DESIGN, DEVELOPMENT OF A NOVEL TRANSDERMAL DRUG DELIVERY SYSTEM AS WOUND DRESSING

Thesis Submitted To

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LIST OF COMMONLY USED ABBREVIATIONS AND SYMBOLS

ANOVA	-	Analysis of Variance
CG	-	Calotropis gigantea
CGfr3	-	Calotropis giagantea fraction 3
cm	-	Centimetre
eg	-	Example
FR	-	Ficus racemosa
FRfr3	-	Ficus racemosa fraction 3
FT-IR	-	Fourier transform infra-red
g	-	Gram
HCl	-	Hydrochloric acid
HPLC	-	High performance liquid chromatography
HPTLC	-	High performance thin layer chromatography
h/hr	-	Hour
ICH	-	International Conference on Harmonization
KBr	-	Potassium bromide
kg	-	Kilogram
L	-	Litre
M	-	Male
MEC	-	minimum effective concentration
mg	-	milligram
mg/kg	-	Milligrams per Kilogram Body Weight of Wistar Rat
min	-	Minutes
mL	-	Millilitre
mL/kg	-	Milliliter per Kilogram Body Weight of Wistar Rat
MS	–	Mass spectrometry
n	-	Number of animals
NAD	-	No Abnormality Detected

NaOH	-	Sodium hydroxide
nm	–	nanometer
NMR	-	Nuclear magnetic resonance
PMMA	-	Poly methyl methacrylate
PVA	-	Poly vinyl alcohol
PVC	-	Poly vinyl chloride
PWC	-	Percent Wound Closure
RH	–	Relative humidity
rpm	-	Revolution per minute
SEM	-	Standard Error Mean
SD	-	Standard deviation
TDDS	-	Transdermal drug delivery systems
TG	-	Treated Group
TLC	-	Thin layer chromatography
UV	-	Ultra violet spectrophotometer
V/V	-	Volume / Volume
WBS	-	Wound Breaking Strength
WA	-	Wound Area
W/V	-	Weight / Volume
W/W	-	Weight / Weight
^{0}C	-	degree Celsius
µg	-	Microgram
%	-	Percentage

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1. INTRODUCTION

Transdermal is one of the common and frequently used drug delivery routes. Transdermal route has gained more attention in drug delivery due to its flexibility in palatability and convenience when compared to other routes of delivery[1]. Transdermal route is one of the suitable, older, convenient, safe and economic ways to deliver drug. Through oral route, the drug is lost in different ways which may lead to increase the drug content in dosage forms. In some route drug may produce toxic effects also. Normal drug delivery systems produce fluctuations in blood concentration which may lead to deviate from minimum effective concentration (MEC)[2]. For all diseases states, it is an important requisite to maintain MEC in blood for a specified length of period. Conventional systems fail to maintain the minimum effective concentration. But novel drug delivery systems like transdermal drug delivery system, controlled drug delivery systems etc., may produce prompt release of drug and maintenance of MEC.

The main objectives of transdermal drug delivery system are targeting to specific site of action and controlling the rate of delivery.

TRANSDERMAL DRUG DELIVERY SYSTEMS:

Transdermal drug delivery systems are self contained, discrete dosage forms which, when applied to the intact skin, deliver the drugs, at controlled rate to the systemic circulation. The development of transdermal drug delivery system may lead to the following advantages: [3,4]

- Improved patient compliance and convenience

- Improved bioavailability
- Minimal or no fluctuation in steady state level.
- Increased safety margin
- Maximum utilization of drug from dosage forms
- Decreased side effects and toxicity
- Permit both local and systemic effects
- Noninvasive drug delivery system
- Provides extended therapy with a single application
- Therapy can be terminated rapidly
- Self administration is possible

Limitations:[4,5]

- Particle size of the drug should be less than 1000 Daltons.
- Limited only to potent drug molecules (drug dose <10 mg)
- Penetration of the drug varies between subjects
- Skin irritation may restricts its usage (penetration enhancers)
- Should meet their physicochemical properties

FACTORS WHICH INFLUENCES THE TRANSDERMAL ROUTE:[6,7]

- Time scale of permeation
- Physicochemical properties of penetrant

  (Eg. pKa, molecular size, stability, solubility, partition coefficient)
- Integrity and thickness of stratum corneum
- Density of sweat glands and follicles
- Vehicle effects

ANATOMY OF SKIN:[8,9]

The skin can be considered to have four distinctive layers of tissue.

a) Non- viable epidermis(stratum corneum)

b) Viable epidermis

c) Viable dermis

d) Subcutaneous connective tissue (hypo dermis)

a) Non- viable epidermis (stratum corneum):

It is the outer most layer of skin. It acts as a physical barrier to most substance that comes in contact with the skin. The stratum corneum is 10- 20 cell layer thick over most of the body with flat plate like structure. Each cell is 34- 44µm long, 25- 36µm wide, 0.5- 0.20µm thick with 750 to 1200µm^2 surface area. It stocked upto each other in brick like fashion.

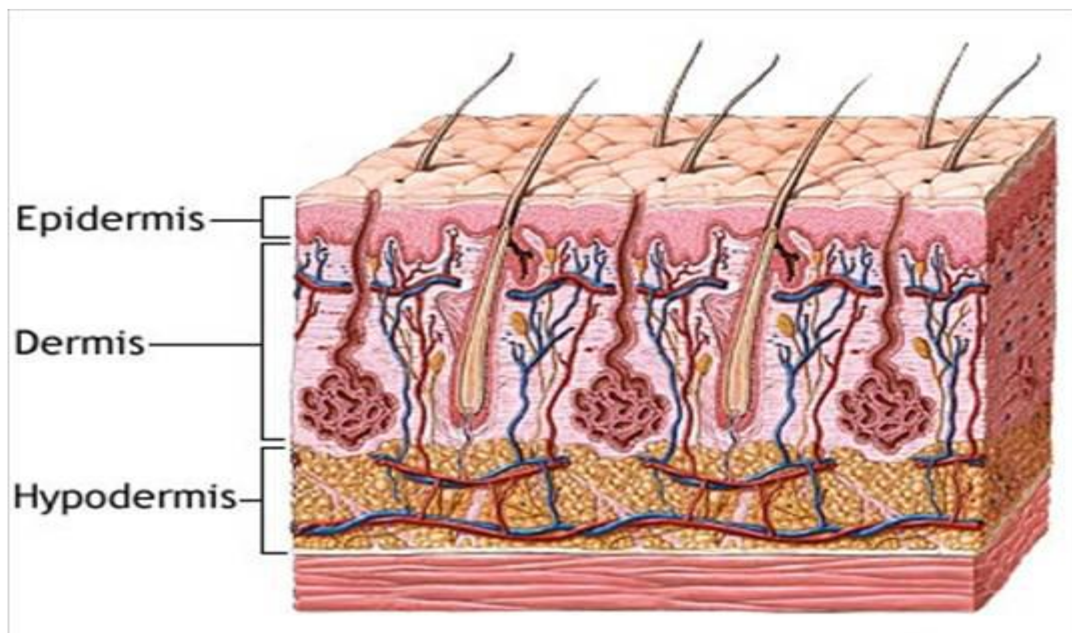


Fig. 1.1. Structure of skin

b) **Viable epidermis:**

Viable epidermis layer of the skin exists between stratum corneum and dermis and has a thickness ranging from 50-100μm.

c) Dermis:

Dermis layer is just below the viable epidermis. It is a structural fibrin and very few cells are like it can be found histologically in normal tissue. The thickness of dermis layer ranges from 2000 to 3000μm.

d) Subcutaneous connective tissue (hypodermis):

The subcutaneous tissue or hypodermis is not actually considered as a true portion of the structured connective tissue. It is composed of loose textured, white, fibrous connective tissue.

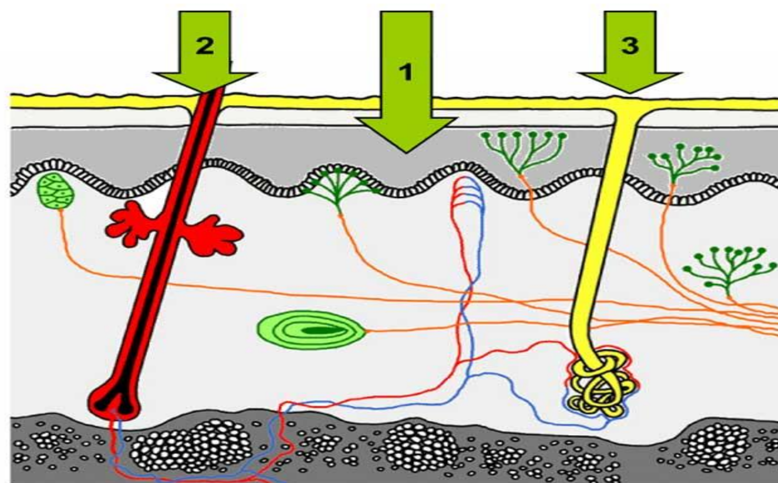


Fig. 1.2. Pathway of transdermal permeation

Permeation can occur by diffusion via

1. Intercellular and transdermal permeation through the stratum corneum
2. Transappendaged permeation through the hair follicle, sebaceous and sweat glands

Most of the chemical molecules penetrate skin via intercellular micro route. Hence many enhancing techniques aim to disrupt or bypass its elegant molecular architecture.

Transcellular and intercellular are the two major pathways by which the drug penetrates the skin and reaches the systemic circulation. By transcellular route, drugs cross the skin, directly by passing through the phospholipids membranes and the cytoplasm of the dead keratinocytes that constitute the stratum corneum. Though this is the shortest distance, the drugs encounter noteworthy resistance to permeation. This is because the drugs must have to cross the lipophilic membrane of each cell, then the hydrophilic cellular contents containing keratin, and then the phospholipid bilayer of the cell. This sequence of steps is repeated numerous times to traverse the full thickness of the stratum corneum.

The other more common pathway through the skin is via the intercellular route. By intercellular route the drugs crossing the skin must pass through the small spaces between the cells, making the route more tortuous. Although the thickness of the stratum corneum is only about 20 µm, the actual diffusional path of most molecules crossing the skin is on the order of 400 µm. The 20-fold increase in the actual path of permeating molecules greatly reduces the rate of drug penetration.

MECHANISM OF DRUG DELIVERY IN TDDS:

A transdermal drug delivery system is a device that is made up of polymer(s) embedded with drug(s), planned to deliver the drug via the skin over a controlled period of time. The concept of transdermal patch was introduced in 1979, with scopolamine patch for motion sickness. The principle mechanism for drug delivery in transdermal drug delivery system is “a slow process of diffusion driven by the gradient between the high concentration in the delivery system and the zero concentration prevailing in the skin”.

Skin is the major barrier in delivering the drug through transdermal route. The mechanism of delivery of drugs in a transdermal patch is diffusion through skin. Usually skin gives out the things not required for body. For example sweat, dirt etc. It also maintains the temperature of the body. The major obstruction is postured by the uppermost epidermal layer of stratum corneum. The stratum corneum is considered as dead skin. It has the unwanted properties as follows:

- Hygroscopic, however impermeable to water
- Tough and flexible membrane
- Lipid rich intercellular space
- Varying thickness for different parts of the body[10]

In spite of its undesirable properties, it allows permeation of certain chemicals to reach onto the tissue and the blood vessels under the skin. The chemical moiety also requires special formulation with low molecular weight, lipophilicity, and effective even at low dose levels for permeation through skin. The obstacle of the stratum corneum can be overcome by use of penetration enhancers[11].

BASIC COMPONENTS OF TRANSDERMAL DRUG DELIVERY SYSTEMS:

An ideal transdermal drug delivery system consists of polymer, drug, permeation enhancer etc in suitable proportion.[12]

a) **Polymer matrix or matrices:**

Usually polymers are the backbone or carriers for the drugs in novel drug delivery system. For transdermal drug delivery systems also polymer acts as a carrier. Polymers used may be of natural, semi-synthetic and synthetic type. Polymers used should be biodegradable. The polymers used should be inert, non-toxic and easy to formulate. The drug is sandwiched between 2 polymeric layers[13]. TDDS helps in achieving release of

drug in controlled manner from the polymer into the blood stream[14]. Examples of polymers include cellulose derivatives, gelatin, shellac gums, starch, silicone rubber, PVA, PVC, polyethylene etc. Poly (urethanes) are used for elasticity, poly (siloxanes) for insulating ability, PMMA for strength and transparency, PVA for strength and hydrophilicity, polyethylene for toughness, PVP for suspension capabilities.[15]

b) **Drug:**

Transdermal delivery is an extremely compatible option for drugs which undergo extensive first pass metabolism, with narrow therapeutic window or drugs with short half-life. To make transdermal system successful, the drug should possess physicochemical properties such as non-ionic, less molecular weight (< 1000 Daltons), low melting point (<200ºC), affinity with lipophobic and lipophilic phases, non-irritating and short half-life and potent.[16,17]

c) **Permeation enhancers:**

Permeation or penetration enhancers are the compounds which enhances the delivery of drugs to skin by altering the skin as a barrier to the flux. They interacts with structural components like proteins or lipids of stratum corneum so that permeability of drugs can be enhanced.[17,18] Permeation enhancers may be of solvents like methanol, ethanol, dimethyl formamide or surfactants like dioctyl sulphosuccinate, sodium lauryl sulphate or chemicals like urea, cyclodextrin, N,N-dimethyl-m-toluamide, eucalyptol etc[19]. They may act by disrupting the lipids of stratum corneum, interaction with cellular proteins or by improved partition of drug and co-enhancer or solvent into the stratum corneum[20].

d) Other Excipients:

Other excipients such as adhesives, backing layer, and release liner may also be used as the case may be.

Pressure sensitive adhesives are defined as a substance that adheres to a substrate when a mild pressure is applied and leaves no residue matter when removed[21]. Acrylic adhesives and silicone adhesives are commonly used as adhesives in TDDS.

Backing Layer used should exhibit low modulus, high flexibility, effective oxygen transmission with high moisture-vapour transmission rate. Also it should be strong and able to withstand without leaching of drug. Further it should be compatible with other excipients. Examples are vinyl, polyethylene and polyester.

Release Liner is the first and top layer in the TDDS. It should be removed before applying to skin. As it is not in direct contact with skin, there is no need bother about the irritability. But it should be inert as it is in direct contact with drug permeation layer. Release liner may be occlusive (eg. PE, PVC) or non-occlusive (eg. Paper fabric)

Other materials used may be polyester foil and metabolised laminates.[22]

Plasticizers such as dibutylphthalate, triethylcitrate, PEG, propylene glycol may be used.[23]

TYPES OF TRANSDERMAL DRUG DELIVERY SYSTEMS:

Based on components of the drug delivery systems, TDDS can be classified as[24]

A. **Reservoir systems**
B. **Matrix systems**
    a. Drug in adhesive system
    b. Matrix dispersion system
C. **Micro reservoir systems**

A. Reservoir systems

In this type of systems, reservoir is embedded between an impermeable backing layer and a rate controlling membrane. Drug release is achieved through the rate controlling membrane which can be non porous or microporous. In the drug reservoir compartment

drug can be suspended in solution form, suspension, gel or solid polymer matrix or a clear solvent.[25]

Examples of marketed formulations: Duragesic, Estradem, Catapress and TransdermScop

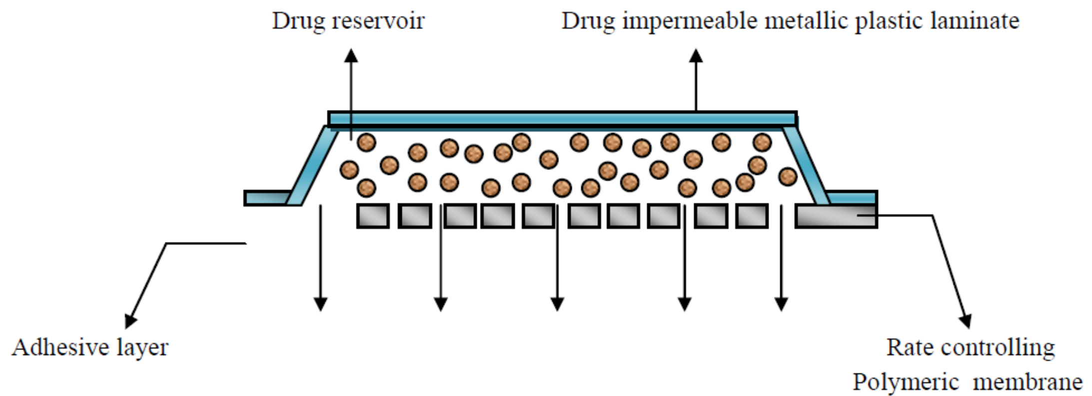


Fig. 1.3. Reservoir system

B. Matrix systems

a. Drug in adhesive system

Drug and selected additives are incorporated into organic solvent based PSA solution, mixed cast as a thin film and dried to evaporate the solvent. Dried drug in adhesive matrix is then sandwiched between release liner and backing layer.[26]

Examples of marketed formulations: Climara, Nicotrol, Deponit

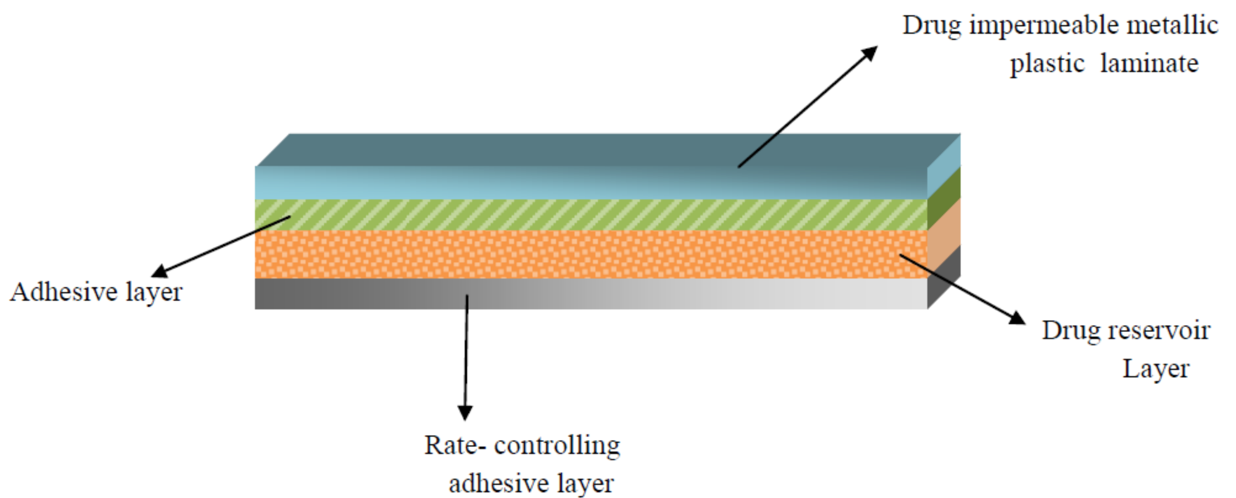


Fig. 1.4. Adhesive system

b. Matrix dispersion system

Drug is homogenously dispersed in a hydrophillic or lipophillic polymer matrix. The required quantity of permeation enhancers and plasticizer are added and mixed properly. Drug encapsulated in the polymer and molded into rings.

Film formed is separated from the rings and mounted onto an occlusive base plate in a compartment fabricated from a drug impermeable backing. Adhesive polymer is then spread along circumference of the film.[27]

Examples of marketed formulations: Nitro-Dur

Fig. 1.5. Drug matrix in adhesive

C. Micro reservoir systems:

These are the combination of reservoir and matrix dispersion systems. Drug reservoir is formed by suspending the drug in aqueous solution of water soluble polymers. Disperse the solution homogenously in a lipophilic polymer to form unleachable microscopic spheres of drug reservoirs.[28]

Example: Nitrodisc

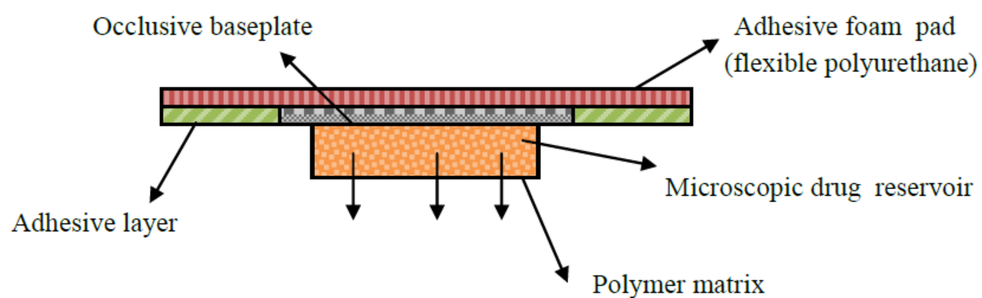


Fig. 1.6 Micro reservoir system

APPROACHES FOR TRANSDERMAL DRUG DELIVERY

During recent years there has been immense development in the field of transdermal delivery devices era; several innovative and more efficient methodologies providing better control, faster onset with larger dose have been developed by technological integration. Some of these technologies are given as below:[29,30,31]

a) Physically enhanced TDDS: Iontophoresis, Sonophoresis and Electroporation.
b) Chemically improved TDDS: Sulphoxides, Pyrrolidines, Fatty acids, Essential Oils, Oxazolidinones, Urea.
c) Vesicular carriers: Liposomes, Transfersomes, Ethosomes Niosomes,
d) Miscellaneous: Prodrugs and Ionpairs, Eutectic mixtures, Complexes etc.

HERBAL DRUGS:

A complementary traditional healing option has become a vital healing option in the present scenario both in developed and developing countries. The healing practices in historical communities serves as an eye opener for today researchers to plunge into practice of the knowledge of the historic community and evidencing the same with valid scientific documentation in order to substantiate the traditional claims on various potent herbs in many ailments. The advancement in sophistication of the scientific technology has made the documentation process simpler and easier.[32]

Herbal drugs are safe drugs due to its prompt therapeutic action without producing side effects. These are also called as botanical medicine or phytomedicine, obtained from any plant parts like seeds, berries, leaves, roots, barks or flowers and flowering tops. From the ancient period herbal drugs were widely used for various types of diseases. Many plants produce substances which include aromatic, phenolic compounds and derivatives, alkaloids, glycosides, tannins, having pharmacological properties. According to World Health Organization around 35% of drugs have been

derived from plant source. Herbal medicines holds lot of advantages including minimum side effects, conversion into any dosage forms etc. Herbal medicines can also be formulated as tablets, capsules, liquids, infusions, ointments, extracts like allopathic systems. The main disadvantage is time-consuming relief from disease state.

Why to use herbal medicine: [33]

- Minimize the undesirable side effects of allopathic systems
- Resistant of allopathic drugs
- Change in consumers attitude
- Alternative to allopathic systems
- Enhance the efficiency and cost effective

Herbal Medicine, referred to as Herbalism 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.

Being one of the oldest forms of health care known to mankind, finds utilization by all cultures throughout history. Herbalism serves as an integral part of the development of modern civilization.

Indeed, well into the 20th century much of the pharmacopoeia of scientific medicine was derived from the herbal lore of native peoples.[34] Many drugs commonly used today are of herbal origin. About 25 percent of the prescription drugs dispensed will contain at least one active ingredient derived from plant material. Such active drug are either made from plant extracts or synthesized to mimic a natural molecule.[35]

The World Health Organization (WHO) evidences that 4 billion people, 80 percent of the world population, currently utilize herbal medicine for some aspects of primary health care needs. WHO evidences about 119 plant-derived pharmaceutical medicines, of which

74 percent existing in modern medicine directly correlating with their traditional uses as plant medicines by native cultures.[36] Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicinal value.[37]

Even today, many plant derived medications serves as a basic tool for a large proportion of the commercial medications used today for the treatment of various ailments viz., heart disease, high blood pressure, pain, asthma, and other problems. For example, ephedra is herb used in Traditional Chinese Medicine for more than two thousand years to treat asthma and other respiratory problems.[38] Interestingly, Ephedrine, the active ingredient in ephedra, is being used in the commercial pharmaceutical preparations for the relief of asthma symptoms and other respiratory problems, making breathing more easily.

Herbal Medicine can be broadly classified into various basic systems: Traditional Chinese Herbalism, being one of the parts of Traditional Oriental Medicine.[39]

Today, Western Herbalism is primarily a system of folk medicine. In ancient times herbal drugs are portrayed as "wonder drugs".

WOUND HEALING:

Wounds are the tissue damage occurred particularly in skin or mucous area. Wound healing is a complex and dynamic process with the wound environment changing with the changing health status of the individual.

Cicatrisation – The process of wound healing, an intricate process which involves repair of skin upon injury. Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers. The human adult wound healing process can be divided into 3 distinct phases: the inflammatory phase, the proliferative phase, and the remodeling phase. Within these 3 broad phases is a complex and coordinated series of

events that includes chemotaxis, phagocytosis, neocollagenesis, collagen degradation, and collagen remodeling.

The epidermis and dermis layer exists as steady state equilibrium. So as to serve as a barrier to external environments. Upon breakage of the protective barrier the wound healing motion sets in to facilitate restoration process.

Factors which interfere with wound healing: [40]

Following are some of the common underlying causes or factors, which may interfere with wound healing:

- Trauma (initial or repetitive)
- Animal bites or insect stings
- Pressure
- Vascular compromise, arterial, venous
- Immunodeficiency
- Malignancy
- Connective tissue disorders
- Metabolic disease, including diabetes
- Nutritional deficiencies
- Psychosocial disorders
- Adverse effects of medications

In many cases the causes may be multifactorial.

Phases of wound healing:

- Hemostasis
- Inflammation
- Proliferation or Granulation

- Remodeling or Maturation

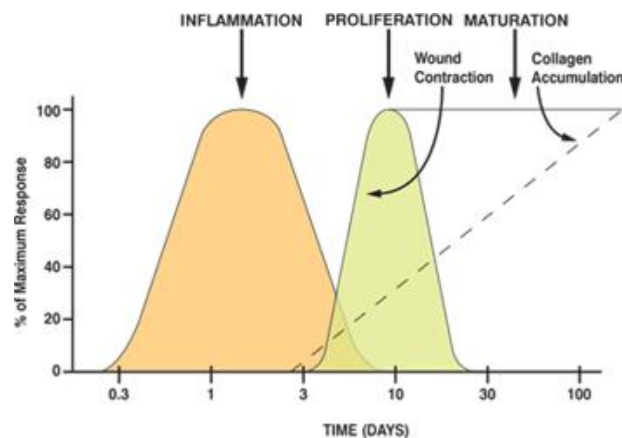


Fig.1.7. Phases of wound healing

Biochemical events upon injury: Immediately within minutes post-injury, fibrin is formed due to aggregation of platelets facilitating control of bleeding. The rate of healing depends on many factors, including the bloodstream levels of hormones such as oxytocin. The inflammatory phase is the body's natural response to injury. After initial wounding, the blood vessels in the wound bed contract and a clot is formed. Once haemostasis has been achieved, blood vessels then dilate to allow essential cells; antibodies, white blood cells, growth factors, enzymes and nutrients to reach the wounded area. This leads to a rise in exudate levels so the surrounding skin needs to be monitored for signs of maceration.[41, 42]

In the maturation and remodeling phase, collagen is remodeled and realigned along tension lines and cells that are no longer needed are removed by apoptosis. Factors which may contribute to this include diabetes, venous or arterial disease, old age, and infection.[43]

During proliferation, the wound is 'rebuilt' with new granulation tissue which is comprised of collagen and extracellular matrix and into which a new network of blood vessels develop, a process known as 'angiogenesis'. Healthy granulation tissue is dependent upon the fibroblast receiving sufficient levels of oxygen and nutrients supplied

by the blood vessels. Healthy granulation tissue is granular and uneven in texture; it does not bleed easily and is pink / red in colour. The colour and condition of the granulation tissue is often an indicator of how the wound is healing.[44]

Maturation is the final phase and occurs once the wound has closed. This phase involves remodelling of collagen from type III to type I. Cellular activity reduces and the number of blood vessels in the wounded area regress and decrease.[45]

TYPES:

Primary intention

- Involves epidermis and dermis without total penetration of dermis healing by process of epithelialization
- When wound edges are brought together adjacently (re-approximated)
- Scarring Minimized
- Majority of the surgical wounds heal by primary intention healing
- Sutures (stitches), staples, or adhesive tape facilitates Wound closure

Examples: well-repaired lacerations, well reduced bone fractures, healing after flap surgery

Secondary intention

The wound is facilitated to granulate

- Wound packed with a gauze or use a drainage system
- Granulation results in a broader scar
- Drainage from infection slows down the rate of healing.
- Wound care encourage wound debris removal and allows granulation tissue formation

Examples: gingivectomy, gingivoplasty, tooth extraction sockets, poorly reduced fractures.[46]

Tertiary intention

- Initially the wound is cleaned, debrided and observed, around 4 - 5 days before closure.
- The wound is left open

Examples: healing of wounds by use of tissue grafts.

The wound edges when not re-approximated immediately, delayed primary wound healing transpire. This type is desired in the case of contaminated wounds. By the fourth day, phagocytosis of contaminated tissues is well underway, and the processes of epithelization, collagen deposition, and maturation are occurring. Foreign materials are walled off by macrophages that may metamorphose into epithelioid cells, which are encircled by mononuclear leukocytes, forming granulomas. Usually the wound is closed surgically at this juncture, and if the "cleansing" of the wound is incomplete, chronic inflammation can ensue, resulting in prominent scarring.[47]

2. AIM AND OBJECTIVE

The aim and objective of the present work was to design, develop and evaluate the transdermal drug delivery system with phytomedicine as wound dressing. Transdermal drug delivery is a commonly used system in the treatment of management of wounds, pain and various inflammations. The following are the specific reasons which demand the development of transdermal drug delivery system for herbal drugs with wound healing efficacy:

i) Generally synthetic drugs are toxic and irritant in nature. Hence a well designed herbal transdermal drug delivery system can reduce the complexity of dosing frequency and side effects.

ii) In comparison to other routes, transdermal route is preferable with respect to quick onset of action, safety and comfort. Possibly it may have quick onset of action and efficacy than oral route and fewer side effects when compared with parenteral. Hence transdermal drug delivery is ideal.

iii) Transdermal release of drugs will reduce the frequency of dosing and increase patient convenience. Practically transdermal drug delivery system holds the advantage of oral and parenteral route and overcomes the disadvantages.

iv) In treatment of wounds, administration of drugs for a longer period during the treatment is unavoidable, hence development of transdermal release of herbal drug with targeted drug delivery is the need for the day.

v) Hence it was decided to formulate a transdermal drug delivery system by incorporating few bioactive compounds for treatment of wound and to evaluate its efficacy by *in vitro* and *in vivo* methods.

The objectives of the work:

- To optimize the dosing period of the bioactive component and targeted drug release for wound healing property.
- To reduce the side effects in the systemic circulation.
- To improve the bioavailability of transdermal drug delivery system.

3. REVIEW OF LITERATURE

Earlier works on *Ficus racemosa* and *Calotropis gigantea* with reference to literature from well established libraries were collected and recorded. The earlier reports on the Pharmacognostical, Phytochemical and Pharmacological studies on the bark of *Ficus racemosa* and aerial parts of *Calotrophis gigantea* were listed below.

PLANT PROFILE

FICUS RACEMOSA

Biological source: *Ficus racemosa*

Family: Moraceae

Parts Used: Bark

Taxonomy:

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Rosales
Family	:	Moraceae
Genus	:	Ficus
Species	:	F. racemosa

Vernacular Names:

Bengali	Dumur
English	Cluster fig
Hindi	Umar, Gular
Malayalam	Atti, Kattatti, Peyatti

Marathi	Umbar
Sanskrit	Udumbara
Tamil	Atti
Telugu	Medi Pandu

Description:

A moderate sized deciduous tree grows up to 20 meters in height. Leaves simple, alternate, ovate or elliptic, acute, and glabrous; receptacles small subglobose or piriform, in large clusters from old nodes of main trunk. Shown in **Fig. 3.1**.

Propagation:

The plant is propagated by using cuttings of stem and root suckers. Seeds can also be used for propagation. The flowers are pollinated by very small wasps.

Distribution:

The plant is a large deciduous tree distributed all over India, from outer Himalayan ranges, Punjab, Orissa, West Bengal, Rajasthan, and common in South India.[48]

Morphology:[49]

The tree is medium to moderate sized deciduous. The rich green foliage provides a good shade. Shown in **Fig. 3.1**

Leaves Leaves are ovate, ovate-lanceolate or elliptic, sub acute, entire and petiolate. The **leaves** are dark green, 7.5-10 cm long, glabrous; receptacles small subglobose or piriform, in large clusters from old nodes of main trunk. **Flowers** are actually a compartment carrying hundreds of flowers.

Fruits are with receptacles of 2-5 cm in diameter, pyriform, in large clusters, arising from main trunk or large branches. The fruits resemble the figs and are green

when raw, turning orange, dull reddish or dark crimson on ripening. The fruit of *F. racemosa* is ¾ inch to 2 inches long, circular and grows directly on the trunk.

Seeds are tiny, innumerable and grain-like.

Bark consists of easily removable translucent flakes grayish to rusty brown, uniformly hard and non-brittle. Bark is reddish grey or grayish green, soft surface, uneven and often cracked, 0.5-1.8 cm thick, on rubbing white papery flakes come out from the outer surface, inner surface light brown, fracture fibrous, taste mucilaginous without any characteristic odour. Shown in **Fig. 3.2.**

Roots are long, brownish in colour. Roots are irregular in shape.

Chemical constituents:

The leaf of this plant contains sterols, triterpenoids (Lanosterol) and alkaloids, tannins and flavonoids. Stem-bark gives gluanol acetate, β-sitosterol, leucocyanidin-3-O-β-D-glucopyrancoside, leucopel- argonidin-3-O-β-D-glucopyranoside, leucopelargonidin -3-O-α-L-rhamnopyranoside, lupeol, ceryl behenate, lupeol acetate and α-amyrin acetate. From trunk bark, lupenol, β-sistosterol and stigmasterol were isolated. It also contains gluanol acetate. Fruit contains gluanol acetate, glucose, tiglic acid, esters of taraxasterol, lupeol acetate, friedelin, higher hydrocarbons (Hentriacontane) and other phytosterols. Aspartic protease was isolated from latex of the plant.[50,51]

Medicinal uses:

In the traditional system of medicine, the plant is used for various health problems and diseases. Root is used in dysentery, pectoral complaints, and diabetes, applied in mumps, other inflammatory glandular enlargements and hydrophobia. The bark is highly efficacious in threatened, abortion and also recommended in urological disorders, diabetes, hiccough, leprosy, dysentery, asthma and piles. The leaves are good wash for wounds, ulcers, dysentery and diarrhea. The infusion of bark and leaves is also employed

as mouth wash to spongy gums and internally in dysentery, menorrhagia, effective remedy in glandular swelling, abscess, chronic wounds, cervical adenitis and haemoptysis. Tender leaves are used in bilious affection and also to improve skin complexion .Tender fruits are astringent, stomachic, refrigerant, dry cough loss of voice ,disease of kidney and spleen ,astringent to bowel ,styptic ,tonic ,useful in the treatment of leucorrhoea, blood disorder, urinary discharges, leprosy burning sensation. Latex is aphrodisiac and administered in hemorrhoids, diarrhea, diabetes, boils, alleviates the edema in adenitis, toothache and vaginal disorders.[52,53]

Ficus racemosa possesses various pharmacological activities; ethanol extract of stem bark of ficus racemosa showing good hypoglycemic activity in the alloxan diabetic albino rats.[54] The methanol extract of stem bark proving its antitussive potential in mice against a cough induced model.[55] Ethanol extract of stem bark showed a potent wound healing in excised and incised wound model in rat.[56] Ethanol extract of leaves exhibited anti-inflammatory effects with carrageenan, serotonin, histamine and dextran induced rat paw edema models.[57,58]

Fig. 3.1. Entire plant (Ficus Racemosa)

Fig. 3.2. Barks (Ficus Racemosa)

FICUS RACEMOSA

PHARMACOGNOSTICAL REVIEW

- **Paarakh** (2009)[59] studied and reported a detailed survey on the literature on its pharmacognosy, phytochemistry, traditional and pharmacological uses of *Ficus racemosa.*

- **Babu, et al.,** (2010)[60] studied a detailed comparative pharmacognostical evaluation of the *Ficus racemosa, F. virens, F. religiosa and F.benghalensis.* The aim was to establish the diagnostic keys based on the macroscopic, microscopic, and HPTLC profiles. The detailed diagnostic and distinctive characteristics were discussed.

PHYTOCHEMICAL REVIEW

- **Li, et al.,** (2004)[61] identified racemosic acid by bioassay-guided fractionation of the ethanol extract of *Ficus racemosa Linn*. as a new anti-inflammatory glucoside. It was further demonstrated for antioxidant activity and cytotoxic effects. Bergenin was also isolated from the same active fraction.
- **Devaraj, et al.,** (2008)[62] has isolated ficins from the latex of *Ficus glabarata* and *Ficus carica.* The purification and characterization of a protease from the latex of Ficus *racemosa* was elucidated.

PHARMACOLOGICAL REVIEW

- **Iweala, et al.,** (2005)[63] **studied** the effect of Vinca rosea and *Ficus racemosa* on hyperglycaemia in rats. It was found the water extract of leaves, stem and roots of *Ficus racemosa* exhibited hyperglycaemic activity.

- **Mehta, et al.,** (2012)[64] studied the experimental models of wound and inflammation to assess the wound healing and anti-inflammatory activity of F.racemosa.

Patil, et al.,(2008)[65] evaluated the anti-hyperglycemic activity of ethanol (95%) petroleum ether extract of Ficus racemosa.

- **Mandal, et al.,** (1998)[66] evaluated antibacterial activity against E.coli, Basillus pumilis, Bacillus subtilis, Pseudomonas aeruginosa and Staphylococcus aureus. The petroleum ether extract was found to be most effective.

- **Mandal , et al.,** (1999)[67] evaluated for hepatoprotective activity in rats by using petroleum ether extract of *Ficus racemosa* leaves by subcutaneous inducing carbon tetrachloride .The biochemical parameters was estimated and the activity was compared with standard liver tonic (Neutrosec) .

- **Kolte, et al.,** (1999)[68] studied immunomodulating effect of dry powder of *Ocimum sanctum* and leaf gall of *Ficus racemosa* leaves in broilers naturally infected with IBD virus. Cellular reaction was found to be intense in *O. sanctum* treated and *O.sanctum* plus leaf gall treated groups.

- **Mandal SC** *et al.,* **(2000)**[69] studied the anti-inflammatory activity of *Ficus racemosa* extract and evaluated on carrageenin, serotonin, histamine and dextran-induced rat hind paw oedema models. The extract at doses of 200 and 400 mg/kg has been found to possess significant anti-inflammatory activity on the tested experimental models.

- **Rao, et al.,** (2002)[70] evaluated glucose lowering efficacy of a methanol extract of stem bark of *Ficus racemosa Linn* in normal and alloxan induced diabetic rats. The MEBFR exhibited hypoglycaemic activity and compared with standard. The investigation claimed MEBFR as an antidiabetic agent.

- **Rao, et al.,** (2002)[71] studied anti pyretic effect of methanol extract of stem bark of *Ficus racemosa Linn* on normal body temperature and yeast induced pyroxia in albino rats .The MEFR exhibited antipyretic effect and compared with standard.

- **Khan, et al.,** (2005)[72] reported modulator effect of Ficus racemosa diminution carbonate included renal oxidative injury and cell proliferation response. The result suggests that Ficus racemosa extract is a potent chemoprotective agent and suppresses potassium bromated mediated nephrotoxicity in rats.

- **Tambekar, et al.,** (2010)[73] reported *Ficus racemosa* leaves as a potential for antibacterial activity.

- **Jahan, et al.,** (2009)[74] has studied the effects of fruit extract and fraction of *Ficus racemosa* on diabetic model rats. The study exhibited antioxidant activity with 1-BuOH soluble part of the ethanol extract and for first time 3 - 0- (E) - Caffeoyl quinate (1) was reported.

- **Ahmed F** *et al* **(2010)**[75] studied and evaluated the radical scavenging and ACE inhibitory activity of cold and hot aqueous extracts of *Ficus racemosa* stem bark. Extracts were standardized using HPLC. This extract can be potentially utilized for hypertension.

- **Patel** *et al*., **(2010)**[76] studied antioxidant activity of alcoholic and aqueous extracts of *Ficus benghalensis* leaf (Moraceae) and *Ficus racemosa* (Moraceae) leaf extracts were carried out by Hydrogen peroxide (H_2O_2), Diphenyl Picryl Hydrazyl (DPPH) and nitric oxide. In alcoholic extract scavenging activity were carried.

- **Krishnamurthi** *et al.,* **(2011)**[77] conducted to evaluate aqueous & ethanolic extracts of *Ficus racemosa* for antibacterial and wound healing properties in presence of dexamethasone depressed healing conditions. Antibacterial properties of the extracts were studied against *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Escherichia coli* bacterial strains, using the well diffusion method. The results were comparatively significant ($P< 0.05$).

- **Krishnamurthi *et al*., (2012)**[78] established the wound healing activity of aqueous and ethanolic extract of roots of *Ficus racemosa* by incision and excision model. In incision model the braking strength was taken into consideration and for excision model percentage wound contraction and period of epithelialization were considered.

ANTI-MICROBIAL REVIEW

- **Mandal, et al.,** (2000)[79] studied petroleum ether extract of *Ficus racemosa* for antibacterial activity against *Escherichia coli*, *Bacillus pumilus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococus aureus*. The effects were further compared with chloramphenicol.
- **Chandrashekhar, et al.,** (2008)[80] evaluated anthelmintic activity using adult earthworms from the crude extracts of *Ficus racemosa.*

CALOTROPIS GIGANTEA

Biological source	**:**	*Calotropis gigantea*
Family	**:**	Apocynaceae
Parts Used	**:**	Aerial
Synonyms	**:**	*Asclepias gigantean; Calotropis procera*

Taxonomy

Kingdom	:	Planatae
Subkingdom	:	*Tracheobionta*
Superdivision	:	*Spermatophyta*
Division	:	*Magnoliophyta*
Class	:	Dicotyledones
Sub class	:	*Asteridae*
Series	:	Bicarpellatae
Order	:	Gentianales
Family	:	Apocynaceae
Subfamily	:	Asclepidiaceae
Genus	:	*Calotropis*
Species	:	*Calotropis gigantea*

VERNACULAR NAMES [81]

English	Crown flower, giant Indian milkweed. Bowstring hemp, crownplant, madar
Hindi	Safed aak, Aak, Alarkh, Madar, Sveta Arka, Akanda, Bara Akand
Sanskrit	Bh¡nu, Ravi, Tapana
Tamil	Erukku, Vellerukku
Telugu	Jilledi Puvvu

DISTRIBUTION

This plant is chiefly found in waste lands in India, Sri Lanka, Southern China, Malaysia, Singapore and Indonesia. It has spread to the South Pacific Islands and Northern Australia.[82]

PROPAGATION

Seeds spread by wind and water over large distances. Local stands increased in size by suckering. Also spread as an ornamental plant

MORPHOLOGY: [83,84]

The genus *Calotropis* comprises of six species all native to North Africa and Southwest Asia. *Calotropis gigantea* is a medium sized shrub or small tree that grows up to 4m high with a generally waxy appearance and copious milky sap. The stem is ash coloured, smooth, branching sometime almost from the base. Shown in **Fig. 3.3**.

The leaves are grey-green, opposite, alternating, waxy, thick and rounded-ovate. They measure 5–15cm x 4–10cm with a short pointed tip and a heart-shaped base partly clasping the stem; a stiff brush of hairs occur at the base of the midvein. Shown in **Fig. 3.4**.

The flowers are white with deep purple blotch at the base of each lobe and deep purple scales between the petals and the stamens; more or less tubular, 5-lobed, 2–3cm across, devoid of milky sap. They are grouped in umbels in which the outer flowers open first while the inner do not develop fully. Shown in **Fig. 3.5**.

The fruit is a grey-green bladdery pod, 8–12 cm long, rounded at the base but shortly pointed at the tip and containing numerous seeds. Shown in **Fig. 3.6**.

The seeds are brown, flattened, with a tuft of long white hair at one end.. Shown in **Fig. 3.7**.

Chemical constituents

Various chemical constituents have been reported from the different parts of the plant. Flowers contain waxy matter which has estersofresinols, α-β-caltropeol, β-amyrin, stigmasterol, giganteol, calotropin, triterpenoid flavonoid, flavonoid glycoside, wax, acids and alcohols. Seeds are rich in amino acids, major being phenylalanine, lysine and histidine. The leaf contain ascorbic acid, ortho–phyro catechic acid and also contains β-amyrin, taxasterol, tarasterol and beta-sitosterol. [85]

Medicinal uses

The plant is a popular remedy for snake-bite and scorpion-sting. A proteolytic enzyme, somewhat similar to papain, has been found in the milky juice of flowers and acting like digitalis on the heart. [86,87] The flower were described in ancients Ayurveda as sweet-bitter, anthelmintic, analgesic, astringent, cures inflammations, tumors, kapha, rat-bite. The flowers are considered as digestive, stomachic, tonic, useful in asthma, catarrh and loss of appetite.[88] Aerial parts of *Calotropis gigantea* reported for anti- diarrheal activity.[89] Latex of *Calotropis gigantea* evaluated for procoagulating activity associated with fibrinolytic activity[90]**.** Alcoholic extract of the dried peeled roots of *Calotropis gigantea* posses CNS activity[91]. Alcoholic extract of the flowers of *Calotropis gigantea*

reported for analgesic activity[92]. Alcoholic extract of roots evaluated for pregnancy intraceptive activity[93]. Aerial parts of total aqueous extract and water soluble fraction of *Calotropis gigantea* were evaluated for immunomodulatory, antiinflammatory, anticancer and antimitotic activity. Alcoholic extract of stems posses hepatoprotective activity.[94]

Fig. 3.3: Entire plant (Calotropis Gigantea)

Fig 3.4: Leaves ((Calotropis Gigantea)

Fig. 3.5: Flowers (Calotropis Gigantea)

Fig. 3.6: Fruits (Calotropis Gigantea)

Fig. 3.7 Seeds (Calotropis Gigantea)

CALOTROPIS GIGANTEA

- **Nalwaya N** *et al* **(2009)**[95] studied the wound healing activity of Calatropis gigantea in albino rats using excision and incision wound models. Latex treated animals exhibit 83.42 % reduction in wound area when compared to controls which was 76.22 %.
- **Rathod NR** *et al* **(2009)**[96] studied the effect of chloroform extracts of *Calotropis gigantea* leaf and flower on free radical scavenging activity, and lipid profile in streptozotozin induced diabetic rats.
- **Gaurav lodhi** *et al* **(2009)**[97] studied the hepatoprotective activity of ethanolic stem extract of Calotropis gigantea against carbon tetrachloride induced liver injury in rats and the effect was compared with that of silymarin.
- **Alum** *et al* **(2009)**[98] studied the insecticidal activity of the chloroform, petroleum ether and methanolic extracts of root bark of Calotropis gigantea and was found to possess fumigant toxicity and repellent effect.
- **Jayakumar** *et al* **(2010)**[99] studied the antioxidant activity and antibacterial activity of Calotropis gigantean and Vinca rosea and indicated. Calotropis gigantea exhibited highest inhibition zone of 14, 14 and 11 mm against Escherichia coli, Salmonella typhi and Shigella sonnei when compared to Vinca rosea.
- **Jagtap V A** *et al* **(2010)**[100] studied the anti inflammatory activity of ethanolic activity of calotropis gigantea leaf extract using albumin degeneration technique and compared it with the effect of ibuprofen which had an effect of 85.71%.

- **Sheela** *et al* **(2010)**[101] studied the vasodilatory effect of latex from Calotropis gigantea in green frog , Rana hexadactyla.and indicated that higher dilutions factor of the latex showed increased activity.
- **Subramanian** *et al* **(2010)**[102] have studied the anti bacterial activity of ethanolic latex extract of Calotropis gigantea and their activity was determined by serial dilution technique and its activity was compared with that of chloramphenicol.
- **Karthick** *et al* **(2010)**[103] studied the anti-candida activity of aqueous, methanolic ethanolic and petroleum ether extracts of Calotropis gigantea and indicated that the aqueous extract had the highest activity compared to other extracts.
- **Joshi A** *et al* **(2010)**[104] studied the ethanolic extract of *Calotropis gigantea* for its antioxidant activity by reducing power, DPPH and nitric oxide method. Hydroalcoholic extract of *Calotropis gigantea* shown significant antioxidant activity.
- **Thosar** *et al (***2011)**[105] studied the antiasthmatic activity of ethanolic extract of Calotropis gigantea root and indicated the basic mechanism by which it produces the relaxant activity. Lower doses showed better therapeutic activity.
- **Bharathi** *et al* **(2011)**[106] studied the anti bacterial activity of the ethy acetate and chloroform extracts of Calotropis gigantean and indicated their activity against Gram positive bacteria like B.subtilis NCIM 2063, Micrococcus luteus NCIM 2704, Staphylococcus aureus NCIM 2079 and Gram negative bacteria namely, K.pneumoniae NCIM 2719, P. vulgaris NCIM 2027 and E.coli NCIM 2118. Their activity was compared against activity of ciprofloxacin.
- **Oskuee** *et al* **(2011)**[107] studied the leishmanicidal activity of fractionated extract of Calotropis gigantea and indicated that the methanol and hexane fractions

which were the non-polar fractions exhibited the highest activity against promastigotes of L. major(13).

- **Kovendan** *et al* **(2012)**[108] studied the mosquitocidal activity of methanolic leaf extract Calotropis gigantean and indicated the larvicidal and pupicidal activity in 24 hrs along with B.thuringiensis.
- **Bharathi** *et al* **(2012)**[109] undertook a comparative study of differential antimycoses activity of crude leaf extracts of calotropis spp. And reported that Calotropis gigantea produced better activity than all other plants of that species of calotropis and chloroform extract had showed higher activity than methanolic and ethyl acetate extract.

TRANSDERMAL PATCHES REVIEW:

- **Waghulkar** *et al (*2011)[110] investigated transdermal films by incorporating herbal drugs Trichodesma indicum and Boswellia serrate. Chloroform extract of the herbal drugs showed a satisfactory flatness in 99.0 %. Overall, it was observed that the herbal drugs have been found to be effective through modern pharmaceutical formulation techniques.
- **Bhujbal** *et al* **(2011)**[111] prepared transdermal films containing the herbal drug fraction from methanolic extract M charantia fruits by using HPMC. The films were evaluated for folding endurance, thickness, weight variation, drug contents and in vitro diffusion studies and in vivo parameterslike acute and sub-acute antihyperglycemic activity in diabetic rats, biochemicalstudies, skin irritation in rats and stability studies.
- **Sarfaraz MD,** *et al* **(2012)**[112] prepared transdermal patches of selegiline hydrochloride by solvent casting method using hydroxy propyl methyl cellulose

(HPMC), polyvinyl alcohol (PVA) and methyl cellulose (MC) in different ratios (1:1, 1:2 and 1:3). Rate controlling membrane was caste by using 2% ethyl cellulose (EC) membrane. The optimized formulation F6 containing PVA (1:3) showed good release rate of 90.08% for 24 h. The patches were seemingly free of potential hazardous skin irritation. FT-IR and DSC studies revealed no interaction between the drug and polymers used.

- **Suruse PB,** *et al* **(2009)**[113] investigation formulation of anti-inflammatory transdermal pad by incorporating herbal drugs such as boswellic acid (*Boswellia serrata* Rox.), shivlingi extract (*Bryonia laciniosa* Linn.), guggul extract (*Commiphora mukul* Hook.) and isolated compounds from raladhupa namely CS1 and CS2 (*Canarium strictum* Rox.). The skin irritation study on albino rabbit skin showed that the formulation does not produce any irritation.

4. PLAN OF WORK

- **Selection of 2 herbal plants with wound healing property**
  - *Ficus racemosa*
  - *Calotropis gigantea*
- **Pharmacognostical Studies**
  - Materials and Methods
  - Macroscopical and microscopical characters of selected plant
    - *Ficus racemosa*
    - *Calotropis gigantea*
  - Physicochemical character studies
    - Ash Values
    - Extractive Values
    - Loss on Drying
- **Phytochemical Studies**
  - Extraction and isolation of 3 fractions from each plant

    Ficus racemosa → FRfr1, FRfr2, FRfr3

    Calotropis gigantea → CGfr1, CGfr2, CGfr3
  - Qualitative estimation
  - Quantitative estimation
    - Total Alkaloid Content
    - Total Flavonoid Content
    - Tannin Content
    - Total Bitters
  - Thin Layer Chromatography Profile

- **Preliminary pharmacological studies for 6 fractions**
  - Anti-inflammatory activity by Carrageen induced acute paw edema model
  - Wound healing activity by Incision and Excision Model
- **Selection of 2 fractions with bio-guided potential for further studies**

  FRfr3 and CGfr3
- **Isolation and Structural elucidation of isolated compounds by spectral analysis**

  FRfr3 and CGfr3
- **Transdermal patches**

  Product development

  Transdermal patch with FRfr3 (TPFRfr3)

  Transdermal patch with CGfr3 (TPCGfr3)

  Transdermal patch with $FRfr_3$ and $CGfr_3$ (TPCGFRfr3)

  ***In vitro* evaluation of formulated transdermal patches**

  Pharmacological studies
- **Stability studies**

  Transdermal patch with $FRfr_3$ (TPFRfr3)

  Transdermal patch with $CGfr_3$ (TPCGfr3)

  Transdermal patch with $FRfr_3$ and $CGfr_3$ (TPCGFRfr3)

5. MATERIALS AND EQUIPMENTS

The materials and equipments used in the research work were given below in table 5.1 and 5.2.

Table 5.1 - MATERIALS USED

S.No	Name of the Material	Source
1	*Ficus racemosa*	Himalaya drug company, Makali, Bangalore, Karnataka (N13,10752 E77.469889)
2	*Calotropis gigantea*	Himalaya drug company, Makali, Bangalore, Karnataka (N13,10635E77.452551),
3	Formalin	Rankem, Mumbai.
4	Ethanol	Loba chemie pvt. Ltd, Mumbai
5	Tertiary-butyl alcohol	Rankem, Mumbai
6	Toluidine blue	In-house
7	Safranin	E.Merck (India) Ltd, Mumbai
8	Fast green	E.Merck (India) Ltd, Mumbai
9	Glycerin	E.Merck (India) Ltd. , Mumbai
10	Hexane	E.Merck (India) Ltd. , Mumbai
11	Chloroform	Ranbaxy Laboratories, Mumbai
12	Ethyl acetate	E.Merck (India) Ltd. , Mumbai
13	Mayer’s reagent	Pub Chem Limited
14	Fehling’s solution A & B	RICCA Chemical Company
15	Benedict’s solution	In-house

S.No	Name of the Material	Source
16	Millon’s reagent	In-house
17	Nin hydrin reagent	In-house
18	Dragendorff’s reagent	In-house
19	A-naphthol	S.D.Fine chemicals, Chennai
20	Sulphuric acid	RFCL Limited, Rankem, Newdelhi
21	benzene	RFCL Limited, Rankem, Newdelhi
22	pyridine	RFCL Limited, Rankem, Newdelhi
23	Sodium nitroprusside	RFCL Limited, Rankem, Newdelhi
24	Lead acetate	S.D.Fine chemicals, Chennai
25	Ferric chloride	S.D.Fine chemicals, Chennai
26	Acetic anhydride	RFCL Limited, Rankem, Newdelhi
27	Copper sulphate	RFCL Limited, Rankem, Newdelhi
28	Thionyl chloride	RFCL Limited, Rankem, Newdelhi
29	Magnesium metal	RFCL Limited, Rankem, Newdelhi
30	Concentrated ammonia	Rankem, Mumbai
31	Sodium sulphate	RFCL Limited, Rankem, Newdelhi
32	luteolin	Tocris biosciences
33	Aluminium chloride	S.D.Fine chemicals, Chennai
34	Tannic acid	S.D.Fine chemicals, Chennai
35	Protanal LF10/60	FMC biopolymer
36	Calcium chloride	S.D.Fine chemicals, Chennai
37	DM water	In-house
38	starch	RFCL Limited, Rankem, Newdelhi

S.No	Name of the Material	Source
39	Potassium ferric cyanide	S.D.Fine Chemicals, Chennai
40	Gelatin	RFCL Limited, Rankem, Newdelhi
41	Paraffin wax	Wax India, Gujarat
42	Acetone	Sisco research laboratories pvt ltd, Mumbai
43	Methanol	Loba chemie pvt ,ltd,Mumbai
44	Potassium Bromide (IR Grade)	Merck, Goa
45	HCl	RFCL Limited, Rankem, Newdelhi
46	Potassium di hydrogen sulphate	Central drug house (p) ltd,Mumbai(andheri)
47	Sodium hydroxide pellets	Merck Ltd., Mumbai

Table 5.2 - EQUIPMENTS USED

S.No.	Name of the Equipment	Model / Source/Supplier
1	Mechanical sieve shaker	Hicon, Grover enterprises New Delhi.
2	Hot air oven for drying	Hicon, Grover enterprises New Delhi.
3	Weighing balance	Sartorious, Germany
4	Ultraprobe sonicator	Electrosonic industries, EI 250 UP, Mumbai
5	Magnetic stirrer	Remi instruments, Mumbai
6	FTIR	Perkin-Elmer , Germany
7	Ultraviolet spectrophotometer	UV-1700, Shimadzu, Mumbai
8	Melting point apparatus	Campbell Electronics, Mumbai
9	Franz diffusion cell	Aar Gee Automation & Control, Mohali, India
10	pH meter	DI-707Digisun Electronics. Hyderabad
11	Stability chamber	Thermolab Scientific equipments Pvt. Ltd., Mumbai
12	Desiccator	Shankar Scientific, Chennai
13	Extraction flask	Sigma-Aldrich, Mumbai
14	Nikon lab photo 2 microscopic unit	Nikon instruments, Mumbai
15	Electronic microscope	Nikon instruments, Mumbai
16	Incinerator	Advance technologies, Chennai
17	HPLC	Waters, Chennai

S.No.	Name of the Equipment	Model / Source/Supplier
18	Waterbath	Thermo scientific, Chennai
19	Remi stirrer	Remi, Chennai
20	Iodine flask	Borosil, Chennai
21	Soxhlet extractor	Sigma-Aldrich, Mumbai
22	TLC	Shankar Scientific, Chennai
23	Plethysmometer	Orchid Scientifics & Innovatives India Pvt. Ltd.
24	Mass spectrometer	Lab India, Chennai
25	NMR	Agilent technologies, Chennai
26	Cell culture media	HIMedia, Mumbai

6. PHARMACOGNOSTICAL STUDIES

Selection of two herbal plants

Two plants with biological potential and its wound healing activity were selected based on the earlier available literature.

Collection

The plants were collected from different regions of the country. *Ficus racemosa* L (Moraceae) from in and around Makali, Bangalore, Karnataka (N13,10752 E77.469889) and *Calotropis gigantea* L (Apocynaceae) was collected from in and around Makali, Bangalore, Karnataka (N13,10635E77.452551), and all the plant drugs were authenticated by the botanist, Himalaya drug company, Bangalore, Karnataka. Specimen samples were preserved in institution laboratory.

MATERIALS AND METHODS

MACROSCOPICAL STUDIES OF THE BARK

Macroscopical characters for the bark of *FICUS RACEMOSA* was studied and shown in Fig. 6.1

Organoleptic characters:

Nature: Bark from trunk and branches

Taste : Not characteristic

Odour : Not characteristic

Colour: Yellowish brown to brown to blackish

Macroscopical characters:

Commercial material consists of bark from trunk and branches. It is 0.5-1.5 cm in thickness, ranges from 4-10 cm in size and flat to curve. It is yellowish in the outer and also dark brown in some places. Inner surface is blackish brown. Lenticels are observed in the dark brown coloured places, where the outer cork is not peel off. Outer surface is smooth and inner surface is rough with ridges and grooves due to the fibres. Fracture is splintery in outer surface and minutely fibrous inner. It is not so hard to break. Fig 6.1

METHODS FOR ANATOMICAL STUDIES

Staining

The dried bark of the plant, *FICUS RACEMOSA* was cut and fixed in FAE (Formalin - 5 ml+ Acetic acid – 5 ml + 70 % Ethyl alcohol – 90 ml). After 24 hrs of fixing, the specimens were dehydrated with graded series of tertiary – butyl alcohol. Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60 ^{o} c) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.[114]

Sectioning

The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the section was 10-12 µm. De waxing of the sections was by customary procedure.[115] The sections were stained with Toluidine blue, since toluidine blue is a polychromatic stain.[116] The staining results were remarkably good and some cytochemical reactions were also obtained. The dye rendered pink color to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. Wherever necessary, sections were also stained with Safranin and Fast green and IKI (for starch). Powdered materials were cleared with NaOH and mounted in glycerin medium after staining.

Photomicrographs

Photographs of different magnifications were taken with Nikon Lab photo 2 Microscopic Unit. For normal observation, bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale-bars.[117]

Fig. 6.1: Macroscopy of the Bark of Ficus racemosa

MICROSCOPICAL STUDIES

Transverse sections of the bark show the general pattern in the tissue arrangements. It has phellem, phellogen, phelloderm and secondary cortex. Phellem is few layered (2-5) in some places where it was peel off and in other places it is more than 15 layers of thickness. The cells are regularly arranged and brownish in colour (Fig. 6.2). Phellogen is 2 or 3 layered. Phelloderm is broad made up of parenchyma cells and stone cells (Fig. 6.3). Stone cells have a large lumen with pitted walls. Comparatively parenchyma cells are lesser than the stone cells (Fig. 6.4). Laticiferous canals are numerous in the phelloderm region **.**Solitary and pyramidal type calcium oxalate crystals are also present in the parenchyma cells of phelloderm (Fig. 6.5).

TRANSVERSE SECTION OF *FICUS RACEMOSA* BARK

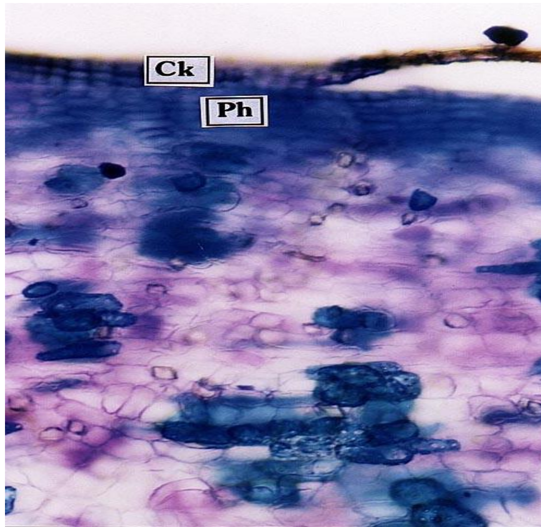


Fig. 6.2: TS of bark (outer region)
Ck: Cork; Ph: Phellogen

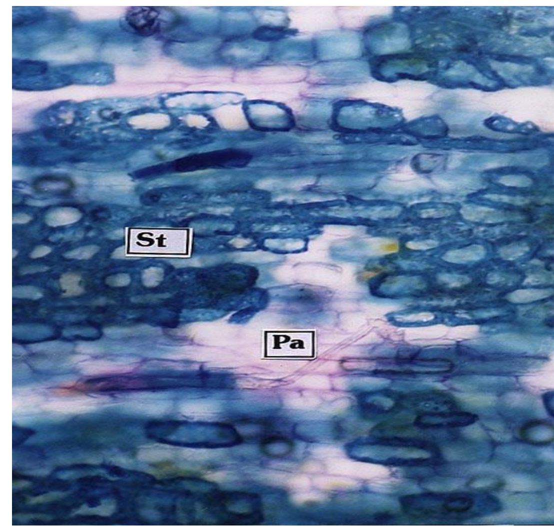


Fig. 6.3: TS of bark (middle region)
St: Stone cells; Pa: Parenchyma cells

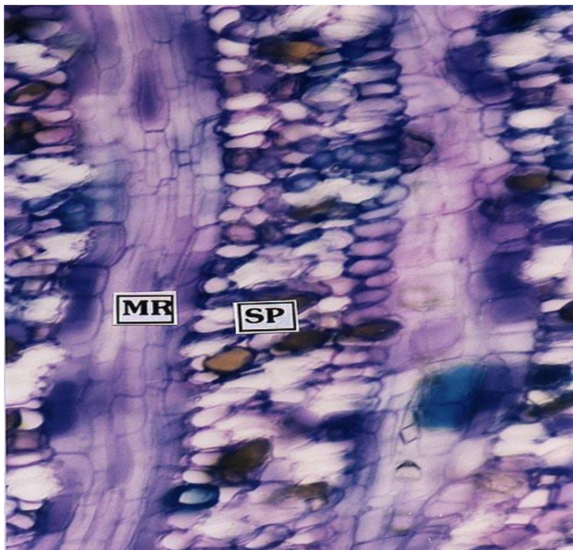


Fig. 6.4: TS of bark (inner region)

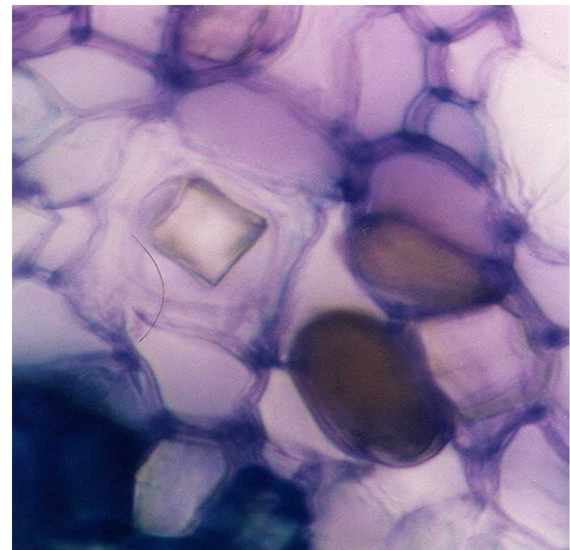


Fig. 6.5: TS of bark showing laticiferous canals

MR: Medullary ray; SP: Secondary phloem

POWDER MICROSCOPY

ORGANOLEPTIC CHARECTERS

Colour : light pink to light brown colour

Odour : faint

Taste : astringent

Fracture: splintery and fibrous and not so hard to break soft

POWDER MICROSCOPICAL CHARACTERS

The powder of the bark exhibits the following characters under the light microscope.

1. **Stone cells**: Presence of Stone cells with large lumen. (Fig. 6.6)
2. **Parenchyma cells:** Presence of large number of stone cells than the parenchyma cells in phelloderm. (Fig. 6.7)
3. **Laticiferous canals**: Presence of laticiferous canals. (Fig. 6.8)

POWDER MICROSCOPY OF ***FICUS RACEMOSA*** BARK

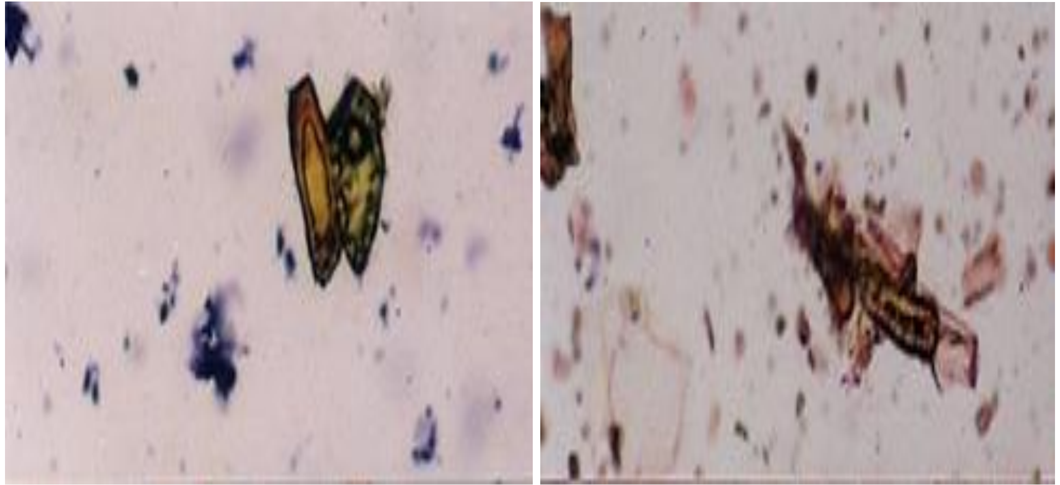


Fig. 6.6: Stone cells with large lumen

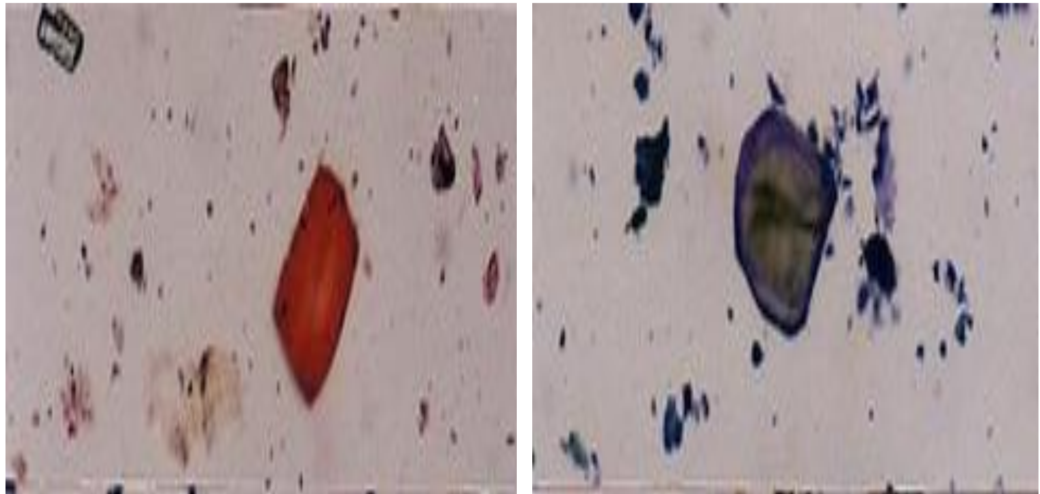


Fig. 6.7: Parenchyma Cells

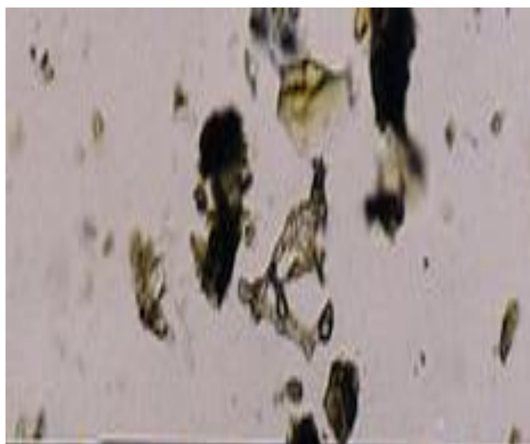


Fig. 6.8: Laticiferous canals

MACROSCOPY AND MICROSCOPY OF AERIAL PARTS OF *CALOTROPIS GIGANTEA*

MICROSCOPICAL STUDIES

T.S. of Petiole:

The leaf consists of a thick plano convex petiole and thick lamina, petiole is flat on the adaxial side and broadly convex on the abaxial side. It is 1.1mm thick and 2.8mm wide. The epidermal layer of the midrib is thin made up of small rectangular cells. The cells in the outer zone are small compact and collenchymatous and the cells in the inner portion are circular, thick walled and parenchymatous, fairly wider and comparatively thick walled circular laticifers are common in the ground tissue. (Fig 6.9)

T.S. of Lamina:

The lamina is smooth and even on both surfaces. It is 420 µm thick. Both epidermal layers are thin with squarish cells and prominent cuticle; epidermis 20 µm thick. The lamina is amphistomatic (stomata occur on both surfaces). The mesophyll tissue is differentiated into adaxial zone of three layers of short cylindrical compact palisade cells and abaxial zone of lobed spongy mesophyll cells which form loosely reticulate aerenchyma. The lateral vein is large circular and collateral; it consists of a few xylem elements and phloem elements surrounded by parenchymatous bundle sheath. (Fig. .10 & 6.11).

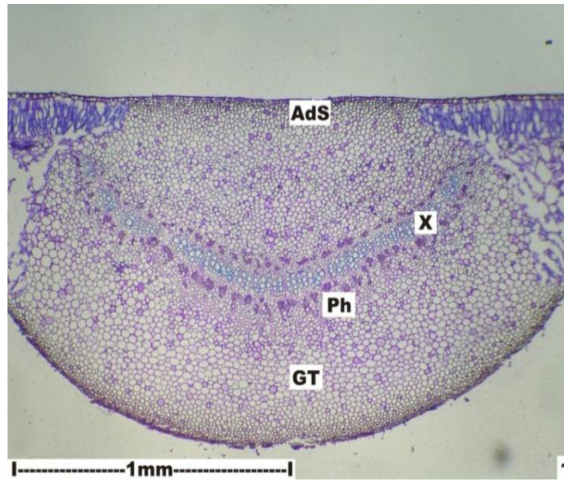


Fig. 6.9: T.S of petiole (40 X)
AdS – Adaxial side; GT – Ground Tissue; Ph – Phloem; X – Xylem

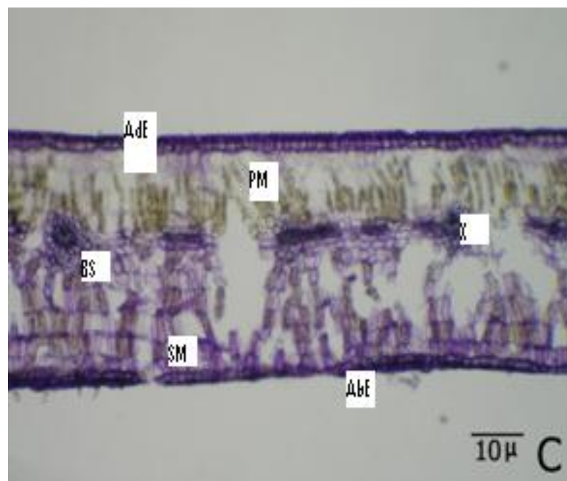


Fig. 6.10: T.S of central part of the lamina
AbE – Abaxial epidermis; AdE – Adaxial epidermis; BS – Bundle sheath;
SM – Spongy mesophyll; X – Xylem

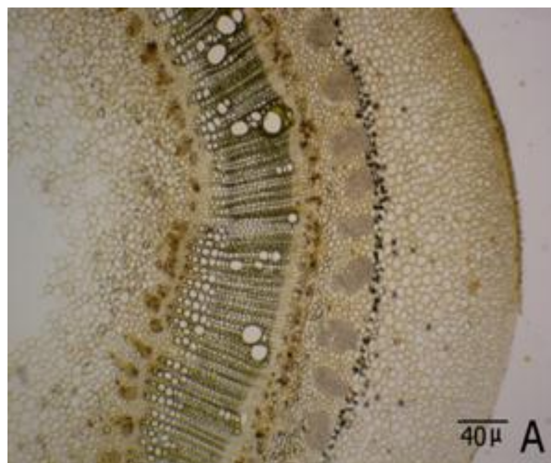


Fig. 6.11: T.S of Stem central part of the lamina

POWDER MICROSCOPY:

Organoleptic characters:

Colour : green to mild brown in color

Odour : faint

Taste : astringent

Texture: velvettty and soft

Powder microscopical characters:

The powder of the aerial parts exhibits the following characters under the light microscope.

The powder showed presence of granular vessels and fibers.

1. Parenchyma cells, epidermal cells and trichomes are abundant, observed almost in every field under microscope. (Fig. 6.12, 6.13 & 6.14)
2. **Laticiferous canals**: Presence of laticiferous canals. (Fig. 6.14)
3. Vessel elements, rosettes of calcium oxalate crystals and fragments of leaves are also observed. (Fig. 6.15, 6.16, 6.17, 6.18, 6.19 & 6.20)
4. **Stone cells**: Presence of Stone cells with large lumen. (Fig. 6.21)

Powder microscopy of *Calotropis gigantea* aerial parts

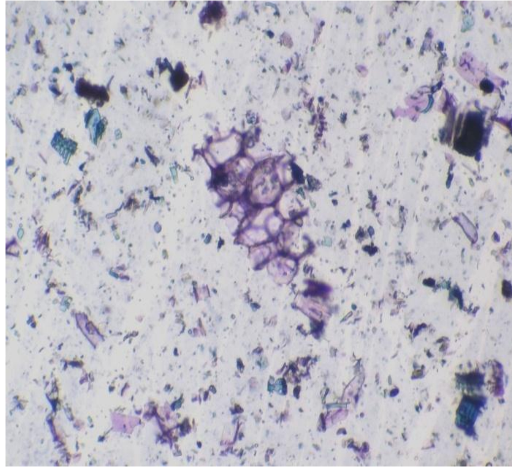


Fig. 6.12: Parenchyma cells

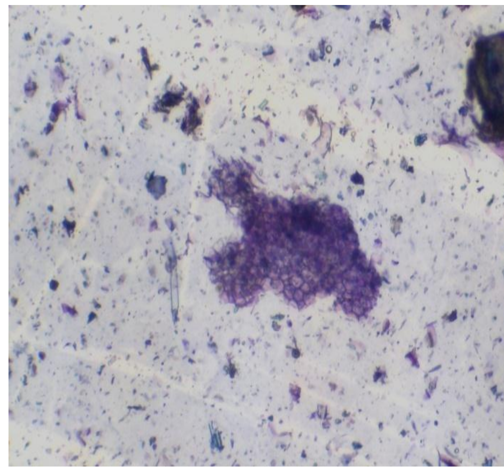


Fig. 6.13: Epidermal cells

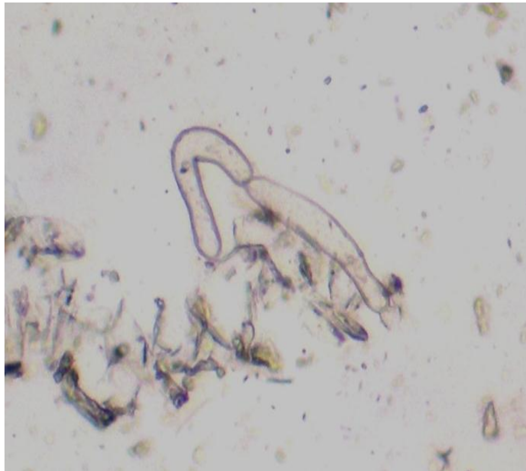


Fig. 6.14: Trichomes

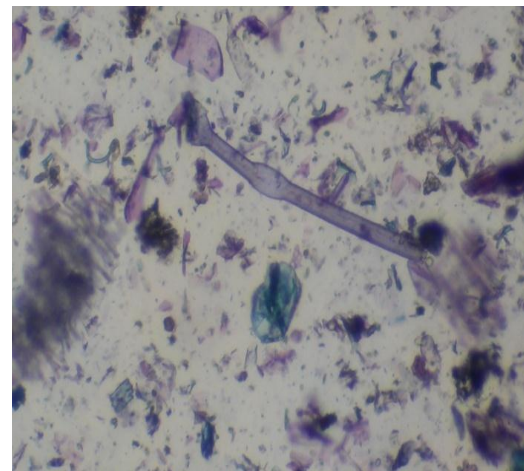


Fig. 6.15: Laticiferous canal

Powder microscopy of *Calotropis gigantea* aerial parts

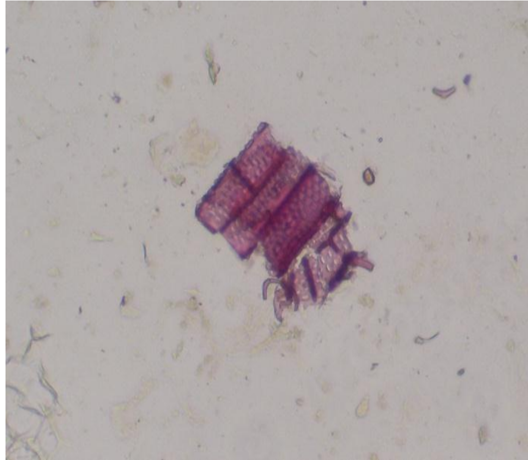


Fig. 6.16: Vessel elements

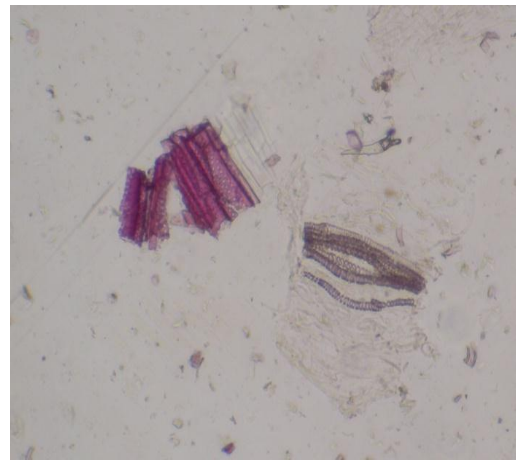


Fig. 6.17: Spiral and pitted vessels

Fig. 6.18: Spiral vessels

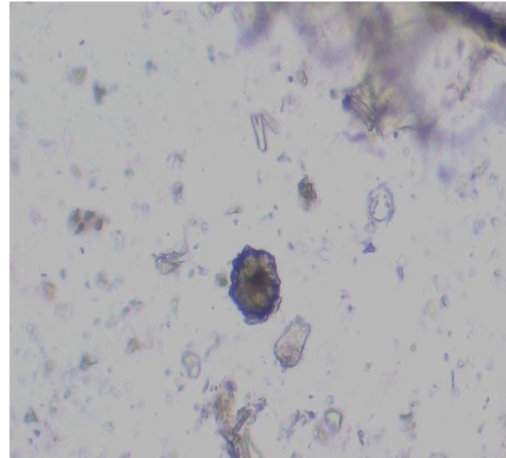


Fig. 6.19: Rosette type of the calcium oxalate crystals

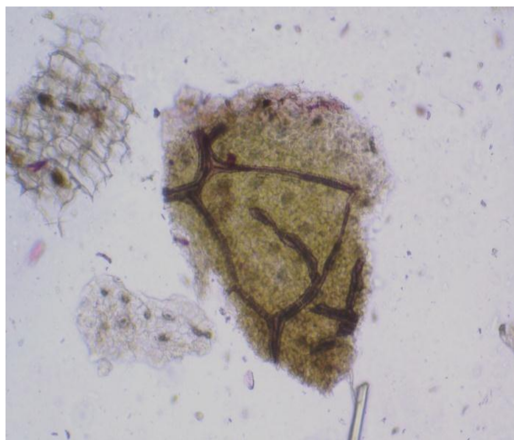


Fig. 6.20: Fragments of leaf

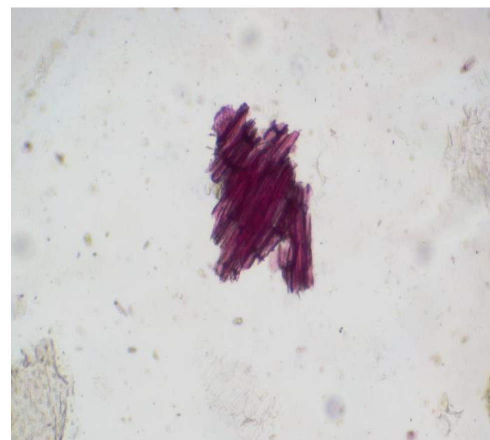


Fig. 6.21: Stone cells from the stem

PHYSICO CHEMICAL CHARACTER STUDIES:

Ash Value:

Ash values are helpful in determining the quality and purity of a crude drug especially in the powdered form. The ash content of a crude drug is generally taken to be the residue remaining after incineration. It usually represents the inorganic salts naturally occurring in the drug and adhering to it, but it may also include inorganic matter fielded for the purpose of adulteration. Dried coarsely powdered bark of *Ficus racemosa* was subjected to the following analysis.

Determination of Total ash:

About 2 grams of dried crude drug was weighed accurately in a tarred silica crucible and incinerated at a temperature not exceeding 450°c until free from carbon. It was then cooled in a dessicator and weighed. The percentage of ash was calculated with reference to air dried drug.

Determination of Water soluble ash:

The total ash was boiled for five minutes with 25 ml of water. The insoluble matter was collected in an ash less filter paper, washed with hot water and ignited for 15 minutes at a temperature not exceeding 450°c. The weight of the insoluble matter was subtracted from the weight of the ash; the difference in the weight of the ash represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the dried drug.

Determination of Acid insoluble ash:

The total ash was boiled with 25 ml of 2 M HCl for 15 minutes. The insoluble matter was collected in an ashless filter paper, washed with hot water, ignited, then cooled in a desiccator and weighed. The percentage of acid insoluble ash was calculated with reference to the dried drug. Results were presented in Table. 6.1.

Extractive Values:

Extractive value of crude drugs is useful for their evaluation especially when the constituents of a drug cannot be readily estimated by any other means. These values are indicative of approximate measures of their chemical constituents and the nature of the constants present in crude drugs.

Determination of Alcohol soluble extractive

About 5 grams of the powder was macerated with 100 ml of alcohol of the specified strength in a closed flask for 24 hours, shaking frequently for 6 hours and allowed to stand for 18 hours. It was filtered rapidly and 25 ml of the filtrate was evaporated to dryness at 105°c and weighed. The percentage of alcohol soluble extractive was calculated with reference to the dried drug.

Determination of Water soluble extractive:

About 5 grams of the powder was macerated with 100 ml of distilled water in a closed flask for 24 hours, shaking frequently for 6 hours and allowed to stand for 18 hours. It was filtered rapidly and 25 ml of the filtrate was evaporated to dryness at 105°c and weighed. The percentage of water soluble extractive was calculated with reference to the dried drug. Results were presented in Table. 6.1.

Loss on Drying:

Loss on drying is the loss of mass expressed as percent w/w and can be determined by the following procedure.

About 2 gm of drug was weighed and transferred to a dry stopper weighing bottle. The weight of the bottle and the drug was taken accurately. After removing the stopper, the bottle containing drug was placed in an oven for 1 hour at 120 ^{0}C. After 1 hr, the bottle was removed and cooled in a dessicator and weighed by replacing the stopper which was continued until difference between two successive weighing was not more than 0.25% of constant weight. Results were presented in Table. 6.1.

RESULTS AND DISCUSSION:

The pharmacognostical investigation of the plants *Ficus racemosa* and *calotropis Gigantea* was carried out with standard procedure. Earlier literature review of the plants *Ficus racemosa* and *calotropis Gigantea* revealed that no work has been carried out with regard to pharmacognostical studies except for its taxonomical identification.

The macroscopy of the bark of ***Ficus racemosa*** *(glomerata)* was greyish green to rusty brown. Surface was soft and uneven, 0.5 – 1.8 cm thick. Outer surface of bark consists of easily removable translucent flakes of whitish tissue, inner surface light brown; fracture fibrous. The entire bark has more or less homogenous leathery texture. Taste astringent without any characteristic odour.

The anatomical character of the bark has the three zones like outer bark, outer secondary phloem, inner secondary phloem. It has phellem, phellogen, phelloderm and secondary cortex. The cells are regularly arranged and brownish in colour. Phellogen is 2 or 3 layered. Phelloderm is broad made up of parenchyma cells and stone cells**.** Stone

cells have a large lumen with pitted walls. Comparatively parenchyma cells are lesser than the stone cells . Laticiferous canals are numerous in the phelloderm region **.** Solitary and pyramidal type calcium oxalate crystals are also present in the parenchyma cells of phelloderm. The powder of the bark exhibits the following characters stone cells, parenchyma and lacticiferous canals.

The calotropis gigantia leaf consists of a thick plano convex petiole and thick lamina, petiole is flat on the adaxial side and broadly convex on the abaxial side. The cells in the outer zone are small compact and collenchymatous and the cells in the inner portion are circular, thick walled and parenchymatous, fairly wider and comparatively thick walled circular laticifers are common in the ground tissue. The lamina is smooth and even on both surfaces. The lamina is amphistomatic (stomata occur on both surfaces). The mesophyll tissue is differentiated into adaxial zone of three layers of short cylindrical compact palisade cells and abaxial zone of lobed spongy mesophyll cells which form loosely reticulate paerenchyma. The powder microscopy of the aerial parts of calotropis exhibits the presence of Parenchyma cells, epidermal cells and trichomes, Laticiferous canals, calcium oxalate crystals, Stone cells.

The ash content of crude drug is generally taken to be the residue remaining after incineration. It usually represents the inorganic salts naturally occurring in the drug and adhering to it, but it may also involve the inorganic matter added for the purpose of adulteration. There is a considerable difference varies with in narrow limits in the case of some individual drug. Hence ash determination furnishes a basis for judging the identity and cleanliness of a drug and gives information related to its adulteration with inorganic matter. Ash standards have been stabilized for a number of official drugs. Usually these standards set a maximum limit on the total ash are on the acid insoluble ash permitted. The ash or residue yielded by an organic chemical compound is a rule to measure the

amount of inorganic matter, which is present as impurity. In most cases the inorganic matter is present in small amounts which are difficult to remove in the purification process and which are not objectionable if only traces and present. Ash values are helpful in determining the quality and purity of the crude drug in powdered form.

Extractive values of crude drugs are useful for the qualitative evaluation of chemical constituents, especially when the constituents of the drugs cannot be readily estimated by any other means. Further these values indicate the nature of constituents present in a crude drug.

The loss on drying is the loss of weight in percentage w/w resulting from water and volatile matter of any kind that can drive under specified conditions.[118]

The physic-chemical parameters of coarse powder of *Ficus racemesa* Bark and the aerial parts of *Calotropis gigantia* were studied. The results revealed that the bark of ***Ficus racemosa*** and *Calotropis gigantia* was having 6.27% w/w&7.5 % w/w of total ash, 3.25% w/w &2.75% w/w of water soluble ash and acid insoluble ash is about 2.93% w/w&2.2% w/w. The water and alcohol soluble extractive value was found as 4.2 % w/w&11.2% w/w, 14.7% w/w and 16.7%w/w respectively. Hence the plant shows high amount of alcohol soluble extractive value. The loss on drying was about 9.48% w/w &6.8% w/w. These data's were helpful for identifying and ascertaining the quality of the collected crude drug. The results were shown in the table 6.1 and 6.2 respectively.

Table 6.1- Analytical Parameters of *FICUS RACEMOSA* bark

S.N	Parameters	Values (%w/w
1.	**Ash Values**	
	Total ash	6.27%
	Water soluble ash	3.25%
	Acid insoluble ash	2.93%
2.	**Extractive Values**	
	Alcohol soluble extractive valu	16.7%
	Water soluble extractive value	4.2%
3.	**Loss on drying**	9.48%

Table 6.2 - Analytical Parameters of *Calotrropis gigantia* aerial parts

S.N	Parameters	Values (%w/w
1.	**Ash Values**	
	Total ash	7.5%
	Water soluble ash	2.75%
	Acid insoluble ash	2.2%
2.	**Extractive Values**	
	Alcohol soluble extractive valu	14.7%
	Water soluble extractive value	11.2%
3.	**Loss on drying**	6.8%

7. PHYTOCHEMICAL STUDIES

Plant material:

The bark of ***FICUS RACEMOSA*** **AND** the Aerial of ***CALTROPIS GIGANTEA*** were supplied and authenticated by the Himalaya drug company, makali, Bangalore, Karnataka with the reference no.N13.10752E77.469889 and N13.10635E77.452551.

Extraction

The shade dried bark of ***FICUS RACEMOSA*** of about 2kg were subjected for size reduction to coarse powder. Each 1 kg of powder was extracted with methanol & water separately for about 16 hrs at room temperature.[119] Both the methanol and water extract was concentrated using water bath at 70-80^{0}C to get the extract. The percentage of yield of methanolic extract was found to be 10 %(w/w) and aqueous extract was found to be 13% (w/w). Fig 7.1 & 7.2.

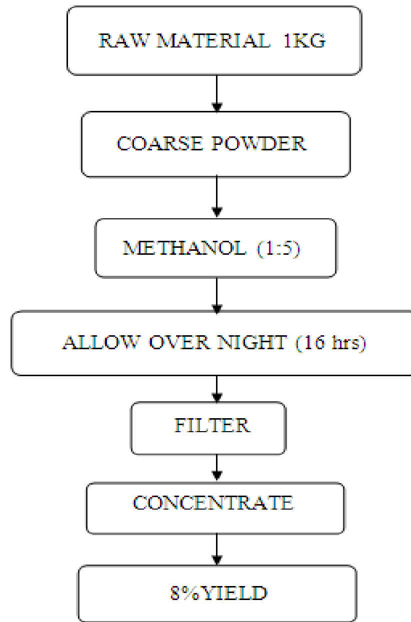


Fig 7.1 Extraction flow chart A: Methanol Extraction

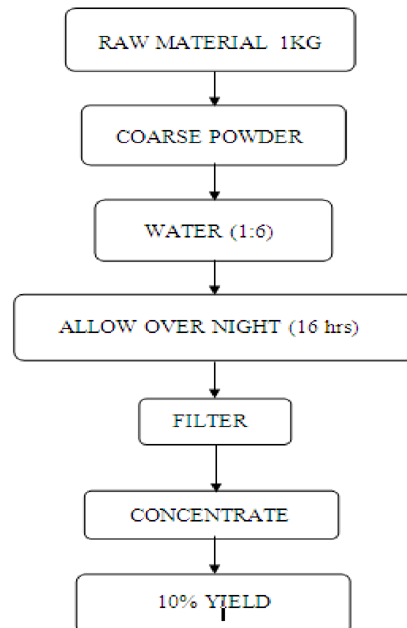


Fig 7.2 Extraction flow chart B: Aqueous Extraction

FRACTIONATION OF METHANOL EXTRACT

- Methanol extract of 50g was treated with 150ml of hexane three times and separated hexane fraction and concentrated using water bath at 70-80^{0}C.The final yield was 10.5g and it's coded as **FRFR-1.**
- Hexane insoluble residue was treated with chloroform, acetone and discarded the mother liquid. The yield of final residue was 15.2g and it's coded as **FRFR-1. FRFR-2 and FRFR-3.**

FRACTIONATION OF AQUEOUS EXTRACT

- Water extract of 100g was treated with 300 ml of 10% aqueous methanol two times and filtered through ordinary filter paper and concentrated the filtrate using water bath at 70-80°C.The yield of final residue was 51g.
- Added 200ml of 10% aqueous methanol in 30g of the residue mixed well and filtered through ordinary filter paper. Filtrate precipitated with 1000ml of acetone by slowly adding filtrate with constant stirring. Collect the precipitate and dried in oven at 60-70°C.[120] The yield of precipitate was 16.4g and it's coded as **FRFR-3.** Fig 7.3

Name: FICUS RACEMOSA

Part: BARK

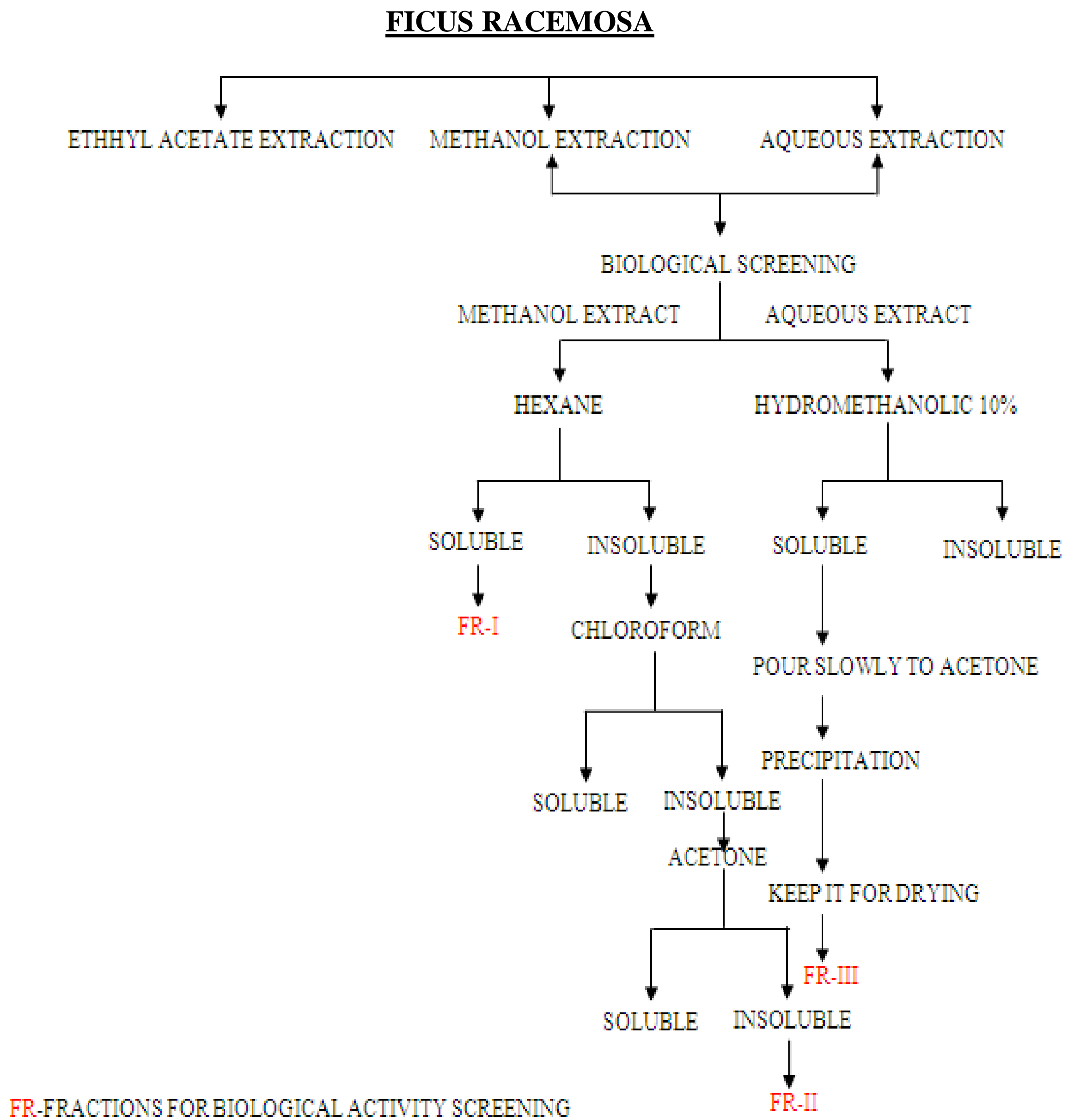


Fig 7.3 Fractionation flow chart for methanol and water extract

Extraction

The shade dried aerial parts *Caltropis* ***GIGANTEA*** of about 2kg were subjected for size reduction to coarse powder. Each 1 kg of powder was extracted with methanol & water separately for about 16 hrs at room temperature. Both the methanol and water extract was concentrated using water bath at 70-80^{0}C to get the extract.[121] The percentage of yield of methanolic extract was found to be 8 %(w/w) and water extract was found to be 10% (w/w). (Fig 7.4 & 7.5)

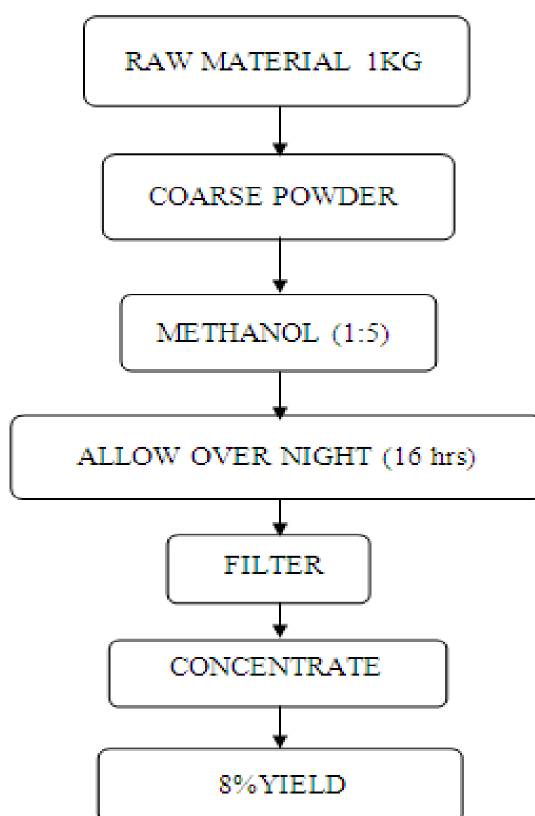


Fig 7.4. Extraction flow chart A: Methanol Extraction

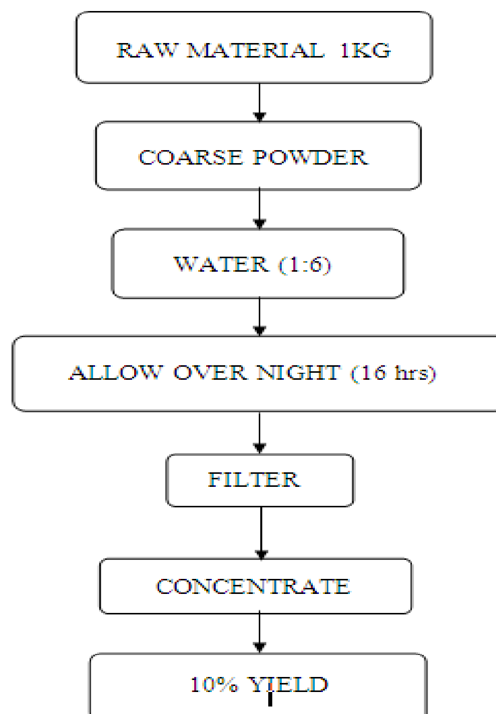


Fig 7.5. Extraction flow chart A: Aqeous Extraction

FRACTIONATION OF METHANOL EXTRACT

- Methanol extract of 50g was treated with 150ml of hexane two times and separated hexane fraction and concentrated using water bath at 70-80^{0}C.The final yield was15g and it's coded as CGFR-1.
- Hexane insoluble residue was treated with chloroform, water and discarded mother liquid. The yield of final residue was 10g and it's coded as CGFR-2.

FRACTIONATION OF AQUEOUS EXTRACT

- Water extract of 80g was treated with 240 ml of 10% aqueous methanol two times and filtered through ordinary filter paper and concentrated the filtrate using water bath at 70-80°C.The yield of final residue was 35g.
- Added 200ml of 10% aqueous methanol in 20g of the residue mixed well and filtered through ordinary filter paper. Filtrate precipitated with 800ml of acetone by slowly adding filtrate with constant stirring. Collect the precipitate and dried in oven at 60-70°C. The yield of precipitate was 14g and it's coded as CGFR-3 (Fig7.6)

Name: CALOTROPIS GIGANTEA

Part: AERIAL

CALOTROPIS GIGANTEA AERIAL

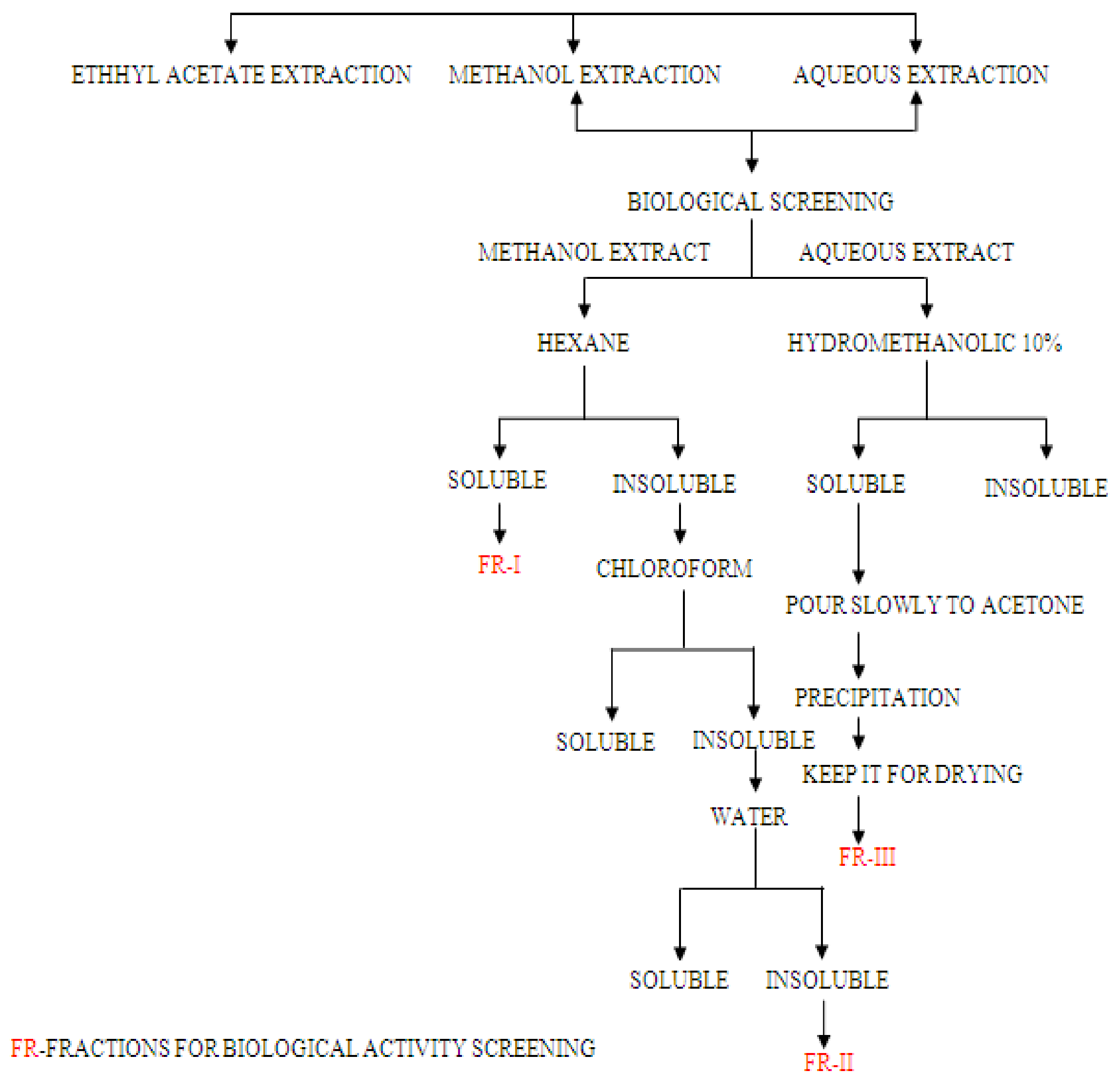


Fig 7.6 Fractionation flow chart for methanol and water extract

Preliminary Chemical Analysis

The total methanol extract and its fractions of the bark of *Ficus racemosa* and aerial part of ***CALTROPIS GIGANTEA*** were subjected to the following chemical tests for identification of phytochemical constituents and the results were tabulated in Table 7.2 & 7.3.

Test for Alkaloids [122, 123, 124]

Mayer's test

A pinch of dried extract was taken and 2 ml of dilute hydrochloric acid was added, mixed, filtered and one or two drops of Mayer's reagent was added.

Formation of white precipitate indicates the presence of Alkaloids.

Dragendorff's test

A pinch of dried extract was taken and treated with 2 ml of 2% v/v of acetic acid, mixed thoroughly and filtered. Two drops of Dragendorff's reagent was added to the filtrate.

Formation of orange brown precipitate indicates the presence of Alkaloids.

Test for Carbohydrates[125, 126,]

Molisch's test

The substance was treated with α-naphthol and conc sulphuric acid.

Formation of violet colour indicates the presence of carbohydrates.

Test for Glycosides[127]

A pinch of extract was taken in a watch glass and 2 drops of alcohol was added to dissolve the extract. An equal quantity of anthrone was added and mixed thoroughly and dried. Then one drop of concentrated sulphuric acid was added, spreaded in a thin film with a glass rod in a watch glass and heated over the water bath.

Formation of dark green colour confirms the presence of glycosides.

Test for Anthroquinone glycosides[128]

Borntrager's test

A pinch of the extract was boiled with dilute sulphuric acid, filtered while hot and filtrate was extracted with solvent like benzene. It was shaken well and the organic layer was separated and to this equal volume of dilute ammonia was added.

Formation of rose pink colour in ammonia layer indicates the presence of anthraquinone glycoside.

Test for Cardiac glycosides[129, 130]

Legal's test

The substance was hydrolysed for few hours in a water bath. The hydrolysate was added with 2 ml of pyridine, sodium nitro prusside solution and was made alkaline with sodium hydroxide solution.

The change of colour from yellow to orange indicates the presence of cardiac glycoside.

Keller killani test

About 1 gram of powder was boiled with 70% ethanol for 3 minutes and filtered. The filtrate was treated with lead acetate solution, shaken well and filtered. The clear filtrate was treated with equal volume of chloroform and the chloroform layer was evaporated. The residue was dissolved in 3 ml of glacial acetic acid and to these 2 drops

of ferric chloride was added. The contents were transferred to test tube containing 2 ml of concentrated sulphuric acid.

Reddish brown layer acquiring bluish green colour after standing indicates the presence of cardiac glycoside.

Test for Sugar[131]

Fehling's test

The extract was treated with equal quantity of Fehling's solution A and B and it was heated on a water bath.

Formation of brick red precipitate indicates the presence of sugar.

Benedict's test

The substance was treated with Benedict's reagent and heated in a water bath.

Formation of reddish brown precipitate indicates the presence of sugar.

Test for Steroids[132, 133]

Liebermann's Burchard test

The extract was dissolved in 2 ml of chloroform and 10 drops of acetic anhydride, 2 drops of concentrated sulphuric acid were added.

Formation of green colour indicates the presence of phytosterols.

Salkowski test:

The extract was dissolved in 2 ml of chloroform and an equal volume of concentrated sulphuric acid was added slowly through the sides of the test tube.

The chloroform layer turns reddish violet colour and the lower layer turns a yellowish colour with green fluorescence which indicates the presence of phytosterols.

Test for Tannins[134, 135]

A pinch of dried extract was dissolved in ethanol, mixed thoroughly and filtered. The filtrate was treated with the following reagents.

1. Lead acetate solution- formation of white precipitate shows the presence of tannins.
2. Ferric chloride solution- formation of deep blue colour shows the presence of tannins.
3. Aqueous gelatin solution- formation of white precipitate shows the presence of tannins.

Test for Saponins[136]

Foam test

One ml extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 min. Formation of foam for about 1 cm layer indicates the presence of saponins.

Test for Proteins[137, 138]

Millon's test

The extract was dissolved in 1 ml of ethanol, filtered and the filtrate was treated with millon's reagent.

Formation of red colour indicates the presence of proteins.

Biuret test

The extract was dissolved in 1 ml of ethanol, filtered and the filtrate was added with 40% v/v sodium hydroxide and copper sulphate solution.

Formation of violet colour indicates the presence of proteins.

Ninhydrin test

The extract was dissolved in 1 ml of ethanol, filtered and the filtrate was treated with ninhydrin reagent.

Formation of purple colour indicates the presence of proteins.

Xanthoprotein test

The extract was dissolved in 1 ml of water, filtered and the filtrate was treated with 1 ml of concentrated nitric acid. The content was made alkaline to litmus paper by adding ammonia solution.

Formation of orange colour indicates the presence of proteins.

Test for Terpenoids[139]

A pinch of dried extract was taken in a dried test tube. A bit of tin foil and 0.5 ml of thionyl chloride was added and heated gently.

Formation of pink colour indicates the presence of terpenoids.

Test for Flavonoids[140]

Shinoda test

A pinch of dried extract was dissolved in ethanol, mixed thoroughly and filtered. To the filtrate, magnesium metal pieces and concentrated hydrochloric acid were added and heated gently.

Appearance of magenta colour indicates the presence of flavonoids.

Test with Sodium Hydroxide solution

A pinch of dried extract was treated with sodium hydroxide solution.

Formation of yellow colour indicates the presence of flavones and yellow to orange colour indicates the presence of flavonones.

Test with Sulphuric acid

The extract was treated with concentrated sulphuric acid.

Yellow to orange colour indicates the presence of flavones and orange to crimson colour indicates the presence of flavonones.

Test for Anthocyanins[141]

1. The extract was treated with sodium hydroxide solution.

   Formation of blue- violet colour indicates the presence of anthocyanins.

2. The extract was treated with concentrated sulphuric acid.

   Formation of yellowish orange colour indicates the presence of anthocyanins.

Test for Quinones[142]

The extract was treated with sodium hydroxide.

Formation of blue green or red colour indicates the presence of quinones.

ESTIMATION OF PHYTOCONSTITUENTS

Plant materials and herbals derived from around 70,000 plant species represents substantial portion of the global market. The W.H.O Assembly currently in number of resolutions emphasized the need to ensure quality control of medicinal plant products by using modern techniques and applying suitable standards.[143]

The purpose of standardization of medicinal plant product is obviously to ensure therapeutic efficacy and to check any adulteration or non deliberate mixing in commercial batches. The *Ficus racemosa* bark and the aerial part of ***c****altropis gigantea* were subjected to the estimation of phytoconstituents and the results were tabulated in Table 7.4.

1. Total alkaloid content
2. Flavonoid content
3. Tannin content
4. Total Bitters

Estimation of total Alkaloids by Gravimetry

Sample preparation: weighed about 10 grams (W) of the fine powdered sample in a 500 ml iodine flask. Added 200 ml of solvent mixture. Shaked well and kept for ten minutes. Added 6 ml of dilute ammonia and shaked for an hour using magnetic stirrer shaker. Kept the flask overnight.

Procedure: To the flask kept overnight, add 10 ml of purified water and shaked vigorously for 1 minute. Filter through a Whatman No.1 filter paper. Taken 100 ml of the filtrate and transferred this filtrate to a separating funnel. Extracted with successive quantities 35 ml, 25 ml and 20 ml of dilute sulfuric acid. Rejected the upper layer and collected the bottom acid layer and transferred this to a clean separating funnel.

Made the acid solution to alkaline (pH-10) with the addition of 10 ml of concentrated ammonia solution. Fractionated the alkaline solution with chloroform in successive quantities of 35ml, 25 ml and 20 ml in a separating funnel.

Rejected the upper layer and collected the chloroform layer. Transferred the chloroform layer into a clean separating funnel. Added approx. 10 ml of distilled water and shake slightly. Kept for 10-15 minutes, and filtered the chloroform layer through cotton Whatman No.1 filter paper into a previously weighed dish or beaker (W_1). Allowed to evaporate the chloroform by keeping on a water bath. The flask containing the residue is kept in oven at 105°C ± 5°C for 30 minutes. Taken the weight (W_2) until a constant reading is obtained. [144]

$$\text{\% w/w of Total Alkaloids} = \frac{W_2 - W_1}{W} \times \frac{200}{100} \times 100$$

W – Weight of the test substance (g), W_1- Weight of empty dish (g)

W_2- Weight of dish or with residue after drying (g)

Estimation of total flavonoids

Total Flavonoid content in dried aerial parts of the plant was estimated by spectrometric method.[145] (Perkin-Elmer UV-Vis spectrometer Lambda 16 (Germany)). Dried powdered plant material (10 gm) was extracted by continuous mixing in 100 ml of 70% ethanol, 24 hr at room temperature. After filtration, ethanol was evaporated untill only water remained. Water phase was subsequently extracted with ethyl acetate. The extract was dried over anhydrous sodium sulphate, filtered and concentrated under vacuum up to a concentration of 1 gm/ ml of extract. They were further diluted with ethyl acetate to obtain 0.01 gm /ml solutions used in the experiments. About 10 ml of the solution was transferred into a 25 ml volumetric flask, 1 ml of 2% $AlCl_3$ was added and the solution was filled to volume with methanol-acetic acid and was kept aside for 30 min, the absorbance was measured at 390 nm against the same solution without $AlCl_3$ being blank. Luteolin was used to construct the calibration curve in the concentration range 1.0-10.0 µg/ml.

Estimation of tannins by spectrophotometry

Sample Preparation: About 100.2mg of finely powdered sample in to 250 ml flat bottomed flask and added 50 ml purified water and reflux at 100^0C ± 2^0C using water bath for 1 hour, cool and decant the dissolved extract into 100 ml volumetric flask. Wash the residue with purified water and make the volume up to the mark with same solvent. Filtered the extract through Whatman No.1 filter paper. Discarded first 25 ml of filtrate; used the subsequent filtrate for analysis.

Standard Preparation (0.01 mg/ml): Weighed accurately about 100 mg of standard tannic acid in 100 ml volumetric flask, dissolve with purified water and made up to volume with purified water (standard stock solution). Pipette out 1 ml from this solution and makeup to 100 ml with purified water (working standard solution).

Preparation for Optical density reading: *Standard solution:* Taken 1 ml of workingnh standard solution in 10 ml volumetric flasks. Added 1 ml of potassium ferri cyanide and 1 ml of ferric chloride reagent. Mixed well and made the volume up to 10 ml with purified water. Exactly 30 minutes after addition of the reagents read the optical density (S) at 720 nm against reagent blank. Reagent blank is prepared by diluting 1 ml of potassium ferri cyanide and 1 ml of ferric chloride to 10 ml with purified water.

Test solution: Taken 0.2 ml of test solution in 10 ml volumetric flasks. Added 1 ml of potassium ferri cyanide and 1 ml of ferric chloride reagent. Mix well and make the volume up to 10 ml with purified water. Exactly 30 minutes after addition of the reagents read the optical density (T) at 720 nm against reagent blank.[146]

Calculation:

$$\%\text{w/w of Tannins} = \frac{\text{Abs. of sample (T-TB)}}{\text{Abs. of std.}} \times \frac{\text{Wt. of std (mg)}}{100} \times \frac{1}{100} \times \frac{\text{Vol. of std. taken for reaction (ml)}}{\text{Vol. of sample taken for reaction (ml)}} \times \frac{\text{Total vol. of sample (ml)}}{\text{Wt. of sample (mg)}} \times \%\text{ Purity of std}$$

T = Test solution absorbance, TB = Test blank, S = Standard absorbance

Estimation of Total Bitters By gravimetry

Weighed accurately about 5.0 g of the finely powdered sample (W) in a 250 ml flat bottomed flask. Added 100 ml of methanol and reflux the content on a water bath at 80^0C ± 2^0C for 30 minutes. Filtered the supernatant through Whatman No.1 filter paper in to 250 ml of beaker. Repeated the above process with each 50 ml methanol until methanolic extract is colorless. Filtered the extract in the same beaker. Concentrated the combined methanolic extract to a thick paste. Dissolve thick paste in a 50 ml hot water. Taken the aqueous extract into a separating funnel and fractionated with 100 ml ethyl acetate.

Separate the ethyl acetate extract and filtered through Whatmann No.1 filter paper in to pre-weighed china dish (W_1). Repeated the fractionation with each 50 ml ethyl acetate until ethyl acetate layer is colorless. Filter in to same china dish and evaporate to dryness. Kept dried china dish in oven at 105^0C for one hour or till constant weight was obtained. Cool in desiccator and weigh (W_2).

$$\% \text{ Bitters} = \frac{\text{Weight of the residue in g } (W_2 - W_1)}{\text{Weight of test substance in g (W)}} \times 100$$

W_1 - Weight of empty beaker or china dish (g)

W_2 -Wight of residue in beaker or china dish after drying (g)

W- Weight of test substance (g)

Thin layer chromatography

- Total extract and fractions of ***FICUS RACEMOSA*** **BARK** and the aerial part of ***CALTROPIS GIGANTEA*** were subjected to thin layer chromatographic studies to support preliminary chemical analysis. Each of the crude and its fractions were spotted on a percolated silica gel $60F_{254}$ plate (Merck).[147]
- The mobile phase employed was Chloroform: Methanol: Water (6:3.5:0.5, v/v/v), Chloroform: Methanol (95:05 v/v), Butanol: Acetic acid: water (4:1:1 v/v/v).
- Reagents such as Vanillin H_2SO_4, Dragendorff, and Ninhydrin were sprayed for the detection of Saponins, Alkaloids and Amino acids respectively.

Number of spots was noted and R_f values were calculated using the formula,

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

The results of thin layer chromatography were given in Fig. 7.7 and 7.8.

High Performance Thin Layer Chromatography

HPTLC method is used for the separation of compounds present in the mixture both qualitatively and quantitatively.[148] This chromatographic technique is still better to separate the various components of a mixture. The present study is to carry out HPTLC quantification of the methanolic and aqueous extracts of the bark of *Ficus racemosa* L and aerial parts of *Calotropis gigantea* L reported here under.

Materials and Methods of Methanolic extract and its fractions of *Ficus racemosa* L

Stationary Phase	:	Pre coated silica gel 60F 254
Mobile Phase	:	Chloroform:Methanol (90:10)
Developing Chamber	:	Twin trough Chamber 20x10
Sample	:	Methanolic extract and its fractions was dissolved in 1ml methanol.
Sample Applicator	:	CAMAG Linomat 5
Dosage Speed	:	150nl/s
Syringe Size	:	10 µl
Number of tracks	:	1
Scanner	:	CAMAG TLC Scanner 3
Wavelength	:	254nm

Materials and Methods of Aqueous extract and its fractions of *Ficus racemosa* L

Stationary Phase	:	Pre coated silica gel 60F 254
Mobile Phase	:	Chloroform:Methanol:water (6:3.5:0.5)
Developing Chamber	:	Twin trough Chamber 20x10
Sample	:	Aqueous extract and its fractions was dissolved in 1ml methanol.
Sample Applicator	:	CAMAG Linomat 5
Dosage Speed	:	150nl/s
Syringe Size	:	10 µl
Number of tracks	:	1
Scanner	:	CAMAG TLC Scanner 3
Wavelength	:	254nm

Materials and Methods of Methanolic extract and its fractions of *Calotropis gigantea*

Stationary Phase	:	Pre coated silica gel 60F 254
Mobile Phase	:	Chloroform:Methanol (95:5)
Developing Chamber	:	Twin trough Chamber 20x10
Sample in	:	Methanolic extract and its fractions was dissolved 1ml methanol.
Sample Applicator	:	CAMAG Linomat 5
Dosage Speed	:	150nl/s
Syringe Size	:	10 µl
Number of tracks	:	1
Scanner	:	CAMAG TLC Scanner 3
Wavelength	:	254nm

Materials and Methods of Aqueous extract and its fractions of *Calotropis gigantea* L

Stationary Phase	:	Pre coated silica gel 60F 254
Mobile Phase	:	Chloroform:Methanol:water (6:3.5:0.5)
Developing Chamber	:	Twin trough Chamber 20x10
Sample	:	Aqueous extract and its fractions was dissolved in 1ml methanol.
Sample Applicator	:	CAMAG Linomat 5
Dosage Speed	:	150nl/s
Syringe Size	:	10 µl
Number of tracks	:	1
Scanner	:	CAMAG TLC Scanner 3
Wavelength	:	254nm

TABLES

Table 7.1 Preliminary Phytochemical Screening of Total methanol extract and its Fractions of

FICUS RACEMOSA bark

Chemical Test	Methanol Extract	Fraction		
		FRFR-1	FRFR-2	FRFR-3
Alkaloids	+	+	-	-
Carbohydrates	-	-	-	-
Steroids	+	+	-	-
Saponins	+	-	+	-
Phenolic compound	+	-	+	+
Tannins	+	-	+	+
Proteins	-	-	-	-
Terpenoids	+	+	+	-
Flavonoids	+	-	+	-
Glycosides	+			-

+ = **Present;** - = **Absent**

Table 7.2 Preliminary Phytochemical Screening of Total methanol extract and its Fractions of aerial part of *CALTROPIS GIGANTEA*

Chemical Test	Methanol Extract	Fraction		
		CGFR-1	CGFR-2	CGFR-3
Alkaloids	+	+	-	-
Carbohydrates	+	-	-	-
Steroids	+	+	-	-
Tannins	+	-	+	+
Proteins	+	+	-	-
Saponins	+	+	-	-
Phenolic compound	+	+	-	-
Terpenoids	+	-	+	-
Flavonoids	+	+	-	-
Glycosides	+			-

+ = **Present;** - = **Absent**

Table 7.3 Quanitative Analysis of Phytochemical in *FICUS RACEMOSA* BARK and the aerial part of *CALOTROPIS GIGANTEA*

PHYTOCHEMICAL	**Assay %**	
	***F**ICUS RACEMOSA*	***C**ALOTROPIS GIGANTEA*
Alkaloid	3.31%w/w	1.5 w/w
Bitters	7.16%w/w	33.7 w/w
Flavonoids	0.64%w/w	0 .27 w/w
Tannins	43.49%w/w	1.37 w/w

RESULTS AND DISCUSSION:

The phytochemical investigation of the bark of *Ficus racemosa* L and aerial parts of *Calotropis gigantea* L was carried out with the standard protocol.

The methanolic and water extract of bark of *Ficus racemosa* L and aerial parts of *Calotropis gigantea* L was prepared by using maceration process. The percentage of yield *Ficus racemosa* L and aerial parts of *Calotropis gigantea* L of methanolic extract was found to be 10 %(w/w) &8 %(w/w) and water extract was found to be 13% (w/w) &10 %(w/w) respectively.

The water and methanolic crude extracts of *Ficus racemosa* L contains alkaloids, phenolic compounds, bitters, flavonoids, saponins, tannins and *Calotropis gigantea* L showed the presence of alkaloids, aminoacids, bitters, flavonoids, saponins, and tannins. Shown in Table 7.1 & 7.2. Further the methanolic and water extract of bark of *Ficus racemosa* L and aerial parts of *Calotropis gigantea* L was subjected to the fractionation. The fractionation was shown in the schematic diagram and fractions were labeled FRFR-1, FRFR-2, FRFR-3 and CGFR-1, CGFR-2, CGFR-3. All the fractions were subjected to the phytochemical studies and the results were shown in the tables 7.1 and 7.2.

The quantitative determination of phytoconstituents was carried out by using methanolic extract of plants. *Ficus racemosa* contains alkaloids 3.31%w/w, bitters 7.16%w/w, flavonoids 0.64%w/w and tannins 43.49% w/w. *Calotropis gigantea* L contains alkaloids 35%w/w, bitters 33.71%w/w, flavonoids 0.27%w/w and tannins 1.37%w/w. Shown in Table 7.3.

The separated fractions were analyzed by thin layer chromatography by using standard markers. The fractions gave good resemblance with the markers. The results were depicted in Fig. 7.7 and 7.8.

Further the extracts and fractions were subjected to the HPTLC finger printing analysis and the presences of phytoconstituents were strengthened. The crude methanolic extract and aqueous extract its fractions of both the plants were subjected for the above studies. In HPTLC fingerprinting the crude extracts and the fractions were shown the presence of various phytoconstituents with their respective Rf values, the results were shown in the fig. 7.9-7.16. HPTLC finger print profile helps for the identification of active biological compounds and further preliminary pharmacological studies and isolation and characterization of bioguided fraction to be carried out.

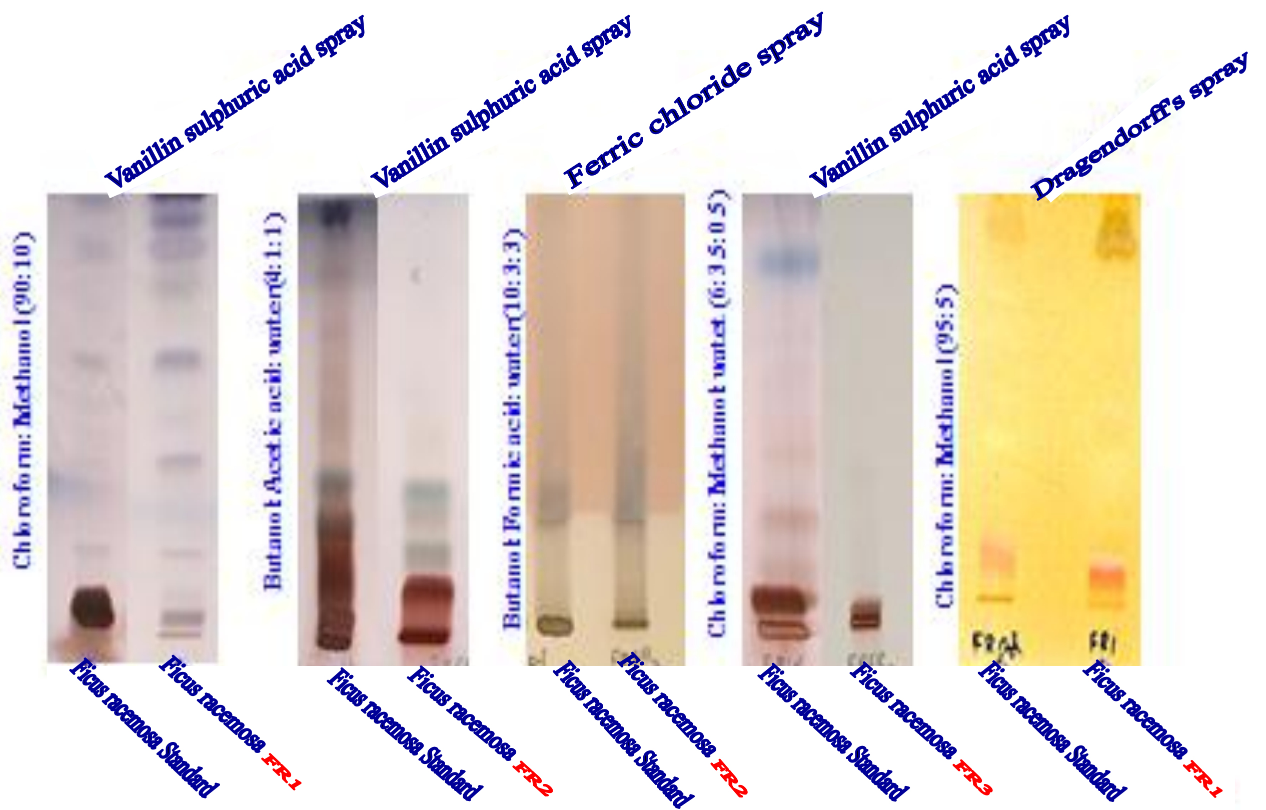


Fig. 7.7: TLC of ***FICUS RACEMOSA*** BARK **Extract/Fractions**

PLATE/**FRI** -INDICATES PRESENCE OF TERPENOIDS

PLATE/**FR2** -INDICATES PRESENCE OF SAPONINS

PLATE/**FR2**- INDICATES PRESENCE OF PHENOLIC COPMPOUND

PLATE/**FR3** -INDICATES PRESENCE OF TANNINS

PLATE/**FR1**-INDICATES PRESENCE OF ALKALOIDS

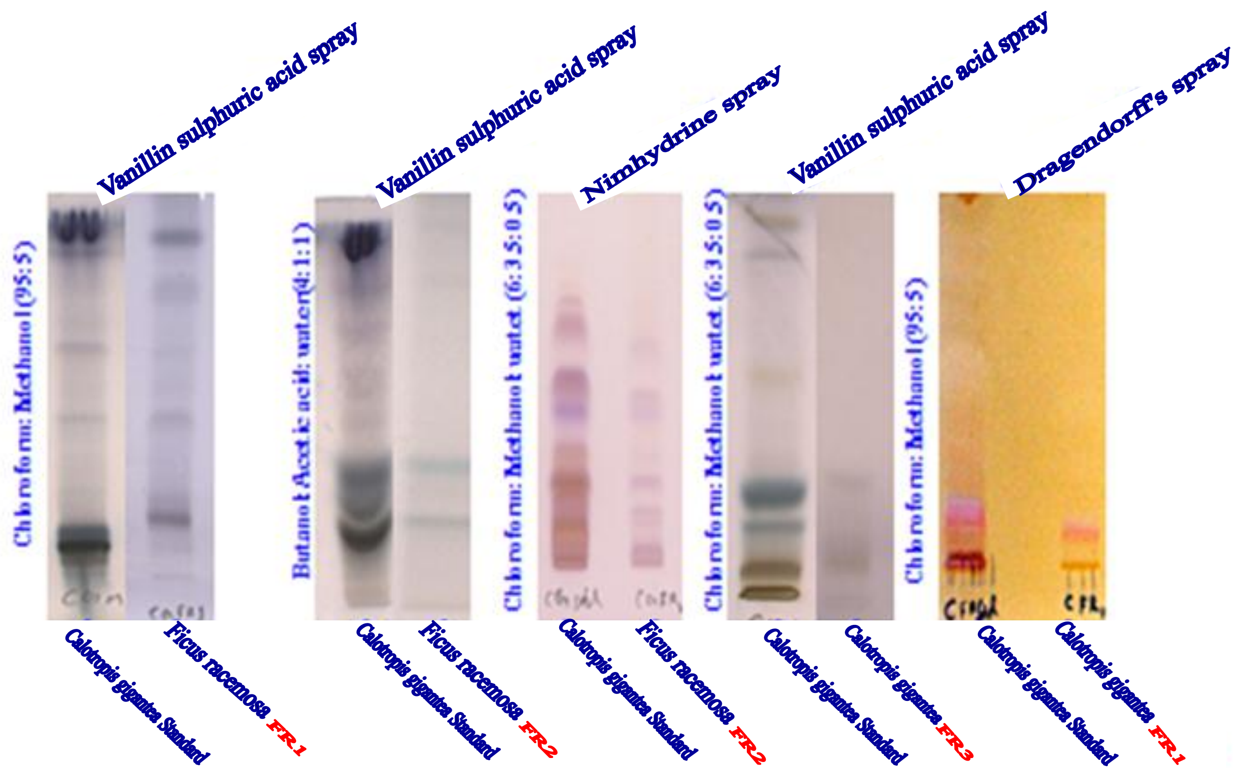


Fig. 7.8: TLC of *CALOTROPIS GIGANTEA* Extract/Fractions

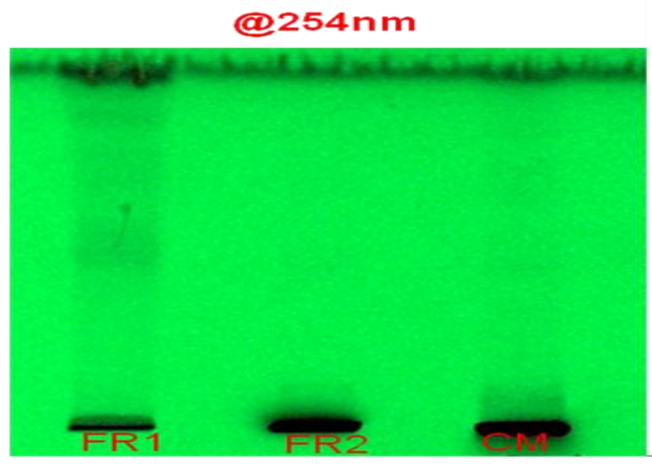
PLATE/**FRI** -INDICATES PRESENCE OF TERPENOIDS

PLATE/**FR2** -INDICATES PRESENCE OF SAPONINS

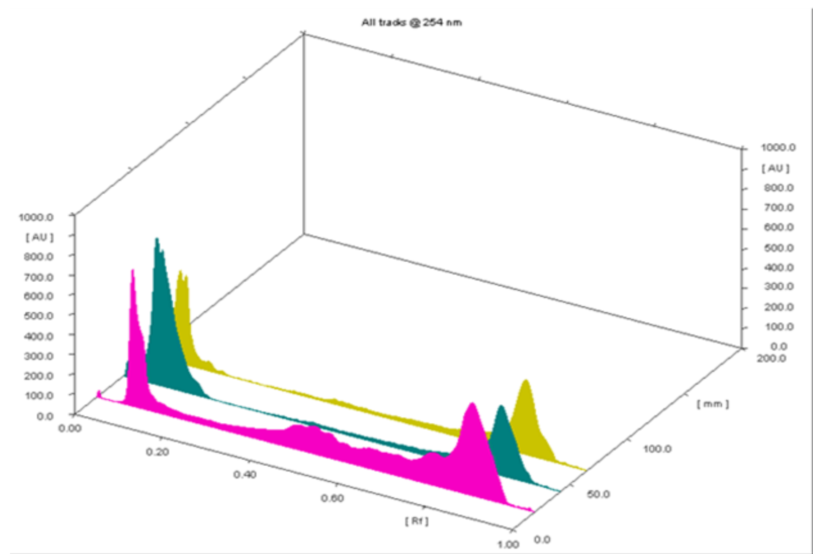
PLATE/**FR2**- INDICATES PRESENCE OF AMINO ACIDS

PLATE/**FR3** -INDICATES PRESENCE OF GLYCOSIDES

PLATE/**FR1**-INDICATES PRESENCE OF ALKALOIDS



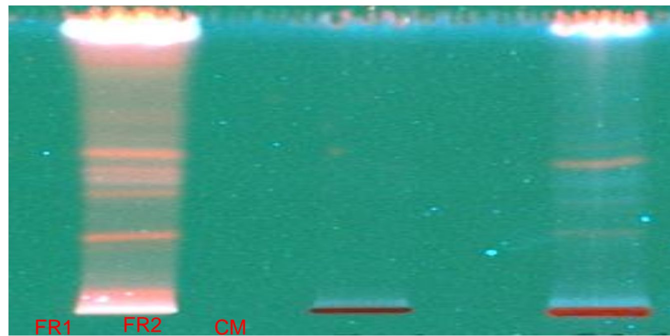
MOBILE PHASE :CHLOROFORM+METHANOL
90 + 10



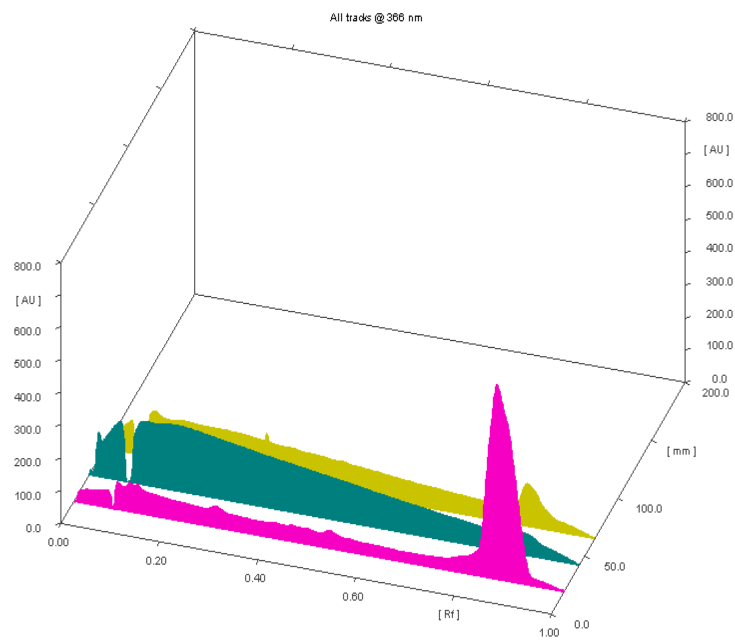
FR1=FRACTION-1
FR2=FRCATION-2
CM =CRUDE METHANOL EXTRACT

Fig 7.9 TLC of Ficus Racemosa bark Methanol Extract and its fractions

@366nm

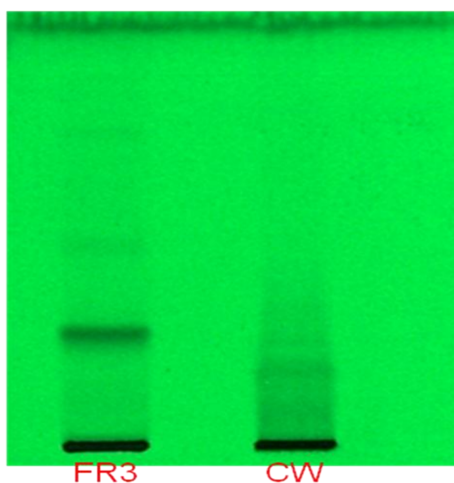


MOBILE PHASE :CHLOROFORM+METHANOL
90 + 10

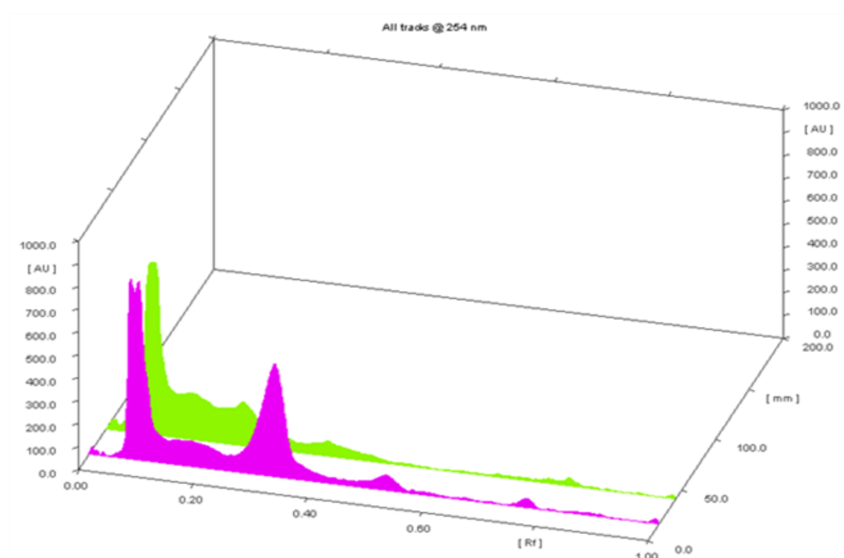


FR1=FRACTION-1
FR2=FRCATION-2
CM =CRUDE METHANOL EXTRACT

Fig 7.10 TLC of Ficus Racemosa bark Methanol Extract and its fractions

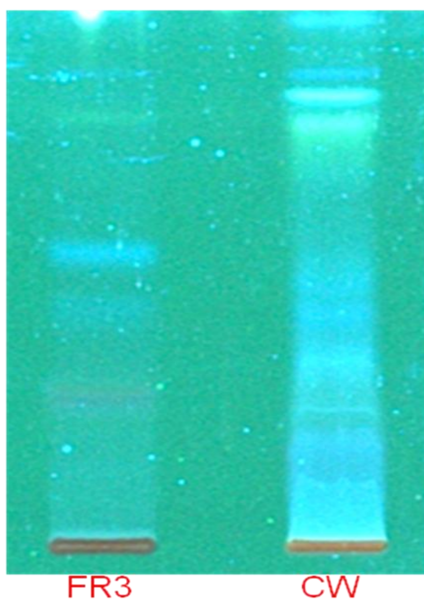


MOBILE PHASE :
CHLOROFORM+METHANOL+WATER
(6 +3.5+0.5ml)

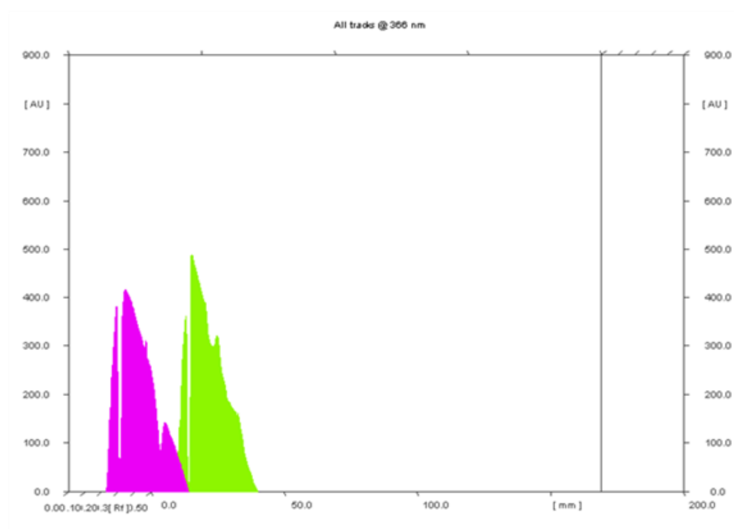


FR3 =FRACTION-3
CW = CRUDE WATER EXTRACT

Fig 7.11 TLC of Ficus Racemosa bark Water Extract and its fractions

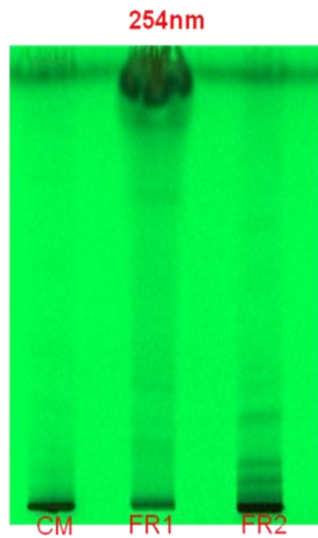


MOBILE PHASE :
CHLOROFORM+METHANOL+WATER
(6 +3.5+0.5ml)

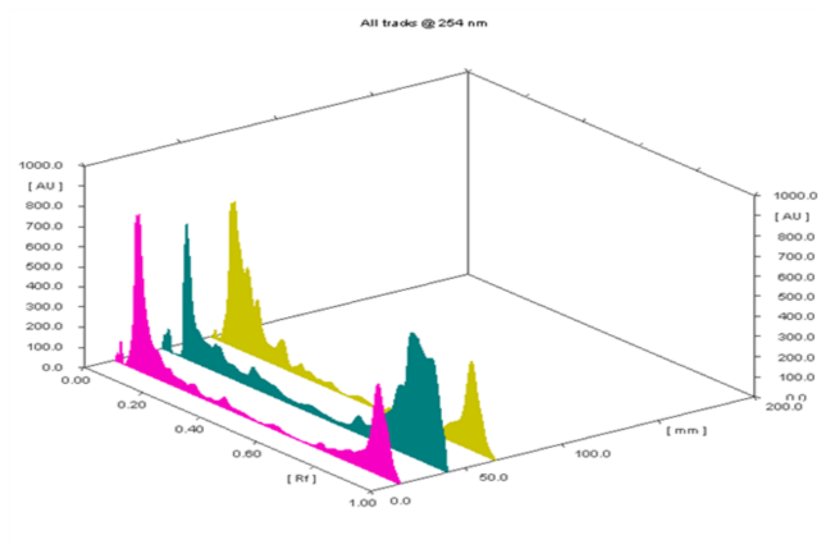


FR3 =FRACTION-3
CW = CRUDE WATER EXTRACT

Fig 7.12 TLC of Ficus Racemosa bark Water Extract and its fractions

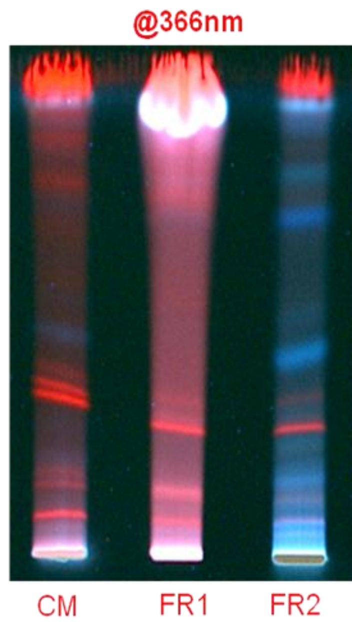


MOBILE PHASE :CHLOROFORM+METHANOL
(95 + 5ml)

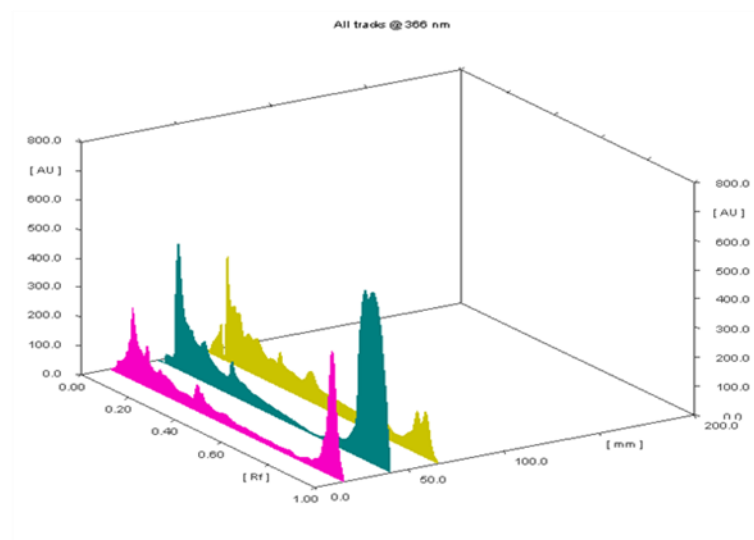


CM =CRUDE METHANOL EXTRACT
FR1=FRACTION-1
FR2=FRCATION-2

Fig 7.13 TLC of Caltropis Gigantea Methanol Extract and its fractions

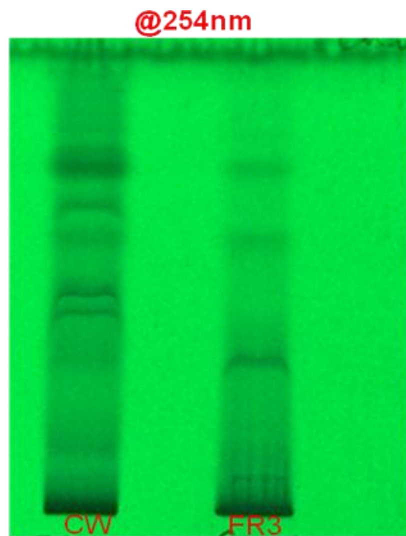


MOBILE PHASE :CHLOROFORM+METHANOL
(95 + 5ml)

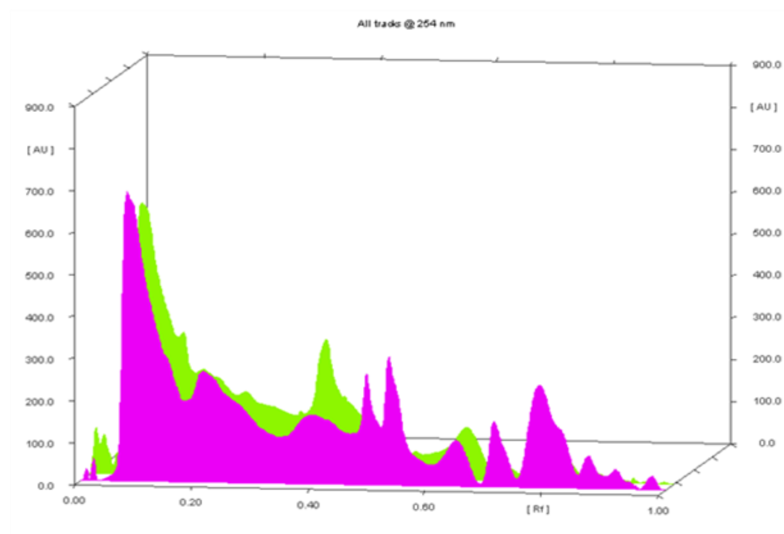


CM =CRUDE METHANOL EXTRACT
FR1=FRACTION-1
FR2=FRCATION-2

Fig 7.14 TLC of Caltropis Gigantea Methanol Extract and its fractions

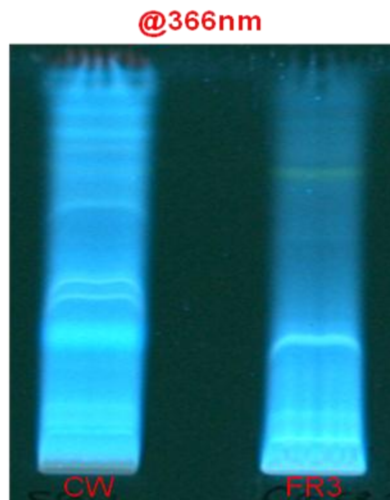


MOBILE PHASE :
CHLOROFORM+METHANOL+WATER
(6 +3.5+0.5ml)

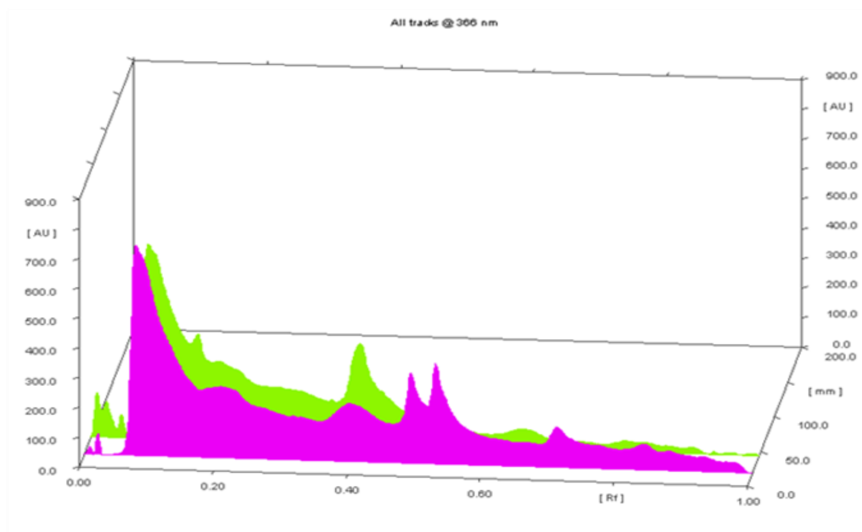


CW =CRUDE WATER EXTRACT
FR3=FRACTION-3

Fig 7.15 TLC of Caltropis Gigantea Water Extract and its fractions



MOBILE PHASE :
CHLOROFORM+METHANOL+WATER
(6 +3.5+0.5ml)



CW =CRUDE WATER EXTRACT
FR3=FRACTION-3

Fig 7.16 TLC of Caltropis Gigantea Water Extract and its fractions

8. PRELIMINARY PHARMACOLOGICAL STUDIES

Preliminary pharmacological studies were carried out for fractions CGFR1, CGFR2, CGFR3 and FRFR1, FRFR2 and FRFR3.

Experimental Animals

Wistar Rats, Male weighing approximately with similar body weights were selected and weight variation should not exceed ±20% of the mean body weight. Animals were obtained from Institutional Animal Breeding House, Vel's College of Pharmacy, Pallavaram, Chennai-117 were used for this work. Animals were housed in plastic cages at an ambient temperature (25±2°C). A 12:12 hr light- dark cycle was maintained during the experiments. They were fed with balanced rodent pellet diet from Poultry Research Station, Nandanam, Chennai-35 and water *ad libitum* throughout the experimental period. Animals were acclimatized to their environment for atleast one week before experimentation and divided into different groups. Each animal was housed separately after recording its body weight and had kept separate marks for identifying the dose level group and individual number. The animal studies were approved by Institutional Animal Ethics Committee (IAEC), Vels College of Pharmacy, Pallavaram, Chennai-117 vide approval number IX/290/CPCSEA/PHA-05-09 dated 16.10.2009.

Grouping:

Table 8.1 - Grouping of animals

Group	Dose (%)	No. of animals/ Group	Animal No
			Male
+ Control	1	4	1-4
- Control	1	4	5-8
CGFR 1	1	4	9-12
CGFR 2	1	4	13-16
CGFR 3	1	4	17-20
FRFR 1	1	4	21-24
FRFR 2	1	4	25-28
FRFR 3	1	4	29-32

Animal Preparation

Excision Wound Model:

Around 5 cm away from the ear and 1 cm away from the vertebral column, an impression was made in anaesthetized rats. The skin from the shaved and disinfected area was excised to obtain an area of about 500 square millimeters. 100mg of dose formulation was applied to each animal on to the wound. The test item was applied once daily for 20 consecutive days. Positive Control group animals were applied standard drug ointment framycetin sulphate cream (1% w/w) and negative control were not be applied any drug.[149,150]

Incision Wound Model:

On the depilated backs of the animals, two paravertebral incisions of 6 cm length were made cutting through the full thickness of the skin. The wound was closed by suturing considering the proper opposition of edges. The test item was applied once daily for 10 consecutive days. Positive Control group animals were applied standard drug ointment framycetin sulphate cream (1% w/w) and negative control were not be applied any drug.

Observations:

All animals were observed for clinical signs and mortality, body weight, percentage wound closure and breaking strength for excision and incision models.

$$\text{Percentage of Wound Closure} = \frac{(\text{Initial area of Wound} - N^{th}\text{ day area of wound})}{(\text{Initial area of Wound})} \times 100$$

The breaking strength of wound will be measured on 11th day by continuous, constant water flow technique as described by *Lee et al., 1969*.[151]

Statistical analysis:

The data was subjected to statistical analysis using GraphPad Prism version 5.00, GraphPad Software. The data on percentage of wound healing, tensile strength and body weight was analyzed statistically. One way ANOVA with Dunnets post test was done for different treatment groups comparing with the positive control and negative control group data separately. All the analyses and comparisons were evaluated at the 95% level of confidence (P<0.05). *: Significant change than the positive control/negative control group (P<0.05).

RESULTS AND DISCUSSION

Clinical Signs and Mortality

All animals were observed once daily for clinical signs and twice daily for mortality and morbidity. There were no treatment related clinical signs observed at any of the test item tested on both the models.

Body Weights:

There were no significant changes observed at any of the test item in both the models when compared to both Positive control and negative control.

Percentage of wound healing in excision wound model:

Table 8.2: Wound healing in excision wound model

Period (Days	Test Article	Healing tendenc
0-4	CGFR 2, CGFR2, FRFR1, FRFR2	↑
4-8	CGFR 2	↑
4-8	CGFR 1	↓
8-12	CGFR 3	↑
8-12	FRFR 1,	↓
12-16	FRFR 2	↑
12-16	CGFR 1, CGFR 2,	↓
16-20	CGFR 1	↓

However, the healing tendency of herbal extracts of all three are comparable with the positive control group as all the compounds irrespective of the nature of extract have shown a significantly higher healing tendency when compared to the negative control (wound without application).

All the compounds have shown an increasingly higher healing tendency when compared with the negative control group.

Negative control group did not shown any infectious condition of the wound throughout the study period.

Breaking strength of wound in incision wound model:

The below indicated test articles have shown a significantly higher breaking strength when compared to the positive control.

Table 8.3 - Breaking strength

Test Article	Breaking strengt
CGFR3	↑
FRFR2, FRFR3	↓

The CGFR3 was showing a significantly better tensile strength of wound when challenged with the positive control groups.

Negative control group did not show any of the post operative complications throughout the study period.

The results for all parameters were given in tables from 8.4 to 8.15.

From the preliminary pharmacological studies it was evident that from the 6 fractions, the fractions FRfr3 and CGfr3 possess predominant wound healing activity. These two fractions were considered for further formulation studies. All the data's of 6 fractions were recorded and summarized.

Table 8.4 - PERCENT HEALING – EXCISION MODEL

Days → / TEST ITEM ↓	0 to 4	4 to 8	8 to 12	12 to 16	16 to 20
PC	18.1	58.3	80.6	97.3	99.7
	1.4	0.7	0.5	0.4	0.6
CGFR 1	19.7	40.9*	78.6	85.7*	97.5*
	3.1	0.5	1.0	3.6	0.3
CGFR 2	46.2*	76.6*	79.6	92.3*	99.5
	4.3	0.7	2.1	1.0	0.6
CGFR 3	34.9*	74.5	86.6*	97.2	99.9
	3.8	1.3	0.9	1.1	0.3
FRFR 1	37.6*	45.4	78.0*	96.5	99.3
	1.2	1.5	1.2	0.5	0.5
FRFR 2	26.9*	56.5	78.4	92.5*	99.1
	3.3	0.7	1.3	1.1	0.6
FRFR 3	21.2	50.2	78.3	95.8	99.8
	2.6	0.9	1.2	1.0	0.5

Values are Mean±SD
*: Significant change than the positive control group ($P<0.05$)

Table 8.5 - PERCENT HEALING – EXCISION MODEL

Days →	0 to 4	4 to 8	8 to 12	12 to 16	16 to 20
TEST ITEM ↓					
NC	1.7	25.8	49.8	59.9	79.1
	1.4	4.2	1.0	0.6	0.5
CGFR 1	19.7*	40.9*	78.6*	85.7*	97.5*
	3.1	0.5	1.0	3.6	0.3
CGFR 2	46.2*	76.6*	79.6*	92.3*	99.5*
	4.3	0.7	2.1	1.0	0.6
CGFR 3	34.9*	74.5*	86.6*	97.2*	99.9*
	3.8	1.3	0.9	1.1	0.3
FRFR 1	37.6*	58.2*	74.1*	96.5*	99.3*
	1.2	1.3	1.6	0.5	0.5
FRFR 2	26.9*	56.5*	78.4*	92.5*	99.1*
	3.3	0.7	1.3	1.1	0.6
FRFR 3	21.2*	50.2*	78.3*	95.8*	99.8*
	2.6	0.9	1.2	1.0	0.5

Values are Mean±SD
*: Significant change than the negative control group ($P<0.05$)

Table 8.6 - TENSILE STRENGTH – INCISION MODEL

TEST ITEM	Day 11
PC	453.0
	13.3
CGFR 1	431.0
	50.5
CGFR 2	402.6
	51.3
CGFR 3	528.9*
	43.6
FRFR 1	396.2
	36.0
FRFR 2	318.9*
	7.4
FRFR 3	379.6*
	11.5

Values are Mean±SD
*: Significant change than the positive control group ($P<0.05$)

Table 8.7 - TENSILE STRENGTH – INCISION MODEL

TEST ITEM	Day 11
NC	363.0
	30.8
CGFR 1	431.0*
	50.5
CGFR 2	402.6
	51.3
CGFR 3	410.3*
	71.4
FRFR 1	396.2
	36.0
FRFR 2	318.9
	7.4
FRFR 3	359.6
	21.2

Values are Mean ± SD
*: Significant change than the negative control group (P<0.05)

Table 8.8 - BODY WEIGHTS- EXCISION MODEL

Treatment	Days					
	0	4	8	12	16	20
PC	227.2	230.7	235.5	240.3	218.5	250.3
	17.3	17.3	16.2	17.6	59.0	17.0
CGFR 1	237.5	240.0	246.6	250.4	255.0	257.6
	9.8	9.7	7.4	8.9	10.9	11.7
CGFR 2	228.1	231.9	237.6	244.4	249.4	257.8
	10.8	10.0	11.7	13.4	15.7	16.9
CGFR 3	217.2	222.3	229.4	235.3	241.4	249.7
	8.8	6.0	8.5	8.6	11.6	13.2
FRFR 1	220.1	224.4	231.0	236.0	240.7	244.0
	13.8	11.4	10.6	12.0	12.9	11.1
FRFR 2	221.8	225.3	231.2	238.3	244.9	243.8
	10.0	9.2	9.2	9.3	8.9	19.4
FRFR 3	231.0	236.2	243.8	249.1	251.7	257.9
	13.7	13.3	15.2	15.4	14.7	15.3

Values are Mean ± SD

Table 8.9 - BODY WEIGHTS- EXCISION MODEL

Treatment	Days					
	0	**4**	**8**	**12**	**16**	**20**
NC	215.8	220.1	224.6	229.9	234.2	237.5
	10.5	10.1	11.9	9.9	6.7	5.7
CGFR 1	237.5	240.0	246.6	250.4	255.0	257.6
	9.8	9.7	7.4	8.9	10.9	11.7
CGFR 2	228.1	231.9	237.6	244.4	249.4	257.8
	10.8	10.0	11.7	13.4	15.7	16.9
CGFR 3	217.2	222.3	229.4	235.3	241.4	249.7
	8.8	6.0	8.5	8.6	11.6	13.2
FRFR 1	220.1	224.4	231.0	236.0	240.7	244.0
	13.8	11.4	10.6	12.0	12.9	11.1
FRFR 2	221.8	225.3	231.2	238.3	244.9	243.8
	10.0	9.2	9.2	9.3	8.9	19.4
FRFR 3	231.0	236.2	243.8	249.1	251.7	257.9
	13.7	13.3	15.2	15.4	14.7	15.3

Values are Mean ± SD

Table 8.10 - BODY WEIGHTS - INCISION MODEL

Treatment	Days			
	0	**4**	**8**	**10**
PC	232.2	233.6	235.3	236.9
	19.4	19.0	19.2	19.4
CGFR 1	247.2	248.7	250.0	251.3
	12.2	12.2	12.3	12.5
CGFR 2	229.9	231.2	232.7	233.8
	9.1	9.5	9.4	9.4
CGFR 3	220.0	221.3	222.8	224.1
	7.2	7.0	7.0	7.1
FRFR 1	221.7	223.3	224.8	226.0
	11.9	11.9	11.9	11.8
FRFR 2	227.6	229.3	230.9	232.0
	9.9	10.1	10.1	10.2
FRFR 3	231.0	232.2	233.7	234.8
	9.3	9.2	9.2	9.1

Values are Mean±SD

Table 8.11 - BODY WEIGHTS - INCISION MODEL

Treatment	Days			
	0	**4**	**8**	**10**
NC	223.3	224.8	226.0	227.4
	6.3	6.2	6.6	6.5
CGFR 1	247.2	248.7	250.0	251.3
	12.2	12.2	12.3	12.5
CGFR 2	229.9	231.2	232.7	233.8
	9.1	9.5	9.4	9.4
CGFR 3	220.0	221.3	222.8	224.1
	7.2	7.0	7.0	7.1
FRFR 1	221.7	223.3	224.8	226.0
	11.9	11.9	11.9	11.8
FRFR 2	227.6	229.3	230.9	232.0
	9.9	10.1	10.1	10.2
FRFR 3	231.0	232.2	233.7	234.8
	9.3	9.2	9.2	9.1

Values are Mean±SD

Table 8.12 - AREA OF HEALING – EXCISION MODEL

Treatment	Days					
	0	4	8	12	16	20
PC	501	401	206	95	12	0
	496	412	204	98	16	0
	499	413	212	99	12	6
	498	408	209	95	14	0
NC	502	486	386	251	201	106
	503	500	387	255	203	108
	500	498	374	256	204	104
	509	496	346	249	200	103
CGFR 1	499	386	294	102	84	12
	503	426	296	105	88	13
	497	394	293	106	49	14
	496	396	297	113	64	11
CGFR 2	506	240	113	89	33	0
	499	275	117	102	45	4
	509	285	121	114	38	0
	498	283	119	106	39	6
CGFR 3	509	307	140	62	13	0
	500	343	124	71	22	0
	508	324	126	68	10	3
	509	344	127	70	12	0
FRFR 1	497	301	276	103	15	0
	499	315	274	106	16	3
	499	314	277	115	18	5
	495	311	259	114	20	5
FRFR 2	496	361	216	101	42	6
	501	366	218	105	40	5
	496	383	211	116	38	6
	494	342	219	108	29	0
FRFR 3	502	384	251	102	26	5
	503	386	256	106	24	0
	504	412	249	112	20	0
	499	400	244	115	15	0

Table 8.13 - TENSILE STRENGTH – INCISION MODEL

Test item	Weight in grams
PC	435.7
	465.8
	460.5
	450.1
NC	339.0
	334.8
	381.2
	396.9
CGFR 1	506.6
	405.6
	402.2
	409.6
CGFR 2	474.3
	352.9
	396.0
	387.1
CGFR 3	594.0
	501.6
	508.3
	511.6
FRFR 1	342.3
	412.2
	417.6
	412.5
FRFR 2	312.6
	329.5
	315.6
	317.9
FRFR 3	389.1
	390.0
	370.5
	368.9

Table 8.14 - BODY WEIGHTS (g) - EXCISION MODEL

Test item	Days					
	0	4	8	12	16	20
PC	252.32	256.32	259.51	265.34	269.24	273.99
	219.29	222.29	228.31	225.34	229.14	236.60
	213.35	218.36	224.35	231.24	133.67	239.39
	223.64	225.97	230.01	239.19	241.87	251.40
NC	204.46	207.46	211.63	218.97	228.34	234.19
	219.93	222.93	226.59	231.99	236.21	241.39
	228.39	231.66	239.87	242.37	242.87	243.11
	210.39	218.34	220.11	226.37	229.39	231.27
CGFR 1	238.80	240.80	248.14	253.44	258.64	261.69
	246.30	249.30	253.66	259.66	266.37	268.36
	223.57	226.47	236.27	238.39	240.48	241.07
	241.27	243.28	248.39	250.30	254.36	259.37
CGFR 2	236.07	237.86	244.63	251.37	260.71	270.94
	238.54	242.54	249.93	258.97	264.33	273.57
	217.39	220.97	224.68	228.91	231.27	240.39
	220.24	226.38	231.24	238.24	241.39	246.28
CGFR 3	222.16	226.14	234.65	240.69	248.99	257.48
	224.47	228.47	238.56	244.47	253.67	264.29
	204.68	215.39	221.67	226.57	230.47	237.17
	217.59	219.37	222.57	229.54	232.58	240.01
FRFR 1	216.95	220.95	231.11	238.55	241.34	243.21
	240.08	241.08	244.65	251.37	258.64	259.92
	215.15	220.34	229.37	230.73	232.57	236.48
	208.39	215.27	218.93	223.27	230.24	236.24
FRFR 2	225.97	228.97	231.95	240.24	246.69	250.22
	232.62	235.62	242.59	250.21	256.67	264.48
	219.27	222.35	230.00	234.48	240.11	218.23
	209.27	214.19	220.27	228.27	236.27	242.33
FRFR 3	244.87	250.87	262.65	268.47	271.22	278.11
	240.80	243.84	248.95	254.22	254.93	261.19
	220.08	226.37	235.39	239.37	240.28	246.28
	218.39	223.59	228.39	234.27	240.27	245.89

Table 8.15 - BODY WEIGHTS (g) - INCISION MODEL

Test Item	Days			
	0	4	8	10
PC	231.31	238.48	249.22	265.87
	248.32	260.51	270.67	227.85
	206.15	221.63	229.37	228.94
	232.48	241.98	250.25	224.97
NC	260.29	269.86	274.69	219.78
	222.71	225.41	238.37	224.85
	240.57	249.55	260.66	234.86
	226.38	236.67	243.38	230.21
CGFR 1	224.18	226.13	234.17	267.94
	216.85	231.91	239.83	252.94
	243.28	250.30	259.37	238.79
	242.54	258.97	273.57	245.38
CGFR 2	232.36	241.31	251.77	233.89
	209.99	217.62	227.19	246.45
	249.93	258.69	269.11	223.82
	232.48	241.98	250.25	230.97
CGFR 3	223.11	225.16	231.08	228.24
	220.52	225.92	236.88	230.48
	220.34	232.57	236.48	214.38
	228.97	246.69	250.22	223.47
FRFR 1	229.07	234.98	241.17	224.86
	243.85	243.80	249.97	242.54
	240.80	253.44	261.69	221.54
	243.28	250.30	259.37	214.86
FRFR 2	217.32	216.78	224.47	234.84
	215.04	217.91	223.89	244.68
	220.08	235.39	240.28	227.84
	231.74	241.15	254.22	220.79
FRFR 3	216.53	218.85	224.40	241.90
	237.38	249.05	251.17	242.94
	224.47	244.47	264.29	229.74
	240.08	251.37	259.92	224.47

ANTI-INFLAMMATORY ACTIVITY:

Carageenan induced acute paw edema model

Wistar rats weighing between 200-300 gms were divided into 6 groups each consisting of 4 animals. The treatment schedules of animals belonging to different groups were shown below.

Group I : Served as control and were administered with water 15ml/kg

Group II : Were administered with CGFR-3 500 mg/kg

Group III : Were administered with CGW 500 mg/kg

Group IV : Were administered with FRFR-3 500 mg/kg (suspension)

Group V : Were administered with FRW 500 mg/kg

Group VI : Were administered with Diclofenac 10 mg/kg

All the animals were administered with the respective treatment for 3 consecutive days. Animals were kept for fasting and deprived of water overnight before starting of the test (Day-2). On Day-3 animal's right hind leg was marked with permanent (OHP) marker above the ankle joint following natural hair lining. The initial Paw volume was measured by dipping the paw in mercury plethysmometer till the mark. After recording initial paw volume animals were administered with respective drug administration in the dose of 15ml/kg body weight. The oral drug administration were followed by 0.1 ml injection of 0.1% carrageenaan in saline in to the subplanter region of right hind paw. The paw volume was recorded at the interval of 1.5 Hr, 3.0 Hr and 5.0 Hr after the injection of carrageenan same like initial. The edema was calculated by subtracting the initial paw volume of respective animal.[152]

The results were represented in **Fig. 8.1**

Statistical analysis:

The values are expressed as Mean ± SEM. The results were analyzed statistically using one-way ANOVA followed by post Dunnet's multiple comparison tests using Prism software package to find out the level of significance. The minimum level of significance was fixed at $p<0.05$.

Results and discussion:

In the experimental model of carrageenan-induced paw inflammation, all the extracts exhibited some degree of anti-inflammatory activity at 1.5 hours but to varying extent. From the results obtained, it can be concluded that, the tested extracts exhibited time dependent anti-inflammatory activity to varying extent.

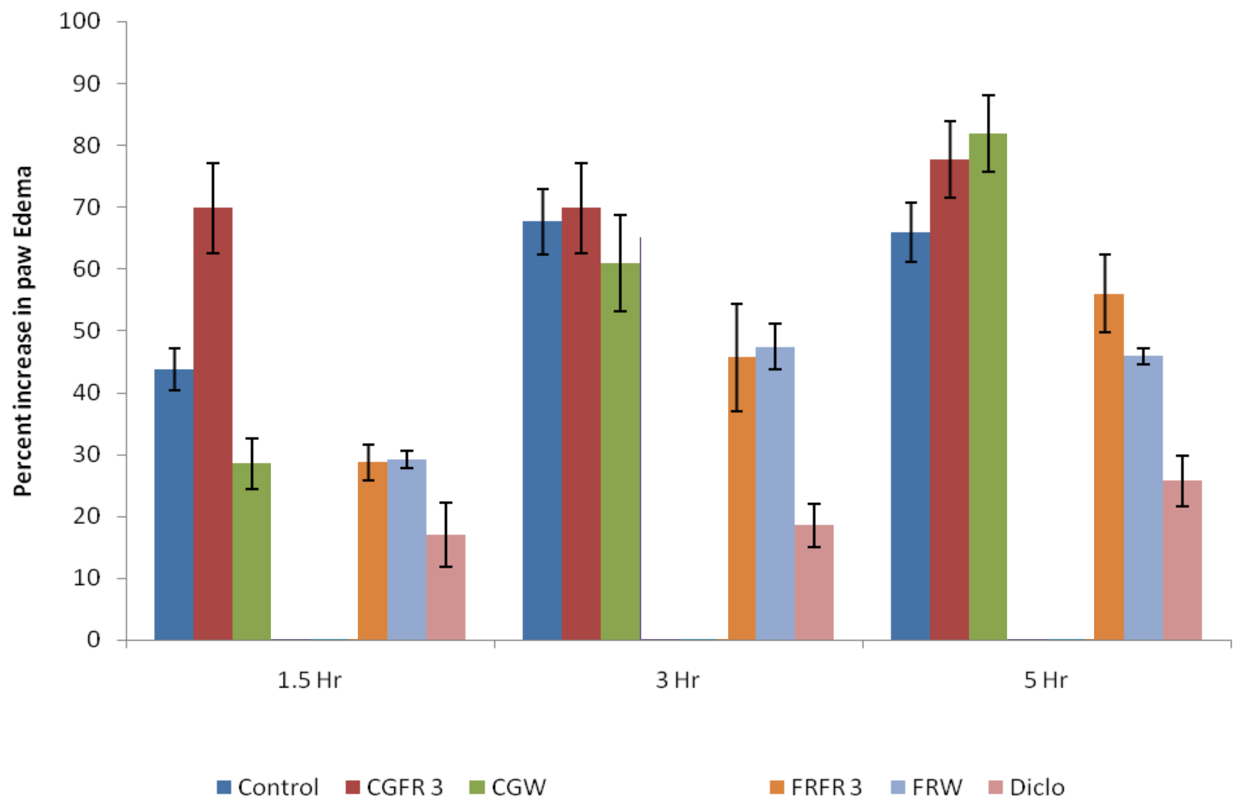


Fig. 8.1. Anti inflammatory activity of fractions in Carrageenan induced paw odema model

9. STRUCTURAL ELUCIDATION OF ISOLATED COMPOUNDS

From the plants the compounds FRfr3 and CGfr3 were isolated from *Ficus racemosa* and *Calotropis gigantea* respectively and the isolated compounds were subjected to HPTLC analysis with their respective fractions (FRFR3 & CGFR3), further structure was elucidated by using various IR, mass and NMR spectral studies.

Materials and methods:

FTIR for CGFR3 and FRFR3:

Potassium bromide (KBr) powder, spectroscopic grade, used for IR analysis was supplied by Anadis Instruments B.V. (Malden, The Netherlands). The sample was analysed by FTIR[153] and the graph were recorded for CGFR3 and FRFR3 in Fig. 9.1 and 9.2.

Mass spectrometer:

The API 2000 (Applied biosystem/MDS SCIEX, Canada) mass spectrometer coupled with ESI (Electrospray ionization) source and a chromatographic system. Batch acquisition and data processing was controlled by Analyst 1.5 version soft ware.

The MS parameters optimization was carried out with the concentration of 1mg/ml of analytes (Methanolic and aqueous extracts) were prepared in Methanol (LCMS grade) and water (LCMS grade) respectively, then filter through 0.2µm syringe filter (Advanced microdevices pvt.Ltd.) and final test solution was infused by syringe pump at a flow rate of 20µl/min continuously into the mass spectrometer. Intensity of ionization response was checked in both positive and negative ionization mode. It was found good intense response in the positive mode and other parameters like declustering potential (DP), Ion source gas (GS1) and (GS2), curtain gas (CUR), focusing potential (FP) and source temperature (TEM) were optimized with respect to ionization intensity

response.[154] (Kindly refer the table-1 below for the optimization values) Acquisition was performed by setting the mass of the analytes with appropriate scan range between 100 – 1000 m/z.

Table 9.1 Conditions for mass spectrometer

Parameter	**Optimization values**
Ion Source	ESI (Turbo spray)
Declustering potential (DP)	20V - 120 V ramping
Focusing potential (FP)	400 V
Entrance potential (EP)	10 V
Curtain gas (CUR)	25 psi
Ion spray voltage (IS)	5500 V
Temperature (TEM)	O ˙C
Source gas (GS1)	30 psi
Source gas (GS1)	40 psi

The spectral data for the fractions were depicted in Fig. 9.3 and 9.4.

NMR

Solvents and chemicals for NMR analysis - acetone, acetone-*d*6, acetonitrile (ACN), chloroform (CHCl3), deuterated chloroform (CDCl3), deuterium oxide (D2O), dimethylsulfoxide(DMSO), MeOH, hydrochloric acid (HCl), C18 material for reversed-phase liquid chromatography, sodium hydroxide (NaOH), sodium sulphate (Na2SO4), trimethylsilyl-*d4*-propanoic acid, sodium salt (TMSP) - were obtained from commercial sources in the best possible quality. The samples were analysed by NMR.[155] The results were given in fig.9.5 and 9.6.

RESULTS AND DISCUSSION:

Compound CGFR3

Table 9.2 Groups present in CGFR3

IR	MASS	NMR	CGFR3-
NH group around 3400 3500 OH- group around 2800 C=N group around 1500 benzene- 800-900	Base peak-301.21 molecular ion peak 701.63 (molwt) base peak-575.34 molecular ion peak 735.62	Cgfr3- 5,3.5-3.8 Ppm values 5 singlet, 3.8 doublet, 3.7doublet multiplet3.6 to 3.7, 3 singlets 3.5 to 3.7	base peak 351.35 molecular ioı peak-717.62

Compound FRFR3

Table 9.3 Groups present in FRFR3

IR	MASS	NMR
- NH group around 3400-350(-C=N group around 1500 benzene- 800-900	base peak-701.52 molecular ion peak-975.76 (molwt base peak-381.13 molecular ion peak -976.73.62 base peak-717.44 molecular ion peak-719.63	Ppm values 5 - singlet, Multiplet 3.5 to 4.: 3.3singlet, 2.1 singlet

The compounds FRfr3 and CGfr3 were isolated from *Ficus racemosa* and *Calotropis gigantea* respectively and the structure was elucidated by IR, LC-MS and NMR spectrum. **Fig. 9.1 to 9.6**.

The possible structure of CGFR3 and FRFR3 were given in fig 9.7 and 9.8 respectively.

IR SPECTRA

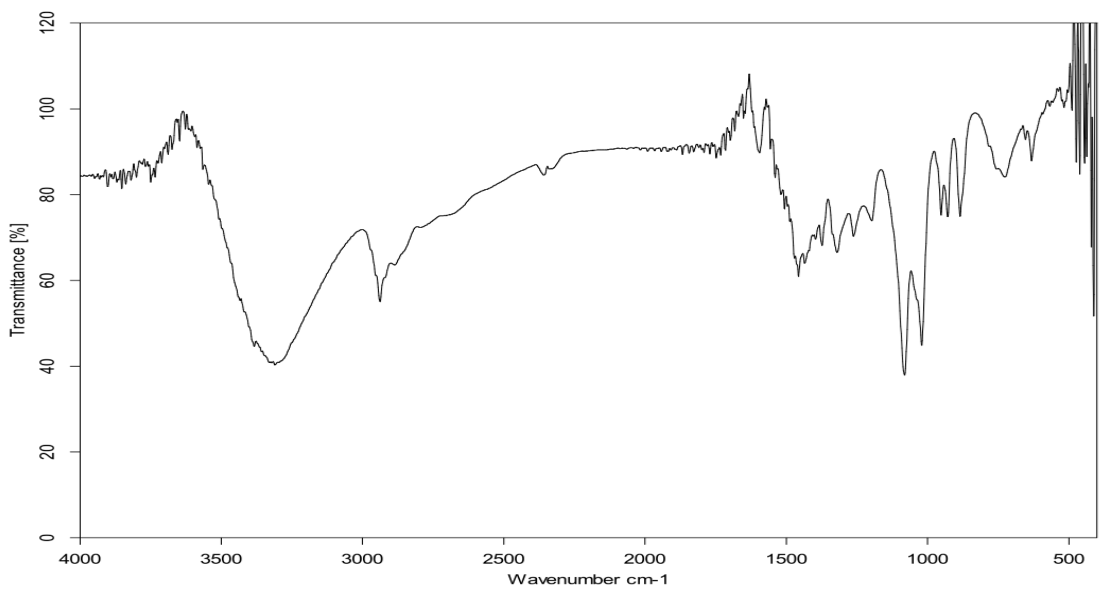


Fig 9.1 CGFR3 – IR spectra

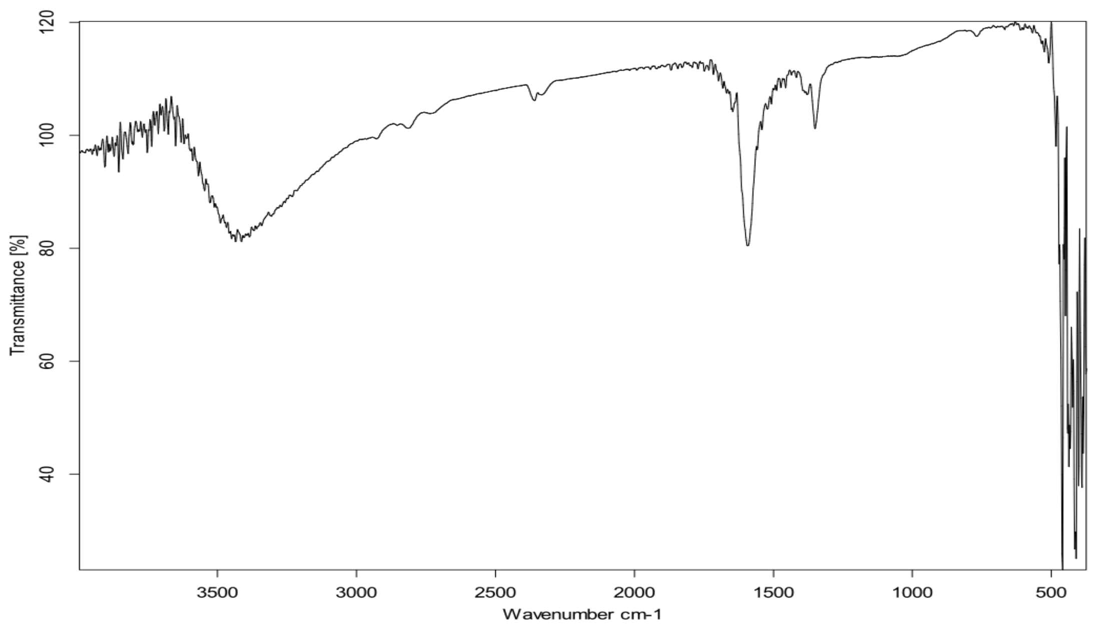
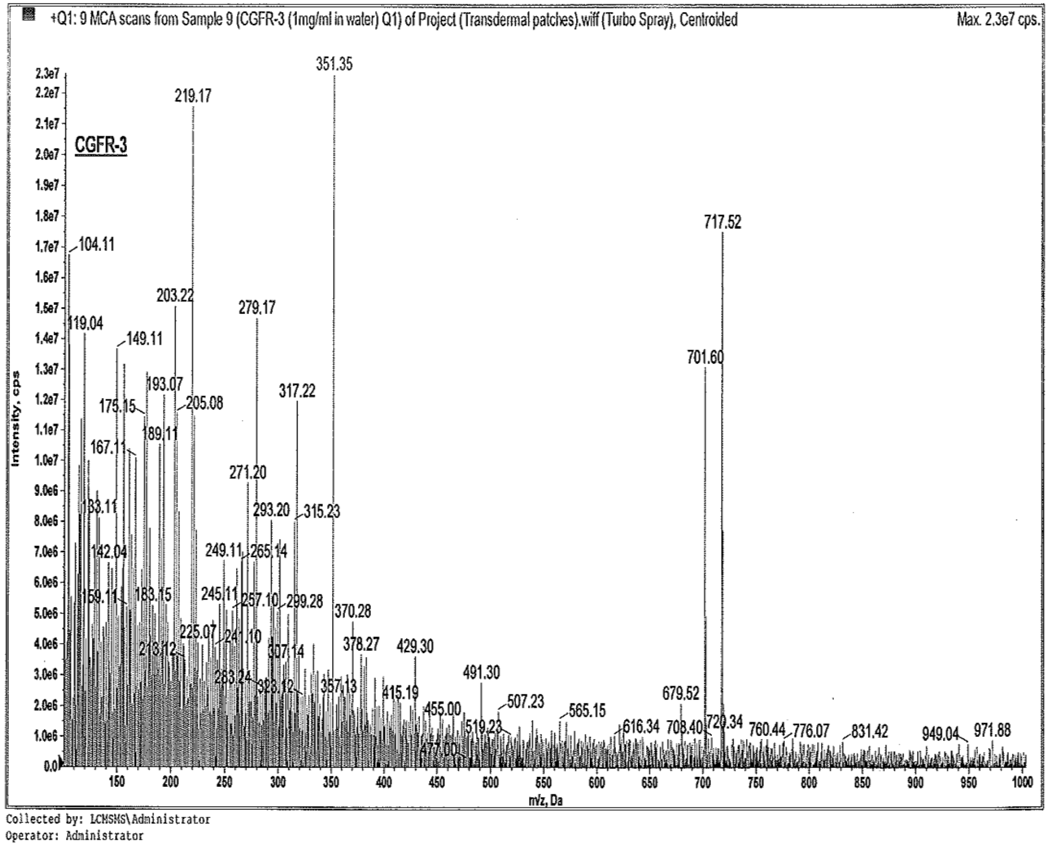


Fig. 9.2. FRFR3- IR spectra

Printing Time: 11:10:23 AM
Printing Date: Tuesday, September 04, 2012

Agrotech Research and Phytochemistry
The Himalaya Drug Company

Analyst Version: 1.5
Workstation: LCMSMS



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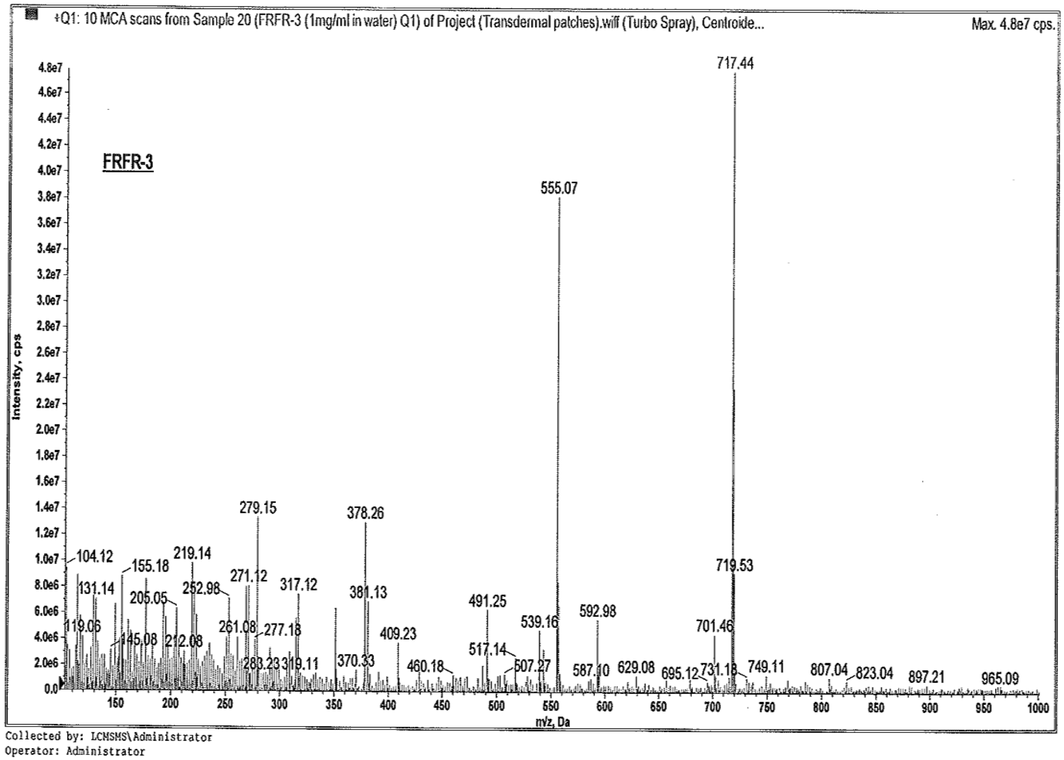
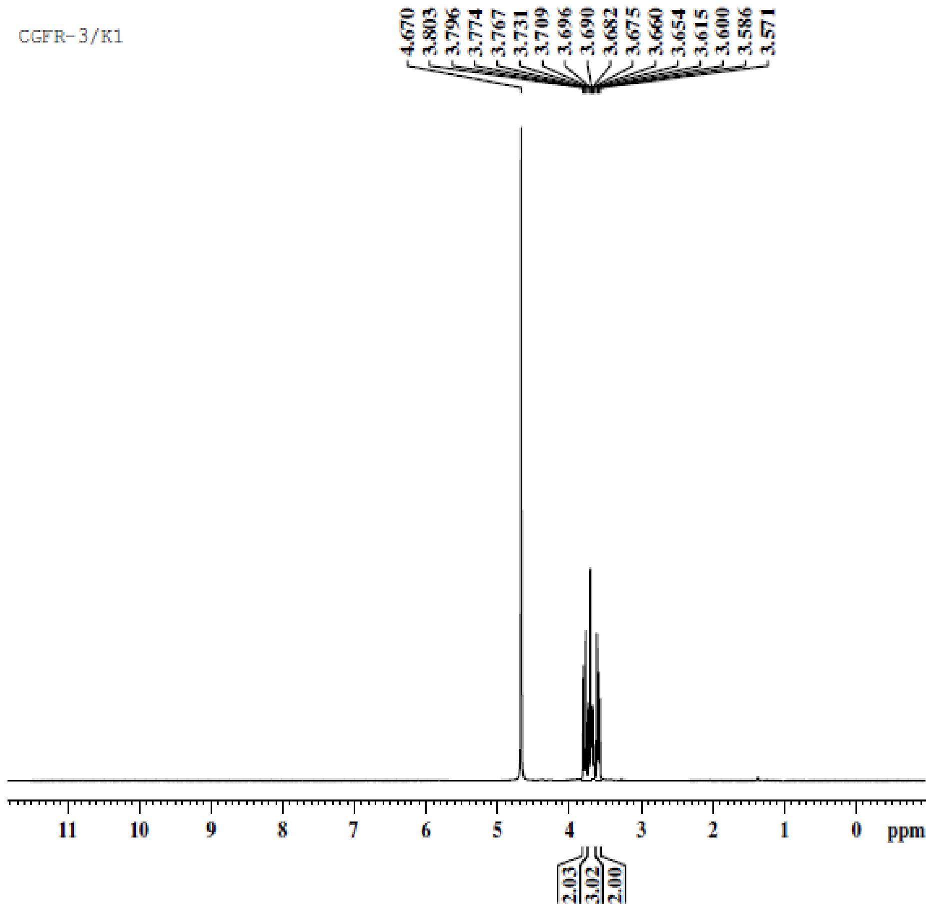
Operator: Administrator

Fig.9.3 LCMS/MS PATTERN OF CGFR-3



Collected by: LCMSMS\Administrator
Operator: Administrator

Fig. 9.4. LCMS/MS PATTERN OF FRFR-3



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EXPNO	1
PROCNO	1
Date_	20120723
Time	12.24
INSTRUM	spect
PROBHD	5 mm PABBO BB-
PULPROG	zg
TD	15580
SOLVENT	D2O
NS	16
DS	2
SWH	5193.906 Hz
FIDRES	0.333370 Hz
AQ	1.4998847 sec
RG	144
DW	96.267 usec
DE	6.50 usec
TE	298.0 K
D1	1.00000000 sec
TD0	1

======= CHANNEL f1 =======

NUC1	1H
P1	14.10 usec
PL1	-3.00 dB
PL1W	13.42244530 W
SFO1	400.2321985 MHz
SI	65536
SF	400.2300146 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

Fig. 9.5 NMR SPECTRUM OF CGFR-3

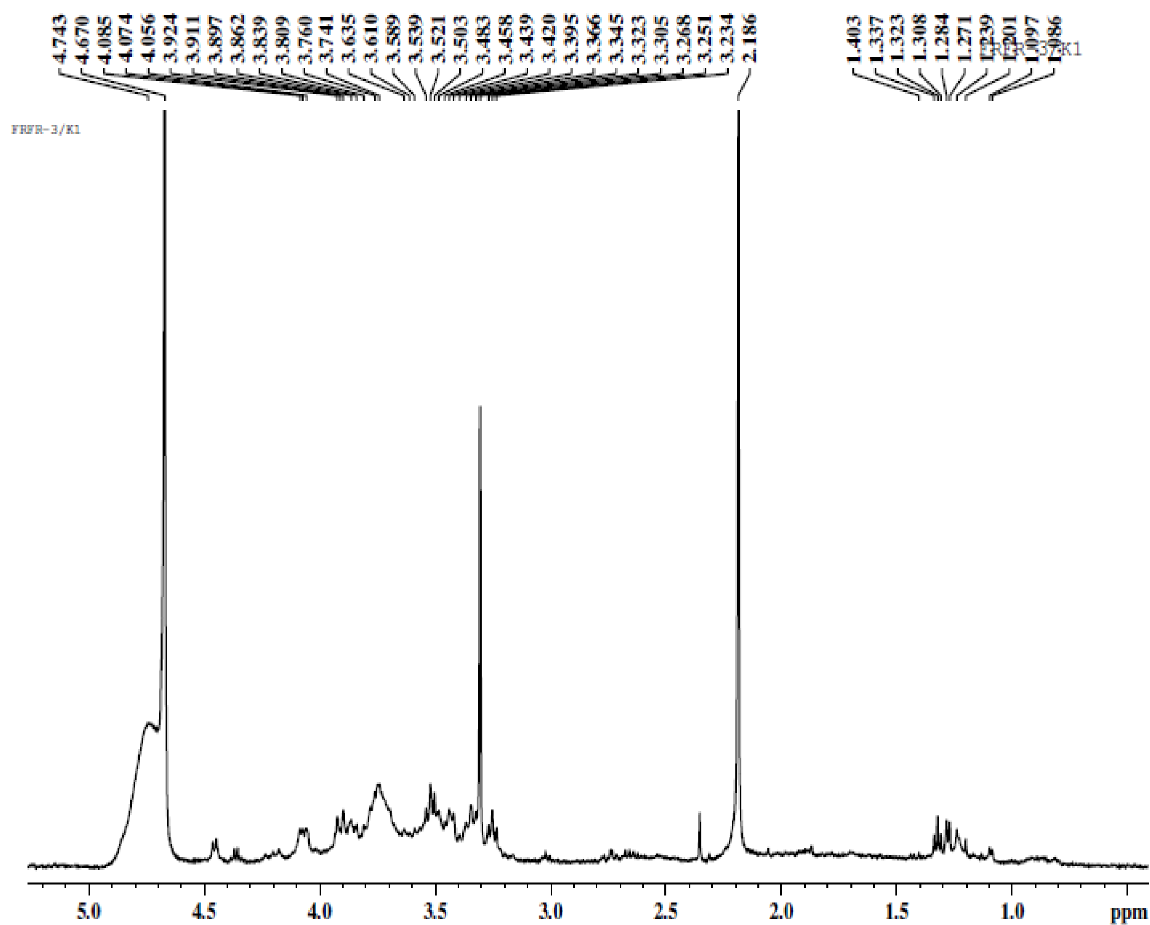

Fig. 9.6 NMR SPECTRUM OF FRFR-3

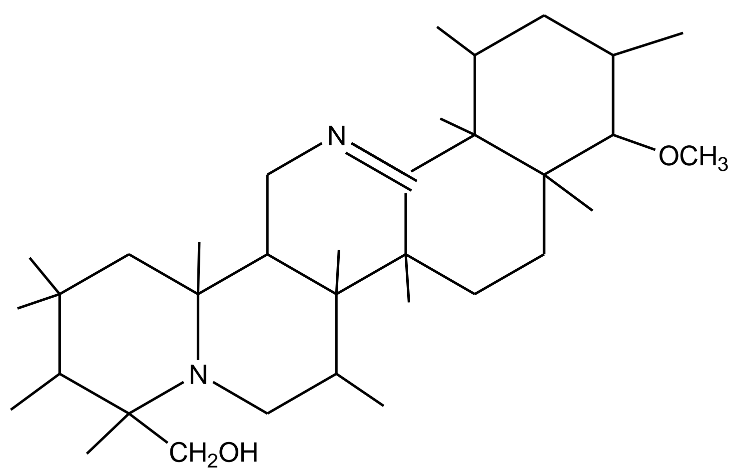

Fig. 9.7 Structure of compound CGFR3

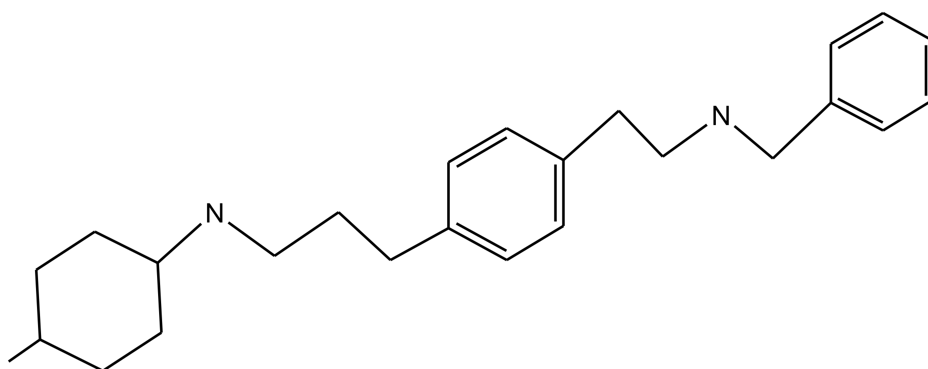

Fig. 9.8 Structure of compound FRFR3

10. HERBAL TRANSDERMAL PATCHES

FORMULATION OF NOVEL HERBAL TRANSDERMAL PATCHES:

Three transdermal patches were designed with individual fractions as well as mixture of fractions as follows:

- Transdermal patches with FRfr3 (TPFRfr3)
- Transdermal patches with CGfr3(TPCGfr3)
- Transdermal patches with FRfr3 and CGfr3(TPCGFRfr3)

Matrix type transdermal patches composed of natural polymer protonal LF10/60 was prepared by using solvent evaporation method.[156,157] Drug matrices were prepared by dissolving required amount of herbal drug (CGfr3, FRfr3and combination of both) in hot water, filtered through filter paper and mixed in natural polymer dispersion. The polymer protonal LF10/60 (optimized concentration of 2.5% solution) was dissolved in water. To this dispersion, starch (0.5% solution in hot water) solution was added and stirred well using Remi stirrer. The uniform dispersion obtained was casted on glass petriplates specially designed for this purpose and dried at RT for 6-8 hrs. The dried films were removed and cut manually for required size and were stored in desiccator until use. The placebo transdermal patches were prepared in a similar method but without incorporation of the fractions.

The working formula was calculated on basis of 300mg of fraction (either alone or in combination) for every formulation except placebo and given in Table 10.1

Table 10.1 Formula for preparation of herbal transdermal patches

S.No	Ingredients	Placebo (%)	TPFRfr3 (%)	TPCGfr3 (%)	TPCGFRfr3 (%)
1.	**Active part**				
	FRfr3	-	0.3		0.15
	CGfr3	-	-	0.3	0.15
	DM water	15.3	7.5	15	15
	Alcohol		7.5		
2.	**Polymer part**				
	Protonal LF10/60	2.5	2.5	2.5	2.5
	DM water	66.7	66.7	66.7	66.7
3.	**Additives part**				
	Starch	0.5	0.5	0.5	0.5
	DM water	15	15	15	15

Development Of Mass Spectrometric Method For Estimation Of Fractions In Transdermal Patches:

The fractions in the transdermal patches were estimated by mass spectrometry[158]. The API 2000 (Applied biosystem/MDS SCIEX, Canada) mass spectrometer coupled with ESI (Electrospray ionization) source with chromatographic system was used for this study. Batch acquisition and data processing was controlled by Analyst 1.5 version soft ware[159].

Condition for estimation of fractions:

The MS parameters optimization was carried out with the concentration of 1mg/ml of analytes (Methanolic and aqueous extracts) by using methanol (LCMS grade) and water (LCMS grade) respectively, then filtered through 0.2µm syringe filter (Advanced micro devices pvt. Ltd.) and final test solution was infused by syringe pump at a flow rate of 20µl/min continuously into the mass spectrometer. Intensity of ionization response was checked both positive and negative ionization mode. It was found good intense response in the positive mode and other parameters like declustering potential (DP), Ion source gas (GS1) and (GS2), curtain gas (CUR), focusing potential (FP) and source temperature (TEM) were optimized with respect to ionization intensity response. Acquisition was performed by setting the mass of the analytes with appropriate scan range between 100 – 1000 m/z.

EVALUATION OF FORMULATED TRANSDERMAL PATCHES:

The formulated patches were evaluated for *in vitro* and *in vivo* tests.

IN VITRO EVALUATION OF FORMULATED TRANSDERMAL PATCHES:

The formulated transdermal patches were evaluated for thickness, weight uniformity, folding endurance, percentage moisture content, water vapor permeability, drug content, *in vitro* permeation of the fractions and *in vitro* cytotoxicity.

a) Thickness of the patch

The thickness of patch was measured in different points by using digital micrometer and the average thickness was calculated.[160]

b) Weight uniformity

The prepared patches were dried at 60°c for 4hrs before testing. A specified area of patch was cut in different parts and weighed in digital balance.[161,162]

c) Folding endurance

A strip of specific area was cut evenly and repeatedly folded at the same place till it broke. The number of times the film could be folded at the same place without breaking gave the value of the folding endurance.[163]

d) Percentage Moisture content

The prepared films were weighed individually and kept in a desiccators containing fused calcium chloride at RT for 24 hrs. After 24hrs the films were reweighed and the percentage moisture content was calculated by the given formula:[164]

Percentage moisture content = initial weight-final weight /initial weight×100

e) Water vapor permeability

Glass vials of 5 ml capacity were washed and dried in an oven to a constant weight. About 1gm of fused calcium chloride was taken in the vials & the polymer films were fixed over the brim with the help of an adhesive tape. Then the vials were weighed and stored in a humidity chamber at 85 % RH condition for a period of 24 hours. The vials were removed and weighed at various time intervals like 3, 6, 12, 18 and 24hrs to note down the weight gain.[165]

f) Drug content

A 1cm^2 area of the prepared patches was dissolved in 10 ml of water. Then the solutions were filtered using Whatman filter paper. The collected filtrate was analyzed for the drug content by mass spectrometry[158].

The results of thickness, weight uniformity, folding endurance, percentage moisture content, water vapor permeability, drug content were given in Table 10.5.

g) *In vitro* Permeation Studies

Condition for in vitro permeation:

In vitro permeation studies were performed by placing each drug patches of 6 numbers by 4” X 2” patches of CGFR-3 (TPCGfr3), FRFR-3 (TPFRfr3) and combination of CGFR-3+FRFR-3 (TPCGFRfr3) in the clean test tube. Hydrophilic condition was maintained for diffusion of biomolecule(s) from the patches by adding water and skin temperature was maintained (32±5°C).

The required quantity of solution were withdrawn at different time intervals of 30min, 1h, 2h… upto 24h. The sample was drawn at regular intervals and quantity of biomolecule(s) was determined by MS. Amount of drug permeated at different time intervals were plotted as time interval v/s percentage release. The results were tabulated in Table 10.6.

The drug diffused through patches was released into the water solution. This solution was directly injected in the mass spectrometry by FIA (Flow injection analysis). The conditions maintained for *in vitro* permeation studies were given in Table 10.2.

Table 10.2 Conditions maintained for In-vitro permeation studies

Parameters	Optimization values
Ion Source	ESI (Turbo spray)
Declustering potential (DP)	20V - 120 V ramping
Focusing potential (FP)	400 V
Entrance potential (EP)	10 V
Curtain gas (CUR)	25 psi
Ion spray voltage (IS)	5500 V
Temperature (TEM)	0ºC
Source gas (GS1)	30 psi
Source gas (GS1)	40 psi

h) *In vitro* cytotoxicity studies of the transdermal patches using Balb/c 3T3: Direct contact method

The direct contact method using Balb/c 3T3 was used to test the *in vitro* cytotoxicity of the prepared transdermal patches.[166,167] The samples CGFR-3 (TPCGfr3), FRFR-3 (TPFRfr3), FRFR-3 + CGFR-3(TPCGFRfr3) and blank (Placebo) were exposed to the in vitro cytotoxicity test. Natural rubber latex and cell culture medium were used as positive and negative control respectively. The samples were stored at room temperature. The cell confluence and morphology were confirmed by microscopy and found to be 80%. The culture media was carefully removed and replaced with fresh media. This confluent culture flask was trypsinised and reseeded on to culture flasks for the cytotoxicity assay at a concentration of approximately 5.5×10^5 cells per culture flask. All the individual test items were treated separately. Triplicate cultures were set up for negative control, positive control and test items. Each individual, negative, positive control and test item measuring 1×2.5 cm^2covering 10% of the total surface area were carefully placed in center of each culture flask. The cell cultures were then incubated at 37°C for 24 h in an atmosphere of CO_2. After 24 h incubation period, the cells were subjected to qualitative and quantitative evaluations viz., cell confluency and morphology and grades of cytotoxicity, respectively.

Qualitative evaluation:

After 24 h incubation, the cultures were analyzed for microscopic evidence of cytotoxicity under microscope. Cell confluency and morphology were specifically looked into using a grading scheme as described in below Table 10.3

Table 10.3 Grading Scheme for cytotoxicity

Grade	Reactivity	Description of reactivity zone
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under specimen
2	Mild	Zone limited to area under specimen
3	Moderate	Zone extending specimen size up to 1.0 cm
4	Severe	Zone extending further than 1.0 cm beyond specimen

Qualitative evaluation was assessed by the above grading system given. If the numerical grade obtained is greater than 2 the test item was considered as cytotoxic.

The results of qualitative evaluation were given in Table 10.7.

Quantitative evaluation:

A decrease in number of living cells results in a decrease in the metabolic activity in the sample. This decrease directly correlates to the amount of the blue-violet formazan formed. The test item is considered non-cytotoxic if the percentage of the viable cell count is equal to or greater than 70% of the untreated control. A reduction of more than 30% viability in the test item treated cultures compared to concurrent untreated culture indicates cytotoxicity. Good scientific judgment was used in interpreting the data. Quantitative evaluation using the MTT assay measured by colorimeter at absorbance at 580 nm was given in Table 10.8.

IN VIVO EVALUATION OF FORMULATED TRANSDERMAL PATCHES:

Animals:

Healthy male Wistar rats (192.34-219.26g for Excision, 181.25 – 219.46g for Incision) obtained from the animal house of Vels College of Pharmacy, Tamilnadu were used for the current study. Animals were kept in polypropylene cages and were left for 5 days for acclimatization to animal room maintained at standard laboratory conditions, air-conditioned with adequate fresh air supply, with 12h fluorescent light and 12h dark cycle. Water was provided ad libitum throughout the acclimatization and experimental period. Animals were used for excision and incision model study. All the animals were taken care under ethical consideration as per the guidelines of CPCSEA with due approval from Institutional Animal Ethics Committee (IAEC), Vels College of Pharmacy, Pallavaram, Chennai-117 vide approval number IX/290/CPCSEA/PHA-05-09 dated 16.10.2009.

Grouping:

No. of groups: 4 (1 Control, 3 Test groups) for both Excision and Incision model.

No. of animals/group: 6 rats/group – Excision and Incision model (24 + 24 rats)

Study Design:

The following study design was followed for the experiment

Table 10.4 Study design for excision and incision model

Grou	**Treatment**	Model	**Animal No**	Model	**Animal No**
G1	Blank **(Placebo)**	Excision	1-6	Incision	25-30
G2	CGFR-3 Patch **(TPCGfr3)**		7-12		31-36
G3	FRFR-3 Patch **(TPFRfr3)**		13-18		37-42
G4	FRFR-3 + CGFR- **(TPCGFRfr3)**		19-24		43-48

Models:

a) Excision Wound Model [168,169]

On last day of acclimatization 24 male Wistar rats were allotted to 4 groups each group consisting of 6 animals. By using electric clipper the dorsal fur of the animal was shaved. Before the experiment each animal was anaesthetized intraperitoneally with ketamine-xylazine cocktail mixture at the dose of 50 and 7.5mg/kg respectively. Approximately 500 mm^2 circular area and 0.2 cm depth, full thickness of excision wound was created along the markings using toothed forceps, a surgical blade and pointed scissors. The entire wound left open and haemostasis was achieved by blotting with normal saline soaked cotton swab and postoperative care was taken until animals get recover. Transdermal patches (3 X 3cm) were secured using gauge and adhesive tape for 21 consecutive days. Each day old patch was removed and fresh patch was applied.

b) Incision Wound Model:[169,170]

On last day of acclimatization 24 male Wistar rats were allotted to 4 groups each group consisting of 6 animals. By using electric clipper the dorso-lateral thoraco lumbar region was clipped. Before the experiment each animal was anaesthetized and a full thickness incision wound was created by a longitudinal paravertebral incision of 6 cm long. After the incision, the parted skin was sutured 1 cm apart using a surgical thread and curved needle. The entire wound left open and haemostasis was achieved by blotting with normal saline soaked cotton swab and postoperative care was taken until animals get recover. Transdermal patches (6 X 2 cm) were secured using gauge and adhesive tape for 21 consecutive days. Each day old patch was removed and fresh patch was applied.

Parameters Considered:

a) General clinical observations:

All animals were observed for

- Clinical signs - once daily
- Mortality and morbidity - twice daily

The results were given in Table 10.9.

b) Body weights:

Body weight of individual animals was recorded before grouping and randomization. After that once in a week body weight was recorded during treatment period. The results were given in Table 10.10.

c) Percentage Of Wound Healing In Excision Wound Model (% Wound Closure):

The wound area was measured on days 1, 5, 10, 15, and 21 for all groups by using a transparent sheet and permanent marker and recording of wound area was measured using graph paper. Falling of scar without any raw wound was considered as end point of epithelisation and the days elapsed for this was considered as period of epithelisation.

$$\text{Percentage of Wound Closure} = \frac{(\text{Initial area of Wound} - \text{N}^{\text{th}}\text{ day area of wound})}{(\text{Initial area of Wound})} \times 100$$

The results were given in Table 10.11, 10.12 and 10.13 and Fig 10.4 and 10.5 and 10.7.

d) Breaking Strength Of Wound In Incision Wound Model:

The breaking strength of wound was measured on 11th day by continuous, constant water flow technique as described by Lee *et al*., 1969.

Wound healing breaking strength was measured as per the procedure given

- The animals were secured to the operation table, under light ether anesthesia on the 11th day

- On normal skin on either side of the wound a line was drawn, 3 mm away from the wound line.
- Two Allis forceps were firmly applied on the lines facing each other.
- One side the forceps was hooked firmly to metal rod and the other was connected to a leak proof polythene container through a string running over a pulley.
- The polythene container was connected to water reservoir
- The tube was released and allowed a constant and continuous water flow for measurement of wound tensile strength
- As the weight gradually increases, it acts as a pulling force to disrupt the wound.
- As soon as the opening of the wound had observed, immediately the rubber tube was clamped and the polythene container was weighed along with water.

The results were given in Table 10.14 and 10.15 and Fig.10.6

Statistical Analysis:

The data were analyzed using one way ANOVA followed by Dunnett post-hoc test. All values were reported as Mean ± SEM. Statistical significance was set at $p \leq 0.05$.

STABILITY STUDIES

The transdermal patches were subjected to stability studies as per ICH guidelines.[171] The samples were stored at 40 ± 0.5 °C and 75 ± 5 % relative humidity (RH) for 6 months. Samples were withdrawn at predetermined time intervals (0, 1,2 3 and 6 months) and then evaluated.

RESULTS AND DISCUSSION:

Formulation of Novel Herbal Transdermal Patches:

In the present investigation, novel herbal transdermal patches were formulated using natural polymer Protanal LF10/60 by solvent evaporation technique. Thin, flexible, smooth and transparent films were obtained with natural polymer Protanal LF10/60 containing wound healing drug ($CGFR_3$, $FRFR_3$ and CGFR3 + FRFR3). The transdermal patches were shown in Fig.10.1 and 10.2.

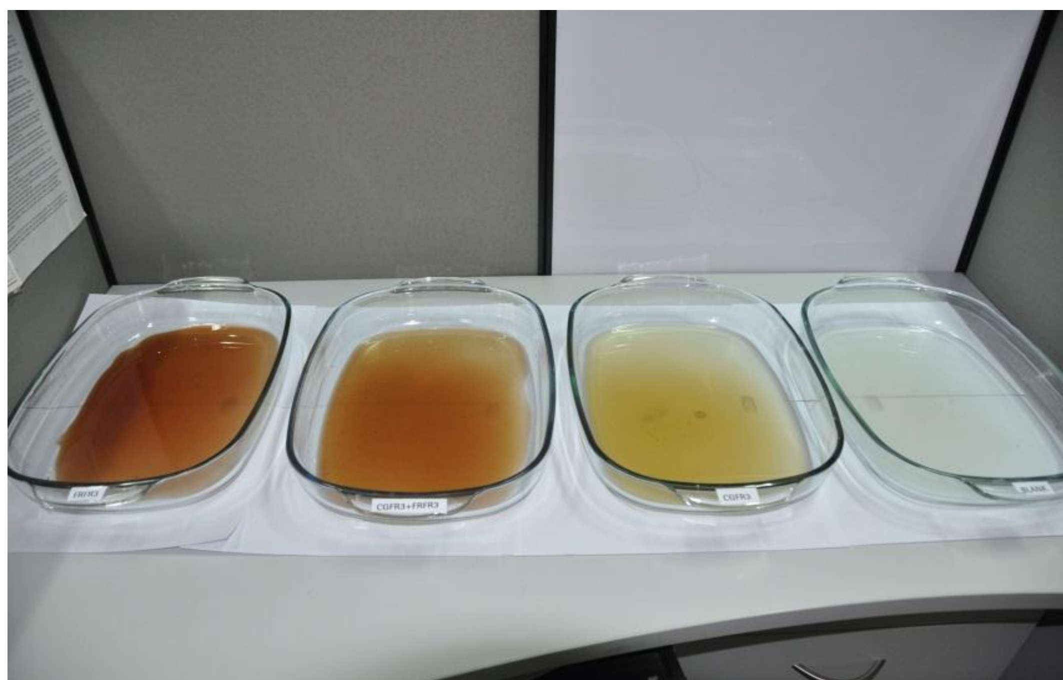

Fig 10.1 Herbal Transdermal Patches – During Formulation

The prepared transdermal patches containing the herbal fractions were pinkish brown to brown colour. They were thin with smooth texture and exhibited good flexibility. No air bubbles or other deformities were identified in any of the batches prepared, including the placebo. The blank/placebo patches were transparent, smooth and flexible.

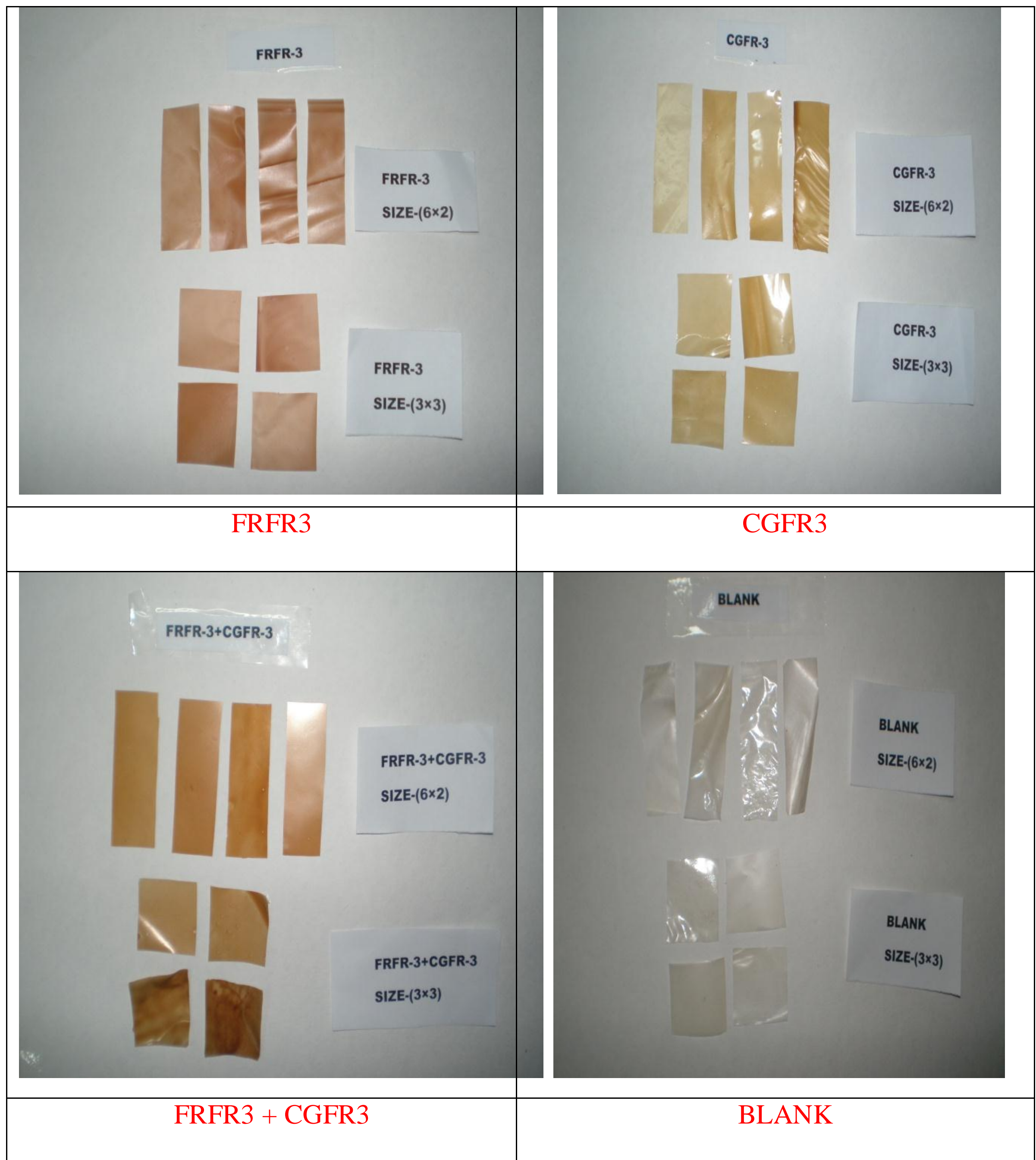

Fig 10.2 Formulated Transdermal Patches

EVALUATION OF TRANSDERMAL PATCHES:

IN VITRO EVALUATION OF FORMULATED TRANSDERMAL PATCHES:

The thickness, weight uniformity, folding endurance, percentage moisture content, water vapor permeability and drug content of the tdds patches were recorded and it was found that the results of all batches of transdermal patches were consistent and were within the specified limits.

The thickness of the patches varied from 0.182 to 0.256 for placebo to TPCGFfr3, respectively. However the batch consistency was high which was well indicated with calculated standard deviations.

The weight uniformity was also consistent among all the batches of the transdermal patches formulated.

The folding endurance was highest in the TPCGFRfr3 with 84% and was lesser for the placebo with 76%, which indicates that the transdermal patches containing the herbal fractions provides better flexibility to the films. These patches are designed for placing over the skin which can normally fold and hence, the greater folding endurance of the patches can adhere to the skin without breaking.

The moisture content in all the transdermal patches were below 1.5%, under the specified storage conditions and this ensure better stability of the patches during storage. Higher moisture contents causes not only physical deformation of the patches but also facilitates chemical degradation of the phytoconstituents.

The water vapour permeability of the prepared films was considerably high and this suggests better release of the therapeutic agents from the patches at a constant rate. Further, it will not cause discomfort to the skin as it do not cause occlusion in the skin.

The drug content in the patches was determined by mass spectrophotometry and it was found to high, reliable and consistent throughout. The drug content is an important

parameter, as it determines the capability of any dosage form to serve as a carrier for the therapeutic agent.

The results of the above mentioned studies were indicated in the table 10.5.

Table 10.5 Results of thickness of the patch, weight uniformity, folding endurance, percentage moisture content, water vapor permeability and drug content.

Test	Placebo	TPFRfr3	TPCGfr3	TPCGFRfr3
Thickness (mm)	0. 182± 0.004	0. 234± 0.006	0. 242± 0.004	0. 256± 0.004
Weight uniformity (mg)	98.1±3.06	104±3.06	102±3.08	106±2.14
Folding endurance	76± 3.1	81± 3.1	82± 2.4	84± 2.1
% Moisture content	1.28±0.04	1.15±0.04	1.08±0.03	1.10±0.02
Water vapor permeability (%)	2.58±1.22	1.56±1.32	1.46±1.22	1.52±1.46
Drug content (%)	0	92.1±0.1	93.3±0.3	94.2±0.1

Values are in ± S.D.

***In vitro* release studies:**

The *in vitro* release studies of the three patches (TPFRfr3, TPCGfr3 and TPCGFRfr3) were carried out by diffusion studies and the samples collected at different time intervals upto 24 hrs. The samples were analyzed by using mass spectrometry. The data of the study reveals that all the three patches released the contents for more than 24 hrs and the release were found to be steady throughout the period of the study. At the end of 24 hrs 82.6±1.6%, 86.6±1.6% and 90.8±1.2% of the fractions were released from TPFRfr3, TPCGfr3 and TPCGFRfr3, respectively. This profile suggests that the release of the fractions form the polymeric matrix takes place by the mechanism of slow diffusion due to the swelling of the polymeric molecules.

Table 10.6 Invitro release studies

Time (h)	Cumulative Percentage Drug Release (%)		
	TPFRfr3	TPCGfr3	TPCGFRfr3
0.5	2.4±0.1	2.2±0.2	8.7±0.4
1	8.5±0.8	9.5±0.7	15.2±0.5
2	11.7±0.9	12.7±0.6	25.1±0.7
3	13.4±1.3	15.4±1.1	32.8±0.6
4	17.9±0.8	19.9±0.9	36.6±1.0
5	24.8±0.8	26.8±0.9	40.1±1.1
6	27.7±1.2	31.7±1.3	42.6±0.9
8	33.8±0.2	36.8±1.5	45.6±1.2
10	38.1±1.8	45.1±1.2	57.6±1.4
12	45.6±1.6	57.6±1.5	65.4±1.4
16	62.3±1.2	71.3±1.3	78.2±1.4
20	78.5±1.6	82.5±1.7	84.3±1.8
24	82.6±1.6	86.6±1.6	90.8±1.2

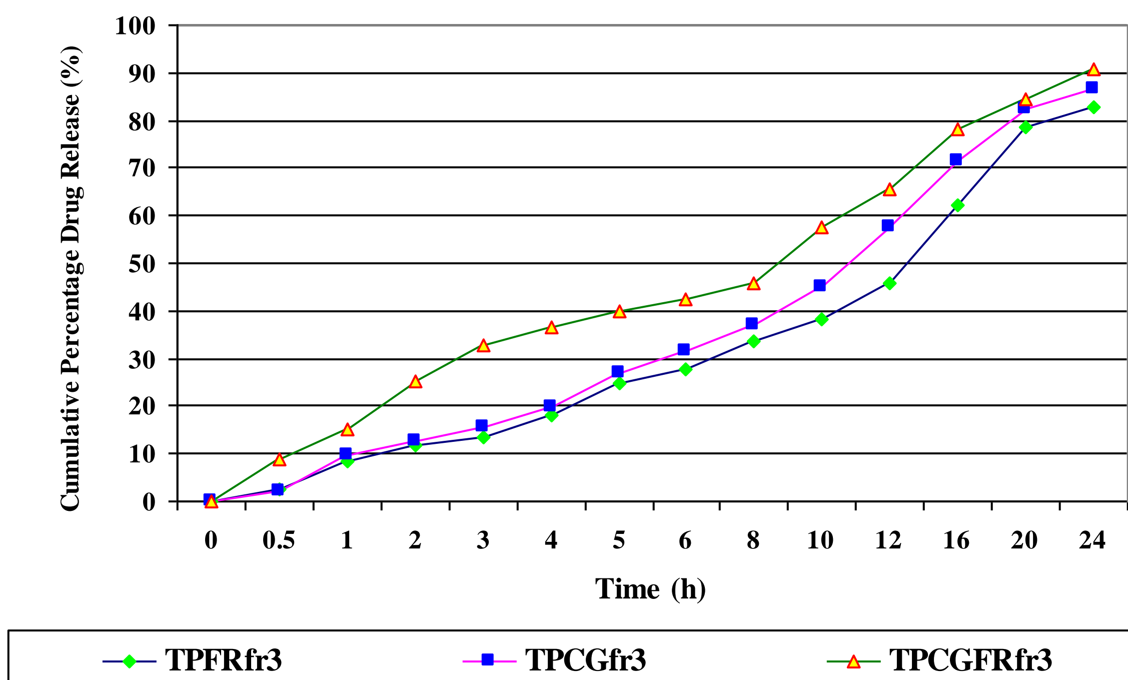

Fig.10.3 In vitro **release of herbal transdermal patches**

***In vitro* cytotoxicity:**

Qualitative evaluation: The cells treated with the positive control showed a complete destruction of cell layer compared with negative control.

Quantitative evaluation: The cells treated with the negative control did not induce any cytotoxicity and the positive control induced 73.68% cytotoxicity.

Based on qualitative and quantitative evaluations, the assay was considered as valid.

The determination of cytotoxicity of the test item was performed by qualitative evaluation were given in table 10.7. Quantitative evaluation using MTT assay for the test item CGFR3 (TPCGfr3), FRFR3 (TPFRfr3) and CGFR3 + FRFR3 (TPCGFRfr3) and Blank (Placebo) showed a viability of 86.84%, 85.53%, 86.84% and 92.76% respectively (Table 10.8.).

Based on the results obtained from the study (test carried out using Balb/c 3T3 cells line), it was concluded that the test items TPCGfr3 (6 x 2cm), TPFRfr3 (6 x 2 cm) and TPCGFRfr3 (6 x 2 cm) were considered non-cytotoxic.

Table 10.7 Qualitative evaluation of cytotoxicity

Sample	Culture	Reactivity	Grade
Negative control	1	None	0
	2	None	0
	3	None	0
CGFR3	1	None	0
	2	None	0
	3	None	0
FRFR3	1	None	0
	2	None	0
	3	None	0
CGFR3 + FRFR3	1	None	0
	2	None	0
	3	None	0
Blank	1	None	0
	2	None	0
	3	None	0
Positive control	1	Severe	4
	2	Severe	4
	3	Severe	4

Table 10.8 Quantitative evaluation of cytotoxicity (absorbance at 580 nm)

Sample	Replicate 1	Replicate 2	Replicate 3	Mean	Viability (%
Negative control	1.53	1.55	1.49	1.52	100
CGFR3	1.34	1.32	1.31	1.32	86.84
FRFR3	1.32	1.27	1.30	1.30	85.53
CGFR3 + FRFR3	1.33	1.30	1.34	1.32	86.64
Blank	1.40	1.42	1.41	1.41	92.76
Positive control	0.41	0.39	0.40	0.40	26.32

RESULTS AND DISCUSSION:

a) Clinical Signs And Mortality:

Table 10.9 Summary of clinical signs and mortality

Group & Formulation	Sex	Clinical signs	Mortality
G1 Blank **(Placebo)**	6 M	NAD	Nil
G2 CGFR-3 Patch **(TPCGfr3)**	6 M	NAD	Nil
G3 FRFR-3 Patch **(TPFRfr3)**	6 M	NAD	Nil
G4 FRFR-3 + CGFR-3 Patch **(TPCGFRfr3)**	6 M	NAD	Nil

NAD: No Abnormality Detected

From the observations it was found that there were no treatment related clinical signs and mortality observed in any of the animals.

b) Body weights:

Table 10.10 Individual Animal Body Weights (g)

Group & Formulation	Animal No.	Sex	Body Weights (g)							
			EXCISION				Animal No.		INCISION	
			Day 1	Day 8	Day 15	Day 21		Sex	Day 1	Day 8
G1 Blank Patch **(Placebo)**	1	M	194.03	194.11	208.50	227.73	25	M	181.86	196.26
	2	M	216.81	234.09	262.57	286.27	26	M	192.92	186.69
	3	M	220.21	224.95	257.62	279.89	27	M	202.82	201.55
	4	M	218.13	219.81	243.87	262.08	28	M	197.04	216.10
	5	M	219.96	216.00	227.33	237.48	29	M	202.10	191.84
	6	M	225.01	237.44	269.08	289.40	30	M	220.60	238.48
G2 CGFR-3 Patch **(TPCGfr3)**	7	M	205.17	216.92	245.93	257.70	31	M	185.47	185.95
	8	M	209.93	229.17	247.68	268.25	32	M	192.02	192.52
	9	M	210.04	215.88	235.60	250.22	33	M	195.48	211.15
	10	M	221.58	238.60	276.99	301.02	34	M	201.37	213.09
	11	M	223.56	242.38	274.68	297.06	35	M	193.14	203.89
	12	M	224.64	244.75	278.11	297.53	36	M	218.54	244.40
G3 FRFR-3 Patch **(TPFRfr3)**	13	M	195.37	207.69	236.69	251.59	37	M	190.19	194.09
	14	M	204.24	221.63	243.46	262.22	38	M	184.86	205.54
	15	M	212.29	222.15	255.04	274.80	39	M	206.17	203.22
	16	M	217.87	227.58	254.98	269.86	40	M	206.09	215.39
	17	M	219.62	229.81	258.25	273.55	41	M	211.56	213.24
	18	M	219.65	221.73	242.55	261.54	42	M	224.97	231.97
G4 FRFR-3 + CGFR-3 Patch **(TPCGFRfr3)**	19	M	223.07	211.84	236.82	256.56	43	M	206.94	225.06
	20	M	218.53	212.96	229.99	244.57	44	M	185.61	200.17
	21	M	204.00	233.35	258.23	289.92	45	M	207.16	209.48
	22	M	212.59	222.64	241.92	270.27	46	M	186.89	199.25
	23	M	227.59	252.60	290.22	320.79	47	M	224.13	220.08
	24	M	234.63	242.78	274.10	296.75	48	M	213.24	220.84

M-Male

From the body weight values there were no treatment related changes.

c) Percentage Of Wound Healing In Excision Wound Model (% Wound Closure):

Table 10.11 Summary of wound area on different days

Groups & Formulations	Wound Area (mm^2)				
	Day 01	**Day 05**	**Day 10**	**Day 15**	**Day 21**
G1 Blank Patch **(Placebo)**	496.33±2.14	348.67 ± 2.5	222.33±1.17	136.17±3.6	86.00 ± 1.71
G2 CGFR-3 Patch **(TPCGfr3)**	498.33±1.17	295.33±1.58*	158.5±2.49*	86.50±3.19*	49.67±1.23*
G3 FRFR-3 Patch **(TPFRfr3)**	497.50±1.98	270.17±3.19*	114.17±2.88*	47.33±1.65*	28.50±1.48*
G4 FRFR-3+CGFR3 **(TPCGFRfr3)**	497.00±0.97	223.67±1.52*	78.33±1.87*	27.17±1.92*	9.67±0.56*

Values are expressed as Mean ± SEM, n = 6

*- Blank Patch Vs CGFR-3 Patch / FRFR-3 Patch / FRFR-3 + CGFR-3 ($p \leq 0.05$)

Table 10.12 Summary of percentage of wound closure on different days

Groups & Formulations	Percentage of Wound Closure (%)			
	Day 05	**Day 10**	**Day 15**	**Day 21**
G1 Blank **(Placebo)**	29.73 ± 0.6	55.22 ± 0.27	72.55 ± 0.76	82.68 ± 0.32
G2 CGFR-3 **(TPCGfr3)**	40.75±0.39*	68.18±0.53*	82.62 ± 0.64*	90.03±0.24*
G3 FRFR-3 **(TPFRfr3)**	45.68±0.59*	77.05±0.61*	90.48 ± 0.35*	94.27±0.31*
G4 FRFR-3 + CGFR-3 **(TPCGFRfr3)**	54.98±0.34*	84.23±0.38*	94.55±0.38*	98.07±0.11*

Values are expressed as Mean ± SEM, n = 6

*- Blank Patch Vs CGFR-3 Patch / FRFR-3 Patch / FRFR-3 + CGFR-3 ($p \leq 0.05$)

The percent of wound closure in CGFR-3, FRFR-3 and FRFR-3+CGFR-3 treated animals was significantly more on 5th,10th,15th, and 21st day as compared to that of control.

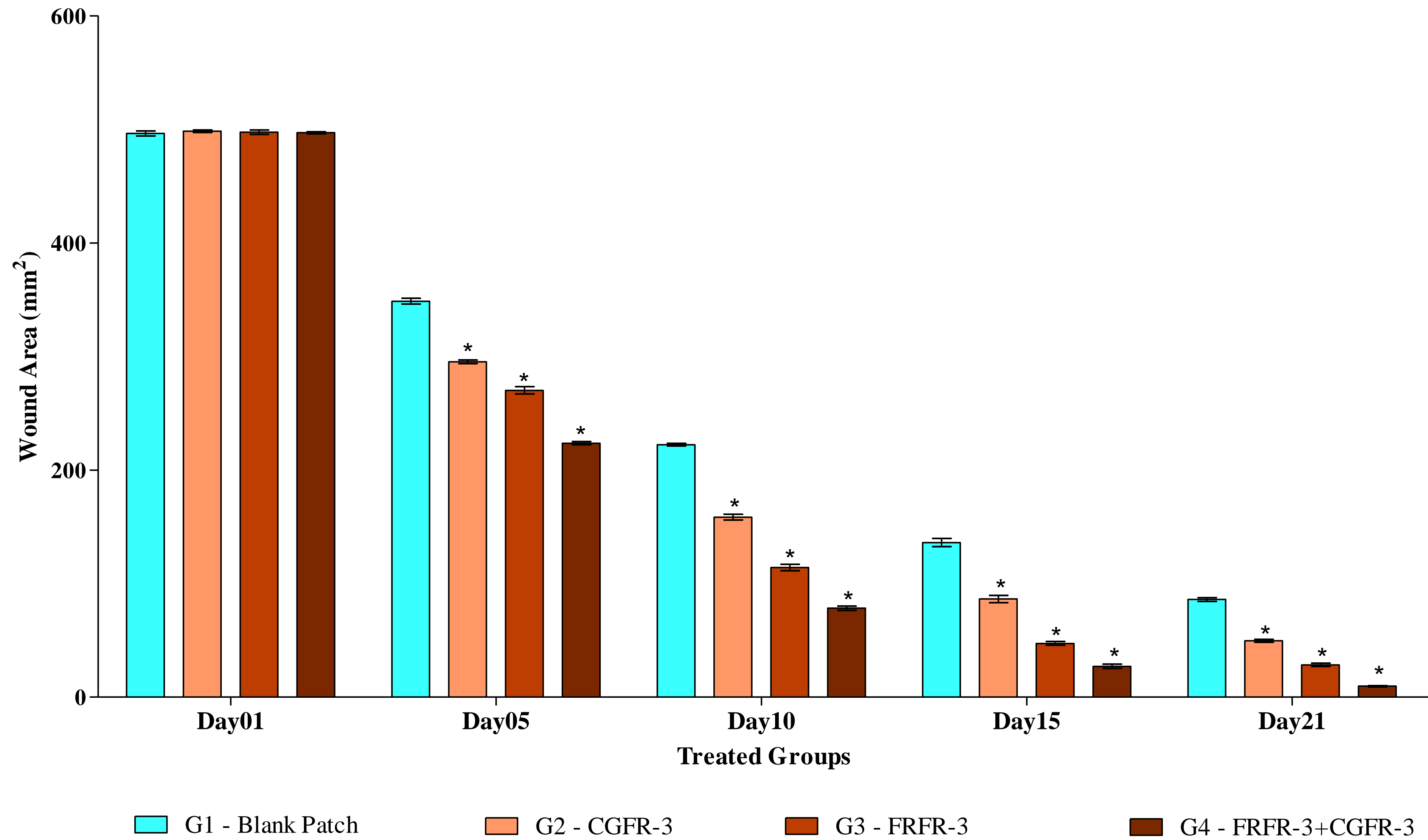

Fig 10.4 WOUND AREA

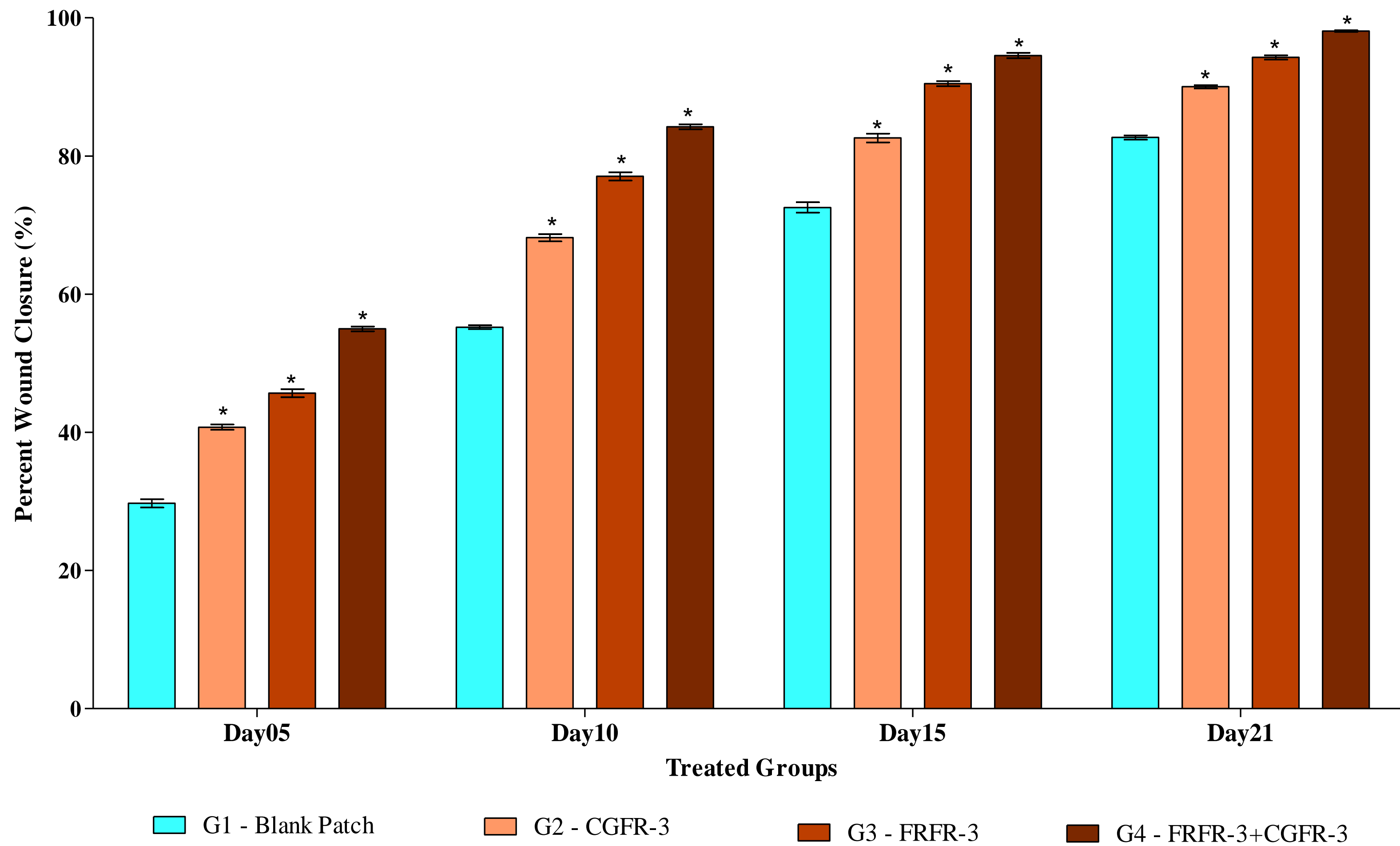

Fig.10.5 PERCENT WOUND CLOSURE

Table 10.13 INDIVIDUAL ANIMAL WOUND AREA AND PERCENTAGE WOUND CLOSURE

Group & Dose	Animal No.	Sex	Wound Area (mm^2)					% Wound Closure			
			Day 01	Day 05	Day 10	Day 15	Day 21	Day 05	Day 10	Day 15	Day 21
G1 Blank **(Placebo)**	1	M	504	356	222	145	91	29.4	56.0	71.2	81.9
	2	M	493	348	225	136	80	29.4	54.4	72.4	83.8
	3	M	490	356	220	145	85	27.3	55.1	70.4	82.7
	4	M	496	342	218	135	83	31.0	56.0	72.8	83.3
	5	M	494	347	224	135	90	29.8	54.7	72.7	81.8
	6	M	501	343	225	121	87	31.5	55.1	75.8	82.6
G2 CGFR-3 **(TPCGfr3)**	7	M	496	299	157	97	46	39.7	68.3	80.4	90.7
	8	M	504	293	156	92	52	41.9	69.0	81.7	89.7
	9	M	498	297	150	78	47	40.4	69.9	84.3	90.6
	10	M	497	295	167	87	48	40.6	66.4	82.5	90.3
	11	M	498	299	157	88	52	40.0	68.5	82.3	89.6
	12	M	497	289	164	77	53	41.9	67.0	84.5	89.3
G3 FRFR-3 **(TPFRfr3)**	13	M	497	263	115	40	25	47.1	76.9	92.0	95.0
	14	M	501	269	123	49	26	46.3	75.4	90.2	94.8
	15	M	492	268	121	47	27	45.5	75.4	90.4	94.5
	16	M	499	285	113	49	28	42.9	77.4	90.2	94.4
	17	M	492	265	108	52	35	46.1	78.0	89.4	92.9
	18	M	504	271	105	47	30	46.2	79.2	90.7	94.0
G4 FRFR-3 + CGFR-3 **(TPCGFRfr3)**	19	M	501	222	74	25	9	55.7	85.2	95.0	98.2
	20	M	496	219	81	22	10	55.8	83.7	95.6	98.0
	21	M	497	228	84	27	9	54.1	83.1	94.6	98.2
	22	M	494	224	76	24	8	54.7	84.6	95.1	98.4
	23	M	498	221	73	35	12	55.6	85.3	93.0	97.6
	24	M	496	228	82	30	10	54.0	83.5	94.0	98.0

M-Male

d) Breaking strength of wound in incision wound model:

The significant increase in breaking strength of incision wounds were observed in CGFR-3, FRFR-3 and FRFR-3+CGFR-3 treated wound of animals compared to that of blank patch.

Table 10.14 Summary of wound breaking strength

Group & formulation	**Wound breaking strength (g)**
G1 Blank **(Placebo)**	268.75±5.41
G2 CGFR-3 **(TPCGfr3)**	303.55 ± 6.97*
G3 FRFR-3 **(TPFRfr3)**	316.98 ± 13.81*
G4 FRFR-3 + CGFR-3 **(TPCGFRfr3)**	386.02 ± 8.06*

Values are expressed as Mean ± SEM, n = 6

*- Blank Patch Vs CGFR-3 Patch / FRFR-3 Patch / FRFR-3 + CGFR-3 ($p \leq 0.05$)

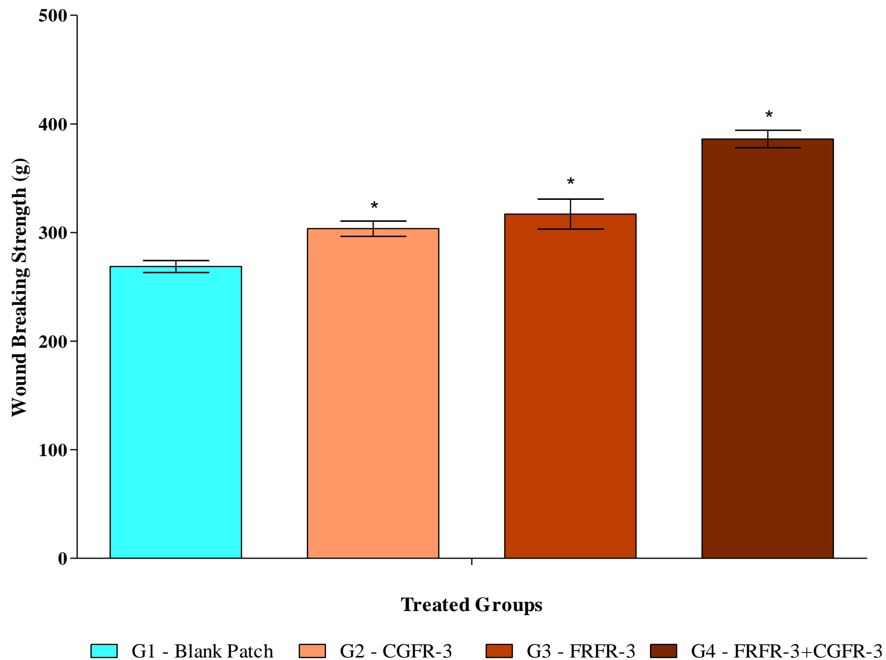

Fig. **10.6 WOUND BREAKING STRENGTH**

Table 10.15 Individual Animal Wound Breaking Strength (for individual animals)

Group and Formulation	Wound Breaking Strength (g)
G1 Blank **(Placebo)**	282.3
	270.5
	270.2
	280.2
	263.6
	245.7
G2 CGFR-3 **(TPCGfr3)**	310.0
	296.4
	290.0
	335.2
	291.2
	298.5
G3 FRFR-3 **(TPFRfr3)**	297.1
	348.2
	295.3
	269.8
	345.0
	346.5
G4 FRFR-3 + CGFR-3 **(TPCGFRfr3)**	388.3
	403.9
	391.7
	401.2
	349.4
	381.6

The transdermal patches CGFR-3, FRFR-3 and FRFR-3+CGFR-3 showed notable wound healing activity in both excision and incision wound models when applied topically.FRFR-3+CGFR-3 Showed the most significant activity followed by FRFR 3& CGFR-3 in both Excision and Incision wound models. This maximal effect of FRFR-3+CGFR-3 might due to the synergistic action of CGFR-3 and FRFR-3.

PHARMACOLOGICAL STUDIES ANIMALS

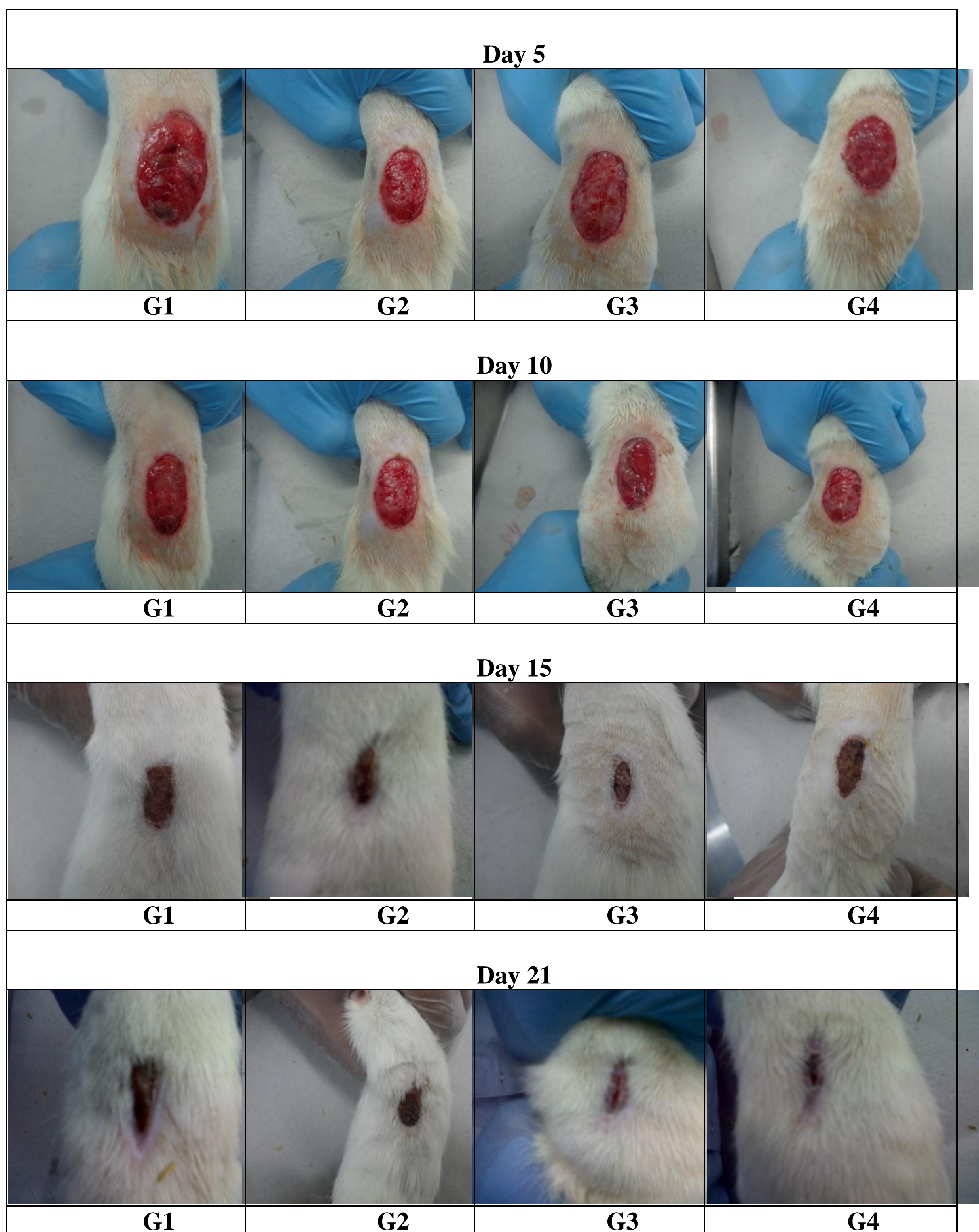

Fig. 10.7 Photo representation of contraction rate on different days in treatment group

STABILITY STUDIES:

Table 10.16 Stability studies of herbal transdermal patches at Temp 40°C /RH75%

Parameters	TPFRfr3 (months)				TPCGfr3 (months)				TPCGFRfr3 (months)			
	Initial	**1**	**2**	**3**	**Initial**	**1**	**2**	**3**	**Initial**	**1**	**2**	**3**
Drug content (%)	92.1 ± 0.1	92.0 ± 0.2	91.9 ± 0.2	91.4 ± 0.4	93.3 ± 0.3	92.9 ± 0.3	92.6 ± 0.4	92.3± 0.3	94.2 ± 0.1	93.9 ± 0.3	93.4 ± 0.4	92.6 ± 0.4
In vitro release at 24 hrs (%)	82.6 ±1.6	82.4 ±1.2	82.2 ±1.2	81.7 ±1.2	86.6 ±1.6	86.4 ±1.2	85.8±1.6	85.4±0.8	90.8 ±1.2	90.6 ±1.2	90.2 ±0.6	89.6 ±1.4

The stability studies of the 3 herbal formulations indicates that, there was no significant change in the physical appearance, drug concentration as well as in the *in vitro* release.

11. SUMMARY AND CONCLUSION

Transdermal delivery is one of the common and frequently used drug delivery routes. Transdermal route has gained more attention in drug delivery due to its flexibility and convenience in comparison to other routes of delivery and it is one of the suitable, convenient, safe and economic way to deliver drug.

Herbal drugs are safe and also called as botanical medicine or phytomedicine obtained from any plant portion. Herbal medicines can also be formulated as tablets, capsules, liquids, infusions, ointments, extracts like allopathic systems. Herbal medicines are commonly used because of the minimized or no side effects and cost effectiveness. Wound healing is a complex and dynamic process with the wound environment changing with the changing health status of the individual.

Transdermal drug delivery is commonly used system in the management of wound, pain and various inflammations. The overall aim and objective of the development of transdermal drug delivery system for herbal drugs with wound healing property were

i) To make an alternate to synthetic/allopathic drugs which may produce severe side effects and toxicity.

ii) Herbal drugs were not successfully employed because of the low bioavailability. So to increase the bioavailability of herbal drugs transdermal drug delivery systems were chosen leading to patient compliance.

iii) Filing of patents may be possible.

To achieve there objectives novel transdermal drug delivery systems for herbal drugs with wound healing property were developed.

Calotropis gigantea and Ficus racemosa were selected for this present study. For calotropis gigantea, aerial parts and for Ficus racemosa, bark were used for the research.

The pharmacognostical studies were performed for the above two plants. Macroscopic and microscopic tests were performed. Ash value, extractive value and loss on drying tests were performed and reported.

Phytochemical factors were studied and reported. The quantitative determination of phytoconstituents was carried out by using methanolic extract of plants. *Ficus racemosa* contains alkaloids 3.31%w/w, bitters 7.16%w/w, flavonoids 0.64%w/w and tannins 43.49% w/w. *Calotropis gigantea* L contains alkaloids 35%w/w, bitters 33.71%w/w, flavonoids 0.27%w/w and tannins 1.37%w/w. The extracts were subjected to fractionation. The bioactive fractions were collected. The active fractions were analyzed by TLC.

Pharmacological studies were carried out for six fractions, 3 from calotropis gigantea and 3 from Ficus racemosa. From the fractions FRfr3 and CGfr3 possessed predominant wound healing activity. So these fractions were considered for further studies.

The wound healing mechanism for these two plants was based on anti-inflammatory activity. The anti-inflammatory activity was determined by carrageenan induced rat model.

The wound healing studies were performed by excision and incision model. The animals were grouped in 8 groups. Positive control, control, CGFR1, CGFR2, CGFR3, FRFR1, FRFR2 and FRFR3. Percentage of wound healing in excision wound model (%

wound closure), breaking strength of wound in incision wound model were determined and reported.

The compounds were isolated from the CGFR3 and FRFR3 fractions and structures were elucidated by interpreting IR, Mass and NMR datas.

Transdermal patches were prepared by solvent evaporation method for TPCGFR3, TPFRFR3 and its combination TPCGFRFR3. A biocompatible nature polymer protonal LF10/60 was used to prepare the matrices. The formulated transdermal patches were evaluated for drug content, thickness, folding endurance, percentage moisture content, percentage moisture uptake, water vapor permeability, in-vitro release and *in vivo* tests. *In vitro* cytotoxicity studies were carried out. From the results it was observed that the physical parameters were found to be satisfactory. The *in vitro* release studies indicated that the drug was released in controlled fashion. Transdermal formulations were exposed to in vitro cytotoxicity studies in Balb/c 3T3 cell line using direct contact method.

Quantitative evaluation using MTT assay for the test item CGFR3 (TPCGfr3), FRFR3 (TPFRfr3) and CGFR3 + FRFR3 (TPCGFRfr3) and Blank (Placebo) showed a viability of 86.84%, 85.53%, 86.84% and 92.76% respectively. The obtained values indicated that the transdermal patches were non-cytotoxic.

In vivo studies, the transdermal patches CGFR3, FRFR3 and FRFR3+CGFR3, significantly improved the wound healing activity in both excision and incision models when compared with control group. All the drug transdermal systems showed significant reduction in wound area and significant increase in wound breaking strength. *In vivo* studies suggested that the combination of the herbals drugs possessed better pharmacological action than the individual drugs formulations.

Stability studies conducted for 90 days at 40ºC ± 2ºC / 75% ± 5% RH confirmed that all the transdermal patches were stable and good release in drug content. The drug content reduced from 92.1 ± 0.1 % to 91.4 ± 0.4, 93.3± 0.3 % to 92.3 ± 0.3% from the period of 3 months for TPFRfr3, TPCGfr3 and TPCGFRfr3 respectively. The in vitro release reduced from 82.6±1.6 % to 81.7±1.2 %, 86.6 ±1.6% to 85.4±0.8 %, 90.8 ±1.2 to 89.6 ±1.4 from the period of 3 months for TPFRfr3, TPCGfr3 and TPCGFRfr3 respectively.

The present studies indicated

- Enhancement in wound healing activity
- Absence of cytotoxicity

Although required qualities were achieved with herbal formulations TPCGFR3 and TPFRFR3, TPCGFRFR3 exhibited better activity in both *in vitro* and *in vivo* studies. The *in vitro* toxicity studies revealed that the formulations were non-cytotoxic. Hence it can be concluded that the newly developed formulation-transdermal drug delivery system of herbal drugs (CGFR3, FRFR3 and CGFR3+FRFR3) was considered to be ideal and effective in the management of wound healing.

CONCLUSION:

The present work is based on the transdermal drug delivery of herbal drugs.

- Two plants *Ficus racemosa* and *Calotropis gigantea* were selected for wound healing activity.
- From each plant, 3 fractions, totally 6 fractions were isolated successfully. From the pharmacological studies, it was proved that both plants possess wound healing activity but fractions FRfr3 and CGfr3 of *Ficus racemosa* and *Calotropis gigantea* respectively were found to possess prominent and predominant wound healing activities.
- Hence transdermal drug delivery systems for fractions of *Ficus racemosa (*FRfr3), *Calotropis gigantea* (CGfr3) and its combination (FRfr3 +CGfr3) were formulated and evaluated. The formulations successfully released active part in a controlled fashion up to 24h.
- The bioavailability of the herbal drugs was enhanced by transdermal drug delivery approach. The three formulations exhibited the ideal characters for transdermal drug delivery systems including *in vitro* and *in vivo* studies.
- *In vitro* cytotoxicity studies confirmed the absence of cytotoxicity for the transdermal patches.
- The transdermal patches CGFR-3, FRFR-3 and FRFR-3+CGFR-3 showed notable wound healing activity in both excision and incision wound models when applied topically. Among three test patches FRFR-3+CGFR-3 exhibited effective wound healing activity, followed by FRFR-3 and CGFR-3 in both Excision and Incision wound models. This maximal effect of FRFR-3+CGFR-3 might due to the synergistic action of CGFR-3 and FRFR-3.

- Stability studies conducted for 90 days at 40ºC ± 2ºC / 75% ± 5% RH confirmed that all the transdermal patches were stable.
- Hence it can be concluded that the newly developed formulation-transdermal drug delivery systems with herbal drugs (*Ficus racemosa* and *Calotropis gigantea*) is considered to be potential and effective in the management of wound healing and its related disease conditions.

12.REFERENCES

1. Chien Y, Robinson J, Lee V. Transdermal Therapeutic Systems. Controlled Drug delivery:Fundamentals and Applications 1987;523–552.
2. Mujoriya R. Dhamande K. A review on transdermal drug delivery system. Research journal of science and technology. 2011;3(5):227-231.
3. Fox LT, Gerber M, Plessis JD. Hamman JH. Transdermal drug delivery enhancement by compounds of natural origin. Molecules. 2011; 16:10507-10540.
4. Gaur PK, Mishra S, Purohit S, Dave K. Transdermal drug delivery system: A review. Asian journal of pharmaceutical and clinical research. 2009; 2(1): 14-20.
5. Monkhouse DC, Huq AS. Transdermal drug delivery – problems and promises. Drug Del ind pharm. 1998; 14(2):183-186
6. Singhal P, Singhal R, Kumar V, Goel KK, Jangra AK, Yadav R. Transdermal drug delivery system; A novel technique to enhance therapeutic efficacy and safety of drugs. Americal journal of Pharmtech research. 2012; 2(1):105-125.
7. Yadav V. Transdermal drug delivery system: Review. IJPSR. 2012; 3(2):376-382.
8. Bouwstra JA. Water distribution and related morphology in human stratum corneum at different hydration levels. Journal of Invest Dermatology. 2003;120:750-758.
9. Venkatarman S, gale R. Skin adhesives and skin adhesion: Transdermal drug delivery systems. Biomaterials 1998;19:1119-1136.

10. Karande P, Jain A, Mitragotri S. Discovery of transdermal penetration enhancers by high-throughput screening. Natural biotechnology. 2004;22(2):192-197.
11. Prausnitz M, Langer R. Transdermal drug delivery. Nature Biotechnology; 2006; 26(11):1261-1268.
12. Priyanka R. Shipra D. Transferosomes: A novel carrier for transdermal drug delivery system. International journal of pharmacy & technology. 2012; 4(1):1854-1865.
13. Jadupati M, Amites G. Kumar NA. Transferosome: An opportunistic carrier for transdermal drug delivery system. IRJP. 2012;3(3):35-38.
14. Snigdha B. Vipin KG, Mayank B, Nitin K. Recent advancement in transdermal drug delivery system: International journal of pharmaprofessionals research. 2011;2(1):247-254.
15. Patel D, Chaudhary SA, Parmar B, Bhura N. Transdermal drug delivery systems: A review. The pharma innovation. 2012; 1(4):66-75.
16. Deepika P, Akhilish D. Prabhakar P, Kamath JV. Transdermal drug delivery system: A novel drug delivery system. IRJP. 2012;3(5):89-94.
17. Pfister W, Hsieh D. Permeation enhancers compatible with transdermal drug delivery systems. Part I: Selection and formulation considerations. Medical Device Technology 1990; 1:48-55.
18. Pathan IB, Setty CM. Chemical penetration enhancers for transdermal drug delivery systems. Tropical journal of pharmaceutical research. 2009;8(2):173-179.
19. Kanikkanan N, Kandimalla K, Lamba S, Singh M. Structure activity relationship of chemical penetration enhancers in transdermal drug delivery. Current medicinal Chemistry. 1999;6:593-608.

20. Williams A, Barry B. Penetration enhancers, Advanced Drug Delivery Reviews 2004; 56: 603-618.
21. Robert L. Transdermal drug delivery; past progress, current status and future prospects. Advnaced drug delivery reviews. 2004;56:557-558.
22. Stanley S. Transdermal drug delivery ; Past, present and future. Molecular intrventions. 2004;4(6):308-312.
23. KelebE,Sharma R, Mosa EB, Aljahwi AZ. Transdermal drug delivery system – design and evaluation. International journal of advances in pharmaceutical sciences. 2010; 1:201-211.
24. Dipen MP and Kavitha K. Formulation and evaluation aspects of transdermal drug delivery system. International journal of pharmaceutical sciences review and research. 2011;6(2):16-22.
25. Murthy SN. Transdermal drug delivery; approaches and significance. Research and reports in transdermal drug delivery. 2012;1-2
26. Kumar JA, Pullakandam N, Prabu SL, Gopal V. Trandermal drug delivery system: An overview. International journal of Pharmaceutical sciences Review and Research. 2010; 3(2):49-53.
27. Mitragotri S. Synergistic effect of enhancers for transdermal drug delivery. Pharmacy research. 2000;17:1354-1359.
28. Sivamani RK, Liepmann D, Maibach HI. Microneedles and transdermal applications. Expert opinion in drug delivery. 2007; 4(1):19-25.
29. Manish G. Vimukta S. Targeted drug delivery system: A review. Research journal of chemical sciences. 2011; 1(2): 135-138.
30. Ravi K, Singh M, Bala R, Seth N, Rana AC. Transferosomes; a novel approach for transdermal drug delivery. IRJP. 2012; 3(1):20-24.

31. Williams AC, Barry BW. Penetration enhancers. Advanced drug delivery reviews. 2004;56(1):603-618.

32. McCaleb R, Research Reviews: Possible Shortcomings of Fertility Study on Herbs,
HerbalGram, 1999; 46.

33. Eldin S, Dunford A, Herbal Medicine in Primary Care, Butterworth-Heinemann, Oxford, UK, 1999.

34. Kirchfield F, Boyle, W, Nature Doctors, MedicinaBiologica, Portland, OR, 1994.

35. Ondrizek PR. An Alternative Medicine Study of Herbal Effects on the Penetration ofZona-free Hamster Oocytes and the Integrity of Sperm Deoxyribonucleic Acid, Fertility, and Sterility 1999; 71(3): 517-522.

36. Hirata JD, Swiersz LM, et al, Does Dong Quai Have Estrogenic Effects in Postmenopausal Women? A Double-Blind Placebo-Controlled Trial, Fertility and Sterility 1997; 68(6): 981-986.

37. Holland BK [Ed.], Prospecting for Drugs in Ancient and Medieval European Texts, HarwoodAcademic Pub, Amsterdam, The Netherlands, 1996.

38. Lueng A, Scientific Studies and Reports in the Herbal Literature: What are we studying and Reporting, HerbalGram. 2000; 48:63-64.

39. Winston D, Dattner A, The American System of Medicine. Clinics in Dermatology, 1999; 17(1): 53-56.

40. Rosenberg L., Torre DJ. (2006). Wound Healing, Growth Factors. Emedicine.com. Accessed January 20, 2008.

41. Stadelmann, WK; Digenis, AG; Tobin, GR (1998). "Physiology and healing dynamics of chronic cutaneous wounds". American journal of surgery 176 (2A Suppl): 26S–38S.

42. Quinn, J.V. Tissue Adhesives in Wound Care. Hamilton, Ont. B.C. Decker, Inc. Electronic book. 1998.

43. Nguyen, D.T., Orgill D.P., Murphy G.F. Chapter 4: The Pathophysiologic Basis for Wound Healing and Cutaneous Regeneration. Biomaterials For Treating Skin Loss. Woodhead Publishing (UK/Europe) & CRC Press (US), Cambridge/Boca Raton. 2009 25-57.

44. Chang HY; Sneddon JB; Alizadeh AA, Sood R, West, RB, Montgomery K, Chi JT, Rijn, VDM Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds". PLoS Biology 2004; 2 (2): E7

45. Midwood KS. Williams LV. Schwarzbauer JE. "Tissue repair and the dynamics of the extracellular matrix". The International Journal of Biochemistry & Cell Biology2004; 36 (6): 1031–1037.

46. Galko MJ, Krasnow MA. "Cellular and genetic analysis of wound healing in Drosophila Larvae". PLoS Biology 2004; 2(8): e239.

47. EnochS. Price P. Cellular, molecular and biochemical differences in the pathophysiology of healing between acute wounds, chronic wounds and wounds in the elderly. Worldwidewounds.com. 2004.

48. The wealth of India-a dictionary of Indian raw materials, vol-4, publications and information directorate, CSIR, New Delhi, 1956; 35-36

49. Warrier PK. Indian Medicinal Plants-A Compendium of 500 species (Vol. III), Orient Longman Ltd: Chennai; 1996; 34-35.

50. Rastogi RP and Mehrotra BN, Compendium of Indian Medicinal Plants, Publication and Information Directorate, CSIR, New Delhi, 1993, Vol.I, 295.

51. Devaraj KB, Gowda LR, Prakash V. An unusual thermostable aspartic protease from the latex of ficusracemosa, Phytochemistry, 2008; 69(3), 647-655.
52. Chopra RN, Chopra IC, Varma BS, supplement to glossary of Indian medicinal plants, Reprinted edition, CSIR, New Delhi, 1992; 29.
53. Chopra RN, Nayar SL and Chopra IC. Glossary of Indian Medicinal Plants. Reprinted edition, CSIR, New Delhi, 1986; 119.
54. Kar A, Choudry BK and Bandyopadhyay NG, Comparative evaluation of hypoglycemic activity of some Indian medicinal plants in alloxan diabetic rats, J. Ethanopharmacol, 2003, 84(1):105-108.
55. Bhaskara RR, Murugesan T, Pal M, Saha BP, Mandal SC, Antitussive potential of methanol extract of stem bark of *Ficusracemosa*, Phytother Res, 2003;17(19):1117-1118.
56. Biswas TK and Mukherjee B. Plant medicines of Indian origin for wound healing activity-A review. Int J Low Extern Wounds. 2003; 2(1): 25-39.
57. Mandal SC, Maity TK, Das J, Saba BP, Pal M. Anti-inflammatory evaluation of *Ficusracemosa*linn. Leaf extract. Journal of Ethno pharmacology. 2000; 72(1-2):87-92.
58. Forestieri AM, Monfotre MT, Ragusa S, Trovato A, Lauk L. Anti-inflammatory, analgesic and antipyretic activity in rodents of plant extracts used in African medicine. Phytother Res, 1996;10(2):100-103.
59. Paarakh PM. Ficusracemosa Linn- An overview. Natural product Radiance. 2009; 8(1):84-90.
60. Babu K, Shankar SG, Rai S. Comparative pharmacognostic studies on the barks of four Ficus species. Turk J Bot. 2010; 34:215-224.

61. Soni V, Jha AK, Dwivedi J, Soni P. A review on ethanomedical, phytochemical and pharmacological profile of Ficusracemosa. The global Journal of Pharmaceutical Research 2012; 1(5): 1401-1409.
62. Devaraj K, Gowda L Prakash V. An usual thermostable aspartic protease from the latex of ficusracemosa. Phytochemistry 2008; 69(3):647-655.
63. Iweala EEJ, Okeke CU. Comparative study of the hypoglycemic and biochemical effects of Catharanthusroseus (Linn) g.apocynaceae (Madagascar periwinkle) and chlorpropamide (diabenese) on alloxan-induced diabetic rats. Biokemistri 2005; 17(2):149-156.
64. Mehta DS, Kataria BC, Chhaiya SB. Wound healing and anti-inflammatory activity of extract of Ficusracemosalinn. Bark in albino rats. Int J Basic ClinPharmacol. 2012; 1(2): 111-115.
65. Patil VV, Pimprikar RB, Sutar NG, Barhate AL. Anti-hyperglycemic activity of Ficusracemosa Linn leaves. Journal of pharmacy research 2009; 2(1):54-57.
66. Mandal SC, Saha BP, Pal M. Studies on antibacterial activity of Ficusracemosa Linn. leaf extract. Phytotherapy Research 2000; 14(4):278-280.
67. Mandal SC, Amity TK, Das J, Saha BP, Pal M. Hepatoprotective activity of Ficusracemosa leaf extract on liver damage caused by carbon tetrachloride in rat. Phytotherapy research 1999; 13(5):430-432.
68. Kolte AY, Sadekar RD, Barmase BS, Desai VF, Lolte BR. Immunomodulating effect of dry powder of Ocimum sanctum and leaf gall of Ficusracemosa leavers in broilers naturally infected with IBD virus. Indian veterinary journal. 76:84-86.
69. Mandal SC, Amity TK, Das J, Saha BP, Pal M. Anti-inflammatory evaluation of Ficusracemosa leaf extract; Journal of Ethnopharmacol. 2000; 72(1-2):87-92.

70. Rao BR, Muirugesan T, Sinha S, Saha BP, Pal M, Mandal SC. Glucose lowering efficacy of Ficusracemosa in normal and alloxan diabetic rats. Phytotherapy Res. 2002;16:590-592.

71. Rao BR, Anupama K, Swaroop KR, Murugesan T, Pal, Mandal SC. Evaluation of anti-pyretic potential of Ficusracemosa bark. Phytomedicine 2002; 9:731-733.

72. Khan N, Sultana S. Chemomodulatory effect of Ficusracemosa extract against chemically induced renal carcinogenesis and oxidative damage response in Wistar rats. Life Sci 2005; 16:590-592.

73. Tambekar DH, Daikar SB. Exploring antibacterial potential of some ayurvedic preparations to control bacterial enteric infections. J Chem. Pharm. Res., 2010; 2(5):494-501.

74. Jahan IA, Nahar N, Mosihuzzaman M, Rokeya B, Hypoglycaemic and antioxidant activities of Ficusracemosa. Nat Prod Res. 2009; 23(4):399-408.

75. Ahmed F, Siddesh JM, Urooj A, Vishwanath BS. Radical scavenging and antiotensin converting enzyme inhibitory activities of standardized extracts of *Ficusracemosa* stem bark. Phytotherapy research. 2010; 24(12):1839-1843.

76. Manish PA, Paras PK, Monang KU, Patel Archita P. In vitro free radical scavenging activity of FicusBengalensislinn and Ficusracemosalinn leaf extracts. Pharmacology online. 2010; 1: 950-957.

77. Krishnamurthi, upendrakumar, Raghuvir Singh. Antimicrobial activity and reversal of dexamethasone depressed healing by roots of ficusracemosalinn. Asian Journal of Traditional medicines. 2011;6(5): 224-230.

78. Krishnamurti, Upendrakumar. Enhancement of woundhealing with roots of *Ficusracemosa* L. in albino rats. Asian pacific journal of tropical biomedicine. 2012;2(4):276-280.

79. MandalSC ,Amity TK, Das J, Saha BP and Pal M, Anti-inflammatory evaluation of *Ficusracemosa* leaf extract , J Ethanopharmacol. 2000;72(1-2):87-92.

80. Chandrashekhar CH, Latha KP, VagdeviHm, Vaidya VP. Anthelmintic activity of crude extracts of Ficusracemosa. International Journal of Green Pharmacy 2008; 2(2):100-103.

81. Atal CK and Kapur BM (Ed). Cultivation and Utilization of Medicinal Plants, Regional Research Laboratory, CSIR, Jammu-Twai. 1982; 514-519.

82. Kritikar K and Basu B.D. Oriental enterprises. 2001; 7: pp. 2218-2221.

83. The Wealth of India, Raw Materials, Vol. II, CSIR, New Delhi, 1959, 20-23.

84. Lewis SN, Richard DS, Micheal JB. Handbook of Poisonous and Injurious Plants. Springer-Verlag Berlin; 2007.

85. Murthip B, Seshadrit R. (1945), Wax and Resin components *of Caltropisgigantea.proc.* Indian academic science. 21: 147-154.

86. Kirtikar KR, Basu BD. Indian Medicinal Plants, Text Vol.III, 2nd ed. International Book Distributors, Dehradun 2005, 1606-1611.

87. Nadkarni AK. Indian MateriaMedica, Vol. I, 3rd ed. Popular Prakashan, Mumbai 1954, 237-241.

88. Chitme HR, Chandra R, Kaushik S. Studies of anti-diarrheal activity of *Calotropis gigantea*R.Br. in experimental animals. J Pharm PharmaceutSci 2004, 7(1): 70-75.

89. R Rajesh, CD RaghavendraGowda, ANataraju, BL Dhananjaya, K Kemparaju, BS Vishwanath. Procoagulant activity of *Calotropis gigantea*latex associated with fibrin (ogen) olytic activity. Toxicon 2005, 46:84-92.

90. Argal A, Pathak AK. CNS activity of *Calotropis gigantea*roots. J Ethnopharmacol 2006, 106: 142-145.

91. Argal A, Pathak AK. Analgesic activity of *Calotropis gigantea*flower. Fitoterpia 2007, 78: 40-42.

92. Sreevastava SR, Keshri G, Bhargavan B, Singh C, Singh MM. Pregnancy interceptive activity of the roots of *Calotropis* gigantea Linn. in rats. Contraception 2007, 75: 318-322.

93. Pardesi GS, Gadgoli C, Vaidya MD, Hasni HY, More BH. Immunomodulatory activity of *Calotropis gigantea*by cyclophosphamide induced myelosuppression. Pharmacologyonline 2008, 2: 164-167.

94. Lodhi G, Singh HK, Pant KK, Hussain Z. Hepatoprotective effects of *Calotropis gigantea*extract against carbontetrachloride induced liver injury in rats. Acta Pharm 2009, 59: 889-896.

95. Narendranalwaya ,Gauravpokharna ,Lokeshdeb ,naveenkumarjain, wound healing activity of latex of calotropisgigantea , International Journal of Pharmacy and Pharmaceutical Sciences, Vol. 1, Issue 1, July-Sep. 2009

96. Rathod NR, Chitme HR, Chandra R. Free Radical Scavenging Activity of *Calotropis gigantea* on Streptozotocin-Induced Diabetic Rats, Indian J Pharm Sci. 2009; 71(6): 615–621.

97. Lodhi G, Singh HK. Pant KK. Hepatoprotective effects of Calotropis gigantea extract against carbon tetrachloride induced liver injury in rats, Acta Pharm. 59 (2009) 89–96.

98. Alam MA, Habib MR, Nikkon F, Khalequzzaman M, Karim MR. Insecticidal Activity of Root Bark of Calotropis gigantea L. Against Triboliumcastaneum (Herbst) , World Journal of Zoology 4 (2): 90-95, 2009.

99. Jayakumar D, Mary SJ, Santhi RJ. Evaluation of antioxidant potential and antibacterial activity of Calotropis gigantean and vincarosea using in vitro model. Indian Journal of Science and Technology 2010; 3(7):720-723.

100. Jagtap VA, Rageeb MD, UsmanMd, Salunkhe PS, Gagrani MB, Anti-inflammatory Activity of Calotropis gigantea Linn. Leaves Extract on In-vitro Models, International Journal of Current Pharmaceutical Review and Research 2010; 1(2):1-5.

101. Sheela B. Hussain SM, Kumar PS, Kalaichelvam VK, Venkatachalam V. Vasodilatation Effect of Latex from Calotropis gigantea in Green Frog Ranahexadactyla, Asian Journal of Medical Sciences 2010; 2(1): 22-24.

102. *Subramanian SP, Saratham V,* Evaluation of Antibacterial Activity of Calotropis gigantea Latex Extract on Selected Pathogenic Bacteria, Journal of Pharmacy Research 2010; 3(3):517-521

103. Karthik L. Rao KVB, Kumar G, In vitro anti-Candida activity of Calotropis gigantea, Journal of Pharmacy Research 2010; 3(3):539-542.

104. Joshi A, Singh N, Pathak AK, TailangM.Phytochemistry and evaluation of antioxidant activity of whole plant of calotropisgigantealinn, International Journal of Research in Ayurveda and Pharmacy 2010; 1(1):120-125.

105. Mayee R, Thosar A, Kondapure A. Evalution of antiasthmatic activity of calotropisgigantea roots, Asian Journal of Pharmaceutical and Clinical Research 2011; 4(2): 33-35.

106. Harathi P, Thomas A, Krishnan S, Ravi TK. Anti-bacterial activity of leaf extracts of calotropisgigantealinn. Against certain gram negative and gram positive bacteria, Int. J. Chem. Sci. 2011; 9(2):919-923.

107. Oskuee RK, Jafari MR, Farzad SA, Ramezani M. In vitro Leishmanicidal activity of Calotropis gigantean and its fractions against Leishmania major, Journal of Medicinal Plants Research 2012; 6(23): 3977-3983.

108. Kovendan K, Murugan K, Kumar KP, Paneerselvam C, Kumar PM, Anerasan D. Mosquitocidal properties of Calotropis gigantea (Family: Asclepiadaceae) leaf extract and bacterial insecticide, Bacillus thuringiensis, against the mosquito vectors, Parasitology Research 2012; 111(2):531-544.

109. Halu B, Vidyasagar M. A comparative study: differential antimycoses activity of crude leaf extracts of calotropis spp., International Journal of Pharmacy and Pharmaceutical sciences 2012; 4(3):705-708.

110. Waghulkar VM, Udasi, Saboo SS. Development of transdermal patches (films) for the anti-rheumatic activity of chloroform extract of Trichodesmaindicum and Boswellia serrate. InventiImpact:Pharma Tech. 2011; 407/11:1-4.

111. Bhujbal SS, HadawaleSSmKulkarni PA, Bidkar JS. A novel herbal formulation in the management of diabetes. Int. J Pharm Investig. 2011; 1(4):222-226.

112. SarfarazMd, Murtale S, Hiremath D, Udipi RH. Design, development and evaluation of Selegiline Hydrochloride transdermal patch. Journal of pharmaceutical sciences. 2012; 2(3):48-56.

113. Suruse PB, Duragkar NJ, Bodele SB. Formulation and development of sustained release anti-inflammatory transdermal pad using herbal extracts. Journal of pharmacy research 2009; 2(3):399-403.

114. Sass JE. Elements of Botanical Microtechnique. First edition. McGraw Hill Book Co, New York, 1940;. 222.

115. Johansen, DA. Plant microtechnique. New York, London, McGraw-Hill Book Company, inc., 1940.

116. O'Brien, T.P, Feder, N, Mccully, M E. Polychromatic staining of plant cell walls by toluidine blue O. *Protoplasma,*1964; 59(2):68-373.

117. Easu, K. Plant Anatomy John Willey and sons. New York Pp. 1964; 767. Easu, K. Anatomy of seed Plants. John Willey and Sons. New York 1979; 550.

118. Jain VC, Shah DP, Sonani NG, Dhakara S, Patel NM. Pharmacognostical and preliminary phytochemical investigation of Lawsoniainermis L. leaf. Rom. J. Biol – Plant Biol. 2010; 55(2): 127-133.

119. BhaskaraRR, MurugesanT,PalM,sahaBPand MandalSC, Antitussive potential of methanol extract of stem bark of ficus racemosa, *phytother Res,*2003,17(19),1117-1118.

120. Rastnasooriya WD, Jayakody JR and Nadarajah T, antidiuretic activity of Aqueous bark extract of SriLankan ficus racemosa in rats.,Acta *Biolhung,*200354(3-4),357-366.

121. Rajpal V., "Standardization of Botanicals: Testing and extraction methods of medicinal herbs" Eastern Publishers, 2002; Volume1: 6.

122. Bratati De and P.C Datta, "Alkaloids in Floral Parts of Strychnos nux-vomica" *Indian. J. Nat. Products.*, 7 (2), 19

123. Indian medicinal plants-a compendium of 500 species, by warrier PK (Ed), orient longman ltd, chennai, 1996, vol.III, PP34-35.

124. Chopra RN, Chopra IC and Varma BS, supplement to glossary of Indian medicinal plants, Reprinted edition, csir, New Delhi, 1992, pp.29.

125. Atal CK and Kapur BM(Ed), cultivation and utilization of medicinal plants, regional research laboratory, csir, Jammu-twai, 1982pp514-519.

126. CookeT,The flora of presidency of Bombay,reprinted edition,botanical survey of india,Calcutta,1967,vol.III,pp.154.

127. MandalSC ,Amity Tk, Das J, Saha BP and Pal M, Anti-inflammatory evaluation of Ficus racemosa leaf extract , *J Ethanopharmacol*,2000,72(1-2),87-92.

128. http://www.ars-grin.gov/cgi-bin/ducke/ethnobot. *Ficusracemosa*.

129. Rajpal V., “Standardization of Botanicals: Testing and extraction methods of medicinal herbs” Eastern Publishers, 2002; Volume1: 215.

130. Rajpal V., “Standardization of Botanicals: Testing and extraction methods of medicinal herbs” Eastern Publishers, 2005; Volume 2: 88-89.

131. Mandal SC,MaityTK,Das J,SahaBPand Pal M,Hepatoprotective activity of ficus racemosa leaf extract on liver dmage caused by carbontetrachloride in rat ,*phytother Res in* 1999,13,(5),430-432.

132. The wealth of India-a dictionary of Indian raw materials, vol-4, publications and information directorate, CSIR, New Delhi, 1956, pp.35-36

133. Chopra RN, Chopra IC, Handa KL and KapurLD, indigenuos drugs of India, U.N.Dhur&sons pvt Ltd., Calcutta, 1958, pp.674-675.

134. A.M. Diaz, Analytical methods of tannins and their application to Myrtus communis seeds extracts, *Fitoterapia*, Volume LVIII, No 6, 1987.

135. Medicinal Plants of India, ICMR, New Delhi, 1956, vol.I, pp.415-416.

136. Khandelwal KR, 2007. Practical Pharmacognosy, 18th edition, Nirali Prakashan, Pune, India, pp 157-161

137. http://www.herbalcureindia.com/herbs/*ficusracemosa* .htm

138. Chopra RN, Nayar SL and Chopra IC,glossary of indian medicinalplants,reprinted edition,csir,new delhi,1986,pp.119.

139. PrabhakarYS and Suresh KD, A survey of cardioactive drug formulations from Ayurveda. II: Porridges, oils, clarified butters,electauries,pastes,ash preparations and calcined powders,*Fitoterapia*,1990,61,395-416.

140. Rastogi RP and Mehrotra BN, Compendium of Indian mdicinal plants, puplication and information directorate, csir, New Delhi, 1993, vol.I, pp.295.

141. O'Brien, T.P, Feder, N, Mccully, M E. Polychromatic staining of plant cell walls by toluidine blue O. *Protoplasma,* v.59, n.2, p.368-373, 1964.

142. Devaraj KB, Gowda LR and prakashV, an unusual thermostable aspartic protease from the latex of ficus racemosa, *Phytochemistry,* 2008, 69(3), 647-655.

143. Kar A, Choudry BK and Bandyopadhyay NG, Comparative evaluvation of hypoglycemic activity of some Indian medicinal plants in alloxan diabetic rats, *j.ethanopharmacol*, 2003, 84(1), 105-108.

144. Biswas TK and MukherjeeB, Plant medicines of Indian orgin for wound healing activity-a review, Int J Low Exterm Wounds, 2003, 2(1), 25-39.

145. Chia-chi chang, Ming-Hua yang, Hwei-mei wen and Jiing-chuan chern, Estimation of Total Flavonoid content in Propolis by two complementary colorimetric methods. *Journal of Food and Drug analysis*, Volume 10, No. 3, 2002 Page No. 178-182

146. ForestieriAM,MonfotreMT,Ragusa S,Trovato A and Lauk L., Anti-inflammatory,analgesic and antipyretic activity in rodents of plant extracts used in African medicine ,*phytother Res*,1996,10(2),100-103.

147. Sass JE. (1940), Elements of Botanical Microtechnique. Mc Graw Hill Book Co, New York, p. 222.

148. Easu, K. 1964. Plant Anatomy John Willey and sons. New York Pp. 767. Easu, K. 1979. Anatomy of seed Plants. John Willey and Sons. New York Pp. 550

149. B.S. Nayak, M .Anderson, L.M. Periara and Pinto, Evaluation of wound-healing potential of Catharanthusroseus leaf extract in rats.Fitoterapia, 78(7-8), 540 (2007).

150. Lee KH. Studies on mechanism of action of salicylates II.Retardation of wound healing by aspirin.*J Pharm Sci 1968*;*57:1042-3*

151. ShahapurkarAparna and Jayanthi. Coriander seeds for wound healing activity in albino rats. 2011(1):1196-1199.

152. Yin W, Wang TS, Yin FZ, Cai B. Analgesic and anti-inflammatory properties of brucine and brucine N-oxide extracted from seeds of Strychnosnux-vomica. J Ethnopharmacol 2003;88:205-14.

153. Yu. P. Synchrotron IR microspectroscopy for protein structure analysis: potential and questions. Spectroscopy. 2006; 20: 229-251.

154. Sridhar L, Karthikraj, Murty MRVS, Raju NP, Vairamani M, Prabhakar S. Mass spectral analysis of N-oxides of nitrogen mustards, and N,N-dialkylaminoethyl-2-chlorides under electrospray ionization conditions. International journal of mass spectrometry. 2012; 333(1):15-20.

155. Yamanobe T, Uehara H, Kakiage M. Chapter 5 – Practical NMR analysis of morphology and structure of polymers. 2010; 70: 203-239.

156. Gannu, R., Vamshi, Y.V., Kishan V., Rao, Y.M., Development of Nitrendipine transdermal Patches:*In vitro* and *Ex vivo* Characterization. Current Drug Delivery 2007; 4:69-76.

157. Shivaraj, A. Selvam, R.P., Mani, T.T., Sivakumar,T., Design and evaluation of transdermal drugdelivery of ketotifenfumarate, Int. J. Pharm.Biomed. Res. 2010; 1(2):42-47.

158. Sakamoto T, Portieri A, Taday PF, Takada Y et al. Detection of tulobuterol crystal in transdermal patches using terahertz pulsed spectroscopy and imaging. Pharmazie 2009; 64(6):361-365.

159. Ji AJ, Lawson GM, Anderson R, Dale LC, Croghan IT. Hurt RD. A new gas chromatrograpy-Mass spectrometry method for simultaneous determination of total and free trans -3-hydroxycotinnne and cotinine in the urine of subjects receives transdermal nicotine. Clincial Chemistry 1999; 45(1):85-91

160. Premjeet S, Bilandi A, Sahil K Akanksha M. Transdemal drug delivery system (patches), applications in present scenario; International journal of research in pharmacy and chemistry 2011; 1(4):1139-1140.

161. Bharkatiya, M., Nema, R.K., Design and characterization of drug free patches fort ransdermal application, Int. J. Pharm, Sci., 2010; 2(1):35-39.

162. Ramkanth. S., Alagusundaram M., Gnanaprakash K., Rao K.M., Mohammed S.T.S., Paneer, K.,Chetty M.C., Design and characterization of matrix type transdermal drug delivery Systemusingmetoprololtartarate, Int. J. Pharm Res.2010; 1(1):1-5.

163. Sanjoy M, Thimmasetty J, Ratan G.N, Kilarimath B.H. Formulation and evaluation of Carvedilol transdermal patches. Int. Res. J. Pharm.2011; 2(1):237-248.

164. Misra AN. Controlled and Novel Drug Delivery. In: Jain NK, editors. Transdermal Drug Delivery. New Delhi, India: CBS Publisher and Distributor 1997:100-101.

165. Chien Y.W. Transdermal therapeutic system: In controlled drug delivery fundamentals and applications. Vol.50. New York, Marcel Dekker.1992: 301-381.

166. Biological evaluation of medical devices part 5. Tests for *in vitro* cytotoxicity. ISO:2009: 10993-10995. (E).

167. Biological evaluation of medical devices – Part 12: Sample preparation and reference materials. ISO 10993-10912: 2007.

168. Aparna S. Jayanthi; Coriander Seeds for wound Healing Activity in Albino Rats Int.J.Ph.Sci. 2011; 3(1):1196-1199.

169. Shetty S, Udupa S, Udupa AL, Vollala VA. Wound healing activities of bark extracts of Jatropha curcas Linn in albino rats of Saudi med j 2006;Vol. 27 (10). 1-12

170. Lee KH (1968). Studies on the mechanism of action of salicylate retardation of wound healing by aspirin, J Pharma Sci.; 57:1042-1043.

171. Gupta V, Yadav SK, Dwivedi AK, Gupta N. Transdermal drug delivery: past, present, future trends. IJPLS. 2011; 2(9):1096-1106.