PHYTOCHEMICAL SCREENING, ISOLATION, CHARACTERIZATION & BIOLOGICAL EVALUATION OF ETHANOLIC EXTRACT OF NELUMBO NUCIFERA LEAF

Dissertation submitted to

The Tamil Nadu Dr. M.G.R. Medical University
Chennai-600 032

In partial fulfillment of the requirements
for the award of the degree of

MASTER OF PHARMACY

APRIL-2012

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

COLLEGE OF PHARMACY
MADURAI MEDICAL COLLEGE
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CERTIFICATE

This is to certify that the dissertation entitled "***PHYTOCHEMICAL SCREENING, ISOLATION, And CHARACTERIZATION & BIOLOGICAL EVALUATION OF ETHANOLIC EXTRACT OF NELUMBO NUCIFERA LEAF"*** was done by **Mr. M. AMARNATH, (Reg. No: 26108631)** in the department of pharmaceutical chemistry, College of Pharmacy, Madurai Medical College, Madurai-625020, in partial fulfillment of the requirement for the Degree of Master of pharmacy in pharmaceutical chemistry under my guidance and supervision for academic year 2011-2012.

This dissertation is forwarded to the Controller of Examination, The Tamil Nadu Dr. M. G. R. Medical University, and Chennai.

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Date:

DR. (Mrs.) R. Ajithadas Aruna, M.Pharm, PhD.,
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Madurai-20

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This dissertation is forwarded to the Controller of Examination, The Tamil Nadu Dr. M. G. R. Medical University, Chennai.

Station: Madurai **DR. (Mrs.). Ajithadas Aruna, M.Pharm, P.hD.**

Date:

Evaluation Certificate

Internal Examiner

External Examiner

ACKNOWLEDGEMENT

I deem our great privilege to acknowledge all this eminent personalities who helped us throughout endeavor.

I extremely thankful to **Dr. A.EDWIN JOE, M.D.(F.M), Dean**, Madurai Medical College, for motivating us with constant encouragement and suggestions to complete this work successfully.

I cordially express my sincere thanks to **DR. (Mrs.) Ajithadas Aruna, P.hD.,** Principal & Head of the Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai for the support and encouragement of this project work.

It is my humble privilege to express to our heartfelt thanks to **Prof**. (**Mrs.) R.Tharabai, M.Pharm**, Professor and Head of the Department of Pharmaceutical Chemistry, College of Pharmacy, Madurai Medical College , Madurai for giving this opportunity to work under her guidance, incessant encouragement, support in topic selection, supervision and completion of my project work in a successful manner.

I also express my heartfelt thanks to the tutors of department of pharmaceutical chemistry, College of Pharmacy Madurai Medical College, Madurai, Mrs. G. Umarani, M. Pharm., Mrs. G. Tamilarasi, M. Pharm., Mr. P. Sivasubramaniyan, M. Pharm.

I express my special thanks to Dr. Jonard, M.V.Sc., Officer in charge, Central Animal house, Madurai Medical College for his valuable support in completion of my project work.

I extend my thanks to Mrs.Indira, B.Sc., Mrs.Dharamambal, B.Sc., Mrs.Lalitha, B.Sc., lab supervisors, Department of Pharmaceutical Chemistry, Madurai Medical College.

I also my express thanks Mrs. Radha, DMLT, Mrs. Sofiya, and DMLT, lab technicians of Department of Pharmaceutical Chemistry, MMC, and Madurai.

I also express special word of thanks to my juniors Mr. T. Thirumalai Nambi, Mr. K. Yuvarajan, Mr. R.Jegadesh, and Miss. J.Soniya in the department of pharmaceutical chemistry and all other department juniors of College of Pharmacy, Madurai Medical College ,for their fruitful discussion and endless help to complete this work successfully.

I express my special thanks to Mr.Joneskumar for supplying the necessary chemicals.

I express my special thanks to all members of, Bose Chemical Laboratory, Madurai for their help to carry out the Antimicrobial Studies. I deem it to my great privilege to be able to acknowledgement this entire endeavor.

AUTHENTICATION CERTIFICATE

Dr. D. STEPHEN M. Sc, Ph.D.,
Taxonomist
Department of Botany
American College (Autonomous)
Madurai

No: CEPSAM/01/2011 Date: 10.11.11

To,

Mr. M. Amarnath,
II yr M. Pharmacy,
College of Pharmacy,
Madurai Medical College,
Madurai.

Sir,

The plant specimen brought for identification is identified as NELUMBO NUCIFERA –FAMILY- NELUMBONACEAE.

Thanking you

Yours faithfully,

(Dr.D.STEPHEN M.sc.ph.D)

Dr. D. STEPHEN, Ph.D.,
ASST. PROFESSOR IN BOTANY
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ANIMAL ETHICAL COMMITTEE CLEARENCE CERTIFICATE

Ref. No. 14024 / E1/4 /2011

ETHICAL CLEARANCE CERTIFICATE

DR. A. Edwin Joe M.D, Dean& Chairman, Animal Ethical committee, Madurai Medical College, Madurai, hereby endorse ethical clearance to the proposal.

""Phytochemical Screening and Anti Inflammatory study of extract of Nelumbo nucifera"

Submitted by

Mr.M.Amarnath,
Post Graduate Student,
Department of Pharmaceutical Chemistry
Madurai Medical College, Madurai.

The study did not violate the regulations and guidelines prescribed by ICMR and are within the permitted norms of animal experimentation in this country. The out come of the study may be beneficial to the human and animals.

Dean& Chairman

Date: .01.2012
Place: Madurai

Office seal:

DEAN, MADURAI MEDICAL COLLEGE
MADURAI-20.

CONTENT

LIST OF ABBREVATIONS

1. ENN - Ethanolic extract of *Nelumbo nucifera*

2. DPPH - (1,1-Diphenyl-2- picryl hydrazyl)

3. V/V - Volume by volume

4. HANN – Hydro alcoholic extract of *Nelumbo nucifera*

5. *HSCCC* - High speed counter current chromatography

6. NMR - Nuclear Magnetic Resonance

7. IR - Infra red values

8. MRSA- Methyl resistance staphylococcus areus

9. CMC - Carboxyl methyl cellulose.

10. SEM - Standard mean error

INTRODUCTION

Introduction

Herbal Medicine sometimes referred to as Herbalist or Botanical Medicine, is the use of herbs for their therapeutic or medicinal value. An herb is a plant or plant part valued for its medicinal, aromatic, or savory qualities. Herb plants produce and contain a variety of chemical substances that act upon the body.

Herbal medicine is the oldest form of healthcare known to mankind. Herbs had been used by all cultures throughout history. It was an integral part of the development of modern civilization. Primitive man observed and appreciated the great diversity of plants available to him. The plants provided food, clothing, shelter, and medicine. Much of the medicinal use of plants seems too been developed through observations of wild animals, and by trial and error. As time went on, each tribe added the medicinal power of herbs in their area to its knowledgebase. They methodically collected information on herbs and developed well-defined herbal pharmacopoeias. Indeed, well into the 20th century much of the pharmacopoeia of scientific medicine was derived from the herbal lore of native peoples. Many drugs commonly used today are of herbal origin. Indeed, about 25 percent of the prescription drugs dispensed in the United States contain at least one active ingredient derived from plant material. Some are made from plant extracts; others are synthesized to mimic a natural plant compound.

The World Health Organization (WHO) estimates that 4 billion people, 80 percent of the world population, presently use herbal medicine for some aspect of primary health care. Herbal medicine is a major component in all indigenous peoples' traditional medicine and a common element in Ayurvedic, Homeopathic,

Naturopathic, traditional oriental, and Native American Indian medicine. WHO notes that of 119 plant-derived pharmaceutical medicines, about 74 percent are used in modern medicine in ways that correlated directly with their traditional uses as plant medicines by native cultures. Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicinal value.

Substances derived from the plants remain the basis for a large proportion of the commercial medications used today for the treatment of heart disease, high blood pressure, pain, asthma, and other problems. For example, ephedra is an herb used in Traditional Chinese Medicine for more than two thousand years to treat asthma and other respiratory problems. Ephedrine, the active ingredient in ephedra, is used in the commercial pharmaceutical preparations for the relief of asthma symptoms and other respiratory problems. It helps the patient to breathe more easily.

Another example of the use of an herbal preparation in modern medicine is the foxglove plant. This herb had been in use since 1775. The powdered leaf of the plant, Digitalis is used as cardiac stimulant, which keeps millions of people alive.

An ancient Egyptian belief said the lotus flower gave life to the Pharaonic Egypt. At the beginning of the world, on the dark waters, a lotus flower floated with closed petals. The petals opened and out of the flower, the Sun God Ra raised, creating the world. In the evening, the Sun went to sleep In the lotus flower, just to rise again next day, Many Mediterranean and Asian civilization took the symbol of the lotus to India, Vietnam, china Laos, Cambodia and Thailand .lotus is the throne on which Buddha seats, the luck bringing collar offered in Ceylon or the passing time

in Tibet, where dalai Lama is called is called “The Master of the White Lotus”. The lotus can be the promise of a successful exam or marriage. In Buddhism, people are compared to lotus flowers, rising from the mud of the deep waters and flowers stages (bud, blossom, seed) represent the past, present and future.

A Buddhist sutra says, “Lotus combines perfumes, purity, grace, and beauty”.

Lotuses belong to the family Nymphaceae and the genus Nymphacea. The white Egyptian lotus, N.lotus, is the real lotus from the Egyptian mythology. Relict lotus population can be found in thermal springs in Europe, like in Romania. There is also a blue Egyptians lotus, N.stellata, the national flower of Ceylon. The Indian red lotus, N.rubra is common in southeastern Asia. The yellow lotus, N.citrina is common in tropical Africa.

Lotuses have huge rhizomes stuck into the mud, of the thickness of a human arm. Various aerial canals, being light and resistant, cross the stems going upward to the water’s surface. The lotus fruit resembles an Apollo special capsules and it is spongy and veracious.

The Chinese folk medicine uses lotus seeds, rhizomes, roots, and flowers against much disease. The seeds are rich in starch and are used as garnishment for roasted chicken as fortifier and, mixed in rice soup, can combat diarrhea while combined with liquorices is effective against kidney diseases. The receptacles of the seeds are used against boils.

Wine macerated flowers are used for treating internal contusions and lotus flower infusion administered at 6 clock in the evening and before sleeping chases away bad dreams, conferring a deep and relaxing sleep.

The rhizomes are employed against cystitis and in Japan, they are used for making a fortifying infusion against lungs conditions.

Inflammation

Protective response intended to eliminate the initial cause of cell injury and the necrotic cells and tissues arising from the injury. Inflammation is intimately associated with the repair process, which includes parenchyma cell regeneration and scarring

Acute inflammation

"The immediate and early response to an injurious agent"

Chronic inflammation

"Inflammation of prolonged duration (weeks or months) in which active inflammation, tissue destruction, and attempts at repair are proceeding simultaneously"

Acute inflammation major components

- Transient vasoconstriction
- Vasodilatation
- Endothelial permeability
- Extravasations' of PM

Five classic local signs of acute inflammation

- Color – vasodilatation
- Rubor – vasodilatation
- Tumor – vascular permeability
- Dolor – mediator release/PMNs
- Functio laesa – loss of function
- Heat
- Redness
- Swelling
- Pain
- Loss of function

Vascular changes

Protein exits vessels:

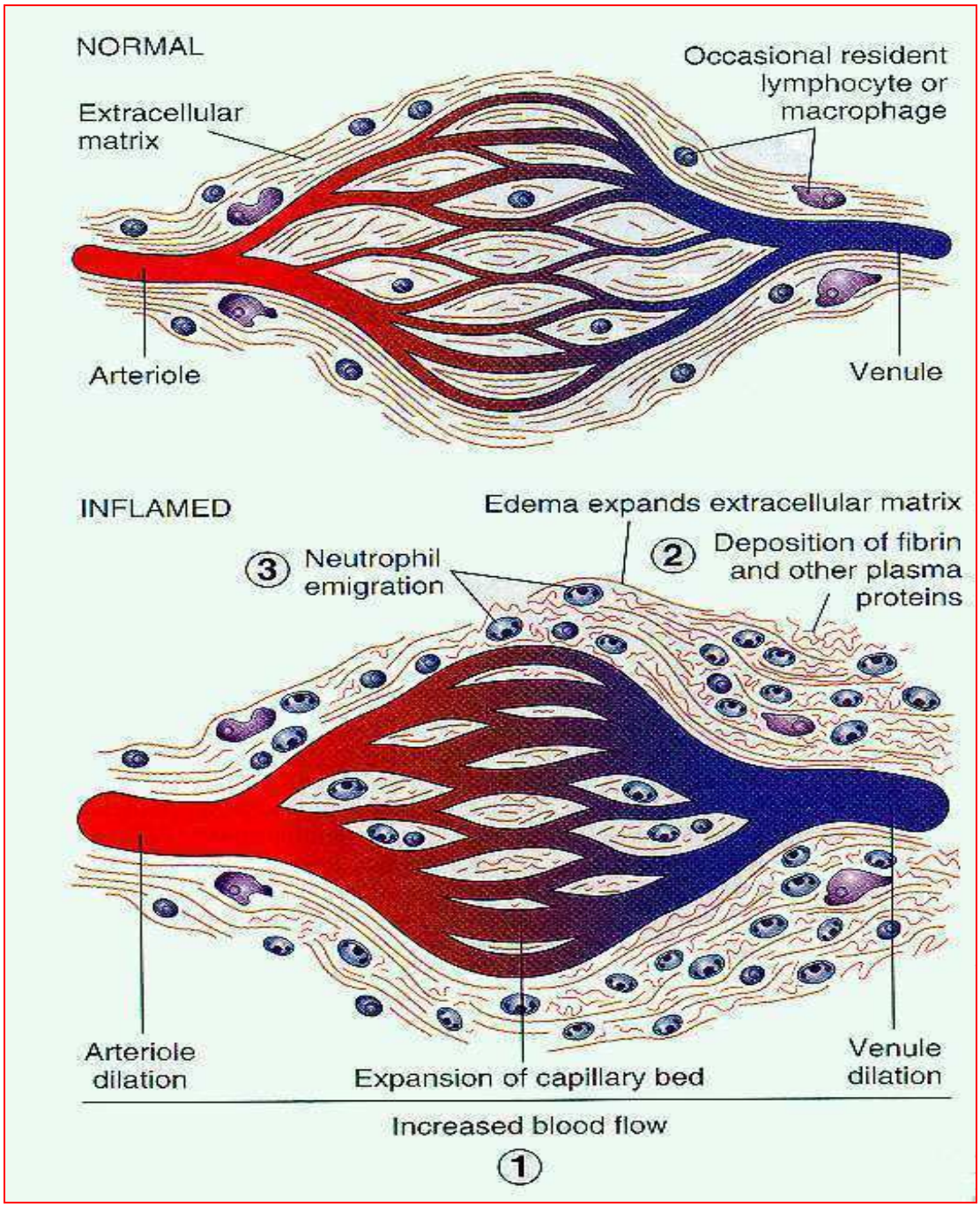
↓ Intravascular osmotic pressure

↑ Intravascular hydrostatic pressure

Endothelial gaps at intercellular junctions:

*** Immediate transient response**

*** histamine, bradykinin, leukotrienes, substance P**


NORMAL
Occasional resident lymphocyte or macrophage
Extracellular matrix
Arteriole
Venule
INFLAMED
Edema expands extracellular matrix
3 Neutrophil emigration
2 Deposition of fibrin and other plasma proteins
Arteriole dilation
Expansion of capillary bed
Venule dilation
Increased blood flow
1

Vascular permeability

Vasodilatation – increased blood flow

Increased intravascular hydrostatic pressure

Transudations - ultra filtrate blood plasma (contains little protein)

Again, this is transient and just gets the process started. Think acute inflammation, think EXUDATE

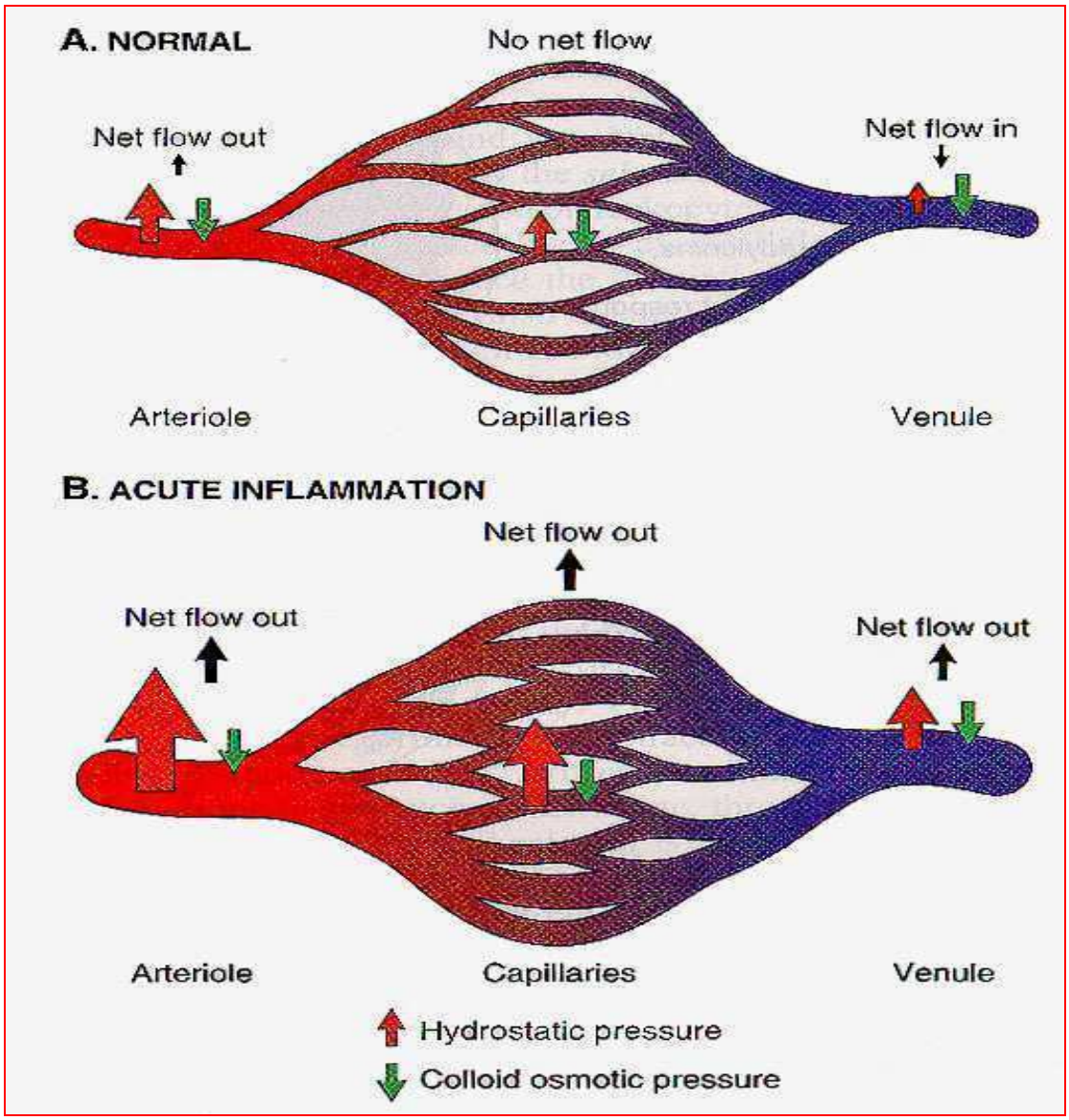
Exudates - (protein-rich with PMNs)

Exudates is the characteristic fluid of acute inflammation

Intravascular osmotic pressure decreases

Osmotic pressure of interstitial fluid increases

Outflow of water and ions - *edema*



Chemical mediators of inflammation

Plasma-derived

Circulating precursors

Have to be activated

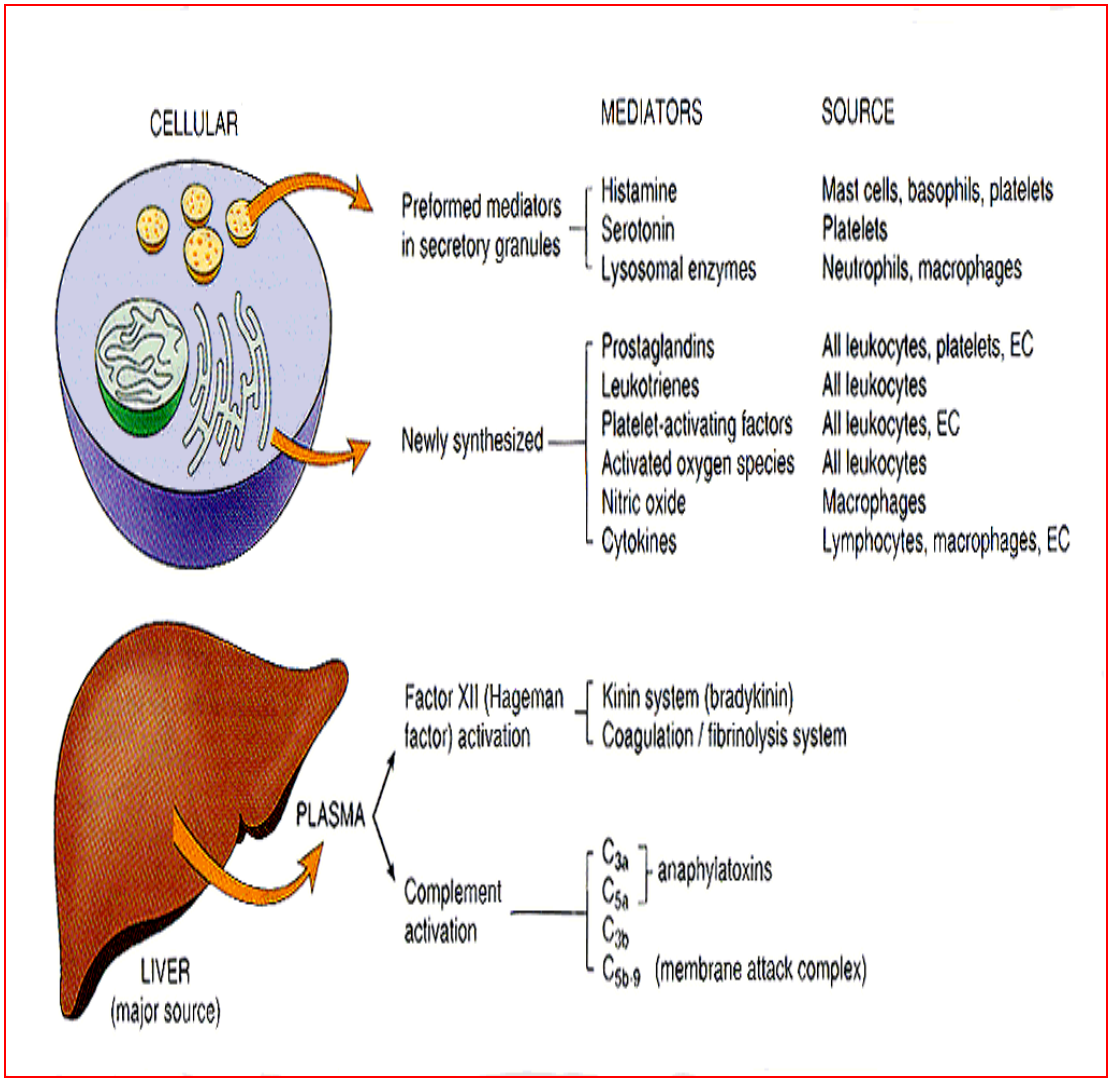
Cell-derived

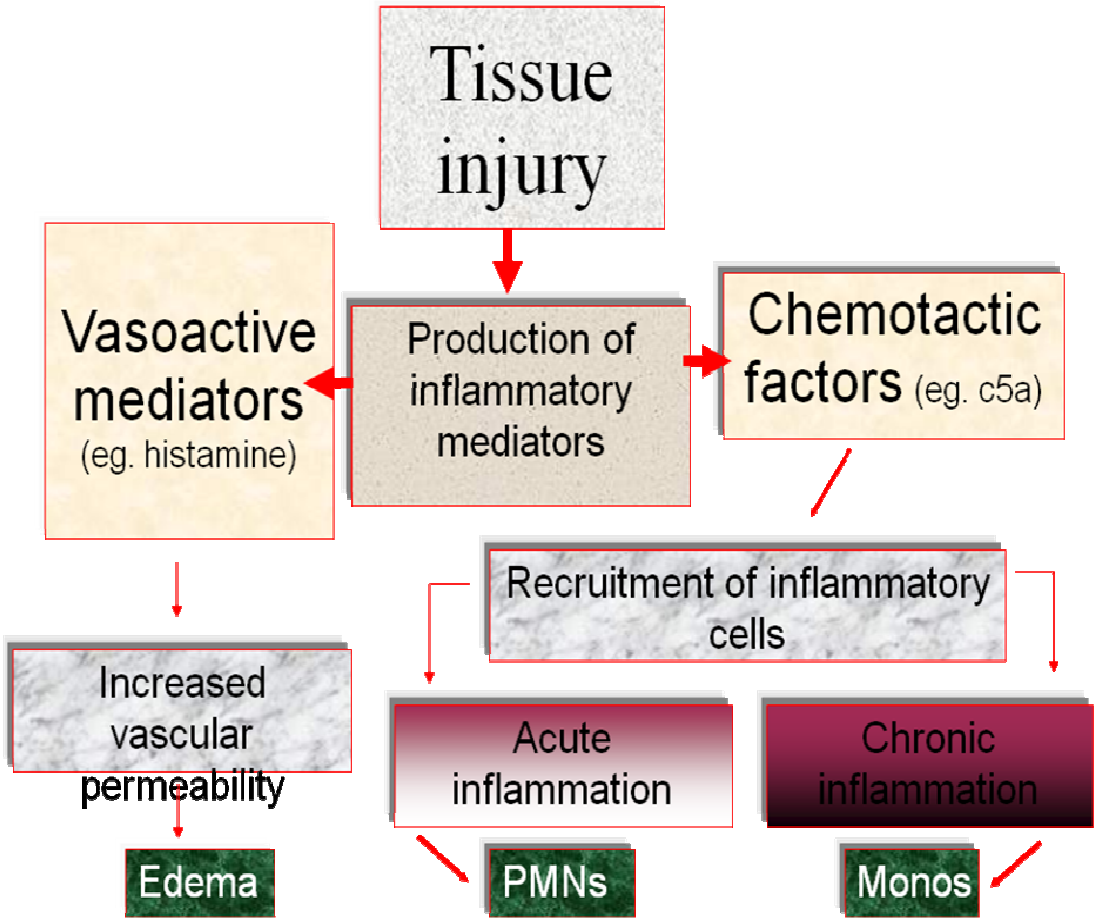
Sequestered intracellular

Synthesized de novo

Most mediators bind to receptors on cell surface but some have direct enzymatic or toxic activity

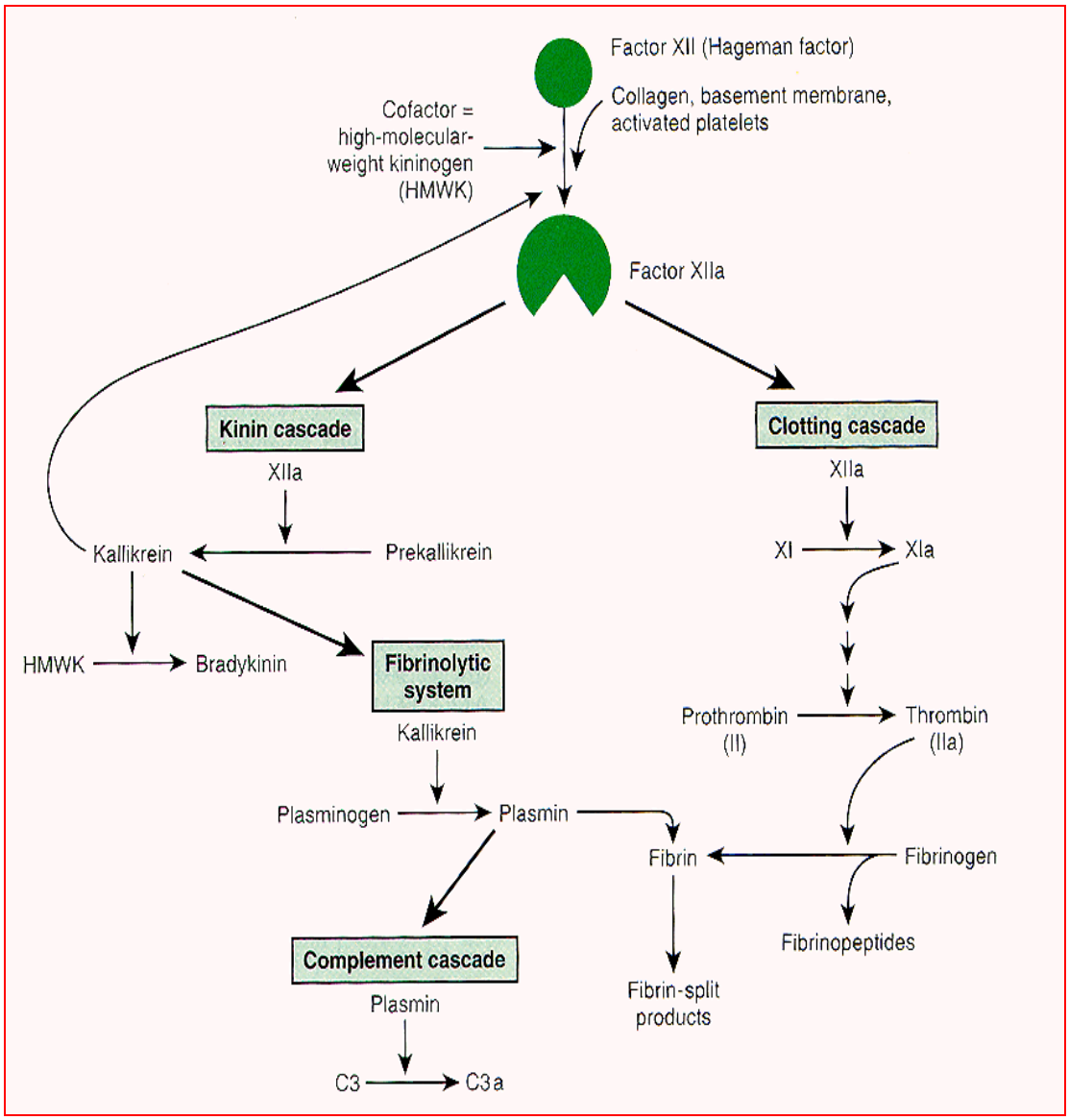
Mediators are tightly regulated





Plasma Mediator Systems - Interaction

1. **Kinin**
2. **Clotting**
3. **Complement**
4. **Fibrinolytic**



Bradykinin

Potent biomolecule

1. Vasodilatation

2. Increased vascular permeability

3. Contraction of smooth muscle

4. Pain on injection

5. Short life, kininase degrade

Factor XII activated by:

1. **Plasmin**
2. **Kallikrein**
3. **Collagen & basement membrane**
4. **Activated platelets**
5. **Co-factor = HMWK**

Vascular Permeability:

- **Bradykinin**
- **Fibrionopeptides**
- **Fibrin Split Prod.**
- **Factor Xa**
- **Leukotrienes**

AA metabolites (eicosanoids) :

Cyclooxygenases synthesize

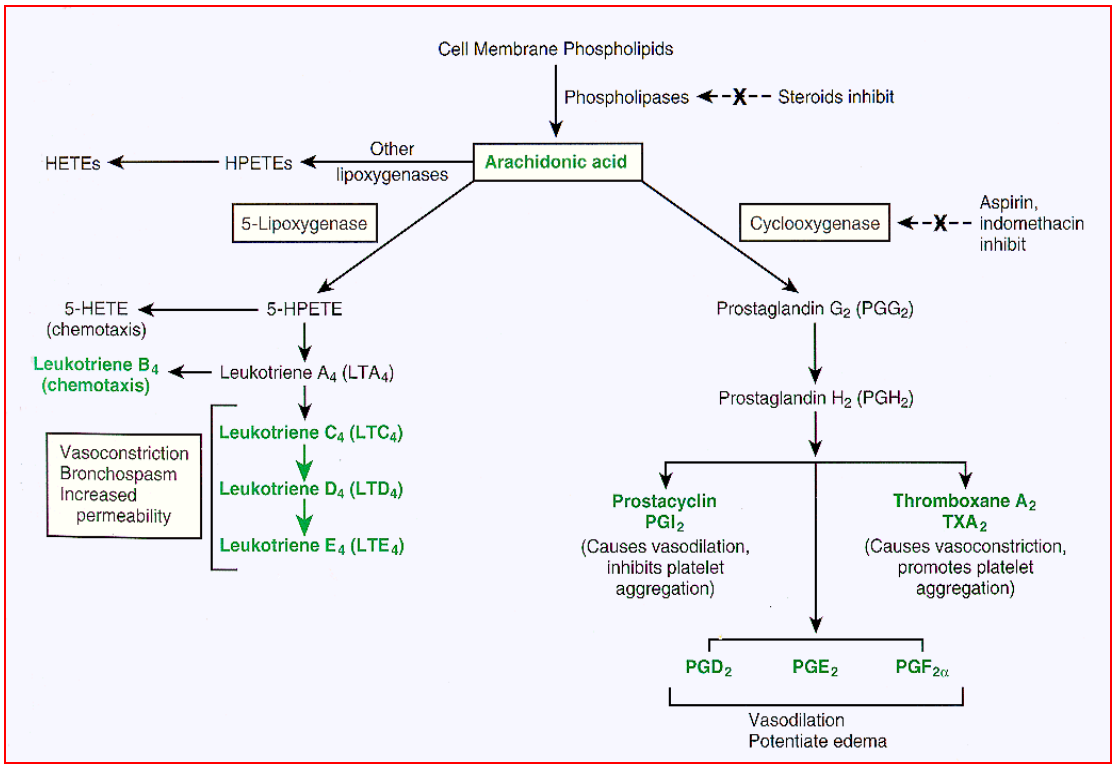
Prostaglandins

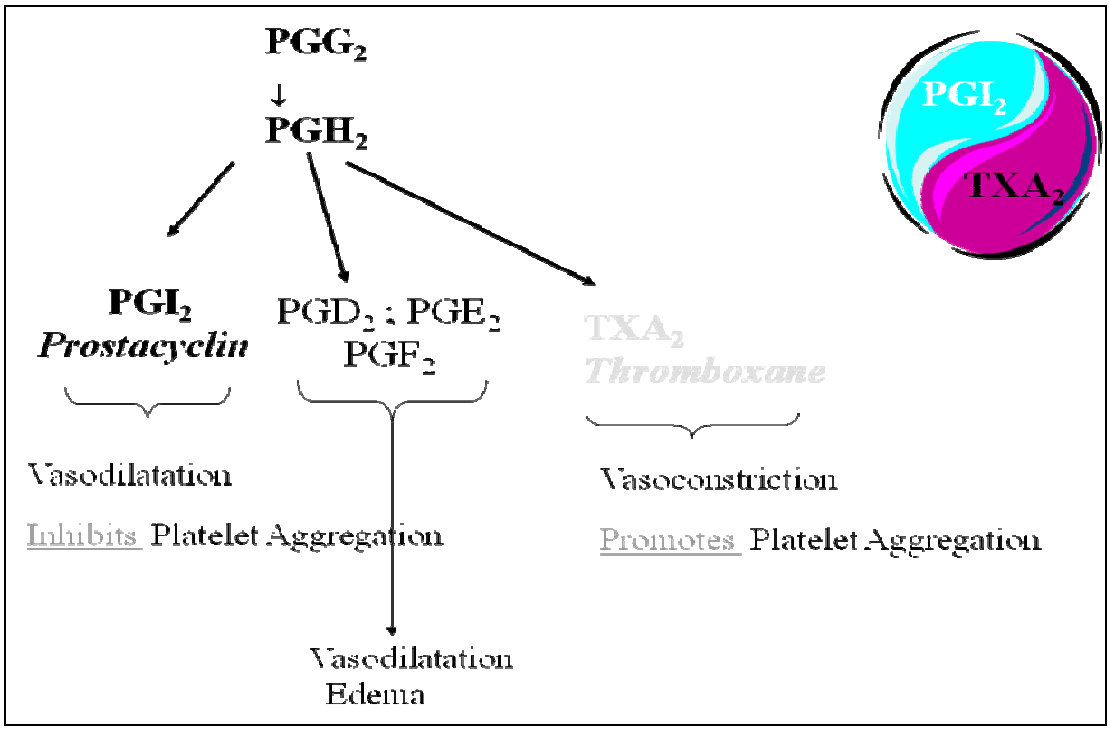
Thromboxanes

Lipoxygenases synthesize

Leukotrienes

Lipoxins


Cell Membrane Phospholipids
Phospholipases
Steroids inhibit
Arachidonic acid
Other lipoxygenases
HPETEs
HETEs
5-Lipoxygenase
Cyclooxygenase
Aspirin, indomethacin inhibit
5-HPETE
5-HETE (chemotaxis)
Leukotriene A4 (LTA4)
Leukotriene B4 (chemotaxis)
Leukotriene C4 (LTC4)
Leukotriene D4 (LTD4)
Leukotriene E4 (LTE4)
Vasoconstriction Bronchospasm Increased permeability
Prostaglandin G2 (PGG2)
Prostaglandin H2 (PGH2)
Prostacyclin PGI2
(Causes vasodilation, inhibits platelet aggregation)
Thromboxane A2 TXA2
(Causes vasoconstriction, promotes platelet aggregation)
PGD2
PGE2
PGF2α
Vasodilation
Potentiate edema


PGG2
PGH2
PGI2
TXA2
PGI2
Prostacyclin
PGD2 ; PGE2
PGF2
TXA2
Thromboxane
Vasodilatation
Inhibits Platelet Aggregation
Vasoconstriction
Promotes Platelet Aggregation
Vasodilatation
Edema

Arachidonic Acid Pathway:

Lipoxygenase

5-HETE

Chemotaxis

5-HPETE

Leukotriene generation

Leukotrienes

Vasoconstriciton

Bronchospasm

Increased vascular permeability

Cyclooxygenase

Prostaglandins

Vasodilatation

Increased vascular permeability

Prostacyclin

Vasodilatation

Inhibits platlelet aggregation

Thromboxane A2

Vasoconstriction

Promotes platlelet aggregation

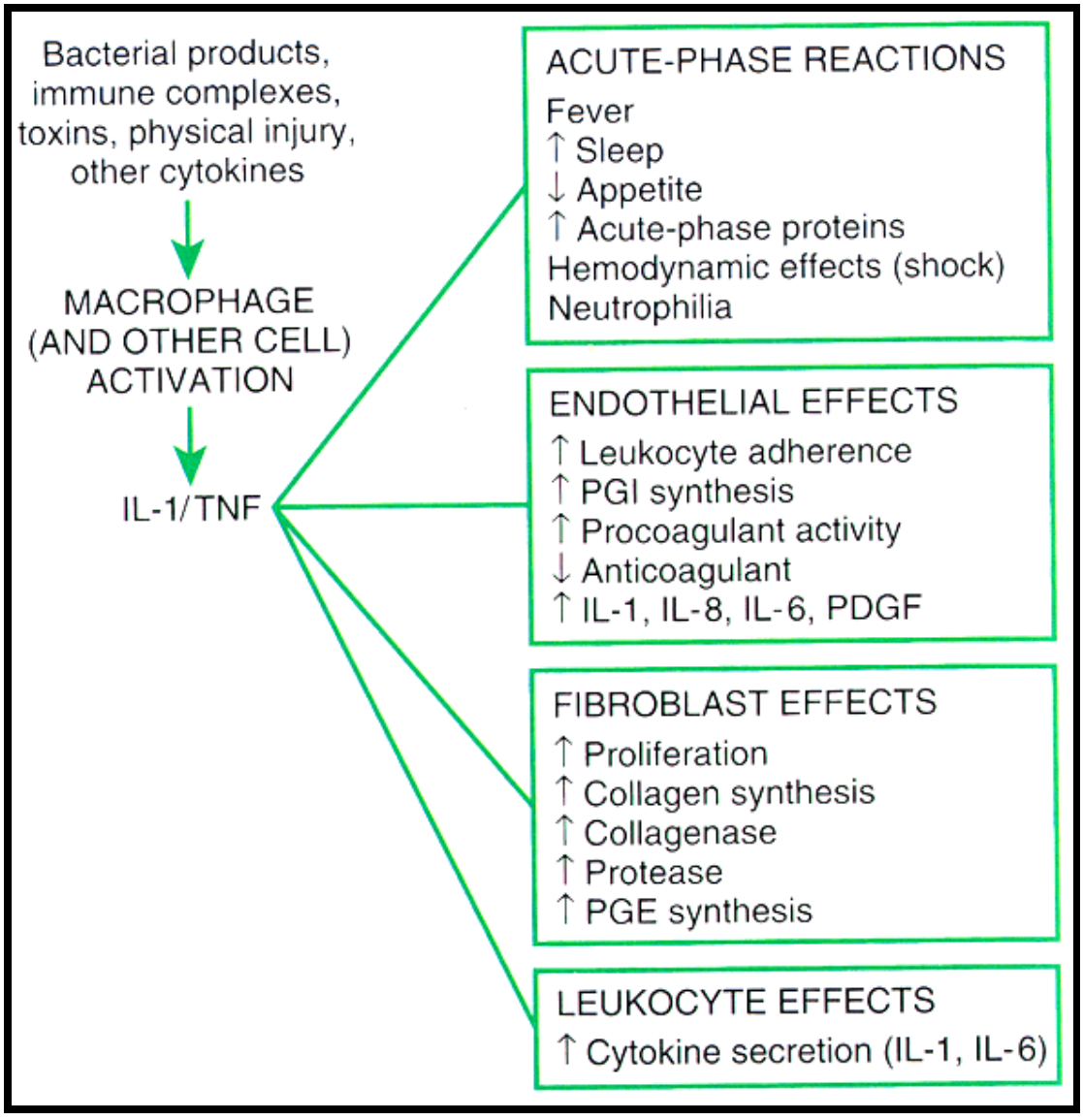
Arachidonic Acid Metabolites

Participate in every aspect of acute inflammation

Effective Anti-inflammatory agents act on AA pathways

Aspirin and Non-Steroidal Anti-inflammatory Drugs (NSAID's) - Cyclooxygenase path

Steroids act, in part, by inhibiting Phospholipase A2



Selected Inflammatory Cells & Target Cell	**Their Chemokines** Important Chemokines
Neutrophils	IL-8, Groα, β, γ, others
Monocytes	MIP-1α, MIP-1β, MCP-1,2,3
Eosinophils	Eotaxin
Lymphocytes	Lymphotaxin

LITERATURE REVIEW

LITERATURE REVIEW

Pulok k. mukherjee, Bishnu Pada saha,kakali mukherjee,Atul Wahile and Sujay Rai(2005) stated in their study that the hydro alcoholic extract of *Nelumbo nucifera* (sacred lotus) seeds exhibited strong free radical scavenging activity as evidenced by the low IC_{50} values in both DPPH and nitric oxide method. The values were found to be less than those of rutin, the standard used. Administration of HANN to *wistar* rats of about 100 to 200 mg/kg body weight for 4days prior to carbon tetrachloride (CCl_4) Treatment caused a significant dose dependent increase. These changes observed at 100mg/kg body weight treatment were comparable to that standard vitamin E at 50mg/kg treatment. *Nelumbo nucifera* seeds contains alkaloids, saponins, phenolics and carbohydrates.The results support significant antioxidant nature of HANN.

Zeyuan Deng, Shengguo Deng,Yawei Fan., *et al*., (2009) used semi-preparative high-speed counter –current chromatography (HSCCC) for successful isolation and purification of flavonoids glycosides from leaves of *nelumbo nucifera* (Lotus) by using a two-phase-solvent system (n-hexane-ethyl acetate –methanol -water(1:5:5:5v/v/v/v). The compounds were isolated, collected, purified and analyzed by using HPLC.The chemical structures of all the three compounds isoquercitrin, hyperoside and astragalin were identified by MS,H ^{1}NMR,^{13}C NMR.

Satheesh kumar, Nandini, sakthi Abirami, Indhumathy and sasikala devi (2011) states that the methanolic extract of *Nelumbo nucifera* were tested for antibacterial efficacy against three gram positive bacteria & three gram negative

bacteria and antifungal activity against three fungal strains by Disc diffusion method which showed significant antimicrobial activity against entire organism tested at the concentration of 150mcg/ml.

In this study, ciprofloxacin (100mcg/ml) and (ketakonazole 100mcg/ml) were used as the standard.

Vijai k. agnihotri, Hala N. Elsohly, shabana I.khan ,Melissa R.Jacob ,*.et al* (2008)., states that from the leaves of Nelumbo nucifera, a new compound, 24(R)-ethylcholest-6-ene-5α-ol-3-o-β-D-glucopyranoside,along with 11 known metabolites were isolated and identified by spectroscopic methods including 1D and 2D NMR. In this study the compound (R) - roemerine was subjected to anti-fungal activity against candida albicans and Anti-malarial activity was done for the same along with another compound N-Methylasimilobine. None of the compound was cytotoxic to in-vitrocells up to concentration of 23.8mcg/ml. An analysis of the structure-activity relationship shows that the substituent's in position C-1 and C-2 of aporhine are crucial for anti-malarial activity.

Brindha Venkatesh and Arthi Dorai (2011) states that the hydroethanolic extract of both white and pink Nelumbo nucifera flower extracts were evaluated at two concentration (5oomcg &1000mcg) against five bacterial strains by the disc diffusion method. The anti-bacterial activity of both N.nucifera flowers extracts was found to be increased in dose dependent manner. The result of the study suggest that white flower of N. *nucifera* extract exert strong anti-bacterial

and potent anti-oxidant activity compared to pink flower of N.nucifera which might be due to the presence of rich phytochemical constituents.

Zhongguo Yao, Li Xue Bao. 1989: They have isolated neferine an alkaloid from the seeds of Neferine, an alkaloid first isolated from the seed embryo of *Nelumbo nucifera* Gaertn in China, possesses an anti-arrhythmic action. The effects on the action potential duration (APD) and the maximal upstroke velocity (Vmax) in different driving rates, the slow response action potentials of K^+-depolarized ventricular myocardium and the ouabain-induced oscillatory potentials were studied in guinea pig papillary muscles.

Yao Xue Xue Bao. 1992; 27(12):881-5. Chinese.Wang JL, Nong Y, Jing MX. : They have Liensinine(Lien), an alkaloid extracted from the green seed embryo of *Nelumbo nucifera* Gaertn, has been shown to have anti-arrhythmic action, its mechanism may be related to blockade of Ca2+, Na+ influx. Lien 3 mg/kg i.v. may temporarily inhibit all parameters of haemodynamics in anesthetized or pithed rats.The inhibitory effects on LVP, +dp/dtmax and SAP in anesthetized rats are slightly stronger than those of quinidine (Qui) 3 mg/kg. Lien 1-30 mg/kg dose-dependently produced these actions.

Zhongguo Zhong Yao Za Zhi. 1993: They have done the Quantitative determination of liensinine in the embryo *Nelumbinis (Nelumbo nucifera Gaertn.)* by TLC-scanning.The content of liensinine in the green seed embryo of *Nelumbo nucifera* was determined by dual-wavelength TLC-scanning. The crude drug extracted with two different methods of impregnating and

refluxing. The content of liensinine was determined to be 0.853% and 0.939% and the average recovery was 97.9% and 100.9% respectively.

Ethano Botany, 1996; Methanolic extract of rhizomes of Nelumbo nucifera (NNRE) investigated for different psychopharmacological actions in rats and mice. The extract was found to cause reduction in spontaneous activity, decrease in exploratory behavioral pattern by the head dip and Y-maze test, reduction in muscle relaxant activity by rotarod, 30 degrees inclined screen and traction test and potentiated the pentobarbitone induced sleeping time in mice significantly.

Physiol Plant. 2001 May;112(1):39-46. Dry seeds of anoxia-tolerant lotus (Nelumbo nucifera Gaertn=Nelumbium speciosum Willd.) have green shoots with plastids containing chlorophyll, so photosynthesis starts even in seedlings germinated under water, namely hypoxia. Here we investigated ant oxidative enzyme changes in N. nucifera seedlings responding to oxygen deficiency. The activity of superoxide dismutase (SOD; EC 1.15.1.1), dehydroascorbate reductase (DHAR; EC 1.8.5.1) and glutathione reductase (GR; EC 1.6.4.2) were lower in seedlings germinated under water (submerged condition) in darkness (SD seedlings) than those found in seedlings germinated in air and darkness (AD seedlings).

Liu CP, Tsai WJ, Lin YL, Liao JF, Chen CF, Kuo YC (2004): They have tested ethanolic extracts of six Chinese herbs for their effects on human

peripheral blood mononuclear cells (PBMC) proliferation in vitro. The results indicated that the extracts from *Nelumbo nucifera Gaertn*, used in treatment of tissue inflammation in traditional Chinese medicine, inhibited PBMC proliferation activated with phytohemagglutinin (PHA). By a bioassay-guided fractionation procedure, NN-B-4 identified from *N. nucifera* ethanolic extracts significantly suppressed activated PBMC proliferation.The inhibitory action of NN-B-4 did not involve direct cytotoxicity.

Wu S, Sun C, Cao X, Zhou H, Hong Z, Pan Y(2004): They state that they havedone the isolation using Preparative counter-current chromatography (CCC) in isolation of liensinine and its analogues, isoliensinine and neferine from embryo of the seed of *Nelumbo nucifera* ,Had been successfully performed for the first time using upright coil planet centrifuge with four multilayer coils connected in series with 1600 mL capacity.Two kinds of two-phase solvent systems were applied to preparative CCC isolation. The first was the system composed of light petroleum (b.p. 60-90 degrees C)-ethyl acetate-tetra chloromethane-chloroform-methanol-water (1:1:4:4:6:2, v/v) which was very suitable for fast and small-scale CCC isolation.

Ling ZQ, Xie BJ, Yang EL. (2005): They state that procyanidins of nonedible parts of lotus (*Nelumbo nucifera Gaertn.)* were determined for the first time. The procyanidins of lotus seedpod were extracted with me (2) CO/H (2) O and purified by Sephadex LH-20 column chromatography, with a purity of >98%. ESI-MS analysis showed that the main molecular weight distribution of procyanidins ranged from 291 to 1155, with M + H peak values of

291.1, 579.2, 731.2, 867.2, 1019.4, and 1155.3, respectively. This indicates that the extract contains monomers, dimers, and tetramers of procyanidins, in which the amounts of dimers are greatest, and catechin and epicatechin are the base units. (1)H NMR and (13)C NMR spectra confirmed that two to four monomers are linked through C(4)-C(8) (or C(6)) bonds. The effects of the procyanidins on lipid autoxidation, lipoxygenase activities, and free radical scavenging were also studied.

J. Ethnopharmacol.2006 Jun 30;106(2):238-44. Epub 2006 Feb 21: They have investigated the pharmacological mechanism of the anti-obesity effect of *Nelumbo nucifera* leaves extract (NNE).They examined the effect of NNE on digestive enzyme activity, lipid metabolism and theromogenesis and evaluated the effects of anti-obesity using high-fat diet-induced obesity in mice that were treated with NNE for 5 weeks. NNE caused a concentration-dependent inhibition of the activities of alpha-amylase and lipase, and up-regulated lipid metabolism and expression of UCP3 mRNA in C2C12 myotubes.NNE prevented the increase in body weight, parametrial adipose tissue weight and liver TGL levels in mice with obesity induced by a high-fat diet. UCP3 mRNA expression in skeletal muscle tended to be higher, when mice were administrated by NNE and were exercised. Therefore, NNE impaired digestion, inhibited absorption of lipids and carbohydrates, accelerated lipid metabolism & energy expenditure. Consequently, NNE is beneficial for the suppression of obesity.

Phytotherapy Res. 2006 Oct;20(10):825-30.: A methanol extract of the stamens of *Nelumbo nucifera* Gaertn. Was shown to exert an inhibitory effect on rat

lens aldose reductase (RLAR), and thus was fractionated using several organic solvents, including dichloromethane, ethyl acetate and n-butanol. The ethyl acetate-soluble fraction, which manifested potent RLAR-inhibitory properties, was then purified further via repeated measures of silica gel and Sephadex LH-20 column chromatography.

Tian N, Liu Z, Huang J, Luo G, Liu S, Liu X. (2007), The Total flavones in the leaves of *Nelumbo nucifera Gaertn* have been studied extensively. At first, crude extract was obtained from lotus leaves by reflux extraction using 60% ethanol for three times. Then, the concentrated crude extract was separated on a D-101 column (eluted with 70% ethanol) and a polyamide column (step gradient 15% to 90% ethanol). The Fr-1 fraction was obtained from the eluate of 45% ethanol and was subjected to a preparative reversed-phase high performance liquid chromatography (RP-HPLC) for the isolation of target components. The preparation of the individual flavonoids was carried out on an RP-HPLC with a Symmetry Prep C18 column, and the mobile phase was water-acetonitrile at a flow rate of 5.0 mL/min. Three compounds were identified with ultra violet absorbance (UV), infrared (IR), nuclear magnetic resonance (NMR) and mass spectrometry (MS). They were hyperin, isoquercetin and astragalin.To our knowledge that astragalin was the first time successfully isolated from this plant. The purity of the three compounds was all over 97%.

AIM

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OBJECTIVE

AIM & OBJECTIVE OF THE WORK

Herbal medicines are in great demand in the developed world for primary health care due their efficacy, safety and lesser side effects. Recently ,considerable attention has been paid to utilize eco-friendly and bio –friendly plant based products. The natural products are undoubtedly valuable as a lead for new medicinal agents. Hence in the present study, is to investigate the Anti-inflammatory (carregeenan induced paw edema method) and Anti-microbial efficacy for the Ethanolic extract of leaves of *Nelumbo nucifera*.

PLAN

OF

WORK

PLAN OF WORK

I. pharmacognostic studies.

a.plant profile

b.collection of plant and authentication

c. extraction of plant material

II. phytochemical studies

Preliminary phytochemical studies involving following steps.

- **Qualitative test**
  - Preliminary phytochemical test
  - Determination of total phenols
  - Determination of total flavonoids
- **Isolation**
- **Characterization**

III. Biological evaluation

- **In vitro microbial assay**

- **In vivo anti-inflammatory activity**

PLANT PROFILE

PLANT PROFILE

Nelumbo nucifera Gaertn.(sacred lotus)

Symbol:	NENU2
Group:	Dicot
Family:	Nelumbonaceae
Duration:	Perennial
Growth Habit:	For/herb

Classification

Nelumbo nucifera Gaertn.

Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* – Vascular plants
Super division	*Spermatophyte* – Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Magnoliopsida* – Dicotyledons
Subclass	*Magnoliidae*
Order	*Nymphaeales*
Family	*Nelumbonaceae* – Lotus-lily family
Genus	*Nelumbo* Adans. – lotus
Species	*Nelumbo nucifera* Gaertn. – sacred lotus

Nelumbo nucifera Gaertn (Family Nymphaeaceae) commonly known as "Padma" (Bengali), "Kanwal" (Hindi) and "Pankaj" (Sanskrit) is an aquatic herb with stout creeping yellowish white coloured rhizome 8. It has reported that rhizome extract showed anti-diabetic and anti-inflammatory effects 9, stalks extract showed anti-pyretic effect 10 leaves and stamens extracts showed anti-oxidant effect 11, 12 and seeds extract showed hepatoprotective and free radical scavenging effects 13. The leaf of *Nelumbo nucifera* is bitter,

sweet and neutral.It is aromatic and blue-green in colour. It is best for cleaning heat, resolving summer heat and stop bleeding 14

. Pharmacological studies of plant revealed that the whole plant possess ant diabetic, antipyretic, anti-inflammatory, anticancerous, antimicrobial, antiviral and anti-obesity properties [7].Furthermore, *N.nucifera* flower has considerable reputations a potent adjunct in the treatment of various ailments such as cancer, hypertension, diarrhea, fever, weakness, infection and body heat imbalance [8].The major constituents isolated from the lotus plant are alkaloids (liens nine, neferine, nuciferine, remrefidine and isoliensinine) and flavonoids ((+)-1(R)-coclaurine,(-)-1(S)-norcoclaurine and quercetin 3-O-b-D-glucuronide).Several previous reports suggested that seed could suppresscell cycle progression, cytokine genes expression and cellproliferation in human peripheral blood mononuclear cells. Recently, the leaf of *N.nucifera* showed the hypotensive effects that were mediated by vasodilatation *via* nitric oxide[9] and betulinic acid isolated from rhizomes and used asanti-tumoral and melanoma specific cytotoxic agent.

N. nucifera has a very long history as a traditional medicine in Asia and all parts of plants can be used for medicine. The medicinal characteristics of lotus are recorded *Compendium of Materia Medica* by a Chinese doctor, Shizhen Li in 1548. Several well-known traditional Chinese medicine formulas include lotus seeds as an important component: Sheng Ling Baizhu San, first described in the Hejiju Fang (1110 A.D.) which tonifies the spleen and aids circulation of moisture, and Jinsuo Gujing Wan, first described in Yifang Jijie (Analytic Collection of Medical Formulas, by Ang Wang, 1682), which has been made into a popular patent remedy.

The whole plant has some ant hemorrhagic effect, but the rhizome nodes are relied upon for that purpose specifically. The active component for reducing bleeding is not yet established, though quercetin and other flavonoids may play a role by improving capillary wall strength.

Lotus seeds are classified as astringents, being sweet and neutral, and benefiting the spleen, kidney, and heart. The seed has also been shown to lower cholesterol levels and to relax the smooth muscle of the uterus. The sweet taste and nourishing qualities of the seeds are responsible for benefiting the spleen and help stop diarrhea associated with qi deficiency. The astringent quality helps prevent loss of kidney essence, so the seeds are used to treat weak sexual function in men and leucorrhea in women.The seed also has calming properties that alleviate restlessness, palpitations, and insomnia (more so in the whole seed with embryo). Lotus embryo ("Lianzixin" in Chinese, heart of the lotus seed), is classified as bitter and cold and benefiting the heart. It dispels pathogenic heat from the heart to treat fidgets and spontaneous bleeding due to heat. The bitter components are isoquinoline alkaloids with sedative and antispasmodic effects. The alkaloids dilate blood vessels and thereby reduce blood pressure.

The flower is used for abdominal cramps, blood discharges, bleeding gastric ulcers, excessive menstruation and post-partum hemorrhage. Lotus stamen is sweet, astringent, and neutral, benefiting the heart and kidney.It is mainly used for preventing discharge, such as treatment of leucorrhea or for frequent urination. The stamen contains flavonoids and a small amount of alkaloids.The fruit is used for agitation and fever.

Materials & Methods

MATERIALS AND METHODS

PHARMACOGNOSTIC STUDIES

Collection of plants and Authentication

Plant was collected from Melur, Madurai district of Tamil Nadu and Authenticated by Taxonomist Dr. D. Stephen, Head of the Department, American College, Madurai, Tamil Nadu.

The Plant was processed, powered coarsely and coarse plant material were used for extraction

PRELIMINARY PHYTOCHEMICAL STUDIES

Ethanolic extraction of Nelumbo nucifera:

The Dried powder of defatted leaf part was taken in soxhlet apparatus. Ethanol (95%) was selected as to prepare the extract. The selection of the solvent was based on their extractive values of the plant in different solvent,and then dried leaf plant material was subjected for extraction. The extract was concentrated in a rotary flask evaporator and dried in dessicator over sodium sulphide. The color and the percentage yield of extract were noted.

Qualitative Preliminary Phytochemical Analysis Extract of *Nelumbo nucifera:*

A) Test for Alkaloids

1) Dragendroff's Test:

1ml of extract was added with 1ml of Dragendroff's reagents. Orange red precipitate was formed, which indicated the presence of Alkaloids.

2) Wagner's Test:

1ml of extract was added with Wagner reagent. Reddish brown precipitate was formed, which indicated the presence of alkaloids.

3) Mayer's Test:

1ml of extract was added with 1or2ml of Mayer's reagent. Dull White precipitate was formed, which indicated the presence of alkaloids.

4) Hager's Test:

1ml of extract was added with 3ml of Hager's reagent. Yellow Precipitate was formed, which indicated the presence of alkaloids.

B) Test for Glycosides:

1) Killer Killiani test:

2ml of extract was dissolved in acetic acid containing trace of ferric chloride and transferred to the surface of concentrated sulphuric acid. At the junction of two liquids reddish brown color was formed which gradually became blue color due to the presence of glycosides.

2) Legal's Test:

2ml of extract was dissolved in pyridine: sodium nitroprusside solution was added and made alkaline. Pink red color was formed, which indicated the presence of Glycosides.

***3) Baljet' tests**:*

2ml of extract was added with sodium picrate solution. Yellow to orange color was formed, which indicated the presence of Glycosides.

***4) Borntrager test**:*

1ml of diluted sulphuric acid was added with 2ml extract . The mixture was boiled, Filtered and extracted. Then filtrated with ether or chloroform. Then organic layer was separated to which ammonia was added ,pink color was produced in organic layer, which indicated the presence of Glycosides.

C) Test for Carbohydrates:

1) Molisch Test:

1ml of extract was treated with the compound of α-napthol and concentrated sulphuric acid along the sides of the test tube. Purple or reddish violet color was formed at the junction between two liquids, which indicated the presence of carbohydrates.

2) Felhing's Test:

1ml of extract was added with 1ml of fehling's solution A and B. It was heated gently, brick red precipitate was obtained, which indicated the presence of carbohydrates.

3) Benedict's test:

5ml of Benedict's reagent was added with 8drops of extract under examination. It was mixed well and boiled vigorously for two minutes and then cooled. Red precipitate was obtained ,which indiacted the presence of carbohydrates.

4) Barfoed's test:

5ml of the Barfoed's solution was added with 0.5 ml of extract under examination, heated or boil. Red precipitate of copper oxide was obtained ,which indicated the presence of carbohydrates.

D) Test for Steroids and Sterols

1) Libermann Burchard test:

1ml of extract was dissolved in 2ml of chloroform in a dry test tube. Then 10 drops of acetic acid anhydride and 2 drops of concentrated sulphuric acid was added .The solution does not changes to red or blue. Indicates the absence of steroids and sterols.

2) Salkowski test:

1ml of extract was dissolved in 1ml of chloroform an added 2ml of conc. sulphuric acid, no bluish red cherry red or purple color was noted in chloroform layer, which indicated the absence of steroids and sterols.

E) Test for Flavonoids:

1) Shinoda test:

1ml of extract was added to magnesium turnings and then concentrated hydrochloric acid was added. Red color was produced ,which indicated the presence of flavonoids.

2) Sulphuric acid test:

To a portion of the ethanolic extract of *Nelumbo nucifera,* concentrated sulphuric acid was added. A Yellow coloration observed indicated the presence of flavonoids.

3) Aluminium trichloride test:

Few drops of 1% Aluminiun trichloride solution were added to a portion of extract of *Nelumbo nucifera*. A yellow coloration observed indiacted the presence of flavonoids

4) Vanillin-Hydrochloric acid test:

To the Ethanolic extract of Nelumbo nucifera,vanillin-hydrochloric acid was added. A Pink color is formed indicates the presence of a flavonoids.

5) Lead acetate solution test:

To the ethanolic extract of Nelumbo nucifera,lead acetate was added. Yellow precipitate is formed indicated the presence of a flavonoids.

F) Test for Tannins and phenolic compounds:

1) Ferric chloride test:

1ml of extract was added with 1ml of ferric chloride solution. No Dark blue or greenish black color was produced, which indicates the absence of Tannins. Violet color shows the presence of phenolic compounds.

2) Potassium cyanide test:

1ml of extract was added with 1ml of potassium cyanide. No Deep red color was produced, which indicates the absence of Tannins.

3) Gelatin test:

1% solution of gelatin containing 10% sodium chloride gives No white precipitate. shows the absence of Taninns.

G) Test for protein and Amino acid:

1) Biuret test:

1ml of extract was added with 1ml of 40% sodium hydroxide and drops of 1% copper sulphate. No violet is formed , indicates absence of protein.

2) Ninhydrin test:

1ml of extract was added with 2 drops of freshly prepared 0.2 % Ninhydrin reagent and heated. No blue color is developed indicating the absence of proteins , peptides or amino acids.

3) Xanthoprotein test:

1ml of extract was added with 1ml of 20% of sodium hydroxide or ammonia. No Orange color is formed, indicating the absence of aromatic amino acid.

The result of Qualitative Preliminary Phytochemical Analysis of Ethanolic Extract of *Nelumbo nucifera*. Is given in the table.

Determination of Total Phenols:

The Phenol content in the raw material of *Nelumbo nucifera* extract was estimated by spectroscopic method.

Standard stock solution:

10mg of Gallic acid in 10 ml of methanol was prepared and used as standard.

***Sample stock solution*:**

100 mg of extract was dissolved in 10 ml of ethanol. 100µl of sample was mixed with 0.2ml Folin-ciocalteu reagent. About 2ml of H_2O and 1ml of 15% sodium carbonate solution was added and the mixture was measured at 765 nm after being kept for 2hrs at room temperature. The mean of triplicate was used and the total phenol content was expressed as milligrams of Gallic acid equivalent 932.1 mg/g extract. The coefficient of determination of was r^2= 0.9930

Determination of Total Flavonoids.

Flavons and flaonols in the ethanolic extract of *Nelumbo nucifera* were estimated as Quercein, Rutin equivalent. Quercetin and Rutin were used to make the calibration curve [10, 20, 30, 40, 50, 60, 70, 80, 90 and 100µg/ml in 99.9% ethanol (v/v)]. The standard solution or extract (0.5 ml) were mixed with 1.5 ml of 95 % ethanol(v/v), 0.1 ml of 10% aluminium chloride,0.1 ml of 1 mol/l sodium acetate and 2.8 ml water. The volume of 10% aluminium chloride was substituted by the same volume of distilled water in blank. After incubation for 30 minutes in room temperature, the absorbance of the above reaction mixture was measured at 415 nm. The mean of above three reading was used and the total Flavonoids content was expressed in milligrams of Quercetin and Rutin equivalent 190.2 mg/g extract. The coefficient of determination was r^2= 0.9985.

For the Flavonoids components:

The solvent system were used for separation in given below.

1. Chloroform: ethyl acetate(60:40)

2. Methanol : water (1:1)

3.Ethyl acetate: formic acid:glacial acid 15%: water(100:11:11:26)

4. Dimethylamine: methanol (85:15)

From the above solvent ,solvent no. 2 and 4 showed the good separation.

In-Vitro* Anti-oxidant Activity of Isolated compounds of *Nelumbo Nucifera

DPPH radical scavenging activity:

The scavenging activity of isolated compound of Nelumbo Nucifera was read out in terms of radical scavenging ability by using the stable radical DPPH. 0.1Mm solution of DPPH in ethanol was prepared and 1.0ml of this solution was added to 3ml of extract solution and standard in water at different concentration (10-100µg/ml). 30 min later, absorbance was measured at 517nm. Lower absorbance of the reaction mixture indicated more free radical scavenging activity. The capability to remove the DPPH radical was calculated using the following equation.

$$\%\text{inhibition} = \frac{A_{CONC} - A_{TEST}}{A_{CONC}} \times 100$$

Where A_{conc} was the absorbance of the control reaction and A_{test} was the absorbance of the sample extracts. The mean values were obtained from the above triplicate experiments. The anti-oxidant activity of the extract was expressed as IC 50. The Ic 50 value was defined as the concentration (in µg/ml) of isolated compounds which prevents the formation of DPPH radicals by 50%.

Isolation

The crude alcoholic extract was chromatographed over a silica gel column built in petroleum ether and eluted with pure as well as mixtures of solvents, petroleum ether, benzene and ethyl acetate in the order of increasing polarity.

Elution of the column with **petroleum ether: benzene (60:40, v/v)** gave a solid, designated as A-1 which was taken up for characterization.

The compound A-1 was found to be homogeneous when tested on TLC in the following solvent systems.

Elution of the column with **benzene (100 %)** gave a semi solid, designated as A-2 which was taken up for characterization.

It was found to be homogenous when tested in TLC in the solvent systems.

Elution of the column with **benzene: ethylacetate (80:20)** gave a solid, designed as A 3, which was taken up for characterization.

It was found to be homogeneous on TLC in the following solvent systems

Elution of the column with **benzene: ethylacetate (60:40)** gave a solid, designed as A 4, which was taken up for characterization.

It was found to be homogeneous on TLC in the following solvent systems

Elution of the column with **benzene ethyl acetate: (40:60)** gave a solid, designed as A-5, which was taken up for characterization.

It was found to be homogeneous on TLC in the following solvent system.

Spectral

Data

SPECTRAL DATA

INFRA RED SPECTRA

The peaks in IR spectrum gave an idea about the probable structure of the compound. IR region ranges between 4000-666 cm^{-1}. The compounds were recorded on Perkin Elmer FT-IR spectrometer, which showed different vibration levels of molecules by using Ker powder technique.

^{1}H NMR SPECTRA

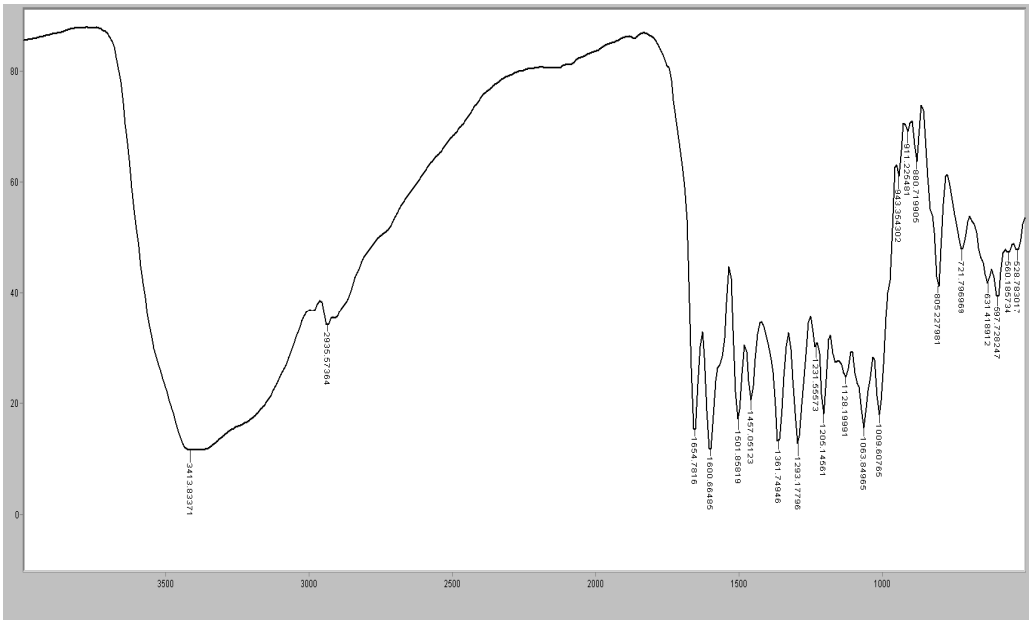
NMR spectroscopy enables us to record differences in magnetic properties of the various magnetic nuclei present and to deduce in the large measure about the position of these nuclei within the molecule. We can deduce how many different kinds of environmental are there in the molecules and also which atoms are present in neighboring groups. The proton NMR spectra, enables us to know different chemical and magnetic environments corresponding to protons in molecules.

^{1}H NMR of the title compounds were recorded in BRUKER AMX-400 MHz

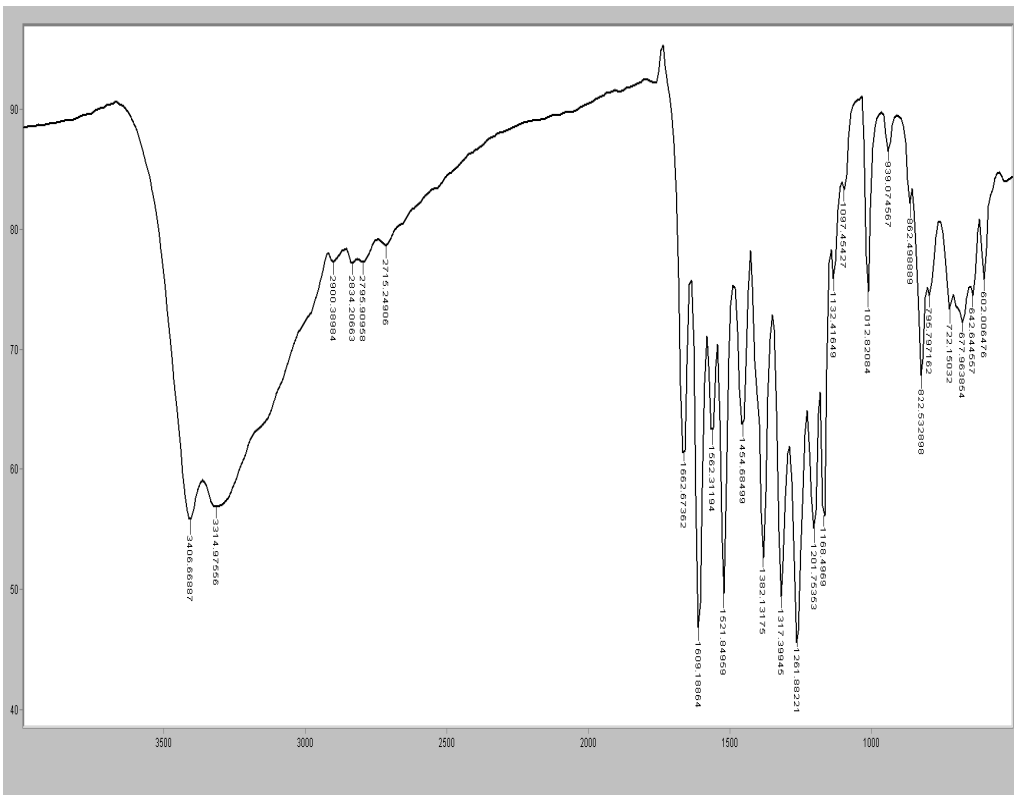
Mass spectra were recorded on a JEOL JMS600 spectrum

INFRA RED SPECTRA

COMPOUND-1

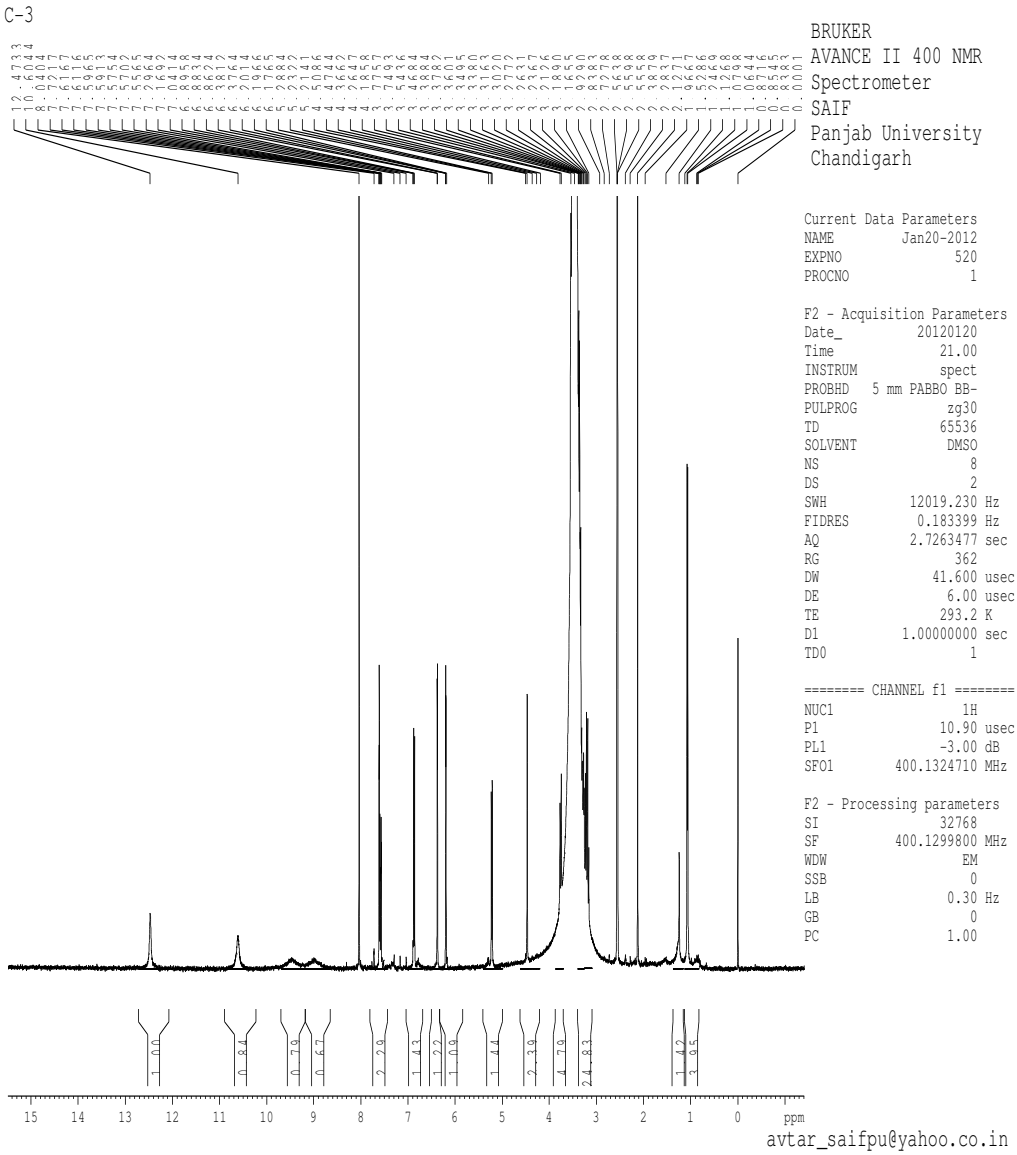


Compound-2

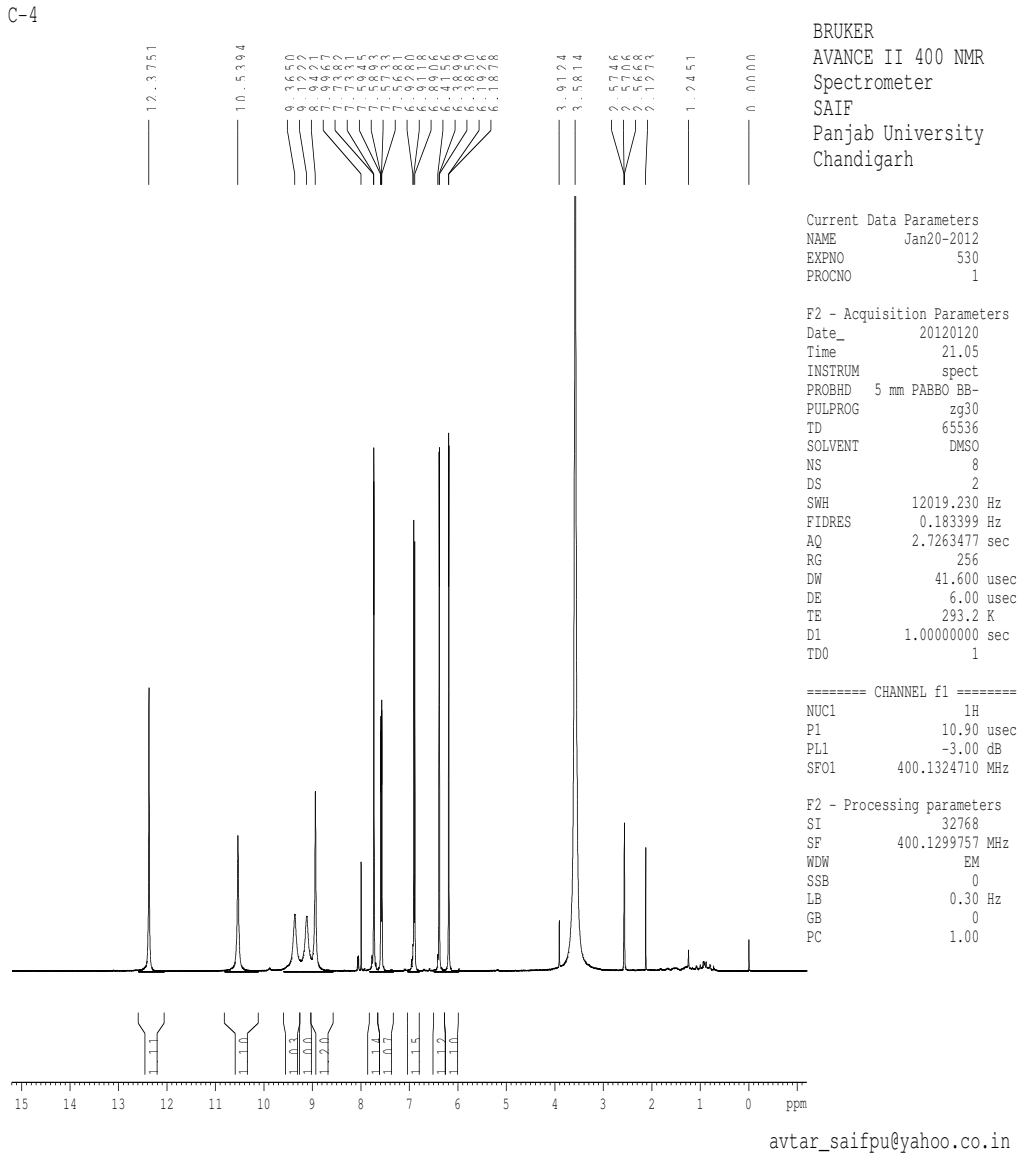


NUCLEAR MAGNETIC RESONANCE SPECTRA ANALYSIS

COMPOUND-1



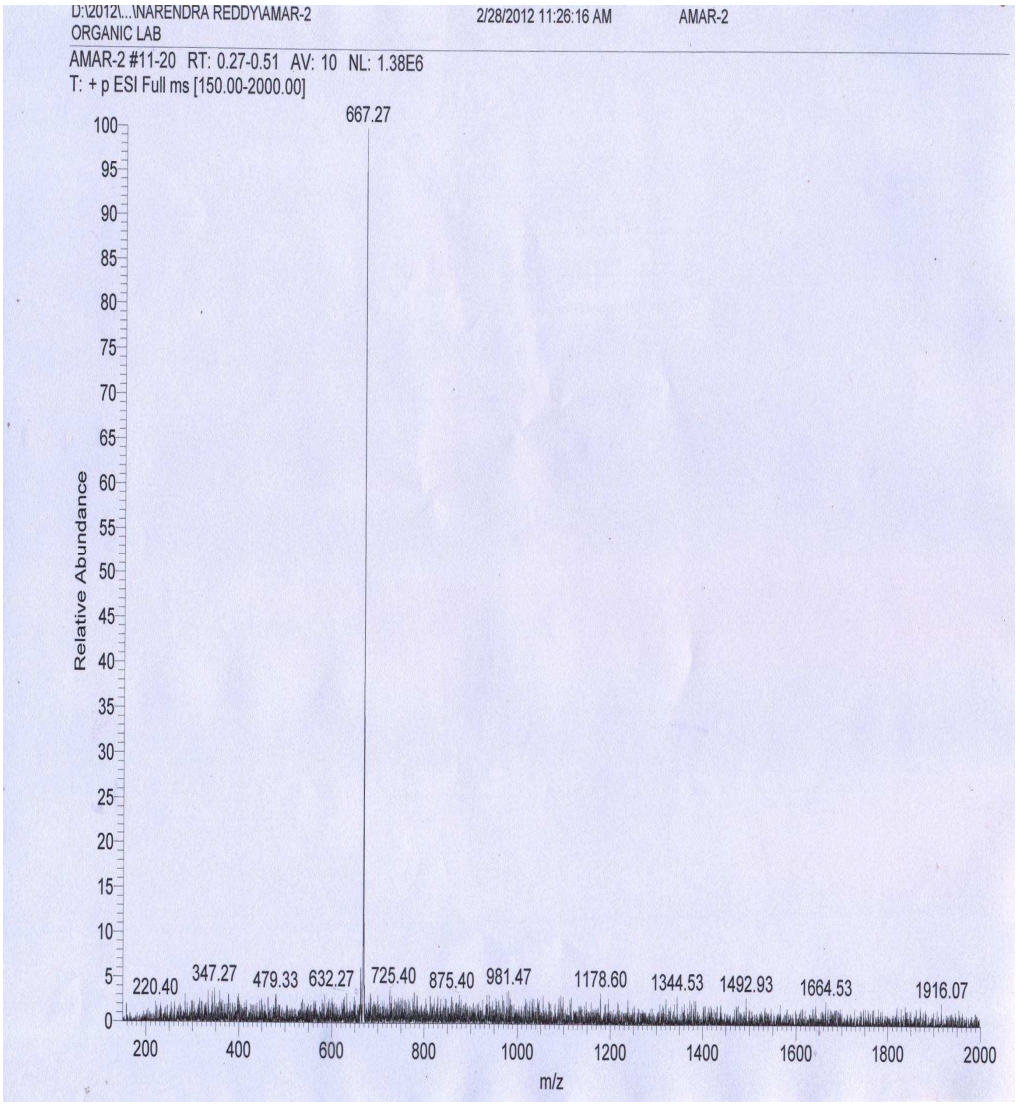
Compound-2



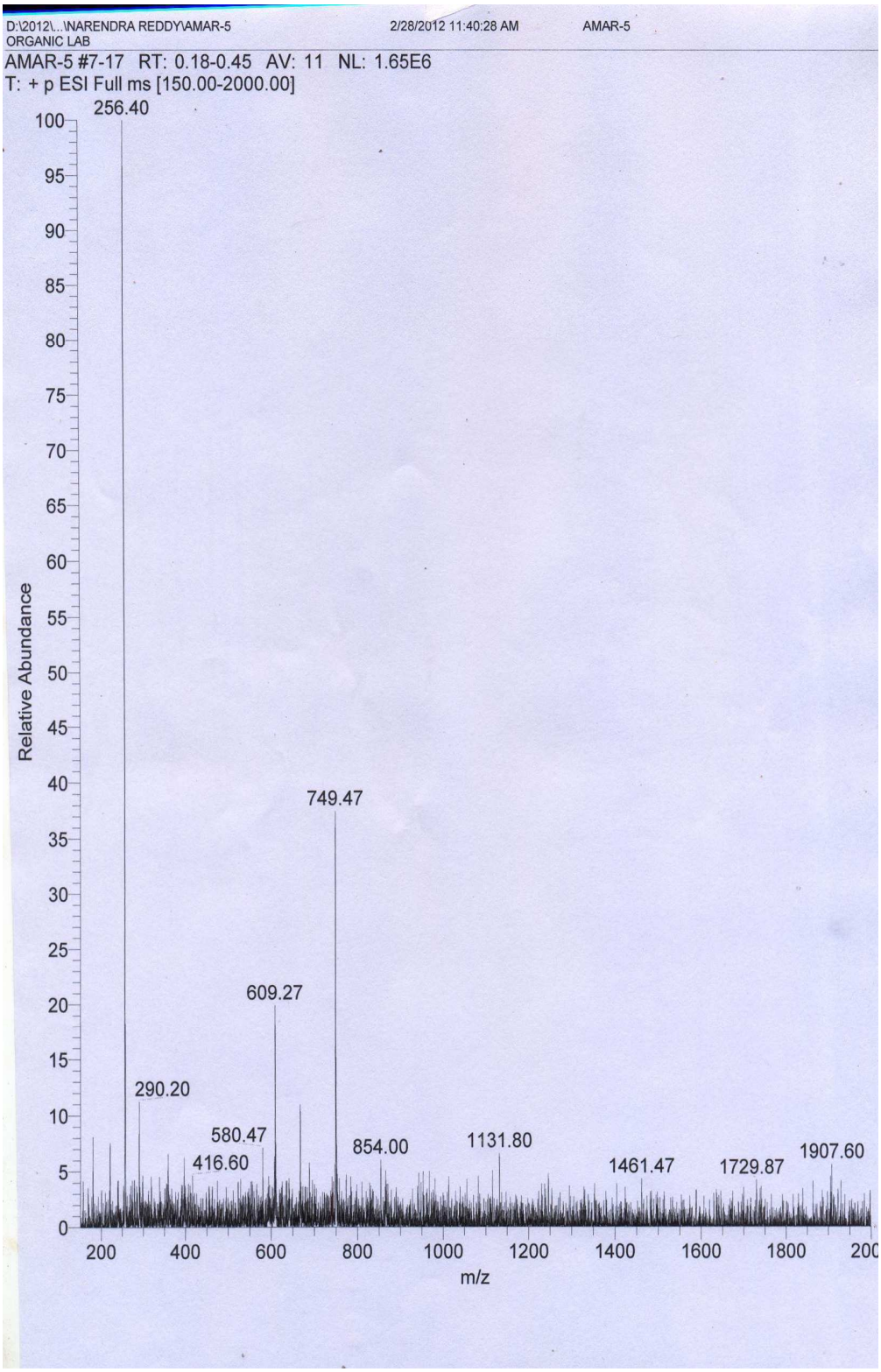
avtar_saifpu@yahoo.co.in

MASS SPECTRA

COMPOUND-1



Compound-2


D:\2012\...\NARENDRA REDDY\AMAR-5
ORGANIC LAB
2/28/2012 11:40:28 AM
AMAR-5
AMAR-5 #7-17 RT: 0.18-0.45 AV: 11 NL: 1.65E6
T: + p ESI Full ms [150.00-2000.00]
Relative Abundance
m/z
256.40
749.47
609.27
290.20
580.47
416.60
854.00
1131.80
1461.47
1729.87
1907.60

BIOLOGICAL EVALUATION

III. BIOLOGICAL EVALUATION

ANTI-MICROBIAL SCREENING

The inhibition of microbial growth under standardized condition may be utilized for demonstrating the therapeutic efficacy of antibiotics. The antimicrobial activity of the ethanolic extract of *Nelumbo nucifera* was screened in the concentration of 50mcg/ml, 100mcg/ml and 150mcg/ml in ethanol against the bacterial organisms like *MRSA (methyl resistance staphylococcus aureus, Streptococci ,Salmonella typhi, shigella.* The anti-microbial activity was evaluated by measuring the zone of inhibition in mm.

Methods used for anti-microbial screening

Filter paper disc method or Disc diffusion method

Materials used

Sterilized petri dishes

Sterilized Filter paper

Muller Hinton Agar medium

Organism used

- *MRSA(Methyl resistance staphylococcus areus)*
- *Streptococci*
- *Salmonell typhi*
- *Shigella*

Standard Drug

Imipenam (30mcg/ml).

Muller Hinton Agar Medium

Formula and Preparation

Ingredient		gm/litre
Beef extract	-	300.00
Casein acid hydrolysate	-	17.50
Starch	-	1.50
Agar	-	17.00
Final pH(at 25^{o}C)	-	7.3±0.2

The Muller Hinton agar media was dissolved in sufficient amount of distilled water (i.e. 38gms in 1000ml of distilled water) and heated in a steam bath to dissolve. The pH of the medium was adjusted to 7.3±0.2 and sterilized by autoclaving at 15lb,121^{o}C for 15 minutes.

Procedure for anti-microbial screening

The sterilized medium was allowed to cool at room temperature .when the medium was in lukewarm condition, it was poured into the sterilized petri dishes and allowed to solidify. Aseptically cultures of *MRSA(methyl resistance staphylococcus aureus, streptococci, salmonella typhi and shigella* were inoculated separately into the sterile Petri dishes containing the medium. The Filter paper discs were prepared from Whatmann No.2filter paper (6mm in diameter) sterilized in a hot air oven (2 hrs at 121^{o}C). The filter paper discs impregnated with the solution of ethanolic extract of the *Nelumbo nucifera* plant and standard disc of Imipenam were placed on the surface of the media. The plates were kept for 1-4 hrs at the room temperature and then incubated for 18hrs at 37^{o}C.

During incubation, the extract diffuses through the media and may prevent the growth of micro organism. Effectiveness of susceptibility is proportional to the diameter of the circular inhibition zones. The zone of inhibition was accurately measured and tabulated.

ANTI-INFLAMMATORY ACTIVITY

Inflammation is a normal, protective response to any noxious stimulus that may threaten the host and may vary from localized reaction to complex response involving the whole organism.

Inflammation has several phases.

1. First phase is caused by an increase in vascular permeability resulting in exudation of fluids from the blood into interstitial space.
2. Second phase involves the infiltration of leucocytes from the blood into the tissue.
3. Third phase represents granuloma formation.

This distinct aspect of inflammation facilitates measurement of anti-inflammatory activity by utilizing the clinical signs such as erythma, oedema and formation of granulation tissue.

Anti-inflammatory assay models

Method for testing acute and sub-acute inflammation are,

1. UV-erythma in guinea pigs
2. Vascular permeability
3. Oxazolone induced ear oedema in mice
4. Croton oil ear oedema in mice
5. Paw oedema in rat
6. Granuloma pouch technique.

In this study, the model used for acute inflammation was carrageenan induced rat paw oedema method.

Materials

1. Instrument : Digital venire caliber.

2. Drugs : a. standard –indomethacin (25mg/kg)

b. Test extract of *Nelumbo nucifera* (100-200mg/kg)

3. Chemicals : 1% carrageenan solution

Solvent –CMC solution

4. Animals : *Albino* rats of either sex.

Treatment protocol

In this method, albino rats of either sex weighing between 150-200gms were divided into four groups. Each group consists of six animals.

Group specification for carrageenam induced rat paw oedema method

Group I : Negative control

Group II : Positive control

Group III : Standard Drug

Group IV : Ethanolic extract of *Nelumbo nucifera*.

Group I received normal CMC solution, Group II received carrageenan alone (0.1 ml 1% solution). Group III received Indomethacin drug (25mg/kg) standard and Group IV received test extract of *Nelumbo nucifera* (orally) respectively. The right paw of the rat was marked with the ink at the level of the lateral malleolus and immerse in the mercury up to this mark each time.

1% solution of carrageenan in distilled water was freshly prepared. After an interval of 30 minutes, a sub plantar injection of 0.1 ml 1% carrageenan solution was administered to all the groups of animals. The paw volume is measured with digital

venire caliber in the normal foot (before treatment) and immediately following carrageenan injection at 0, 30,1,2,3,4,5,6 hrs. The average paw volume in the standard and test extract treated group was compared with that of the positive control group and the percentage inhibition of edema was determined and statistically evaluated. The anti-inflammatory activity was calculated by using the formula,

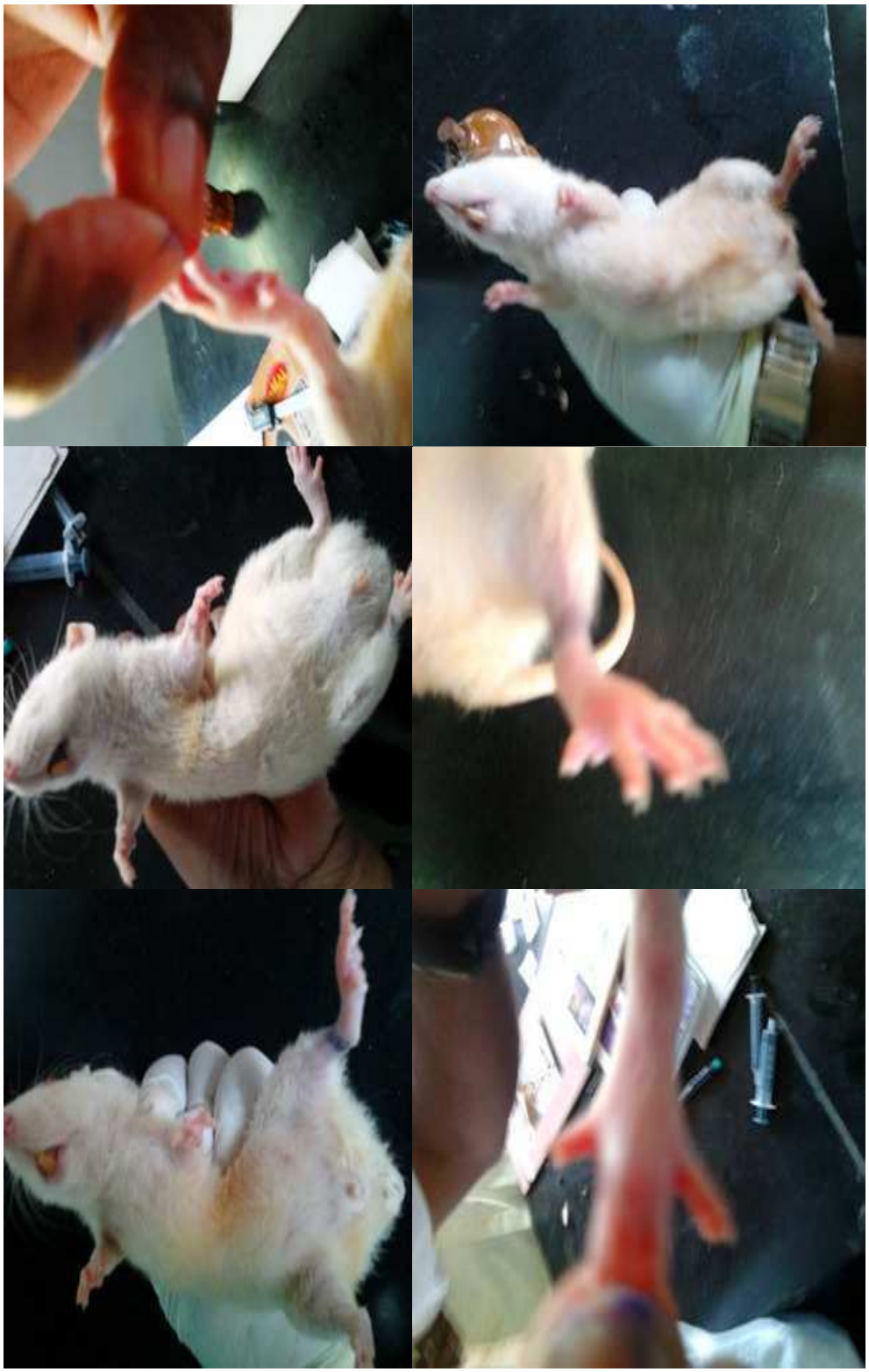
Percentage inhibition of oedema = $1-(V_d-V_p/V_c-V_p) \times 100$

Where,

Vd-Vp= Difference in paw volume after carrageenan injection and Initial paw volume for drug treated groups.

Vc-Vp= Difference in paw volume after carrageenan injection and Initial paw volume for control groups.

Anti-inflammatory photos



RESULTS

&

TABLES

Results & Discussion

Table -1: The colour and percentage yield of Ethanolic Extract of *Nelumbo nuceifera*:

Name of the Extract	**Amount of extract Yield (gms)**	**Percentage of yield**	**colour**
NN-ethanolic extract	26	5.76 %W/W	Dark greenish brown

Table: 2, Rf value of Isolated compounds

S. No	**Substance**	**R_f Value**
1	Compound 1	0.42
2	Compound 2	0.5

Table-3:

Qualitative Preliminary Phytochemical Analysis of Ethanolic Extract of *Nelumbo nucifera*

S.NO	Chemical Test	Ethanolic Extract of Nelumbo nucifera
1	**Test for Alkaloids**	
	Dragendroff's Test	+
	Wagner's Test	+
	Mayer's Test	+
	Hager's Test	+
2	**Test for Glycosides**	
	Killer killiani Test	+
	Legal's Test	+
	Baljet's Test	+
	Borntrager's Test	+
3	**Test for Carbohydrates**	
	Molisch's Test	-
	Fehiling's Test	-
	Benedict's Test	-
	Barfoed's Test	-
4	**Test for Steroids and Sterols**	
	Libermann Burchard Test	-
	Salkowski Test	-

5	**Test for Flavonoids**	
	Shinoda Test	+
	Sulphuric Acid Test	+
	Aluminium trichloride Test	+
	Vanillin-Hydrochloric Test	+
6	**Test for Tannins and Phenolic compound**	+
	Ferric chloride Test	+
	Potassium cyanide Test	+
	Gelatin Test	
7	**Test for protein and amino acid**	
	Biuret Test	-
	Ninhydrin Test	-
	Xanthoprotein Test	-

Table 4: Determination of Total Phenol content of Ethanolic Extract of *Nelumbo nucifera*

S. No	Sample	Concentration (mg/ml)	Absorbance
1.	Gallic acid	30	0.208
		40	0321
		50	0.467
		60	0.535
		70	0.601
2.	*Nelumbo nucifera*	100	0.625

Report: The total phenolic content in ethanolic extract of Nelumbo nucifera was expressed in milligrams of Gallic acid equivalent 220.02 mg/g extract. The coefficient of determination was r^2 =0.9930

Table 5: Determination of Total Flavonoids Content of Ethanolic Extract of *Nelumbo nucifera*

S.No	Sample	Concentration (µg/ml)	Aborbance
1.	Quercetin	20	0.340
		40	0.510
		60	0.792
		80	0.962
		100	0.998
2.	Nelumbo nucifera	100	0.310

Report: Flavonoids content was expressed in milligrams of Quercetin and Rutin

Equivalents 28.09 mg/g extract. The coefficient of determination was r^2=0.9985.

Table :6:

DPPH radical scavenging activity of NN compound-1

DPPH(control-0.8761)

Concentration	Absorbance
	Test
100	0.8101,0.8134,0.8062
200	0.7949,0.7912,0.7926
300	0.7544,0.7521,0.7532
400	0.7214,0.7230,0.7248
500	0.6869,0.6863,0.6821

TABLE-7

DPPH radical scavenging activity of NN comp-1 in percentage Inhibition

Conc (µg/ml)	Percentage Inhibition		
	Test	**Conc (µg/ml)**	**Standard**
100	7.5333,7.1567,7.9755	20	52.8457,53.3386,53.2016
200	9.2683,9.6906,9.1192	40	64.3762,64.6159,64.4332
300	13.8911,14.1536,14.0280	60	71.1790,70.9964,70.8937
400	17.6578,17.4751,17.2697	80	77.0345,76.9889,76.8177
500	21.5957,21.6641,22.1435	100	81.5660,81.5774,80.7556

TABLE-8

DPPH Radical scavenging activity of compound -1

Conc (µg/ml)	Percentage Inhibition	
	Test	Standard
100	7.55167±0.23635	53.1294±0.14622
200	9.35937±0.17105	64.4751±0.07229
300	14.0242±0.07577	71.0230±0.08344
400	17.4675±0.11205	76.9470±0.06602
500	21.8011±0.17227	81.2997±0.27206
EC-50	11.245µg/ml	17.84µg/ml

Table-9

DPPH radical scavenging activity of NN compound-2

DPPH(control-0.8761)

Concentration	Absorbance
	Test
100	0.8002 ,0.8164, 0.7658
200	0.7984 , 0.7132 ,0.7458
300	0.7321, 0.7258 ,0.7415
400	0.7112, 0.7012 ,0.7088
500	0.6987, 0.6874 ,0.6554

Table-10

DPPH radical scavenging activity of NN comp-2 in percentage Inhibition

Conc (µg/ml)	Percentage Inhibition				
	Test			Conc (µg/ml)	Standard
100	8.663395	6.814291	12.5896	20	52.8457,53.3386,53.2016
200	8.868851	18.59377	14.87273	40	64.3762,64.6159,64.4332
300	16.43648	17.15558	15.36354	60	71.1790,70.9964,70.8937
400	18.82205	19.96347	19.09599	80	77.0345,76.9889,76.8177
500	20.24883	21.53864	25.19119	100	81.5660,81.5774,80.7556

Table-11

DPPH radical scavenging activity of NN comp-2 in percentage Inhibition

Conc (µg/ml)	Percentage Inhibition	
	Test	**Standard**
100	9.355857± 1.389843	53.1294±0.14622
200	14.11178±2.312274	64.4751±0.07229
300	16.31853±0.424963	71.0230±0.08344
400	19.29384±0.280794	76.9470±0.06602
500	22.32622±1.20803	81.2997±0.27206
EC-50	10.58µg/ml	17.84µg/ml

CHARACTERISATION:

IR-SPECTRA

S.NO	COMPOUNDS	IR VALUES	POSSIBLES GROUPS
1.	SAMPLE-1	3413.63	O-H stretch(alcohol)
		2935.57	C-H stretch(alkane)
		1654.78	N-H stretch(amide)
		1129.98	C-O stretch(ether)
		721.79	C-H bending(aromatic)
		631.41	C-Cl stretch(halogen)
2.	SAMPLE-2	3314.97	O-H stretch(hydroxyl)
		3406.66	N-H stretch(amines)
		{2900.29, 2834.4}	C-H stretch(aldehyde)
		1662.67	C=O stretch(ether)
		{795.95, 822.53}	C-H bending(aromatic)

NMR-SPECTRA

S.NO	COMPOUNDS	NMR VALUES	POSSIBLES GROUPS
1.	SAMPLE-1	(6-8) (4-5) (3-4) (3-4) (3-5)	Aromatic RCH2=CH2 Cl-CH Ether R-O-CH Alcohols HO-CH
2.	SAMPLE-2	(6-8) (3-4) (3-4) (3-5) (5.5-8) (9-10) (10.5)	Aromatic ring Cl-CH Ether R-O-CH Alcohols HO-CH R-CO-NH CHO Aldehyde COOH Carboxylic acid)

MASS SPECTRA

S.NO	COMPOUNDS	MOLECULAR WT
1.	SAMPLE-1	667.7
2.	SAMPLE-2	749.47

IN-VITRO ACTIVITY:

ANTI-MICROBIAL ACTIVITY RESULT:

Table: 12

ZONE OF INHIBITIONS IN MM					
S.NO	**DRUG**	**GRAM POSITVE**		**GRAM NEGATIVE**	
		S.Aureus	***S.cocci***	***S.typhi***	***shigella***
1.	IMIPENUM	22**	20**	20**	21**
2.	Extract 50	6	5	6	5
3.	Extract 100	9	8	10	8
4.	Extract 150	15**	19**	17**	16**

** Test drug is significant with standard drug

IN-VIVO ACTIVITY

ANTI-INFLAMMATORY ACTIVITY RESULTS

Table: 13 showing group, drug treatment and paw volume indifferent time interval

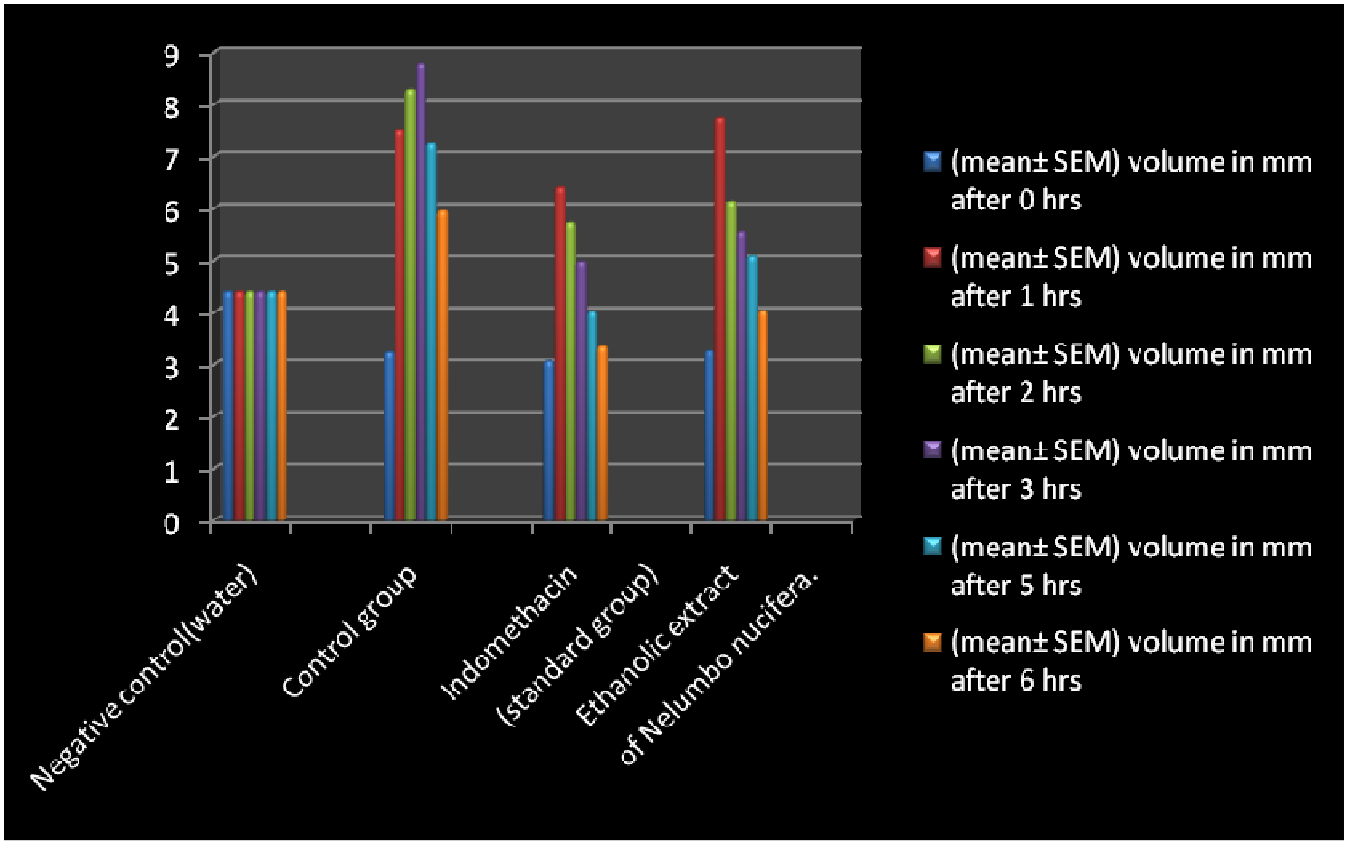
Group	Treatment &dose in (mg/kg)	(mean± SEM) volume in mm after						% Inhibition At 6th hr.
		0 hrs	1 hrs	2 hrs	3 hrs	5 hrs	6 hrs	
I	Negative control(water)	4.3885 ±0.1367	4.3885 ±0.1367	4.3885 ±0.1367	4.3885 ±0.1367	4.3885 ±0.1367	4.3885 ±0.1367	0
II	Control group	3.2233 ±0.2410	7.505 ±0.1306	8.2733 ±0.7840	8.786 ±0.1011	7.235 ±0.1596	5.941 ±0.2894	0
III	Indomethacin (standard group)	3.0554 ±0.4307	6.395 ±0.2585	5.7183 ±0.2997	4.971 ±0.9942	4.0052 ±0.6978	3.3316 ±0.4173	89.83
IV	Ethanolic extract of *Nelumbo nucifera.*	3.2483 ±0.3379	7.7342 ±0.7541	6.1266 ±1.2269	5.5516 ±0.6443	5.075 ±0.3967	4.0235 ±0.2141	71.02

n=6, Results were presented as mean ± standard error of mean (SEM) and the statistical analysis was done using one way analysis of variance (ANOVA).

A p-value of $p < 0.01$ was considered to be statistically significant.

ANTI-INFLAMMATORY ACTIVITY RESULTS:

Tables showing group, drug treatment and paw volume indifferent time interval



Discussion

Discussion

The Aim of the present investigation was to study the Isolation, characterisation and biological evaluation of ethanolic extract of the leaf of *Nelumbo Nucifera.*

In this context, a new oral crude drug of ethanolic extract of *nelumbo nucifera* provides interesting therapeutic properties.

Phenolics and flavonoids normally scavenge the free radicals and play an essential role in prevention and therapy of cancer, cardiovascular disease and inflammation by Inducing Anti-oxidant defence system, drug metabolizing enzymes. Modulating diverse events in cellular inhibiting inflammation, hyperplasia, proliferation and oxidative DNA damage.

Polyphenolic compounds (quercetin, protocatachuic acid, Gallic acid) are natural anti-oxidant, which decreases oxidation of bio molecules essentials.

The isolation was carried out using the various solvent systems. In that, predominantly two individual compounds were isolated, which were characterised using the IR, NMR and MASS spectra.

Infra Red Spectra

Infra red spectroscopy was taken for isolated compounds .The characteristics absorption peaks were observed for all relevant groups. The absorption peak around 600 -1200 cm-1 indicates the presence of C-CL stretching, C-H bending , vibration around 1654.78 cm-1 ,N-H stretching was observed. At 2500-3500 cm-1, C-H stretching (alkane), O-H stretching was observed .At 720 cm-1, aromatic spectra was found .

In the compound -2, at 700-850 cm-1 C-H bending for aromatic spectra were observed. At 1662.67 C=O stretching for ether was observed. From 2800-2900 cm-1 C-H stretching for aldehyde group was observed . At 3300-3500 cm-1 O-H and N-H stretching was observed . All other relevant groups absorption were observed for the isolated compounds.

^{1}H Nuclear Magnetic Resonance

^{1}H nuclear magnetic spectra were taken for the isolated compounds. For compound -1, Aromatic protons were observed at 6.68-8.138 ppm and (RCH2=CH2 ,CH-OH) proton was observed at 4-5ppm, (CL-CH, R-O-CH) were observed at 3-4 ppm for the compound-1.

For compound -2, COOH & CHO group were observed at 9-10.5 ppm Aromatic ring were observed at 6-8ppm, CH-Cl & R-O-CH were observed at 3-4 ppm.

Mass Spectra

The mass spectra of these compounds showed molecular ion peaks.

The various functional groups were identified by interpretation of the obtained values of spectra. The results were tabulated.

In-vitro anti-oxidant activity:

The isolated compounds were investigated for the *in-vitro* anti-oxidant activity using DPPH radical scavenging method. The DPPH radicals absorbed at 517nm and this absorption is inhibited in the presence of possible anti-oxidant compound. This reduction in absorbance is related to the anti-radical efficiency of the compound. The results were shown in the table No. 6-11.

The isolated compounds were in negligible quantity. Hence, this study was further continued using the crude drug of the extract.

***Anti-Microbial Activity*:**

The crude extract of the *Nelumbo Nucifera* was then subjected to the Anti-microbial activity on various Gram positive and Gram negative strains like ***S.aureus, Streptococci* , *MRSA* and *Shigella***. The results were then compared with the standard drug Imipenam. Results showed that the crude extract possess significant Anti-microbial activity at 150µg/ml dose. The results were shown in the table No. 12.

***Anti-inflammatory activity*:**

The crude ethanolic extract of *Nelumbo nucifera* was further investigated for its Anti-inflammatory effect using **carregenan induced paw edema method**. Acute oral toxicity of ENN was found to be non-toxic up to higher concentration of 2gm/kg. (Reference: Journal of ethanopharmacology 104(2006)322-327). The activity was carried out using indomethacin as the standard drug.

The results revealed that the crude extract possess significant biological action on the inflammation in the paw. The onset of the action of the crude drug was gradually slow when compared with the standard drug yet possess significant activity. The results were shown in the table No.13.

Hence, this **study reveals that the ethanolic extract of leaf of *Nelumbo nucifera* has the significant anti-microbial and anti- inflammatory effect.**

CONCLUSION

Conclusion

The plant *Nelumbo nucifera* was highly praised for its beauty and ornamental values, yet it is highly considered for its medicinal values. Various parts of the plant like leaf, flower, seeds and rhizomes were subjected for various *in-vivo* and *in-vitro* activities, it shows excellent pharmacological actions on the diseases like obesity, hyperlipdemia, various cancers, anti-HIV and various anti-bacterial activities.

In present study, crude ethanolic extract of leaf of NN was subjected for the isolation of a probable flavonoid compound, which was characterised using spectral data's. The compound posses potential *DPPH* radical scavenging activity, which was further evident for the anti-inflammatory effect of the crude extract using carragenam induced paw oedema method.

The extract was also investigated for its Anti-microbial activity, particularly for (MRSA), which is highly resistant for various anti-biotic. From the results obtained, it may be concluded that extract of leaf of NN, could reduce the inflammation and considered for its anti-microbial activity.

It seems promising that these data will be validated in the future molecular level studies to establish its potential medicinal values.

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