13	40	55	32
		50	07	21	25	15
3.	Chloroform	100	37	**67**	**79**	**62**
		50	21	32	28	38
4.	Methanol	100	54	12	23	34
		50	26	05	12	17
5.	Pet-ether	100	**67**	**52**	**26**	32
		50	46	28	12	23

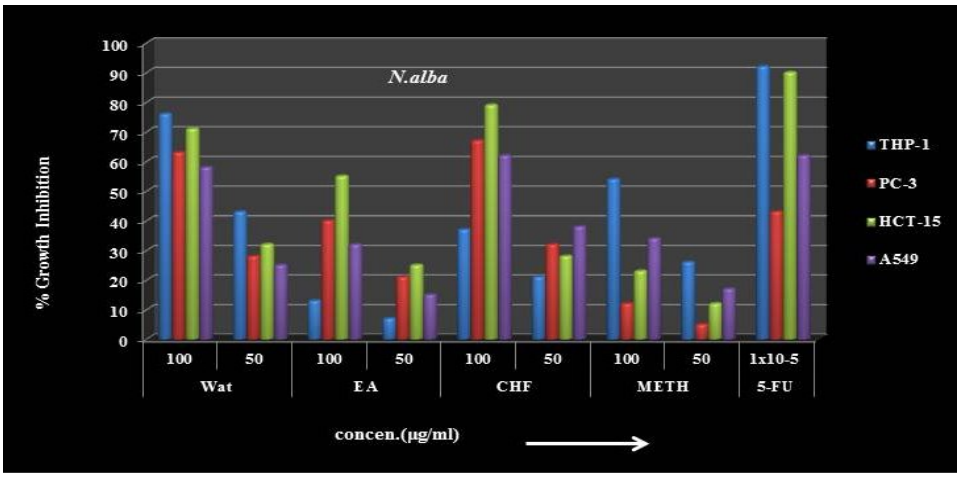


Fig.2.5: % age growth inhibition of *Nymphaea alba* against different human cancer cells in comparison to the control 5-Fluoro Uracil

Observation/Assessment

The results depict that the inhibition of different cancer cell lines of varying tissue origin with 100µg/ml and 50µg/ml of extract dissolved in DMSO imparted significant cellular cytotoxic effects on almost all the cell lines that were tested. However the most appreciable results were obtained by the ethyl acetate and methanol

extract of *Nymphaea mexicana* while in *Nymphaea alba* aqueous, chloroform and pet ether extracts showed most promising activity against almost all the cell lines that were tested.

The demonstration of promising cytotoxic activity by various extracts of *Nymphaea mexicana* and *Nymphaea alba* may help to discover new chemical classes of cytotoxic substances that could serve as selective agents for chemotherapy and control. Results of these studies indicate that further screening and characterization of *Nymphaea alba* and *Nymphaea mexicana* for cytotoxic compounds are warranted. As the search for new anticancer agents intensifies, plant extracts may provide attractive alternate sources of molecules for consideration. As drug resistance becomes an increasing problem and consumer demand for products with natural preservatives grows on each passing day, therefore *Nymphaea alba* and *Nymphaea mexicana* can form the basis of future anticancer research efforts.

2.3.3.3 Antimicrobial activity

Extracts of both species were evaluated for their antimicrobial activity by following Minimum Inhibitory concentration Assay as per the guidelines of clinical and laboratory standard institute (formerly the national committee for clinical laboratory standards). The primary screening of the samples was done at 256 µg/ml concentration. The results are depicted in the table 2.8

Table 2.8: Antimicrobial activity of *Nymphaea alba* and *Nymphaea mexicana*

S. No	Extracts	*S. Aures ATCC 29213*		*E .Coli ATCC 25922*		*C. albicans ATCC 90028*		*A. fumigatus*	
		alba	***mexicana***	***alba***	***mexicana***	***alba***	***mexicana***	***alba***	***mexicana***
1	Methanol	--	--	--	--	--	--	--	--
2	Pet-ether	--	--	--	--	--	--	--	--
3	Chloroform	--	--	--	--	--	--	--	--
4	Ethyl acetate	--	--	--	--	--	--	--	--
5	Water	--	***Active***	--	--	--	--	--	--

Observation

Out of all the extracts, only aqueous extract of *N. mexicana* was found to be active and showed promising antibacterial activity against *Staphylococcus Aures (ATCC 29213).* The antibacterial activity of the active sample (aqueous extract) of *Nymphaea mexicana* was expressed *in terms of* **M**inimum **I**nhibitory **C**oncentration (MIC) in µg/ml against the standard *Ciprofloxin*. Table 2.9

Table 2.9 MIC determination of the active sample Viz-a-Viz the standard *Ciprofloxin*

S.No	Samples	MIC µg/ ml	
		***S. aures* (ATCC 29213)**	***E.coli* (ATTCC) 5922**
1	Aqueous extract	32	□256
2	Ciprofloxin	0.125	0.007

From the MIC assay it can be concluded that aqueous extract of *Nymphaea mexicana* showed fairly good antimicrobial activity against *S. aures* with MIC value of (32 µg/ml), vis-a-vis the standard Ciprofloxin (MIC 0.125 µg/ml).

2.4 Experimental

2.4.1 Methodology

Physical constants

Melting points (corrected and un-corrected) were determined in glass capillary tubes using Labotech-perfit melting point apparatus.

Spectroscopy

^{1}H NMR and ^{13}C NMR were run on 300MH Bruker Daltonics Spectrometers respectively. The chemical shifts δ were measured in Hertz.

Chromatography

Column chromatography was carried out on silica gel (Qualigens, 60-120 mesh). Pre-coated silica gel preparative plates were used for separation. The purity of samples was also checked on the same pre-coated plates. Solvent LR grade (Qualigens) was used for isolation.

Detection of compound on chromatogram

Thin layer chromatographic plates were viewed under ultra violet light at 254 nm for fluorescence quenching spots and at 366 nm for fluorescent spots. Cerric ammonium sulphate, anisaldehyde and Iodine (Merck) were used to detect the spot. `

2.4.2 Plant material

The flowers of *Nymphaea mexicana* were collected from Khuaja-Yaarbal area of Saida Kadal, Nigeen Lake and also from Dal Lake, on 26 May 2011. The flowers of *Nymphaea alba* were collected from Hookarsar, (wetland) on 11 June 2011. Both plants were identified in the Institute of Plant taxonomy, Department of Botany, University Of Kashmir under the voucher specimen No KASH 36358 and were deposited at KASH herbarium in Centre of plant Taxonomy, University of Kashmir, Srinagar. The plant material was shade dried and the dried material was crushed into a fine powder.

2.4.4 Extraction

The powdered plant material (260 g of *Nymphaea alba* and 614 g of *Nymphaea mexicana*), was subjected to successive soxhlet extraction, separately, using organic solvents in increasing order of their polarity e g, pet-ether → chloroform → ethyl acetate → methanol → water.

2.4.3.1 Pet- ether extraction.

260 g of *Nymphaea alba* and 614 g of *Nymphaea mexicana* was extracted (hot extraction) separately with petroleum ether, using soxhlet extractor at boiling temperature for about 9-10 hours. The extract was then collected, filtered, vacuum dried and extractive value was determined.

2.4.3.2 Chloroform extraction

After de-fatting the plant material with pet-ether, the dried plant material was extracted with chloroform (hot extraction), using soxhlet apparatus at boiling temperature, for about 15-20 hrs. The extract was carefully filtered using what man's filter paper and concentrated in vacuum under reduced pressure using rotatory evaporator and extractive value was determined.

2.4.3.3 Ethyl-Acetate extraction

Chloroform residue was completely dried and then extracted with ethyl acetate in the same manner as above at boiling temperature for about 15-20 hrs. The extract was collected, filtered and then concentrated in vacuum.

2.4.3.4 Methanol extraction

Ethyl-acetate residue was dried completely and then extracted with methanol, at boiling temperature for 48 hrs. After filtering, it was vacuum dried and extractive value was determined for both the plant material.

2.4.3.5 Aqueous extraction

22 g dried flower powder of *Nymphaea alba* and 107g dried flower powder of *Nymphaea mexicana* was weighed out and soaked in 150 ml of distilled water in separate 300 ml round bottom flasks, which was thereafter stoppered with a rubber cork and left for 24 hours. After this, the extract was filtered and dried over water bath.

All these extracts were dried completely, stored in refrigerator, maintained at 4^0C, in labelled sterile bottles, till further evaluation.

2.4.4 Physicochemical parameters of the plants

The physicochemical parameters of the plants such as the extract values and qualitative analysis were done using standard procedures in the literature.

2.4.5 Protocol for Biological activities

2.4.5.1 Determination of antioxidant activity.

Free radical scavenging activity of various extracts of *Nymphaea alba* and *Nymphaea mexicana* was measured in terms of hydrogen donating or radical scavenging ability, using stable DPPH radical. Quercitin was used as a reference compound. Antioxidant activity was determined according to methods of *Alasarvar et.al* and *Collin.* [45] DPPH radical is scavenged by antioxidants present in these extracts through the donation of proton forming $DPPH_2$. The colour changes from purple to pink and then to yellow after reduction, which can be quantified by decrease in the absorbance at 517 nm. Radical scavenging activity increases with the increasing percentage of free radical. A 0.05 mM solution of DPPH in methanol was prepared. Stock solutions at conc. 1mg/ml of 10 sample extract (5 from each plant) were prepared in methanol. Different concentrations (10 – 100 µg/ml) of the extracts in methanol were added to 1 ml (0.05 mM DPPH) and the final volume was made to 3ml with methanol. The mixture was shaken vigorously and kept standing at room temperature in dark for 30 mints. The

absorbance was then measured at 517 nm on spectrophotometer. The decrease in absorbance indicates an increase in radical scavenging ability. The percentage ability was calculated by the following equation.

$$\textbf{DPPH radical scavenging activity (\%)} = A_C - A_S / A_C \times 100$$

Where A_C was the absorbance of the control (blank, without extract) and A_S was the absorbance in presence of extract. All these tests were performed in triplicate and graph was plotted with the mean values.

2.4.5.2 Determination of anticancer activity

Anticancer activity of various extracts of *Nymphaea alba* and *Nymphaea. mexicana*, was determined by SRB (Sulpharhodamine–B) assay, against the human cancer cell lines, which include cultured THP-1 (leukemia), A-549 (lung), colon (HCT-15) and prostrate (PC- 3) cell lines. These cell lines were exposed to concentrations of 100µg/ml and 50µg/ml of the extracts for 48 hours, which reduced the viability of these cell lines. These extracts, as per the table 2.6 and 2.7, displayed variable activity against majority of the cancer cell lines.

Human cell lines and cultures

The optimum density of seeded cell suspensions were introduced to each well of 96-well plates (Iwaki) and exposed to range of concentrations of the plant extract (samples). The cells were incubated with samples for 48-hrs and were fixed in ice cold TCA for one hour at 40^0 C. The plates were washed with distilled water and allowed to dry in the air. Sulpharhodamine-B (SRB) solution (0.4%) was added to each well of dry 96-well plate and allowed to stain at room temperature for 30 minutes. The unbound SRB solution was removed by washing the plates quickly with 1% (v/v) acetic acid. The bound SRB dye was solubilized by adding 100µl of 10 mM

un buffered Tris base (pH = 10.5) to each well and shaking for 5 minutes on shakers platform. The plates were read in a 96-well plate reader at 540 nm.

2.4.5.3 Antimicrobial activity

Minimum Inhibitory Concentration assay

All the extracts prepared were tested against following microbial strains: *S. aures, C. albicans, A. fumigatus and E.coli.* The microbial cultures were maintained on tryptone soya agar and stored at -70 ^{0}C containing 50% glycerol. Ciprofloxin obtained from Sigma Aldrich was used as standard antibacterial agent for this study. Stock solution was prepared at 1mg/ml. MIC was determined as per the guidelines of clinical and laboratory standard institute (formerly the national committee for clinical laboratory standards). Bacterial suspensions were prepared by suspending 18-24 hrs grown bacterial cultures in sterile normal saline. The turbidity of the bacterial suspension was adjusted to 0.5 McFarland standards (equivalent to 1.5 × 108 CFU/ ml) at wave length 625 nm. The two fold serial of extracts (stock solution prepared in DMSO) were prepared in MHB (Muller Hilton Broth: DIFCO laboratories) in 100 µl volume in 96-well U bottom microliter plates (Tarson, Mumbai, India). The above mentioned bacterial suspensions were further diluted in the MHB and 100µl volume of this dilute inoculum was added to each well of the plate resulting in the final inoculum of 5 × 10^{5} CFU /ml in the well and the final concentration of the sample ranging from 2000 to 3.9 µg/ml till the 10^{th} column. Column 11 and Column 12 containing 100µl and 200µl of the medium without drug served as growth and media control respectively. The plates were incubated at 37 ^{o}C for 18 hrs and were read visually and the minimum concentration of the compounds showing no turbidity was recorded as MIC.

Chapter 3

Phytochemical investigation of genus Inula

3.1 Introduction of genus *Inula*

Inula an ornamental plant, belonging to asteraceae and of great medicinal value, represents one such genera which has a worldwide distribution, comprising about 90 species of flowering plants.[46] India is represented by 20 species and Pakistan along with Kashmir is represented by 11 species. Out of 11 species of genus *Inula* which occur in the area under consideration, five species *Inula koelzic dewar*, *Inula stewartie dawar*, *Inula obtisifolia. Kern, Inula accuminata* and *Inula falconeri* are typically Irano-Turanian elements. However 3 species *Inula racemosa*, *Inula grandifolia* and *Inula clarkei* may be classified biregional. They occur equally in Irano-Turanian and Sino-Japanese region. Some species also occur in N. Africa, Arabia, Europe, Asia.[47] The chemistry of genus Inula is diverse and represents many important compounds from the chemical world, many of whose activity has been determined. On extraction of the plants belonging to this genus, major compounds isolated are mainly sesquiterpene lactone e.g. dihydroisoalantolactone, isoalantolactone and alantolactone.[48] Lignans and essential oils also form an important constituent of the genus Inula.[49]

3.2 Review of literature

Phytochemical investigation of many *Inula* species reported in the literature of past five decades revealed the presence of many compounds, major compounds being sesquiterpene e.g. Alantolactone, dihydroalantolactone and isoalantolactone. Besides inulin and essential oils, Inula also contains some alkaloids, tannins, flavones and illicic acid. General overview of important constituents reported in some of its species is represented in the tabulated form in. 3.3

3.3 Phytoconstituents of genus *Inula*

S. No	Species	Class of compounds/ Compounds	References
1.	*Inula Japonica*	Skin beautifying agents containing titanium seed extract	*Lishizhen Medicine and Materia Medical Research*. 2007-11
		Inulicin, tannins, antidiabetic polysaccharides	*Phytotherapy research*. 24, (II)
2.	*Inula racemosa*	Oxygenated allantolides e.g. 4(15)-α-epoxy isotelekin and perhydro epoxyalantolide.	*Phytochemistry*1990, **29**(7), 2341-3
		sesquiterpene lactones, lignans	*Journal of phytochemistry* 1998. **49**(1), 157-61
		inunolide and dihydro inunolide	*Planta Med*, 1988, **54**(2), 186-87
3.	*Inula britanica*	Polyphenolic constituents, exdate flavonoids	*Free radical biology*, (2002)
		Pulchellin C, Kaurane glcosides	*Phytochemistry. **42**.*(3). 783-86.1996
		Sesquiterpene lactones.	*Journal of Natural Products.*, **9.(**2006)
		carotenes, Terpene derivatives britanin	*Chinese Journal of natural medicine. **6*** (1). (2008)
4.	*Inula salcina*	Sesquiterpene lactones, thymol derivatives, sterols	*Sci. Biol*. **14** (9), 645-9 (1966)
		aromatic esters	*Anthonsem, Thorleif, Kjoesens, Berit Acta. Chem. Scand. **25**.(2) 390-2.(1971)*
5.	*Inula germanica*	Melampolides	*Planta Med*, 1985, (3), 261-2

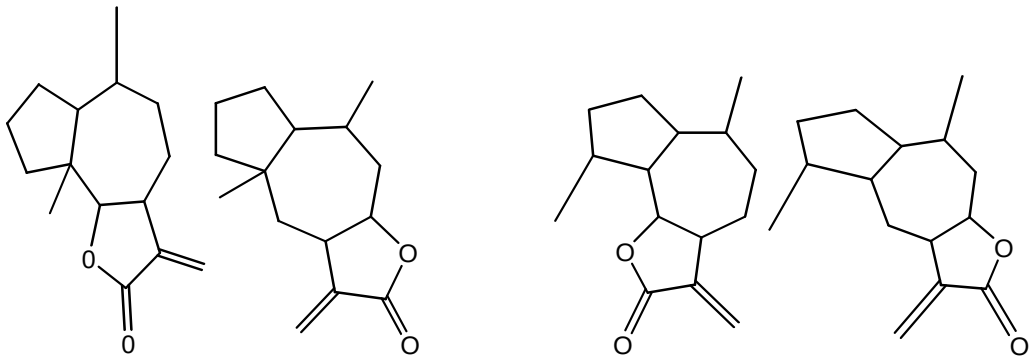
		sesquiterpenoid lactones	*Structure* of new SL's from *Inula germanica. Chemistry of natural compounds.* **10**.(5) 591-99
6.	*Inula crithmoides*	Methoxylated flavonols, Chlorinated thymol derivatives, Sesquiterpene lactones.	*Phytochemistry*. **24** (6) 1377-78. (1985)
7.	*Inula grantiodes*	Triterpenes, sitosteryl glucoside and olefins.flavones, fatty acids.	*Pak. J. Sci. Ind. Res* ***35****(9), 352-4. (1992),*
8.	*Inula Caspica*	Incapsin-a new sesquiterpene lactone and britanin	*Deposited Doc.1984, VINITI 5036-84, 127- 29*
		inuchinenolide C and Pulchellin C, Sesquiterpene lactones.	*Khem. Prir. Soedin*. **6,** 798-8. (1984)
9.	*Inula Indica*	Germacranolides	*Bhimsen A. Nagasampagi, Uppoor. Bhatt, Ferdinand Bholmann, Christa Zdero. Phytochemistry. 20 (8), (1981)*
10.	*Inula royleana*	quinones, abietane Triterpenoids, β-Caryophyllene, epoxides, Diterpenoids	*Edwards, O. E.,Feniak, G and Los, M. Can. j. Chem.* ***40****. 1540-46. (1962*
11.	*Inula Viscosa*	Sesquiterpenoids	*J. Nat. Prod. 1998,* ***61****(6) 798-800*
		Flavonoids	*Planta. Med. Phytother (1991),* ***25****(4),****P:*** *170-6*
		Eucalptol, Thymol content, eudesmane acids, oxygenated nerolidol esters, Carboxy eudesmadiene	*Biol. Sper. 1990,* ***66****(9), 829-34*
12.	*Inula graveolens*	Sesquiterpenes, Eudesmanolides, illicic acid, graveolides	*Fitoterapia 1979,* ***50****(1), 3-4*
13.	*Inula macrophylla*	Sesquiterpenes and monoterpenes, inulin	*Rastit Rasur. 1992,* ***28****(3), 71-72*

3.4 Introduction to sesquiterpene lactones

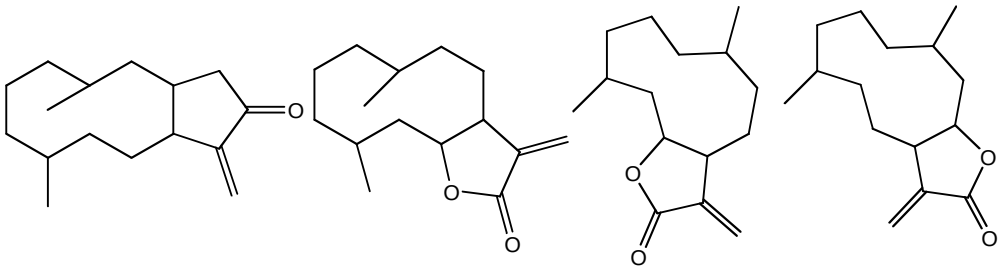
Sesquiterpene are C-15 terpenoids which occur as hydrocarbons or in oxygenated forms such as alcohols, aldehydes, ketones, acids or lactones in nature. They are important constituents of essential oils which have many applications in medical and also in soap and perfume formulations. Moreover they are also found as flavour compounds in aroma mixtures. Sesquiterpene lactones (SLs) have been isolated from numerous genera of the family asteraceae (compositae) and can also be found in other angiosperm families. They are described as the active constituents of a variety of medicinal plants used in traditional medicine for the treatment of inflammatory diseases. They are known to possess wide variety of biological and pharmacological activities such as antimicrobial, cytotoxic, anti inflammatory, antiviral, antibacterial, antifungal, effects on the central nervous and cardiovascular systems as well as allergenic potency. Their wide structural diversity and potential biological activities have generated further interest among the chemist.[50]

Sesquiterpene lactones are a class of naturally occurring plant terpenoids that represent a diverse and unique class of natural products and are important constituent of essential oils, which are formed from head-to-tail condensation of three isoprene units and subsequent cyclization and oxidative transformation to produce a *cis* or *trans*-fused lactone. These secondary compounds are primarily classified on the basis of their carbocyclic skeletons into pseudoguainolides, guaianolides, germacranolides, eudesmanolides, heliangolides and hyptocretenolides.

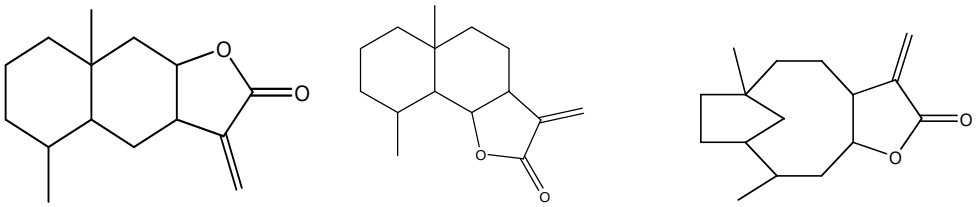
An important usual feature of the SLs is the presence of a γ-lactone ring (closed towards either C-6 or C-8) containing in many cases, an α-methylene group. Among other modifications, the incorporation of hydroxyls or esterified hydroxys and epoxide ring are common. A few SLs occur in glycoside form and some contain halogen or sulphur atoms.[51]



Pseudoguaianolides Guaianolides



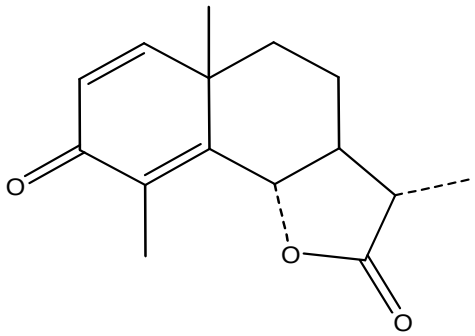
Germacronolides



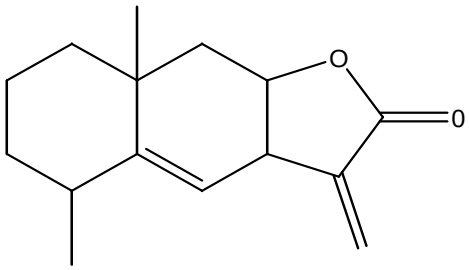
Eudesmanolides Helangolides

Fig.3.1 Basic skeleton of sesquiterpene lactone

Several main group of sesquiterpene lactones may be distinguished. Santonins (I), which is found in large numbers in *asteraceae* species and alantolactones (II) from *Inula species*, represent lactones of eudesmane series or eudesmanolides.

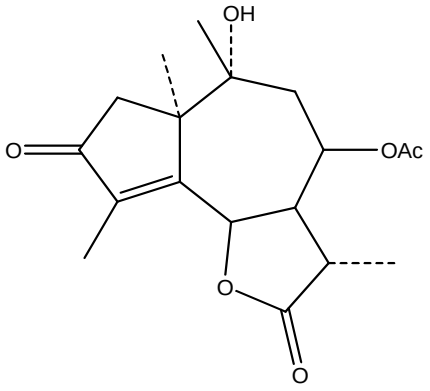


(I)

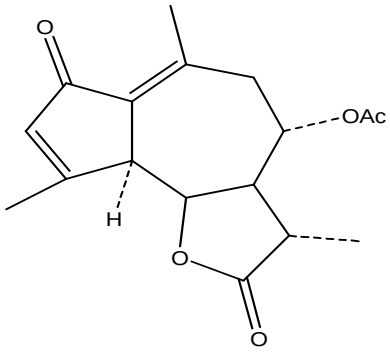


(II)

A second group of lactones represent the guainolides, typical examples are geigerin (III), constituent of South Africa *Geigria* species[52-54)] and Matricarin (IV) which is found distributed in Achila[55-57)] Artemisia and Matricaria species.[58)]

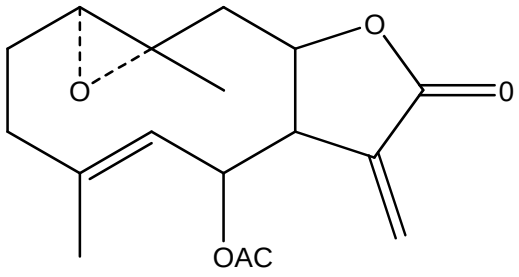


(III)

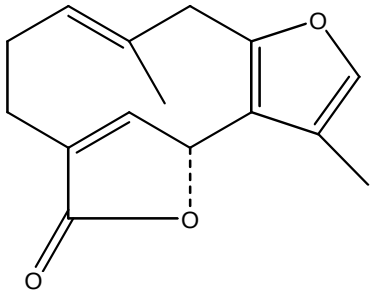


(IV)

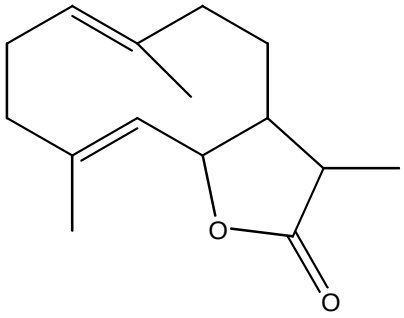
Another discovery of late nineties were lactones of germacrane series, so called germacranolides. Illustrated are Pyrethrosin (V),[59-61)] a non-insecticidal constituent of *chrysanthemum*, *cinerarium,* linderalactone (VI) a type of compound found in some *lauraceae* and costunolide (VII),[62-64)] an ingredient of costus oil (*Saussurae Lappa Clarke)*. Lastly small group of lactones based on drimane series is illustrated by Iresine (VIII) from Iresine elosiodes (*Amaranthaceae*)[65, 69)]



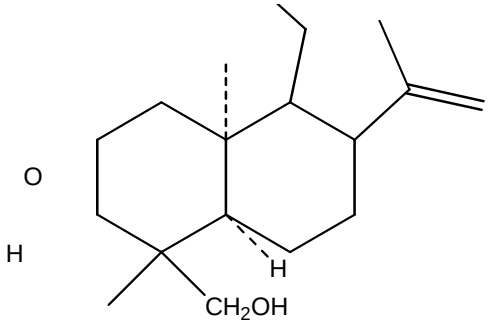
(V)



(VI)



(VI)



(VIII)

3.4.1 Review of application of analytical techniques to sesquiterpene lactones

Within the group of sesquiterpene, their lactones deserve special interest. These secondary metabolites derive from the basic sesquiterpene carbon skeleton in which one of the methyl groups of the isopropyl group is oxidised to the lactone group.[70] These compounds are of interest not only from chemical and chemo taxonomical stand points, but also because many of them possess biological and therapeutic activities including anti- inflammatory, antitumor, antimicrobial, anthelmintic and antifeeding.[71] Because of these effects, preparations of these plants containing sesquiterpene lactones are often used in traditional medicines. To guarantee the same quality of medicinal plants chromatographic techniques including qualitative and

quantitative analysis are used. Moreover these methods are also helpful tools in isolation and identification of these compounds.[72)] Some common hyphenated techniques used for the characterization and identification of sesquiterpene lactones are as under.

3.4.1.1 HPLC with UV detection

Sesquiterpene lactones (SLs) are compounds with low volatility and many of them are thermolabile. Because of these properties, HPLC mainly on reversed phase, is the analytical method of choice for SL analysis in crude plant extracts. However normal phase gradient run HPLC by n-Hexane-acetonitrile-isopropanol maybe some times preferable.[73-74] HPLC has been applied successfully to the analysis of 33 pseudoguainolides and xanthanolides of the genus *parthenium* as well as 21 pseudo-guainolides of *Arnica chamissonis* and 15 of *Arnica montana* acetonitrile-water gradient or methanol-water gradient was used respectively.[75-76] In order to avoid severe peak leading, especially in early peaks, SL's should be solved in the same ratio of solvent mixture rather than in only one solvent. HPLC is a valuable tool for quantitative analysis too. The content of single SL as well as total amount can be determined as shown with the complex mixture of SLs in flowers of *Arnica chamissonis* and *Arnica montana*, which are used as Arnicae flos in German pharmacophore. However the problem with most of the sesquiterpene lactones is their low absorption maximum in UV. This property is often a limiting factor in carrying out successful HPLC analysis of SLs in crude plant extracts, especially for minor components.[77] A solution to this problem, but only for SLs containing α-methylene butyrolactone functions is derivatization by 9-thiomethylanthracene. The thiol containing reagents reacts in a Michael type addition with methylene butyrolactone of

SLs and increase the sensitivity, so that routinely nanogram quantities of the lactones can be detected by HPLC and monitored at 369 nm.[78]

3.4.1.2 Combined techniques with HPLC: HPLC-MS and HPLC- NMR

UV detection is sometimes unsuccessful for SL analysis, because several SLs show weakly absorbing chromatophoric groups or no chromophores at all. Therefore other detection methods, such as evaporative light scattering detection (ELSD) can be used to overcome this limitation.[79-80] Where as these detection techniques are not yet widespread in HPLC analysis of SLs, the combination with mass spectrometry has become extremely helpful in the detection and identification of SLs in raw plant extract. Nevertheless HPLC–ESI gains more and more importance in the structure elucidation of isolated SLs for information of molecular mass, because the molecular mass is often missed in EI mass.[81]

LC-NMR is a further advanced technique which combines high performance technique with a structurally important informative spectroscopic method. It allows extracts to be screened not only for structural classes but also for compounds without isolation of individual compounds. In the beginning, this method has achieved limited success due to lack of sensitivity. Recently this situation has changed as new solvent suppression techniques have been introduced. The successful application of LC-NMR is demonstrated with *Vernonia fastigiata* extracts, in which nine sesquiterpene including minor constituents could be identified.[82]

3.4.1.3 Super Critical Fluid Chromatography

Sesquiterpene lactones can be separated by SCF chromarography. This method is suitable for thermolabile SLs. Packed columns in SFC has a sample capacity similar to HPLC, operates with the same columns, but requires shorter analysis time and

allows more consistent column conditions during repeated analysis. SFC has the disadvantage that polar stationary phases can retain more polar components. Detection can be carried by UV spectroscopy as demonstrated with *Artemisia umbelliformis* and *Carduss benedictus* extracts[83] or by ELSD as proven with bilobalides in *Ginkgo biloba* and *Artemisia annua.*

3.4.1.4 Gas Chromatography

Gas chromatography analysis are often used in the routine analysis of the plant extracts which are screened for SLs. However thermal degradation has to be considered. Owing to this property, many heteroatoms-substituted sesquiterpene lactones cannot be analysed by GC without derivatization and trimethylsilyl ether have to be prepared. [84] Nevertheless special applications of GC and GC-MS to underivatized SLs not being thermolabile have been reported in the literature[67-68]. Comparing separation power of GC and HPLC analysis, it was concluded that the use of both systems in conjugation with one another is a powerful technique for the rapid analysis of plant samples for these SLs.

3.4.1.5 Thin layer chromatography

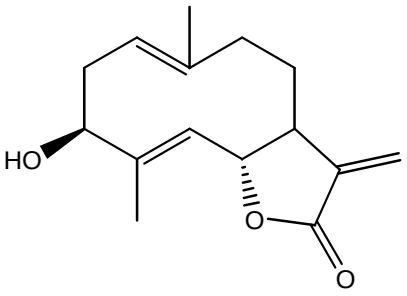
TLC was one of the first chromatographic method and was also applied to analysis of SLs. Its importance has decreased nowadays. However in combination with different spray reagents TLC can provide valuable information about the type of SL. Visualization reagents are different and numerous, e.g Vanillin/O-phosphoric acid, anisaldehyde, p-dimethyl amino benzaldehyde-sulphuric acid, resorcin-sulphuric or phosphoric acid, aluminium chloride or hydroxylamine[65,66]. SLs lacking an exocyclic α-methylene group can be visualized by dimethyl amine followed by Dragondorff reagent.[66] In general it depends on the skeleton which of the reagents is more favourable. TLC is here advantageous because of its rapid, easy and cheap

performance without the need of large instrumental equipments. However, compared with HPLC or GC-MS, this chromatographic method provides less information. However TLC can be used to separate diastereomers such as parthenin and hymenin by multiple developments of TLC plates in the same solvent system.[85] These two SLs differ in the configuration of cyclopentenone ring. A special TLC method, worth mentioning is over pressured layer chromatography (OPLC)[65,66]. As separation in OPLC chamber is much faster than in normal TLC, the separation time is relatively short and in closed systems there is no or limited chance providing artefacts. Therefore OPLC is especially suitable for studying big number of samples.

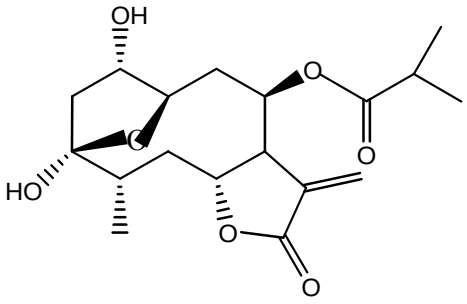
3.5 Biological activity of Sesquiterpene lactones

An important usual feature of the SLs is the presence of a γ-lactone ring (closed towards either C-6 or C-8) containing in many cases, an α-methylene group. Among other modifications, the incorporation of hydroxyls or esterified hydroxyls and epoxide ring are common. A few SLs occur in glycoside form and some contain halogen or sulfur atoms. Majority of SLs have shown cytotoxic activity (KB and P388 leukemia *in vitro*) and activity against *in vivo* P388 leukemia. Structure activity relationship studies showed that various cytotoxic SLs react with thiols, such as cystiene residues in the protien, by rapid Michael type of addition. These reactions are mediated chemically by α, β-unsaturated carbonyl systems present in the SLs. These studies support the view that SLs inhibit tumour growth by selective alkylation of growth regulatory biological macromolecules such as key enzymes, which controls cell division, thereby inhibiting a variety of cellular functions, which directs the cell into apoptosis. Differences in activity between individual SLs may be explained by different number of alkylating structural elements. However, other factors, such as lipophilicity, molecular geometry, and chemical environment or the target sulfhydryl

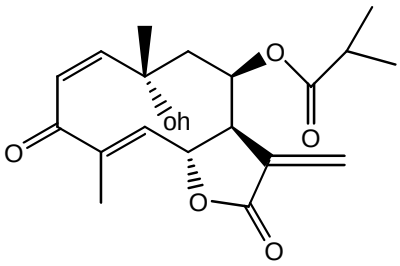
may also influence the activity of sesquitepene lactones.[86] Few examples of structurally diverse sesquiterpene lactones are given in Fig 3.2.



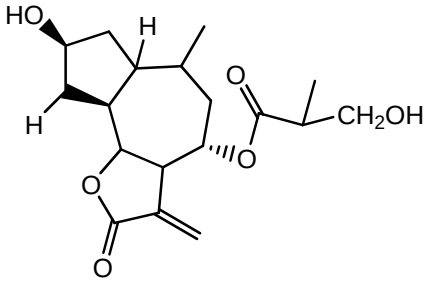
Costunolide



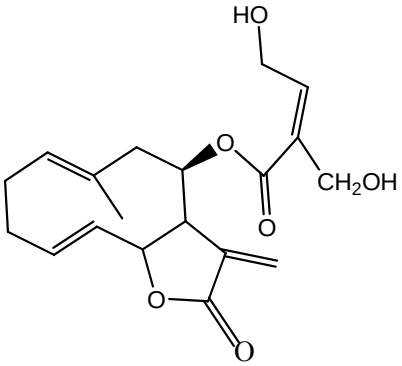
Tagitinin A



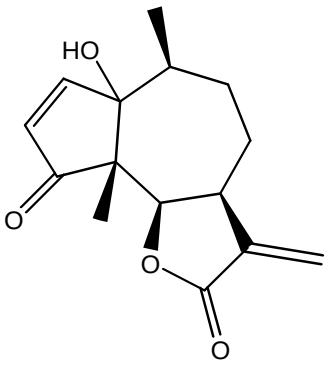
Tagitinin C



Cynaropicrin



Eupatoriopicrin



Parthenin

Fig 3.2: Structural diversity of sesquiterpene lactones

In recent years, many researchers over the world have reported that sesquiterpenes lactones possess a wide range of bioactivities which include potential anticancer activity, anti-inflammatory, antimalarial, antiviral and antifungal activities.

Some examples of the well-known SLs with their wide spectrum of activities has been reported as below.

(I) Costunolide

Costunolide is an active component from the crude extract of *Saussurea lappa* roots, a traditional Chinese medicinal herb. costunolide is a potent apoptotic inducer in cancer cells, *via* multiple pathways. It has been reported that costunolide readily depletes intracellular GSH and disrupts the cellular redox balance.[87] It triggers an intracellular reactive oxygen species (ROS) burst which leads to mitochondrial dysfunction: loss of mitochondrial membrane potential, onset of mitochondrial membrane transition, and release of mitochondrial pro-apoptotic proteins[88]. Costunolide also displays potential anti-inflammatory activity through NF-kB pathway. Since NF-kB plays a central role in most disease processes, and since it can regulate the expression of many key genes involved in iflammatory as well as in a variety of human cancers,[89] NF-kB represents a relevant and promising target for the development of new chemopreventive and chemotherapeutic agents.

(II) Parthenolide

Parthenolide, is the major SL responsible for bioactivity of feverfew (*Tanacetum parthenium*), a traditional herb plant which has been used for the treatment of fever, migraine and arthritis for centuries,[90] one of the well explored bioactivity of parthenolide is its potent anti-inflammatory effects which is mainly achieved through its strong inhibitory effect on NF-KB activation. The anticancer activity of parthenolide has been pursued in a number of laboratories. A large number of studies have been undertaken to investigate the mechanism of action of parthenolide at molecular levels in the different phases of Carcinogenesis. The data were obtained using different tumour cell systems. Parthenolide induced apoptosis in pre-B acute

lymphoblastic leukemia lines, including cells carrying chromosomal translocations.[91] Parthenolide induced rapid apoptotic cell death distinguished by loss of nuclear DNA, externalization of cell membrane phosphatidyl-serine, and depolarization of mitochondrial membranes. Besides this, it is also used in folklore medicine for their anti-inflammatory and analgesic properties. Several *in vitro* studies have shown that a great part of the anti-inflammatory action of this compound appears to be related to its ability to inhibit the NF-kB pathway.

(III) Helenalin

Helenalin, is another SL, from *Arnica* species, which has been reported to possess cytotoxicity and anti-cancer activity. Earlier studies demonstrated its potent activity to inhibit nucleic acid and protein synthesis. Similar to other anticancer SLs, mechanism of action mainly involve: (i) thiol depletion, (ii) inhibition of NF-kB, and (iii) induction of apoptosis.[92] Besides this Helenalin also possess anti-inflammatory activity and Recently, Lyu *et al*. provided evidence that a sesquiterpene lactone, helenalin containing two functional groups, namely α,β-unsaturated carbonyl group and α-methylene-δ-lactone ring, exerts its effect by direct alkylation of the p65 subunit of NF-kB without inhibition of IkB degradation.[93] Anti-viral activity of various sesquiterpene lactones was reported by Hsieh and their co-workers against hepatitis C virus ,wherein they found the best anti-HCV activity was shown by helenalin.with Ec 50 =1.25μm.

(IV) Artemisinin and its derivatives

In 1972, a group of Chinese researchers isolated a new anti-malarial drug (+)-artemisinin a sesquiterpene lactone from the hexane extract of a traditional Chinese medicinal plant *Artemesia annua* (Asteraceae) - a plant which has been used for the treatment of fever and malaria since ancient times.[94] Artemisinin is a sesquiterpene

lactone containing an endoperoxide linkage in it. This highly oxygenated sesquiterpene lactone peroxide, unlike most other anti-malarials, lacks nitrogen containing heterocyclic ring systems and was found to be superior plasmocidal and blood schizontocidal agent to conventional antimalarial drugs, such as chloroquine, quinine *etc* against malaria strains, without obvious adverse effects in patients. Recently, there was also report about anti-HBV activity of artemisinin.[95]

3.5.1 Structural-activity relationships (SAR) of sesquiterpene lactones

It is generally believed that the bioactivity of SLs is mediated by alkylation of nucleophiles through their α, β or α, β, γ-unsaturated carbonyl structures, such as α-methylene-γ-lactones or α,β-unsaturated cyclopentenones. These structure elements react with nucleophiles, especially the cysteine sulphahydryl groups by Michael-type addition. Therefore, it is widely accepted that thiol groups such as cysteine residues in proteins, as well as the free intracellular GSH, serve as the major targets of SLs. In essence, the interaction between SLs and protein thiol groups or GSH leads to reduction of enzyme activity or causes the disruption of GSH metabolism and vitally important intracellular cell redox balance. The relationship between chemical structure and bioactivity of SLs has been studied in several systems, especially with regards to cytotoxicity. Biologically active sesquiterpene lactones mainly possess anti-inflammatory and antitumor activity. It is believed that the *exo*-methylene group on the lactone is essential for cytotoxicity because structural modifications such as saturation or addition to the methylene group resulted in the loss of cytotoxicity and tumor inhibition. However, it has also been shown that the factor responsible for the cytotoxicity of SLs might be the presence of the O=C-C=CH2 system, regardless of lactone or cyclopentenone. It was latter demonstrated that the presence of additional alkylating groups greatly enhanced the cytotoxicity of SLs. Furthermore, it was

established that the α-methylene-γ-lactones and α, β-unsaturated cyclopentenone ring (or α- epoxycyclopentenone) present in SLs is essential for their *in vivo* anti-tumor activity. It has been confirmed through various published reports that the various kinds of biological activities displayed by SLs is due to presence of either α-methylene-γ-lactones and α,β-unsaturated cyclopentenone ring. In summary, the differences in activity among individual SLs may be explained due to the different nature and the number of alkylating elements, lipophilicity, molecular geometry, and the chemical environment of the target sulfhydryl group.

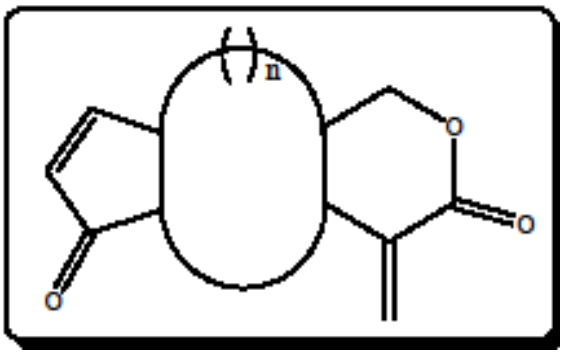


Fig 3.3: General Structure Of Sesquiterpene lactones

3.5.2 Chemical transformation of Alantolactone

Chemical or biochemical modification of an active molecule is a tool to obtain more active molecule from its natural counterpart.[96] Several bioactive molecules have been converted to their analogues in recent years by chemical or biochemical means. Medicinally important sesquiterpene lactones in general have been found to be of interest for their antibacterial,[97] antifungal,[98] antiparasitic, antihelmintic[99-101] and cytotoxic activities[102] besides this, sesquiterpene lactones with an α-methylene-γ-lactone moiety such as alantolactone isolated from many species of asteraceae are emerging as a group of plant growth regulators.[103-105] Alantolactone like many other sesquiterpene lactones have been chemically modified to form various analogues and their SAR has been studied so far. One of such transformations is the chemical

conversion of alantolactone into its pyrazoline derivatives with enhanced bioactivity.[106] My present work aimed at isolation of sesquiterpene lactone with a special reference to alantolactone from the roots of *Inula racemosa* and to do its chemical transformation through a series of simple well known reaction steps followed by the condensation of the products with some aromatic carbonyl compounds (aldehydes/ketones) like acetophenone and its derivatives, to form some pharmacodynamics compounds and study their SAR .

3.6 Results and discussion

3.6.1 Observation based on literature/present studies.

Genus *Inula* is a rich source of sesquiterpene lactones[107] and some of its species also contain terpenoids, alkaloids and steroids. Sesquiterpene lactones are also important constituent of essential oil, obtained from most of its species. In the present study, Pet ether extract of *Inula racemosa* was phytochemically screened for the presence of steroids, sesquiterpenoids and alkaloids, following the standard procedure in literature.[108]. Table 3.2

Table 3.1 Phytochemical screening of pet ether extract of *Inula racemosa*

S. No	Test for	Pet ether extract
1	Terpenoids	+++
2	Sesquiterpenoids	+++
3	Alkaloids	---

Since terpenoids in general and sesquiterpene lactones in particular are responsible for broad pharmacological profile, including antimicrobial and anticancer activities, therefore the pet ether extract of *Inula racemosa* was screened for its possible anticancer and antimicrobial activities.

3.6.2 Antimicrobial assay

Antimicrobial activity of petroleum ether extract was performed by following **M**inimum **I**nhibitory **C**oncentration Assay as per the guidelines of clinical and laboratory standard institute (formerly the national committee for clinical laboratory standards). The extract showed good activity against various microbial strains. Gram

positive bacteria *Staphy-lococcus aures (*ATCC 9213), *Candida albicans (ATCC 90028)*, gram negative bacteria *Escherichia coli* (ATCC 25922) and the fungal strain *A. fumigatus* were used to test the activity. The cultures were maintained as per standard procedures. The preliminary screening of the sample was done at 256 µg/ml concentration. The pet ether extract was found to be active against all the test microbes. The results of the antimicrobial assay are depicted in the table 3.2 and 3.3

Table 3.2 In vitro antimicrobial activity of Pet-ether extract of *Inula racemosa* (Preliminary investigation)

Extract	***S. aures*** **ATCC 29213**	***E.Coli*** **ATCC 25922**	***C. albicans*** **ATCC 90028**	***A.fumigatus***
Pet ether	Active	Active	Active	Active

Table 3.3 MIC of the active sample and the reference antibiotic Ciprofloxin

S. No	**Tested samples**	**MIC in µg/ ml**	
		S. aures ATCC 29213	***E. coli ATCC 25922***
1.	Pet ether extract	128	>256
2.	Ciprofloxin	0.125	0.007

The antimicrobial profile of Pet ether extract of *Inula Racemosa* showed desirable results, being active against all the test microbes. The extract showed an appreciable activity against *S. aures* (**MIC** 128 µg/ml) as compared to *E. coli (***MIC** >256 µg/ml).

3.6.3 Cytotoxic assay

Invitro cytotoxic assay of pet-ether extract of *Inula racemosa* was performed following sulpharhodamine-B assay against human cancer cell lines namely THP-1 (leukaemia), PC-3 (prostrate), HCT-15 (colon), and A-549 (lung), which revealed increase in growth of inhibition during 48 hour incubation at concentration of 100 µg/ml and 50µg/ml of sample. DMSO was used as a negative control, and was used as a solvent to dissolve sample homogenously. The result of cytotoxic activities is depicted in the table 3.4.

Table 3.4 Cytotoxic activity of Pet Ether extract of *Inula racemosa* against various human cancer cell lines

Tissue type			**Leukaemia**	**Prostrate**	**Colon**	**Lung**
Cell line type			**THP-1**	**PC-3**	**HCT-15**	**A-549**
S. No	Test samples	concentrations	% growth inhibition			
1	Pet ether extract	100µg/ml	**79**	56	64	**73**
		50 µg/ml	**56**	34	35	**38**
2	(Standard) 5-Fluoro uracil	10^{-5} M	92	43	90	62

The result depicted that Pet ether extract of *Inula racemosa* imparted significant cellular cytotoxic effects on all cell lines that were tested. However most promising results were obtained against cell lines THP-1 and A-549 corresponding to the tissue type leukaemia and lung respectively.

3.7 Isolation and characterisation

Observation based on literature /present studies

Inula racemosa is a rich source of sesquiterpenoids and several sesquiterpene lactones from this species have already been reported by the previous workers. However the

present investigation afforded the isolation of Alantolactone using column chromatography and TLC and its chemical transformation to study the Structure Activity Relationship (SAR) of the compound.

3.7.1 Identification of C-1

Hydro distillation of fresh rhizomes of *Inula racemosa* yield a crystalline substance N-2 and cold extraction of dried rhizomes of the same plant with petroleum ether yield M-1 which on column chromatography yield N-1. N-1 and N-2 were found to be identical, based on their co-TLC behaviour in various solvent systems.

Re-column chromatography of N-1 on $AgNO_3$ impregnated column, yield C-1, which was characterised using various spectral techniques.

Compound C-1

Physical state: White crystalline compound. Crystallized in pet ether-ethyl acetate (1:1)

Melting point: 77 – 78 ^{o}C

UV- λ max ($CHCl_3$): 211 nm

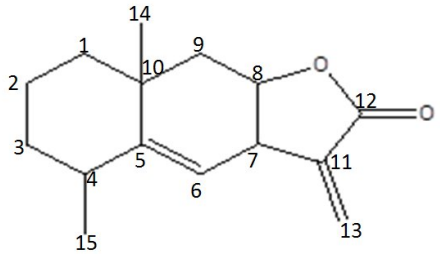


Fig 3.4 Structure of Alantolactone

The structure of C-1, based on comparative study of its physical constants and spectral data with that given in literature, suggests its identity with alantolactone (Fig 3.4)

Spectroscopic studies

IR Spectra

IR is characterised by a prominent band at 1740 cm^{-1}, characteristic of α-β-unsaturated-γ-lactone, besides other peaks at 2860, 2921, 2951 and 2981cm^{-1} which corresponds to C-H stretching of $–CH_3$ group. Another prominent peak at 1245 cm^{-1} in the fingerprint region (500-1500 cm^{-1}) corresponds to O-C stretching. Peak at 1655 cm^{-1} and corresponding peak at 890 cm^{-1} corresponds to the presence of exocyclic double bond in the molecule. (Fig. 3.5)

^{13}C Spectra

The spectra shows about 15 peaks which means the molecule may be C-15 moiety. Peak at 172 ppm corresponds to carbonyl carbon of lactone. The presence of vinyl carbons in the molecule is represented by resonance at 141, 152, 125 and 128 ppm. (Fig. 3.6)

^{1}H Spectra

The spectra show prominent peaks at δ 1.12 (d) and δ 1.54 (s) which corresponds to the methyl protons. Resonance at 6.17 (d), 5.59 (d), 5.12 (d) corresponds to the presence of vinyl protons in the molecule. (Fig.3.7)

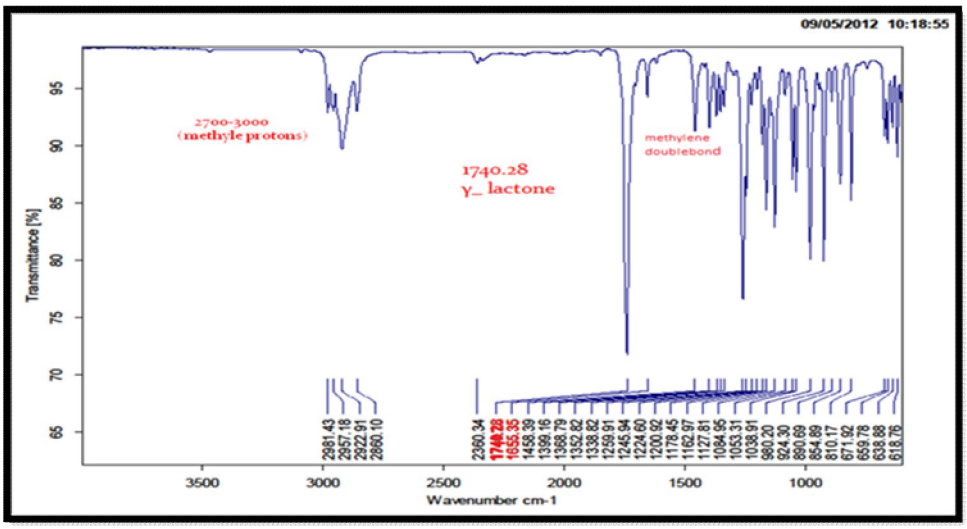


Fig 3.5 IR spectra of C-1

Prominent peaks in the spectra and their assignments

1740 cm $^{-1}$	- Corresponds to lactone carbonyl
1655 cm $^{-1}$	- Corresponds to double bond
2981 – 2860 cm $^{-1}$	- Corresponds to alkyl group

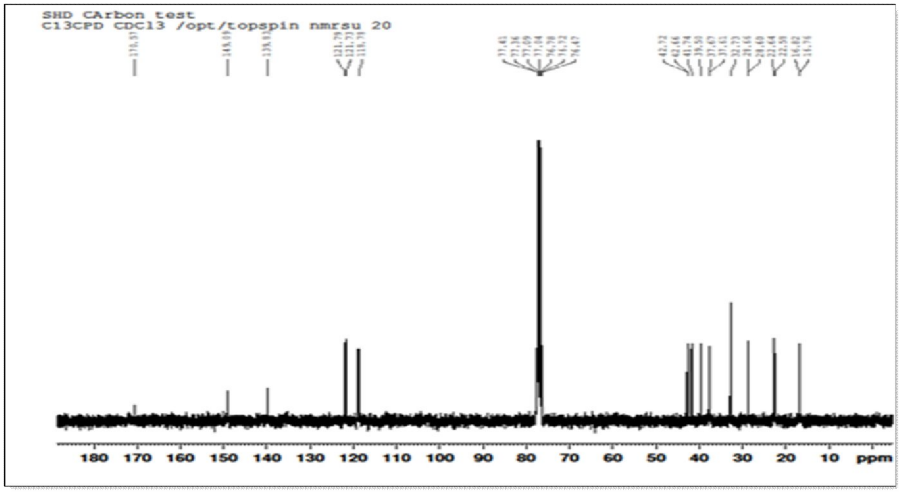


Fig 3.6: ^{13} C Spectra of C-1 in $CDCl_3$

Table 3.5 ^{13} C NMR data of C-1 ($CDCl_3$)

Position	δ C	Position	δ C	Position	δ C
1	28	**6**	120	**11**	141
2	38	**7**	41	**13**	125
3	23	**8**	78	**14**	18
4	34	**9**	46	**15**	22
5	150	**12**	172		

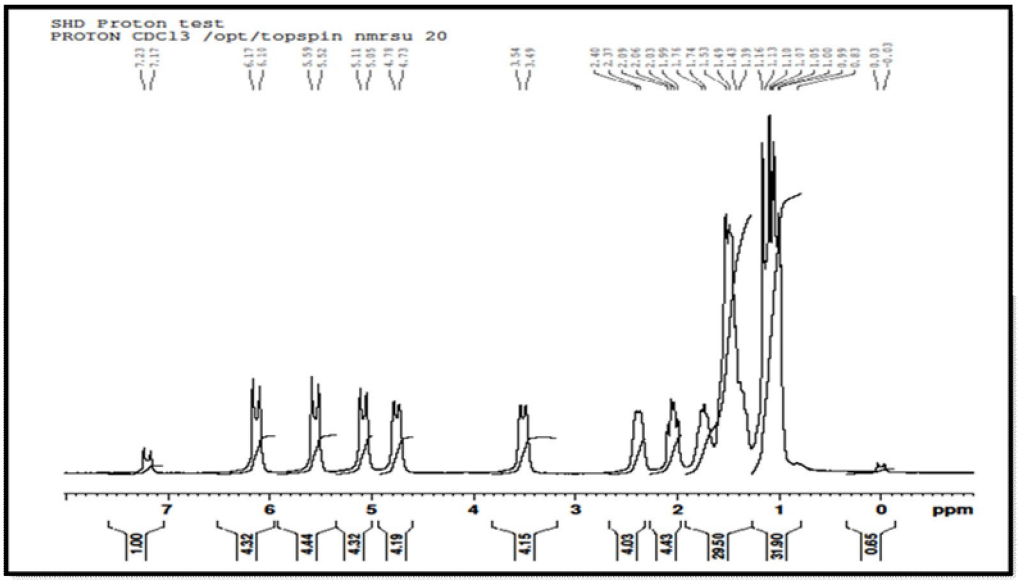


Fig 3.7: ^{1}H Spectra of C-1 in $CDCl_3$

Table 3.6: ^{1}H NMR data of C-1 ($CDCl_3$)

δ	No of protons	Multiplicity	Proton assignment
1.12	3	d	H-15
1.54	3	s	H-14
6.17	1	d	H-13a
5.59	1	d	H-13b
3.5	1	d	H-7
5.1	1	d	H-6

3.7.2 Chemical transformation of C-1

Reaction 1:

3.7.2.1 Hydrogenation Of C-1

Catalytic hydrogenation of C-1 over 10% Pd /C in ethyl acetate after usual workup yielded a crystalline compound C-2, which was UV inactive and melting point was found to be 140 - 143 ^{0}C.

Spectral data

IR Spectra: The IR spectra exhibited prominent peaks at 2819–2928 cm $^{-1}$ characteristic of C-H stretching of methyl group in the molecule. An examination of IR spectra of C-2 shows the absence of prominent bands at 1655 cm $^{-1}$ and 890 cm $^{-1}$ due to exocyclic double bond, thus the presence of such bond in C-2 is ruled out. Shift of the band from 1740 cm^{-1} in C-1 to 1755 cm^{-1} in C-2 (saturated lactone) further supports the formation of hydrogenated product C-2. Based on comparative spectral studies and melting point 140-143 ^{o}C, with those given in the literature, C-2 is suggested to be tetrahydroalantolactone. [109] (Fig 3.8)

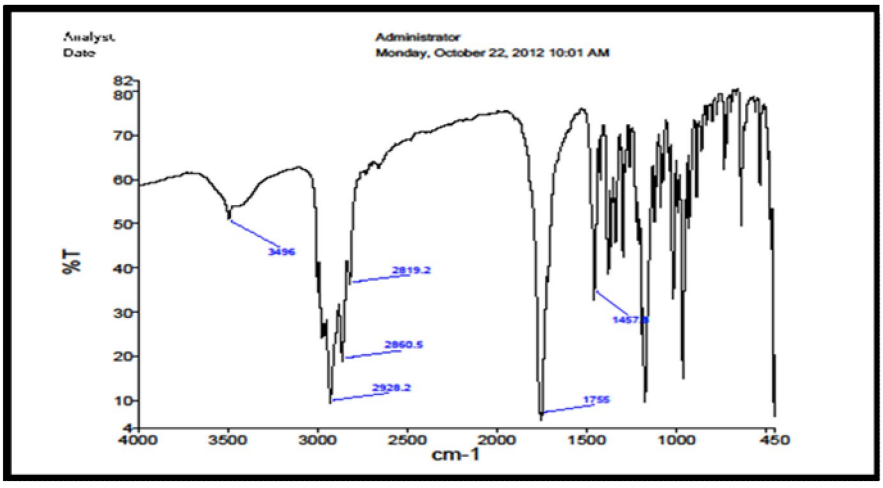


Fig 3.8: IR Spectra of C-2

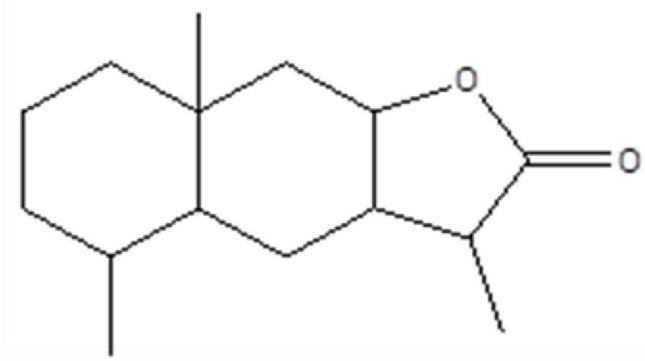


Fig 3.8.1 Structure of C-2

Reaction II

3.7.2.2 $LiAlH_4$ reduction of C-2:

Hydrogenated product C-2, on reduction with $LiAlH_4$ in dry THF and usual workup at room temperature yielded a compound C-3, with the melting point of 107^{o}C.

Spectral data:

A prominent band visible in the region 2800-2900 cm^{-1} of the IR spectrum (Fig.3.6), corresponds to the presence of methyl group, a characteristic broad band at 3300 - 3400 cm^{-1} and corresponding peak at 1040 cm^{-1} corresponds to the presence of hydroxyl group in the molecule. Also absence of the peak at 1755 cm^{-1} in the IR spectrum of C-3 suggests that lactone ring has now been cleaved. $LiAlH_4$ reduction of C-2 and the spectral data of the product further substantiates proof in favour of the assigned structure to C-2 as tetrahydroalantolactone. Hence the possible structure of the diol could be as shown in Fig.3.7

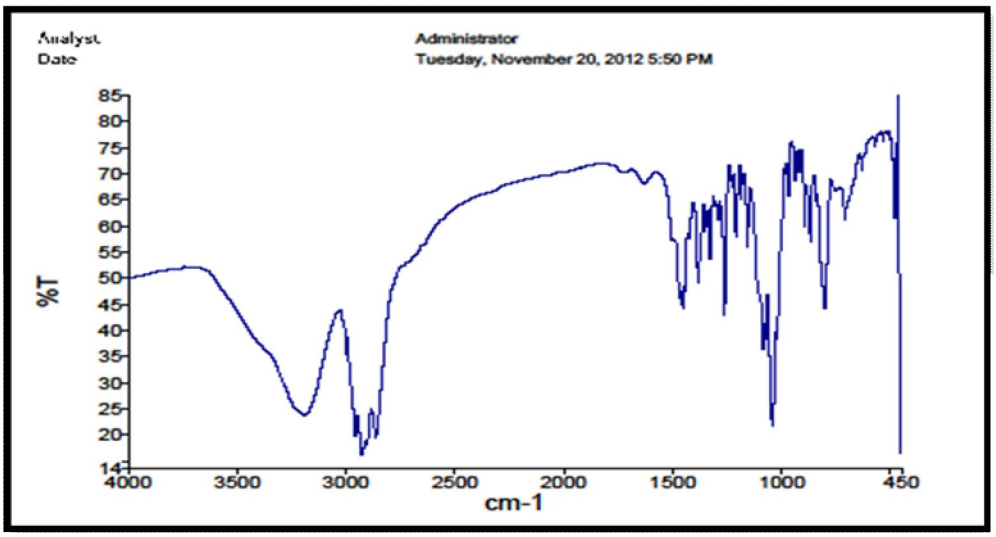


Fig 3.9: IR Spectra of C- 3

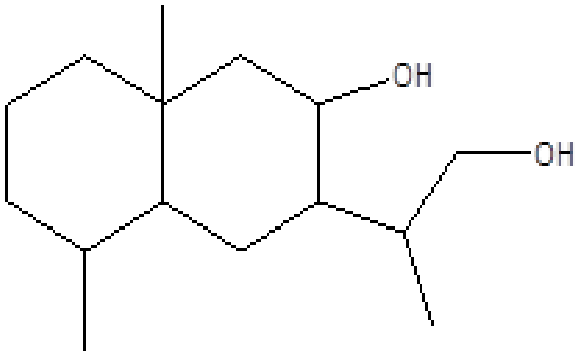


Fig 3.8.2 Structure of C-3

3.8 Experimental

3.8.1 Hydrodistillation

1.5 kg rhizomes of *Inula racemosa* was subjected to hydrodistillation for 5 hours in a 5 litre flask, fitted with a Clevenger type apparatus. After 3 hours some solid substance (28.5 mg) got separated out. This solid substance upon crystallization with (pet-ether-ethyl acetate) resulted in the isolation of some needle like crystals (N-2) which were found to be pure on TLC.

3.8.2 Cold extraction

Dried rhizomes (3 Kg) of the same plant were grind to small pieces and then subjected to cold extraction with Pet-ether for 90 hours. The extract was then vacuum evaporated with rota vapour which resulted in the isolation of some golden brown residue (34 g), giving an extract value of 1.1%. The extract was solvent extracted with petroleum ether many times, with the result some crystalline, solid residue (M-1) was obtained and the solvent was steam distilled to yield a viscous oil (M-2).

3.8.3 Isolation and separation:

3.8.3.1 Column chromatography

The mixture M-1 was analysed by TLC. It showed the presence of some complex mixture of various components which gave overlapping spots in polar, non-polar and combination of both solvent systems. The extract was then dissolved in a minimum amount of DCM and adsorbed on silica gel to form slurry. The dried slurry was then loaded on column having height 6 ft. and diameter 2 inches. Silica gel 60- 120 mesh was used as adsorbent. The column was first eluted with pure pet-ether. Fractions were continuously monitored with TLC in various solvent systems. First 15 fractions gave no spots on TLC, hence were discarded. Polarity of the eluent was increased to

1% (chloroform in pet- ether) and various fractions then collected showed the presence of single spot on TLC. These fractions were pooled, distilled and left to self-crystallize at room temperature which afforded (2.4 g), extra pure, white crystalline compound (N-1). M.p 54 ^{0}C. N-1 showed identical TLC behaviour as (N-2) in different solvent systems, indicating N-1 and N-2 the same compounds.

3.8.3.2 Re-column of N-1

The crystalline mixture (N-1) was analysed on 1% $AgNo_3$ impregnated TLC [110] where the single spot got resolved into two, R_F values 0.67 and 0.37 (pet ether- ethyl acetate 9:1), indicating (N-1) to be the mixture of two compounds (probably the isomers). In order to separate the isomers, the crystalline mixture (N-1) was subjected to repeat column chromatography on 1% $AgNo_3$ impregnated silica gel. The column was wrapped with silver foil, so as to avoid its exposure to light which may cause decomposition of $AgNo_3$. The slurry was prepared in DCM and the column loaded with this slurry was eluted with Pet-ether. First 7 fractions (25 ml) each showed an identical TLC behaviour, with a single spot for one of the isomer separated. These fractions were pooled and vacuum evaporated, leaving behind a colourless residue which upon crystallization with Pet-ether and Ethyl acetate afforded an extra pure crystalline compound (C-1). Elution of the column after analysis on TLC, showed the presence of both the isomers, hence they were pooled and discarded.

3.8.4 Chemical transformation (Derivatization)

3.8.4.1: Reaction I

Catalytic hydrogenation Of C-I

700 mg of C-1 was subjected to catalytic hydrogenation over 10% Pd /C in ethyl acetate. The reaction was carried out at room temperature and high pressure. The course of the reaction was monitored with the help of TLC and ceric ammonium

sulphate as visualizing agent. Catalyst was removed from the reaction mixture by filtration and filtrate was vacuum evaporated leaving behind a white residue, which on crystallization with Pet ether- Ethyl acetate yielded a compound C-2 M. p 140 - 143 ^{o}C. (570 mg)

3.8.4.2 Reaction II

$LiAlH_4$ reduction

This reaction was specifically carried out in high purity dry solvent, THF (tetrahydrofuran). 305 mg of C-2 was dissolved in dry THF and 70-100 mg of $LiAlH_4$ was added slowly, at room temperature .The course of the reaction was monitored with the help of TLC. Once the reaction was complete (as revealed by the TLC), the reaction mixture was diluted with ethyl acetate (work up), in order to remove excess of $LiAlH_4$.[111-112] After the usual work up, the filtrate (THF) was vacuum dried, leaving behind a white solid which upon crystallization with pet ether-ethyl acetate yield a compound C-3. M. p 107 ^{o}C (232 mg).

Summary

Summary

The main objective of the present study was phytochemical investigation of two aquatic species of *Nymphaea* in Kashmir, i.e. *Nymphaea alba* and *Nymphaea mexicana* followed by bioactivity evaluation and the comparative study of antioxidant, anticancer and antimicrobial activity. The powdered plant material (flowers) of the species was extracted with solvents in order of their increasing polarities and then these extracts were subjected to bioactivity evaluation. Free radical scavenging activity of these extracts was measured in terms of hydrogen donating ability, using stable DPPH radical, while anticancer activity was determined by SRB (Sulpharhodamine B) assay against the human cancer cell lines which include THP-1 (leukaemia), A-549 (lung), colon (HCT-15) and prostrate (PC-3). 5-Fluoro Uracil was used as the positive control. While antimicrobial activity was tested against *S. aures, C. albicans, A. fumigatus and E.coli* following **M**inimum **I**nhibitory **C**oncentration assay. Ciprofloxin was used as the positive control. The results of DPPH free radical scavenging assay suggest that methanol, ethyl acetate and aqueous extract of both species show most promising antioxidant activity while Pet ether extract in both species show least activity. A comparative study of EC_{50} values of active extracts with the standard Quercetin further suggests that Aqueous extract of *N. mexicana* is the most active extract with Ec_{50} value of 3µg/ml as compared to Quercetin with E_C50 value of 1.66µg/ml. The active extracts especially aqueous extract of *Nymphaea mexicana* and methanol extracts of both species possess strong free radical scavenging activity which could exert a beneficial action against liver damage and many other ailments induced by different exogenous and endogenous sources. Out of all the extracts, only aqueous extract of *N. mexicana* was found to be active and showed promising antibacterial activity against *Staphylococcus Aures (ATCC 29213).*

The antibacterial activity of the active sample (aqueous extract) of *Nymphaea mexicana* was expressed *in terms of* **M**inimum **I**nhibitory **C**oncentration (MIC) in µg/ml against the standard *Ciprofloxin***.** The **MIC** value was found to be 32µg/ml. The results of anticancer activity, depict that with 100µg/ml and 50µg/ml of extract dissolved in DMSO imparted significant cellular cytotoxic effects on almost all the cell lines that were tested. However the most appreciable results were displayed by ethyl acetate and methanol extract of *Nymphaea mexicana* while in *Nymphaea alba* aqueous, chloroform and pet ether extracts showed most promising activity. The demonstration of promising cytotoxic and antioxidant activity by various extracts of *Nymphaea mexicana* and *Nymphaea alba* may help to discover new chemical classes of cytotoxic and antioxidant substances that could serve as selective agents for chemotherapy and control. Results of these studies indicate that further screening and characterization of *Nymphaea alba* and *Nymphaea mexicana* for these compounds are warranted. As drug resistance becomes an increasing problem and consumer demand for products with natural preservatives grows on each passing day, therefore *Nymphaea alba* and *Nymphaea mexicana* can form the basis of future anticancer research efforts.

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Appendices

Appendix I

List of tables

Appendix II

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Appendix III

List of symbols and abbreviations

ATC	American type culture collection
CC	Column Chromatography
$CDCl_3$	Deuterated chloroform
CFU	Colony forming Units
cm^{-1}	Centimetre Inverse
^oC	Degree Celcius
d	Doublet
DEPT	Distortionless Enhancement By Polarization Transfer
DMSO	Deuterated Dimethyl Sulphoxide
Hz	Hertz
IR	Infrared
J	Coupling Constant
m	Multiplet
M	Molecular Mass
mg	Milligram
µg	Microgram
MHz	Mega Hertz
MIC	Minimum Inhibitory Concentration
Ml/ml	Milliliter
mM	Millimolar
M.p	Melting Point
NMR	Nuclear Magnetic Resonance
ppm	Parts Per Million
Rf	Retention factor
s	Singlet
TLC	Thin Layer Chromatography
UV	Ultra Violet
α	Alpha
β	Beta
δ	Delta
λmax	Lambda maximum

Appendix IV
Phytochemical Tests
Tests For Steroids and Terpenoids:

Libermann Burchards Tests: Treat the extracts with a few drops of acetic anhydride, boil and cool. Then add concentrated sulphuric acid, from the side of the test tube, brown ring is formed at the junction of two layers and upper layer turns green which shows presence of steroids and formation of deep red colour shows the presence of Triterpenoids.

Salkowski Test: Add few drops of concentrated sulphuric acid to the extract, red colour at the lower end indicates the presence of steroids and formation of yellow colour indicates the presence of Triterpenoids.

Tests for Flavonoids:

To an aqueous filtrate of the extract, add 5ml of dilute ammonia and 1ml of concentrated sulphuric acid. A yellow colouration that disappeared on standing indicate the presence of flavonoids.

Tests for glycosides:

To a small quantity of the extract , add 1 ml of water , followed by the addition of NaOH solution. Appearance of Yellow colour indicate the presence of glycosides.

Tests for alkaloids:
Draggendorff's test:
Dissolve few mg of extract in 5 ml of distilled water. Add 2ml HCl until an acid reaction occurs, then add 1 ml of Draggendorff reagent, formation of orange red precipitate, indicate the presence of alkaloids.

Appendix V
Spray Reagents:

i. **Anisaldehyde-sulphuric acid reagent:** 0.5ml anisaldehyde is mixed with 10 ml glacial acetic acid, followed by 85ml methanol and 5ml concentrated sulphuric acid.

ii. **Cerric Sulphate reagent:** 1 gm of $Ce(SO_4)_2$ is dissolved in 10 ml of concentrated H_2SO_4 and made upto 100ml with distilled water.

iii. **Ferric chloride reagent**: To 5% solution of $FeCl_3$, Ammonium hydroxide solution is added with shaking drop by drop till the permanent precipitate is obtained.