PRECLINICAL EVALUATION OF ANALGESIC, ANTI-INFLAMMATORY & ANTI-RHEUMATIC ACTIVITIES OF SIDDHA HERBAL FORMULATION *"KEELVAYU NIVARANA CHOORANAM"* IN ANIMAL MODEL

The dissertation Submitted by

Dr. T. GIFTILLDA SELVA ELSEE

Reg, No. 321412103

Under the Guidance of

Prof. Dr. M.D. SARAVANA DEVI, M.D(S).,

Dissertation submitted to

THE TAMILNADU DR. MGR MEDICAL UNIVERSITY

CHENNAI - 600032

In partial fulfilment of the requirements

for the award of the degree of

DOCTOR OF MEDICINE (SIDDHA)

BRANCH-II-GUNAPADAM

POST GRADUATE DEPARTMENT OF GUNAPADAM

GOVERNMENT SIDDHA MEDICAL COLLEGE

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GOVT. SIDDHA MEDICAL COLLEGE,

ARUMBAKKAM, CHENNAI-106.

DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation entitled **"Preclinical evaluation of Analgesic, Anti-inflammatory & Anti-Rheumatic activities of Siddha herbal formulation *Keelvayu Nivarana Chooranam* in animal model"** is a bonafide and genuine research work carried out by me under the guidance of **Prof**. **Dr. M.D. Saravana Devi, M.D(S).,** Department of *Gunapadam*, Govt.Siddha Medical College, Arumbakkam, Chennai-106 and the dissertation has not formed the basis for the award of any Degree, Diploma, Fellowship or other similar title.

Date: **Signature of the Candidate**

Place: Chennai **DR. T. GIFTILLDA SELVA ELSEE**

GOVT. SIDDHA MEDICAL COLLEGE,

ARUMBAKKAM, CHENNAI-106.

CERTIFICATE BY THE GUIDE

This is to certify that the dissertation entitled **"Preclinical evaluation of Analgesic, Anti-inflammatory & Anti-Rheumatic activities of Siddha herbal formulation *Keelvayu Nivarana Chooranam* in animal model"** is submitted to The Tamilnadu Dr.M.G.R. Medical University in partial fulfillment of the requirements for the award of degree of M.D (Siddha) is the bonafide and genuine research work done by **Dr. T. Giftillda Selva Elsee** under my supervision and guidance and the dissertation has not formed the basis for the award of any Degree, Diploma, Associateship, Fellowship or other similar title.

Date: **Signature of the Guide**

Place: Chennai

GOVT. SIDDHA MEDICAL COLLEGE,

ARUMBAKKAM, CHENNAI-106.

ENDORSEMENT BY THE HOD

PRINCIPAL/HEAD OF THE INSTITUTION

This is to certify that the dissertation entitled **"Preclinical evaluation of Analgesic, Anti-inflammatory & Anti-Rheumatic activities of Siddha herbal formulation *Keelvayu Nivarana Chooranam* in animal model"** is a bonafide work carried out by **T.Giftillda Selva Elsee** under the guidance of **Prof. Dr.M.D.Saravana Devi, M.D(S).,** Department of Gunapadam, Govt.Siddha Medical College, Chennai - 106.

Signature of the HOD **Signature of the Principal**

Date:

Place: Chennai

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CONTENT

ABBREVIATIONS

Alb	**Albumin**
ALP	**Alkaline phosphatase**
ALT	**Alanine transaminase**
ANA	**Anti-nuclear antibodies**
ANOVA	**Analysis Of Variance**
ANTI CCP	**Anti-Cyclic Citrullinated Peptide**
AST	**Aspartate transaminase**
BUN	**Blood urea nitrogen**
CIA	**Collagen Induced Arthritis**
CMC	**Carboxy Methyl Cellulose**
CPCSEA	**Committee for the purpose of control and supervision of experimental animals.**
CRP	**C reactive protein**
DC	**Differential count**
Dep.	**Deposits**
E	**Eosinophil**
ED_{50}	**Effective dose**
EDAX	**Energy dispersive X-Ray analysis**

ESR	**Erythrocyte Sedimentation Rate**
FPC	**Few Pus Cells**
FTIR	**Fourier Transform Infrared Spectroscopy**
GSH	**Reduced Glutathione**
Hb	**Haemoglobin**
HDL	**High density lipo protein**
IAEC	**Institutional Animal Ethical Committee**
ICMR	**Indian Council of Medical Research**
IR	**Infrared**
KVNC	***Keelvayu Nivarana Chooranam***
L	**Lymphocyte**
LD_{50}	**Lethal dose**
LDL	**Low density Lipoprotein**
LFT	**Liver function test**
NOAEL	**No- observed Adverse Effect Level**
OECD	**Organisation for Economic Co-Operation & Development**
P	**Polymorphs**
RA	**Rheumatoid Arthritis**
RBC	**Red blood cell**
RFT	**Renal function test**

SEM	**Scanning Electron Microscope**
SEM	**Standard error mean**
SGOT	**Serum Glutamic Oxaloacetic Transeaminase**
SGPT	**Serum Glutamic Pyruvic Transaminase**
SOD	**Superoxide Di Mutase**
TA	**Total Protein**
TB	**Total Bilirubin**
TC	**Total count**
TGL	**Triglycerides**
TP	**Total Protein**
TWBC	**Total white blood cell count**
VLDL	**Very low density lipo protein**
UV	**Ultra Violet**
WHO	**World Health Organization**
XRD	**X-Ray Diffraction**

FIGURE CONTENTS

TABLE CONTENTS

TABLE CONTENTS

GRAPH CONTENTS

INTRODUCTION

Humans are the greatest of all organisms living in the world. It is not as easy to born as a human. Poet Auvaiyar puts it as follows:

"Human Birth Is Indeed A Rarity"

The ability to achieve self – realization is given to humans alone. The creative energy called God and the energy of inanimate things remain latent in humans. But not many of us either try to understand the hidden creative energy in us or use it properly. The object of human life is to realize the power in us and use it for the benefit if mankind.

Medicine is one of the greatest feats of mankind which brings health and happiness. It is very much important to note that the growth of medicine started based on the nature, the customs and the civilization of the respective peoples of the world. As the child called society grows along in the cradle called civilization, medicine has stood by nourishing and fostering the child. All systems of medicine are unique in their own ways. Medicine has evolved along with humans through changes and improvements in theory and practice. [1]

The Siddha System of Medicine is one of the ancient systems contemporaneous with those of the submerged lands, Egyptian, Mesopotamian, Chinese and Grecian medicines. The unique nature of this system is its continuous service to humanity for more than five thousand years in combating diseases and in maintaining its physical, mental and moral health, while many of its contemporaries had completed their courses long ago. [2]

The word "Siddha" not only denotes simplicity, uniqueness, ancientness, nobility, truth and purity but includes all these senses and stands a unique, lofty entity. "Siddhi" refers to a yogic state. "Siddhars" are said to be the yogis, having lived a complete life. [1-A] They were revealed and offered the medicines to the world.

They were well aware of the nature of illness and to save the patient and they contributed drugs to the world. Most of the Siddha medicines are prepared from the natural herbs and it has the power to demolish the disease cause, it is an effective curative dose with less toxicity. They gave the internal medicines in the form of

*chooranam, kudineer, mathirai, vadagam, manapagu, legium, parpam, chendooram, chunnam, kattu, kalangu, e*tc.,

Siddha medicine is meant to be a **"TREASURE MEDICINE"** for multiple diseases to get cured.

In Siddha, the term "Arthritis" is compared with *"Keelvayu". Keelvayu* is a disease caused due to the derangement of *Vadham* or *Vali* humour mainly. It is classified into 10 different types. Of them, one of the commonest types is *Vali Azhal Keelvayu*, which can be compared to Rheumatoid Arthritis as per Modern medicine and it is caused due to derangement of *Vali* and *Azhal* humour.

Vali or *Vatham* is one of the three humors (*Vali, Azhal, Iyam*) among five elements. *Vali* is formed by air and sky. In a healthy individual the existence of the three humours are in the ratio of 1:1/2:1/4 respectively. These are all the three pillars unite of functioning human body. When they de-arranged they are called *Kuttram*. *Vatham* is the most important humour because it regulates the equilibrium of the dearranged Kuttrams. In *Vali Azhal Keelvayu* (Rheumatoid Arthritis), *Vali a*nd *azhal* humours are affected. [3]

Rheumatoid Arthritis (RA) is an inflammatory disease that causes pain, swelling, stiffness and loss of function in the joints. It occurs when the immune system, which normally defends the body from invading organisms, turns it attack against the membrane lining the joints. [4] It occurs in all races and ethnic groups. Although the disease often begins in middle age and occurs with increased frequency in older people, teenagers and young adults may also be diagnosed with the disease.

Generally, a joint (the place where two bones meet) is surrounded by a capsule that protects and supports it. The joint capsule is lined with a type of tissue called synovium, which produces synovial fluid that lubricants and nourishes joint tissues. In rheumatoid arthritis, the synovium becomes inflamed causing warmth, redness, swelling and pain. As the disease progresses, the inflamed synovium invades and damages the cartilage and bone of the joint. Surrounding muscles, ligaments and tendons become weakened. It also can cause more generalised bone loss that may lead to Osteoporosis. (Fragile bones those are prone to fracture).

RA affects people differently. Some people have mild or moderate forms of the disease with periods of worsening symptoms called flares and periods in which they feel better called remissions. Others have a severe form of the disease that is active most of the time last for many years or a lifetime and leads to serious joint damage and disability. Peoples are also experience issues related to depression, anxiety, feeling of helplessness and low self-esteem.

RA is the third most common type of arthritis behind Osteoarthritis (prevalence 26.9 million). The incidence (new cases per year) of RA increases with increasing age in most populations until about the eighth decade of life when it declines. [5]

The prevalence varies between 0.3% and 1%. It is more common in women and in developed countries. [6] Women are about two and half times more likely to get RA than men. Hormones in both genders may play a role in either preventing or triggering it. RA generally starts between the ages of 30 and 60 in women and somewhat later in life in men. The lifetime risk of developing RA is 4% for women and 3% for men. It can strike at any age even small children can get it. More than 3, 00,000 children have the juvenile form of the disease. [7]

In economic terms Rheumatoid Arthritis is a costly disease, both at personal and at a societal level. It is a condition which has a peak onset during middle age, therefore predominantly affecting individuals of working age. Approximately 75 % of new diagnosis of individuals who are working at the time of diagnosis. [8]

Rheumatoid awareness day to be held each year on February 2nd giving people with the chronic illness known as rheumatoid arthritis or rheumatoid disease a day of recognition. Because the disease is commonly presumed to be a type of arthritis awareness is lacking. [9]

In 1981, World Health Organization (WHO) and International League Associations for Rheumatology (ILAR) launched a special program for rheumatoid diseases called COPCORD (Community Oriented Program for Control of Rheumatic Disease). [10]

The word “Rheumatology has its origin in the word “rheuma” which means flowing and it is mentioned in Hippocratic corpus. Hippocrates made several

observations about gout, popularly known as "aphorism of gout". Many famous pointing in the medieval era depict joint disease. Hand lesions resembling those of RA are found in painting of the Flemish school. The famous portrait of Federigo de montefeltre, thought to have been painted by joos (Justus) van gent, shows arthritis of the proximal interphalangeal joint of the left index finger. [11]

Rheumatology developed as a well-recognized specialty of medicine in the 20th century. American physician Bernard Comroe and Joseph lee Hollender coined the term Rheumatology in 1949. Guillaume de baillou (1538-1616) a French physician introduced the term rheumatism who was the father of rheumatology. He tried to distinguish gout from other rheumatic disorders.

Rheumatoid arthritis is a challenge to the world of medicine, because it affects the patient mentally, physically, economically and his domestic life. It affects 1% of people in world population and the number of affected patients is also getting increased. The genetic factor HLA-DR4, HLA-DR1 plays important role. [12]

Many drugs are used for managing the pain and slowing the progression of Rheumatoid arthritis, but none completely cure the disease. The drug categories are used for RA include: Non – Steroidal Anti-inflammatory drugs (NSAIDs), Disease modifying anti rheumatic drugs(DMARDs), Biologic Response Modifiers (Biologic DMARDs), Anti TNF Drugs.

NSAIDs are the least potent drugs used for RA. These drugs relieve pain by reducing inflammation. DMARDs are the main drugs used in the treatment of RA. They slow the progression of the disease. It is much more effective than NSAIDs but also have more side effects. All DMARDs may produce stomach and intestinal side effects and serious drug reactions.

NSAIDs produces adverse effects in GIT (Peptic ulcer, bowel ulceration/ perforation, colitis, stomatitis, oseophagitis), Renal diseases (Acute renal failure, interstitial nephritis, hyponatraemia, Hyperkalaemia, transient risk in serum creatinine), Cardiovascular (interference with actions of anti-hypertensive and anti-cardiac failure drugs), Hepatic failure, Cholestasis, CNS (Head ache, Insomnia, abnormal behaviour), skin rashes, erythema, bone marrow suppression and anaemia. The symptoms may

subside with administration of medicines but it may reoccur with more severity as the disease becomes chronic finally leading to permanent debility. [13]

Because of this condition patients suffer physically and mentally. So obviously they are in the period of expecting an alternative medicine. While in this situation, they are so many medicines are available in Siddha system of medicine which was all given by the Siddhars to help us to live a disease free life.

The people are in need to have easily available medicines to cure many diseases. Nature has given us a wide variety of medicine to preserve good health and cure diseases. The Siddha is dealing with natural system of medicines with minimal side effects. So Siddha system of medicine is a living science forever.

There are many drugs are available in Siddha for the treatment of RA. The Author has chosen the compound drug ***"KEELVAYU NIVARANA CHOORANAM"*** which is going to be the best drug in the treatment of Rheumatoid Arthritis. And the present study is carried out to validate the Anti-Rheumatic potential through Standardization, Instrumental analysis, Toxicological profile and Pharmacological screening.

2. AIM AND OBJECTIVES

AIM

There is a need for valuable Anti Rheumatoid drugs in this present medical world. The aim of this study is to validate the analgesic, anti-inflammatory (acute and chronic) and anti-rheumatic activity of ***Keelvayu Nivarana Chooranam*** in animal model and also validate the safety profile of the trial drug through acute and repeated oral toxicity studies.

OBJECTIVES

The key objectives of the study are:

- To collect various literature which include Siddha & Modern aspect of drug and disease review, pharmacological review and pharmaceutical review.
- To prepare the trial drug according to Siddha classical literature.
- To standardize the trial drug right through physico-chemical analysis.
- To analyze the drug chemically for detection of acid, basic radicals and heavy metals.
- To assess the Acute and 28 days repeated oral toxicity profiles of *Keelvayu Nivarana Chooranam* according to OECD guidelines. (OECD 423 and OECD 407)
- To determine the Analgesic, Anti-inflammatory and Anti Rheumatic activity of *Keelvayu Nivarana Chooranam.*

3. REVIEW OF LITERATURE

3.1. DRUG REVIEW

3.1.1. GUNAPADAM ASPECT

Nannari (Hemidesmus indicus)

Synonyms : *Angarimooli, Pathalamooli, Kopagu, Saripam, Parrkodi, Neerundi, Kanannusari, Krishnavalli, Saariyam.*

Vernacular Names

English	:	Indian sarasaparilla
Malayalam	:	Nannari
Kannada	:	Sugandha - palada
Telugu	:	Sugandhi
Sanskrit	:	Sariba
Hindi	:	Magrabu
Parts used	:	Root
Taste	:	Sweet, Mild bitter
Character	:	Coolant
Division	:	Sweet
Action	:	Alternative
		Tonic
		Diuretic
		Diaphoretic

General Character

"சலதோடம் பித்தமதி தாகம் உழலை
சலமேறு சீதமின்னார் தஞ்சு – டுலகமதிற்
சொன்னமது மேகம் புண் சுரமிவையெ லாமொழிக்கும்
மென்மதுர நன்னாரி வேர்."

- தேரையர் குணவாகடம்[14]

Indications

Thirst, diabetes and anti-dote for insects bite.

Medicinal uses

Root of *Nannari* therapeutically used in treatment used in treatment of Diabetes mellitus, *Pittha* disease, Tumour, Migraine, Arthritis, Indigestion. Also used in the treatment of urinary tract infection. [14-a]

Parangipattai (Smilax chinensis)

Synonyms : *Madhusmigam, Madhusmeegi, chinnapattai, Parangichakkai*

Vernacular Names

English : China root

Malayalam : Pavu

Telugu : Pirangi-chekka

Sanskrit : Madusnuhi

Hindi : Chobchini

Parts used : Rhizome

Taste : Sweet

Character : Coolant

Division : Sweet

Action : Alternative

Anti-syphilitic

Aphrodisiac

Depurative

Tonic

General Character

"தாகம் பலவாதந் தாதுநட்டம் புண்பிளவை
மேகங் கடிகிரந்தி வீழ்மூலந்- தேகமுடன்
குட்டை பகந்தமேற் கொள்வமனம் போம்பறங்கிப்
பட்டையினை யுச்சரித்துப் பார்."

- தேரையர் குணவாகடம்[14-b]

Indications

Thirst, various *Vadha* diseases, diabetes, anti-dote for insects bite, scabies, piles,

Medicinal uses

Parangipattai decoction therapeutically used in the treatment of *Vadha* diseases, Eczema, Psoriasis, Pain, Diarrhoea, Abdominal distension, Leucorrhoea, Diabetes mellitus.

Amukkara (Withania somnifera)

Synonyms : *Amukkiri, Amukkuravi, Amukkuravu, Amukkinangkizhangu, Ashwagandham, Ashwagandhi, Ashuvam, Irulichevi, Kidichevi, Varagakarni.*

Vernacular Names

English	:	Winter cherry
Malayalam	:	Amukkuram
Kannada	:	Sogade-beru
Telugu	:	Penneru- gadda
Sanskrit	:	Aswagandha
Urdu	:	Asgandh
Parts used	:	Leaves, Seeds, Rhizome
Taste	:	Bitter

Character : Heat

Division : Pungent

Action **:** Febrifuge

Diuretic

Sedative

General Character

"கொஞ்சந் துவர்ப்பாங் கொடியகயம் சூலையரி
மிஞ்சுகரப் பான்பாண்டு வெப்பதப்பு- விஞ்சி
முசுவுறு தோடமும்போ மோகம்அன லுண்டாம்
அசுவகந் திக்கென் றறி"

- அகத்தியர் குணவாகடம்[14-c]

Indications

Various *Vadha* diseases, *Kapha* diseases, fever, swelling.

Medicinal uses

Amukkara powder used as *kayakarpam*. It therapeutically used in treatment of Vadha diseases, Pain, Swelling, Obesity, Leucorrhoea, and Diabetes mellitus.

Taking *Amukkara chooranam* with ghee increases the sperm count.

Chittraratthai (Alpinia officinarum)

Synonyms : *Aratthai*

Vernacular Names

English : Galangal the lesser, Java galangal

Malayalam : Arattha

Kannada : Rasmi

Telugu : Sanna- rashtramu, Thumpa rashtramu

Sanskrit : Rasna

Urdu	:	Khulanjan
Parts used	:	Root
Taste	:	Pungent
Character	:	Heat
Division	:	Pungent
Action	:	Expectorant
		Ferbrifuge
		Alternative

General Character

"வாதபித் தங்கரப்பான் வாதஞ் சிரோரோகஞ்
சேர்ந்தகப முத்தோடஞ் சீதமொடு- நேர்ந்தசுரம்
மற்றரத்தைக் காட்டி வருமிரும லுந்தீரும்
சிற்றத்தை வன்மருந்தாய் தேர் ."

- தேரையர் குணவாகடம்[14-d]

Indications

Kapha Diseases (Cough with expectoration, Tuberculosis), Eczema, Chest pain, Swelling, Dental Disease.

Medicinal uses

On chewing a small piece of *Chittraraththai* cures vomiting and coughs with expectoration. Root of *Chirraraththai* therapeutically used in treatment of *Vadha* diseases, cold, fever, vomiting, head ache.

Decoction of *Chittraraththai* cures fever, cough, pain, swelling, head ache.

3.1.2. BOTANICAL ASPECT

Hemidesmus indicus

Taxonomical classification

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order : Gentianales

Family : Apocynaceae

Genus : *Hemidesmus*

Species : *indicus*

Distribution

Throughout India

Description

A perennial, slender, lacticiferous, twining or prostate, wiry shrub, with woody root, stock and numerous, slender, terete, stems having thickend nodes, leaves simple, opposite, very variable from elliptic-oblong to linear lanceolate, variegated with white above, silvery white and pubescent beneath, flower greenish purple crowed in subsessile cymes in the opposite leaf axils; fruits slender follicles, cylindrical, 10cm long, tapering to a point, at the apex, seeds flattenend, black, ovate, oblong, coma silvery white.

The tuberous root is dark- brown, coma silvery white, tortuous with transversely cracked and longitudinally fissured bark. It has a strong central vasculature and a pleasant smell and taste. [15]

Anatomy

Transverse section of the fresh root is circular with a fairly regular outline. It shows a slightly compact porous strand of wood at the centre enveloped by a massive cream coloured starchy tissue and a peripheral strip of light reddish brown rind.

Parts used

Roots, Leaves, Stem.

Chemical constituents

Different parts of the plant especially root contain various compounds such as 2-hydroxy 4-methoxy benzaldehyde, 4-hydroxy 3-methoxy benzaldehyde, lupeol, ledol, nerolidol, linalyl acetate, dihydrocarvyl acetate, cis-caryophyllene, isocaryophyllene, β-selinene, dodecanoic acid, hexadecanoic acid, camphor, borneol, dehydrolupanyl-3 acetate, dehydrolupeol acetate, 3-hydroxy 4-methoxy benzaldehyde, hexadecanoic acid, hexatriacontane, lupeol octacosanoate, β-amyrin acetate, lupeol acetate, α-amyrin, β-amyrin, sitosterol, drevogenin β-3-O-β-D-oleandropyranosyl, hemidesmin-1, hemidesmin-2, hemidesminine, phytosterols, triterpenes, saponin, resin acid, tannins, tetracyclic triterpene alcohols, fatty acid**s**, glycosides, 16-dehydropregnenolone, a new pregnane ester diglycoside (desinine), indicine, hemidine and rutin are the chief components present in the plant.[16]

Properties and uses

The roots are bitter, sweet, astringent, aromatic, refrigerant, emollient, depurative, aphrodisiac, carminative, appetizer, anthelmintic, alterant, demulcent, diaphoretic, febrifuge, experctorant and tonic.

They are useful in vitiated conditions of pittha, burning sensations, leucoderma, leprosy, skin diseases, pruritis, asthma, bronchitis, hyperdipsia, opthalmopathy, emicranians, epileptic fits, dyspepsia, helminthiasis, dysentery, haemorrhoids, strangury, leucorrhoa, syphilis, abscess,arthralgia, fever and general debility.

The leaves are useful in vomiting, wounds and leucoderma.

The stems are bitter, diaphoretic, laxative and are useful in inflammations, cerebropathy, nephropathy, syphilis, leucoderma, odontalgia, cough, asthma.

The latex is good for conjunctivitis. [15]

Smilax chinensis

Taxonomical classification

Kingdom : Plantae

Division : Tracheophyta

Class : Magnoliopsida

Order : Liliales

Family : Liliaceae

Genus : *Smilax*

Species : *chinensis*

Distribution

In China and Japan

Description

A hard tendril climber with sparsely prickled or unarmed stems and thick tuberous rhizomes.

Leaves simple, alternate, elliotic, rounded at the base, prominently nerved.

Flowers many, small, white inumbels.

Fruits red berries.

Parts used

Rhizomes

Chemical constituents

Thirteen compounds were obtained and identified as kaemperol-7-O-beta-D-glucopyranoside, engeletin, isoengeletin, kaempferol, dihydrokaempferol, dihydrokaempferol-5-O-P-D-glucopyranoside, rutin, kaempferol- 5-O-beta-D-

glucopyranoside, 3, 5, 4'-trihydroxystibene, vanillic acid, 3, 5-dimethoxy4-O-beta-D-glu-copyranosylcinnamic acid, beta-sitosterol, and beta-daucosterol, respectively.[18]

Properties and uses

The rhizomes are bitter, acrid, thermogenic, anodyne, anti-inflammatory, digestive, laxative, depurative, aphrodisiac, diuretic, sudorific, febrifuge and tonic.

They are useful in syphilis, leprosy, skin diseases, epilepsy, insanity, scrofula, vitiated conditions of vadha, flatulence, dyspepsia, colic, neuralgia, constipation, helminthiasis, psoriasis, fever, strangury, seminal weakness and general debility. [17]

Withania somnifera

Taxonomical classification

Kingdom	:	Plantae
Division	:	Tracheophyta
Class	:	Magnoliopsida
Order	:	Solanales
Family	:	Solanaceae
Genus	:	*Withania*
Species	:	*somnifera*

Distribution : Throughout the drier parts of India, in waste places also.

External Morphology[18]

Plant

The plant is an erect, perennial, much branched undershrub growing to1-3m in height

Stem

It is terate, branched, cylindrical, solid, clothed with mealy, stellate-hoarytomentun, at length somewhat glabrous

Root

Roots straight, unbranched, thickness varying with age, roots bear fibre like secondary roots, outer surface buff to gray yellow with longitudinal wrinkles;crown consists of 2-6 remains of stem base; stem bases variously thickened;nodes prominent only on the side from where petiole arises, cylindrical, greenwith longitudinal wrinkles; fracture, short and uneven; odour, characteristic;bitter and acrid. The roots when dry are cylindrical, gradually tapering down with a brownish white surface and pure white inside when broken

Leaves

Leaves are cauline and ramal, simple, exstipulate, petiolate, ovate, acute, entire,and up to 10 cm long. Petioles up to 1.25 cm long. The leaves on vegetative shoots are alternate and large while those on floral branches are opposite,rounded or somewhat produced at base, pubescent on lower surface and glabrous on upper surface unicostate, reticulate venation. Arranged in pairs of one large and one small leaf and arranged somewhat laterally, having in their axila cymose cluster of 5-25 inconspicuous pale green flowers.

Inflorescence

It is solitary, axillary

Flowers

Flowers are ebracteate, pedicellate, complete, hermaphrodite, pentamerous, actinomorphic and hypogynous, gamosepalous, 4-6 mm in diameter, lucid-yellow or greenish.

Calyx

Sepals are five, fused, tubular,persistent, green, hairy.

Corolla

Petals are five, united, tubular, gamopetalous, lobes spreading or recurved, acute,pubescent and greenish yellow. Aestivation is valvate or imbricate.

Androecium

Stamens are five, attached near the base of the corolla, epipetalous, anthers oblong, dehiscing longitudinally, introrse, dithecous, filament deeply inserted in corolla tube ,linear slender

Gynoecium

It is bicarpellary, syncarpous, composedof minute swollen ovary, ovary superior, placed obliquely, placentation axile,with many ovules in each locule, style simple, stigma shortly bifid

Flowering time

Winter

Fruits

Fruitis a berry enclosed in the green persistent calyx, 5 mm in diameter, smooth,more or less globose, green when unripe, orange-red coloured in ripening stage

Seeds

Bean shaped, endospermic, yellow andorange-red coloured, somewhat scurfy. Fruit contains numerous small Capsicum like seeds .

Pollination

Entomorphilous.

Parts used

Roots and Leaves. [19]

Chemical constituents

Alkaloids: Withanine, Withaninine, Somniferine, Tropeltigloate, Somniferinine, Somninine, Nicotine, Visamine, Withasomine

Salts: Cuscohygrine, Anahygrine, Tropine, Pseudotropine, Anaferine

Steroidal Lactones: Withaferin-A, Withanone, WS-1, Withanolide E C28H38O7, Withanolide F C28H38O6, Withanolide G C28H36O4, Withanolide H C28H36O5, Withanolide I C28H36O5, Withanolide J C28H36O5, Withanolide K C28H36O5, Withanolide L C28H36O5, Withanolide M

Nitrogen containing compounds: Withanol C25H34O5, Somnisol C32H46O, Somnitol C33H46O7

Steroids:Cholesterol, β-sitosterol, Stigmasterol, Diosgenin, Stigmastadien, Sitoinosides VII, Sitoinosides VIII, Sitoinosides IX, Sitoinosides X

Flavonoids: Kaempferol, Quercetin.[20]

Uses

The tuberous roots are astringent, bitter, acrid, somniferous, thermogenic, stimulant, aphrodisiac, diuretic and tonic.

They are useful in vitiated conditions of vadha, leucoderma, constipation, insomnia, tissue building and nervous breakdown.

The leaves are bitter and are recommended in fever, painful swellings and opthalmitis.

A paste of the roots and bruised leaves are applied to carbuncles, ulcers and painful swellings. [19]

Medicinal uses

- Ashwagangha is a revitalizing herb that maintains proper nourishment of tisuues, particularly muscle and bones.
- It increases resistance to stress and mainly beneficial in stress related disorders such as arthritis, hypertension, tremors, diabetes, general debility and inflammation. It increases body's resistance towards adverse influence.
- It restores the neurotransmitters and hence useful in various mental disorders.

- ❖ It is also used for treating memory loss.
- ❖ It supports proper function of adrenals and reproductive system.
- ❖ As it is powerful aphrodisiac, it is also used for sexual vitality and as an adaptogen.
- ❖ It is blood tonifier, that improves circulation and absorption of nutrients from the cells

Medicinal properties

- ❖ It has anti-stress, adaptogenic, aphrodisiac, sedative, diuretic, antispasmodic, germicidal, anti-inflammatory action.
- ❖ It is a nervine tonic.
- ❖ It enhances immunity and endurance.
- ❖ It is a natural nutrient for insomnia
- ❖ It is good hypnotic in Alcoholism.
- ❖ It is bitter in taste and hot in potency so it alleviates vata and kapha.
- ❖ It stimulates thyroid activity.
- ❖ Enhances anti-peroxidation of liver

Alpinia officinarum

Taxonomical classification

Kingdom : Plantae

Division : Tracheophyta

Class : Magnoliopsida

Order : Zingiberales

Family : Zingiberaceae

Genus : *Alpinia*

Species : *officinarum*

Distribution

Alpinia officinarum Hance (Zingiberaceae), commonly known as lesser galangal, is an important plant from the ginger family that originates in southern China and is cultivated in Southeast Asia. [21]

Description

The branched pieces of rhizome are from 1 1/2 to 3 inches in length, and seldom more than 3/4 inch thick. They are cut while fresh, and the pieces are usually cylindrical, marked at short intervals by narrow, whitish, somewhat raised rings, which are the scars left by former leaves.

They are dark reddish-brown externally, and the section shows a dark centre surrounded by a wider, paler layer which becomes darker in drying. [15-a]

Microscopic studies

T.S of rhizome

Under a microscope, transverse section reveals epidermal cells often containing resin-like substances; cortex, endodermis and stele present beneath the epidermis; cortex and stele divided by endodermis; vascular bundles surrounded by fibers, scattered throughout the cortex and stele, cortex and stele composed of parenchyma interspersed with oil cells; parenchymatous cells containing solitary crystals of calcium oxalate and starch grains, starch grains generally simple (sometimes 2 to 8 compound), ovate, oblong or narrowly ovate, 10 to 40 mm in diameter and with an eccentric navel.

Powder microscopy

Powdered microscopy revealed the presence of parenchymatous cells in surface view with fragments of epidermal cells. Fragments of vessels having scalariform thickening are presents. Many circular starch grains are presents.

Parts used

Roots

Chemical constituents

Four crystalline substances were isolated from rhizome of Alpinia officinarum Hance. They were identified as beta-Sitosterol (I), Galangin (II), Emodin (III) and Quercetin (IV), I and III. [22]

Properties and uses

The rhizomes are bitter, acrid, nervine tonic, carminative, expectorant, anti-inflammatory and tonic. They are useful in vitiated conditions of *Vadha* and *Kapha*, Rheumatoid Arthritis, Inflammations, Cough, Asthma, Bronchitis, Dyspepsia And Intermittent Fevers.[15-a]

3.2. DISEASE REVIEW

3.2.1. SIDDHA ASPECT- VALIAZHAL KEELVAYU

Other names [23]

Mudakku vayu, Sandhu vadham, Mootu vali, Maega soolai, Aama vadham.

"வளியு மையுந் தன்னிலை கெட்டு
வளியுடன் வீக்கச் சுரமும் காய்ந்து
மூட்டுக டோறும் முடுக்கியே நொந்து
மூட்டுக டன்னின் நீரும் சுரந்து
தாங்கொணா வலியுடன் நொந்திடு மம்மே"

(சபாபதி கையேடு)

Types

- *Valikeelvayu*
- *Theekkeelvayu*
- *Iyakeelvayu*
- *Valitheekkeelvayu*
- *Valiiyakkeelvayu*
- *Theevalikeelvayu*
- *Theeiyakkeelvayu*
- *Iyavalikeelvayu*
- *Iyatheekkeelvayu*

- *Mukkuttrakeelvayu*

Valitheekkeelvayu **(வளித்தீக்கீல்வாயு)**

"வாதபித் தக்கில் வாய்வின்
வருங்குறி சாற்றிக் கேளாய்
ஏதமார் மந்த மேப்பம்
இரைச்சலும் வயிற்றிற் காணும்
ஓதருங் குத்தல் வீக்கம்
ஓய்தலில் எரிச்ச லுண்டாம்
காதறு முறக்க மின்மை
காய்ச்சலும் காணுங் கண்டாய்"

Symptoms

- Elevation of Vadha and Pittha kuttram causes swelling in the wrist joint, ankle joints (including all major and minor joints).
- Reddness
- Pain
- Fever
- Insomnia

According to Siddha text causes for *Vadha* diseases

"நோயினுற் பத்திகேள் நோன்மை கூர்மைந்தனே
நோயாளி யென்பதறு நோயாளி முன்னே
நோய்கள் வினை காதரமு நோய்கள் வரவேது
நோனாமை யால்வேகுளி நோனாத பசியால்
நோனாத நீர்பருகல் நோனாத உணவால்
நோக்கமரு ணம்தாக நோக்கியொழி தொழிதலால்
நோக்கரென நடமாடி நோக்கி நடமிடலால்
நோட்டக்கா ரரிநாடி நோட்டமொழி குரலால்
நோவயர் மாதரோடு நோவ மருவுதலால்
நோன்புவிர தாதிபல நோற்கவரு மெலிவால்
நோஞ்சையென வணுகுணவு நோவ வயிலுதலால்
நோனாத வீரமடி நோனவணி குதலால்
நோய்களுற் பத்திவழு நோய்கள் வினைவிதையாய்
நோய்கள் வினை வயலாய் மெய்நோய்கள் வளர்பயிராய்

நோயுதவு பலனாகி நோய்கள் வெகுமேலாம்
நோதக உரைத்தணமிந் நோய்களற மேலே"

- தேரன் கரிசல்

-

"தக்கவாயு கோபித்தால் சந்து அளைந்து தலைநோவாம்
மிக்கமுர் கொட்டாவி விட்டது கெரிய மலங்கட்டும்
ஒக்கநரம்பு தான் முடங்கு மூலமந்து வாய் நீருறிவரும்
மிக்கு குளிரும் நடுக்கமாம் மேனிகுன்றி வருங்காணே"

- தேரையர் வாகடம்

Theraiyar vagadam discusses that the vitiated conditions of *vali* as follows:

- Pain in the joints
- Head ache
- Excessive yawning
- Burning sensation of the body
- Constipation
- Chillness
- Rigor

Symptoms

"அறிய இம்மூன்றின் தன்மை சொன்னார் என நந்தி
எரி அனல் வாதம் எரிக்கும் குணம் கேளு
குறி எனக் கைகால் குளைச்சு விலாச் சந்து
பறி என நொந்துடல் பச்சை புண் ஆகுமே
புண்ணாய், வலிக்கும் பொருமும் குடல் ஓடித்
தண்ணா மலம் அதனைத் தம்பிக்கும் போக்காது
ஒண்ணா அசனம் உறவே கருக்கிடும்
பன்னார் குளிர்- சீதம் பகுத்திடும் வாதமே"

-திருமூலர் திருமந்திரம்.

- Pain in the metacarpal and metatarsal joints
- Pain in the intercostal area
- Pain all over the body
- Dyspepsia

- Constipation
- Loss of appetite

In Siddha system treatment for Rheumatoid Arthritis

Internal medicine

Chooranam

- *Amukkara Chooranam*
- *Parangipattai Chooranam*
- *Thirikaduku Chooranam*

Parpam

- *Sangu Parpam*
- *Velli Parpam*
- *Palagarai Parpam*
- *Thanga Parpam*
- *Pavazha Parpam*
- *Muttaiottu Parpam*

Chendhooram

- *Ayaveera Chendhooram*
- *Arumuga Chendhooram*
- *Chandamarutha Chendhooram*
- *Kaalamega Narayana Chendhooram*
- *Poorana Chandhrothayam*
- *Thanga Chendhooram*

Chunnam

- *Velvanga Chunnam*
- *Rasa Chunnam*

Mezhugu

- *Veera Mezhugu*

- *Nandhi Mezhugu*
- *Rasagandhi Mezhugu*
- *Vaan Mezhugu*

External Medicines

Pattru

- *Moosambara Pattru*
- *Kazharchi Pattru*
- *Kaavikkal Pattru*

Thylam

- *Vadhakesari Thylam*
- *Etti Thylam*
- *Kukil Thylam*
- *Sadamaanjil Thylam*
- *Ulunthu Thylam*
- *Meruguli Thylam*
- *Mezhugu Thylam*

3.2.2. MODERN ASPECTS – RHEUMATOID ARTHRITIS

Definition

Rheumatoid arthritis is a chronic systemic inflammatory disease of joints characterized by symmetrical relapsing ankylosing polyarthritis affecting mainly the peripheral small joints initially, associated with varied constitutional symptoms and presence of Rheumatic factor. [24]

It affects the lining of joints, causing a painful swelling that can eventually result in bone erosion and joint deformity.

An auto immune disorder rheumatoid arthritis occurs when immune system mistakenly attacks own body's tissue. In addition to causing joint problems, rheumatoid arthritis sometimes can affect other organs of the body such as the skin, eyes, lungs and

blood vessels. RA can occur at any age, it usually begins after age 40. The disorder is much more common in women.

Rheumatoid arthritis is a disease affecting the connective tissue of the whole body with focalized involvement of the musculoskeletal system. [25]

According to modern medicine Rheumatoid arthritis is a disease with genetically susceptible. Rheuma means fluid; it affects the synovial fluid of the joints. RA is a chronic auto immune disease with inflammation of joints and marked deformities, something triggers an attack on the synovium by the immune system which release cytokines that stimulates the inflammatory reaction that can lead to the destruction of all components of the joints. It affects 1% of people in world population. The genetic factor HLA-DRB 4 plays an important role in this disease. The human leukocyte antigen system is the locus of genes that encode for proteins and surface of cell that are responsible for immune system in human, any alteration in this system caused several disease. Auto immune diseases also caused by alteration in these Human leukocytes antigen systems. [26]

Aetiology

RA is an autoimmune disease. Concordance rates are higher in monozygotic twins (12-15%) than in dizygotic twins (3%) and frequency of disease is increased in first degree relatives of patients with RA. Up to 50% of the genetic contribution to susceptibility is due to genes in the HLA region. HLA-DR4 is the major susceptibility haplotype in most ethnic groups, occurring, for example, in 50-75% of Caucasian patients with RA compared to 20-25% of the normal population. However, DRI is more important in Indians and Israelis and DW15 in Japanese. It is likely that genetic factors influence both susceptibility and severity, with DR4 positivity more common in those with severe erosive disease.

Female gender is a risk factor and this susceptibility is increased post-partum and by breast feeding. No infectious agents have been consistently isolated and there is no evidence of disease clustering. Cigarette smoking is a risk factor for RA and for positivity for rheumatoid factor in non-RA subjects. [27]

Although the cause of rheumatoid arthritis remains obscure, there is increasing evidence that the disease is triggered by T- lymphocytes in genetically predisposed individuals with define HLA class II haplotypes.HLA-DR4 is the major susceptibility haplotype in most ethnic group but DR1 is more important in Indians.HLA-DR4 sub types results from only few amino acid difference in the third hyper variable region of the amino acid sequence a though several other HLA allies that have more recently been associated with RA in some population.[28]

Pathology

RA is characterized by persistent cellular activation, autoimmunity and the presence of immune complexes at sites of articular and extra-articular lesions. This leads to chronic inflammation, granuloma formation and joint destruction. The earliest change is swelling and congestion of the synovial membrane and the underlying connective tissues, which become infiltrated with lymphocytes (especially CD4 T cells), plasma cells and macrophages. Effusion of synovial fluid into the joint space takes place during active phases of the disease. Hypertrophy of the synovial membrane occurs, with the formation of lymphoid follicles resembling an immunologically active lymph node. Inflammatory granulation tissue (pannus) spreads over and under the articular cartilage, which is progressively eroded and destroyed. Later, fibrous or bony ankylosis may occur. Muscles adjacent to inflamed joints atrophy and there may be focal infiltration with lymphocytes.

Subcutaneous nodules consist of a central area of fibrinoid material surrounded by a palisade of proliferating mononuclear cells. Similar granulomatous lesions may occur in the pleura, lung, pericardium and sclera. Lymph nodes are often hyperplastic, showing many lymphoid follicles with large germinal centres and numerous plasma cells in the sinuses and medullary cords. Immunofluorescence confirms ‘Rheumatoid factor’ autoantibody synthesis by plasma cells in synovium and lymph nodes.

The earliest findings include micro vascular injury and proliferation of synovial cells accompanied by interstitial oedema and perivascular infiltration. [29]

In synovial fluid, immune complexes activate the complement system. Kinins, phagocytic cells and the release of lysosomal enzyme and oxygen free radicals.

Mediators produced in this process stimulate synovial cells to proliferate and to produce proteinases and prostaglandins. These products cause dissolution of the connective tissue macromolecules as well as articular cartilage. [30]

The ultimate destruction of cartilage, bone, tendons and ligaments probably result from a variety of proteolytic enzymes, metalloproteinases and soluble mediators. Collaginase, produced at the interface of pannus and cartilage is proably largely responsible for the typical erosions.

Criteria for diagnosis of Rheumatoid arthritis [26-a]

Diagnosis of RA is made with four or more of the following,

1. Morning stiffness (>1 hour)
2. Arthritis of three or more joint areas
3. Arthritis of hand joints
4. Symmetrical arthritis
5. Rheumatoid nodules
6. Rheumatoid factor
7. Radiological changes
8. Duration of 6 weeks or more.

Predisposing causes:[24-a]

- Heredity : It may play a part in 5% to 10% of cases
- Trauma : In many cases history of trauma is present
- Climate : It was thought to be more common in temperate climates. However, it is equally prevalent in India also.
- Psychic factors: Psychic upsets may aggravate the disease.
- Infections :Existence of an infetcious agent Mycoplasma Epstein barr virus, Cyto Megalo virus or rubella virus either local/ systemic prior to attack of RA. Inflammatory fluid contains lymphocytes plasma cells and some macrophages at places forming lymphoid follicles. These is foci fibroid necrosis and fibrin deposition.

- Inflammation: Inflammation of synovial membrane oedematous thick with inflammatory exudation. It follows 3 stage synovitis, destruction and deformity. Microscopy shows lymphoid follicles forming nodules with scattered cells.

Clinical features [31]

Age: Common in adults and middle aged subjects (20-40 years).

Sex: Common in females (3:1)

- Morning stiffness
- Involves small joints of hands and feet later spread to proximal joint like knee, hips, elbow and shoulder
- Occasionally mono articular joint affected
- Inflammation –spindle shaped
- Joint line tenderness and movements are painful and limited
- There is effusion in to the joint
- Synovium becomes thick and tender
- If presence of knee joint swelling its look like fusiform shape with pericapsular swelling
- Tennis elbow present

Extra articular manifestation

Skin: Subcutaneous nodules, palmar erythema and fragility of the skin. Rheumatoid vaculitis.

Neurologic manifestation [32]

Peripheral neuropathy produced by proliferating synovium causing compression of nerves carpal tunnel syndrome, tarsal tunnel syndrome offer associated with wrist or foot drop.

Ophthalmic manifestation [33]

Siogrens syndrome cause corneal damage associated with dryness of the eyes. Scleritis may result in visual impairment.

Felty's syndrome [34]

Triad of chronic rheumatoid arthritis, splenomegaly and neutropenia is associated with lymphadenopathy

Causes of RA [35]

Rheumatoid arthritis occurs when immune system attacks the synovium which is the lining of the membrane that surround joints.

The resulting inflammation thickens the synovium, which can eventually destroy the cartilage and bone within the joint

The tendons and ligaments that hold the join together weaken and stretch. Gradually the joint loses its shapes and alignment.

Genetic component actually causes rheumatoid arthritis .they can make more susceptible to environmental factors, such as infection with certain viruses and bacteria that may trigger the disease. [36]

Symptoms of RA

- Swelling, warm and stiffness around the affected joints
- Loss of appetite that results in weight loss
- Fever and fatigueness
- Continuity of stiffness in the morning for around 1-2 hrs
- Firm bumps of tissue under the skin on arm (rheumatoid nodules)

Early RA tends to affect the small joint first particularly the joints that attach fingers to hands toes and feet. As the disease progresses symptoms often spread to the wrists, knee ankles elbows, hips and shoulders in more cases symptoms occurs in the same joints on both sides of the body.

Signs and symptoms may vary in severity and may even come and go .periods of increased disease activity, called flases, alternate with periods of relative remission, when the swelling and pain fade or disappear. Over time, RA can cause joints to deform and shift out of place

Onset

Insidious -70%

Oligo articular - 44%

Poly articular - 35%

Mono articular - 21%

Acute - 15%

Systemic - 10%

Palindromic - 5%

Insidious

The majority of RA develops insidiously over weeks or months with gradually increasing joint involvement.

Poly articular onset

This onset is very rapid, even over night with severe symmrtrical poly articular involvement.

Mono articular onset

In young women initially very limited joint involvement particularly involving the knee.

Systemic onset

Common middle aged women, Non-articular features may dominate the clinical picture, fever, Myalgia, Weight loss, anaemia, pleural effusion, vasculitis lesion may be severe

Palindric onset

Patient with RA persistent joint disease may be antedated by repeated attacks of acute self- limiting synovitis affecting variable number of joints.

Risk factors

Factors that may increase risk of rheumatoid arthritis include

Sex: women are more likely to develop rheumatoid arthritis

Age: rheumatoid arthritis can occur at any age but it most commonly begins between the age of 40 and 60

Familial history: If a member of family has rheumatoid arthritis, it may have an increased risk of the disease

Rheumatoid nodules

Rheumatoid nodules are the most common dermatologic manifestation of Rheumatoid arthritis (RA), occurring in approximately one-fourth of RA patients. Similar to other autoimmune diseases, RA affects women three times as often as men. Antibodies created by the immune system against one's own cells cause inflammation and pain in the synovial lining of joints and other tissues. The results of this inflammation include morning stiffness, joint destruction, symmetric multi-joint arthritis and sometimes lung, heart or other organ complications. Severity of this disease is wide ranging from mild discomfort to complete debilitation and the presence of nodules may signify a more severe and destructive form of RA.

Rheumatoid nodules are well-demarcated; flesh colored, subcutaneous lumps or masses which are usually freely movable, though attachment to underlying tissues is possible. The nodules can vary in size from small, pea sized lesions up to the size of a lemon. RA nodules are most often round though sometimes taking a linear shape. To the touch, nodules may feel doughy or firm however are usually not tender unless there is inflammation, ulceration or impingement of underlying structures such as nerves. Typically rheumatoid nodules are distributed over areas of repeated trauma or pressure and occur adjacent to joints on extensor surfaces, such as the elbow, fingers and forearms. In patients who are bedridden, areas of prolonged pressure such as the heel, Achilles tendon, posterior scalp, hip and sacrum are often affected.

Rheumatoid nodules may also occur internally, in sites unassociated with joints, such as in the sclera of the eyes, lungs or vocal cords, however diagnosis in those

locations is difficult. In patients treating their RA with methotrexate (an immune modulating drug), accelerated formation and growth of rheumatoid nodules, most commonly in the joints of the hands, may occur. This is known as accelerated nodulosis. Discontinuation of methotrexate or the addition of another medication may reverse or shrink nodules due to methotrexate therapy, though collaboration with the patient's rheumatologist is important before any medication changes. Interestingly, rheumatoid nodules occur almost exclusively in seropositive patients, meaning patients who are positive for a marker of RA known as Rheumatoid Factor (RF).

Diagnosis of rheumatoid nodules can typically be made based on a positive history of RA and thorough physical exam demonstrating asymptomatic, slow growing, moveable subcutaneous nodules on extensor surfaces. This is especially true when the patient's rheumatoid factor titer is high. In most patients, the arthritis symptoms precede the development of rheumatoid nodules, however in a small percentage; nodules are present on initial diagnosis. Biopsy of mature lesions, though often not necessary, show a very typical histological appearance of central necrosis surrounded by palisading histiocytes and macrophages with an outer layer of fibroblasts, lymphocytes and plasma cells owing to the rich vascular supply.

Of note, other immune-mediated diseases, such as systemic lupus erythematosus or ankylosing spondylitis, may produce lesions with a very similar histological appearance. Additionally, in young healthy children, a similar benign often self-resolving dermatologic entity occurs known as subcutaneous granuloma annulare or pseudorheumatoid nodules. The differential diagnosis for subcutaneous nodules is extensive and includes fibromas, tophi (gout or pseudogout), metastatic lesions, xanthomas, epidermoid cysts, subcutaneous granuloma annulare, basal cell carcinoma and a variety of other lesions.

Treatment of rheumatoid nodules is not always necessary, especially when they are mostly a cosmetic concern. Symptomatic lesions however, such as those infected, ulcerated, impinging of underlying nerves, or causing dysfunction due to location (e.g. the bottom of the foot), deserve more aggressive therapy. This is particularly true if a patient is on immunosuppressive medications for their RA. Injection with corticosteroids such as methylprednisolone is one treatment option that is usually

successful in decreasing the size of the nodules; however increased risk of infection or ulceration occurs with any type of injection. If lesions are already ulcerated, infected, entrapping peripheral nerves, limiting range of motion or in a location that incurs repeated trauma, then surgical excision is indicated. Unfortunately, nodules often recur at sites of excision. Patients should talk with their physician and decide the best course of action in each individual case. Though rheumatoid nodules typically are benign in nature, negative psychological effects due to the physical limitation and deformity are not uncommon. Patients should collaborate with their physician to receive treatment appropriate for their physical and psychological needs.

Complications

- Joint destruction and deformity: In rheumatoid arthritis, the body's immune system attacks the joint lining (synovium), leading to cartilage and bone damage. If untreated, chronic joint inflammation can lead to permanent joint damage and deformity. The best way to prevent deformities of the hands, fingers, feet, and toes is treatment early in the disease.

- Loss of function and disability: Rheumatoid joint inflammation affects tissues surrounding the joint, including tendons, ligaments, and muscles that stabilize joints. This weakens the joints and can lead to a loss of function and disability.

- Osteoporosis: People who have rheumatoid arthritis are at increased risk for the bone-thinning condition osteoporosis. Bone loss associated with the inflammation of rheumatoid arthritis is one cause for this increased risk. Joint damage and disability may also contribute to inactivity, which increases the risk of osteoporosis. Moreover, corticosteroids that are used to treat rheumatoid inflammation can also promote bone loss.

- Coronary artery disease: People who have rheumatoid arthritis have an increased risk of coronary artery disease (hardening of the arteries to the heart) compared to those who don't have rheumatoid arthritis. Inflammation is associated with both rheumatoid arthritis and coronary artery disease. Researchers suspect that inflammation related to rheumatoid arthritis triggers the buildup of plaque in the arteries.

- Anemia: Many people who have rheumatoid arthritis may also have anemia, a condition in which the body lacks enough red blood cells. Red blood cells are needed to deliver oxygen to the body tissues. Anemia often causes fatigue and other symptoms. The inflammation of chronic rheumatoid arthritis decreases the formation of red blood cells in the bone marrow, which leads to anemia.

- Early death: Untreated rheumatoid arthritis increases the risk of mortality. People who have untreated rheumatoid arthritis are twice as likely to die compared to age-matched controls that do not have the disease. At least 25% of people with RA die from cardiovascular disease, and another 25% die from infections.

- Depression and nervous system: People with RA commonly develop depression. Changes in behavior, cognitive dysfunction, problems with peripheral nerves, and spinal cord compression may also occur. The inflammatory process of RA affects the brain and nerves, which leads to neuropsychiatric symptoms.

Others

Osteoporosis: Some medications used for treating rheumatoid arthritis,can increase risk of osteoporosis which is a condition that weakens bones and makes them more prone to fracture.

Carpal tunnel syndrome: If RA affects wrists the inflammation can compress the nerve that serves most of hand and fingers

Heart problems: RA can increase risk of hardened and blocked arteries, as well as inflammation of the sac that encloses heart

Lung disease: People with RA have an increased risk of inflammation and scarring of the lung tissues, which can lead to progressive shortness of breath.

Investigation [37]

1. Haematological:
    a) ESR- increased in active stage.

Determination of erythrocyte sedimentation rate by Westergren method is a sensitive indicator of the presence of systemic or locally severe inflammation

b) Serum protein-Hyper globulinaemia with elevation of gamma &alpha-2 globulin and hypoalbuminaemia during active phase.

- ❖ TRBC
- ❖ TWBC
- ❖ Platelets
- ❖ ESR
- ❖ RF
- ❖ CRP
- ❖ Antinuclear antibodies (ANA)
- ❖ Anti-CCP.

Rheumatoid factor

Rheumatoid factor is an immunoglobulin M (IgM) antibody diretced against normal human immunoglobulin it is usually measured by agglutination test (IgG). (Agglutination of IgG coated latex particles) and reported as either negative or positive with titres up to 1:320.

The rheumatoid factor is an antibody present in the blood of many patients with rheumatoid arthritis. Doctors measure the level of rheumatoid factor by performing a blood test. A positive rheumatoid factor test means that the patient's level of rheumatoid factor in their blood is considered high.

Rheumatoid factor was first described in connection to rheumatoid arthritis in 1940. For decades, a positive rheumatoid arthritis test was used to diagnose those with symptoms of rheumatoid arthritis.

Today, a positive rheumatoid factor test is mostly used as a supportive tool in helping doctors reach a diagnosis. Yet, it must be combined with other tools and

criteria. A positive rheumatoid factor test is also used to determine a general prognosis for rheumatoid arthritis in adults and children.

All immune systems contain healthy antibodies that fight off infections caused by bacteria and viruses. Sometimes, however, bad antibodies are produced by the immune system which can attack healthy antibodies. In other words, the antibodies turn against the person's system damaging their health.

When antibodies begin to attack the person, it can result in triggering an immune response. This immune response can turn into an autoimmune disorder which in turn can produce a variety of different symptoms, ultimately damaging that person's health.

Rheumatoid factor is a bad protein antibody produced by a person's immune system. If triggered, rheumatoid factor antibodies attack the healthy tissue in the patient's body. Rheumatoid factor attacks result in a variety of inflammatory symptoms.

Typically, these symptoms cause inflammation in the synovium of the patient's joints, which leads to rheumatoid arthritis. Rheumatoid factor can also cause other symptoms seen in autoimmune disorders like Sjogren's Syndrome or Hepatitis C.

The reason rheumatoid factor is commonly associated with rheumatoid arthritis specifically is because when the antibody was discovered it was used to diagnose rheumatoid arthritis. Approximately 80% of adult rheumatoid arthritis patients test positive for rheumatoid factor. However, people without any autoimmune disorders can also test positive for rheumatoid factor.

It is unknown what causes rheumatoid factor to develop in someone's blood. It is thought to be however a combination of genetics and other external risk factors. Certain people have low levels of rheumatoid factor, which may not ever be enough to trigger an autoimmune response.

On the other hand, some people have high levels of rheumatoid factor and go on to develop autoimmune disorders like rheumatoid arthritis. It isn't known what triggers the autoimmune response that causes rheumatoid arthritis.

Testing for rheumatoid factor is like any other blood test. The rheumatoid factor test is done by drawing blood and analyzing it for the presence of this particular protein antibody. If the results show a certain level of rheumatoid factor, then the test reads positive for rheumatoid factor. Rheumatoid factor tests can also detect lower levels of rheumatoid factor that don't result in it being declared a positive reading.

Testing positive for rheumatoid does not necessarily mean the patient has rheumatoid arthritis. A positive for rheumatoid factor test results means that it can lead to or is the cause of inflammatory symptoms from an autoimmune disorder. In certain cases, however, patients may test positive for rheumatoid factor and remain healthy never to experience symptoms.

Rheumatoid factor can be present in patients several months or even years before clinical rheumatoid arthritis symptoms develop. Depending on the level of symptoms a patient exhibits, the rheumatoid factor test results can assist doctors in reaching a rheumatoid arthritis diagnosis.

The amount of rheumatoid factor in blood can be measured in two ways:

- **Agglutination tests.** One test method mixes blood with tiny rubber (latex) beads that are covered with human antibodies. If RF is present, the latex beads clump together (agglutinate). This method is best used as a first-time screening test for rheumatoid arthritis. Another agglutination test mixes the blood being tested with a sheep's red blood cells that have been covered with rabbit antibodies. If RF is present, the red blood cells clump together. This method is often used to confirm the presence of RF.
- **Nephelometry test.** This test mixes the blood being tested with antibodies that cause the blood to clump if RF is present. A laser light is shined on the tube containing the mixture, and the amount of light blocked by the blood sample is measured. As levels of RF increase, more clumping occurs, causing a cloudier sample and less light to pass through the tube.

Positive rheumatoid factor results in someone who has been clinically diagnosed with rheumatoid arthritis may also indicate the potential for a more aggressive disease course. This is possible in both children and adult patients.

Patients who test negative for rheumatoid factor but still exhibit symptoms and meet other diagnostic criteria may still be diagnosed with rheumatoid arthritis.

Patients who test positive for rheumatoid factor aren't necessarily diagnosed with rheumatoid arthritis right away. There must also be a clear history of consistent rheumatoid arthritis symptoms.

Suspects symptoms of rheumatoid arthritis, other criteria must first be taken into consideration before reaching a diagnosis. The presence of rheumatoid factor alone does not typically lead to an immediate rheumatoid arthritis diagnosis. Doctors look at physical symptoms, medical history and may conduct other blood tests, like anti-CCP, and imaging scans to help identify the condition.

Depending on the final diagnosis, the presence of rheumatoid factor in a patient's blood may help determine the type of treatment that will be provided to that patient, such as disease-modifying antirheumatic drugs (DMARDs).

A positive rheumatoid factor test along with a rheumatoid arthritis diagnosis can potentially help the doctor determine a prognosis, although this depends on a variety of factors. Determining a prognosis for rheumatoid arthritis patients is difficult. The disease affects different people in different ways. A prognosis can depend largely on the types of symptoms the patient displays as well as their medical history.

Many doctors and researchers feel that a positive rheumatoid factor test result may predict a more severe pattern of symptoms and overall disease course. Extra-articular symptoms like rheumatoid nodules may be more likely to form in patients who have positive rheumatoid factor blood test results. Other aggressive symptoms, though rare, could be more likely to occur in rheumatoid factor positive patients. These may include lung and heart conditions.

Rheumatoid factor positive patients may also have a higher disease activity score, meaning frequent flare-ups and fewer remission periods. Keep in mind this isn't always the case. If rheumatoid factor is tested and symptoms are detected early, a diagnosis can be quickly reached. Treatment can begin as soon as possible to prevent further progression of joint damage, swelling and pain.

a) Rose waaler test:

A special type of passive haem agglutination test is rose waaler test. In RA autoantibodies appears in the serum, which acts as an antibody to gamma globulin. The RA factor is able to agglutinate red cells coated with globulin. The antigen used for the test is suspension of sheep erythrocytes sensitized with subagglutinating dos of rabbit anti-sheep erythrocyte antibody. (amboceter).

b) Latex agglutination test (latex fixation test)

c) Differential agglutination test(DAT)

d) Human erythrocyte agglutination test(HEAT)

e) Anti-nuclear antibodies.

Routine determination of the presenceof anti-nuclear antibodies is performed by the indiretc immunofluorescent technique.

C reactive protein [38]

CRP is produced in the liver and is normally found in serum in minute amounts (less than 0.6 mg/dl). In conditions characterized by inflammation with tissue destruction, the CRP level may increase. Although CRP has many effects in the immune system, its specific primary role is still unclear.

C-reactive protein (CRP) is a substance produced by the liver in response to inflammation. Other names for CRP are high-sensitivity C-reactive protein (hs-CRP) and ultra-sensitive C-reactive protein (us-CRP).

A high level of CRP in the blood is a marker of inflammation. It can be caused by a wide variety of conditions, from infection to cancer. High CRP levels can also indicate that there's inflammation in the arteries of the heart, which can mean a higher risk for heart attack. However, it's important to remember that the CRP test is an extremely nonspecific test, and CRP levels can be elevated in any inflammatory condition.

C-reactive protein is measured in milligrams of CRP per liter of blood (mg/L). In general, a low C-reactive protein level is better than a high one, because it indicates less inflammation in the body.

According a reading of less than 1 mg/L indicates you're at low risk of cardiovascular disease. A reading between 1 and 2.9 mg/L means you're at intermediate risk. A reading greater than 3 mg/L means you're at high risk for cardiovascular disease. A reading above 10 mg/L may signal a need for further testing to determine the cause of such significant inflammation in your body.

A CRP reading of greater than 10 mg/L is especially high and may indicate:

- a bone infection, or osteomyelitis
- an autoimmune arthritis flare-up
- tuberculosis
- lupus, connective tissue disease, or other autoimmune diseases
- cancer, especially lymphoma
- pneumonia or other significant infection

Note that CRP levels may also be elevated if you're on birth control pills. However, other markers of inflammation are not necessarily abnormal in these individuals. Elevated CRP values in pregnancy may be a marker for complications, but more studies are necessary to fully understand the role of CRP and pregnancy.

If pregnant or have any other chronic infection or inflammatory disease, a CRP test is unlikely to accurately assess your risk for heart disease. Before having a CRP test, speak to your doctor about any medical conditions that may skew the test results. Since there are other blood tests that can be performed instead, you might wish to forego a CRP test altogether.

Anti-citrullinated protein antibodies (ACPA) or anti-cyclic citrullinated protein antibodies (anti-CCP) are autoantibodies (antibodies directed against one or more of an individual's own proteins) that are frequently detected in the blood of rheumatoid arthritis patients.

ANA

The antinuclear antibody (ANA) test is used as a primary test to help evaluate a person for autoimmune disorders that affect many tissues and organs throughout the

body (systemic) and is most often used as one of the tests to help diagnose systemic lupus erythematosus (SLE).

ANA are a group of autoantibodies produced by a person's immune system when it fails to adequately distinguish between "self" and "nonself." They target substances found in the nucleus of a cell and cause organ and tissue damage. Depending on a person's signs and symptoms and the suspected disorder, ANA testing may be used along with or followed by other autoantibody tests. Some of these tests are considered subsets of the general ANA test and detect the presence of autoantibodies that target specific substances within cell nuclei, including anti-dsDNA, anti-centromere, anti-nucleolar, anti-histone and anti-RNA antibodies. An ENA panel may also be used in follow up to an ANA.

These supplemental tests are used in conjunction with a person's clinical history to help diagnose or rule out other autoimmune disorders, such as Sjogren syndrome, polymyositis and scleroderma.

Different laboratories may use different test methods to detect ANA. Two common methods include immunoassay and indirect fluorescent antibody (IFA). IFA is considered the gold standard. Some laboratories will use immunoassay to screen for ANA and use IFA to confirm positive or equivocal results.

- Indirect fluorescent antibody (IFA)—this is a method in which a person's blood sample is mixed with cells that are affixed to a slide. Autoantibodies that may be present in the blood react with the cells. The slide is treated with a fluorescent antibody reagent and examined under a microscope. The presence (or absence) and pattern of fluorescence is noted.

- Immunoassays--these methods are usually performed on automated instrumentation but may be less sensitive than IFA in detecting ANA.

Other laboratory tests associated with the presence of inflammation, such as erythrocyte sedimentation rate (ESR) and/or C-reactive protein (CRP), may also be used to evaluate a person for SLE or other autoimmune disease. The ANA test is ordered when someone shows signs and symptoms that are associated with a systemic

autoimmune disorder. People with autoimmune disorders can have a variety of symptoms that are vague and non-specific and that change over time, progressively worse, or alternate between periods of flare ups and remissions. Some examples of signs and symptoms include:

- Low-grade fever
- Persistent fatigue, weakness
- Arthritis-like pain in one or more joints
- Red rash (for lupus, one resembling a butterfly across the nose and cheeks)
- Skin sensitivity to light
- Hair loss
- Muscle pain
- Numbness or tingling in the hands or feet
- Inflammation and damage to organs and tissues, including the kidneys, lungs, heart, lining of the heart, central nervous system, and blood vessels

A positive ANA test result means that autoantibodies are present. In a person with signs and symptoms, this suggests the presence of an autoimmune disease, but further evaluation is required to assist in making a final diagnosis.

Tests for ANA

- Immunoassay (enzyme linked immunosorbent assay, ELISA, or enzyme immunoassay, EIA)—the results are usually reported as a number with an arbitrary unit of measure (abbreviated as a "U" on the report, for example).

- Indirect fluorescent antibody (IFA)—the results of this method are reported as a titre. Titers are expressed as ratios. For example, the result 1:320 means that one part blood sample was mixed with 320 parts of a diluting substance and ANA was still detectable.

In addition to a titer, positive results on IFA will include a description of the particular type of fluorescent pattern seen. Different patterns have been associated with different autoimmune disorders, although some overlap may occur. Some of the more common patterns include:

- Homogenous (diffuse) associated with SLE, mixed connective tissue disease, and drug-induced lupus
- Speckled associated with SLE, Sjgren syndrome, scleroderma, polymyositis, rheumatoid arthritis, and mixed connective tissue disease
- Nucleolar associated with scleroderma and polymyositis
- Centromere pattern (peripheral) associated with scleroderma and CREST (Calcinosis, Raynaud syndrome, Esophogeal dysmotility, Sclerodactyly, Telangiectasia)

A positive result from the ELISA or EIA method will be a number of units that is above the laboratory's reference number (cutoff) for the lowest possible value that is considered positive. An example of a positive result using the IFA method would give the dilution titer and a description of the pattern, such as "Positive at 1:320 dilution with a homogenous pattern."

For either method, the higher the value reported, the more likely the result is a true positive. ANA test results can be positive in people without any known autoimmune disease and thus need to be evaluated carefully in conjunction with an individual's signs and symptoms.

An ANA test may become positive before signs and symptoms of an autoimmune disease develop, so it may take time to tell the meaning of a positive ANA in a person who does not have symptoms.

Conditions associated with a positive ANA test

The most common condition is SLE.

- SLE- ANA are most commonly seen with SLE. About 95% of those with SLE have a positive ANA test result. If someone also has symptoms of SLE, such as arthritis, a rash, and skin sensitivity to light, then the person probably has SLE. A positive anti-dsDNA and anti-SM (often ordered as part of an ENA panel) help confirm that the condition is SLE.

Other conditions in which a positive ANA test result may be seen include:

- Drug-induced lupus - a number of medications may trigger this condition, which is associated with SLE symptoms. When the drugs are stopped, the symptoms usually go away. Although many medications have been reported to cause drug-induced lupus, those most closely associated with this syndrome include hydralazine, isoniazid, procainamide, and several anticonvulsants. Because this condition is associated with the development of autoantibodies to histones, an anti-histone antibody test may be ordered to support the diagnosis.
- Sjogren syndrome - 40-70% of those with this condition have a positive ANA test result. While this finding supports the diagnosis, a negative result does not rule it out. A health practitioner may want to test for two subsets of ANA: Anti-SS-A (Ro) and Anti-SS-B (La). About 90% or more of people with Sjögren syndrome have autoantibodies to SSA.
- Scleroderma (systemic sclerosis) - About 60-90% of those with scleroderma have a positive ANA. In people who may have this condition, ANA subset tests can help distinguish two forms of the disease, limited versus diffuse. The diffuse form is more severe. The limited form is most closely associated with the anticentromere pattern of ANA staining (and the anticentromere test), while the diffuse form is associated with autoantibodies to Scl-70.
- Less commonly, ANA may occur in people with Raynaud syndrome, arthritis, dermatomyositis or polymyositis, mixed connective tissue disease, and other autoimmune conditions. For more on these, read the article on Autoimmune Diseases.

A health practitioner must rely on test results, clinical symptoms, and the person's history for diagnosis. Because symptoms may come and go, it may take months or years to show a pattern that might suggest SLE or any of the other autoimmune diseases.

A negative ANA result makes SLE an unlikely diagnosis. It usually is not necessary to immediately repeat a negative ANA test; however, due to the episodic nature of autoimmune diseases, it may be worthwhile to repeat the ANA test at a future date if symptoms recur.

Aside from rare cases, further autoantibody (subset) testing is not necessary if a person has a negative ANA result.

ANA testing is not used to track or monitor the clinical course of SLE, thus serial ANA tests for diagnosed patients are not commonly ordered.

Use of a number of drugs, some infections, autoimmune hepatitis and primary biliary cirrhosis as well as other conditions mentioned above can give a positive result for the ANA test.

About 3-5% of healthy Caucasians may be positive for ANA, and it may reach as high as 10-37% in healthy individuals over the age of 65 because ANA frequency increases with age. These would be considered false-positive results because they are not associated with an autoimmune disease. Such instances are more common in women than men.

Anti – CCP

CCP (cyclic citrullinated peptide) antibody is an autoantibody against citrullinated proteins (ACPA). The anti-CCP test is able to detect the autoantibodies against citrullinated proteins which have a relatively high sensitivity (reportedly between 50 and 75 percent) for rheumatoid arthritis and extremely high specificity (about 90 percent) for rheumatoid arthritis. Its high specificity is why the anti-CCP test has become an important part of the diagnostic process for rheumatoid arthritis.

Anti-CCP Test: Its Diagnostic and Prognostic Value

Anti-CCP test helps to distinguish rheumatoid arthritis from other inflammatory types of arthritis. It is also extremely valuable in diagnosing people who are seronegative for rheumatoid factor. Not only does the anti-CCP test have diagnostic value, it also has prognostic value. If anti-CCP is present at a moderate to high level, it not only helps to confirm the diagnosis, it suggests there may potentially be a more destructive and severe disease course (i.e., progressive joint damage). Low levels of the antibody are not as telling or predictive.

Usually, the anti-CCP test is ordered along with the rheumatoid factor test, since neither test alone can definitively confirm the diagnosis of rheumatoid arthritis. According to rheumatologist Scott J. Zashin, MD, "While rheumatoid factor is more common in people who have rheumatoid arthritis, there are people who are positive for rheumatoid factor who do not have rheumatoid arthritis.

Furthermore, the presence of the rheumatoid factor has less prognostic significance than ACPA. Also, if a person is negative for rheumatoid factor, they are less likely to be positive for ACPA."

Identifying Autoantibodies

The importance of identifying autoantibodies in rheumatic diseases had been recognized for decades, but identifying those that were clinically relevant in terms of specificity and sensitivity took time. There have been three generations of the anti-CCP test. With each generation of testing the specificity and sensitivity have improved.

According to Kelley's Textbook of Rheumatology, more than 90 percent of people with undifferentiated arthritis who test positive for anti-CCP develop rheumatoid arthritis within 3 years. Only about 25 percent of those with undifferentiated arthritis who test negative for anti-CCP develop rheumatoid arthritis.

Autoantibodies may be detectable before the clinical onset of certain autoimmune diseases, including rheumatoid arthritis. Autoantibodies can precede the onset of seropositive rheumatoid arthritis by 2 to 6 years, according to Kelley's Textbook of Rheumatology. Reportedly, anti-CCP that precedes the diagnosis of rheumatoid arthritis is twice as prevalent as rheumatoid factor that precedes the diagnosis. While rheumatoid factor usually remains consistently present, the presence of anti-CCP can vary in rheumatoid arthritis patients—even disappearing in some cases.

Significance of Anti-CCP

As researchers work to determine the cause (i.e., etiology and pathogenesis) of rheumatoid arthritis, defining which antigens promote the formation of autoantibodies against citrullinated proteins is an important task.

Peptidylarginine deiminase (PAD) enzymes, which catalyze the conversion of peptidylarginine to peptidylcitrulline, have a significant role in generating autoantigens in rheumatoid arthritis. Also, more research is needed to determine what causes the pre-symptomatic phase of rheumatoid arthritis to shift into a symptomatic, full-fledged disease process.

While the specificity of anti-CCP is very high for rheumatoid arthritis, positive results can occur with other autoimmune rheumatic diseases, tuberculosis, and chronic lung disease. Anti-CCP antibodies have been reported in systemic lupus erythematosus and primary Sjogren's syndrome, typically when erosive arthritis is present.

It has also been found in up to 16 percent of people with psoriatic arthritis- most often with erosive or polyarthritis. Sometimes, it accompanies severe psoriasis without arthritis.

Anti-CCP antibodies are a highly specific marker for RA in several diverse patient groups. This specificity extends to patients with early disease, in whom a timely diagnosis is most needed. The low sensitivity of the test (40–50% in most published cohorts) indicates that a negative anti-CCP antibody test does not exclude disease, but its high specificity means that a positive result markedly increases the probability that the patient will have RA. Anti-CCP antibodies also identify a subset of patients who are likely to have substantial ongoing disease activity, accrue more damage, and who will probably benefit most from early aggressive treatment. A significant number of these patients do not have rheumatoid factor, and may not otherwise have been expected to develop severe aggressive disease. Anti-CCP antibodies tend to remain stable or decline slightly with treatment, and have not been found frequently in non-RA inflammatory or arthritic diseases. The presence of anti-CCP antibodies in serum years before the onset of RA suggests the possibility of pre-clinical detection, and may provide information about early events in the pathogenesis of the disease.

Special investigation

Synovial fluid analysis confirms the presence of the inflammatory arthritis. Fluid may show positive rose-waaler test in joint fluid before it can be detected in blood. Also it may show neutrophils or monocytes containing cytoplasmic inclusion bodies.

Gross test of inflammation in synovial fluid found in patient with RA has a WBC count of greater than 2000/cumm and may be either translucent or opaque.

Synovial membrane biopsy

Villus formation with thickening of synovial layer and infiltration with abnormal cells.

Radiographic evaluation

The primary value of radiography is to determine the extent of cartilage destruction and bone erosion produced by the disease.

Stages of x ray progression in RA

- Peri articular osteoporosis.
- Loss of articular cartilage(joint space)
- Erosions
- Subluxation and ankylosis

Arthroscopy

In acute RA synovium is odematous, diffusely erythematous and friable. In more chronic conditions, it becomes thickened.

Renal biopsy

Indicated in cases of reduced tubular or glomerular function.

Pulmonary biopsy

Used to distinguishing rheumatoid nodules from carcinoma or to establish diagnosis of fibrosing alveolitis.

Others

- Ultra sound
- Biochemical analysis
- Urine analysis
- CT Scanning

- Scintigraphy
- MRI

Treatment [39]

Drugs used in the treatment of Rheumatoid arthritis are

I. NSAIDS
   - Aspirin
   - Ibuprofen
   - Diclofenac
   - Naproxen
   - Piroxicam

II. DMARDs
   1. Immunosuppressants:
      - Methotrexate
      - Cyclophosphamide
      - Azathioprine
      - Cyclosporine
      - Leflunomide
   2. Biological agents:
      - TNF – alpha blockers
        - Ethanercept
        - Infliximab
        - Adalimumab
      - Inhibitors of T cell activation
        - Abataceot
      - IL – 1 antagonist
        - Anakinra
      - Anti B lymphocyte antibody
        - Rituximab
   3. Gold salts:
      - Auronofin
      - Aurothiomalate
   4. Others:

- ❖ Penicillamine
- ❖ Sulphasalazine
- ❖ Chloroquine
- ❖ Hydroxychloroquine

III. Adjuvants:

- ❖ Glucocorticoids

3.3. PHARMACEUTICAL REVIEW

CHOORANAM

Definition

Chooranam is a fine powder s of drugs. The "Chooranam" may be applied to the powders of a single drug or a mixture of two or more drugs, which are powdered separately prior to their being mixed to homogeneity.

Method of preparation

Equipment required

1. The drug enumerated in the recipe in clean and well dried state.
2. A mortar and pestle.
3. A fine sieve or fine cloth of close mesh.

Process of preparation

The drugs which are to be used in the preparations should be taken from recently collected material. Drugs which are aged by prolonged storages or changed in colour, taste and scent, and those that are insects infested or attacked by fungi should be positively rejected.

However drugs like Embelia fruits, Senna, Long Pepper, Jaggery and cows ghee are preferred from fairly aged stock, provided they are not infested with pests, deteriorated or spoiled or developed racidity.

In general the aromatic drugs are slightly fried in order to enhance their aroma and milling properties. Any extraneous material, organic or inorganic, should be removed from the drugs by close inspection.

The Chooranam should be so fine to be called amorphous and should never be damp. The fineness of the sieve should be 100 mesh or still finer.

Purification of the prepared chooranam

தானென்ற சூரணத்தின் சுத்திக்கேளு
தப்பாதே சரக்கெல்லாஞ் சூரணித்து
நானென்ற வாவின் பாலாற் பிசைந்து
நலமான சட்டியிலே பாலைவிட்டு
வானென்ற சுத்தசலம் பாதிவிட்டு
வளமாக மேற்சீலை கோடு கட்டிப்
பானென்ற சூரணத்தைப் பிட்டுபோல் வைது
பதறாதே வெந்தெடுக்கச் சித்தியமே!

- அகஸ்தியர் வைத்திய இரத்தினச் சுருக்கம்

The prepared chooranam is mixed with the milk in pot half quantity milk and half quantity water is taken. The mouth of the pot is covered with a thin cloth material. Above this cloth the mixed chooranam is placed. The pot is placed over the stove and heated.

ஆமப்பா ரவியுலர்த்திப் பொடிதான் செய்து
அப்பனே சமனாய்ச் சர்க்கரையைச்சேர்த்து
நாமப்பா கொண்டு வர தோஷம் போச்சு
நன்றாக்ச் சுத்தி செய்யாச் சூரணந்தான்
தாமப்பா ரோகத்தை வெல்லா தப்பா
தளமான வியதி யெல்லாம் பாரிக்கும் பார்
வேமப்பா சுத்தி செய்து கொண்டாயனால்
வெகுசுறுக்காய் தீருமா வியாதி கேளு

- அகஸ்தியர் வைத்திய இரத்தினச் சுருக்கம்
-

Then the Chooranam is placed in the sunlight and powdered. Equal amount of sugar is added and taken internally. All type of diseases get cured. If the drug is taken without purification the disease does not cure. If taken after purification the disease cures easily.

Storage

The prepared Chooranam should be allowed to cool by spreading and mixing, prior to packing. They should be stored in tightly stoppered glass, polythene or tin containers, or in polythene or cellophane bags and sealed. These bags should in turn be enclosed in cardboard boxes.

The Chooranam to facilitate easy handling and to assure exact dosage administration, could be pressed into tablets, could be packed in bottles or tubes made either of glass or plastic or packed in strip of metal foil or plastic sheets.

In industry the tablets are made, counted & packed by electronic devices.

Then Chooranam is said to retain its potency for 2 months and then gradually deteriorate. However if properly packed & stored they keep good for a year.

According to AYUSH guidelines shelf life of Chooranam is one year.

Table No: 1 Analytical specifications of *Churna/Choornam* [40]

Sl.No	TESTS
1.	Description
	Macroscopic, Microscopic
2.	Loss on drying at 1050 C
3.	Total – ash
4.	Acid – insoluble ash
5.	Water-soluble extractive
6.	Alcohol – soluble extractive
7.	Particle size (80-100 mesh for Churna; 40-60 mesh for churna)
8.	Identifications, TLC/HPTLC-with marker (wherever possible)
9.	Test for heavy/Toxic metals
	Lead
	Cadmium
	Mercury
	Arsenic
10.	Microbial contamination
	Total bacterial count
	Total fungal count
11.	Test for specific Pathogen
	E. coli
	Salmonella spp.
	S.aureus
	Pseudomonas aeruginosa
12.	Pesticide residue
	Organochlorine pesticides
	Organophosphorus pesticides
	Pyrethroids
13	Test for Aflatoxins (B1,B2,G1,G2)

3.4. PHARMACOLOGICAL REVIEW

EVALUATION OF ANALGESIC ACTIVITY

A) Acetic acid induced writhing test

Principle

Painful reactions in animals may be produced by chemicals also. Intraperitoneal injection of phenylquinone, bradykinin or acetic acid produces pain reaction which is characterized as a writhing response.Abdominal constriction,turning of trunk(twist) and extension of hind legs(stretching) responses by the animal are taken as reaction to chemically induced pain. Analgesics both narcotic and non-narcotic type, inhibit writhing response.

Procedure

Swiss albino mice were divided into four groups (n = 6). Group I received acetic acid (1% v/v, 10 ml/kg b.w., i.p.) and writhing reflex was noted for the period of 15 minutes. Group II received aspirin (100 mg/kg b.w.p.o.) Group III and IV received trial drug at the doses of 100 mg/kg and 200 mg/kg b.w., p.o. respectively. 30 min after aspirin and trial drug administration, group II and III received acetic acid (1% v/v, 10 ml/kg b.w., i.p.) and writhing reflex was noted for the period of 15 min. [41, 42]

Writhing test is a chemical method used to induce pain of peripheral origin by injection of irritant principles like phenylquinone, acetic acid in mice. Analgesic activity of the test compound is inferred from decrease in the frequency of writhings. The manifestations of abdominal writhings in mice were first described by *Sigmund et al* as an arching of back, extension of hind limbs and contraction of abdominal musculature. The writhing response is considered as a reflexive test and is without clinical counterparts as it cannot be performed in human and sensations involved are unknown. Further, authors pointed out that the writhing test produced severe pain in mice which raises ethical concern regarding its use. Stevens has commented on the report of one pharmaceutical company, Warner–Lambert Research Institute, Morris Plains N.J. which company has conducted about 100 experiments on 5000 mice. 0.1 ml of phenylquinone was injected intraperitoneally to induce writhing and animals began stretching their

abdomen in few minutes. He wrote that writhing, of course, means agony, a standard test that should not be tolerated in any civilized country.

Writhings generated by parenteral administration of acetic acid in mice, are due to profound pain of endogenous nature which recur for a prolonged period of time. Due to irritant nature, these principles are also prone to induce lesions. Writhing is an overt response to the intense pain induced by irritant principles via nociceptors characterized by episodes of retraction of abdomen and stretching of hind limbs. The signals transmitted to central nervous system in response to pain due to irritation, cause release of mediators such as prostaglandins which contributes to the increased sensitivity to nociceptors. Writhing test was in practice for the evaluation of analgesic effect till 2004. However, the test was withdrawn from Sept. 2004, soon after implementation of report of ministry of environmental and forest, animal welfare division, Govt. of India. In CPCSEA report it was stated that laboratory animals used for the experimentation should be properly used and pain and sufferings inflicted in animals should be avoided or minimized if avoidance is not possible. Scientists and investigators should proceed on the basis that experimental procedures that cause pain or sufferings in human beings will also cause similar pain or sufferings in animals.

Despite government's decision, number of scientific journals publish papers in which writhing test is employed. It is suggestive to kindly adhere to the government's notification of avoidance of tests causing longer lasting painful infliction and to perform in this context, triple analgesic test using thermal, mechanical and electrical methods in which animals are exposed to momentary pain or sufferings. The inferences drawn from these tests can be used to evaluate analgesic effect of new active principles.

B) Hot plate assay

Principle

In this method heat is used as a source of pain. Animals are individually placed on a hot plate maintained at constant temperature (55^0C) and the reaction of animals such as paw licking or jump response is taken as the end point. Analgesic increases the reaction-time. The method was first described by Eddy and Leimbach.

Procedure

Four groups of mice (n = 6) were treated orally with trial drug (100 and 200 mg/kg b.w. p.o.), morphine sulphate (10 mg/kg b.w. p.o.) and normal saline (5 ml/kg b.w.). Mice were placed on a hot plate (Bibby Sterilin, UK) maintained at 55 ± 1°C and the reaction latency (in seconds) for licking of hind paw or jumping noted. The mice which reacted within 15 sec and which did not show large variation when tested on four separated occasions were selected for studies. Recordings were taken before treatment with the different drugs and 1, 2, 3, 4, 5 h post treatment. Results were expressed as the difference between the baseline reaction latency and the reaction latency at recorded times [43, 44].

During the experiment, the rat or mouse is introduced into an open-ended cylindrical space with a floor consisting of a heated plate. The plate heated to a constant temperature produces two behavioral components that can be measured in terms of their reaction times, namely paw licking and jumping. Both are considered to be supraspinally integrated responses.

One particularity of this test is that it can only be performed once in each animal when the jumping response is evaluated. Indeed, when the animal is exposed to the first time to the test, it learns that the experimenter takes it out of the plate as soon as the jumping behavior is done. So, when the animal is place again on the plate, it jumps after some few seconds without performing the primary licking responses.

C) Tail-flick method:

Principle

Analgesia is defined as a state of reduced awareness to pain, and analgesica are substance which decrease pain sensation (pain-killers) by increasing threshold to painful stimuli. The commonly used analgesics are aspirin, paracetamol (non-norcotic type) and morphine. (narcotic type)

Painful reaction in experimental animals can be produced by applying noxious (unpleasant) stimuli such as

1. Thermal (radiant heat as a source of pain).

2. Chemical (irritant such as acetic acid and bradykinin)

3. Physical pressure (tail compression).

In laboratory, commonly used procedures are tail flick (tail withdrawal from the radiant heat) method using analgesiometer, hot plate (jumping from the hot plate at 55^0C) method and acetic acid induced writhing. [45, 46]

D) Formalin assay

The formalin test was carried out as described by previous workers. Four groups of mice (n = 6) were treated orally with trial drug (100 and 200 mg/kg), aspirin (100 mg/kg) and normal saline (5 ml/kg b.w.). Formalin solution (1% v/v) was injected into the sub-plantar region of the right hind paw of the animals 30 min post treatment. The number of times paw was licked/bitten within the time frames of 0-5 min (neurogenic phase) and 15-30 min (inflammatory phase) after formalin administration was counted. [47, 48]

EVALUATION OF ANTI-INFLAMMATORY ACTIVITY

A) Carrageenan induced rat paw oedema

Principle

Inflammation is a tissue-reaction to infection, injury, irritation or foreign substance. It is a part of the host defence mechanism but when it becomes uncontrolled it is a hopeless condition. Aging is also considered to be an inflammatory response. There are several tissue factors or mechanism that are known to be involved in the inflammatory reactions such as release of histamine,bradikinin and prostaglandins. The development of non-steroidal anti –inflammatory agents in recent years has contributed a lot in not only overcoming the human suffering such as arthritis but also has helped in understanding the tissue mechanisms of inflammation. The student is advised to study the pathophysiology and the mechanism of action of anti-inflammatory drugs before performing this experiment for better understanding.

The inflammatory reaction is readily produced in rats in th form of paw oedema with the help of irritants. Substances such as carrageenan,formalin,bradykinin, histamine, 5 –hydroxytryptamine, mustard, or egg white when injected in the dorsum of

the foot of rats they produce acute paw edema within a few minutes of the injection. Carrageenan –induced paw oedema is the most commonly used method in experimental pharmacology. Carrageenan is a sulphated polysaccharide obtained from sea weed (rhodophyceae), and by causing the release of histamine, 5-HT, bradykinin and prostaglandins, it produces inflammation and oedema [49, 50].

Procedure

The rats were divided into four groups containing six rats in each group. 0.1 ml of 1.0% carrageenan in normal saline (0.9% w/v NaCl) was injected to the sub plantar region of right hind paw. The trial drug was administered to the rats 1 h before carrageenan injection. Different groups were treated as follows:

Group I: Carrageenan (0.1 ml of 1.0% carrageenan/rat to the sub plantar region).

Group II: Carrageenan + Indomethacin (10 mg/kg b. w., p. o.)

Group II and IV: Carrageenan + trial drug (100 mg/kg and 200 mg/kg b. w., p. o.respetcively).

The paw volume was measured initially and at 1, 2, 3 and 4 h after carrageenan injection, using Plethysmograph, inflammation was calculated for comparison.

B) Dextran induced rat paw oedema

The rats were divided into four groups containing six rats in each group. 0.1 ml of 1.0% dextran in normal saline (0.9% w/v NaCl) was injected to the sub plantar region of right hind paw [51]. The trial drug was administered to the rats 1 h before dextran injection. Different groups were treated as follows:

Group I: Dextran (0.1 ml of 1.0% dextran/rat to the sub plantar region).

Group II: Dextran + Indomethacin (10 mg/kg b. w., p. o.)

Group III and IV: Dextran +trial drug (100 mg/kg and 200 mg/kg b.w., p.o.respetcively).

The paw volume was measured initially and at 1, 2, 3 and 4 h after dextran injection, using Plethysmograph,inflammation was calculated for comparison[52,53]

.C) Histamine induced rat paw oedema

The rats were divided into four groups containing six rats in each group. 0.1 ml of 1.0% histamine sulphate in normal saline (0.9% w/v NaCl) was injected to the sub plantar region of right hind paw. The test drug was administered to the rats 1 h before histamine injection. Different groups were treated as follows:

Group I: Histamine (0.1 ml of 1.0% histamine/rat to the sub plantar region).

Group II: Histamine + Indomethacin (10mg/kg b. w., p. o.)

Group III and IV: Histamine + trial drug (100 mg/kg and 200 mg/kg b.w., p.o. respectively).

The paw volume was measured initially and at 1, 2, 3 and 4 h after histamine injection, using Plethysmograph, inflammation was calculated for comparison [54, 55]

ANTI RHEUMATIC ACTIVITY

Collagen-induced arthritis (CIA)

- The CIA model in rats is induced by intradermal injections of heterologous type II collagen in IFA, followed by a booster injection on day 7.
- Heterologous type II collagen is highly arthritogenic in DA, BB-DR and LEW, but not in BN or F344, strains of inbred rats. Homologous rat type II collagen is also arthritogenic in DA but not in other rat strains.[56]
- Erosive polyarthritis typically develops 10-14 days after the primary immunization.
- Auto reactive T cells, as well as B cells, which produce antibodies to type II collagen, play a critical role in disease progression.
- Similar to RA, females tend to be more susceptible than males, and arthritic rats also produce rheumatoid factor and antibodies against heat shock protein Hsp65[57,58,59]

Adjuvant-induced arthritis (AIA)

- Experimental arthritis was induced in rats according to method of Newbould.
- The right footpad of each rat was injected subcutaneously with 0.1 ml of Freund's Complete Adjuvant agent (FCA, sigma).
- The extract at three dose levels (100, 200 mg/Kg.i.p), distilled water and Acetyl salicylic acid (ASA) at 10mg/Kg were given daily for 16 consecutive days to the treatment groups, control group and reference group respectively.
- Treatment started from day 8^{th} after FCA injection.
- The assessment of theantiarthritic activity was done by measuring the mean paw edema on the 8th, 12, 16 and 24 days post injection of FCA[60,61]

Formalin –induced Arthritis

- The test was performed according to Dubuissson and Dennis .
- The extract was dissolved in a mixture of propylene glycol and water (1:4).
- The extracts (100, 200 mg/Kg) and ASA (10mg/Kg.) were administered i.p in a volume of 1.5-2ml.
- Control group received only vehicle (1.5-2ml) Briefly 30 min after treatment, 50 µl (2.5% v/v in distilled water) of formaldehyde was injected subcutaneously into the plantar surface of the hind paw of rats.
- The behavioral responses to nociception were noted and the time spent licking, biting or scratching the injected hind paw was recorded up to 30min.
- The first 5 min was considered as early phase (neurogenic phase) and the period of 15-30 min as late phase (inflammatory phase).

Agar- induced edema of the rat paw

- The rat paw edema method was used, whereby agar is the edematogenic agent.

- Acute inflammation was measured in terms of change in volume of the rat hind paw induced by subplantar injection of agar.
- Animals (n =5 group) received 100, 200mg/Kg of MFB administrated orally.
- Edema was induced one hour later with agar (0.1ml) injected into the sub plantar region of the right hind paw of the rats.
- The volume of distilled water displaced by the treated paw was measured before and 1,2,3 and 4 hr after induction of edema using a plethysmometer (model 7159, Ugo Basile, Varese ,Italy).
- Control groups received either equivalent volume of the vehicle (distilled water) or indomethacin (100mg/Kg).
- Inflammation was assessed as the difference between the zero time volume of the treated paw (Vo) and the volume at the various times (Vt) after the administration of the phlogistic agent.
- Percent inhibition of edema Inhibition of edema (%) = 100 [1-{(a-x)/ (b-y)}].
- Where a= mean paw volume of treated rats at various time after agar injection, x = mean paw volume of treated rats before agar injection, b=mean paw volume of control rats at various time after agar, y = mean paw volume of control rats before agar injection.

3.5 LATERAL RESEARCH

Hemidesmus indicus: [62]

- Administration of aqueous extract of *Hemidesmus indicus* roots (500mg/kg/day) for a period of 12 weeks decreased lipid peroxidation index which is attributed to its **antioxidant** action.
- Ethanolic extract of *Hemidesmus indicus has* protective effect against ethanol induced liver injury **(hepato protective)**. *H. indicus* extract significantly decreased level of liver collagen and hydroxyproline content, lipid peroxidation and increases solubility of liver collagen and ascorbic acid level.
- 95% ethanolic extracts of roots of *H. indicus* possess **antifungal activity** against *Aspergillus niger.*

- *Hemidesmus indicus* extract inhibited tumor growth in mouse skin and hence can be considered as a potent chemopreventive agent (**anti-carcinogenic activity).**

Smilax chinensis:

- The ethyl acetate, butanol and aqueous extracted fractions from *S. china* root showed high levels of DPPH free radical scavenging activity(**anti-oxidant**). [63]
- Experimental studies reveals that the aqueous and alcoholic extracts from *Smilax china* roots (200 mg/kg) orally administered for 7 days produced a significant decrease in the blood glucose level in the model of alloxan-induced diabetes in rats **(anti-diabetic activity).** [64]
- *Smilax chinensis* L. extract in affording the **hepatoprotective activity** against carbon tetrachloride may be due to the cell membrane stabilization and hepatic cell regeneration. [65]

Withania somnifera: **[66]**

- *W. somnifera* is known to alter the oxidative stress markers of the body. The root extract has found to significantly reduce the lipid peroxidation and increase the superoxide dismutase (SOD) and catalase activities, thus carrying free radical scavenging property.
- It is been proved to have **hepatoprotective** effect against radiation induced and iron induced toxicity *Withania somnifera* helped in decreasing the significantly altered levels of Bilirubin thus bringing liver to function normally. Withaferin A at 10mg/kg dose showed significantly protective effect against CCl4-induced hepatotoxicity in rats.
- Withaferin A, withanolide D & E exhibited significant **antitumour activity** in vitro against cells derived from human epidermoid carcinoma of nasopharynx (KB) and in vivo against Ehrlich ascites carcinoma, Sarcoma 180, Sarcoma Black (SBL), and E 0771 mammary adenocarcinoma in mice.

Alpinia officinarum

- **Anti-oxidant activity**

  Radical scavenging activities of galanga extracts were performed by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging assays. This study clearly showed that galanga had marked antioxidant, anticholinergic effect, reducing ability, radical scavenging and metal binding activities. [67]

- **Anticholinergic activity**

  Inhibition effects of galanga extracts on AChE activities were measured. AChI, and DTNB (Product no: D8130-1G, Sigma–Aldrich) were used for the determination of the AChE activity. Namely, 100 ml of buffer (Tris/HCl, 1 M, pH 8.0), 10 mL of sample solution dis-solved in deionised water at different concentrations. [67-a]

- **Expectorant activity**

  It was studied on rabbits partially anaesthetised with intravenous injection of urethane. [68]

4. MATERIALS AND METHODS

4.1. DRUG SELECTION

In this dissertation the ***Keelvayu Nivarana Chooranam*** was taken as a compound drug for Rheumatoid arthritis from the literature **The Pharmacopoeia of Siddha Research Medicine** (chapter 2-14) written by **Dr. M. Shanmugavelu & Dr.G.D.Naidu**. Published by: G.D.Naidu Charities, 84, President hall, Coimbatore-18. Page no: 118

Ingredients

1. *Nannariverpattai Chooranam (Hemidesmus indicus*) - 116 g
2. *Parangipattai Chooranam* (*Smilax chinensis*) - 116 g
3. *Seemai Amukara Chooranam (Withania somnifera)* - 116 g
4. *Chittaraththai Chooranam (Alpinia officinarum*) - 58 g

Source of Collection

All the raw drugs were bought from Ramasamy chetty country drug shop at Parry's corner, Chennai, Tamilnadu, India.

Identification and Authentication of the drug

All the raw drugs were identified and authenticated by the *Gunapadam* experts in Government Siddha Medical College, Arumbakkam, Chennai – 106. The specimen sample of all the herbs have been preserved in PG *Gunapadam* department individually for future reference.

Ref No: GSMC/PGGM/014-017/2014-2017

Purification of the drugs

All the drugs mentioned here were purified as per the Siddha literature.

- *Nannariverpattai* were washed in the running tap water to remove the soil and impurities.

- *Parangipattai* was dried and powdered and then it was purified by *Pittaviyal* method (steam cooking in milk). A mud pot was taken and it was half filled by milk and half filled by pure water. The mouth of the pot was sealed by a cloth. This *chooranam* then placed over the cloth and the pot was heated. The same drug was later dried and powdered then sieved again.
- *Amukara* was dried and powdered and then it was purified by *Pittaviyal* method (steam cooking in milk). A mud pot was taken and it was half filled by milk and half filled by pure water. The mouth of the pot was sealed by a cloth. This *chooranam* then placed over the cloth and the pot was heated. The same drug was later dried and powdered then sieved again.
- *Chittaraththai* were washed in the running tap water to remove the soil and impurities.

Preparation of the trial drug – *Keelvayu Nivarana Chooranam*

Procedure

All the above purified ingredients were powdered in an iron mortar separately and it was sieved by a cotton cloth. Then these powders were mixed together and bottled up. It was labeled as *Keelvayu Nivarana Chooranam* (KVNC).

Purification of the *Chooranam*: Steaming process (*Pittaviyal murai*)

The ***Keelvayu Nivarana Chooranam*** was purified by *pittaviyal* method (steam cooking in milk) as per Siddha classical literature. A mud pot was taken and it was half filled by milk and half filled by pure water. The mouth of the pot was sealed by a cloth. This chooranam then placed over the cloth and the pot was heated. After this process the drug was later dried and powdered then sieved again. It was used for the further study.

Storage of the drug

The prepared test drug was stored in a clean, air tight glass container. The contents were inspected frequently to avoid moisture and insects.

Administration of the drug

Form of the medicine : *Chooranam*

Route of Administration : Enteral

Dose : 1g twice a day

Anubanam : Honey

4.2. STANDARDIZATION OF *KEELVAYU NIVARANA CHOORANAM* BY USING MODERN TECHNIQUES

Standardization of drug helps to authenticate and determine its quality and efficiency. Standardization of herbal drug is rooted in qualitative and quantitative analysis by means of Physico chemical properties and instrumental analysis.

The Physico-chemical analysis of *Keelvayu Nivarana Chooranam* was done.

The chemical finger print was engaged by using modern analytical technique FTIR (Fourier Transform Infra-Red Spectroscopy).

The particle size and qualitative analysis of chemical elements of *Keelvayu Nivarana Chooranam* were also assessed by Scanning Electron Microscope (SEM).

The quantitative and qualitative analysis of chemical elements was carried out by using inductively X-Ray Fluorescence Spectroscopy (XRF).

ORGANOLEPTIC CHARACTERS

The organoleptic characters of the sample drug were evaluated. 1gm of the KVNC was taken and the colour, texture, particle size and other morphological characters were viewed by naked eye under natural light. Then the result is tabulated in Table No: 5

4.2.1. PHYSICO-CHEMICAL ANALYSIS

Quantitative analysis

Total Ash

3gm is accurately weighed and incinerated in a crucible dish at a temperature not exceed 450$_o$C until free from carbon. It is then cooled and weighed. The % w/w of ash with reference to the air-dried powder is calculated [69].

Water Soluble Ash

The total ash is obtained as the above method for preparation of total ash. The ash is boiled for 5 minutes with 25ml water. The insoluble ashes is collected using filter paper and washed with hot water and then transferred to the silica crucible then ignite for 15 minutes at temperature not exceeding 450^{o}C. The silica crucible and residue are weighed until constant weight is attained for determination of weight of insoluble ash. The weight of the water soluble ash was determined by subtracting the weight of insoluble ash from the weight of total ash.

Acid insoluble Ash

The total ash was obtained as the above method for preparation of total ash. The ash was boiled for 5minutes with 25ml 10% Hcl. The insoluble ashes was collected using filter paper and washed with hot water and then transferred to the silica crucible then ignite for 15 minutes at temperature not exceeding 450$_{o}$C. The silica crucible and residue are weighed until constant weight is attained.

Determination of Extractive value

Alcohol Soluble Extractive Value

3g of KVNC was weighed and macerated with 100ml of ethanol in a closed container for 24 hours. The resulting solution was shaken continuously for 6 hours. It was then allowed to stand and soak for 18 hours. The solution was filtered and evaporated of the filtrate in a flat bottomed shallow dish and dried at 105^{o}C. Then the content was cooled and weighed.

Water soluble Extractive value

3g of KVNC was weighed and macerated with chloroform and water, respectively, at 80^{o}C for 24 hrs. The resulting solution was shaken continuously for 6 hours and allowed to stand and soak for 24hrs then filtered. The solution from both chloroform and water respectively was filtered and evaporated of the filtrate in a flat bottomed shallow dish. It was dried at 105^{o}C then cooled and weighed.

Loss on Drying

The powdered drug is dried in the oven at 100- 105°C to constant weight.

Physical Characterization

Solubility

a. A little of the sample was shaken well with distilled water.

b. A little of the sample was shaken well with con Hcl and Con H_2SO_4. Sparingly soluble character indicates the presence of Silicate.

pH value

Potentiometrically pH value determined by a glass electrode and a suitable pH meter.

Action on heat

A small amount of the sample was taken in a dry test tube and heated gently. If strong white fumes evolve indicate the presence of Carbonate.

Flame test

A small amount of the sample was made into a paste with con.Hcl in a watch glass and introduced into non-luminous part of the Bunsen flame. Appearance of bluish green flame indicates the presence of Copper.

Ash Test

A filter paper was soaked into a mixture of sample and cobalt nitrate solution and introduced into the Bunsen flame and ignited. Appearance of yellow colour flame indicates the presence of Sodium [70].

All the results were noted and tabulated in Table No: 6

4.2.2 PHYTOCHEMICAL ANALYSIS

The Phytochemical screening of the extract gives general idea regarding the nature of chemical constituents present in the crude drug. The phytochemical tests were done as the method illustrated in Prashant Tiwari et al., 2011 and Harborne, 1973.

Test for Alkaloids

A small portion of solvent free extracts were stirred separately with few drops of dilute hydrochloric acid and filtered & tested carefully with various alkaloidal reagents.

Mayer's reagent - Cream precipitate

Dragendroff's reagent - Orange brown precipitate

Hager's reagent - Yellow precipitate

Wagner's reagent - Reddish brown precipitate

Test for Carbohydrates and Reducing Sugars

The minimum amount of extracts were dissolved in 5ml of distilled water & filtered. The filtrate was subjected to test for carbohydrates & glycosides.

a) Molisch's test

The filtrate 1 ml was treated with 2-3 drops of 1% alcoholic alpha naphthol & 2ml concentrated sulphuric acid was added along the sides of test tube. Violet ring was observed at the junction of 2 layers which showed the presence of carbohydrate.

b) Benedict's test

The filtrate 1 ml was treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

c) Fehling's test

The filtrate 1 ml was treated with equal volume of Fehling's solution A and B and heated gently. Orange red precipitate indicates the presence of reducing sugars.

Test for Glycosides

The extract was hydrolyzed with dil. HCl and subjected to test for glycosides.

a) Modified Borntrager's test

To the hydrolysate extract, 1 ml of Ferric chloride solution was added and immersed in boiling water for about 5 min. The mixture was cooled and extracted with equal voulme of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose pink colour in the ammoniacal layer indicates the presence of Anthranol glycosides.

b) Legal's test

The hydrolysate extract was treated with Sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of Cardiac glycosides.

Test for Saponins

The extract 0.5 ml was shaken with 5 ml distilled water. The presence of saponins was indicated by formation of copious lather.

Test for Tannins

Gelatin test

To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

Test for Phenolic compounds

To 0.5 ml of extract, 1 ml of alcoholic ferric chloride solution was added. Formation of bluish green or bluish black indicates the presence of Phenolic compounds.

Test for Phytosterol

Ferric chloride – acetic acid test

1 ml of extract was treated with 1 ml of chloroform and then, 2 ml of ferric chloride acetic acid reagent was added followed by 1 ml of conc. sulphuric acid. Appearance of reddish pink colour shows the presence of phytosterol.

Test for Diterpenes

Copper acetate test

1 ml of extract was dissolved in water and treated with 3-4 drops of Copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes.

Test for Triterpenes

Salkowski's test

1 ml of extract was treated with 1 ml of chloroform followed by 1 ml of conc. sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour shows the presence of triterpenes.

Test for Flavonoids

a) Alkaline reagent test

To 1 ml of extract, 1 ml of 10% sodium hydroxide solution was added. Formation of dark yellow colour indicates the presence of flavonoids.

b) Lead acetate test

To 1 ml of extract, 3-4 drops of 10% lead acetate solution was added. Formation of yellow precipitate indicates the presence of flavonoids.

c) Ferric chloride test

To 1 ml of extract, 3-4 drops of ferric chloride solution was added. Formation of dark green colour indicates the presence of flavonoids.

d) Shinoda test

To 1 ml of extract, few mg of magnesium turnings was added followed by few drops of conc. hydrochloric acid and boiled for five minutes in a boiling water bath. Formation of red colour indicates the presence of flavonoids.

Test for Proteins and Free Amino Acids

a) Xanthoproteic test

To 1 ml of extract, 3-4 drops of conc. nitric acid was added. Formation of yellow precipitate indicates the presence of proteins.

b) Million's test

To 0.5 ml of extract, 2.5 ml of Million's reagent was added. Formation of white precipitate and the precipitate warmed indicates the presence of proteins.

c) Biuret test

To 0.5 ml of extract, 2.5 ml of diluted Biuret reagent was added. Appearance of purple colour or brick red precipitate showed the presence of proteins and free amino acids.

Test for Quinones

Sodium hydroxide test

To 0.5 ml of extract, 1 ml of 10% sodium hydroxide was added. Appearance of blue or green or red colour shows the presence of quinones.

Results of phytochemical analysis were noted and tabulated in Table No: 7

4.2.3. TLC/HPTLC FINGER PRINT STUDIES

Preparation of spray reagent-vanillin-sulphuric acid reagent

Vanillin (1g) was dissolved in ice cold ethanol (95ml). Add to 5ml of cooled concentrated sulphuric acid. Ice was added and stirred well. The solution was stored in refrigerator.

Chromatographic conditions

Instrumen	: CAMAG (Switzerland).
Sample applicator	: Camag Linomat - IV applicator with N_2 gas flow
Photo documentation System	: Digi store - 2 documentation system with Win Cat and video scan software.
Scanner	: Camag HPTLC scanner - 3 (030618), Win Cats - IV.
Development Chamber	: Camag HPTLC 10X10, 10 X 20 twin trough linear development chamber.
Quantity applied	: 5, 10 µl for extracts and 5 µl for standards
Stationary phase	: Aluminium plate pre-coated with silica gel 60(E. Merck)
Plate thickness	: 0.2 mm.
Mobile Phase	: For Chloroform extract - Toluene: Ethyl acetate (9:1) Ethanol extract - Toluene: Ethyl acetate (1:1).
Scanning wavelength	: 254 nm
Laboratory condition	: 26 ± 5^{o}C and 53 % relative humidity

The plate was developed up to a height of 8 cm, air dried, spots were observed under the UV light at 254 and 366 nm. Finally the plates were derivatized using vanillin-sulphuric acid reagent heated at 105° till colour spots appeared.

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Retention Factor

After a separation is complete, individual compounds appear as spots separated vertically. Each spot has a retention factor (Rf) which is equal to the distance migrated over the total distance covered by the solvent.

The Rf formula is Rf = distance traveled by sample /distance traveled by solvent

The Rf value can be used to identify compounds due to their uniqueness to each compound. When comparing two different compounds under the same conditions.

The compound with the larger Rf value is less polar because it does not stick to the stationary phase as long as the polar compound, which would have a lower Rf value.

Method

Extract 4 g of sample in alcohol (25 ml x 3) under reflux on a water-bath for 30 min, filter, concentrate to 10 ml and carry out the thin layer chromatography.

Apply 10 µl of the extract on TLC silica gel precoated Aluminium plate and develop the plate to a distance of 8 cm using toluene: ethyl acetate (5: 3) as mobile phase. After development allow the plate to dry in air and examine under ultraviolet (254 nm & 366 nm).

HPLTC results were noted and tabulated in Table No: 8

4.2.4. BIO-CHEMICAL ANALYSIS [71]

Methodology for chemical analysis

Preparation of extract

- 5gm of KVNC was weighed accurately and placed in a 250ml clean beaker and added with 50ml of distilled water.
- Then it was boiled well for about 20 minutes.
- Then it was cooled and filtered in a 1000ml volumetric flask and made up to 100ml distilled water.

Table No.2. Test for basic radicals

PROCEDURE	OBSERVATION	INFERENCE
Test for Potassium: A pinch of sample is treated with 2ml of sodium nitrate solution and then treated with 2ml of cobalt nitrate in 30% of glacial acetic acid.	Formation of Yellow colour precipitate	Presence of Potassium
Test for Calcium: Taken 2 ml of extract in a clean test tube. Then acetic acid and potassium chromate solution were added	No Yellow precipitate	Presence of Calcium
Test for Magnesium: 2ml of extract was taken in a clean test tube, few drops of Magnason reagent was added in drops.	Formation of Blue colour precipitate	Presence of Magnesium
Test For Ammonium: 2ml of extract was taken in a test tube and added few ml of Nessler's reagent.	Appearance of Brown colour	Presence of Ammonium
Test For Sodium: 2 pinches of *Keelvayu Nivarana Chooranam* was mixed with HCl and made it into paste. And introduced into the blue flame of Bunsen burner.	Appearance of intense Yellow colour	Presence of Sodium
Test for Iron (Ferrous): 2ml of extract was taken in a clean dried test tube and conc. HNO_3 and ammonium thiocyanate were added.	Appearance of Blood red colour	Presence of Ferrous iron
Test For Zinc: 2 ml of the extract was taken in a test tube and Potassium ferro cyanide solution was added.	Formation of White colour precipitate	Presence of Zinc

PROCEDURE	OBSERVATION	INFERENCE
Test For Aluminium: To the 2m1of the extract was taken in a test tube sodium hydroxide drops were added to it.	White precipitate obtained	Presence of Aluminium
Test For Lead: 2 ml of extract was taken in a test tube and added with 2ml of potassium iodide solution	Formation of yellow colour precipitate	Presence of Lead
Test for Copper: To a small portion of a extract dilute hydrochloric acid was added and then hydrogen sulphide gas is passed through the solution.	Black precipitate	Presence of Copper
Test For Mercury: 2m1 of the extract was taken in a test tube and treated With 2ml of sodium hydroxide solution.	Formation of Yellow precipitate	Presence of Mercury
Test for Arsenic: 2m1 of the extract was taken in a test tube and treated with 2ml of sodium hydroxide solution.	Formation of brownish red precipitate	Presence of Arsenic

Results were noted and tabulated in Table No: 10

Table No.3. Test for acidic radical

PROCEDURE	OBSERVATION	INFERENCE
Test for Sulphate: 2 ml of the extract was taken in clean, dry test tube and 5 % barium chloride solution was added to it.	Formation of white precipitate	Presence of Sulphate
Test for Chloride: The extract was taken in a test tube and then treated with Silver nitrate solution.	Formation of White precipitate	Presence of Chloride
Test for Phosphate: The extract was taken in a test tube and treated with ammonium molybdate and conc. HNO_3.	Formation of Yellow precipitate	Presence of Phosphate
Test for Carbonate: The substance was taken in a clean dry test tube and then treated with Conc. HCl.	Formation of Effervescence	Presence of Carbonate
Test for fluoride &oxalate: 2ml of extract was taken in a test tube and added with 2ml of dil.acetic acid, 2ml calcium chloride solution and then heated.	Formation of cloudy appearance	Presence of Fluoride & Oxalate
Test For Nitrate: 1gm of the KVNC was heated with copper turnings and concentrated H2SO4 and observed the test tube vertically down.	Characteristic changes	Presence of Nitrate

Results were noted and tabulated in Table No: 11

4.2.5. MICROBIAL LOAD

Enumeration of bacteria by plate count – agar plating technique

The plate count technique was one of the most routinely used procedures because of the enumeration of viable cells by this method.

Principle

This method is based on the principle that when material containing bacteria are cultured, every viable bacterium develops into a visible colony on a nutrient agar medium. The number of colonies therefore is the same as the number of organisms contained in the sample.

Dilution

A small measured volume is mixed with a large volume of sterile water or saline called the diluent or dilution blank. Dilution is usually made in multiples of ten.

A single dilution was calculated as follows:

$$\frac{\text{Volume of the sample}}{\text{Total volume of the sample and the diluent}}$$

Requirements

- Sample or Bacterial suspension
- 9 ml dilution blanks (7)
- Sterile petri dishes (12)
- Sterile 1 ml pipettes(7)
- Nutrient agar medium (200 ml)
- Colony counter.

Procedure

- Label the dilution blanks as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}.
- Prepare the initial dilution by adding 1 ml of the sample into a 9 ml dilution blank labeled 10^{-1} thus diluting the original sample 10 times.
- Mix the contents by rolling the tube back and forth between hands to obtain uniform distribution of organisms.

- From the first dilution transfer 1 ml of the suspension while in motion, to the dilution blank 10^{-2} with a sterile and fresh 1 ml pipette diluting the original specimen to 100 times.
- From the 10^{-2} suspension, transfer 1 ml of suspension to 10^{-3} dilution blank with a fresh sterile pipette, thus diluting the original sample to 100 times.
- Repeat this procedure till the original sample has been diluted 10,000,000 times using every time a fresh sterile pipette.
- From the appropriate dilutions transfer 1ml of suspension while in motion, with the respective pipettes, to sterile petri dishes. Three petri dishes are to use for each dilution.
- Add approximately 15 ml of the nutrient medium, melted and cooled to 450c, to each petri dish containing the diluted sample. Mix the contents of each dish by rotating gently to distribute the cells throughout the medium.
- Allow the plates to solidity.
- Incubate these plates in an inverted position for 24-48 hours at 370c.

Observation

Observe all the plates for the appearance of bacterial colonies. Count the number of colonies in the plates.

Calculate the number of bacteria per ml of the original suspension as follows:

$$\frac{\text{Number of colonies (average of 3 replates)}}{\text{Amount of plated} \times \text{dilution}}$$

Results were noted and tabulated in Table No: 12

4.2.6. INSTRUMENTAL ANALYSIS

FTIR analysis

Principle

FTIR microscopy is the conjunction of optical microscopy with IR spectroscopy. IR is dedicated to identify the chemical composition of samples on the basis of functional groups of molecules. The knowledge of these characteristic frequencies of peaks allows us to perform bio-chemical analysis of a sample. The FTIR determines the differences in the chemical composition between samples [72].

Infrared spectrum was useful in identifying the functional groups like -OH, -CN, -CO, -CH, -NH2, etc. FTIR spectrum is the most reliable method of reflecting the chemical constituents present in a complex system for validating herbal medicines.

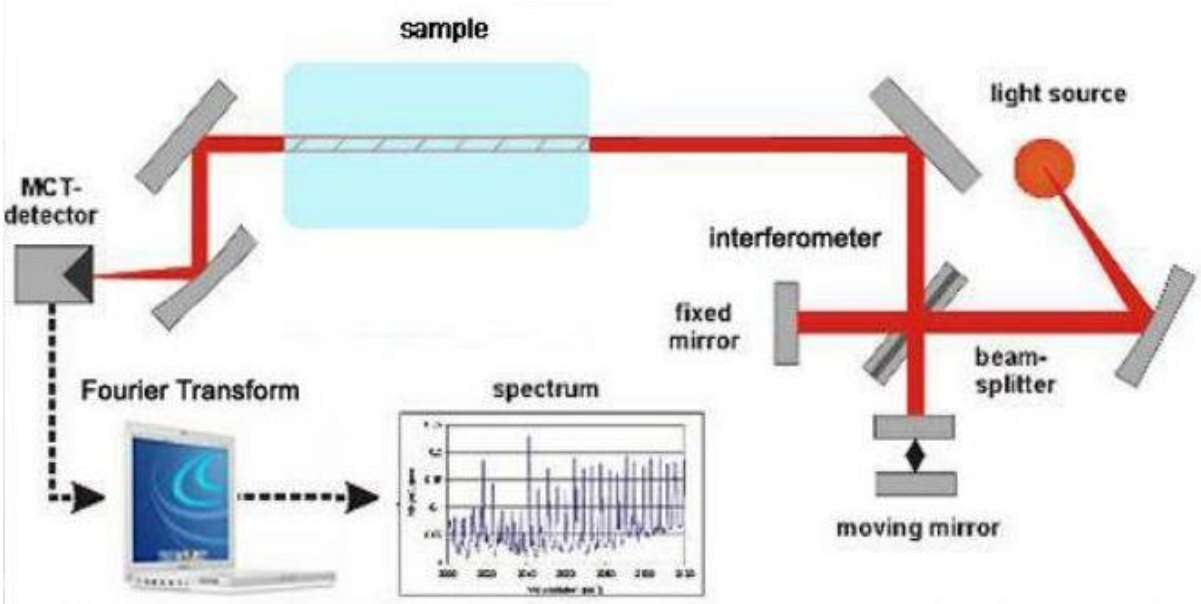


Fig.No.4. Showing the picture of FTIR mechanism

Instrument details

Model	:	Spectrum one: FT-IR Spectrometer
Scan Range	:	MIR 450-4000 cm-1
Resolution	:	1.0 cm-1
Sample required	:	50 mg, solid or liquid

Procedure

KBr Pellet procedure

About 1/8th of the solid KVNC was taken on a microspatula and about 0.25-0.50 teaspoons of KBr was added and thoroughly ground in a mortar with the pestle until it became very fine. It was placed in a pellet die.

The sample was pressed at 5000-10,000 psi and the sample was removed carefully from the die and placed in the FTIR sample holder.

The computer was turned on and the software was launched and certain fine details of the working method were done. The sample was placed on ZnSe crystal with a spatula until the pressure marker showed 12. The computer display showed spectrum of graphs with peaks and the results were printed.

Results were noted and tabulated in Table No: 13

XRD (X-RAY POWDER DIFFRACTION)

Definition

X-ray powder diffraction is most widely used for the identification of unknown crystalline materials (e.g. minerals, inorganic compounds). Determination of unknown solids is important to studies in geology, environmental science, material science and biology.

Applications

- Characterization of crystalline materials [73]

- Identification of fine-grained minerals such as clays and mixed layer clays that are difficult to determine optically
- Determination of unit cell dimensions.

With specialized techniques, XRD can be used to:

- Determine crystal structures using Rietveld refinement
- Determine of modal amounts of minerals (quantitative analysis)
- Characterize thin films samples by:
    - determining lattice mismatch between film and substrate and to inferring stress and strain
    - determining dislocation density and quality of the film by rocking curve measurements
    - measuring super lattices in multilayered epitaxial structures
    - determining the thickness, roughness and density of the film using glancing incidence X-ray reflectivity measurements
    - Make textural measurements, such as the orientation of grains, in a polycrystalline sample.

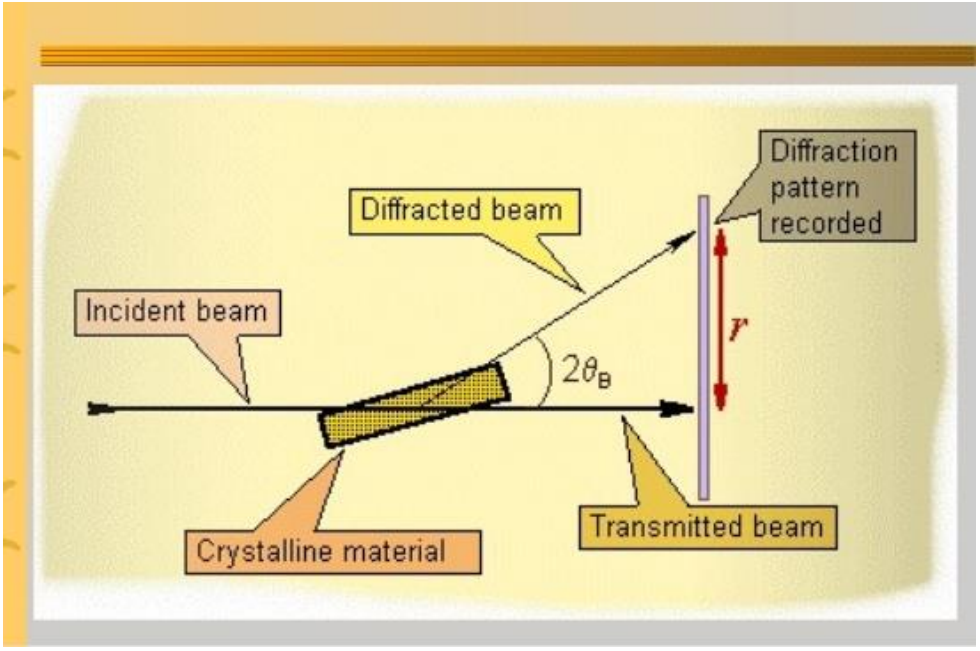


Fig No.5. Showing the picture of XRD Mechanism

Strengths and Limitations of X-ray Powder Diffraction

Strengths

- Powerful and rapid (< 20 min) technique for identification of an unknown mineral
- In most cases, it provides an unambiguous mineral determination
- Minimal sample preparation is required
- XRD units are widely available
- Data interpretation is relatively straight forward.

Limitations

- Homogeneous and single phase material is best for identification of unknown
- Must have access to a standard reference file of inorganic compounds
- Requires tenths of a gram of material which must be ground into a powder
- For mixed materials, detection limit is ~ 2% of sample
- For unit cell determinations, indexing of patterns for non-isometric crystal systems is complicated.

Sample Collection and Preparation

Determination of an unknown requires: the material, an instrument for grinding, and a sample holder.

- Obtain a few tenths of a gram (or more) of the material, as pure as possible
- Grind the sample to a fine powder, typically in a fluid to minimize inducing extra strain (surface energy) that can offset peak positions, and to randomize orientation.
- Powder less than ~10 μm(or 200-mesh) in size is preferred place into a sample holder or onto the sample surface.

SEM (SCANNING ELECTRON MICROSCOPE) [74]

In scanning electron microscope high-energy electron beam is focused through a probe towards the sample material. Variety of signals was produced on interaction with the surface of the sample. This results in the emission of electrons or photons and it is collected by an appropriate detector.

The types of signal produced by a scanning electron microscope include

- Secondary electrons
- back scattered electrons
- characteristic x-rays, light
- specimen current
- Transmitted electrons.

This gives the information about the sample and it includes external morphology, texture, its crystalline structure, chemical composition and it displays the shape of the sample.

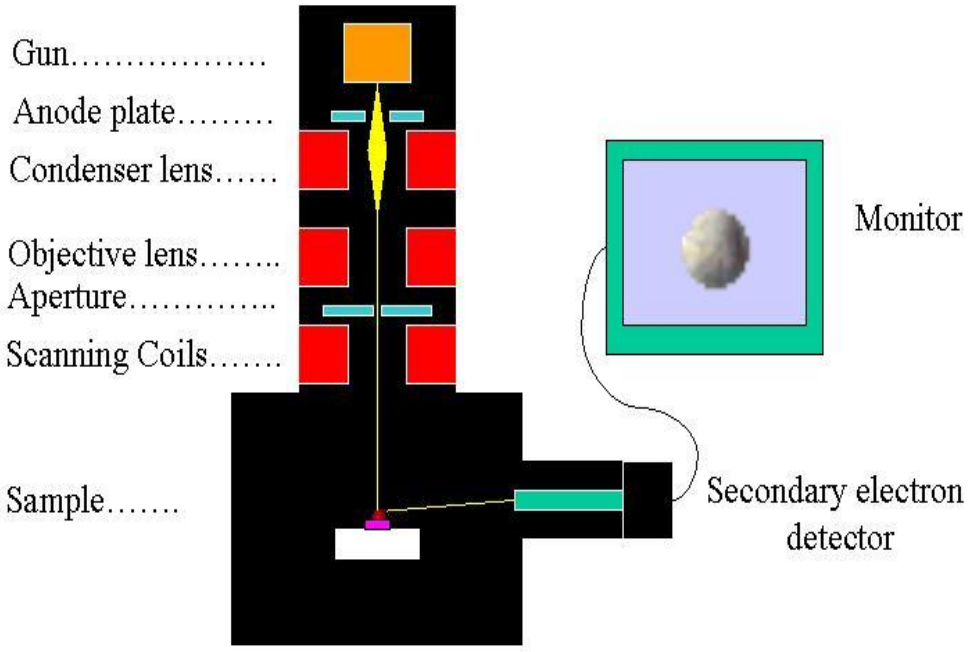
Instrument details

Model	: SEM-Hitachi
Scan Range	: S-3400n
Resolution	: 1.2 nm gold particle separation on a carbon substrate
Magnification	: From a min of 12 x to greater than 1, 00,000 X

Procedure

- The study was conducted in a very fine powder of the drug and the sample was quick frozen in liquid nitrogen.
- The sample was mounted rigidly on a specimen holder called specimen stub.
- The mounted sample was placed inside the microscope's vacuum column evaporator through an air tight door.

- On expelling air from the air pump, a beam of electrons passed from an electron gun. This beam travelled through a series of magnetic lenses designed to focus the electrons.
- The focused beam moved back across the mounted sample row by row by a set of scanning coils.
- As the electron beam hit each spot on the sample, secondary electrons are backscattered from its surface.
- A detector counts these electrons and sends the signals to an amplifier. The final image was built up from the no of electrons emitted from each spot on the sample.
- The micrographs obtained give sufficient data about the topography of the subjected sample.



Schematic diagramme of a scanning electron microscope

Fig. No.6. Showing schematic diagramme of SEM

ICPOES (INDUCTIVELY COUPLED PLASMA OPTIC EMISSION SPECTROMETRY) [75]

Manufacturer: Perkin Elmer

Model: Optima 5300 DV ICP-OES Inductively Coupled Plasma Spectrometer (ICP)

Principle

An aqueous sample is converted to aerosols via a nebulizer. The aerosols are transported to the inductively coupled plasma which is a high temperature zone (8,000–10,000ºC). The analysts are heated (excited) in different (atomic and/or ionic) states and produce characteristic optical emissions (lights).

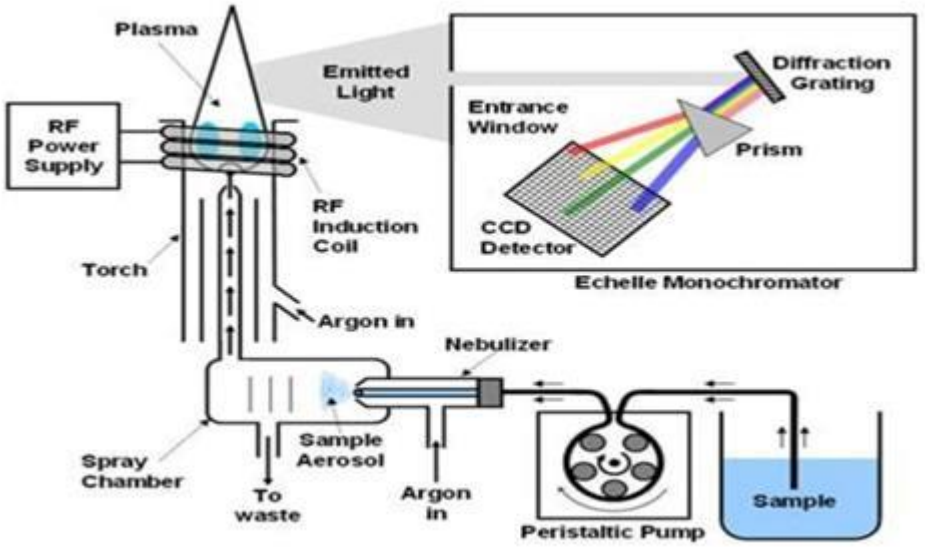


Fig. No.7. Showing schematic diagramme of ICPOES

These releases are separated based on their respective wavelengths and their strengths are measured (spectrometry). The intensities are proportional to the concentrations of analyses in the aqueous sample.

The quantification is an external multipoint linear standardization by comparing the emission intensity of an unknown sample with that of a standard sample.

Multi-element calibration standard solutions are prepared from single- and multi element primary standard solutions. With respect to other kinds of analysis where chemical speciation is relevant (such as the concentration of ferrous iron or Ferric Iron), only total essential concentration is analysed by ICP-OES.

Application

The analysis of major and minor elements in solution samples.

Objectives

- Determine elemental concentrations of different metals.
- Learn principles and operation of the ICP-OES instrument
- Develop and put on a method for the ICP-OES sample analysis
- Enhance the instrumental conditions for the analysis of different elements
- Probes the outer electronic structure of atoms.

Mechanism

In plasma emission spectroscopy (OES), a sample solution is presented into the core of inductively coupled argon plasma (ICP), which generates temperature of approximately 8000°C.

At this temperature all elements become thermally excited and emit light at their characteristic wavelengths.

This light is collected by the spectrometer and passes through a diffraction grating that serves to resolve the light into a spectrum of its essential wavelengths.

Within the spectrometer, this deflected light is then collected by wavelength and amplified to yield strength of measurement that can be converted to an elemental concentration by comparison with standardization values.

The Inductively coupled plasma optical emission spectrometric (ICP-OES) analysis was done in SAIF, IIT MADRAS, Chennai-36 using Perkin Elmer Optima 5300 DV.

Results were noted and tabulated in Table No:14

4.3. TOXICOLOGICAL STUDIES

4.3.1. Acute Oral Toxicity Study (OECD Guideline – 423) [76]

Introduction

- The acute toxic class method is a stepwise procedure with the use of 3 animals of a single sex per step.
- Depending on the mortality and/or the moribund status of the animals, on average 2-4 steps may be necessary to allow judgement on the acute toxicity of the test substance.
- This procedure is reproducible, uses very few animals and is able to rank substances in a similar manner to the other acute toxicity testing methods.
- The acute toxic class method is based on biometric evaluations with fixed doses, adequately separated to enable a substance to be ranked for classification purposes and hazard assessment.
- In principle, the method is not intended to allow the calculation of a precise LD_{50}, but does allow for the determination of defined exposure ranges where lethality is expected since death of a proportion of the animals is still the major endpoint of this test.
- The method allows for the determination of an LD_{50} value only when at least two doses result in mortality higher than 0% and lower than 100%.
- The use of a selection of pre-defined doses, regardless of test substance, with classification explicitly tied to number of animals observed in different states improves the opportunity for laboratory to laboratory reporting consistency and repeatability.

Principle of the Test

It is the principle of the test that based on a stepwise procedure with the use of a minimum number of animals per step; sufficient information is obtained on the acute toxicity of the test substance to enable its classification. The substance is administered

orally to a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, each step using three animals of a single sex. Absence or presence of compound-related mortality of the animals dosed at one step will determine the next step, i.e.

− No further testing is needed

− dosing of three additional animals, with the same dose

− dosing of three additional animals at the next higher or the next lower dose level. The method will enable a judgment with respect to classifying the test substance to one of a series of toxicity classes.

Methodology

Selection of Animal Species

The preferred rodent species is the wistar albino rat, although other rodent species may be used. Healthy young adult animals are commonly used laboratory strains should be employed. Females should be nulliparous and non-pregnant. Each animal, at the commencement of its dosing, should be between 6 to 8 weeks old and the weight (150-200gm) should fall in an interval within±20 % of the mean weight of any previously dosed animals.

Housing and Feeding Conditions

The temperature in the experimental animal room should be 22ºC ± 3ºC. Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be group-caged by dose, but the number of animals per cage must not interfere with clear observations of each animal.

Preparation of animals

The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least 7 days prior to dosing to allow for acclimatization to the laboratory conditions

Test Animals and Test Conditions

Sexually mature Female Wistar albino rats (150-200gm) were obtained from TANUVAS, Madhavaram, and Chennai. All the animals were kept under standard environmental condition (22±3 ° C). The animals had free access to water and standard pellet diet (Sai meera foods, Bangalore).

Preparation of animals

The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least 7 days prior to dosing to allow for acclimatization to the laboratory conditions

Preparation for Acute Toxicity Studies

Rats were deprived of food overnight (but not water 16-18 h) prior to administration of ***Keelvayu Nivarana Chooranam*.** The principles of laboratory animal care were followed and the Institutional Animal Ethical Committee approved the use of the animals and the study design.

IAEC No: IAEC/XLVIII/05/CLBMCP/2016

Test Substance	:	*Keelvayu Nivarana Chooranam*
Animal Source	:	Kings Institute, Chennai.
Animals	:	Wister Albino Rats (Female-3+3)
Age	:	>6 weeks
Body Weight on Day 0	:	150-180 gm.
Acclimatization	:	Seven days prior to dosing.
Veterinary examination	:	Prior and at the end of the acclimatization period.
Identification of animals	:	By cage number, animal number and individual marking by using Picric acid.

Number of animals	: 3 Female/group,
Route of administration	: Oral
Diet	: Pellet feed supplied by Sai meera foods Pvt Ltd, Bangalore.
Water	: Aqua guard portable water in polypropylene bottles.
Housing & Environment	: The animals were housed in Polypropylene cages provided with bedding of husk.
Housing temperature	: between 22ºC ± 3ºC.
Relative humidity	: between 30% and 70%,
Air changes	: 10 to 15 per hour and
Dark and light cycle	: 12:12 hours.
Duration of the study	: 14 Days

Administration of Doses

Keelvayu Nivarana Chooranam was suspended in water and administered to the groups of wistar albino rats in a single oral dose by gavage using a feeding needle. The control group received an equal volume of the vehicle.

Animals were fasted 12 hours prior to dosing. Following the period of fasting, the animals were weighed and then the test substance was administered. Three Female animals are used for each group. The dose level of 20, 100 and 200 mg/kg body weight was administered stepwise.

After the substance has been administered, food was withheld for a further 3-4 hours. The principle of laboratory animal care was followed. Observations were made and recorded systematically and continuously as per the guideline after substance administration.

The visual observations included skin changes, mobility, aggressively, sensitivity to sound and pain, as well as respiratory movements. Finally, the number of survivors was noted after 24 hrs and these animals were then monitored for a further 14 days and observations made daily.

The toxicological effect was assessed on the basis of mortality.

Observations

Animals are observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days, except where they need to be removed from the study and humanely killed for animal welfare reasons or are found dead.

It should be determined by the toxic reactions, time of onset and length of recovery period, and may thus be extended when considered necessary. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed.

All observations are systematically recorded with individual records being maintained for each animal.

Observations include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavior pattern. Attention was directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

The principles and criteria summarized in the Humane Endpoints Guidance Document taken into consideration. Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress was humanly killed.

When animals are killed for human reasons or found dead, the time of death was recorded.

4.3.2. Repeated Dose 28-Day Oral Toxicity Study

Test Substance	: *Keelvayu Nivarana Chooranam*
Animal Source	: Kings institute, Chennai.
Animals	: Wister Albino Rats (Male -24, and Female-24)
Age	: >6 weeks
Body Weight	: 160-180 gm.
Acclimatization	: Seven days prior to dose.
Veterinary examination	: Prior and at the end of the acclimatization period.
Identification of animals	: By cage number, animal number and individual marking by using Picric acid
Diet	: Pellet feed supplied by Sai meera foods Pvt Ltd, Bangalore
Water	: Aqua guard portable water in polypropylene bottles.
Housing & Environment	: The animals were housed in Polypropylene cages provided with bedding of husk.
Housing temperature	: between 22ºC $\pm$ 3ºC.
Relative humidity	: between 30% and 70%,
Air changes	: 10 to 15 per hour
Dark and light cycle	: 12:12 hours.
Duration of the study	: 28 Days.

Table No.4. 28-days repeated oral toxicity study grouping

Groups	**No of Rats**
Group I Vehicle control (Normal Saline)	12(6male,6 female)
Group II *Keelvayu Nivarana Chooranam* 20 mg/kg	12 (6male,6 female)
Group III *Keelvayu Nivarana Chooranam* 100 mg/kg	12 (6male,6female)
Group IV *Keelvayu Nivarana Chooranam* 200 mg/kg	12(6male,6female)

Methodology

Randomization, Numbering and Grouping of Animals

48 Wistar Albino Rats (24M + 24F) were selected and divided into 4 groups. Each group consists of 12 animals (Male -6, and Female-6). First group treated as a control and other three groups were treated with test drug (low, mid, high) for 28 days. Animals were allowed acclimatization period of 7 days to laboratory conditions prior to the initiation of treatment. Each animal was marked with picric acid. The females were nulliparous and non-pregnant.

Justification for Dose Selection

As per OECD guideline three dose levels were selected for the study. They are low dose (20 mg/kg), mid dose (100 mg/kg), high dose (200 mg/kg). X is calculated by multiplying the therapeutic dose of human (1000mg/kg) and the body surface area of the rat (0.018). i.e X dose is 20 mg/kg/animal, 5X mid dose is 100 mg/kg, 10X high dose is 200 mg/kg.

Preparation and Administration of Dose

Keelvayu Nivarana Chooranam suspended in with water, It was administered to animals at the dose levels of 20, 100 and 200 mg/kg. The test substance suspensions were freshly prepared every two days once for 28 days. The control animals were administered vehicle only. The drug was administered orally by using oral gavage once daily for 28 consecutive days.

Observations

Experimental animals were kept under observation throughout the course of study for the following

Body Weight

Weight of each rat was recorded on day 0, at weekly intervals throughout the course of study.

Food and water Consumption

Food and water consumed per animal was calculated for control and the treated dose groups.

Clinical signs

All animals were observed daily for clinical signs. The time of onset, intensity and duration of these symptoms, if any, were recorded.

Mortality

All animals were observed twice daily for mortality during entire course of study.

Necropsy

All the animals were sacrificed by excessive anaesthesia on day 29. Necropsy of all animals was carried out.

Laboratory Investigations

Following laboratory investigations were carried out on day 29 in animals fasted over-night. Blood samples were collected from orbital sinus using sodium heparin (200IU/ml) for Bio chemistry and potassium EDTA (1.5 mg/ml) for Hematology as anticoagulant. Blood samples were centrifuged at 3000 r.p.m. for 10 minutes.

Haematological Investigations

Haematological parameters were determined using Haematology analyzer.

Biochemical Investigations

Biochemical parameters were determined using auto-analyzer.

Histopathology

Control and highest dose group animals will be initially subjected to histopathological investigations. If any abnormality found in the highest dose group than the low, then the mid dose group will also be examined. Organs will be collected from all animals and preserved in 10% buffered neutral formalin for 24 h and washed in running water for 24 h. The organ sliced 5 or 6µm sections and were dehydrated in an auto technicon and then cleared in benzene to remove absolute alcohol. Embedding was done by passing the cleared samples through three cups containing molten paraffin at 50°C and then in a cubical block of paraffin made by the "L" moulds. It was followed by microtome and the slides were stained with Haematoxylin-eosin red.

Statistical analysis

Findings such as body weight changes, water and food consumption,hematology and blood chemistry were subjected to One-way ANOVA followed by dunnett test using a computer software programme – Graph pad version 5.0 .

4.4. PHARMACOLOGICAL ANALYSIS

4.4.1. Evaluation of Analgesic activity

Acetic acid induced writhing test

Wistar albino rats were divided into four groups (n = 6). Group I received acetic acid (1% v/v, 10 ml/kg b.w., i.p.) and writhing reflex was noted for the period of 15 minutes. Group II received aspirin (100 mg/kg b.w.p.o.)

Group III and IV received KVNC at the doses of 100 mg/kg and 200 mg/kg b.w., p.o. respectively. 30 min after aspirin and KVNC administration, group II and III received acetic acid (1% v/v, 10 ml/kg b.w., i.p.) and writhing reflex was noted for the period of 15 min.

4.4.2. Evaluation of Acute Anti-inflammatory activity

Carrageenan induced rat paw oedema

The rats were divided into four groups containing six rats in each group. 0.1s ml of 1.0% carrageenan in normal saline (0.9% w/v NaCl) was injected to the sub plantar region of right hind paw. The trial drug KVNC was administered to the rats 1 h before carrageenan injection. Different groups were treated as follows:

Group I: Carrageenan (0.1 ml of 1.0% carrageenan/rat to the sub plantar region).

Group II: Carrageenan + Indomethacin (10 mg/kg b. w., p. o.)

Group II and IV: Carrageenan + KVNC (100 mg/kg and 200 mg/kg b. w., p. o.respectively).

The paw volume was measured initially and at 1, 2, 3 and 4 h after carrageenan injection, using Plethysmograph, inflammation was calculated for comparison[75,76].

Evaluation of Chronic Anti-inflammatory activity

Cotton pellet granuloma pouch method

Chronic inflammation was induced by cotton pellet granuloma method. Rats were divided into four groups. First two groups received oral doses of 100 mg/kg and

200 mg/kg of KVNC respectively. The reference drug indomethacin (10mg/kg) was used as a positive control and the other negative control group received saline solution. Sterilized Cotton pellets 50 mg were implanted under light ether anesthesia in the axilla and groin region of each rat by making a small incision Drugs (KVNC 100, KVNC 200 and Indomethacin) and saline (5ml/kg) for control group were administered orally to four groups of rats once daily for 7 consecutive days from the day of cotton pellet implantation. The 8th day, the animals were sacrificed and cotton pellets were removed and dried in an oven at 600C for 24 hours.

They were then weighed. The granuloma formation was calculated as a measure of increment in the dry weight of the pellet. The percentage of inhibition of granuloma was calculated using the following formula. P = (1 - Wt /Wc) × 100, where, Wt – Dry weight of the cotton in test animals and Wc - Dry weight of the cotton in control animals.

4.4.3. Evaluation of Anti-Rheumatic activity

Collagen induced arthritis in rats

Collagen induced arthritis is an experimental auto immune disease that can be elicited in susceptible strains of rodent and non-human primates by immunization with type II collagen, the major constituent protein of articular cartilage.

Collagen-induced arthritis (CIA) shares both immunological and pathological features with human rheumatoid arthritis (RA) therefore it has been used extensively as a model to study the pathogenesis of RA and for testing therapeutics.

The development of arthritis is associated with high levels of both cell mediated and humoral immunity to type II collagen. The arthritic response appears to be due to collagen immunity. In addition, the histopathology of collagen induced arthritis resembles that seen in human rheumatoid arthritis in that lesion is one of synovial proliferation that progresses to pannus formation and results in marginal erosions with extensive destruction of cartilage.

5. RESULTS AND DISCUSSION

There are many studies have been carried out to bring the potency, efficacy and safety of the drug *Keelvayu Nivarana Chooranam.*

This study includes:

- Literary collections
- Organoleptic character
- Physicochemical analysis
- Phytochemical analysis
- Instrumental analysis
- Toxicological study
- Pharmacological study

The drug *Keelvayu Nivarana Chooranam* has been selected for Anti-Rheumatic activity.

Standardization of the test drug KVNC

Standardization of the drug is more essential to derive the efficacy, potency of the drug by analyzing it by various studies. Following are the results of physicochemical and phytochemical analysis. Physical characterization and estimation of basic and acidic radicals have been done and tabulated.

Organoleptic Character

The following characters have been noted in *Keelvayu Nivarana Chooranam.*

Table No.5. Results of Organoleptic characters

Colour	Brown
Odour	Pleasant
Taste	Bitter
Texture	Fine powder
Particle size	Completely pass through sieve no 88

PHYSICOCHEMICAL ANALYSIS

Table No.6. Results of Physicochemical analysis

S.NO	PARAMETER		RESULT
1	pH		5.58
2	Total ash		4.78
3	Water soluble ash		2.75
4	Acid soluble ash		0.40
5	Loss on drying (at 105°C)		5.20
5	Water soluble extractive		16.65
6	Alcohol soluble extractive		16.10
7	Solubility		+ve
	i	Distilled water	Soluble
	ii	Benzene	Soluble
	iii	Chlroform	Soluble
8	Action on heat		- ve
9	Flame test		- ve
10	Ash test		- ve

Interpretation

- The physical characterization of the drug result reveals the solubility, pH, action on heat, flame test and ash test.

- **pH:** It is a measure of hydrogen ion concentration; it is the measure of the acidic or alkaline nature. 7.0 is neutral, above 7.0 is alkaline and below is acidic.
- The pH of the drug *is* 5.58 which are slightly acidic in nature and it is essential for its bioavailability and effectiveness.
- The result concludes that the oral bioavailability of the drug *Keelvayu Nivarana Chooranam* is very high.
- Ash is the inorganic residue left after ignition at 650-700°C. The ash content is an approximate measure of the mineral content and other inorganic matter in biomass.
- The ash content is a measure of the total amount of minerals present within a food, whereas the mineral content is a measure of the amount of specific inorganic components present within a food, such as Ca, Na, K and Cl.
- The quality of drugs depends on the concentration and type of minerals they contain, including their taste, appearance, texture and stability.
- Ash is one of the components in the proximate analysis of biological materials, consisting mainly of salty, inorganic constituents.
- It includes metallic salts which are important for processes requiring ions such as Na^+ (Sodium), K^+ (Potassium), and Ca^{2+} (Calcium).
- The ash value of *Keelvayu Nivarana Chooranam* indicates the presence of minerals such as Sodium, Potassium and Calcium.
- The acid insoluble value of the drug *Keelvayu Nivarana Chooranam* is 0.40%
- Solubility is the basic requirement for the absorption of the drug from GIT. Here the water soluble nature of *Keelvayu Nivarana Chooranam* is 16.65 %.
- This nature might be helpful for the better absorption.
- To determine the moisture content of a sample, although occasionally it may refer to the loss of any volatile matter from the sample.
- Here the loss of drying value of the trial drug *Keelvayu Nivarana Chooranam* is 5.20% at 105°C (%).

PHYTOCHEMICAL ANALYSIS

Table No: 7 Results of Phytochemicals screening

PHYTOCHEMICALS	TEST	RESULT
1. Alkaloids	a. Mayer's test	++
	b. Wagner's test	-
	c. Dragendroff's test	-
	d. Hager's test	-
2. Carbohydrates	a. Molisch's test	+
3. Reducing sugars	a. Benedicts test	-
	b. Fehling's test	-
4.Anthranol Glycosides	Modified Borntrager's test	+
5. Saponins	a. Froth test	+
	b. Foam test	-
6. Tannins	Gelatin test	++
7. Phenols	Alcoholic Ferric chloride test	+
8. Phytosterols	Ferric chloride acetic acid test	-
9. Diterpenes	Copper acetate test	-
10. Triterpenes	Salkowski's test	-
11. Flavanoids	Alkaline reagent test	+++
	b. Lead acetate test	-
	c. Ferric chloride test	-
	d. Shinoda test	+
12. Proteins	a. Xanthoproteic test	-
	b. Biuret's test	+
	c. Million's test	-

Interpretation

Phytochemicals are natural bioactive compound found in plants and fibers which act as a defense system against diseases and more accurately, to protect against diseases. The phytochemical analysis reveals the presence of alkaloids, tannins, flavonoids, anthral glycosides, cardiac glycosides, saponins, phenols, proteins and carbohydrates.

Flavonoids

- It is the most important group of polyphenolic compounds in plants.
- Flavonoids have potent Anti-Oxidant activity and it is its important function.
- It also possesses anti-microbial activity which is confirmed by the various anti-microbial assays.

Tannins

- Reported to have anti-arthritic activity.
- They restore the Anti-Oxidant status of the organs to almost normal levels.
- Increases the cellular Anti-Oxidant enzymes.
- Helps in healing of wounds and inflammation of mucous membrane.

Phenols

- They possess rich Anti-Oxidant property and protect body from oxidative stress.
- Phenol groups are the essential part of many anti-oxidant compounds

Alkaloids

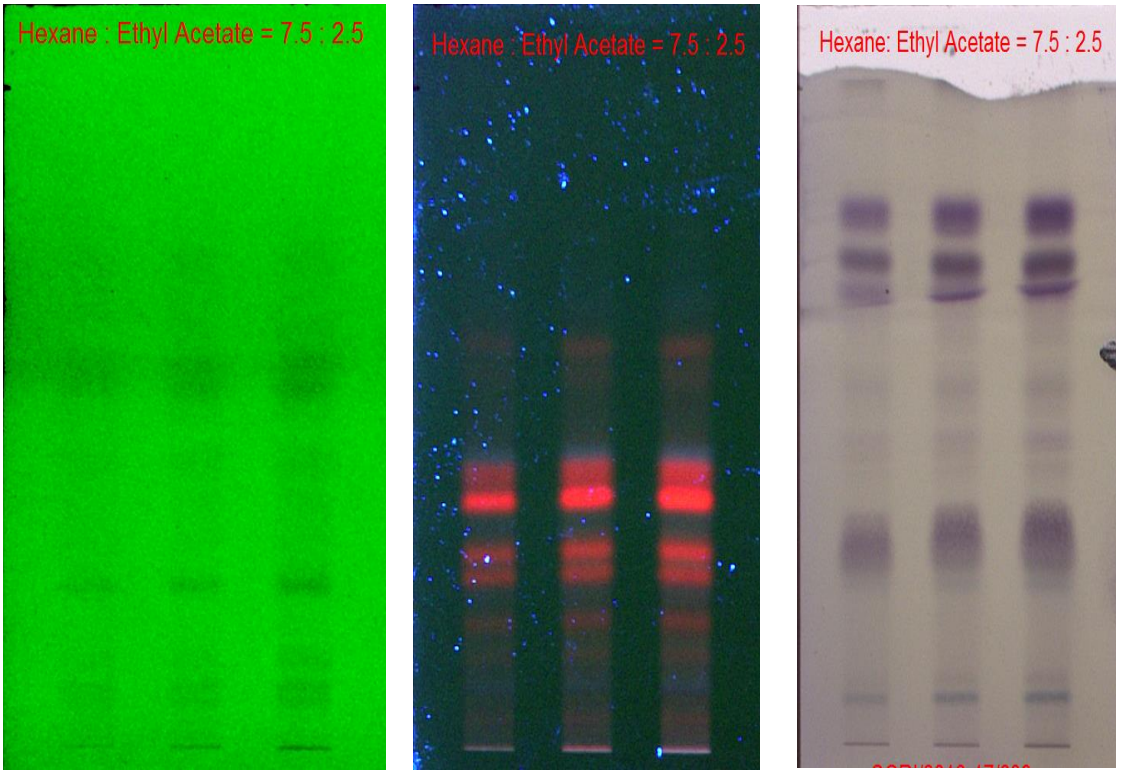
- Alkaloids possess antispasmodic, analgesic, anti-inflammatory, bactericidal effects.
- Alkaloids are the active principles producing many essential effects in protecting the body.

A synergistic effect of all these flavonoids, alkaloids, glycosides, tannins, phenols, saponins, increases the potency of the drug against Rheumatoid Arthritis.

HPTLC (HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY)

Stationary Phase - Silica Gel 60 F_{254}

Mobile Phase - Hexane: Ethyl Acetate: (7.5:2.5 v/v)



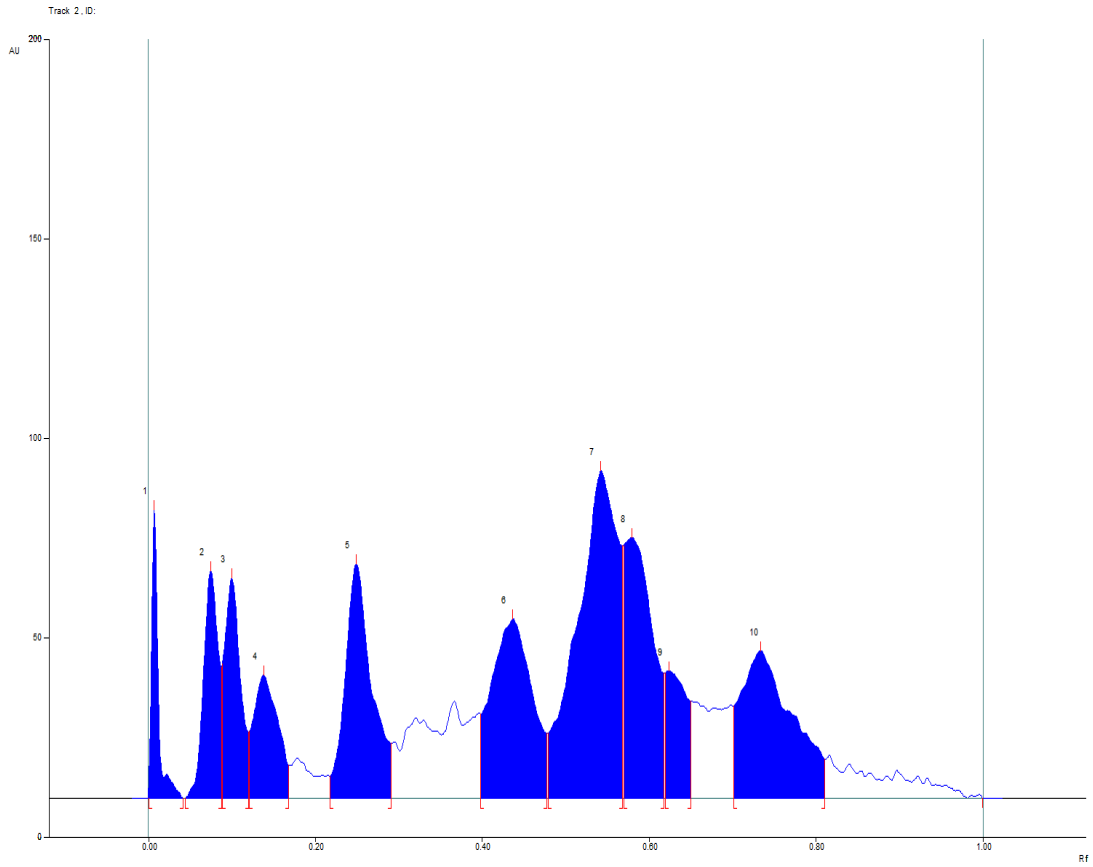
λ = 254 nm **λ= 366 nm** **λ=520nm (Derivatized)**

Fig. No.8. Results of HPTLC

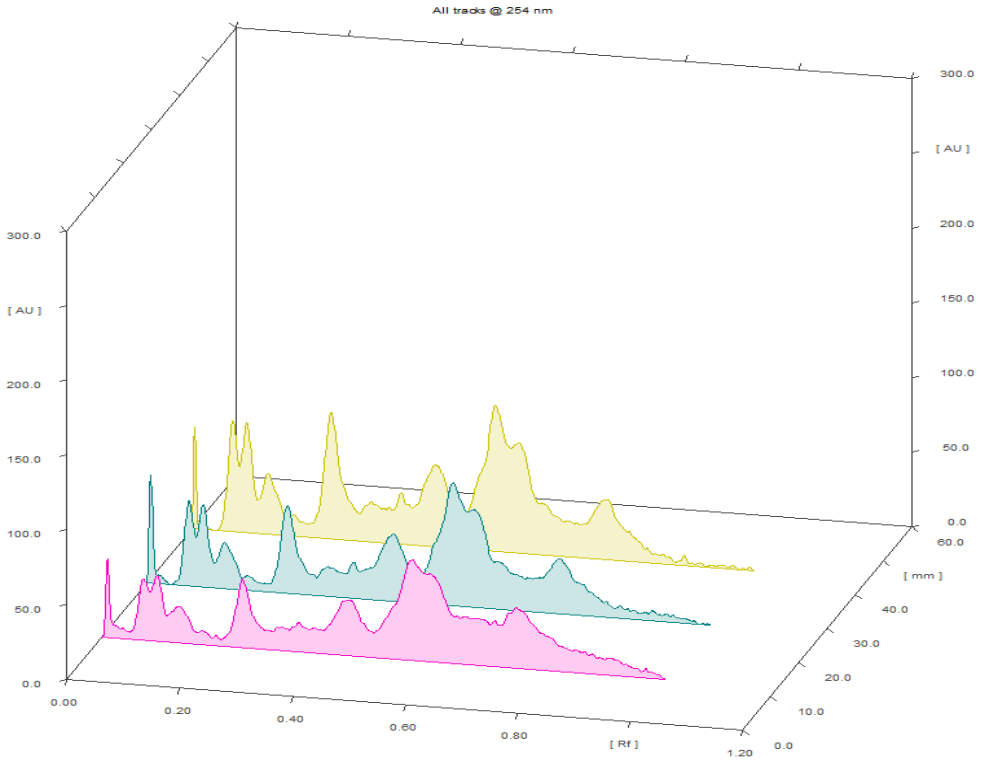
Table No.8. Rf value for chloform extract

Color	R_f value(s)	Color	R_f value(s)	Color	R_f value(s)
Dark	0.07	Red	0.05	Ash	0.08
Dark	0.10	Red	0.14	Violet	0.11
Dark	0.14	Red	0.20	Ash	0.24
Dark	0.25	Red	0.27	Violet	0.31
Dark	0.44	Red	0.30	Violet	0.45
Dark	0.54	Red	0.37	Light Blue	0.55
Dark	0.58	Bluish Red	0.39	Ash	0.69
Dark	0.50	Red	0.62	Ash	0.74
Dark	0.54	Yellow	0.73	Dark grey	0.82

- Under UV 254 nm, it shows major spots at Rf 0.07, 0.10, 0.14, 0.25, 0.44, 0.54, 0.58, 0.50, 0.54 major compounds are found.
- Under UV 366 nm, it shows major spots at Rf 0.05, 0.14, 0.20, 0.27, 0.30, 0.37, 0.62,0.73,



Graph No.1. TLC Chromatogram at 254 nm



Graph No.2. 3D Chromatogram at 254 nm

Table No.9. Peak value at 254 nm

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.00 Rf	0.0 AU	0.01 Rf	72.4 AU	13.52 %	0.04 Rf	0.1 AU	535.5 AU	3.39 %
2	0.04 Rf	0.1 AU	0.07 Rf	57.2 AU	10.67 %	0.09 Rf	33.0 AU	939.8 AU	5.94 %
3	0.09 Rf	33.7 AU	0.10 Rf	55.2 AU	10.30 %	0.12 Rf	16.6 AU	969.5 AU	6.13 %
4	0.12 Rf	16.8 AU	0.14 Rf	31.0 AU	5.78 %	0.17 Rf	8.2 AU	845.4 AU	5.34 %
5	0.22 Rf	5.5 AU	0.25 Rf	58.7 AU	10.95 %	0.29 Rf	13.7 AU	1749.1 AU	11.06 %
6	0.40 Rf	21.1 AU	0.44 Rf	45.0 AU	8.40 %	0.48 Rf	16.4 AU	2050.6 AU	12.96 %
7	0.48 Rf	16.4 AU	0.54 Rf	82.1 AU	15.32 %	0.57 Rf	63.2 AU	3746.2 AU	23.68 %
8	0.57 Rf	63.4 AU	0.58 Rf	65.4 AU	12.20 %	0.62 Rf	31.3 AU	2085.9 AU	13.19 %
9	0.62 Rf	31.5 AU	0.62 Rf	32.0 AU	5.97 %	0.65 Rf	24.4 AU	754.4 AU	4.77 %
10	0.70 Rf	23.2 AU	0.73 Rf	36.9 AU	6.89 %	0.81 Rf	9.7 AU	2142.1 AU	13.54 %

Interpretation

- The quantitative analysis of compounds present in the KVNC has been performed by HPTLC.
- The method may be applied to identify the KVNC from other manufacturing process.
- It provides the identification of constituents, determination of impurities and quantitative determination of active substance present in KVNC. [77]
- The Rf value of the KVNC supports the better standardization of the drug.
- The present study revealed that KVNC showed best results in Toluene: Ethyl Acetate (9:1) Solvent system.
- After scanning and visualizing the plates in absorbance mode at 254nm, 366nm and 520nm. Best results were shown at visible light range.
- TLC plate showed different colour phyto constituents of chloroform extract of KVNC.
- The bands revealed presence of six green, two blue and one fluorescent yellow, bands showing the presence of alkaloids, glycosides, phenols, triterpenes, flavonoids and quinines.
- The results from HPTLC finger print scanned for chloroform extract of KVNC.
- There are thirteen polyvalent phytoconstituents and corresponding ascending order of Rf values start from 0.02 to 0.92 in which highest concentration of the phyto constituents was found to be 28.96% and 27.95% with its corresponding Rf value found to be 0.02 and 0.92 respectively.

BIOCHEMICAL ANALYSIS

Table No: 10 Results of Basic radicals studies

S.NO	PARAMETER	OBSERVATION	RESULT
1	Test for Potassium	Yellow colour precipitate	+ ve
2	Test for Calcium	White colour precipitate	+ve
3	Test For Magnesium .	White colour precipitate	+ve
4	Test For Sodium	Intense yellow colour	+ ve
5	Test for Iron (Ferrous)	Blood red colour	+ve

Interpretation [78]

- The basic radical test shows the presence of **Potassium, Calcium, Magnesium, Sodium and Iron.**
- **Potassium** is extending to improving the health of bones. There are certain qualities of potassium which neutralize various acids throughout the body which retain and preserve calcium, making it inaccessible to use for bone strength and durability.
- **Calcium** strengthens the backbone and ensures the right shape to the body, as well as helping to alleviate the presence of joint pain. It helps to keep the bones in their proper shape and prevents many skeletal complaints like arthritis and osteoporosis, which could hamper your freedom of movement, as well as being extremely painful.
- **Sodium** helps to facilitate the absorption of glucose by cells, resulting in the smooth transportation of nutrients in the body's cell membranes.

- **Iron** is a vital element for muscle health. It is present in the muscle tissues and helps to provide the supply of oxygen required for contraction of muscles. Without it, muscles lose their tone and elasticity.

Table No.11. Test for Acid radical studies

S.NO	PARAMETER	OBSERVATION	RESULT
1	Test for Sulphate	Formation of white precipitate	+ ve
2	Test for Phosphate	Formation of Yellow precipitate	+ ve

Interpretation

- The acidic radicals test shows the presence of Sulphate and Phosphate.
- **Sulphates** are important in forming proteins in the joints**.**
- **Phosphate** works in association with calcium to create strong bones, which can withstand the normal wear and tear of human life. It also helps in relieving serious problems like bone loss or the loss of mineral density, also known as osteoporosis. This mineral lays the foundation of a strong skeletal structure to ensure health and functional living.

MICROBIAL LOAD

Table No.12. Bacterial and fungal dilutions

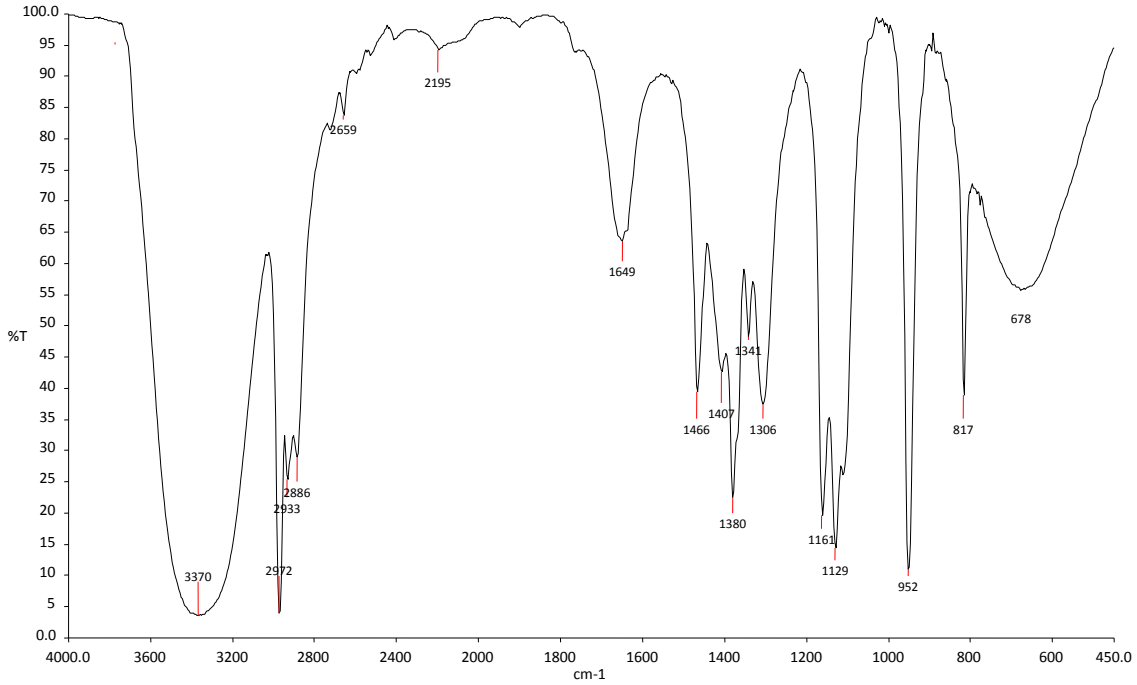
MICROBES	DILUTION	RESULT
Bacteria	**10^{-4}**	**15**
Bacteria	**10^{-6}**	**11**
Fungi	**10^{-2}**	**8**
Fungi	**10^{-3}**	**4**

Interpretation

- The availability of bacterial load in the KVNC has been performed by Agar plate technique.
- As KVNC is made from plant material it is more prone to contamination. The contamination of herbal drugs by microorganism not only cause bio deterioration but also reduces the efficacy of drugs.
- The toxin produced by microbes makes herbal drugs unfit for human consumption because the contaminated drug may develop unwanted disease instead of disease being cured.
- The contamination of KVNC has been examined by bacterial and fungal load.
- Total bacterial load in 10^{-4} dilution is 15 and 10^{-6} dilution 11.
- Total fungal load in 10^{-2} dilution is 8 and 10^{-3} dilution is 4.

This result shows the presence of bacterial and fungal load in the trial drug (KVNC). The load of bacteria and fungi are within the limits of WHO norms.

FTIR (FOURIER TRANSFORM INFRARED SPECTROSCOPY)



Graph No.3. FTIR Spectrum

Table No.13. Interpretation of FTIR Spectrum

ABSORPTION PEAK CM^{-1}	STRETCH	FUNCTIONAL GROUP
3370	N-H stretch	1,2 Amines, Amide
2972	C-H Stretch	Alkanes
2933	C-H Stretch	Alkanes
2886	C-H Stretch	Alkanes
2195	-C=C Stretch	Alkenes
1649	-C=C Stretch	Alkenes
1466	C-H bend	Alkanes
1161	C-H way ($-CH_2X$)	Alkyl halides
1129	C-H way ($-CH_2X$)	Alkyl halides
952	=C-H bend	Alkenes
817	C-Cl Stretch	Alkyl halides
678	C-Br Stretch	Alkyl halides

Interpretation

FTIR instrumental analysis was done. The test drug was identified to have 15 peaks. They are the functional groups present in the trial drug *Keelvayu Nivarana Chooranam*.

The above table shows the presence of amide, phenols, alkanes, alkyl halides alkynes, alkenes, ester, aromatics and alcohol groups which represents the peak value.

- ❖ OH group has higher potential towards inhibitory activity against microorganisms.
- ❖ Phenols possess highly Anti-Oxidant property which enhances the drug effect against the disease.

ICP-OES (INDUCTIVELY COUPLED PLASMA ATOMIC EMISSION SPECTROSCOPY)

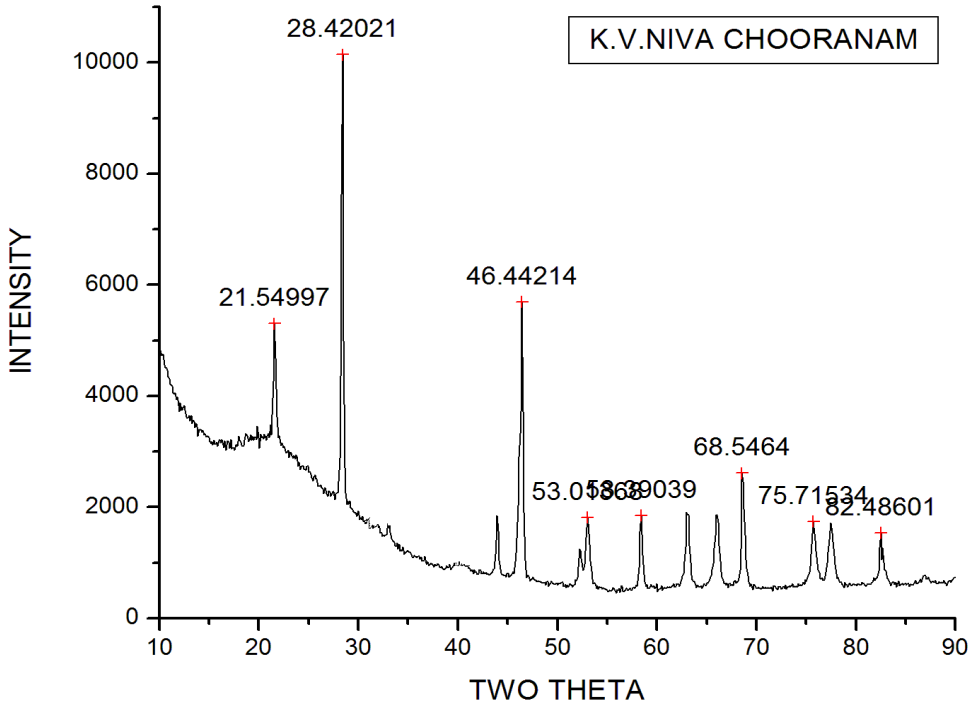
Table No.14. ICPOES Interpretation

S. NO	ELEMENTS	DETECTED LEVELS
1	Al 396.152	BDL
2	As 188.979	BDL
3	Ca 315.807	221.160 mg/L
4	Cd 228.802	BDL
5	Cu 327.393	BDL
6	Hg 253.652	BDL
7	K 766.491	43.114 mg/L
8	Mg 285.213	01.324 mg/L

S. NO	ELEMENTS	DETECTED LEVELS
9	Na 589.592	05.310 mg/L
10	Ni 231.604	BDL
11	Pb 220.353	BDL
12	P 213.617	104.341 mg/L
13	Zn 206.200	01.208 mg/L

- From the above results, the heavy metals Aluminium, Cadmium, Hg, and Lead were found below detection level.
- Calcium, potassium, Magnesium, Sodium is found to have remarkable benefit in anti-arthritic therapy.
- Calcium is crucial in growing new bone and maintaining bone strength. Calcium supplements are standard for treating and preventing osteoporosis.
- Magnesium's benefits can include reduced symptoms from conditions such as chronic pain, fatigue and insomnia. Magnesium may also provide protection from a number of chronic diseases, especially those associated with arthritis. [79]

XRD (X-Ray diffraction)



Graph No: 4 XRD - Interpretation

Interpretation

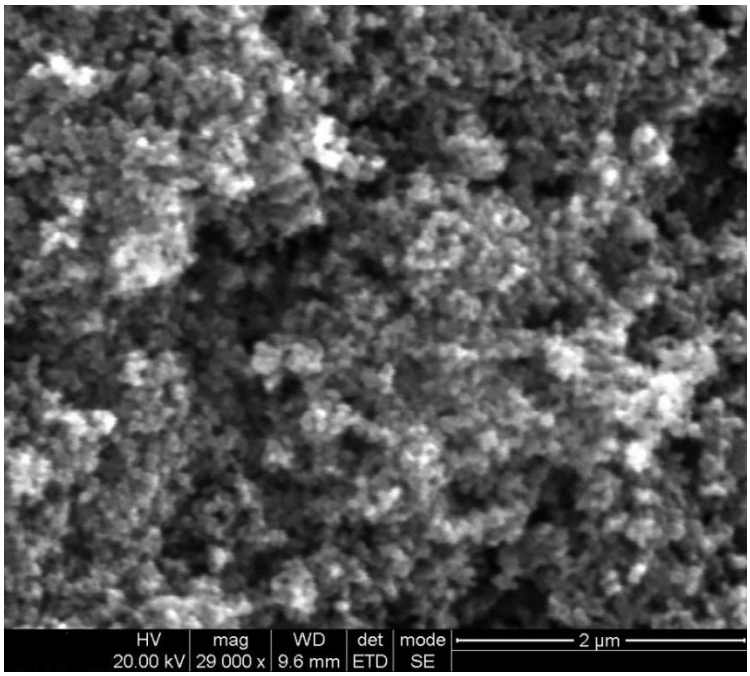
The structure, the size and shape of the particles are highly dependent on the route of synthesis and high lights the efficacy of the drug. The micro particles may enhance bio absorption of the drug.

The major diffraction peaks are identified after XRD analysis KVNC concluded that range 48-75nm is association with organic molecules probably plays an important role in making it biocompatible and nontoxic at therapeutic doses.

Other elements present in KVNC act as additional supplement and possibly helps in increase the efficacy of the formulation.

SEM (Scanning Electron Microscope)

In addition, the particle size and chemical elements were assessed by Scanning Electron Microscope SEM is one of the most widely used instruments in research areas.



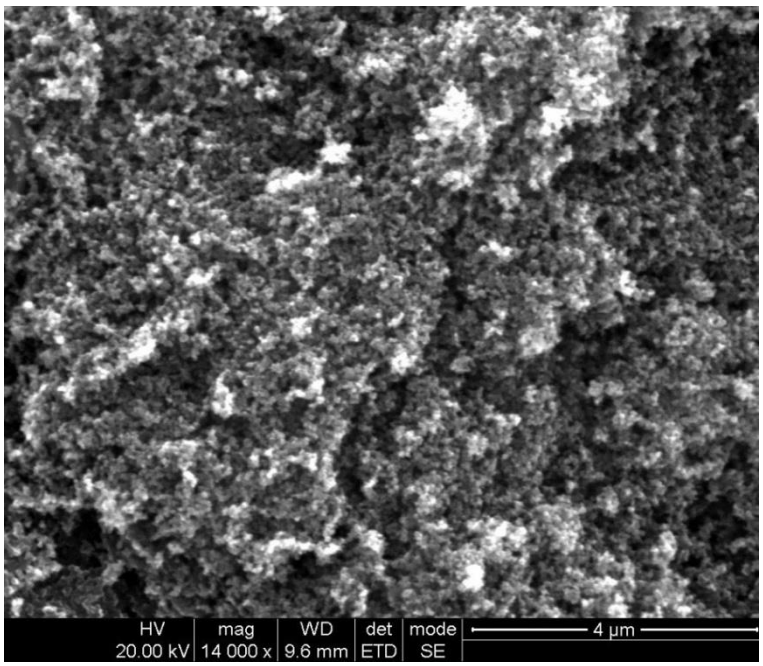


Fig. No.9. Showing of micro particles of KVNC

The above SEM studies of microscopic resolution of 1.00kx and examining surface area of 800x800µm^2. The surface of the sample grains is uniformly arranged in agglomerates. They are micro particles ranging from 500nm - 1µm.

Size and surface of micro particles can be easily manipulated to achieve both active and passive drug targeting. They

- control release of the drug
- sustain release of the drug during the transportation
- alters distribution of the drug
- subsequently clear the drug
- increase drug therapeutic efficacy
- increase bio-availability
- reduces side effects

TOXICITY STUDY RESULTS OF KVNC

Acute oral toxicity study of Keelvayu Nivarana Chooranam

Wistar albino rat was treated with the test drug *Keelvayu Nivarana Chooranam* of single dose of 200mg/kg. This study was conducted as per the OECD guidelines.

The result of acute toxicity of *Keelvayu Nivarana Chooranam* has been tabulated below.

Table No.15. Dose finding experiment and its behavioral Signs of acute oral Toxicity (Observation)

S.No	Group CONTROL	Observation	S.No	Group TEST GROUP	Observation
1	Body weight	Normal	1	Body weight	Normally increased
2	Assessments of posture	Normal	2	Assessments of posture	Normal
3	Signs of Convulsion Limb paralysis	Normal	3	Signs of Convulsion, Limb paralysis	Absence of sign (-)
4	Body tone	Normal	4	Body tone	Normal
5	Lacrimation	Normal	5	Lacrimation	Absence
6	Salivation	Normal	6	Salivation	Absence
7	Change in skin color	No significant color change	7	Change in skin color	No significant color change
8	Piloerection	Normal	8	Piloerection	Normal
9	Defecation	Normal	9	Defecation	Normal
10	Sensitivity response	Normal	10	Sensitivity response	Normal
11	Locomotion	Normal	11	Locomotion	Normal
12	Muscle gripness	Normal	12	Muscle gripness	Normal
13	Rearing	Mild	13	Rearing	Mild
14	Urination	Normal	14	Urination	Normal

Table No.16. Dose finding experiment and its behavioural Signs of Toxicity for KVNC

No	Dose mg/kg	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1.	**Control**	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2.	**2000 mg/kg**	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

1..Alertness 2. Aggressiveness 3. Pile erection 4. Grooming 5. Gripping 6. Touch Response 7. Decreased Motor Activity 8. Tremors 9. Convulsions 10. Muscle Spasm 11. Catatonia 12. Muscle relaxant 13. Hypnosis 14. Analgesia 15.Lacrimation 16. Exophthalmos 17. Diarrhea 18. Writhing 19. Respiration 20. Mortality.

(+ Present, - Absent)

Interpretation

The Acute toxicity result shows no mortality rate up to 2000mg/kg. It showed changes in alertness, grooming and touch response. The behavioural changes are normal. This dose level did not produce signs of toxicity, behavioral changes, and mortality in the test groups as compared to the controls when observed during 14 days of the acute toxicity experimental period. Hence the test drug *Keelvayu Nivarana Chooranam* is a safe herbal drug and can be used for long time administration.

Table No.17. Body weight Observation

DOSE	DAYS		
	1	**7**	**14**
Control	176.21± 3.22	177.2± 4.27	179.2 ± 4.82
200 mg/kg	172.5± 3.18	174.2± 3.26	175.4 ± 3.27

*N.S- Not Significant, ** (p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test)*

Table No: 18 Water intakes (ml/day) of Wistar albino rats group exposed to KVNC

DOSE	DAYS		
	1	**6**	**14**
Control	38.7 ± 2.74	32.9± 4.33	33.4± 4.13
200 mg/kg	32.4±1.34	33.5±1.11	35. 9± 4.19
P value (p)*	NS	NS	NS

*N.S- Not Significant, ** (p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test)*

Table No.19. Food intakes (gm/day) of Wistar albino rats group exposed to KVNC

DOSE	DAYS		
	1	7	14
Control	32.56±2.16	32.92±3.26	30.92±3.26
200 mg/kg	34.12±8.64	34.31±1.22	35.22±2.24
P value (p)*	NS	NS	NS

*N.S- Not Significant, ** (p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test)*

Interpretation

- The result of the body weight of rats exposed to control and the trial drug of *Keelvayu Nivarana Chooranam* 200 mg exhibited overall mild weight gain throughout the dosing period of 14 days.
- The quantity of water and food intake taken by the animals from the group and the control is comparably normal.

REPEATED DOSE 28- DAY ORAL TOXIC STUDY OF KVNC

Table No: 20 Body weight of wistar albino rats group exposed to KVNC

DOSE	DAYS				
	1	7	14	21	28
CONTROL	165.6± 2.76	166.4 ± 3.42	167.7 ± 3.26	169.2 ± 3.73	170.7 ± 1.31
LOW DOSE	165.2 ± 4.12	166.7 ± 2.64	166.9± 1.51	172.9 ± 1.66	174.42± 2.76
MID DOSE	168.6± 1.24	168.9 ± 4.74	170.4 ± 8.92	171.1 ± 6.36	174.7 ± 9.12
HIGH DOSE	171.4± 3.74	173.6 ± 6.32	174.6 ± 2.86	175.1± 8.82	175.92 ± 6.42
P value (p)*	NS	NS	NS	NS	NS

*NS- Not Significant, ** (p > 0.01),*(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test)*

Table No: 21 Water intakes (ml/day) of Wistar albino rats group exposed to KVNC

DOSE	DAYS				
	1	6	14	21	28
Control	31.5 ± 8.95	32.0 ±6.23	28.5±6.23	29.12±8.19	31.5±3.96
Low Dose	29.5±3.31	29.9±6.62	31.7±4.02	32.2±4.29	34.9±3.13
Mid Dose	31.7±3.93	32.3±3.11	34.1±2.83	32.4±4.11	34.4±2.14
High Dose	32.1±1.12	33.2±2.43	34.7±2.53	35.2±1.89	36.4±2.45
P value (p)*	NS	NS	NS	NS	NS

*N.S- Not Significant, ** (p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test)*

Table No.22. Food intake (gm/day) of Wistar albino rats group exposed to KVNC

DOSE	DAYS				
	1	7	14	21	28
Control	37.12 ±5.37	38.5±3.22	39.5±3.37	38.5±3.37	37.12±3.12
Low Dose	33.7±2.12	35.3±1.42	35.9±1.68	36.4±2.62	35.9±8.42
Mid Dose	34.2±3.64	35.9±3.64	36.2±6.15	37.4±2.18	35.2±2.64
High Dose	36.2±2.14	36.2±2.18	37.6±2.14	38.2±4.28	39.2±2.18
P value (p)*	NS	NS	NS	NS	NS

*N.S- Not Significant, ** (p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test)*

Table No.23. Haematological parameters of Wistar albino rats group exposed to KVNC

CATEGORY	CONTROL	LOW DOSE	MID DOSE	HIGH DOSE
Hb(g/dl)	14.8±1.88	12.98±1.28	13.01±1.26	14.18±3.96
Total WBC $\times 10^3$	10.91±2.59	12.25±3.53	12.18±3.61	12.96±3.47
Neutrophils (%)	32.65±1.06	34.23±2.54	34.91±1.36	33.40±2.80
Lymphocyte (%)	69.34±2.48	70.22±3.42	71.48±2.66	71.20±3.96
Monocyte (%)	0.78±0.17	0.81±0.12	0.84±0.11	0.95±0.16
Eosinophil (%)	0.64±0.09	0.19±0.12	0.78±0.06	0.42±0.04
Platelets cells10^3/µl	687.17±8.76	698.71±8.16	705.18±4.0	712.16±4.6
Total RBC 10^6/µl	7.99±0.12	6.82±1.87	6.92±0.59	6.18±0.72
PCV%	37.79±0.6	36.35±1.53	38.2±1.18	36.82±2.14
MCHC g/dl	33.6±2.23	34.19±1.19	35.18±1.92	34.13±1.94
MCV (µm^3)	49.17±3.64	48.20±1.24	49.28±1.24	49.99±1.84

*N.S- Not Significant, ** (p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test)*

Table No: 24 Biochemical parameters of Wistar albino rats group exposed to KVNC

BIOCHEMICAL PARAMETERS	CONTROL	LOW DOSE	MID DOSE	HIGH DOSE
Glucose (r) (mg/dl)	76.45±13.4	76.16±8.54	79.64±9.20	77.42±11.6
T.Cholesterol (mg/dl)	115.26±1.83	112.45±1.13	112.42±1.98	115.22±1.83
Tri Glycerides (mg/dl)	46.35±1.48	45.32±1.48	45.58±1.26	46.66±1.45
LDL	72.81±2.13	70.14±2.34	71.8±2.94	72.64±6.12
VLDL	15.2±2.44	14.42±4.63	14.44±6.64	14.94±5.14
HDL	26.66±6.88	27.96±2.34	27.88±5. 66	29.78±6.22
Ratio 1(T.CHO/HDL)	4.42±2.44	4.36±1.44	4.84±2.44	4.86±1.92
Ratio 2(LDL/HDL)	2.83±4.22	3.02±1.52	2.96±4.80	2.86±3.82
Albumin(g/dL)	3.63±0.17	3.13±1.12	3.10±1.92	2.94±3.86

*NS- Not Significant** (p > 0.01), * (p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test)*

Table No 25: Renal function test of of Wistar albino rats group exposed to KVNC

PARAMETERS	CONTROL	LOW DOSE	MID DOSE	HIGH DOSE
Urea (mg/dl)	13.35±0.99	12.91±1.86	13.16±1.98	13.18±3.92
Creatinine(mg/dl)	0.28±0.08	0.16±1.16	0.12±0.14	0.18±1.22
Bun(mg/dl)	15.02±0.10	14.80±1.20	14.66±0.44	15.10±2.32
Uric Acid(mg/dl)	5.17±0.35	5.25±1.43	5.02±1.35	5.18±1.08

*NS- Not Significant, ** (p > 0.01), * (p >0.05) , n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test)*

Table No 26: Liver Function Test of of Wistar albino rats group exposed to KVNC

PARAMETERS	CONTROL	LOW DOSE	MID DOSE	HIGH DOSE
T.BILIRUBIN(mg/dl)	0.48±0.07	0.43±1.26	0.64±1.28	0.68±1.25
AS(U/L)	79.95±1.39	77.15±1.31	78.71±1.83	80.35±3.03
ALT(U/L)	31.23±1.28	31.81±3.52	30.14±3.18	31.9±1.88
ALP(U/L)	143.25±8.70	141.9±8.17	142.16±4.10	144.33±4.25
T.PROTEIN(g/dL)	5.32±0.38	5.28±0.34	5.21±1.33	5.13±1.06

*NS- Not Significant, ** (p > 0.01), * (p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test)*

From the maximum tolerable dose 200mg/kg 1/5, 1/10 and 1/20th dose were selected for further sub-acute toxic study. In sub-acute toxicity study 28 days duration was followed. The signs and symptoms of toxicity were noted. The animals treated with *Keelvayu Nivarana Chooranam* 200mg/kg showed no statistically significant body weight changes during four weeks of drug treatment. There was no major change in the haematological parameters. After the experimental period the biochemical results indicates that there were no significant changes in Total protein, Hb, Globulin, GGT, Chloride, HDL, VLDL, total bilurubin, and Triglycerides. From the urine routine analysis the drug treatment indicates no significant changes in the pH of urine, bilurubin and Ketones.

Body weight

The result of the body weight of rats exposed to control and the trial drug of different dose groups exhibited overall mild weight gain throughout the dosing period of 28 days.

The quantity of food taken by the animals from different dose groups and the control is comparably normal.

Haematological investigation interpretation

The haematological investigation results of the rats conducted on 28th day after the repeated dose of the drug revealed the values of different parameters.

The increase and decrease in the values obtained were all within the normal biological and laboratory limits.

Biochemical investigation interpretation

The biochemical investigations were conducted on 28th day and the result is produced.

The results revealed there is no significant change in the values of different parameters with that of the control. All the values were within the normal biological and laboratory limits.

Renal Function Test analysis

Renal Function Test analysis data of control group and the test groups of animals taken on 28th day showed no abnormal results.

Liver Function Test analysis

Liver Function Test analysis data of control group and the test groups of animals taken on 28th day showed no abnormal results.

Interpretation

The above slides show the histopathology studies of sub-acute toxicity. There is no toxicological abnormality seen in the vital organs after administration of the test drug *Keelvayu Nivarana Chooranam*.

Mortality in the repeated oral toxicity test was not seen in the limit test up to high dose. Maximum tolerable dose was considered for the further pharmacological studies. No other toxic symptoms were observed in any of the dose treated animals.

Thus the safety of the drug is revealed so that it can be administered for long time without any side effects.

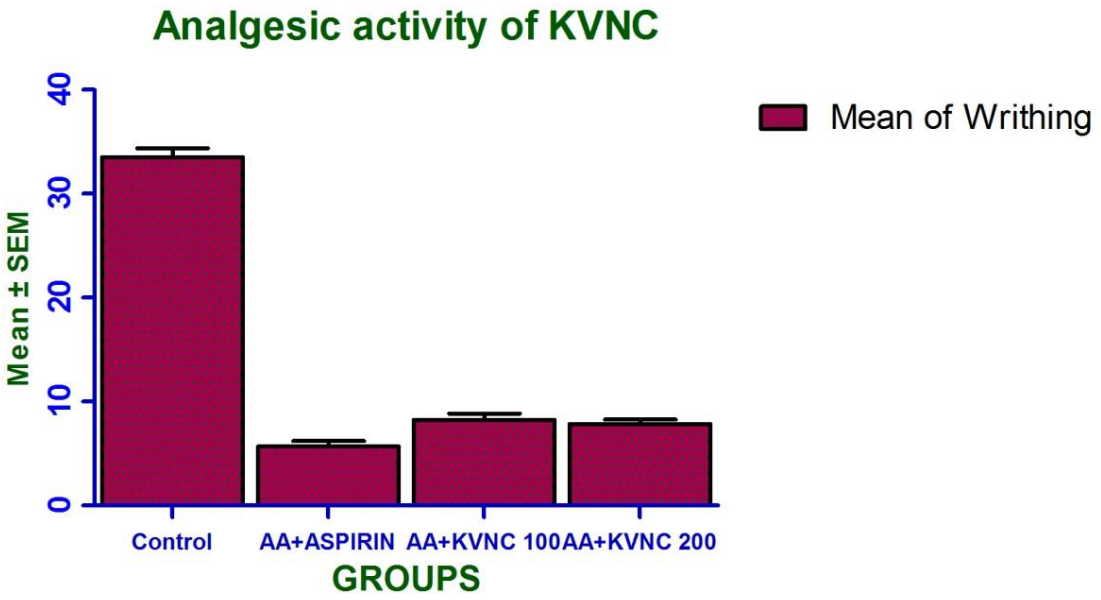
ANALGESIC ACTIVITY OF KVNC

Acetic acid induced writhing in rat

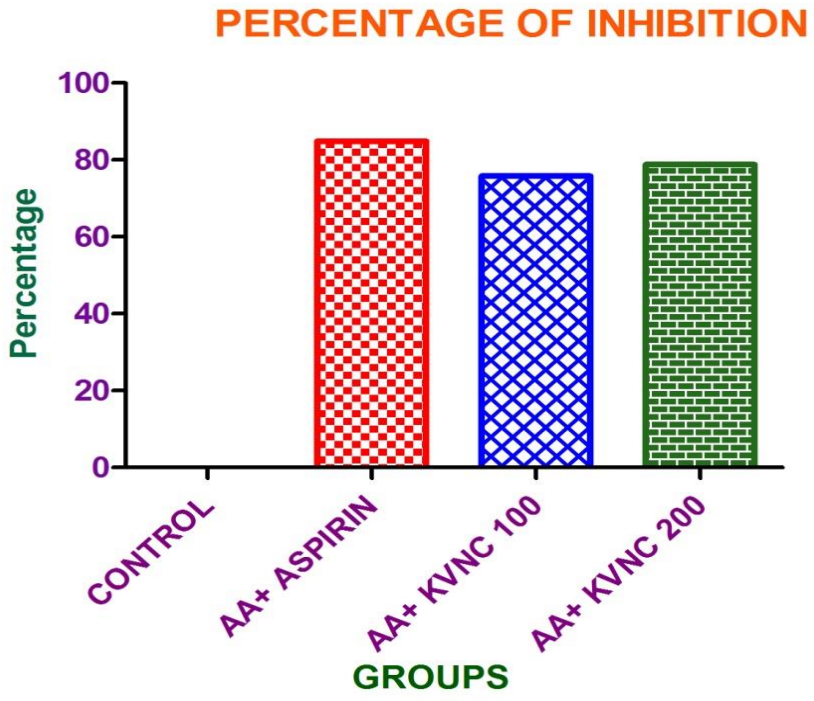
Table No: 27 Analgesic effect of KVNC on acetic acid induced writhing in rat

Groups	Treatment	Dose	Mean of writhing ± SEM (sec)	% of Inhibition
Group I (control)	Acetic acid (1% v/v)	10 mg/kg	33.53±0.87	_
GroupII (standard)	Acetic acid + Aspirin	100 mg/kg	5.68±0.53***	84.85%
Group III	Acetic acid + KVNC	100 mg/kg	8.25±0.60***	75.76%
Group IV	Acetic acid + KVNC	200 mg/kg	7.85±0.42***	78.79%

*Values are mean ± SEM (n = 6 Dunnett' test). ***p< 0.001 when compared to control.*



Graph No.5. Analgesic activity of KVNC



Graph No.6. Percentage inhibition of KVNC

Writhing method is the most common test for evaluating the analgesic efficacy of drugs/compound in rodents. Abdominal constrictions in rats were caused by the intraperitoneal injection of acetic acid.

The animals were previously treated, by oral administration (p.o.) with *Keelvayu Nivarana Chooranam* 1 h before the stimulation with acetic acid. Control animals received the same volume of vehicle. Five minutes after the acetic acid injection, the number of times that each animal presented abdominal constriction was counted for 20 consecutive minutes. The abdominal constriction response induced by glacial acetic acid is a sensitive procedure to establish peripherally acting analgesics. This response is thought to involve local peritoneal receptors.

The number of writhing observed during a 20 min period in control group was 33.53±0.87. It was also observed that animals in test group showed delayed onset of writhes (after 10min) as compared to other groups in which onset of writhes was within 5 min. The *Keelvayu Nivarana Chooranam* (100 mg/kg, p.o.) showed the significant ($P<0.001$) reduction in the number of writhes induced by acetic acid. Aspirin significantly reduced the number of writhes ($P<0.001$). There was a significant, inhibition of pain response in rats.

The Acetic acid -induced writhing response is believed to be produced by the liberation of endogenous substance, notably metabolites of the arachidonic cascade. Hence, Acetic acid causes analgesia by liberating endogenous substances including serotonin, histamine, bradykinin which stimulates pain nerve endings. Local peritoneal receptors are postulated to be partly involved in the abdominal constriction response.

The method has been associated with prostanoids in general, i.e. increased levels of PGE_2 and $PGF_2\alpha$ in peritoneal fluids as well as lipoxygenase products. Therefore, the *Keelvayu Nivarana Chooranam* might inhibit the synthesis and/or release of these endogenous substances. Significant reduction in abdominal constriction compared with vehicle treated animals was considered as antinociceptive response. So it can be concluded that the Siddha drug *Keelvayu Nivarana Chooranam* possess excellent peripheral analgesic property which is equipotent to standard drug used in this study.

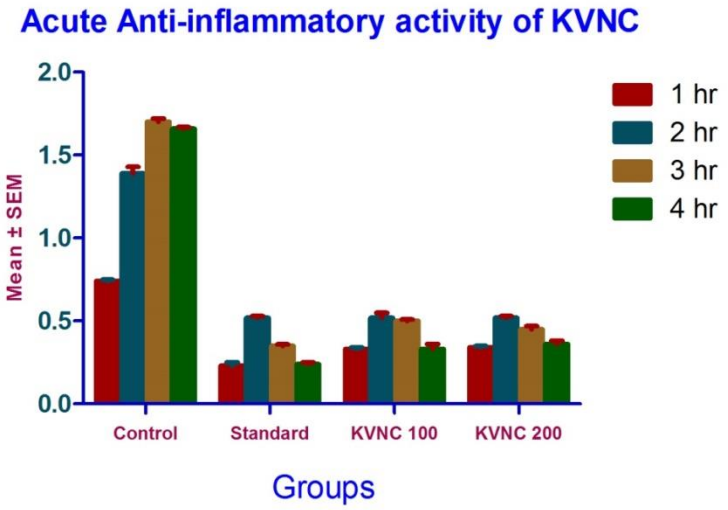
ACUTE ANTI-INFLAMMATORY ACTIVITY OF KVNC

Carrageenan induced rat paw oedema

Table No: 28 Results of the acute anti-inflammatory activity of KVNC

Groups	Treatment	1h	2h	3h	4h	% of Inhibition
Group I (Control)	Carrageenan (1% w/v)	0.74±0.01	1.39±0.04	1.70±0.02	1.66±0.01	_
Group II (Standard)	Carrageenan + Indomethacin (10 mg/kg)	0.23±0.01	0.52±0.01	0.35±0.01	0.24±0.01***	85.55%
Group III	Carrageenan + KVNC (100 mg/kg)	0.33±0.01	0.52±0.01	0.50±0.01	0.33±0.01***	80.13%
Group IV	Carrageenan + KVNC (200 mg/kg)	0.34±0.01	0.52±0.01	0.45±0.01	0.36±0.01***	81.33%

*Values are mean ± SEM (n = 6) (Dunnett' test). *** p < 0.001 when compared to control*



Graph No.7. Acute Anti-inflammatory activity of KVNC

The carrageenan-induced hind paw oedema model in rats is known to be the acute inflammatory model sensitive to cyclooxygenase (COX) inhibitors and has been used to evaluate the effect of nonsteroidal anti-inflammatory agents (NSAID), which primarily inhibit the cyclooxygenase involved in prostaglandin (PG) synthesis. In case of the time course of oedema development in carrageenan induced paw edema model in rats is generally two phases are found.

The first phase, which occurs between 0 to 2.5 h of injection of the phlogistic agent, has been attributed to the release of histamine or serotonin. The edema volume reaches to its maximum approximately 3 h post treatment and then begin to decline. The second phase of inflammatory reaction which is measured at 3h is caused by the release of bradykinin, protease, prostaglandin and lysosome.

Therefore, it can be inferred that the inhibitory effect of the extract on the carrageenan induced inflammation could be due to the inhibition of enzyme cyclooxygenase leading to inhibition of prostaglandin synthesis. Thus, the results of the present study demonstrate that the *Keelvayu Nivarana Chooranam* exhibited acute anti-inflammatory activity in the tested models which was found to be the most effective at higher concentrations employed.

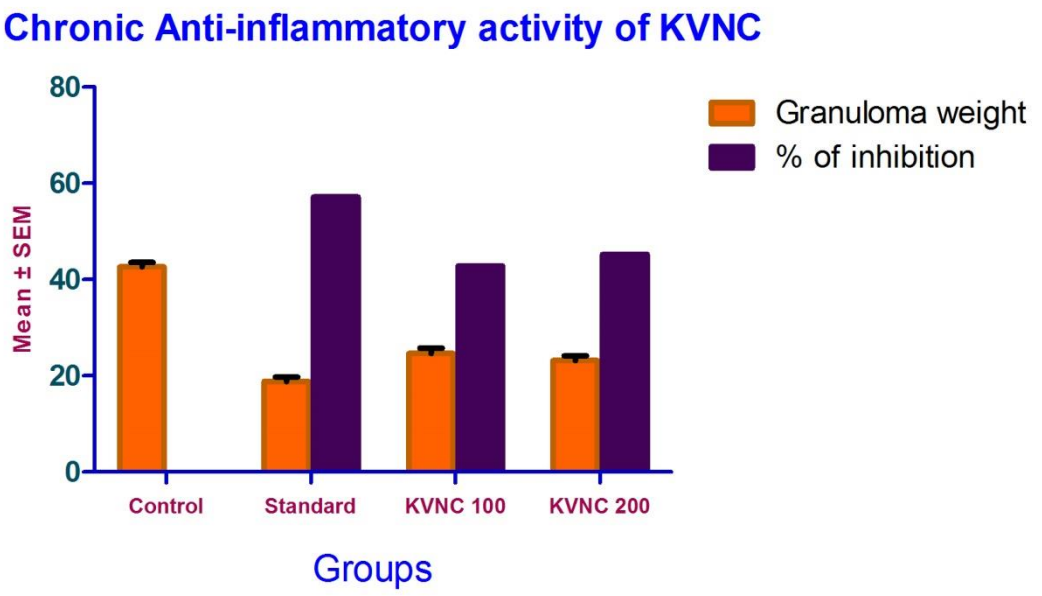
CHRONIC ANTI- INFLAMMATORY ACTIVITY OF KVNC

Cotton pellet granuloma pouch method

Table No: 29 Results of the Chronic Anti-inflammatory activity of KVNC

S.No	Treatment	Dose (mg/kg)	Granuloma weight (mg)%	% of Inhibition
1.	Control	5ml/kg	42.66±0.88	--
2.	Indomethacin	10mg/kg	18.83±0.94***	57.15%
3.	KVNC	100mg/kg	24.66±1.14***	42.86%
4.	KVNC	200mg/kg	23.16±1.01***	45.24%

*Values expressed in mean ±SEM (Dunnett' test), ***P<0.001 compared to control.*



Graph No.8. Chronic Anti-inflammatory activity of KVNC

Cotton pellet granuloma pouch method

Chronic inflammation was induced by cotton pellet granuloma method. Rats were divided into four groups. First two groups received oral doses of 100 and 200 mg/kg of KVNC respectively. The reference drug indomethacin (10mg/kg) was used as a positive control and the other negative control group received saline solution. Sterilized Cotton pellets 50 mg were implanted under light ether anesthesia in the axilla and groin region of each rat by making a small incision Drugs (KVNC 100mg, KVNC 200mg and Indomethacin) and saline (5ml/kg) for control group were administered orally to four groups of rats once daily for 7 consecutive days from the day of cotton pellet implantation.

The 8th day, the animals were sacrificed and cotton pellets were removed and dried in an oven at 600C for 24 hours. They were then weighed. The granuloma formation was calculated as a measure of increment in the dry weight of the pellet. The percentage of inhibition of granuloma was calculated using the following formula.

P = (1 - Wt /Wc) × 100, where, Wt – Dry weight of the cotton in test animals and Wc - Dry weight of the cotton in control animals. Statistical Data were presented as

mean ± S.E.M. Statistical differences between control and treated groups were tested by one way ANOVA followed by dunnett's test.

The percentage of inhibition of granuloma in Cotton pellet granuloma pouch method is shown in table. From this result it was observed that both doses of *Keelvayu Nivarana Chooranam* (KVNC 100 mg and KVNC 200 mg) significantly inhibited granuloma weight ($P<0.05$ and $P < 0.01$ respectively) when compared to the control group.

The percentage of inhibition of KVNC 100 mg and KVNC 200 mg were 42.86%and 45.24% respectively which indicated the dose dependent activity of *Keelvayu Nivarana Chooranam*. KVNC 200mg exhibited percentage of inhibition more than KVNC *100 mg* and slightly less than the reference drug Indomethacin (10mg/kg) which produced 57.15% of inhibition.

ANTI RHEUMATIC ACTIVITY OF KVNC

Collagen Induced Arthritis in Rats

Preparation of emulsion

Chicken sternal collagen type II was dissolved in 0.1M acetic acid at a concentration of 2mg/ml, kept overnight at 40 C. This solution is added drop wise to an equal volume of chilled Incomplete Freunds Adjuant to produce the inducing agent and stored on ice before use. The glass wares used in this process were pre- chilled

Induction of arthritis [80]

1. On day 1 each rat receives a total of 0.5mg collagen in 0.5ml equally divided in five sites (base of the tail, and region above each limb).

2. Administration of booster injection on day 7. The same concentration of the emulsion is used as for the primary immunization. Injected 0.1ml of booster emulsion in the base of the tail, but different location from the first injection site.

All injections were given in intra-dermal route.

Arthritic index

Arthritic index of animals in all groups were recorded on day 1, 14, 21, 28, 35 and 40. Data obtained were tabulated in table.

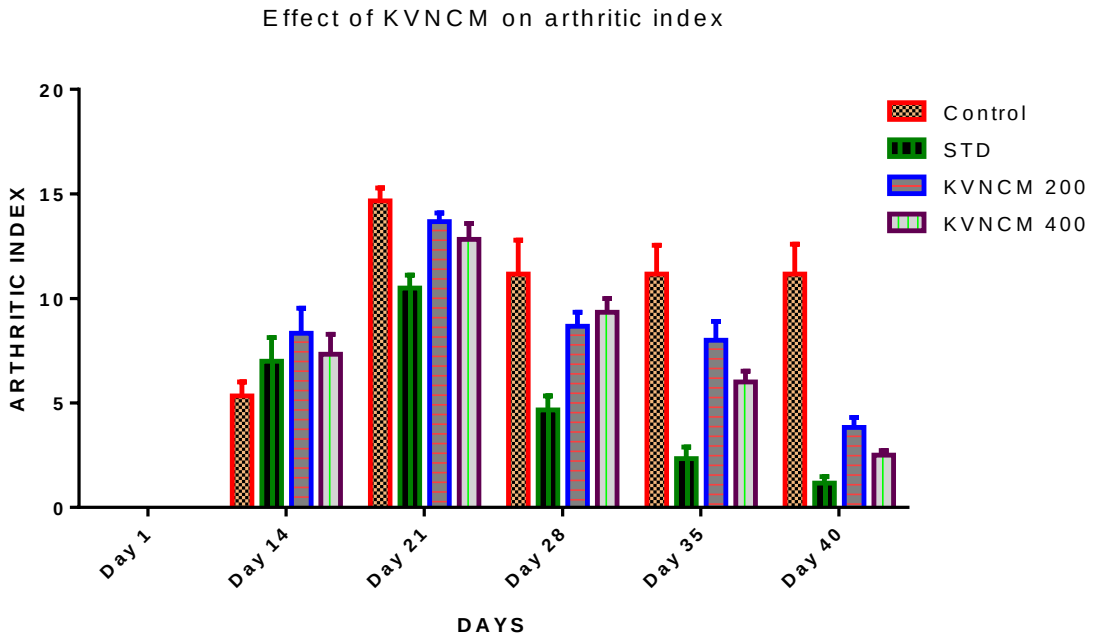
Following the injections of collagen emulsion, rats developed arthritis beginning from day 8 onwards.

After the onset of treatment the KVNC treated rats show marked reduction in the arthritic score CIA rats.

Table No.30. Effect of KVNC on arthritic index in collagen induced arthritic rats

ARTHRITIC INDEX				
Days	**Group I** (Control)	**Group II** (Prednisolone)	**Group III** (KVNC 100mg/kg)	**Group IV** (KVNC200mg/kg)
Day 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Day 14	5.33±0.67	7.00±1.12	8.33±1.20	7.33±0.95
Day 21	14.67±0.61	10.50±0.61	13.6±0.42	12.83±0.74
Day 28	11.17±1.62	4.67±0.66***	8.66±0.66	9.33±0.66
Day 35	11.17±1.38	2.33±0.55***	8.00±0.89	6.00±0.51**
Day 40	11.17±1.42	1.16±0.30***	3.83±0.47***	2.50±0.22***

*Values plotted were the mean ± SEM (n=6). Significant reductions in score were analyzed by using one-way ANOVA followed by Dunnett's multiple comparisons test. *-represent statistical significance. **-p<0.01, ***-p<0.001 when compared with control*

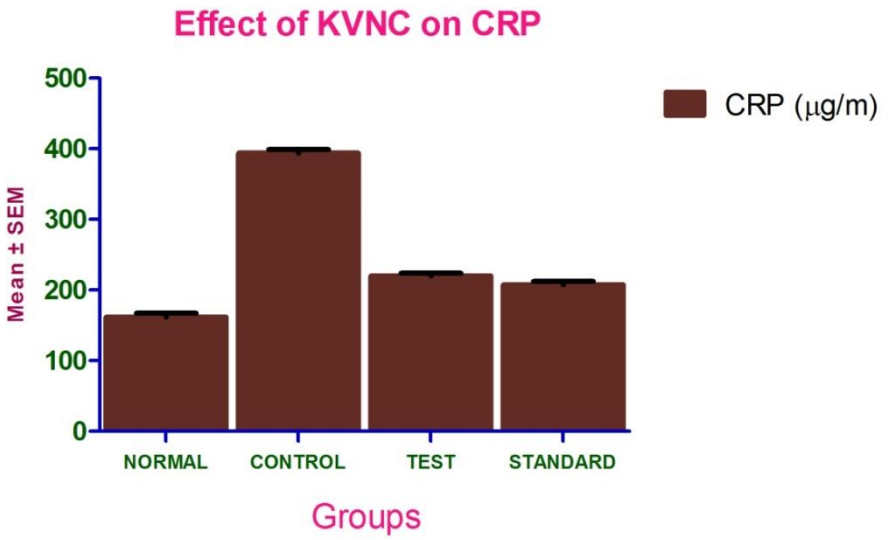


Graph No: 9 Effect of KVNC on arthritic index

Table No: 31 Effect of KVNC effect on CRP in CIA induced arthritic rats

Group	Treatment	Dose	CRP (µg/ml)
Normal	Saline	1ml/kg	166.05±1.46
Control (RA)	Honey alone	1ml/kg	396.59±1.03
Standard	Indomethacin	10mg/kg	204.84±0.65***
Test I	KVNC+Honey	100mg/kg	225.89±0.41***
Test II	KVNC+Honey	200mg/kg	210.84±0.68***

*Values are as Mean ± SEM (Dunnett' test); N = 6; *P<0.05, **P <0.01,***P<0.001 vs Normal*



Graph No: 10 Effect of KVNC on CRP

Table No: 32 Effect of KVNC on heamatological parameters in arthritic rats

Group	Treatment	WBC (Cells/cu.m)	RBC count (millions/cu. mm)	Hb (gm%)	ESR (mm/hr)
Normal	Saline (1ml/kg)	7.36±0.21	5.89±0.17	14.02±0.04	2.23±0.07
Control (RA)	Honey (1ml/kg)	7.44±0.21**	5.23±0.03***	12.37±0.08***	4.11±0.06***
Test	KVNC (100mg/kg)	7.53±0.13*	5.55±0.27***	13.75±1.20***	3.12±0.46***
Test	KVNC (200mg/kg)	7.55±0.06	5.54±0.18***	13.59±0.16**	3.19±0.19***
Standard	Indomethacin 10mg/kg	7.60±0.07	5.53±0.16***	14.59±0.15**	3.22±0.19***

*Values are as Mean ± SEM (Dunnett' test); N = 6; *P<0.05, **P <0.01,***P<0.001 vs Normal*

The results of the present study show that the *Keelvayu Nivarana Chooranam* exhibits anti-arthritic effect in rats with collagen-induced arthritis, either on its acute and chronic phase. The model of collagen-induced arthritis in rats has been extensively used in the study of inflammatory processes and validated as a model of chronic pain. In addition, altered sleep pattern has been observed in these animals. The significant difference for acute and chronic treatments, demonstrating that the method used for induction of arthritis was effective, reducing the pain threshold in the injected animals, thus, revealing the applicability of induced arthritis as an experimental model of chronic pain.

RA develops as a result of interaction of many factors, which include genetic (inherited) factors, environmental (viral or bacterial) factors and hormonal factors. A variety of tools are used to diagnose RA and these include; medical history, physical examination, laboratory test (rheumatoid factor, white blood cell count and erythrocyte sedimentation rate, measurement of Hb calculated) and X-rays. The goals of managing arthritis are basically the same no matter what treatment approach is chosen; these are to relieve pain, reduce inflammation, slow down or stop joint damage and improve a sense of wellbeing and ability to function. Rheumatoid arthritis is a progressive, disabling, chronic multisystem disease of unknown cause characterized by pain and stiffness of synovial joints.

On day 1 each rat receives a total of 0.5mg collagen in 0.5ml equally divided in five sites (base of the tail, and region above each limb). Administration of booster injection on day 7. The same concentration of the emulsion is used as for the primary immunization. Injected 0.1ml of booster emulsion in the base of the tail, but different location from the first injection site. Deregulation of IL-6 expression causes the synthesis and release of many inflammatory mediators, which may result in pain and edema.

Due to its multiple stimulatory effects on cells of the immune system and vascular endothelial cells, it is believed that excess IL-6 plays a pathogenic role in the development of inflammation, resulting in hyperalgesia and edema. Modulation of immune responses to alleviate pain and inflammation has been of interest for many years [81]. Due to the central role played by IL-6 in a number of manifestations of

inflammatory diseases, therapeutic inhibition of IL-6 represents a novel approach to the treatment of chronic inflammation.[82]

Studies have demonstrated that some symptoms of inflammation with a significant cytokine component, such as rheumatoid arthritis, can be treated by inhibition of IL-6. However, IL-6 has been shown to elicit both pro- and anti-inflammatory effects.

The erythrocyte sedimentation rate is elevated in various stress conditions, cell necrosis and inflammation.

The ESR is an indirect method for the measurement of inflammation in the body. Due to protein production in inflammation, erythrocytes move closer, stack up in a group, become denser and settle faster.

With the increase in erythrocyte settling rate, erythrocyte sedimentation rate also increases. The significant low level of ESR in the KVNC treated arthritis rats indicates their anti-inflammatory potential.

Rats exhibited a gradual increase WBC, ESR compared with control group, whereas RBC count and haemoglobin percentage decreased compared with control group. Here attempt has been made to evaluate pharmacological potential of *Keelvayu Nivarana Chooranam* after stability study by using parameters likes change in arthritic score in collagen induced arthritis.

The result of Anti-Rheumatic activity of KVNC after stability studies showed that *Keelvayu Nivarana Chooranam* is having better anti-rheumatic potential as compare to control.

The tissue sections of CIA injected control revealed the pathological changes that can be correlated with arthritis as compared to the normal control. In particular, marked hyperkeratosis of skin of footpad, infiltration of leukocytes and eosinophilic inflammatory exudates, edema in deeper subcutaneous tissues and proliferation of collageneous tissues was evident.

The treatment with KVNC 200mg/kg showed marked reduction of the injury to tissue sections and most of the histological changes were minimized and found negligible as compared to the arthritis control. In particular, marked reduction in hyperkeratosis of skin of footpad, leukocytic and eosinophilic infiltration, edema and proliferation of collageneous tissues was evident. [83]

The most probable mechanism of this drug might be the inhibition of proinflammatory cells by KVNC which could have led to an alteration in the immunological milieu during the delayed phase of the response.

The results obtained in the present study demonstrate beneficial effects of KVNC during recovery from arthritis by including Hb, ESR and body weight along with clinical signs histopathological examination. On the basis of the results obtained in the present study we suggest that possibly, the anti-arthritic potential of KVNC. May be through protection of synovial membrane, vascular permeability, prevention of cartilage destruction, thereby improving the health status.

The results demonstrate the Anti-rheumatic effects of *Keelvayu Nivarana Chooranam* in the collagen induced arthritis in rats. The most probable mechanism of this drug might be the inhibition of proinflammatory cells by *Keelvayu Nivarana Chooranam* which could have led to an alteration in the immunological response.

6. CONCLUSION

The test drug ***Keelvayu Nivarana Chooranam*** was taken as a compound drug for Rheumatoid arthritis from the literature **The Pharmacopoeia of Siddha Research Medicine** (chapter 2-14) written by **Dr. M. Shanmugavelu & Dr.G.D.Naidu**, to validate the safety and its efficacy of drug to treating Analgesic, Anti-inflammatory, and Anti Rheumatic activity in animal model.

The trial drug was subjected to various studies through which the efficacy of the drug is proved.

The preparation of trial drug was standardized by physico chemical and bio chemical analysis.

The Physico chemical analysis the drug shows the presence of sulphur, calcium. phosphate. These elements are responsible for anti-rheumatic activity.

FTIR analysis revealed the presence of O-H groups, C-C group, which indicates functional groups present in the sample. FTIR Interprets the moleculate structure of the sample.

SEM analysis showed the size of the drug in micro particles which denotes that the trial drug could have potent drug delivery.

XRD analysis disclosed the percentage of elements presence in the drug.

The acute and sub-acute toxicological studies proved that the drug is nontoxic and safe.

The pharmacological studies conducted by in vivo method revealed that the drug has effective Analgesic, Acute and Chronic Anti-Inflammatory, Anti-Rheumatic activity as well. The ESR values are also greatly reduced and moved towards normal.

7. SUMMARY

The trial drug of **"*Keelvayu Nivarana Chooranam*"** has been selected and its efficacy was analyzed for the preclinical study to establish the analgesic, acute and chronic anti-inflammatory, anti-rheumatic activity. The drug is easily prepared and preparation is very simple when compared to modern drugs. The trial medicine is cost effective. It contains essential elemental, organic, constituents for its basic and potentiality and it represented the micro particles of its content.

The ingredients of the test drug was identified and authenticated by Siddha experts. The drug was prepared as per the procedure and subjected to various studies to reveal its potency and effectiveness against the disease.

The phytochemical analysis reveals the presence of alkaloids, tannins, flavonoids, cardiac glycosides, saponins, phenols, proteins and carbohydrates. From the above analysis we came to know the presence of active ingredients responsible for its activity.

Biochemical analysis showed the presence of Potassium, Calcium, Magnesium, Sodium and Iron. Thus from these results we come to know the effectiveness of the drug is due to the presence of these constituents and it has a synergistic effect in acting against the disease.

The FTIR analysis construe the results that showed the presence of functional groups like alcohols, esters, alkanes, aromatic amines which might be responsible for the presence of anti-rheumatic action of the drug. SEM picture described its morphology and the particle size.

Toxicological study of both acute and sub-acute toxicity study were carried out in animal model Wister albino rat according to the OECD guidelines. The toxicity helped to fix the doses as 1/5th and 1/10th for pharmacological activities that were carried out.

The treated rats in the toxicity studies did not show any mortality, any untoward clinical sign, any behavioural signs, and alterations in body weight and necropsy findings at

the end of the study. This indicates that the dosages administered were below toxic level and proves the safety of the drug.

Further acute and repeated 28 day oral toxicity studies were done to evaluate the safety of the drug. The mortality, functional observations, hematological and biochemical investigations were made. There was no significant change seen in the normal values.

Thus the toxicological study of the test drug greatly establishes the safety and gives the justification for long time administration.

The pharmacological study was carried out in the animal model Wistar albino rats. Three activities were seen in the drug *Keelvayu Nivarana Chooranam*. The activities were

- Analgesic activity
- Anti-inflammatory activity
- Anti-rheumatic activity

Analgesic activity was carried out in Acetic acid induced writhing test the test drug KVNC 100 mg/kg b.wt showed marked analgesic activity. Thus this activity reveals the effect of the drug against Rheumatoid arthritis.

Anti-inflammatory of the trial drug *Keelvayu Nivarana Chooranam* was carried out. . This activity reveals the anti-inflammatory property of the test drug.

Anti-rheumatic activity was carried out in collagen induced arthritis in Wistar albino rat models. The test drug showed significant decreases inflammation and decreased level of CRP in blood. Thus anti-rheumatic activity of the drug is justified.

The anti-rheumatic activity of the drug *Keelvayu Nivarana Chooranam* is mainly of the presence of the active principles. The analgesic and the anti-inflammatory property of the test drug support the drug in treating RA.

In RA is always accompanied with increased level of CRP. The drug showed significant decrease in the level of mentioned the entire above drug compared with than the standard drug.

Thus by scrutinizing all the above mentioned factors it is concluded that the test drug *Keelvayu Nivarana Chooranam* a safe and a potent anti-rheumatic drug.

To conclude, the drug *Keelvayu Nivarana Chooranam* beneficiary values for the therapeutic efficacy for anti-rheumatic activity. It also possesses analgesic, anti-inflammatory activities which support the effective treatment for managing Rheumatoid arthritis.

8. FUTURE SCOPE

The trial drug ***Keelvayu Nivarana Chooranam*** was taken from the literature **The Pharmacopoeia of Siddha Research Medicine** (chapter 2-14) written by **Dr. M. Shanmugavelu & Dr.G.D.Naidu.** Its validation for analgesic, anti-inflammatory and anti-rheumatic activity was completed at preclinical level. The result showed the assurance of Anti-Rheumatic activity against Rheumatoid Arthritis. Clinical trials are required to understand the exact molecular mechanism of action and it would be a safer drug for Rheumatoid Arthritis in worldwide.

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Introduction

Aim & Objectives

Review of Literature

Materials & Methods

Results & Discussion

Conclusion

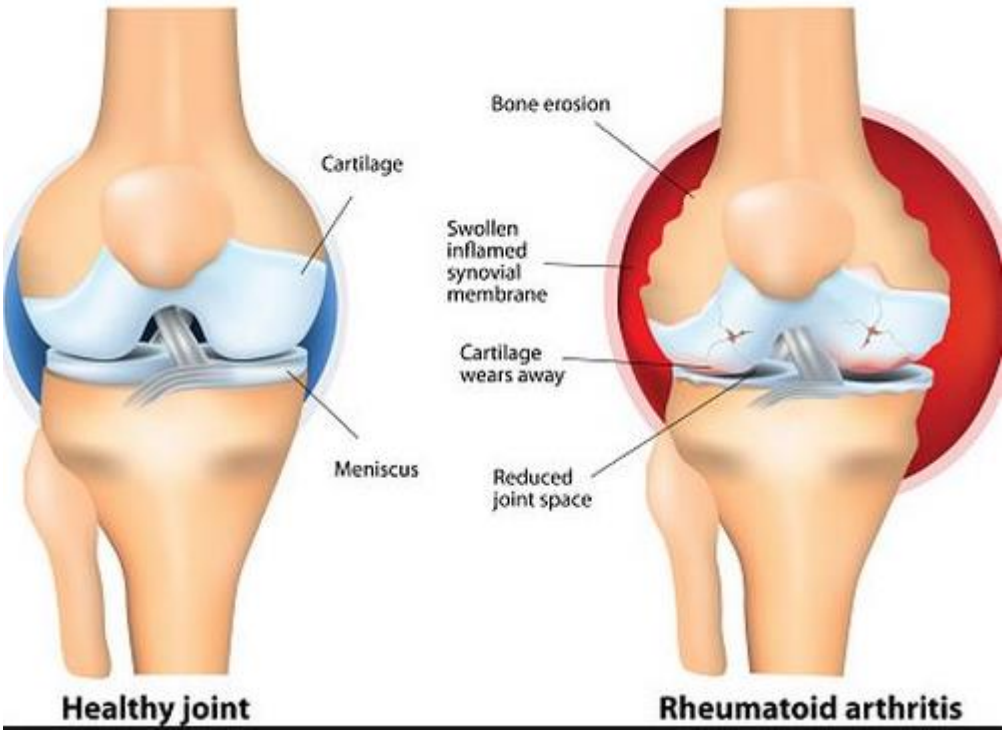
Summary

Future Scope

Bibliography

Annexure

RHEUMATOID ARTHRITIS



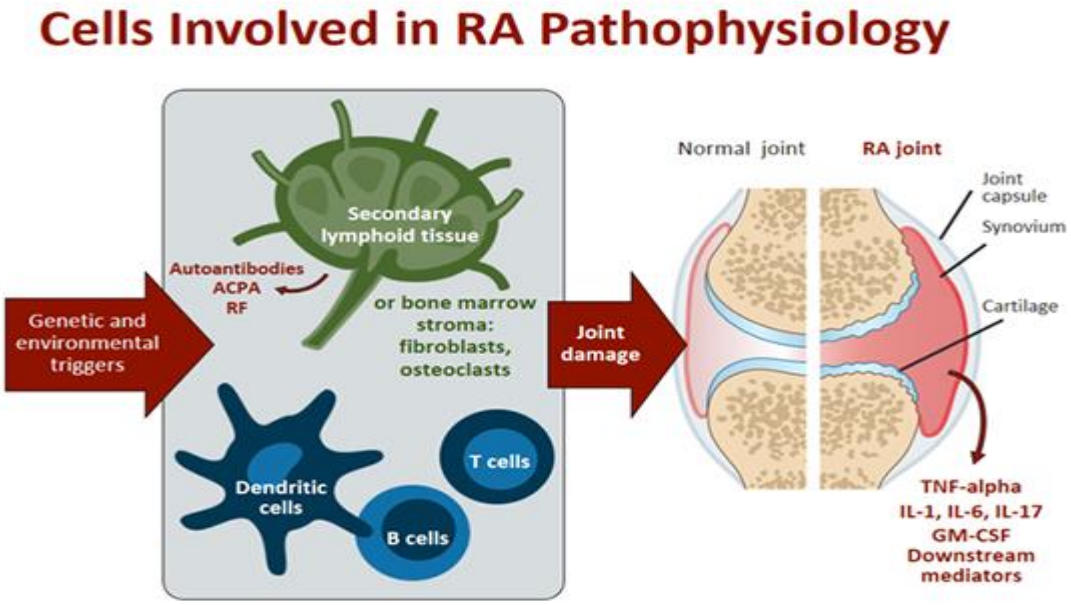


Fig. No.1. Rheumatoid Arthritis – Pictures

INGREDIENTS OF *KEELVAYU NIVARANA CHOORANAM*

A) Hemidesmus indicus

B) Simlax chinensis

C) Withania somnifera

D) Alpinia officinarum

Fig No.2. Ingredients of *Keelvayu Nivarana Chooranam*

PREPARATION OF *KEELVAYU NIVARANA CHOORANAM*

Pounding

Sieving process

Keelvayu Nivarana Chooranam

Fig No. 3. Preparation of *Keelvayu Nivarana Chooranam*

HISTOPATHOLOGY – 28 DAYS REPEATED ORAL TOXICITY STUDY

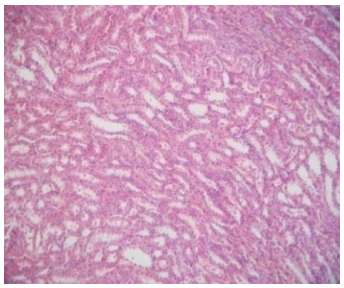
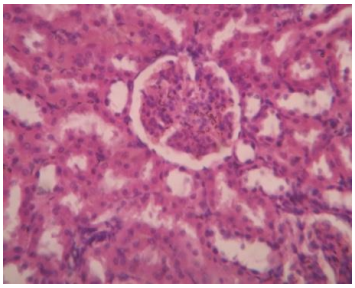
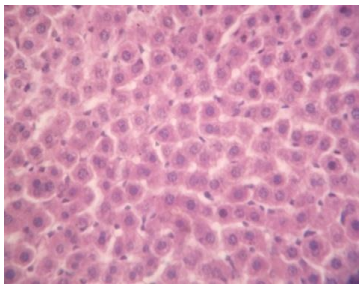
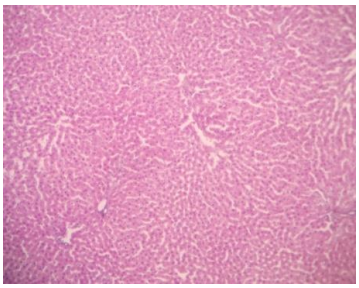
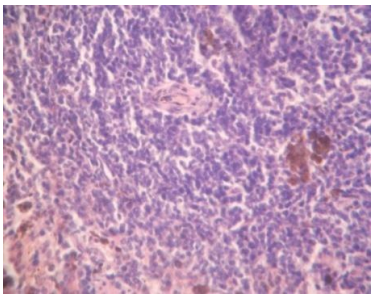
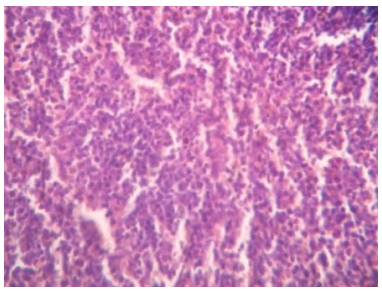
Control Group	Test Group (High Dose)
Kidney	**Kidney**
	
Liver	**Liver**
	
Spleen	**Spleen**
	

Fig No.10. Histopathology study of KVNC- Toxicity

The Tamil Nadu Dr.M.G.R.Medical University

69,Anna Salai,Guindy,Chennai - 600 032.

This Certificate is awarded to Dr/Mr/Mrs....T.:..Giftillda...Selva Elsee.....

for participating as ~~Resource~~ ~~Person~~ / Delegate in the Eighteenth Workshop on

" RESEARCH METHODOLOGY & BIOSTATISTICS "

FOR AYUSH POST GRADUATES & RESEARCHERS

Organized by the Department of Siddha

The Tamil Nadu Dr. M.G.R. Medical University from 20 th to 24 th July 2015.

Dr.N.KABILAN, M.D.(Siddha)
READER,DEPT.OF SIDDHA

Prof. **Dr.P.ARUMUGAM,** M.D.,
REGISTRAR i/c

Prof. **Dr.D.SHANTHARAM,** M.D., D.Diab.,
VICE - CHANCELLOR

C.L.BAID METHA COLLEGE OF PHARMACY

(An ISO 9001-2000 certified institute)

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Thoraipakkam, Chennai – 600 097

CERTIFICATE

This is to certify that the project entitled, **Toxicological and Pharmacological study on KEEL VAAYU NIVARANA CHOORANAM & KUTTHUKAR CHAMMATTI (*Indigofera oblongifolia*) CHOORANAM** in rats submitted in partial fulfilment for the degree of **M.D. (siddha)** was carried out at C.L. Baid Metha college of Pharmacy, Chennai-97, in the Department of Pharmacology during the academic year of 2016-2017. It has been approved by the **IAEC No: IAEC/XLVIII/05/CLBMCP/2016**

(Dr.P.Muralidharan)

IAEC Member Secretary

C.L. Baid Metha College of Pharmacy ★ CHENNAI-97 ★ Thoraipakkam