Development and evaluation of polyherbal formulation for inflammatory conditions

Synopsis of the Ph.D. thesis submitted to
The Maharaja Sayajirao University of Baroda

For the degree of
Doctor of Philosophy
in
Pharmacy

By
Preeti Siddharthsinh Jadeja
(Registration number- FOPH/23)

Under the supervision of
Dr Rajashree C. Mashru
Associate Professor

Research Guide	Head	Dean
(Dr. R. C. Mashru)	(Prof. Krutika K. Sawant)	(Prof. Krutika K. Sawant)

Table of Contents

1. Introduction

a. Herbal drugs

Medicinal plants provide a host of chemical compounds, which have been optimized on the basis of their biological activities. Chemical compounds present in medicinal plants have shown great promise in the management of various inflammatory disorders and have continued to serve as alternative and complementary therapies.

b. Inflammation

Inflammation is a localized physical condition in which the inflamed part of the body develops swelling, redness, pain, etc. in response to an infection or injury. Infections, wounds and tissue damages cannot heal without an inflammatory response. This response is mediated by two main components of the host's defense mechanisms: innate and adaptive immune response. The innate immune response is the primary host response to any foreign material which then is acted upon by granulocytes, phagocytes and other cells which are a part of the adaptive immune response. The adaptive immunity is characterized by specificity and helps in the elimination of pathogens in the later phase as well as the generation of immunological memory. However, sometimes the inflammatory response persists longer than necessary which causes more harm than benefit. [1]

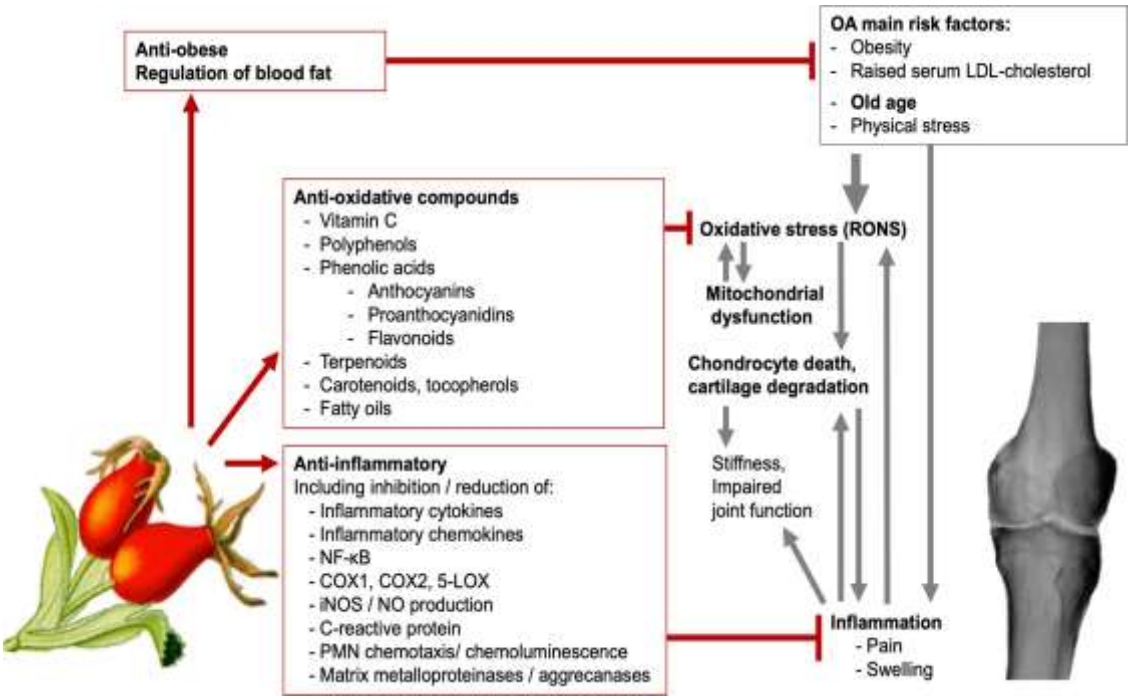


Fig. 1. The progression of Arthritic symptoms is based of several vicious cycles. [2]

c. Medicinal Plants

1) Calotropis proceras

Common names [4]

Arabic: Dead sea plant, debaj, usher, oshar, kisher;

English: Clotrope, calotropis, dead Sea fruit, desert wick, giant milkweed, swallow-wort, mudar fibre, rubber bush, rubber tree, sodom apple;

French: Womme de Sodome, algodón de seda, arbre á soie, coton soie, arbre a soie du Senegal;

German: Wahre mudarpflanzer, gomeiner;

Hindi: Madar, akada, akdo,aak; Italian: calotropo;

Sanskrit: Arka, alaka, ravi;

Spanish: Bomba, algodón extranjero, cazuela; and

Urdu: Madar, aak

Classification of *Calotropis procera* [5]

Kingdom - Plantae

Division - Magnoliophyta

Class - Magnoliopsida

Subclass - Asteridae

Order - Gentianales

Family - Asclipiadaceae

Subfamily - Caesalpinioideae

Genus - *Calotropis*

Species – *procera*

MORPHOLOGICAL CHARACTERISTICS OF PLANT

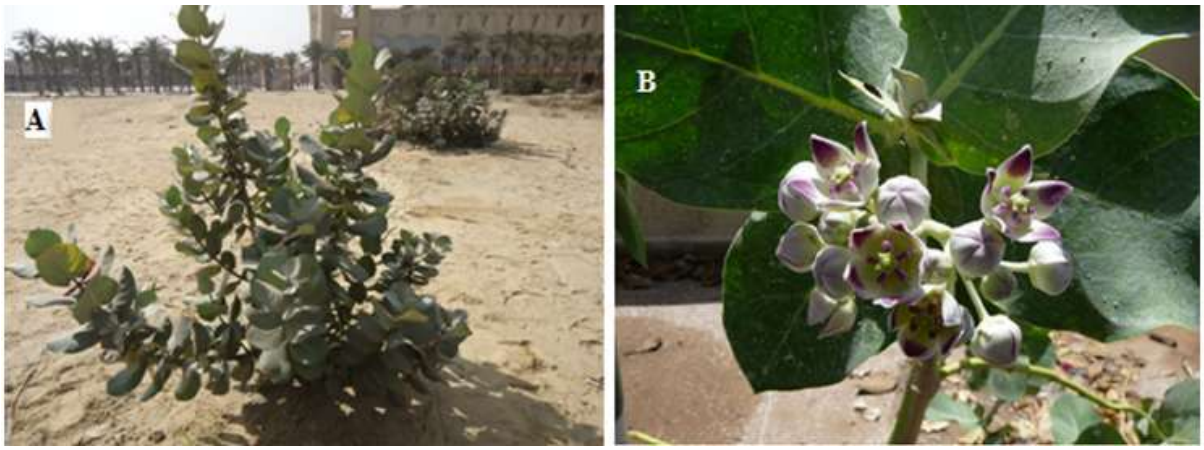


Figure 2. Morphology of *Calotropis procera*

The morphological studies revealed the plant is erect, tall, large, much branched and perennial with milky latex throughout. *Calotropis procera* have large bushy shrub, leaves decussate, inflorescence extra axillary umbellate panicale, corolla purple, lobes erect. The leaves are sub-sessile, 6-15 cm by 4.5-8 cm, broadly ovate, ovate-oblong, elliptic or obovate acute, pubescent; when young and glabrous on both sides when mature. [6, 7]

(2) *Rosa indica*

Rosa indica belongs to the family of Rosaceae. It is known for various pharmacological activities, and the presence of colored pigments and chemical constituents like flavonoids. It is also valued for their culinary, medicinal, cosmetic and aromatic properties.

Common Names [8]

Region	Name
Sanskrit	*Taruni, Shatapatri, Karnika, Charukeshara, Laksha, Gandhadhya*
Hindi, Marathi, Gujarathi	Gulab
Bengal	Golap
Tamil	Irasha
Telugu	Gulabi
Arabi	Varde ahmar
Farasi	Gulesurkh
English	Rose

Classification of *Rosa indica*

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliolpsida

Order: Rosales

Family: Rosaceae

Genus: *Rosa*

Species: *indica*

MORPHOLOGICAL CHARACTERISTICS OF PLANT

The plant is shrubby and is 6.15cm to 3 meters in height. Branches bear thorns. Leaves have serrate margins. Flowers have many shades of colors. Fruit – oval and becomes red on ripening.

Figure 3. Morphology of *Rosa indica*

Medicinal Actions of Rose- Antidepressant, Antispasmodic, Aphrodisiac, Astringent, Antibacterial, Antiviral, Antiseptic, Anti-inflammatory, Blood tonic, Cleansing, Digestive stimulant, Expectorant, Increases bile production, Kidney tonic, Menstrual regulator

3)*Adhatoda vasica*

Common names
Hindi: Adosa, adalsa, vasaka
Sanskrit: Amalaka, bashika,
Bengali: Basak
Tamil: Adatodai
Marathi: Vasuka
Telugu: Adasaram
Malayalum: Ata-lotakam

Classification of *Adhatoda vasica* [9]
Kingdom: Plantae
Order: Lamiales
Family: Acanthaceae
Genus: Justicia
Species: J. adhatoda
Common name: Adulsa (Vasaka)

MORPHOLOGICAL CHARACTERISTICS OF PLANT

The leaves of *Adhatoda vasica* are light green in colour, characteristic odour, taste is bitter, size is 10-13 cm long. The shape of leaves is ovate-lanceolate, apex is acuminate, margin slightly crenate to entire, base is symmetric, venation is pinnate, and texture is leathery as shown in Figure [10]

Figure 4. Morphology of *Adhatoda vasica* plant

Phytochemistry

Vasica contains phytochemicals such as alkaloids, glycosides, sterols, and phenolic acid. Alkaloids (quinazoline) (vasicine, vasicinone, 7-hydroxyvasicine, vasicinolone, 3-

deoxyvasicine, vasicolinone, vasicol, vasicoline) betaine, steroidscarbohydrate and alkanes are the most common constituents. The major pharmacological actions are due to the presence of alkaloidal content specially vasicine (7.5%) in the plant. Besides vasicine, the leaves include alkaloids (Vasicinone, Adhatodine, Vasicinol, Adhvasinone, Anisotine Adhatonine, and Hydroxypeganine), betaine, steroids, alkanes, kaempferol and quercetin. The leaves are high in vitamin C and carotene, making this plant a potential essential oil source. In addition, it contains amino acids and proteins. Triterpenes and flavonoids are abundant in flowers. Apigenin, astragalin, kaempferol, quercetin, and vitexin are flavonoids.

Pharmacological Properties- Antibacterial activity**,** Anti-asthmatic activity**,** Anti-diabetic activity**,** Anticancer activity**,** Insecticidal activity**,** Thrombolytic and cardioprotective activity, Antitussives**,** Anti-tuberculosis activity**,** Analgesic activity**,** Uterine Activity**,** Hepatoprotective Activity**,** Anti-inflammatory**,** Antioxidant activity**,** Radio modulatory activity**,** Immuno-modulatory activity**,** Anthelmintic activity**,** Antimutagenic activity**,** Wound healing activity**,** Anti-Alzheimer Activity**,** Anti-Ulcer activity**,** Antiviral activity**,** Anti-allergic activity**,** Anticholinesterase activity, Antifungal activity [(11)]

Aim and Objectives

Fifty percent of all prescribed drugs throughout the world are derived or synthesized from natural products, the only available sources for which are animals, marine, plants and micro-organisms. It is considered that because of the structural and biological diversity of their constituents, plants offer a unique and renewable resource for the discovering of potential new drugs and biological entities. Between 1983 and 1994, 41% of new approved drugs have natural products as their source, which indicates that natural products still play a very important role in the development of new medicine.

The present work therefore aimed to evaluate and standardize the selected plant drugs such as ***Rosa indica, Calotropis procera*** and ***Adhatoda vasica*** for the claims made under traditional systems for their **anti-inflammatory activities** and prepare **polyherbal formulations**. It was observed that herbal formulations contain a number of constituents which have a very narrow therapeutic index. Thus, development of quality control methods and safety as well as efficacy studies are important for such multi-component therapies in the present scenario. Although, the physical parameters prove to be important standardization tools, the quantitative assessment of bioactive molecules (marker compounds) have been empirically and scientifically proven to be better standardization parameter. Therefore, there is an urgent need to develop analytical methods for quantification of the active constituent in the polyherbal formulations.

The present study is thus aimed at developing new standardization tools for assessment of safety, quality and efficacy for polyherbal formulation. The present study was planned in the following manner:

The specific objectives of the work undertaken were:

1. Preliminary pharmacognostical parameters:

a) Collection and authentication of plant materials.

b) Physico-chemical analysis of extracts.

c) Qualitative Phytochemical Screening of extracts

2. In-vitro anti-inflammatory activity of extracts.

3. In silico-molecular docking studies of extracts.

4. Method development and validation for the quantitative analysis of active constituents using analytical methods.

5. Preparation and optimization of formulations Gel and Spray using Design of experiment.

6. Evaluation of the formulation

7. In vivo studies for anti-inflammatory activity of developed formulations

Outcome of the Project

Ayurveda, a natural system of medicine, originated in India more than 3,000 years ago. The term Ayurveda is derived from the Sanskrit words ayur (life) and veda (science or knowledge). The developed formulations, owing to their derivation from natural sources, can be included under the category of Ayurvedic Proprietary medicine. The developed formulations can be used for treating different inflammatory conditions mentioned in the list below.

- Ankylosing Spondylitis
- Antiphospholipid Antibody Syndrome
- Autoimmune Encephalitis
- Chronic Recurrent Multifocal Osteomyelitis
- Gout
- Juvenile Dermatomyositis
- Juvenile Idiopathic Arthritis
- Juvenile Lupus (SLE)
- Juvenile Scleroderma
- Juvenile Vasculitis
- Lupus (Systemic Lupus Erythematosus)
- Mixed Connective Tissue Disease
- Myositis
- Poststreptococcal Inflammatory Syndromes
- Psoriatic Arthritis
- Reactive Arthritis
- Rheumatoid Arthritis
- Scleroderma
- Sjogren's Syndrome
- Spondylarthritis
- Systemic Juvenile Idiopathic Arthritis
- Undifferentiated Connective Tissue Disease
- Uveitis
- Vasculitis

Experimental Work

Collection and authentication of Plant materials

The leaves of *Calotropis procera* and leaves of *Adhatoda vasica* were obtained from medicinal garden of The Maharaja Sayajirao University of Baroda and flower petals of *Rosa indica* were obtained from market at Vadodara. All the plant materials were identified by Botany Department, The M. S. University of Baroda. The voucher specimens of the herbs have been deposited in the Pharmacy department, The M.S. University of Baroda.

Preparation of powdered material

The selected plant materials were collected, cleaned to remove any adhering material and then dried in shade. The large dried plant parts were then subjected to size reduction to coarse powder and used for further studies.

Standardization of the plant materials as per the WHO guidelines

Determination of ash

After ignition, the remaining ash of medicinal plant materials was determined by in terms total ash, acid-insoluble ash and water-soluble ash.

Total ash

About 2 gm of the ground drug was weighed accurately in a previously ignited and tarred crucible. The material was spread in an even layer and ignited by gradually increasing the heat to 500 to 600°C until it was white in colour that indicates the absence of carbon. Then ash was cooled at room temperature and weighed. The total ash was calculated in % of air-dried material.

Acid-insoluble ash

About 25 ml 70% Hydrochloric acid (HCl) was added to the total ash and then covered with a watch glass. After boiling the mixture for 5 min, the insoluble matter was collected on an ash less filter paper and transferred to the crucible and ignited to constant weight. The residue was weighed, and the acid-insoluble ash was calculated in % of air-dried material.

Water-soluble ash

The mixture containing 25 ml of water and total ash was boiled for 5 min. The insoluble matter was collected on an ash less filter paper. The crucible was ignited to constant weight. The

weight of the residue was subtracted from the weight of total ash and the content of water-soluble ash in % of air-dried material was calculated.

Preparation of Extracts

The commonly employed technique for separation of active substance from crude drug is called as 'Extraction' which involves the use of different solvents. The plant material used for extraction should be properly authenticated or identified. The choice of the plant material for extraction depends upon its nature and the components required being isolated. The dried powdered plant material is commonly used for extraction. The solvent used for extraction is called menstrum and the residue is known as marc.

- **Soxhlet extractor**

A Soxhlet extractor is a piece of laboratory apparatus invented in 1879 by Franz Von Soxhlet. It was originally designed for the extraction of a lipid from a solid material. However, a Soxhlet extractor is not limited to the extraction of lipids. Typically, a Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a high solubility in a solvent, then a simple filtration can be used to separate the compound from the insoluble substance. Principle and working of Soxhlet apparatus normally a solid material containing some of the desired compound is placed inside a thimble made from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor is placed onto a flask containing the extraction solvent. The Soxhlet is then equipped with a condenser. The solvent is heated to reflux. The solvent vapor travels up a distillation arm and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapor cools, and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle may be allowed to repeat many times, over hours or days. The non-soluble portion of the extracted solid remains in the thimble, and is usually discarded.

Extracts:

The dried powder of plant was extracted with various solvents. Aqueous extract and methanolic extract of three selected plants were obtained using Soxhlet apparatus. About 20 gm of dried powder of plant part was subjected to Soxhlet apparatus for 36 hours. The temperature was maintained at 40 degrees centigrade. The solvents were removed by heating at low temperature on water bath and got semi solid mass.

Qualitative Phytochemical Screening of extract

Phytochemical examinations were carried out for the extracts as per the standard methods.

- **Detection of alkaloids:** Extracts were dissolved individually in dilute Hydrochloric acid and filtered.
  - **Mayer's Test:** Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a syellow-coloured precipitate indicates the presence of alkaloids.
  - **Wagner's Test:** Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.
  - **Dragendroff's Test:** Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.
  - **Hager's Test:** Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow coloured precipitate.
- **Detection of carbohydrates:** Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.
  - **Molisch's Test:** Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. Formation of the violet ring at the junction indicates the presence of Carbohydrates.
  - **Benedict 's test:** Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

- ❖ **Fehling's Test:** Filtrates were hydrolysed with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

- **Detection of glycosides:** Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.
  - ❖ **Modified Borntrager's Test:** Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.
  - ❖ **Legal's Test:** Extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of cardiac glycosides.
- **Detection of saponins**
  - ❖ **Froth Test:** Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.
  - ❖ **Foam Test:** 0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.
- **Detection of phytosterols**
  - ❖ **Salkowski's Test:** Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.
  - ❖ **Libermann Burchard's test:** Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was added. Formation of brown ring at the junction indicates the presence of phytosterols.
- **Detection of phenols**
  - ❖ **Ferric Chloride Test:** Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

- **Detection of tannins**
  - **Gelatin Test:** To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.
- **Detection of flavonoids**
  - **Alkaline Reagent Test:** Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.
  - **Lead acetate Test:** Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.
- **Detection of proteins and amino acids**
  - **Xanthoproteic Test:** The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.
  - **Ninhydrin Test:** To the extract, 0.25% w/v Ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.

Cell viability study and Evaluation of anti-inflammatory activity of extracts by in-vitro method

In inflammation, macrophage plays an important role by producing reactive oxygen species (ROS), reactive nitrogen species (RNS), cytokines such as interleukin-1β (IL-1ß), IL-6, tumor necrosis factor-α (TNF-α), IL-4, IL-10 and inflammatory mediator nitric oxide (NO) and prostaglandin (PGE). Exposure of bacterial lipopolysaccharides (LPS) has been found to increase the mRNA expression of those inflammatory cytokines and mediators. LPS is a bacterial endotoxin which stimulates innate immunity by regulating inflammatory mediator such as TNF-α, IL-6, and IL-4. The suppression of inflammatory mediator synthesis has been known to be one of the useful therapeutic strategies in the treatment of inflammatory diseases.

Cell culture

Human monocyte (THP-1) cell line was obtained from National Centre for Cell Science (NCCS), Pune, Maharashtra, India and maintained in RPMI-1640 medium (HiMedia Laboratories, Mumbai, India) supplemented with L-glutamine (2 mmol/l), 10% FBS (Gibco, Thermo Fisher Scientific, USA) and 1× antibiotic–antimycotic solution (HiMedia Laboratories, Mumbai, India) in a humidified atmosphere with 5% CO2 at 37 °C. For the induction of cell differentiation, THP-1 cells (1.5 × 106 per ml) were seeded in serum-free RPMI-1640 with 50 nM PMA for 24 h. After incubation, non-adherent cells were removed by aspiration, and the adherent THP-1 derived macrophages (TDMs) were washed with PBS before experimental treatments.

Cell viability assay

Cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. After experimentation, the cells were washed with PBS, followed by addition of 0.5 mg/ml MTT to each well and incubation at 37 °C for 4 h. The purple formazan crystals formed were dissolved in DMSO and the absorbance was recorded at 590 nm using Synergy HTX Multimode Microplate Reader (BioTek Instruments Inc., USA). The results were represented as percentage cell viability with respect to control.

ELISA of TNF-α, IL-4, IL-6 and IL-10

Conditioned media from cells was collected and centrifuged at 800×g for 5 min to remove cellular debris. Levels of TNF-α, IL-4, IL-6 and IL-10 in conditioned media were detected using an ELISA kit according to manufacturer's protocol (Krishgen). The concentration of TNF-α, IL-4, IL-6 and IL-10 in samples was determined from standard curve using GraphPad Prism 6.0.

In silico-molecular docking studies

Inflammation is a key etiological factor for several disease conditions such as hypersensitivity, asthma, Inflammatory Bowel Disease (IBD), rheumatoid arthritis and many others. Most of the currently marketed therapeutic drugs are associated with adverse side effects and are not suitable for chronic therapies and so some of them were withdrawn from the markets. For instance, Non-Steroidal Anti-Inflammatory (NSAID's) drugs are reported to have adverse drug interactions and hence are not

prescribed along with warfarin, antihypertensives and diuretics. Thus, treatment of these inflammatory disorders still remains a growing health concern and has become a major challenge to the health professionals. Molecular docking studies were carried out to explore the binding affinity of active constituents of three plants to different mediators of inflammatory pathway and to arrive at possible anti-inflammatory targets for constituents.

Methodology

Docking studies were carried out by using the software Pyrx, where ligand molecule in an arbitrary conformation, orientation and position was used to find its favourable dockings in a protein-binding site using both simulating annealing and genetic algorithms. The program Pyrx, which has been released as an extension suite to the PyMoL Molecular Viewer, was used to prepare the protein and the ligand. Active chemical constituents of the three plants have been docked against different classes of inflammatory mediators such as cytokines/chemokines (IL-6, IL-4), transcription factors (TNF-α, NF Kappa B), signalling kinases (Protein Kinase C (PKC), Syk kinase, ERK and p38 MAP Kinase) and other important inflammatory mediators-Cox-2 and lipoxygenase and their respective binding affinities were recorded. All these proteins play an important, unique role in different stages of inflammatory cascade. The X-Ray crystallographic structures of these proteins were obtained from RCSB Protein Data Bank (www.pdb.org).

Development of Polyherbal formulations by DOE approach

Preparation of Optimized Polyherbal gel-

The gel was prepared using the dried extract of *Adhatoda vasica*, *Rosa indica* and Calotropis procera. The gel was prepared using Carbapol-934 (1%), polyethylene glycol, methyl paraben, propyl paraben, tri-ethanolamine and distilled water in a quantity sufficient to prepare 100 g of gel. Water required for these formulations was divided in to two parts. In one part the exact amount of extracts were dissolved and in other part, carbapol-934 was dissolved and to this solution methyl paraben and propyl paraben were added. Both of these solutions were mixed in a beaker and tri-ethanolamine was added to the mixture dropwise to obtain the gel consistency.

Preparation of Optimized Polyherbal Spray-

Ethyl alcohol 95% and distilled water were selected as solvents for formulating of spray due to the good solubility of all three extracts. First dissolve weighed quantities of all three extracts in 10 ml of distilled water. Shake it well for 5 -10 minutes for proper mixing of the extracts. Then add 90 ml of ethanol to the solution. Mix thoroughly then filter the solution and fill it in the spray bottle.

Evaluation of topical herbal gel formulation

pH measurement

pH measurement of the gel was carried out using a digital pH meter by dipping the glass electrode completely into the gel system to cover the electrode. The measurement was carried out in triplicate and the average of the three readings was recorded.

Appearance and Homogeneity

Physical appearance and homogeneity of the prepared gels were evaluated by visual perception.

Viscosity

Viscosity of gel was determined using Brookfield viscometer (S-62, model DV-1) at 25 ℃ with a spindle speed of the viscometer rotated at 12 rpm.

Spreadability

Two sets of glass slides of standard dimensions were taken. The herbal gel formulation was placed over one of the slides. The other slide was placed on the top of the gel, such that the gel was sandwiched between the two slides. Hundred g weight of gel was placed on the upper slides so that the gel was between the two slides was pressed uniformly to form a thin layer.

Development and validation of analytical methods

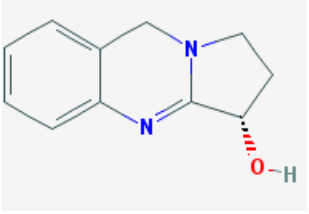
HPTLC method development and validation for Vasicine in *Adhatoda vasica*

Vasicine

Chemical Names: vasicine, Peganine, Vasicin

Molecular Formula: $C_{11}H_{12}N_{20}$

Molecular weight: 188.23 g/mol



Preparation of Standard Solution: Weigh accurately 1 mg of Standard Vasicine in 1 mL volumetric flask. To it add 0.5 mL of Methanol and sonicate till the standard gets dissolved completely. Then, make up the volume up to 1 mL with Methanol. Use the standard solution thus obtained for HPTLC fingerprinting.

Preparation of Test Solution (Extract and Gel): Weigh accurately 1 g of sample in a 250 mL reflux flask. To it add 10 mL of Methanol and reflux on water bath for 60 minutes. On completion of time, remove the reflux flask from the water bath and allow to cool. Filter with the help of Whatman filter paper no. 1 in a 10 mL volumetric flask. Then, make up the volume up to 10 mL with Methanol. Use the Test Solution thus obtained for HPTLC fingerprinting.

Preparation of Test Solution (Spray): Weigh accurately 1 g of sample in a 10 mL volumetric flask. To it add 5 mL of Methanol and mix well for 1 minute on a cyclo mixer. Then, make up the volume up to 10 mL with Methanol. Use the Test Solution thus obtained for HPTLC fingerprinting.

Chromatographic Conditions:	
Application Mode	CAMAG Linomat 5 - Applicator
Filtering System	Whatman filter paper No. 1
Stationary Phase	MERCK - TLC / HPTLC Silica gel 60 F_{254} on Aluminum sheets
Application (Y axis) Start Position	10 mm
Development End Position	80 mm from plate base
Standard Application Volume	10.0 µL
Sample Application Volume	10.0 µL
Distance Between Tracks	13.3 mm
Development Mode	CAMAG TLC Twin Trough Chamber
Chamber Saturation Time	30 minutes
Mobile Phase (MP)	Dioxane: Toluene: Methanol: Ammonia (5: 2: 2: 1 v/v)
Visualization	@ 254 nm
Quantification	@ 254 nm
Drying Mode, Temp. & Time	TLC Plate Heater Preheated at 100 ± 5^0C for 3 minutes

HPTLC method development and validation for Rutin in *Rosa indica*

Preparation of Standard Solution: Weigh accurately 2 mg of Standard Rutin in 1 mL volumetric flask. To it add 1 mL of Methanol and sonicate till the standard gets dissolved completely. Then, make up the volume up to 1 mL with Methanol. Use the standard solution thus obtained for HPTLC fingerprinting.

Preparation of Test Solution (Extract and Gel): Weigh accurately 1 g of sample in a 250 mL reflux flask. To it add 10 mL of Methanol and reflux on water bath for 60 minutes. On completion of time, remove the reflux flask from the water bath and allow to cool. Filter with the help of Whatman filter paper no. 1 in a 10 mL volumetric flask. Then, make up the volume up to 10 mL with Methanol. Use the Test Solution thus obtained for HPTLC fingerprinting.

Preparation of Test Solution (Spray): Weigh accurately 1 g of sample in a 10 mL volumetric flask. To it add 5 mL of Methanol and mix well for 1 minute on a cyclo mixer. Then, make up the volume up to 10 mL with Methanol. Use the Test Solution thus obtained for HPTLC fingerprinting.

Chromatographic Conditions:	
Application Mode	CAMAG Linomat 5 - Applicator
Filtering System	Whatman filter paper No. 1
Stationary Phase	MERCK - TLC / HPTLC Silica gel 60 F254 on Aluminum sheets
Application (Y axis) Start Position	10 mm
Development End Position	80 mm from plate base
Standard Application Volume	10.0 µL
Sample Application Volume	10.0 µL
Distance Between Tracks	13.3 mm
Development Mode	CAMAG TLC Twin Trough Chamber
Chamber Saturation Time	30 minutes
Mobile Phase (MP)	Toluene: Ethyl acetate: Formic acid: Methanol
Visualization	@ 254 nm
Quantification	@ 257 nm
Drying Mode, Temp. & Time	TLC Plate Heater Preheated at 100 ± 5^0C for 3 minutes

HPTLC method development and validation for Gallic acid in *Calotropis procera*

Preparation of Standard Solution: Weigh accurately 2 mg of Standard Gallic acid in 2 mL volumetric flask. To it add 1 mL of Methanol and sonicate till the standard gets dissolved completely. Then, make up the volume up to 2 mL with Methanol. Use the standard solution thus obtained for HPTLC fingerprinting.

Preparation of Test Solution (Extract and Gel): Weigh accurately 1 g of sample in a 250 mL reflux flask. To it add 10 mL of Methanol and reflux on water bath for 60 minutes. On completion of time, remove the reflux flask from the water bath and allow to cool. Filter with

the help of Whatman filter paper no. 1 in a 10 mL volumetric flask. Then, make up the volume up to 10 mL with Methanol. Use the Test Solution thus obtained for HPTLC fingerprinting.

Preparation of Test Solution (Spray): Weigh accurately 1 g of sample in a 10 mL volumetric flask. To it add 5 mL of Methanol and mix well for 1 minute on a cyclo mixer. Then, make up the volume up to 10 mL with Methanol. Use the Test Solution thus obtained for HPTLC fingerprinting.

Chromatographic Conditions:	
Application Mode	CAMAG Linomat 5 - Applicator
Filtering System	Whatman filter paper No. 1
Stationary Phase	MERCK - TLC / HPTLC Silica gel 60 F254 on Aluminum sheets
Application (Y axis) Start Position	10 mm
Development End Position	80 mm from plate base
Standard Application Volume	10.0 µL
Sample Application Volume	10.0 µL (Extract and Spray); 20.0 µL (Gel)
Distance Between Tracks	13.3 mm
Development Mode	CAMAG TLC Twin Trough Chamber
Chamber Saturation Time	30 minutes
Mobile Phase (MP)	Toluene: Ethyl acetate: Formic acid (10: 7: 1 v/v)
Visualization	@ 254 nm
Quantification	@ 278 nm
Drying Mode, Temp. & Time	TLC Plate Heater Preheated at 100 ± 50C for 3 minutes

Assessment of anti-inflammatory effect of formulations

- **Skin irritation study**

  This test was performed on Wistar/Sprague-Dawley rats. The animals given standard animal feed and had free access to water ad libitium. Animals were divided into three groups, each batch containing six animals. Dorsal hairs at the back of the rats were removed one day prior to the commencement of the study and kept individually in cages to avoid contact with the other rats. Two groups of each were used for control and standard irritant. Another group was used as a test. The 50 mg of the formulation was applied over one square centimeter area of whole and abraded skin of different animals. Aqueous solution of 0.8% formalin was used as standard irritant. The animals were observed for days for any signs of edema and erythema.

	Groups	No. of animals
Group I	Normal control	6
Group II	Standard irritant	6
Group III	Polyherbal Gel	6
Group IV	Polyherbal Spray	6
	Total No. of animals	24

Evaluation of anti-inflammatory activity

Male or female Sprague-Dawley or Wistar rats are used. The animals are starved overnight. To ensure uniform hydration, the rats receive 5 ml of water by stomach tube (controls) or the test drug dissolved or suspended in the same volume. Thirty minutes later, the rats are challenged by a subcutaneous injection of 0.05 ml of 1% solution of carrageenan into the plantar side of the left hind paw. The paw is marked with ink at the level of the lateral malleolus and immersed in mercury up to this mark. The paw volume is measured plethysmographically immediately after injection, again 3 and 6 h, and eventually 24 h after challenge.

Groups		**Administered samples**	**No. of animals**
I	Normal Control	Purified water	6
II	Test I (Gel)	Polyherbal Gel (Extract of leaves of *Adhatoda vasica*-1/3, Extract of flowers of *Rosa indica* 1/3 and Extract of leaves of *Calotropis procera* 1/3)	6
III	Test II (Spray)	Polyherbal Spray (Extract of leaves of *Adhatoda vasica*-1/3, Extract of flowers of *Rosa indica* 1/3 and Extract of leaves of *Calotropis procera* 1/3)	6
IV	Test III (Diclofenac Gel)	Diclofenac Gel (1%)	6
V	Test IV (Diclofenac Spray)	Diclofenac Spray (4%)	6
Total No. of Animals			30

Results and Discussion

Physico-Chemical Parameters

The physico-chemical parameters like ash value, acid insoluble ash and water-soluble ash, were observed as shown in Table.

Name of Extract	Total ash (%)	Water-soluble ash (%)	Acid insoluble ash (%)
Rosa indica extract	8.34	5.44	2.21
Calotropis procera extract	19.51	3.92	2.52
Adhatoda vasica extract	12.53	4.41	1.52

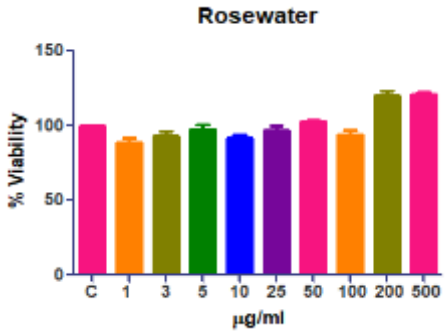
Qualitative Phytochemical Screening of extracts

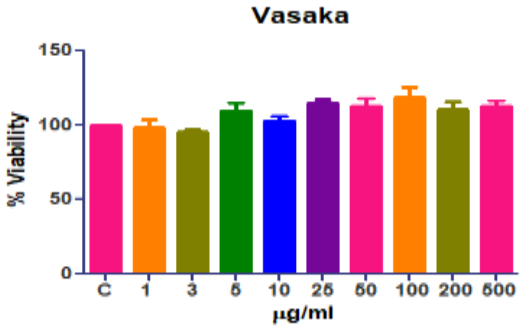
Preliminary phytochemical screening was performed to find out the phytoconstituent present in the extracts.

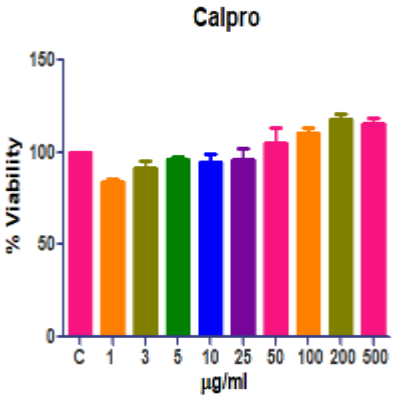
	AVM	AVA	RIM	RIA	CPM	CPA
Alkaloids	+ve	+ve	+ve	+ve	-ve	-ve
Saponin	+ve	+ve	+ve	+ve	+ve	+ve
Carbohydrates	+ve	+ve	+ve	+ve	+ve	-ve
Phenolic glycoside/Tannins	+ve	+ve	-ve	-ve	+ve	+ve
Proteins	-ve	-ve	-ve	-ve	+ve	+ve
Flavonoids	+ve	+ve	+ve	+ve	+ve	+ve
Volatile Oil	-ve	-ve	+ve	+ve	+ve	-ve
Fixed Oil	-ve	-ve	+ve	+ve	+ve	-ve

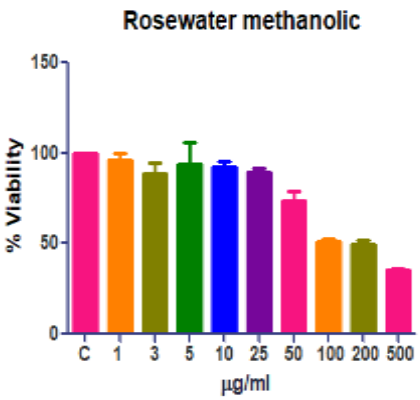
- AVM- *Adhatoda vasica* Methanolic extract
- AVA- *Adhatoda vasica* Aqueous extract
- RIM- *Rosa indica* Methanolic extract
- RIA- *Rosa indica* Aqueous extract
- CPM- *Calotropis procera* Methanolic extract
- CPA- *Calotropis procera* Aqueous extract

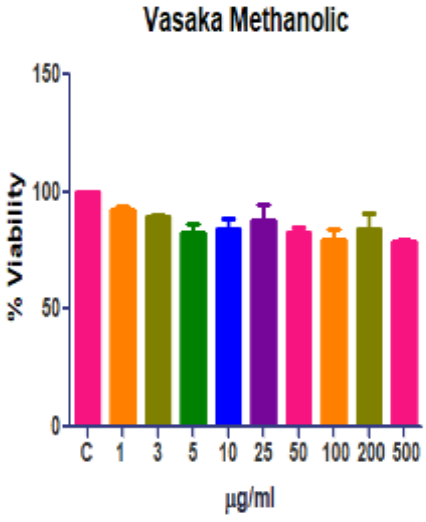
Cell viability assay

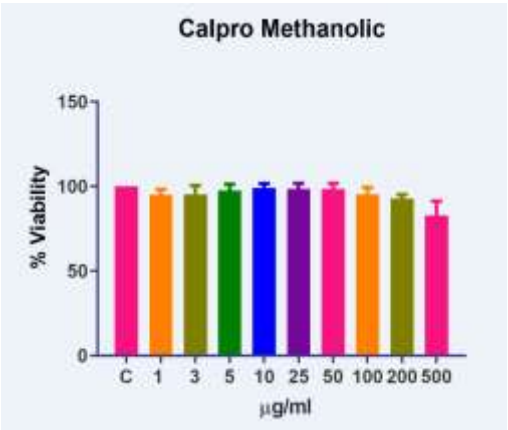


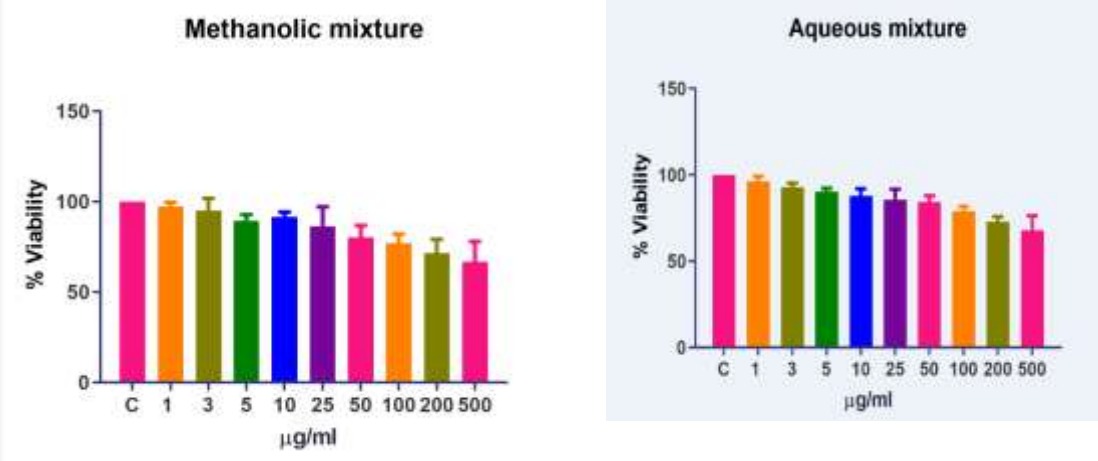








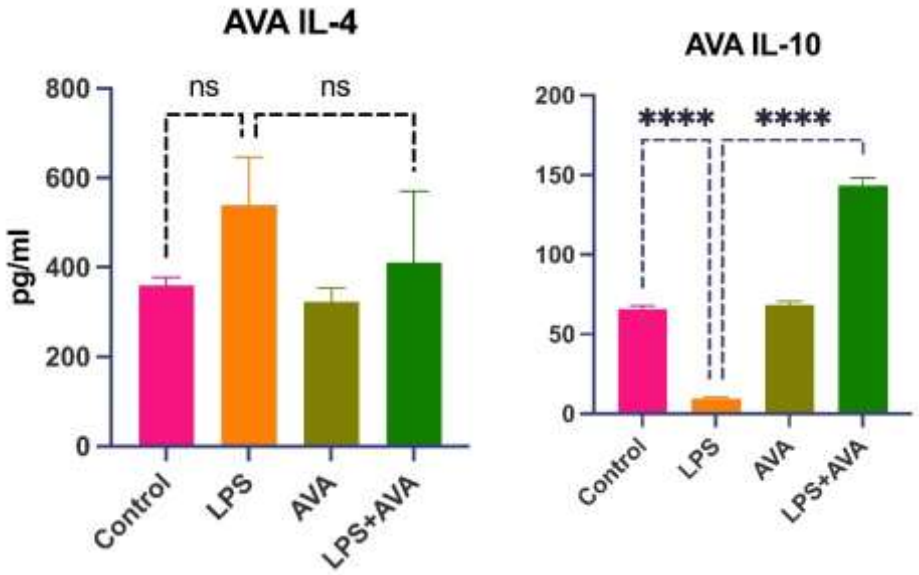


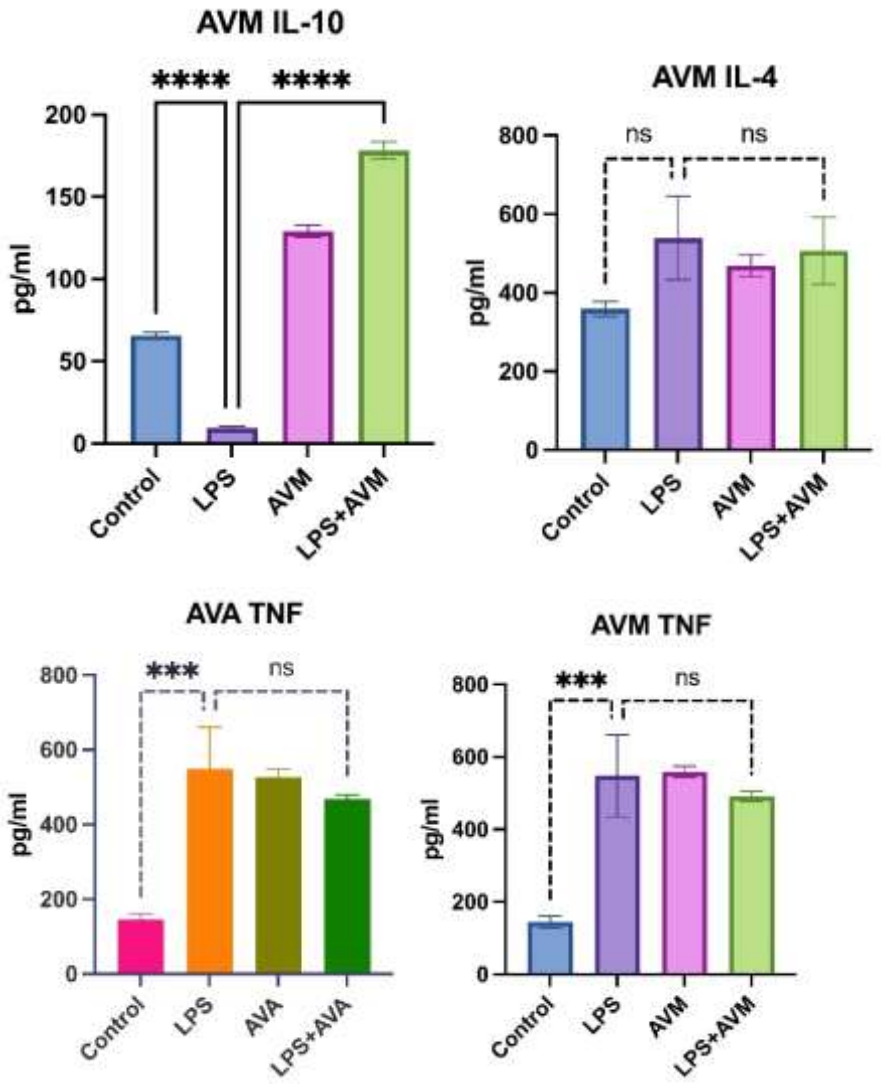


Conclusion of MTT assay

Cell viability was not affected by any of the plant extract and also mixture of all three plant extracts as measured by MTT assay. These results indicating that no lethality or local toxicity were observed after administration 500 μg/ml of dose. Only there was some lethality with the *Rosa indica* methanolic extract above 100 μg/ml of dose.

ELISA of TNF-α, IL-4, IL-6 and IL-10





IL-4 assay

Based on graphs, *Rosa indica* aqueous extract treatment showed higher IL-4 inhibition activity compared to the other extracts. It can be seen that all other aqueous extracts were able to inhibit IL-4 production in LPS-induced cells. The LPS induction was successfully increase the IL-4 concentration, showed by significantly high IL-4 level in positive control (LPS-induced cells without treatment) compared to the negative control (normal cells without LPS induction).

IL-10 assay

Based on graphs, *Calotropis procera* aqueous extract treatment showed higher IL-10 concentration as compared to the other extracts. So, it indicates best anti-inflammatory activity than other extracts. It can be seen that other aqueous extracts were able to increase IL-10 production in LPS-induced cells. The LPS induction was successfully decrease the IL-10 concentration, showed by significantly low IL-10 level in positive control (LPS-induced cells without treatment) compared to the negative control (normal cells without LPS induction).

Conclusion of in-vitro assay

Aqueous extracts of *Rosa indica*, *Adhatoda vasica* and *Calotropis procera* possess anti-inflammatory activity showed by significantly decrease in production of pro-inflammatory cytokines IL-4 and TNF-α level in activated THP-1 cells. We suggest that the mechanisms of extracts may be associated with the inhibition of inflammatory mediator overproduction, including, TNF-α, and IL-4. Simultaneously these extracts increase the levels of IL-10 which act as anti-inflammatory.

In silico-molecular docking studies

Molecular Docking against TNF-α Receptor

Ligand	Target	Binding Energy
Artemiseole_1_1_uff_E=1435.64	1tnf_chainABC_Apo	-5.35
Dihydromyrcene_1_1_uff_E=74.18	1tnf_chainABC_Apo	-3.48
AC1NSUBD_1_1_uff_E=545.08	1tnf_chainABC_Apo	-6.1
Anisotine_1_1_uff_E=690.04	1tnf_chainABC_Apo	-5.97
Calotropagenin_1_1_uff_E=1013.94	1tnf_chainABC_Apo	-7.41
Hexopyranose_1_1_uff_E=187.66	1tnf_chainABC_Apo	-3.52
Benzoyllineolone_1_1_uff_E=770.17	1tnf_chainABC_Apo	-4.38
Isosteviol_1_1_uff_E=610.83	1tnf_chainABC_Apo	-7.3
Peganine_1_1_uff_E=261.62	1tnf_chainABC_Apo	-4.66
Daucosterol_1_1_uff_E=1015.91	1tnf_chainABC_Apo	-1.86
VASICINONE_1_1_uff_E=293.59	1tnf_chainABC_Apo	-5.09
Phytosterols_1_1_uff_E=798.26	1tnf_chainABC_Apo	-5.93
Phytosterols_1_1_uff_E=807.66	1tnf_chainABC_Apo	-6.5
Vasicinol_1_1_uff_E=264.57	1tnf_chainABC_Apo	-4.94
Syriogenin_1_1_uff_E=1029.49	1tnf_chainABC_Apo	-7.94
Phytosterols_1_1_uff_E=807.66_1	1tnf_chainABC_Apo	-7.65
Vasicinolone_1_1_uff_E=319.30	1tnf_chainABC_Apo	-5.44
Vasicol_1_1_uff_E=283.46	1tnf_chainABC_Apo	-4.43
alpha.-Amyrin_1_1_uff_E=1093.65	1tnf_chainABC_Apo	-7
betaine_1_1_uff_E=96.88	1tnf_chainABC_Apo	-3.74

beta.-Amyrin_1_1_uff_E=1104.74	1tnf_chainABC_Apo	-8.17
mol38_1_1_uff_E=11.99	1tnf_chainABC_Apo	-4.66
mol11_1_1_uff_E=920.88	1tnf_chainABC_Apo	-2.06
mol24_1_1_uff_E=1475.62	1tnf_chainABC_Apo	-5.74
mol12_1_1_uff_E=163.30	1tnf_chainABC_Apo	-0.72
mol10_1_1_uff_E=77.53	1tnf_chainABC_Apo	-0.52
mol39_1_1_uff_E=2537.19	1tnf_chainABC_Apo	-5.91
mol2_1_1_uff_E=497.37	1tnf_chainABC_Apo	-5.13
mol44_1_1_uff_E=1516.15	1tnf_chainABC_Apo	-3.76
mol19_1_1_uff_E=1243.37	1tnf_chainABC_Apo	-3.55
mol42_1_1_uff_E=59.47	1tnf_chainABC_Apo	-1.31
mol40_1_1_uff_E=175.08	1tnf_chainABC_Apo	-3.93
mol43_1_1_uff_E=53.98	1tnf_chainABC_Apo	-2.43
mol5_1_1_uff_E=77.60	1tnf_chainABC_Apo	-1.43
mol8_1_1_uff_E=81.62	1tnf_chainABC_Apo	-1.29
mol9_1_1_uff_E=92.88	1tnf_chainABC_Apo	-0.77
Diclofenac	1tnf_chainABC_Apo	-8.2

Molecular Docking against TNF-α Receptor

From the above table it can be seen that Beta-Amyrin had highest binding affinity towards TNF-α receptor whereas Syriogenin, phytosterol, α-Amyrin, Calaptropagenin and Isosteviol has comparable binding affinity towards TNF-α.

Molecular Docking against COX-2 Receptor

Artemiseole_1_1_uff_E=1435.64	3ln1_chainA_Apo	-4.91
Dihydromyrcene_1_1_uff_E=74.18	3ln1_chainA_Apo	-3.99
AC1NSUBD_1_1_uff_E=545.08	3ln1_chainA_Apo	-4.67
Anisotine_1_1_uff_E=690.04	3ln1_chainA_Apo	-7.15
Calotropagenin_1_1_uff_E=1013.94	3ln1_chainA_Apo	-5.15
Hexopyranose_1_1_uff_E=187.66	3ln1_chainA_Apo	-2.83
Benzoyllineolone_1_1_uff_E=770.17	3ln1_chainA_Apo	-4.65
Isosteviol_1_1_uff_E=610.83	3ln1_chainA_Apo	-6.55

Peganine_1_1_uff_E=261.62	3ln1_chainA_Apo	-5.43
Daucosterol_1_1_uff_E=1015.91	3ln1_chainA_Apo	-3.62
VASICINONE_1_1_uff_E=293.59	3ln1_chainA_Apo	-5.36
Phytosterols_1_1_uff_E=798.26	3ln1_chainA_Apo	-5.53
Vasicinol_1_1_uff_E=264.57	3ln1_chainA_Apo	-5.43
Phytosterols_1_1_uff_E=807.66	3ln1_chainA_Apo	-5.19
Syriogenin_1_1_uff_E=1029.49	3ln1_chainA_Apo	-6.08
Phytosterols_1_1_uff_E=807.66_1	3ln1_chainA_Apo	-4.77
Vasicinolone_1_1_uff_E=319.30	3ln1_chainA_Apo	-5.24
betaine_1_1_uff_E=96.88	3ln1_chainA_Apo	-2.59
Vasicol_1_1_uff_E=283.46	3ln1_chainA_Apo	-4
alpha.-Amyrin_1_1_uff_E=1093.65	3ln1_chainA_Apo	-9.22
beta.-Amyrin_1_1_uff_E=1104.74	3ln1_chainA_Apo	-7.08
mol38_1_1_uff_E=11.99	3ln1_chainA_Apo	-2.93
mol11_1_1_uff_E=920.88	3ln1_chainA_Apo	-2.83
mol24_1_1_uff_E=1475.62	3ln1_chainA_Apo	-6.08
mol12_1_1_uff_E=163.30	3ln1_chainA_Apo	-1.73
mol10_1_1_uff_E=77.53	3ln1_chainA_Apo	-0.56
mol39_1_1_uff_E=2537.19	3ln1_chainA_Apo	-5.88
mol2_1_1_uff_E=497.37	3ln1_chainA_Apo	-3.84
mol19_1_1_uff_E=1243.37	3ln1_chainA_Apo	-4.35
mol44_1_1_uff_E=1516.15	3ln1_chainA_Apo	-4.26
mol42_1_1_uff_E=59.47	3ln1_chainA_Apo	-1.95
mol40_1_1_uff_E=175.08	3ln1_chainA_Apo	-3.68
mol43_1_1_uff_E=53.98	3ln1_chainA_Apo	-2.52
mol5_1_1_uff_E=77.60	3ln1_chainA_Apo	-2.21
mol8_1_1_uff_E=81.62	3ln1_chainA_Apo	-4.56
mol9_1_1_uff_E=92.88	3ln1_chainA_Apo	0.06
Diclofenac	3ln1_chainA_Apo	-9.8

The above results shows that α-Amyrin has highest binding affinity towards COX-2 receptor followed by β-Amryin and Anisotine.

Molecular Docking against IL-6 Receptor

betaine_1_1_uff_E=96.88	1alu_chainA_Apo	-2.36
AC1NSUBD_1_1_uff_E=545.08	1alu_chainA_Apo	-4.51
Anisotine_1_1_uff_E=690.04	1alu_chainA_Apo	-5.8
Phytosterols_1_1_uff_E=807.66	1alu_chainA_Apo	-5.24
mol5_1_1_uff_E=77.60	1alu_chainA_Apo	-0.98
mol2_1_1_uff_E=497.37	1alu_chainA_Apo	-1.93
mol8_1_1_uff_E=81.62	1alu_chainA_Apo	-0.46
Daucosterol_1_1_uff_E=1015.91	1alu_chainA_Apo	-3.52
Peganine_1_1_uff_E=261.62	1alu_chainA_Apo	-4.05
mol11_1_1_uff_E=920.88	1alu_chainA_Apo	-1.86
mol12_1_1_uff_E=163.30	1alu_chainA_Apo	-1.42
Vasicinol_1_1_uff_E=264.57	1alu_chainA_Apo	-4.3
mol10_1_1_uff_E=77.53	1alu_chainA_Apo	0.55
VASICINONE_1_1_uff_E=293.59	1alu_chainA_Apo	-4.02
Vasicinolone_1_1_uff_E=319.30	1alu_chainA_Apo	-4.38
mol9_1_1_uff_E=92.88	1alu_chainA_Apo	1.28
Vasicol_1_1_uff_E=283.46	1alu_chainA_Apo	-2.7
alpha.-Amyrin_1_1_uff_E=1093.65	1alu_chainA_Apo	-5.98
beta.-Amyrin_1_1_uff_E=1104.74	1alu_chainA_Apo	-6.43
mol24_1_1_uff_E=1475.62	1alu_chainA_Apo	-5.28
Phytosterols_1_1_uff_E=798.26	1alu_chainA_Apo	-5.06
Hexopyranose_1_1_uff_E=187.66	1alu_chainA_Apo	-1.76
Calotropagenin_1_1_uff_E=1013.94	1alu_chainA_Apo	-4.09
Benzoyllineolone_1_1_uff_E=770.17	1alu_chainA_Apo	-4.34
mol19_1_1_uff_E=1243.37	1alu_chainA_Apo	-3.68
Syriogenin_1_1_uff_E=1029.49	1alu_chainA_Apo	-4.62
Artemiseole_1_1_uff_E=1435.64	1alu_chainA_Apo	-3.64
Isosteviol_1_1_uff_E=610.83	1alu_chainA_Apo	-5.81

mol38_1_1_uff_E=11.99	1alu_chainA_Apo	-3.71
mol39_1_1_uff_E=2537.19	1alu_chainA_Apo	-4.65
Dihydromyrcene_1_1_uff_E=74.18	1alu_chainA_Apo	-2.88
mol44_1_1_uff_E=1516.15	1alu_chainA_Apo	-2.9
mol43_1_1_uff_E=53.98	1alu_chainA_Apo	-1.7
mol40_1_1_uff_E=175.08	1alu_chainA_Apo	-2.16
mol42_1_1_uff_E=59.47	1alu_chainA_Apo	-1.68
Diclofenac	1alu_chainA_Apo	-5.4

From the above results it can be seen that molecular docking performed against IL-6 receptor gave relatively poor results for all the ligands. However, β-Amyrin had the highest binding affinity among all ligands for respective IL-6 receptor.

Molecular Docking against COX-1 Receptor

betaine_1_1_uff_E=96.88	3n8y_chainA_Apo	-2.46
AC1NSUBD_1_1_uff_E=545.08	3n8y_chainA_Apo	-4.37
Anisotine_1_1_uff_E=690.04	3n8y_chainA_Apo	-4.38
Phytosterols_1_1_uff_E=807.66	3n8y_chainA_Apo	-4.96
mol5_1_1_uff_E=77.60	3n8y_chainA_Apo	-1.56
mol2_1_1_uff_E=497.37	3n8y_chainA_Apo	-2.45
Daucosterol_1_1_uff_E=1015.91	3n8y_chainA_Apo	-2.72
mol8_1_1_uff_E=81.62	3n8y_chainA_Apo	-1.5
Peganine_1_1_uff_E=261.62	3n8y_chainA_Apo	-5.12
mol11_1_1_uff_E=920.88	3n8y_chainA_Apo	-1.95
mol12_1_1_uff_E=163.30	3n8y_chainA_Apo	-1.81
mol10_1_1_uff_E=77.53	3n8y_chainA_Apo	-0.01
mol9_1_1_uff_E=92.88	3n8y_chainA_Apo	0.68
Vasicinol_1_1_uff_E=264.57	3n8y_chainA_Apo	-5.71
VASICINONE_1_1_uff_E=293.59	3n8y_chainA_Apo	-5.18
Vasicinolone_1_1_uff_E=319.30	3n8y_chainA_Apo	-5.25
Vasicol_1_1_uff_E=283.46	3n8y_chainA_Apo	-3.8
alpha.-Amyrin_1_1_uff_E=1093.65	3n8y_chainA_Apo	-7.13
beta.-Amyrin_1_1_uff_E=1104.74	3n8y_chainA_Apo	-7.36
mol24_1_1_uff_E=1475.62	3n8y_chainA_Apo	-6.12

Phytosterols_1_1_uff_E=798.26	3n8y_chainA_Apo	-5.3
Calotropagenin_1_1_uff_E=1013.94	3n8y_chainA_Apo	-5.36
Benzoyllineolone_1_1_uff_E=770.17	3n8y_chainA_Apo	-4.8
Hexopyranose_1_1_uff_E=187.66	3n8y_chainA_Apo	-2.34
mol19_1_1_uff_E=1243.37	3n8y_chainA_Apo	-4.99
Artemiseole_1_1_uff_E=1435.64	3n8y_chainA_Apo	-4.84
Isosteviol_1_1_uff_E=610.83	3n8y_chainA_Apo	-6.01
Syriogenin_1_1_uff_E=1029.49	3n8y_chainA_Apo	-5.61
mol38_1_1_uff_E=11.99	3n8y_chainA_Apo	-3.74
mol39_1_1_uff_E=2537.19	3n8y_chainA_Apo	-5.01
Dihydromyrcene_1_1_uff_E=74.18	3n8y_chainA_Apo	-4.19
mol44_1_1_uff_E=1516.15	3n8y_chainA_Apo	-4.4
mol42_1_1_uff_E=59.47	3n8y_chainA_Apo	-1.79
mol40_1_1_uff_E=175.08	3n8y_chainA_Apo	-6.24
mol43_1_1_uff_E=53.98	3n8y_chainA_Apo	-2.27
Diclofenac	3n8y_chainA_Apo	-7.8

The results of molecular docking performed against COX-1 receptor showed α and β-Amyrin to possess maximum binding affinity of -7.13 and -7.36 respectively.

Conclusion of in silico studies

Molecular docking for compounds of all three plants was performed against various receptors like COX-1, COX-2, IL-6 and TNF-α. From the results it can be concluded that TNF-α had best results in terms of maximum number of ligands showing higher binding affinity followed by COX-2 and COX-1 receptors. Molecular docking results of IL-6 were least satisfactory since most ligands showed poor binding affinity towards IL-6.

The comparison among the binding capacity of particular ligand towards various receptor lead to the conclusion of α and β-Amyrin to be the best ligands having highest binding affinity towards all kind of receptors. Other active compounds that showed relatively good binding affinity towards maximum number of receptors are Syriogenin, phytosterol, Calaptropagenin and Isosteviol. Hence it can be concluded that all three plants have components that can be responsible for potential anti-inflammatory activity.

Formulation development by DOE approach

Formula of Optimized Polyherbal Gel

Name of Ingredients	**Quantity of Ingredient**
Carbopol 934	1%
Adhatoda vasica extract	1%
Rosa indica extract	1%
Calotropis procera extract	1%
Polyethylene glycol	5 gm
Triethanolamine	4-5 drops
Methyl paraben	0.1%
Propyl paraben	0.05%
Water	Quantity sufficient to 100 gm

Gel formulation is more preferred, among the other topical semisolid preparations, since it has long residence time on the skin, high viscosity, moisturizing effect on flaky skin due to their occlusive properties, more bio adhesiveness, less irritation, independent of water solubility of active ingredient, ease of application and better release characters.

Formula of Optimized Polyherbal Spray

Name of Ingredients	**Quantity of Ingredient**
Distilled water	10 ml
Adhatoda vasica extract	1%
Rosa indica extract	1 %
Calotropis procera extract	1 %
Ethanol	Quantity sufficient to 100 ml

Evaluation of topical herbal gel

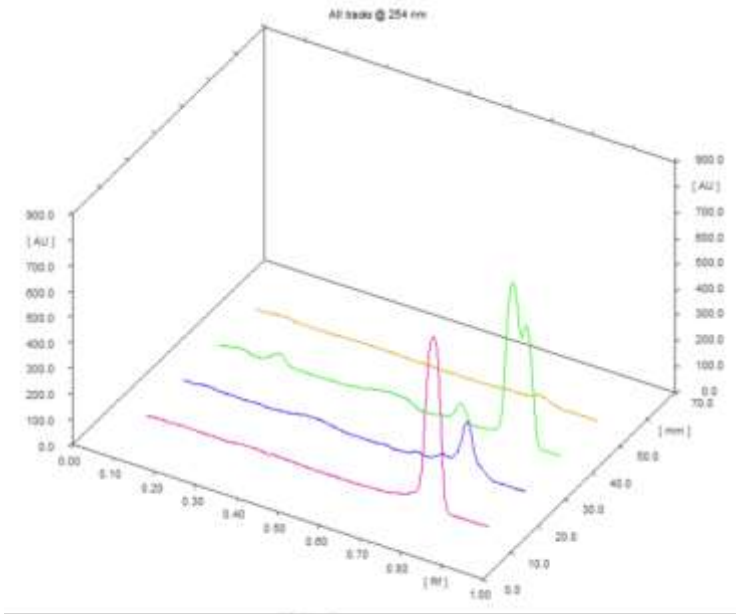
pH- Prepared gels were found to be homogeneous and in good appearance and consistency. The pH values of all the formulations were in the close range of neutral pH 7.42-7.88 and hence it caused no skin irritation, which is also supported by skin irritation study.

Viscosity- Polymers were included in the designed topical formulations in order to provide a prompt release of drug and to achieve as well as to maintain the drug concentration within the therapeutically effective range. The value between 5200-6000 centi poise was reported for topical gel formulation developed using Carbopol polymers.

Spreadability- Values of the spreadability indicated that the gel formulations are easily spreadable.

Evaluation parameter	**Value of parameter**
Appearance and Homogeneity	Brownish Transparent
pH	7.42-7.88
Viscosity	5600 cp
Spreadability	45mm

HPTLC method development and validation of vasicine in *Adhatoda vasica*



3D Chromatogram

Parameters	**Standard**	**Vasaka Extract**	**Polyherbal Gel**	**Herbal Spray**
Weight	1.1 mg	1293 mg	2073 mg	1125 mg
Area	19860	16732.4	5819.7	789.7
% Vasicine	--	**0.716 %**	**0.151 %**	**0.038 %**

R_f value

Spot No.	T1	T2	T3	T4
1	--	--	0.13	--
2	--	--	0.23	--
3	--	0.38	--	--
4	--	0.40	--	--
5	--	--	0.50	--
6	--	--	0.51	--
7	--	0.65	0.65	--
8	--	0.72	--	--
9 (Vasicine)	**0.78**	**0.78**	**0.78**	**0.78**
10	--	--	0.83	--

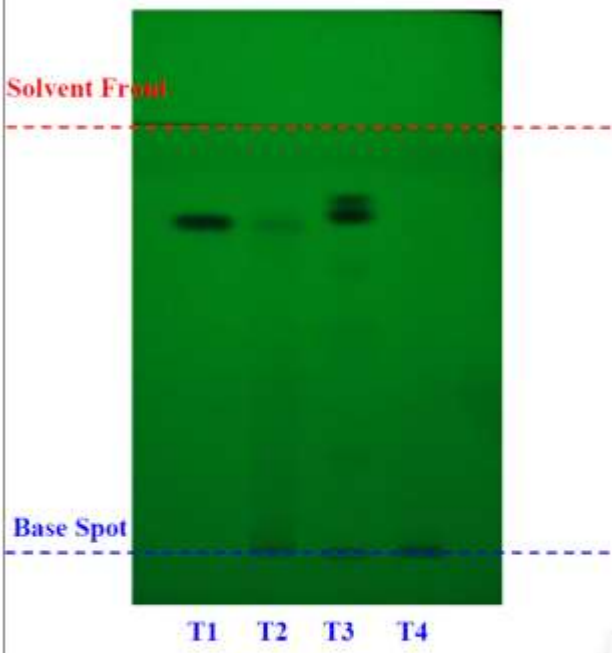
Track T1: Standard Vasicine

Track T2: Vasaka Extract

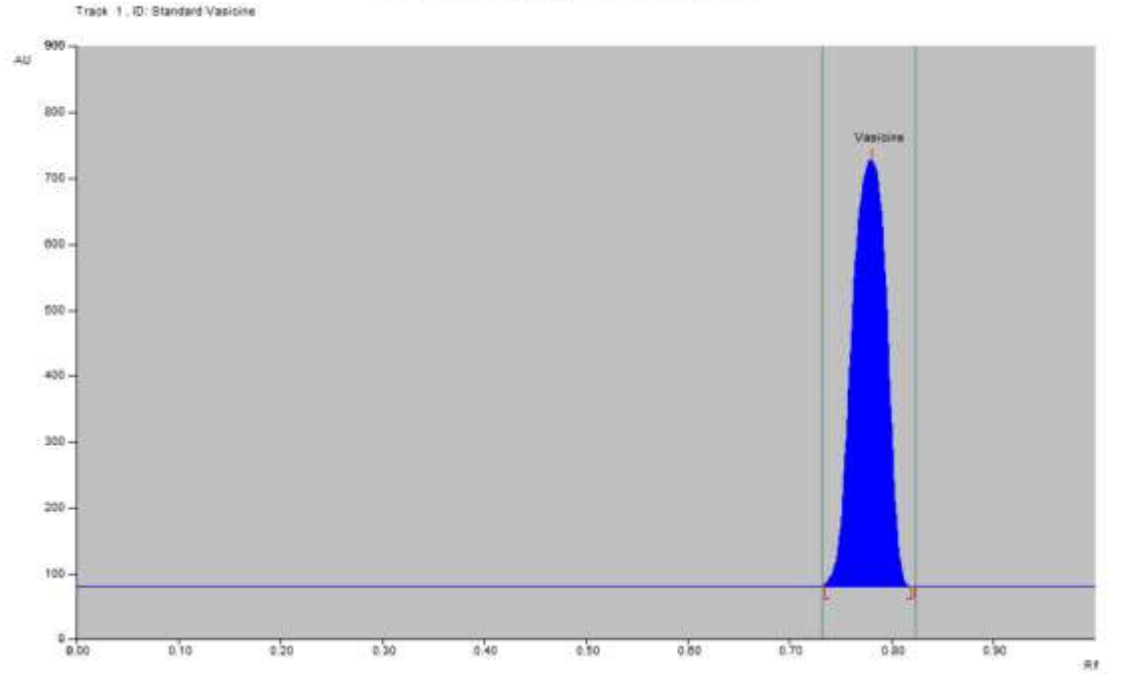
Track T3: Polyherbal Gel

Track T4: Herbal Spray

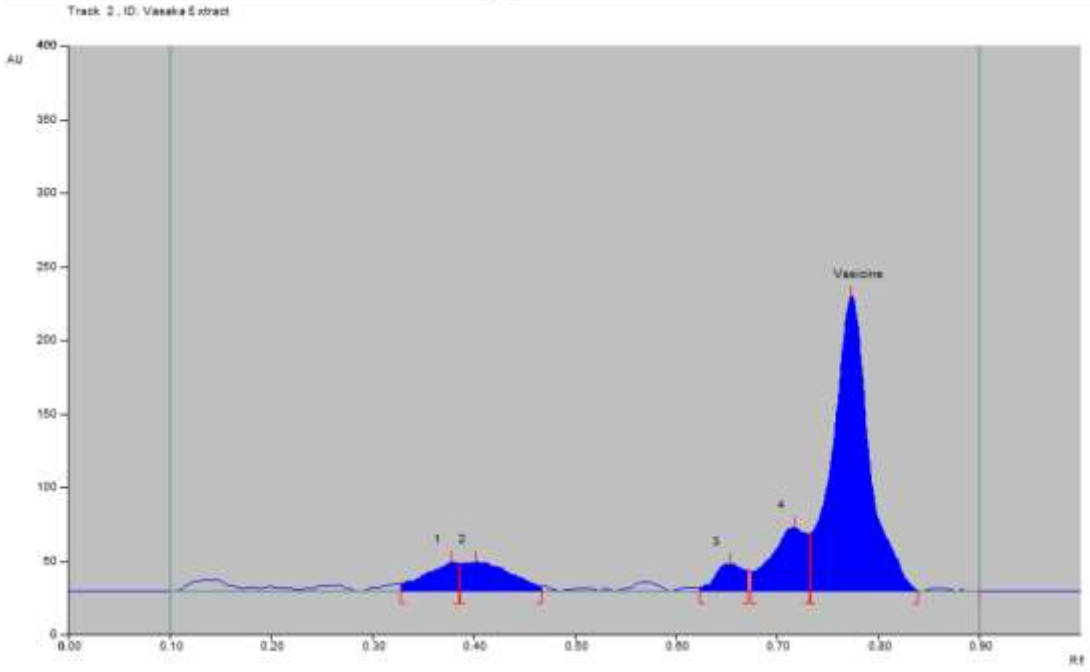
HPTLC Plate



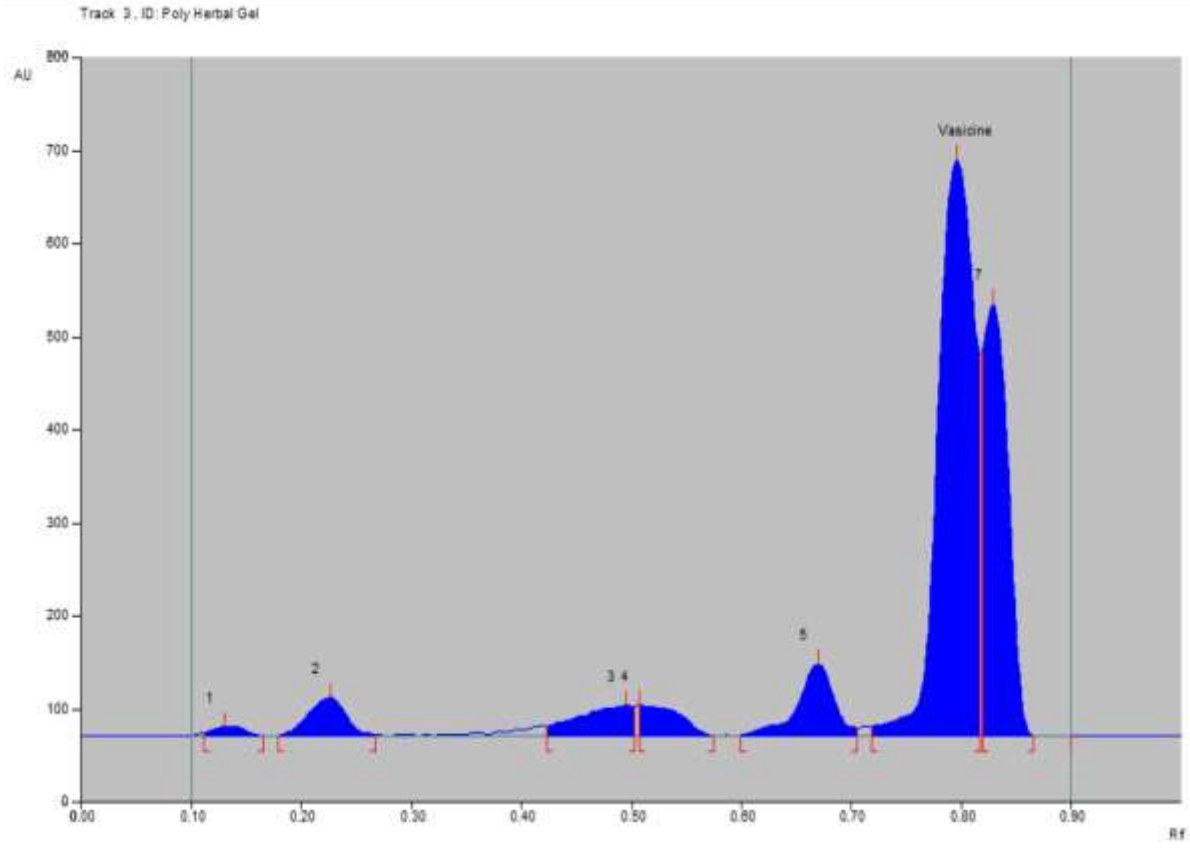
2D Chromatogram of Standard

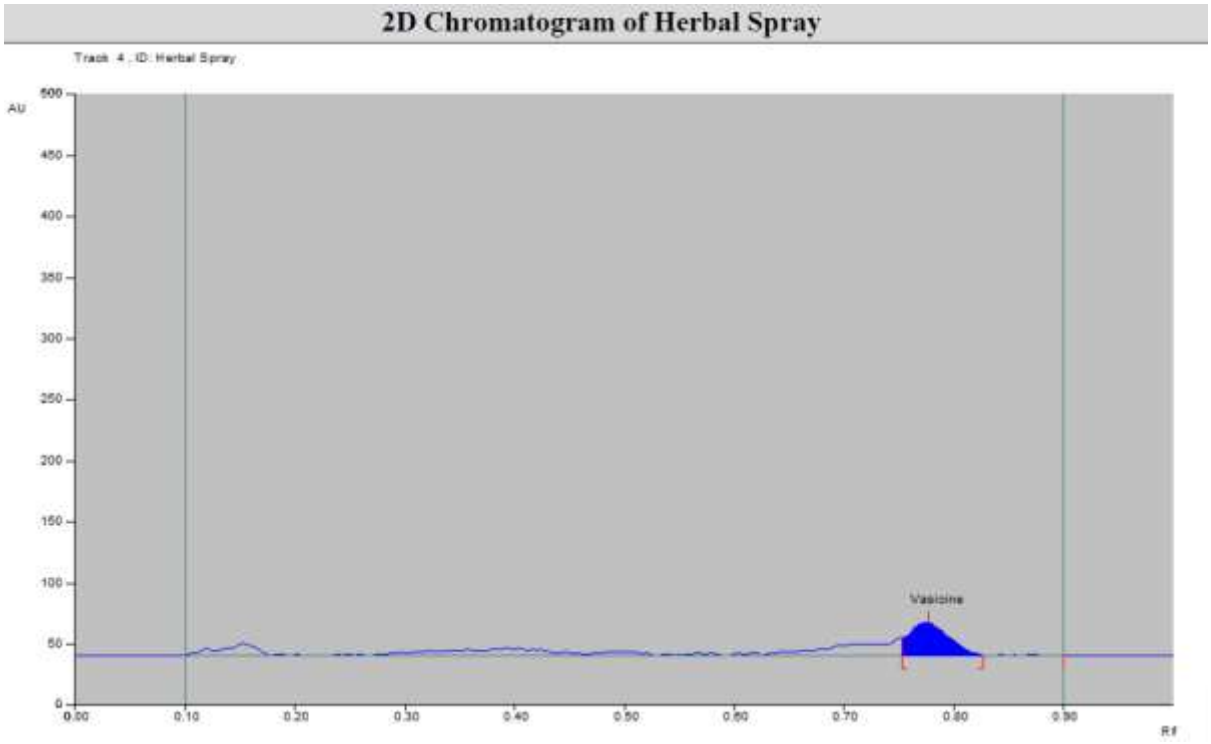


2D Chromatogram of Vasaka Extract

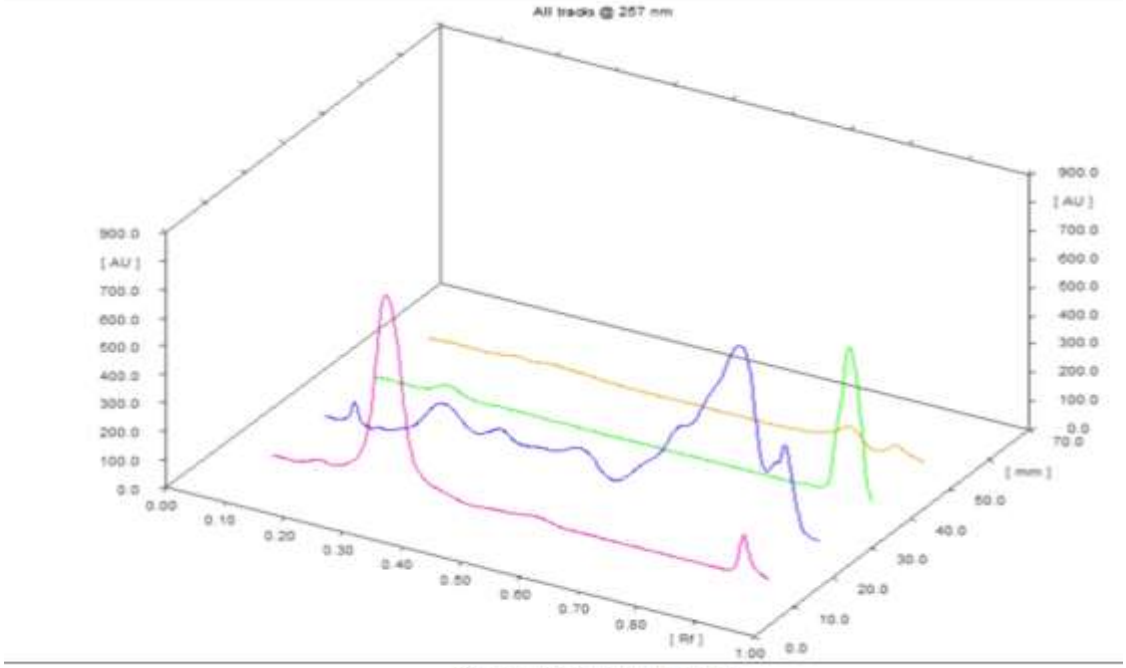


2D Chromatogram of Polyherbal Gel





HPTLC method development and validation for Rutin in *Rosa indica*



Parameters	Standard	Rose Extract	Polyherbal Gel	Herbal Spray
Weight	2.2 mg	1013 mg	1293 mg	1125 mg
Area	27535.4	9115.4	1149.4	399.2
% Rutin	--	**0.319 %**	**0.031 %**	**0.012 %**

R_f value

Spot No.	T1	T2	T3	T4
1	--	0.13	--	--
2 (Rutin)	**0.25**	**0.25**	**0.25**	**0.25**
3	--	0.38	--	--
4	--	0.51	--	--
5	--	0.79	--	0.79
6	--	0.86	0.86	0.86

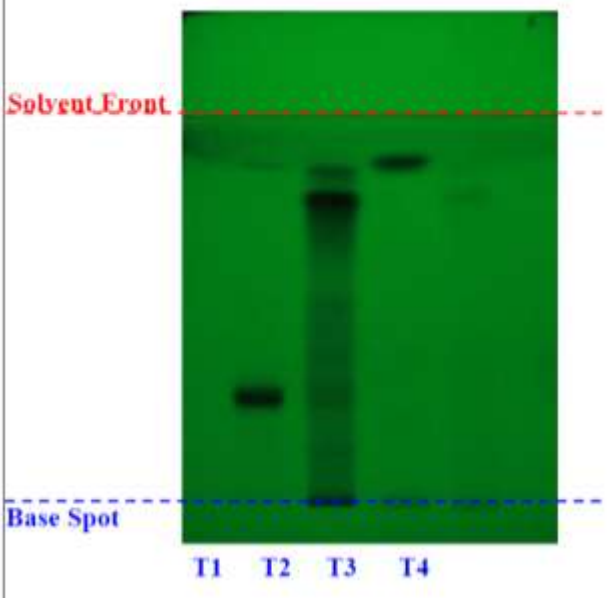
Track T1: Standard Rutin

Track T2: Rose Extract

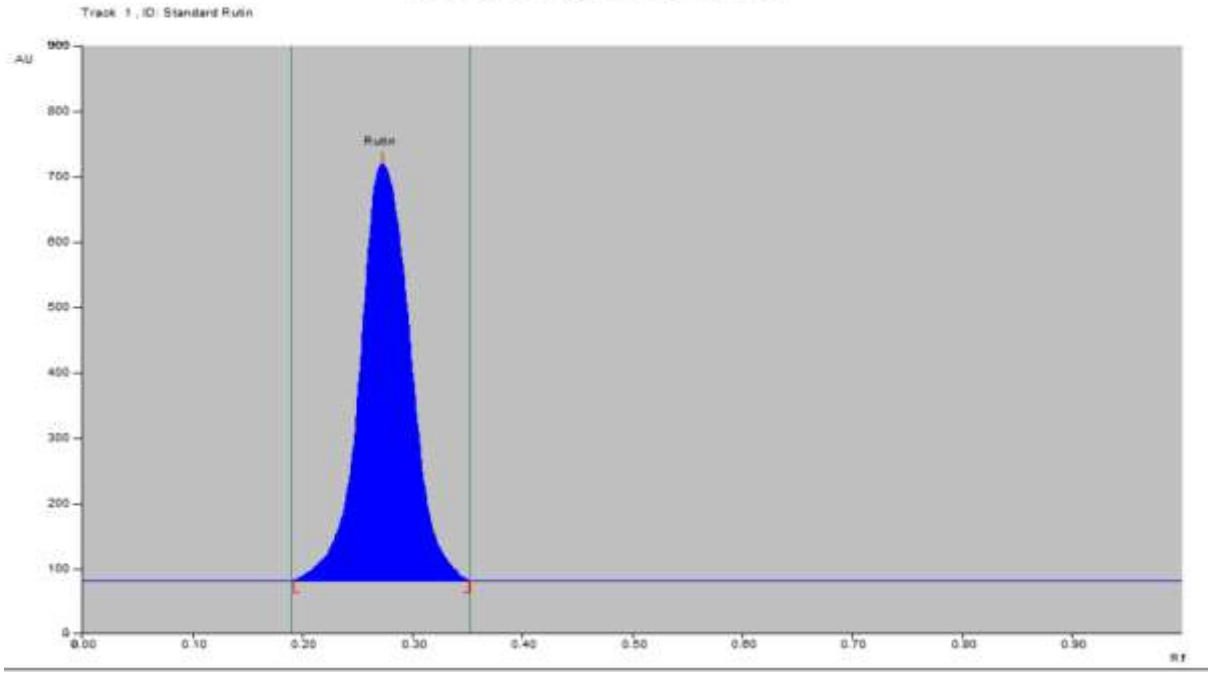
Track T3: Polyherbal Gel

Track T4: Herbal Spray

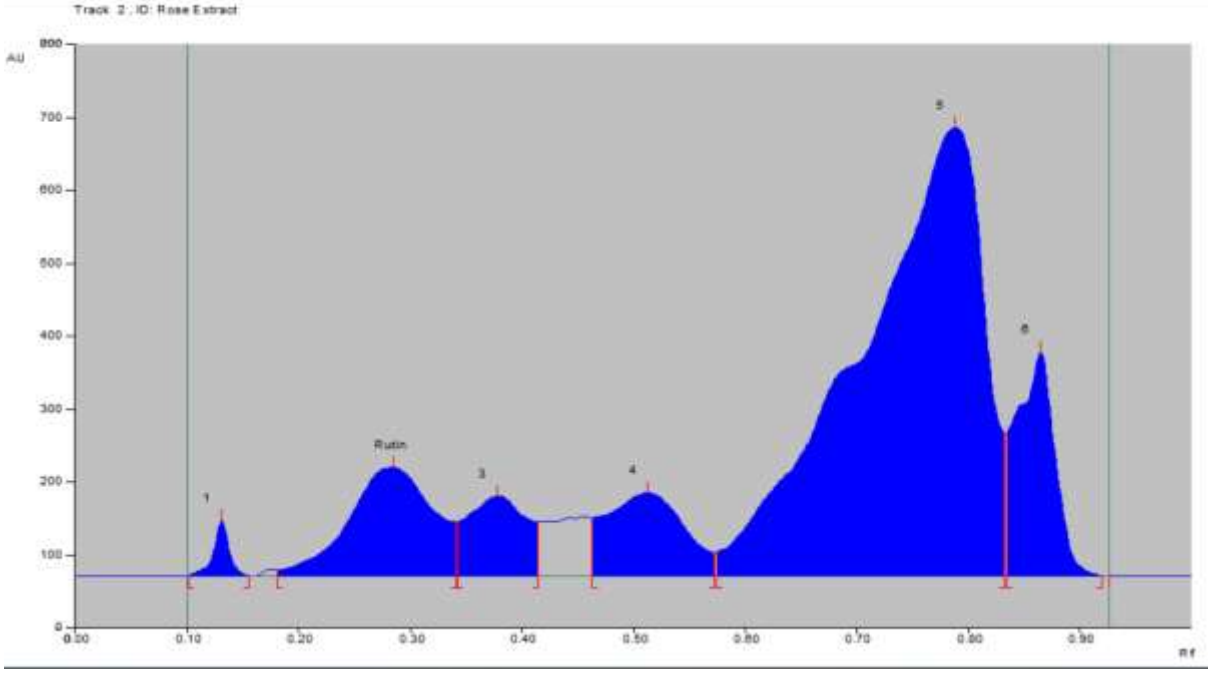
HPTLC Plate



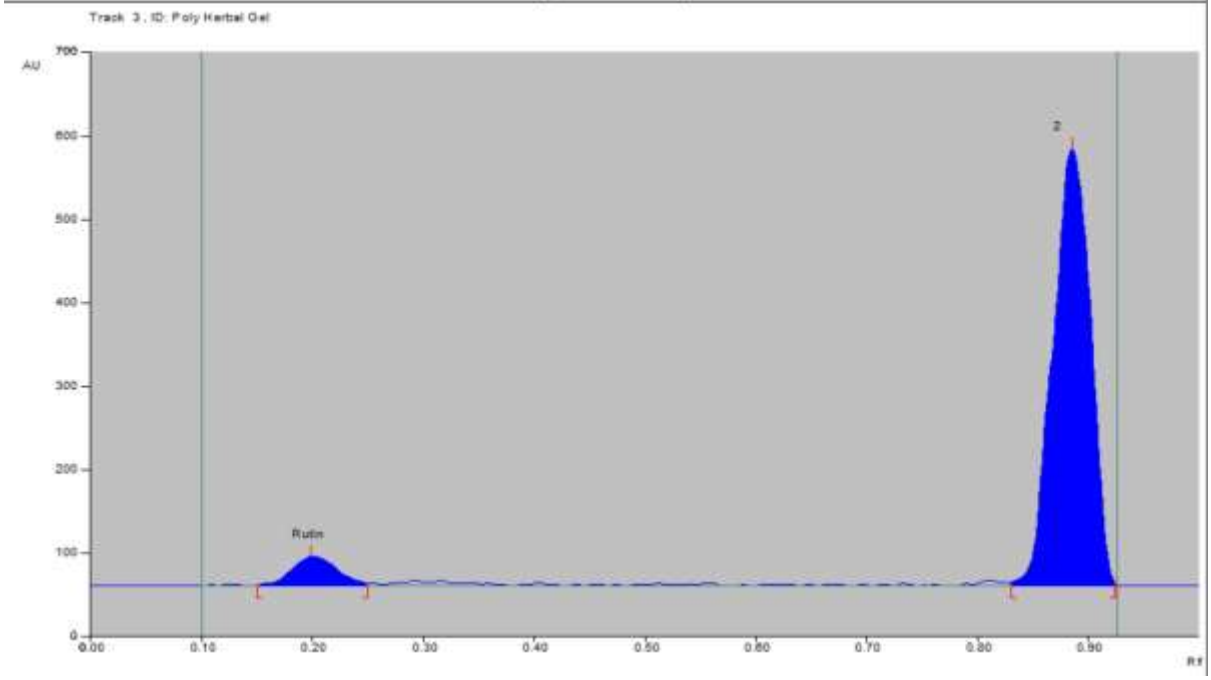
2D Chromatogram of Standard

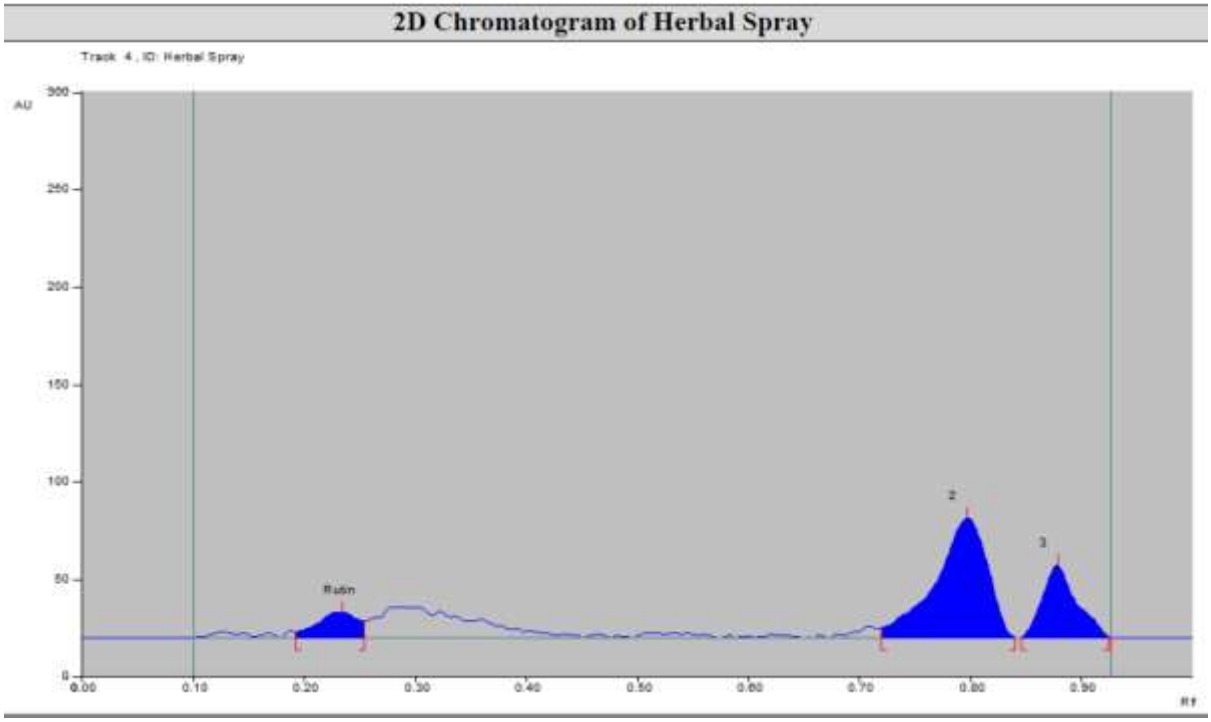


2D Chromatogram of Rose Extract

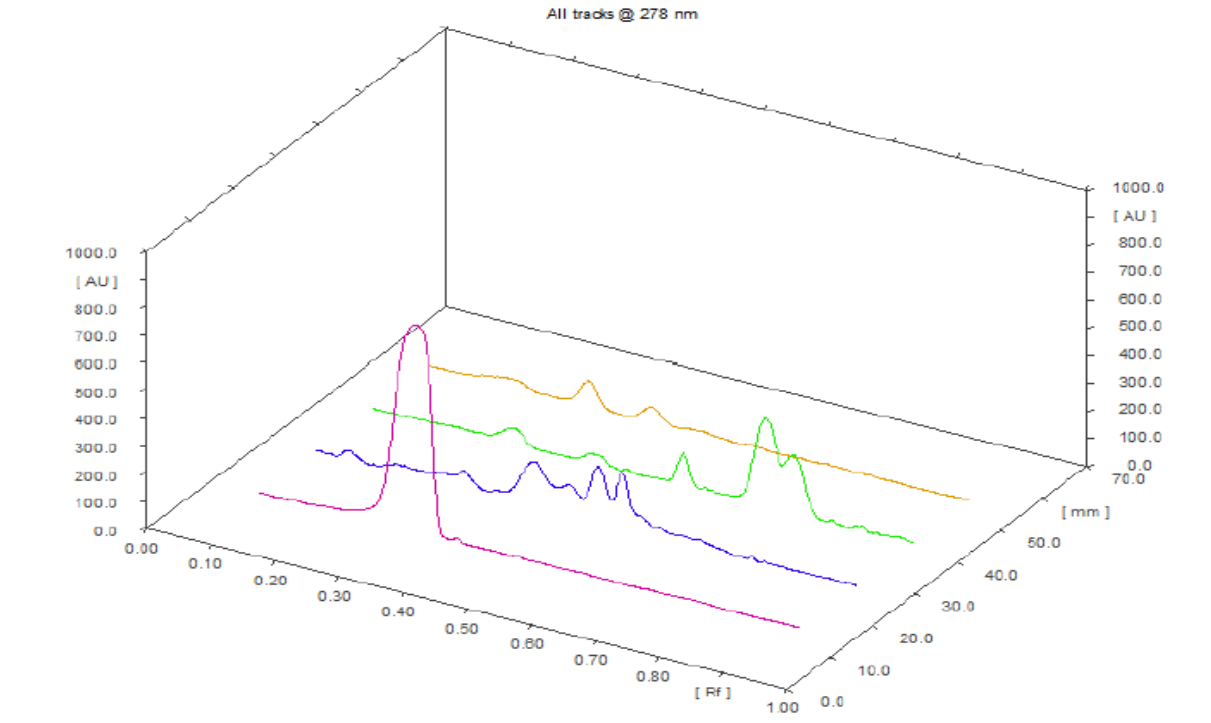


2D Chromatogram of Polyherbal Gel





HPTLC method development and validation for Gallic acid in *Calotropis procera*



3D Chromatogram

Parameters	Standard	*Calotropis procera* Extract	Polyherbal Gel	Herbal Spray
Weight	2.9 mg	1125 mg	1029 mg	1293 mg
Area	34346.7	2084.8	1440.7	1614.6
% Gallic acid	--	**0.077 %**	**0.058 %**	**0.026 %**

3D Chromatogram

R_f value

Spot No.	T1	T2	T3	T4
1	--	0.13	--	--
2	--	0.20	--	0.20
3	--	--	--	0.21
4	--	0.27	--	--
5 (Gallic acid)	**0.31**	**0.31**	**0.31**	**0.31**
6	--	0.42	0.42	0.42
7	--	0.47	0.47	--
8	--	0.52	--	0.52
9	--	0.56	0.56	--
10	--	0.61	0.61	--
11	--	0.68	0.68	--
12	--	--	0.73	--
13	--	0.76	0.76	--
14	--	--	0.84	--

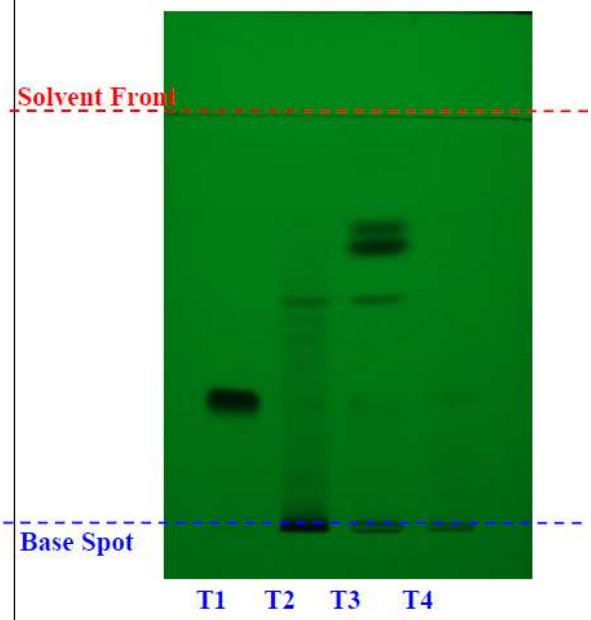
Track T1: Standard Gallic acid

Track T2: *Calotropis procera* Extract

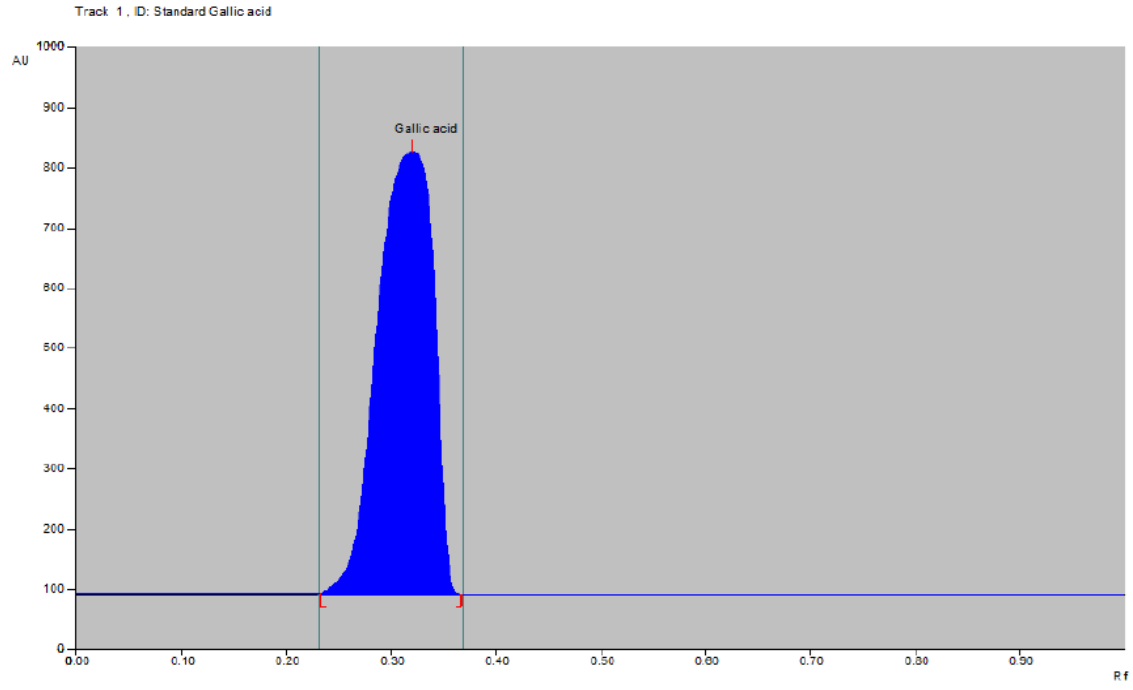
Track T3: Polyherbal Gel

Track T4: Herbal Spray

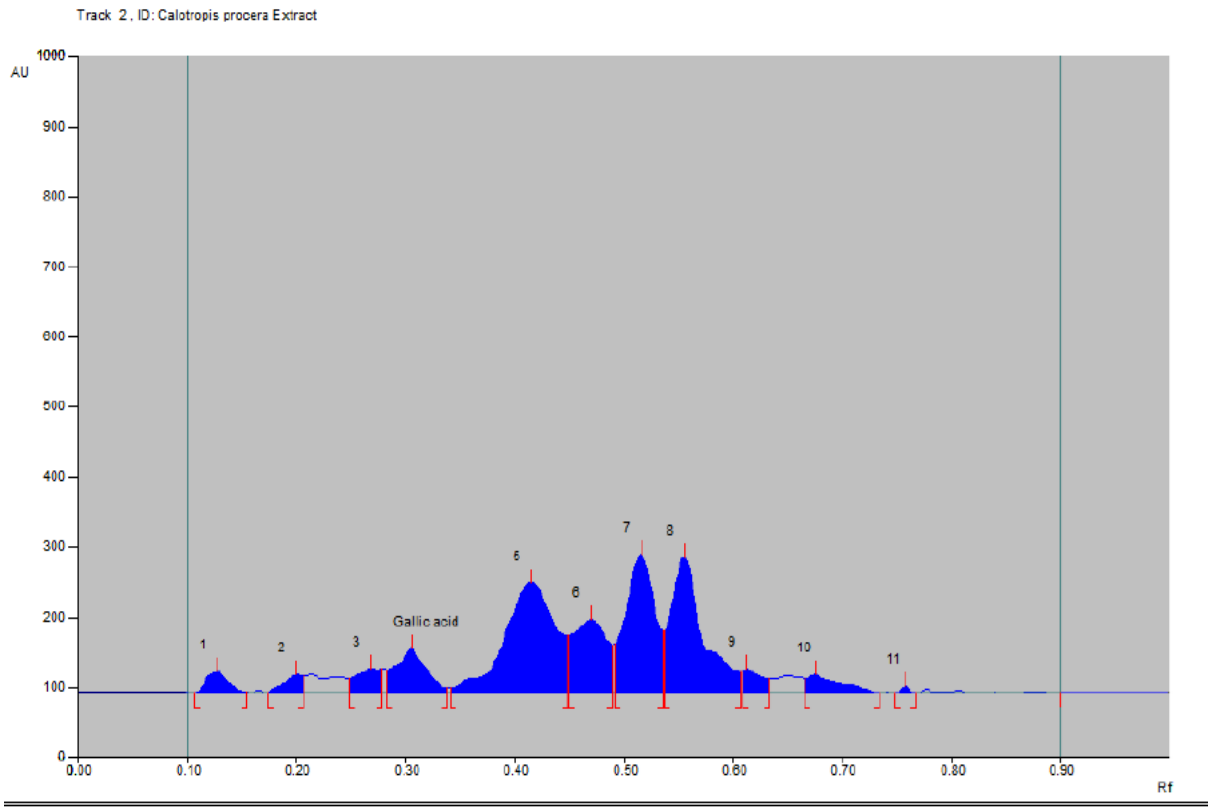
HPTLC Plate



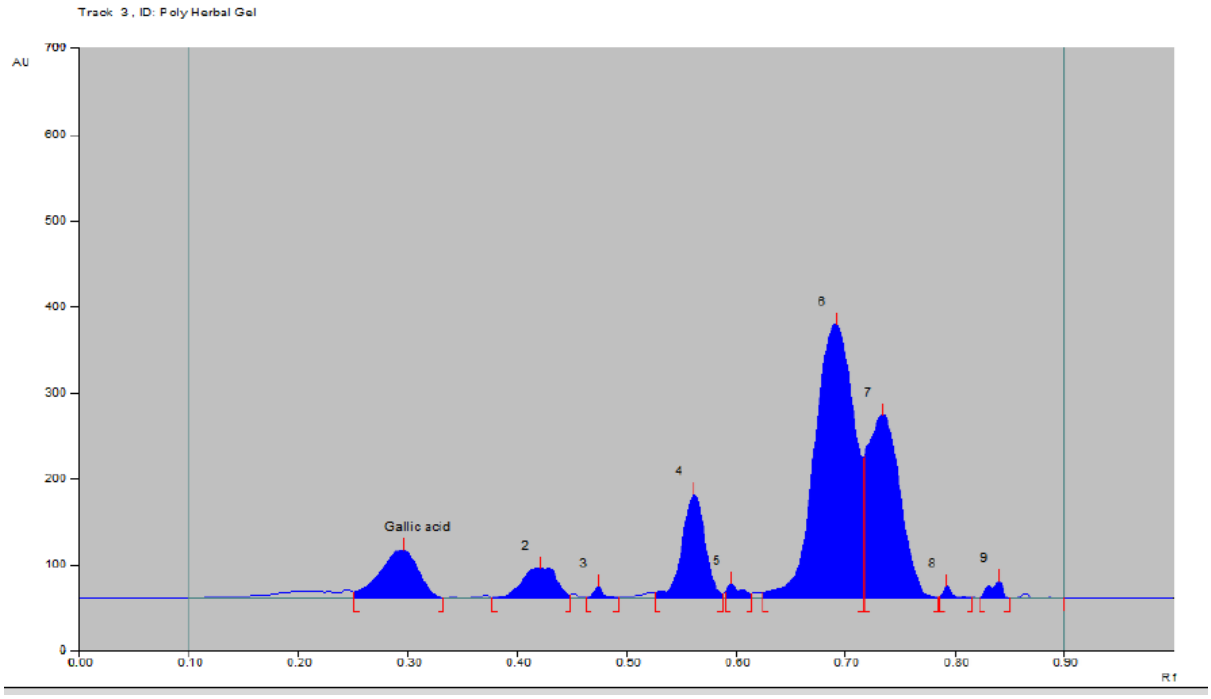
2D Chromatogram of Standard

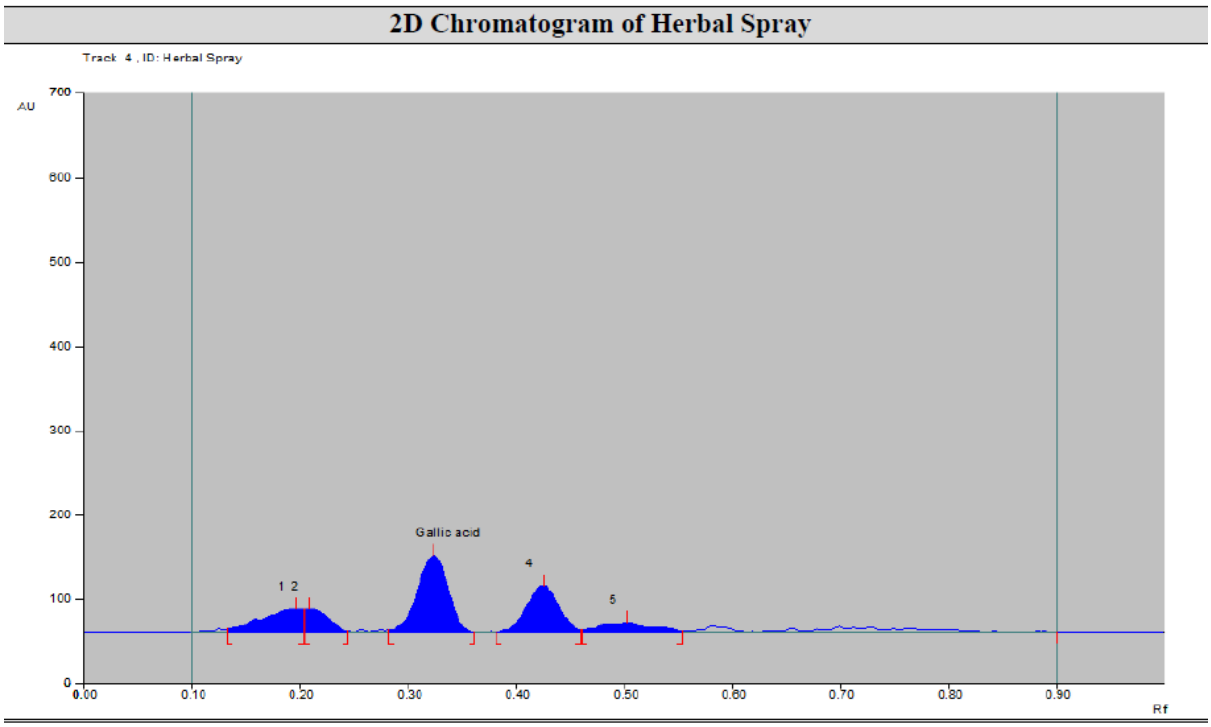


2D Chromatogram of *Calotropis procera* Extract



2D Chromatogram of Polyherbal Gel





Skin irritation test

The prepared herbal gel and herbal spray were evaluated for its skin irritant effect, where no erythema or edema was observed for all the formulations, even after 10 days of study, indicating that the prepared herbal gel formulation and spray were found to be safe.

Evaluation of anti-inflammatory activity

Observation Table

	Inhibition of edema (%)					
Groups (n=6)	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
Carrageenan	-	-	-	-	-	-
Carrageenan+ Diclofenac Gel	60.0	63.0	62.0	65.0	61.0	59.0
Carrageenan +Diclofenac Spray	55.0	56.0	58.0	57.0	54.0	53.0
Carrageenan+ Polyherbal Gel	65.0	67.0	68.0	69.0	68.0	67.0
Carrageenan + Polyherbal Spray	51.0	52.0	53.0	52.0	54.0	50.0

The pharmacological screening was carried out by using the carrageenan-induced edema model to evaluate the possible anti-inflammatory activity of the two formulations. As shown in Table, the inhibition of edema was 67.3% for Herbal Gel, highly effective to that produced by the

standard Diclofenac Gel 61.66%, indicating an anti-inflammatory effect. The inhibition of edema of Diclofenac Spray was 55.5% and Herbal Spray was 52%. From this study it can also be concluded that Carbopol 934 successfully used as gel base and Ethanol used as solvent for Spray. It can be concluded that both the developed formulations Gel and Spray have an anti-inflammatory effect better than respective anti-inflammatory formulation of diclofenac available in the market. However, developed Gel has better activity than developed Spray formulation.

Summary

The current work of development of poly-herbal formulation for inflammatory conditions involves usage of three plants*:* ***Adhatoda Vasaka***, ***Calotropis procera*** and ***Rosa indica***. The selection of these plants for development was done based on relative activity obtained through extensive literature review. The physico-chemical parameters evaluation showed presence of saponins, alkaloids, flavonoids glycosides and other chemical classes in both aqueous and alcoholic extracts of all three plants.

Analytical method development was done for qualitative and quantitative estimation using HPTLC. **Vasicine**, **Rutin** and **Gallic acid** were used as marker for estimation for *Adhatoda Vasaka*, *Rosa indica* and *Calotropis procera* respectively. Analytical method was validated as per ICH guidelines. Later on, cell viability assay was performed to determine the potential level of toxicity produced by all extracts on **THP-1 cell line**. The results for cell viability assay showed that individual aqueous extracts of all three plants were better than individual alcoholic extracts in terms of cell survival at higher concentration ranges. The cells were found to be viable in the presence of mixture of all three aqueous extracts of plants however mixture of methanolic extracts of were found to be toxic to cells. The in-vitro studies performed to assess anti-inflammatory activity of all plants were done using assay of two pro-inflammatory cytokines **IL-4** and **TNF-α** and two anti-inflammatory cytokines **IL-10** and **IL-6**. The experiment was performed using both alcoholic and aqueous extracts of all three individual plants. The levels of IL-4 were considerably reduced in the presence of aqueous extracts of all three plants whereas results for TNF-α were ambiguous since it showed increased levels in presence of all extracts. The levels of IL-6 were not detected in control as well as LPS induced inflammation hence was dropped out due to not satisfactory results. The levels of IL-10 were increased more in presence of aqueous extracts of all three plants as compared to alcoholic

extracts. Hence it was concluded that effect of all three aqueous extracts had better anti-inflammatory effect as compared to alcoholic extracts. The results of cell viability studies and in-vitro anti-inflammatory activity led to selection of aqueous extracts of all three plants.

Molecular docking studies were performed to provide additional evidence of possibility of anti-inflammatory activity of all three plants. The molecular docking studies was performed on five receptors as follows: TNF-α, COX-1, COX-2, IL-6 and IL-10. The results showed that αand β-amyrin had highest binding affinity towards all the receptors. Other active compounds with good binding affinity were found to be Syriogenin, phytosterol, Calatropagenin and Isosteviol. From the molecular docking studies, it was concluded that all three plants have active constituents that can be responsible for potential anti-inflammatory activity. Based on the conclusion of in-vitro and in-silico studies, mixture of aqueous extracts of all three plants was selected for formulation development and in-vivo studies.

Gel and spray were the two formulations selected for development owing to ease of patient compliance. Poly-herbal gel was composed of base consisting of Carbopol 934, Triethanolamine, Polyethylene glycol Methyl paraben, Propyl paraben, water and mixture of aqueous extracts of all three plants. Spray was composed of all three aqueous extracts, water and ethanol. Development of formulation was done using DOE approach. Evaluation of poly-herbal gel was performed and satisfactory compliance for evaluation parameters was obtained. In-vivo studies were performed on rats to assess the efficacy of both formulations. Skin irritation studies and Carrageenan induced paw edema model were implemented for assessing the anti-inflammatory activity. Results obtained from in-vivo studies showed that effect of poly-herbal gel was comparable to effect of diclofenac used as standard and was better than poly-herbal spray. The developed poly-herbal formulation can be said to have advantages in terms of plausible anti-inflammatory activity, usage of natural sources without any side-effects and ease of patient compliance and economical at same time.

Ongoing work

Evaluation of various parameters of Herbal Spray formulation

Presentation

1. Oral presentation in GUJCOST and DST sponsored two days International E-Conference on "Revolutionary Applications of 3D-Printing Technology in Pharma and Healthcare System" organized by Arihant School of Pharmacy and Bioresearch Institute, Adalaj, Gandhinagar on 12th and 13th August 2021

Publications

1. **Jadeja P**.*, Kadam A., Chokshi P., Dr. Mashru R. C., Insights Into the Ethnopharmacological Features of Purple Pitcher Plant, International Journal of Pharmacognosy and Phytochemical Research 2022; 14(3); 38-47
2. Anokhi Patel, **Preeti Jadeja** and Rajshree Mashru, Development And Validation Of New Smartphone Based Colorimetric Method For Vildagliptin In Bulk And Tablet Dosage Form, World Journal Of Pharmacy And Pharmaceutical Sciences, Volume 11, Issue 7, XXX-XXX, DOI: 10.20959/wjpps20227-22559
3. Saiyed SA, **Jadeja P**, Mashru R, Development and Validation of Extractive Spectrophotometric Methods for the Estimation of Telmisartan by Using Smartphone Application, Journal of Drug Delivery and Therapeutics. 2022; 12(3-S):178-190 DOI: http://dx.doi.org/10.22270/jddt.v12i3-s.5527
4. Anokhi Patel, **Preeti Jadeja** and Dr. Rajashree Mashru, Analytical Method Development And Validation For Simultaneous Estimation Of Dapagliflozin And Teneligliptin Hydrobromide Hydrate From Synthetic Mixture By Three Different Uv Spectrophotometric Methods, World Journal of Pharmaceutical Research, Volume 11, Issue 7, XXX-XXX, DOI: 10.20959/wjpr20227-24349
5. Samar Saiyed, **Preeti Jadeja** and Dr. Rajashree Mashru, A Review On The Novel Mind Technique For Treating Neurodegenerative Disorders, World Journal of Pharmaceutical Research, Volume 11, Issue 5, 1049-1066. DOI: 10.20959/wjpr20225-23988

REFERENCES

1. Lawrence, T., Willoughby, D. A., & Gilroy, D. W. (2002). *Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nature Reviews Immunology, 2(10), 787–795.* doi:10.1038/nri915
2. Joerg Gruenwald, Ralf Uebelhack and Margret Irmgard Moré, Phytomedicine, https://doi.org/10.1016/j.phymed.2019.152958
3. Mali RP, Rao PS, Jadhav RS, A Review on Pharmacological Activities of *Calotropis Procera*, Journal of Drug Delivery and Therapeutics. 2019; 9(3-s):947-951 **http://dx.doi.org/10.22270/jddt.v9i3-s.2870**
4. Ali Esmail Al-Snafi, The constituents and pharmacological properties of *Calotropis procera* - An overview, International Journal of *Pharmacy Review & Research,* Vol 5, Issue 3, 2015, 259-275
5. Bhumika Yogi*, Sujeet Kumar Gupta and Ashutosh Mishra, *Calotropis procera* (Madar): A Medicinal Plant of Various Therapeutic Uses-A Review, Bull. Env. Pharmacol. Life Sci., Vol 5 [7] June 2016: 74-81
6. Rastogi, R. & Mehrotra, B. (1999). Compendium of Indian medicinal Plant. Central drug research Institute Lucknow, National Institute of Science, New Delhi, Vol. 2, 1st Edn., p. 147
7. Murti, Y., Yogi, B. & Pathak, D. (2010). Pharmacognostic Standardization of Leaves of *Calotropis procera* (Ait.) R.Br. (Asclepiadaceae). Inter. J. Ayur. Res., 1(1): 14-17
8. Thakare Priya Ashokrao, Deshbhratar Kiran, Suryawanshi M.N. A Brief Review on Therapeutic Effects of – "Ornamental Plant" Rose. International Journal of Ayurveda and Pharma Research. 2017;5(12):46-52.
9. Md. Tofazzal Hossain Md., Obydul Hoq, Therapeutic use of *Adhatoda vasica,* Asian J. Med. Biol. Res. 2016, 2 (2), 156-163; doi: 10.3329/ajmbr.v2i2.29005
10. Shah Unnati, Pharmacognostical and Phytochemical Evaluation of *Adhatoda vasica* Leaf, *International Journal of Research Studies in Biosciences (IJRSB) Volume 2, Issue 11, December 2014, PP 144-148*
11. Ambreen Shoaib, A systematic ethnobotanical review of *Adhatoda vasica* (L.), Nees., Cellular and molecular biology.
12. World Health Organization, Quality Control methods for medicinal plant materials, Geneva 1998. 198.

13. World Health Organization, *Guidelines for the assessment of Herbal medicines*, Annex 11, WHO Technical Report series No.863, (1996).
14. Indian Pharmacopoeia, Government if India, Ministry of Health and Welfare, Controller of publication, Delhi, (1966), 708 pp.
15. Ajay Kumar Meena *, Ajay Yadav, M M Rao, Ayurvedic uses and Pharmacological activities of *Calotropis procera* Linn. / Asian Journal of Traditional Medicines, 2011, 6 (2)
16. Prasanth Ghanta , Shweta Sinha , Mukesh Doble & Basavaraju Ramaiah(2020): Potential of pyrroquinazoline alkaloids from *Adhatodavasica* Nees. as inhibitors of 5-LOX– a computational and an *in-vitro* study, Journal of Biomolecular Structure and Dynamics, DOI:10.1080/07391102.2020.1848635
17. Syed Murthuza, B.K. Manjunatha, In vitro and in vivo evaluation of anti-inflammatory potency of Mesua ferrea, Saraca asoca, Viscum album & Anthocephalus cadamba in murine, macrophages raw 264.7 cell lines and Wistar albino rats, Beni-Suef University Journal of Basic and Applied Sciences, 7 (2018) 719–723
18. Sandhiutami NMD, Moordiani M, Laksmitawati DR, Fauziah N, Maesaroh M, Widowati W. In vitro assesment of anti-inflammatory activities of coumarin and Indonesian cassia extract in RAW264.7 murine macrophage cell line. Iran J Basic Med Sci 2017; 20:99-106; http://dx.doi.org/10.22038/ijbms.2017.8102
19. THP-1 curcumin--Bisht, A., Dickens, M., Rutherfurd-Markwick, K., Thota, R., Mutukumira, A. N., & Singh, H. (2020). *Chlorogenic Acid Potentiates the Anti-Inflammatory Activity of Curcumin in LPS-Stimulated THP-1 Cells. Nutrients, 12(9), 2706.* doi:10.3390/nu12092706
20. THP-1 vanillin ---Zhao, D., Jiang, Y., Sun, J., Li, H., Huang, M., Sun, X., & Zhao, M. (2019). *Elucidation of The Anti-Inflammatory Effect of Vanillin In Lps-Activated THP-1 Cells. Journal of Food Science.* doi:10.1111/1750-3841.14693
21. The Anti-Inflammatory Effect of Algae-Derived Lipid Extracts on Lipopolysaccharide (LPS)-Stimulated Human THP-1 Macrophages Ruairi C. Robertson 1,2, Freddy Guihéneuf 3, Bojlul Bahar 4, Matthias Schmid 3, Dagmar B. Stengel 3, Gerald F. Fitzgerald 2,5, R. Paul Ross 5,6 and Catherine Stanton 1,5, Mar. Drugs 2015, 13, 5402-5424; doi:10.3390/md13085402
22. David Izuchukwu Ugwu, Uchechukwu Christopher Okoro, Pius Onyeoziri Ukoha, Astha Gupta & Sunday N. Okafor (2018) Novel anti-inflammatory and analgesic agents: synthesis, molecular docking and *invivo* studies, Journal of Enzyme Inhibition

and Medicinal Chemistry, 33:1, 405-415, DOI: 10.1080/14756366.2018.1426573

23. El Gizawy, H.A.; Boshra, S.A.; Mostafa, A.; Mahmoud, S.H.; Ismail, M.I.; Alsfouk, A.A.; Taher, A.T.; Al-Karmalawy, A.A. Pimenta dioica (L.) Merr. Bioactive Constituents Exert Anti-SARS-CoV-2 and Anti-Inflammatory Activities: Molecular Docking and Dynamics, In Vitro, and In Vivo Studies. Molecules 2021, 26, 5844. https://doi.org/10.3390/molecules26195844
24. Prathap *et al.* Bioinformation 17(2): 363-368 (2021)
25. Rakesh Kumar, Saurabh Sharma, Swati Sood, Vijai K. Agnihotri, Virendra Singh & Bikram Singh (2014) Evaluation of several Rosa damascena varieties and Rosa bourboniana accession for essential oil content and composition in western Himalayas, Journal of Essential Oil Research, 26:3, 147-152, DOI: 10.1080/10412905.2013.829004
26. Sharad Srivastava, Adarsh Pratap Singh, A. K. S. Rawat, Comparative botanical and phytochemical evaluation of *Calotropis procera* Linn. and Calotropis gigantea Linn. Root, Journal of Applied Pharmaceutical Science Vol. 5 (07), pp. 041-047, July, 2015 Available online at http://www.japsonline.com, DOI: 10.7324/JAPS.2015.50707
27. Kiran Zahid, Maqsood Ahmed & Farah Khan (2017): Phytochemical screening, antioxidant activity, total phenolic and total flavonoid contents of seven local varieties of *Rosa indica* L., Natural Product Research, DOI: 10.1080/14786419.2017.1331228
28. Valiollah Hajhashemi, Alireza Ghannadi and Mohammad Hajiloo**,** Analgesic and Anti-inflammatory Effects of Rosa damascena Hydroalcoholic Extract and its Essential Oil in Animal Models**,** Iranian Journal of Pharmaceutical Research (2010), 9 (2): 163-168
29. Drug discovery and evaluation, Pharmacological evaluation, Second edition by H. Gerhard Vogel, Springer Publication, Page number 759**.**
30. Rajendran Vadivu and K.S. Lakshmi, In vitro and In vivo anti-inflammatory activity of leaves of Symplocos cochinchnensis (Lour) Moore ssp laurina, A Journal of the Bangladesh Pharmacological Society (BDPS), 2008; 3: 121-124
31. Divya Jyothi, Marina Koland, Formulation and evaliation of herbal anti-inflammatory gel containing Trigonella Foenum greatum tract, International Journal of Pharmacy and Pharmaceutical Sciences, Vol 8, Issue 1, 2016, 41-44
32. Juanjuan Cheng, Tingyun Ma, Wei Liu, Hanxue Wang, In in vivo evaluation of the anti-inflammatory and analgesic activities of compound Muniziqi granule in experimental

animal models, BMC Complementary and Alternative Medicine volume 16, Article number: 20 (2015)

33. Jian-HuaShang, Xiang-HaiCaiTaoFeng,ˈYun-LiZhao, Pharmacological evaluation of Alstonia scholaris: Anti-inflammatory and analgesic effects, Journal of Ethnopharmacology, Volume 129, Issue 2, 27 May 2010, Pages 174-181
34. Sumana Sarkhel, Evaluation of the anti-inflammatory activities of Quillaja saponariaMol. saponin extract in mice, Toxicology Reports, 3 (2016) 1–3
35. H M Arif Ullah, Sayera Zaman, Fatematuj Juhara, Lucky Akter, Syed Mohammed Tareq, Emranul Haque Masum and Rajib Bhattacharjee, Evaluation of antinociceptive, in-vivo & in-vitro anti-inflammatory activity of ethanolic extract of Curcuma zedoaria rhizome, BMC Complementary and Alternative Medicine 2014, 14:346.
36. Jacinthia B. Xavier-Santosa,1, Juliana Félix-Silvaa,1, Júlia G.R. Passosa, Jacyra A.S. Gomesa, Júlia M. Fernandesb, Development of an effective and safe topical anti-inflammatory gel containing Jatropha gossypiifolia leaf extract: Results from a pre-clinical trial in mice, Journal of Ethnopharmacology, 227 (2018) 268–278 (%w/w)
37. Gailkwad D.D. and Banerjee S. K, Development and evaluation of herbal gel formulation of Magnifera indica linn extract, Department of Pharmaceutical Sciencs, Karpagam University, Coimbatore-641021, Tamil Nadu, India, International Journal of Research in Pharmaceutical Sciences.4(2) 260-265.
38. Rajashekaran A, Subramaniam I, Govindarajan A, Ramasamy A (2017) Formulation and Evaluation of Novel Herbal Aerosol for Arthritis. J Rheumatol Arthritic Dis 2(4): 1-12.
39. Zdzis1awa Nowakowska, A review of anti-infective and anti-inflammatory chalcones, European Journal of Medicinal Chemistry 42 (2007) 125e137.
40. Venkata, M., R. Sripathy, D. Anjana, N. Somashekara, *In Silico*, *In Vitro* and *In Vivo* Assessment of Safety and Anti-inflammatory Activity of Curcumin**,** American Journal of Infectious Diseases 8 (1): 26-33, 2012
41. Bhaskar karmakar, Partha Talukdar, Soumendra Nath talapatra, An *in silico* study for two anti-inflammatory flavonoids of *nerium oleander* on proinflammatory receptors, 2019 Jan – Feb RJLBPCS 5(1) page no.582, doi:10.26479/2019.0501.49
42. Happy Agarwal, Amatullah Nakara, Venkat Kumar Shanmugam, Anti-inflammatory mechanism of various metal and metal oxide nanoparticles synthesized using plant extracts: A review, Biomedicine & Pharmacotherapy 109 (2019) 2561–2572

43. Toby Lawrence, Derek A.Willoughby and Derek W. Gilroy, ANTI-INFLAMMATORY LIPID MEDIATORS AND INSIGHTS INTO THE RESOLUTION OF INFLAMMATION, NATURE REVIEWS, IMMUNOLOGY, VOLUME 2,OCTOBER 2002, 787-795.
44. Chittasupho, C.; Ditsri, S.; Singh, S.; Kanlayavattanakul, M.; Duangnin, N.; Ruksiriwanich, W.; Athikomkulchai, S. Ultraviolet Radiation Protective and Anti-Inflammatory Effects of Kaempferia galanga L. Rhizome Oil and Microemulsion: Formulation, Characterization, and Hydrogel Preparation. Gels 2022, 8, 639. https://doi.org/10.3390/gels8100639
45. Patel, N. A., Patel, N. J., & Patel, R. P. (2009). Design and Evaluation of Transdermal Drug Delivery System for Curcumin as an Anti-Inflammatory Drug. Drug Development and Industrial Pharmacy, 35(2), 234–242. doi:10.1080/03639040802266782
46. Yatoo, M. I., Gopalakrishnan, A., Saxena, A., Parray, O. R., Tufani, N. A., Chakraborty, S., Iqbal, H. M. N. (2018). Anti-Inflammatory Drugs and Herbs with Special Emphasis on Herbal Medicines for Countering Inflammatory Diseases and Disorders – A Review. Recent Patents on Inflammation & Allergy Drug Discovery, 12. doi:10.2174/1872213x12666180115153635