# “PHARMACOGNOSTICAL AND PHARMACOLOGICAL INVESTIGATIONS ON SOME IMPORTANT MEDICINAL PLANTS FROM ZINGIBERACEAE FAMILY”

**A THESIS SUBMITTED TO**
**BHARATI VIDYAPEETH DEEMED UNIVERSITY, PUNE**
**FOR AWARD OF DEGREE OF**
**DOCTOR OF PHILOSOPHY IN PHARMACOGNOSY**
**UNDER THE FACULTY OF PHARMACEUTICAL SCIENCES**

**SUBMITTED BY**
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# CERTIFICATE

This is to certify that the work incorporated in the thesis entitled **"Pharmacognostical and Pharmacological Investigations on Some Important Medicinal Plants from Zingiberaceae Family"** for the degree of 'Doctor of Philosophy' in the subject of **Pharmacognosy** under the faculty of Pharmaceutical Sciences has been carried out by **Mr. Vijaykumar Manoharrao Kale** in the Department of Pharmacognosy at Bharati Vidyapeeth Deemed University, Poona College of Pharmacy, Pune during the period from November 2011 to November 2015 under the guidance of **Dr. A. G. Namdeo**.

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# CERTIFICATION OF GUIDE

This is to certify that the work incorporated in the thesis entitled **"Pharmacognostical and Pharmacological Investigations on Some Important Medicinal Plants from Zingiberaceae Family"** Submitted by **Mr. Vijaykumar Manoharrao Kale** for the degree of 'Doctor of Philosophy' in the subject of Pharmacognosy under the faculty of Pharmaceutical Sciences has been carried out in the Department of Pharmacognosy, Bharati Vidyapeeth Deemed University, Poona College of Pharmacy, Pune during the period from November 2011 to November 2015, under my direct guidance.

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# DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled **"Pharmacognostical and Pharmacological Investigations on Some Important Medicinal Plants from Zingiberaceae Family"** submitted by me to the Bharati Vidyapeeth Deemed University, Pune for the degree of Doctor of Philosophy (Ph.D.) in Pharmacognosy under the Faculty of Pharmaceutical Sciences is original piece of work carried out by me under the supervision of **Dr. A. G. Namdeo.** I further declare that it has not been submitted to this or any other university or Institution for the award of any degree or Diploma.

I also confirm that all the material which I have borrowed from other sources and incorporated in this thesis is duly acknowledged. If any material is not duly acknowledged and found incorporated in this thesis, it is entirely my responsibility. I am fully aware of the implications of any such act which might have been committed by me advertently or inadvertently.

Place: Pune

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Date:

Place: Pune

Mr. Vijaykumar M Kale


DEDICATED TO
MAHILA UTKARSH PRATISTHAN,
MY GUIDE AND MY FAMILY

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## List of Abbreviations

| | |
|---|---|
| AA | Arachidonic Acid |
| ACA | 1'-acetoxychavicol acetate |
| AEAG | Acetone extract of *A. galanga* |
| ALP | Alkaline phosphatase |
| ALT | Alanine transaminase |
| AOT | Acute oral toxicity |
| AST | Aspartate transaminase |
| BAP | Benzylaminopurine |
| BM | Basal medium |
| $^{0}C$ | Degree centigrade |
| $^{13}C$-NMR | Carbon nuclear magnetic resonance |
| $^{1}H$-NMR | Proton nuclear magnetic resonance |
| cm | Centimeter |
| CNS | Central nervous system |
| COX | Cyclooxygenase |
| CPCSEA | Committee for the Purpose of Control and Supervision on Experiments of Animals |
| CRP | C-reactive protein |
| DEPT | Distortion-less enhancement by polarization transfer |
| DW | Dry weight |
| EDTA | Ethylene diamine tetraacetic acid |
| FCA | Freund's complete adjuvant |
| FW | Fresh weight |
| g | Gram |
| g/l | Gram/litre |
| GA | Gibberelic acid |
| GIT | Gastrointestinal tract |
| GSH | Glutathione |
| Hb | Haemoglobin |

| | |
|---|---|
| IAA | Indole-3-acetic acid |
| IBA | Indole-3-butyric acid |
| i.p. | Intraperitoneal |
| i.v. | Intravenous |
| IAEC | Institutional animal ethics committee |
| IFN | Interferon |
| IL | Interleukin |
| iNOS | Inducible nitric oxide synthase |
| IR | Infra red |
| IU | International unit |
| J | Coupling constant |
| Kn | Kinetin |
| kg | Kilogram |
| LC-MS | Liquid chromatography/ mass spectroscopy |
| LPS | Lipopolysaccharide |
| MDA | Malondialdehyde |
| mg/dl | Milligram/ deciliter |
| mg/kg | Milligram /kilogram |
| Min. | Minutes |
| mm | Millimeter |
| MS | Mass spectroscopy |
| MS | Murashige and Skoog medium |
| NAA | Napthalene-3-acetic acid |
| NF-κB | Nuclear factor kappa B |
| NMR | Nuclear magnetic resonance |
| NO | Nitric oxide |
| NSAID | Non steroidal anti-inflammatory drug Multiple sclerosis |
| OECD | Organization for economic co-operation and development |
| p.o. | Per oral |
| PAF | Platelet aggregation factor |
| PBS | Phosphated buffer saline |

| | |
|---|---|
| PG | Prostaglandin |
| PK | Protein kinase |
| RA | Rheumatoid arthritis |
| RBC | Red blood cell |
| Rf | Retention factor |
| ROS | Reactive oxygen species |
| rpm | Rotations per minute |
| RT | Room temperature |
| Sec | Seconds |
| S.D | Standard deviation |
| S.E.M. | Standard error mean |
| SOD | Superoxide dismutase |
| SOP | Standard operating procedure |
| TGF-α | Transforming growth factor α |
| TLC | Thin layer chromatography |
| TMB | Tetra methyl benzidine |
| TNF-α | Tumour necrosis factor α |
| UV | Ultraviolet |
| w/v | Weight/volume |
| WBC | White blood cell |
| WHO | World Health Organization |
| µg/ml | Microgram/milliliter |
| µIU/ml | Micro international units/milliliter |
| µl | Microlitre |
| 2,4-D | 2, 4-Dichlorophenoxyacetic acid |

# INTRODUCTION

## 1. INTRODUCTION

The family Zingiberaceae is distributed widely throughout the tropics, particularly in Southeast Asia and it is well known for its immense medicinal values. India is one of the richest and diverse regions for Zingiberaceae, having 22 genera and about 170 species (Tushara et al., 2010). Zingiberaceae family constitutes a fundamental group of rhizomatous medicinal and aromatic plants which were characterized by presence of volatile oils and oleoresins, due to its high export value. Zingiberaceae family is a vital natural resource useful in many products for food, spices, medicines, dyes and perfume. The rhizomes and fruits are aromatic, tonic and stimulant; occasionally they are nutritive. A few are used as food as they contain starch in huge quantities while others yield an astringent and diaphoretic juice. The important genera coming in Zingiberaceae are *Curcuma, Kaempferia, Hedychium, Amomum, Zingiber, Alpinia, Elettaria* and *Costus.* In the genus *Alpinia, A. galanga* is the most significant one, which finds different uses in ayurvedic preparations such as "Rasnadi powder" (Joy et al., 1998). Numerous Zingiberaceae species have been studied through *in vitro* rhizome bud multiplication which is a simple and safe method for obtaining uniformity and it also guarantee the consistent production of true-to-type plants within a short span of time (Selvakkumar et al., 2007).

Rhizomes of certain ginger species like *A. officinarum, A. galanga, A. calcarata, Kaempfera galangal* have more medicinal values (Indranyl et al., 2009). Zingiberaceae family constitutes about 50 genera usually found all over the warm regions of both hemispheres. Out of these 50 genera only nine genera are reported to constitute potential medicinal and aromatic plants as listed below:

| *Genus* | *Species* |
|---|---|
| *Curcuma* | *C. amada, C. longa, C. zedoaria, C. aromatic* |
| *Kaempferia* | *K. rotunda, K. galanga* |
| *Hedychium* | *H. spicatum, H. coronarium* |
| *Amomum* | *A. subulatum* |
| *Zingiber* | *Z. officinale, Z. zerumbet* |
| *Alpinia* | *A. galanga, A. calcarata, A. allughas* |
| *Elettaria* | *E. cardamomum* |
| *Costus* | *C. speciosus* |
| *Gastrochilus* | *G. pandurata* |

## 1.1: *Alpinia galanga*

### 1.1.1 Taxonomy of *A. galanga*

Botonical name- *Alpinia galanga*

Family - Zingiberaceae

Genus - *Alpinia*

Species - G*alanga*

Synonyms- *Languas galanga*

Common name- Greater galanga

### 1.1.2 Morphology of *A. galanga*

Root stocks of *A. galanga* are tuberous and to some extent aromatic. Leaves are oblong lanceolate, glabrous, acute, green above, paler beneath, with somewhat callus white margins, sheaths are long and glabrous, ligule are short and rounded. Flowers of the plant are greenish white, in dense flowered, 30 cm Panicles; bracts ovate-lanceolate. Calyx is tubular, unevenly 3-toothed. Corolla lobes oblong, claw green, blade white, striated with red, more than 1 cm long, broadly elliptic, shortly 2-lobed at the apex part with a pair of subulate glands at the base of the apex and with a pair of subulate glands at the base of claw. Fruit is look like small cherry, orange red (Joy et al., 1998)

**Fig. 1.1.2a: Morphological features of plant and rhizome of *A. galanga***

**Fig. 1.1.2b: Rhizomes of *A. galanga***

### 1.1.3 Chemical constituent

The compounds to be present in the plant viz. camphene, myrcene, 1, 8-cineole, α-fenchol, camphor, α-fenchyl acetate, carotol, guaiol etc are recognized to be responsible for the characteristic odour in addition to have the medicinal properties. It also contains phenylpropanoids like acetoxychavicol acetate (ACA), acetoxyeugenol acetate and p-coumaryl diacetate, liable for the anti-tumour, anti- HIV and anti-parasitic activities (Rao et al., 2011).

### 1.1.4 Uses

*A. galanga* Linn is an aromatic rhizomatous herb and the main important crop plant of family Zingiberaceae which is cultivated in India, China, Thailand, Malaysia and Indonesia. Many *Alpinia* species are valued for their medicinal properties and are also used in traditional medicines as a spasmolytic, hypotensive, anti-emetic, anti-oxidant, anti-inflammatory, bacteriostatic, fungistatic property in India, China and other regions (Parida et al., 2011).

*A. galanga* is commonly known as greater galangal. It's harvesting management at 3 months-interval from 6 to 48 months subsequent to planting. Harvesting the crop at 42 months after planting was the most excellent for realizing greatest rhizome and for obtaining oils of superior quality (27.1% cineole [Eucalyptol]). The shoot was developed maximum at 18 months subsequent to planting. *A. galanga* reached a maximum height of 129.4 cm with more than 48 tillers per clump and 13 leaves per tiller in the trial location. Its taxonomy, botanical distribution and morphology were given by (Verma et al., 2011).

*A. galanga* is mostly used in cooking as an aromatic stimulant or flavoring agent and as folklore medicine for the treatment of bronchial catarrh, rheumatism and respiratory ailment like asthma. *A. galanga* is used for ingredients in polyherbal preparations for relieving pain of various etiologies such as rheumatoid arthritis, back pain and pain in persons who had chikungunya fever in South India (Acharya et al., 2011).

*A. galanga* willd, is usually used in the Arabian and in Unani systems of medicine for the management of dyspepsia, gastralgia, chronic enteritis and sea sickness. The rhizome is also said to help digestion and is useful in the treatment of abdominal colic. In China it is used for the treatment of stomach cancer (Al-Yahya et al., 1990). The rhizomes of *A. galanga* treat the problems related with digestive system, and relieve bronchitis, measles, rubella and cholera.

1'S-1' Acetoxychavicol acetate (ACA) is isolated from the rhizomes of *A. galanga* is used as a ginger replacement and a stomach medicine in Southeast Asia. ACA exerts its antitumor action by inducing apoptosis in a variety of cancer cells such as *Ehrlich ascites*, rat and human hepatocellular carcinoma cells, human colon cancer cells, and human myeloid leukemia cells (Azuma et al., 2006). The polysaccharide fractions from the medicinal plants *A. galanga* may include active principles with immune enhancing effects on both phagocytic and lymphocytic systems (Bendjeddou et al., 2003).

### 1.2: *Alpinia officinarum*

#### 1.2.1 Taxonomy of *A. officinarum*

Botonical name: *A. officinarum* Hance

Family: Zingiberaceae

Genus: *Alpinia*

Species: *Officinarum*

Synonyms: *Languas officinarum* (Hance), *galanga, lesser Galangal*

Common name: Lesser galanga

#### 1.2.2 Morphology of *A. officinarum*

*A. officinarum* can grow up to several feet high, with long leaves and reddish-white flowers. The leaves are long and thin; the flowers are white with streaks of red,

growing from a spike at the top. Its odour is aromatic, and their taste pungent and spicy. The rhizomes are reddish brown and about 2 cm in diameter.

**Fig. 1.2.2: Rhizomes of *A. officinarum***

### 1.2.3 Chemical constituents

Diarylaheptanoid from this plant was 7-(3,4-dihydroxyphenyl)-1-(4-hydroxy-3-methoxyphenyl)-4-en-3-heptanone produced moderate cytotoxicity against human tumor cell lines, HepG2, MCF-7 and SF-268 (An et al., 2008) and potent inhibitor of prostaglandin synthase enzymatic activity. [7-(4'-hydroxy-3'-methoxyphenyl)-1-phenylhept-4-en-3-one] HMP, isolated from *A. officinarum* for treatment of its anti-inflammatory properties (Yadav et al., 2003).

The bioassay-guided purification of ether extracts of *A. officinarum* contains two new compounds 6- hydroxy-1,7-diphenyl-4-en-3-heptanone (1) and 6-(2-hydroxy-phenyl)-4-methoxy-2-pyrone (4) as well as three known compounds 1,7-diphenyl-4-en-3-heptanone (2), 1,7-diphenyl-5-methoxy-3-heptanone (3), and apigenin (5). Their structures were recognized on the basis of spectral methods (Fan et al., 2007).

### 1.2.4 Uses

*A. officinarum* Hance (Lesser galangal), family Zingiberaceae is an aromatic perennial herb spread throughout the tropical and subtropical Asian region. They are widely used in India, China and other parts of Asia for a broad range of diseases like respiratory tract infections, arthritis, and cancer. It was also used for treatment of microbial and gastrointestinal disorders. Diarylheptanoids has been reported to reduce the pro inflammatory mediators like the Nuclear Factor kappa B (NF-kB) phosphorylation of

MAPK, P44/42 and also potential inhibitors of leucotriene, biosynthesis and prostaglandin synthase enzymatic activity (Selvakkumar et al., 2007).

*A. officinarum* is a traditional, perennial herb mostly used in China and India for treating arthritis, gastrointestional disorders. The ethyl acetate extract of *A. officinarum* efficiently suppressed enteropathogenic *Escherichia coli* lipopolysaccharide induced inflammatory response (Subramanian et al., 2008).

*A. officinarum*, a pungent and aromatic rhizome was used in a traditional Chinese medicine with anti-inflammatory, antioxidant, antiproliferative, anticancer and antiemetic activities. The three new diarylheptanoid compounds from this plant showed strong action against *Helicobactor pylori* (Zhang et al., 2010).

The rhizomes of *A. officinarum* has been commonly used as a traditional medicine in China for relieving stomach-ache, treating colds, invigorating the circulatory system, and reducing swelling. Its rhizome has been used as anti-arthritic, antiphlogistic, analgesic, anti-emetic, stomachic, carminative and anti-spasmodic medicine. Some bioactive components of this plant have been reported for diarylheptanoids, flavonoids, and essential oils as 5'-reductase inhibitors, PGD2 inhibitors, anti-emetic and anti-oxidative components; but, *in vivo* anti-inflammatory and anti-nociceptive activities for treatment of arthritis have not been reported (Lee et al., 2009).

### 1.3: *Alpinia purpurata*

#### 1.3.1 Taxonomy

Botanical name- *A. purpurata*

Family: Zingiberaceae

Genus: *Alpinia*

Species: Purpurata

Synonym: *Guillainia purpurata*

Common names: red ginger, ginger, pink cone ginger

#### 1.3.2 Morphology

*A. purpurata* (Vieillard) K. Schumann commonly known as Red ginger is a tall, herbaceous, upright, evergreen plant from the South Pacific, with inconspicuous white flowers and bright red floral bracts. It is widely cultivated in the tropics and subtropics. It grows well in rich soil and in wet environments, but it can grow in dry areas as well

(Kobayashi et al., 2007). Leafy, cane-like stems begin from rhizomes to form a plant 3–15 feet tall and 2–4 feet wide. A stem's single inflorescence can be up to about 12 inches long. The rhizomes were spread laterally in or thick clusters. The rhizomes and stalks are aromatic. The deep green leaves of the plant are alternate and sessile (lacking a petiole), with a long sheath that wraps around the stem. The seeds of this plant are long, black, oily, and may have a red aril. Red ginger is grown in Hawaii as field crop. The plant stems, which begin from the rhizome system, may vary in height 1 to 5 m; the floral spike, composed of red bracts, is at the end of a leafy stem and may be as long as 30 cm. The inflorescence is produced throughout year if moisture, nutrition and temperature are adequate, and crop is harvested as a cut flower prior to the inflorescence is about two thirds open (Hansen 1993).

**Fig 1.3.2 Morphological features of *A. purpurata***

### 1.3.3 Chemical constituents

Leaves of *A. purpurata* contain flavonoids, rutin, kaempferol-3-O-rutinoside and kaempferol-3-Oglucuronide validating their therapeutic value (Kochuthressia et al., 2010). The compositions of essential oil of *A. purpurata* were β-pinene, 1, 8 cineoleand α- pinene (Nana et al., 2004).

### 1.3.4 Uses

*Alpinia* is the biggest genus in ginger family in which *A.purpurata* (Vieill.) K. Schum. is a very popular garden plant in India. Rhizome are sharp odour, improves appetite, taste and voice. It was also used for headache, rheumatism, sore throat and renal disease. Phytochemical studies on *A. purpurata* exposed that it possess flavonoids, rutin, kaempferol-3-rutinoside and kaempferol-3-oliucronide. The phytochemical constituents of *A. purpurata* support antimicrobial activity against certain microorganisms. In addition

to the purported anti- inflammatory activity, its phytomedicinal potential is to treat tuberculosis. *A. purpurata* may provide as potential dietary sources of natural antioxidants. The plant-derived extracts containing antioxidant principle showed cytotoxicity against tumour cells. Although information is existing regarding the composition of the essential oil derived from flowers of *A. purpurata*, the pharmacological activities of extracts of this species have established little attention. It is known that essential oils in general exhibit a broad variety of biological effects including antibacterial, antiviral, antifungal and antiparasitic activities. Flowers of *A. purpurata* that are not of adequately high quality for direct commercialization can be hydro distilled to provide an essential oil and an aqueous extract, both of which could be exploited as larval insecticides for the purpose in the fight against the spread of *A. aegypti*. Furthermore, the floral oil from *A. purpurata* exhibits huge potential as an antibacterial agent that could be employed in pharmacological formulations (Santos et al., 2012).

The flowers used as decoction are a release against cough. It is broadly used globally due to the exquisite inflorescences and therapeutic potential. In addition, this plant is main sources of raw material for many valuable products such as foods, spices, medicines, perfumes, dyes and fiber paper.

The flowers stalks of red ginger are attractive, cutting flowers can also be used in floral arrangements have long vase life, which is inadequate by bract browning. An extension of vase life is achieved in red ginger through using sucrose and ascorbic acid (Islam, et al., 2013).

The *in vitro* screening of the ethylacetate extract of *A. purpurata* showed possible anticancer activity against the ovarian cancer cells. *A. purpurata* has fairly antioxidant and anticancer activity. The anticancer activity of this plant may be due to the presence of alkaloids, flavonoids and terpenoids present in the leaves of *A. purpurata* respectively.

Murashige and Skoog medium supplemented with BA (3.0 mg/l) and Kn (2.0 mg/l) exhibited good regeneration rate up to 6.4±0.32 shoots/explants. Spontaneous rooting of shoots of the plant occurred in the same concentration of cytokinins. The number of leaves (4.8±0.67) and abundant rooting (8.4±0.70) facilitated 100% of plant recovery on acclimatization (Kochuthressia et al., 2010).

*Azospirillum* and *Azobacter* induced a higher survival rate in micro propagated plant of *A. purpurata*. The survival rate of the plantlets was bigger when increasing the amount of bacterial inoculums. The survival was improved from 13 to 23%, depending on the treatment, regarding the un-inoculated plantlets. The plantlets inoculated with $10^8$ or $10^9$ cells of *Azospirillum* had 100% of survival rate (Ovando et al., 2007).

**1.4 Standardization**

Standardization is an important tool for herbal drugs in order to establish their identity, purity, safety and quality (Mukherjee, 2002). In order to standardize a drug, various macroscopic, physicochemical analysis, phytochemical analysis and fluorescence analysis were done. The quantitative determination of some pharmacognostical parameters is useful for setting standards for crude drugs. (Ravichandra and Parakh 2011). In order to assure a consistent and acceptable quality herbal product, care should be taken right from the identification and authentication of herbal raw materials to the verification process of final product (Mosihuzzaman and Choudhary, 2008).

**1.5 Inflammation and arthritis**

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease affecting about 1% of the population in developed countries (Cardinali and Esquifino 1985). Although the disease can start at any age, the peak onset is between 25 and 55 years, women being affected about three times more frequently than men (Vierboom et al. 2007). It is characterized by progressive joint destruction, deformity, disability and premature death in most patients (Iain and Schett 2011). Medication like non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and analgesics are used to suppress the symptoms but they may cause side effects (Srivastava et al. 2012). NSAIDs help to relieve pain and stiffness and reduce inflammation; however, they do not slow down the progression of rheumatoid arthritis (Mali et al. 2011). It seems for that reason of relevance to develop new strategies for treating pain in muscle and joints, therefore need to develop medication that should be affordable and associated with a minimum of side effects (Willich et al. 2010). However, chronic use of NSAIDs is associated with gastrointestinal toxicity, which has limited their use and leads to the search for safer alternative agents.

### 1.6: Plant tissue culture

Plant tissue culture refers to the *in vitro* cultivation of plant cells/tissues on an artificial nutrient media under controlled aseptic environmental conditions. The idea of *in vitro* culture was proposed by German botanist Gottlieb Haberlandt (1845-1945). During the period of 1940-1970, suitable nutrient media to culture plant tissue, embryo, anthers, pollen, cells, protoplasts and the regeneration of complete plants from culture tissues and cells were developed. Murashige and Skoog (MS) medium is very widely used in different culture systems as it gives satisfactory results.

Application of plant tissue culture:

- Plant regeneration
- Induction of somatic embryos/shoots
- *In vitro* mutagenesis and mutant selection
- Genetic transformation
- Production of secondary metabolites
- Protoplast culture can be used for various biochemical and metabolic studies, to create somatic hybrids and cytoplasmic hybrids.

### 1.6.1 Advantages of plant tissue culture systems over conventional cultivation techniques

- The process offers the prospect of absolutely uniform biomass obtainable at all times and manageable under regulated and reproducible conditions.
- This technique is used for synthesis of those medicinal compounds, which are too difficult or impossible to synthesize chemically.
- The useful natural compounds could be produced under controlled environmental conditions, independent soil conditions and change in climatic conditions.
- Used to study biogenesis of secondary metabolites, it is possible to feed labelled precursors to cell cultures and deduce interpretations pertaining to metabolic pathway of desired compound.
- It is possible to attempt biotransformation reactions in plant cell cultures.
- The cells of any plants, tropical temperature, could be multiplied to yield specific metabolites produced by them.

- The culture cells could be maintained free from any microbial contamination and insect attack.
- Defined production systems as and when required.
- Another important application of plant tissue culture technique is immobilizations of cells, which could be used for biotransformation or biochemical reactions (Kokate et al., 2002).
- Despite a number of advantages listed above, plant cell and tissue culture technologies also suffer from some drawbacks (Zafar et al., 1992).
- Since plant cells are much larger in size, they have a bigger surface area and hence lower metabolic activity resulting in slower growth rates as compared to smaller sized microbial cells.
- Plant cells tend to grow together as cell clumps rather than single cells posing some problems for their cultivation in bioreactors.
- The yield of secondary metabolites *in vitro* cannot be predicted beforehand. Many times the compounds of interest are not produced *in vitro* or if produced are present in extremely low quantities.
- High genetic and epigenetic instability of cell cultures can lead to loss of phytochemical production capacities of cells.
- Differentiation related products are not produced in unorganized tissues that are easier to manipulate *in vitro*.
- Culture conditions may trigger new pathways producing novel but useless products.
- Empirical methods developed for cell cultures of a particular plant cannot be extrapolated to a wide range of plants and culture systems.

Secondary metabolites were obtained by conventional method of cultivation, collection, extraction and isolation, which has its limitations like unforeseen environmental conditions, time consuming, desired quality, and a gap between the demand and supply. Therefore micropropagation through plant tissue culture can be an attractive alternative method. By suitable manipulation of hormones and contents of the medium, it is possible to initiate the development of roots, shoots and complete plants from callus cultures (Evans, 2001).

### 1.6.2. Culture medium

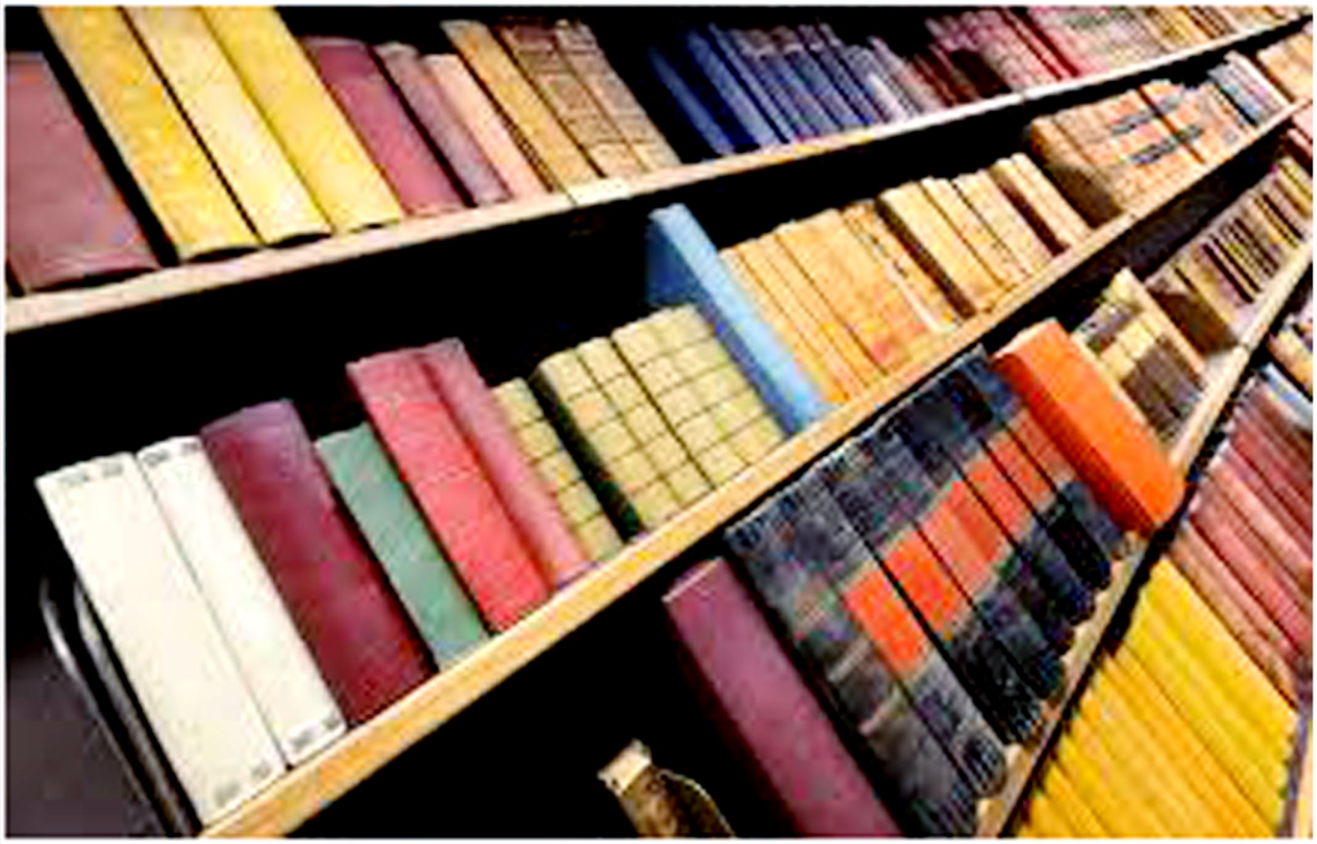
A culture medium composed of inorganic nutrients, an iron source, carbon source, vitamin, growth regulators and some organic supplements. Several well defined standard nutrient media that has been used most widely in basic or modified forms such as Gautheret (1942), White (1943), Hildebrandt (1946), Heller, (1953), Nitsch and Nitsch (1956), Murashige and Skoog (1962), Eriksson (1965) and B5 (1968) (Kokate et al., 2002). Murashige and Skoog (MS) medium is very widely used in different culture systems as it gives satisfactory results.

### 1.6.3: Growth regulators

Growth regulators are organic compounds that have been naturally synthesized in higher plants, which influence growth and development. These are usually active at different sites from where they are produced and active in very small quantities.

In the present study, the pharmacognosy, pharmacology and biotechnological strategies of selected medicinal plants were performed on the basis of extensive literature survey and ethno pharmacological uses. The extracts were studied for phytochemical evaluation followed by pharmacological studies. Extract showing significant pharmacological activity was fractionated using bioactive guided fractionation method and the most bioactive fraction was further evaluated for its pharmacological study and characterization was done by LC-MS, IR, $^{1}$H-NMR, $^{13}$C-NMR and DEPT. Micropropagation through plant tissue culture was also studied for selected plant according to literature and analysis was carried out for determination of concentration of secondary metabolite in tissue culture grown plant with that of normal grown.

# REVIEW OF LITRETURE



## 2. REVIEW OF LITERATURE

*Alpinia* is the major and most common genus of the *Zingiberaceae* family. The Latin generic name "*Alpinia*" was given by Italian botanist who specialized in exotic plants. This review collects the information on phytochemistry, analysis, micropropogation and pharmacological activity of selected medicinal plants was focused on the basis on many sources. Commercially these species have been used in the food and cosmetic industries. These species contains essential oils such as flavonoids, phenylpropanoids and terpenoids, which are medicinally important for the treatment and managament of ulcers, whooping cough, throat infections, rheumatism, bad breath, incontinence, fever, headache and microbial infections. Traditionally these species were also used for the treatment of various ailments including analgesic, anti-inflamantory, anti-tumor, rheumatic pains, allergy, fever, antidiabetes. The result of statistical analysis on the phytochemical, analytical, pharmacological activities of these species shows that these species has many beneficial applications. Based on useful information from this genus, present review will be help to provide the latest and broad knowledge on these species for some suggestions for further research with special emphasis on its phytochemistry, pharmacology, analysis and micropropogation.

For the treatment of various diseases of man and animals, plants have been extensively used since ancient times (Nadkarni, 1954; Hamdard's, 1969; Lewis and Elvin-Lewis, 2003). From ancient times world population was widely used traditional medicines for health and treatment of disease (WHO, 1993). *Alpinia* is the most important genus from the Zingiberaceae, mostly these were found in the region of Sri Lanka, Western Ghats of India to China and Japan. Also these are found in all of Southeast Asia, Samoa, and the Caroline Islands, and Australia. Rhizomes of certain gingers like *A. officinarum, A. galanga, A. calcarata, Kaempfera galangal* have high medicinal values (Indranyl et al., 2009).

### 2.1 *A. galanga* (L.) Willd

*A. galanga*, commonly used as traditional medicines for treatment of diseases such as skin, indigestion, colic, dysentery, enlarged spleen, respiratory, mouth and stomach cancer. Rhizomes of *A. galanga* find varying medicinal uses for the treatment as analgesic (Acharya et al., 2011), bactericidal action on food borne bacteria (Okonogi et

al., 2011), neuroprotective (Reddy et al., 2011), antileishmanial (Singh et al., 2010 ), antiallergic (Yoshikawa et al., 2003), immunostimulating (Bendjeddou et al., 2003), hypoglycemic (Akthar et al., 2002), antimicrobial (Gasaluck et al., 2006), antitumor (Itokawa et al., 1987), antifungal (Janssen and Scheffer, 1985), inhibitors of nitric oxide production (Yoshikawa et al. 2005) and antioxidant activity (Nopparat and Siree, 2009).

The major active constituent of this plant is 1'S-1'-acetoxychavicol (Ye and Li, 2006), dihydrogalangal (Xiaogen et al., 2009), acetoxy-1, 8-cineoles (Kikue et al., 1998), essential oils (Jirovetz et al., 2003), galangoflavonoid (Jaju et al. 2009), ACA (Janssen AM and Scheffer JJ 1985), 1'S-1'-Acetoxyeugenol acetate (Noor et al., 2010).

### 2.1.1 Phytochemistry

This plant contains various flavones such as galangin, alpinin, kampferide and 3-dioxy-4-methoxy flavones, essential oils such as cineole, 1; 8-cineole, methyl cinnamate, myrecene, and methyl eugneol and galangoflavonoids were isolated from *A. galanga* (Jaju et al. 2009). Phenylpropanoides isolated from *A. galanga* were trans-p-hydroxycinnamaldehyde, trans-p-coumaryl alcohol, hydroxychavicol, eugenol, trans-cinnamic acid, trans-p-hydroxycinnamyl acetate, trans-p-coumaryl diacetate, ACA, 1'S-1'-acetoxyeugenol acetate, HCA and eugenol acetate (Sabu, 2006). Acetoxy-1, 8-cineoles was evaluvated as odorous principle from *A. galanga* (Kikue et al., 1998). 4, 4'-[(2E, 2'E)-bis (prop-2-ene)-1, 1'-oxy]-diphenyl-7, 7'-diacetata was characterised as a new phenylpropanoid (Zhu et al., 2009).

### 2.1.2 Pharmacological activities and clinical trials of *A. galanga*

#### 2.1.2.1 Analgesic and anti-inflammatory activity

*A. galanga* was observed for analgesic effect in mice by using analgesic model and used in ethno medicine for the treatment of pain (Acharya et al., 2011). Chavicol analogues (ACA and HCA) from *A. galanga* posses powerful antioxidant and anti-inflammatory actions, HCA was useful to suppress T-bet expression and used for treatment of inflammatory immune disorders caused by excessive activation (Min et al., 2009). Structural requirements for inhibition of degranulation and release of TNF-and IL-4 of phenylpropanoids were studied (Yoshikawa et al., 2003). Antidiabetic and anti-inflammatory activities from the phenolic and methanolic extracts of rhizome of *A. galanga* were evaluated (Shivkanya et al., 2009). Herbal drug with antibacterial,

antioxidant, and anti-inflammatory action jointly is highly useful to patient of acute inflammation and also in chronic inflammatory disorders (Ghosh et al., 2011). *A. galanga* was effective for nitric oxide (NO) production inhibitory activities in mouse peritoneal macrophages and this effect was observed in 80% aqueous acetone extract from the rhizomes of *A. galanga* (Matsuda et al., 2005). *A. galanga* extracts contains p-hydroxycinnamaldehyde and it was beneficial for acting on human chondrocytes which is highly effective therapeutic plant for treatment of osteoarthritis (Phitak et al., 2009).

**2.1.2.2 Anti-cancer activity**

1'S-1'-acetoxyeugenol acetate isolated from *A. galanga* was reported as new chemotherapeutic agent useful against MCF-7 human breast cancer cells and according to study 1'S-1'-acetoxyeugenol acetate has a potential for treatment against human breast cancer cells with higher cytotoxic activity than ACA (Noor et al., 2010). Apoptotic effect of ACAs was observed against human leukemia HL-60 cells (Azuma et al., 2006). ACA was studied for its enantiomer. (S) ACA and (R)-ACA were inhibiting tumor cells proliferation via different mechanisms (Xu et al., 2008). Comparison of glutathione reductase activity and the intracellular glutathione reducing effects of 13 derivatives of ACA in *Ehrlich ascites* tumor cells was studied and the study revealed that structural factors regulating activity were the para or 1'-position of acetoxyl group (or other acyl group) was essential and the presence of a C2'–C3' double or triple bond was essential, and the S configuration of the 1'-acetoxyl group was preferable (Xu et al., 2010). ACA used as a new therapeutic agent in patients with multiple myeloma (Ito et al., 2005).

**2.1.2.3 Antimicrobial activity**

Antimicrobial activity of greater galanga showed broad spectrum of antimicrobial activity against Gram-positive bacteria, but it has slight antimicrobial value against Gram-negative bacteria (Hsu et al., 2010).

Antimicrobial effect was observed under the transmission electron microscopy for *A. galanga* Linn on *Staphylococcus aureus* and it was clearly confirmed that the galangal extract caused both outer and inner membrane damage, and cytoplasm coagulation (Gasaluck et al., 2006). Bactericidal effect of *A. galanga* on food borne bacteria was observed for essential oils (Okonogi et al., 2011).

### 2.1.2.4 Antiplasmid activity

Antiplasmid effect of 1-acetoxychavicol acetate was evaluated (Latha et al., 2009). According to conclusion ACA mediated R-plasmid curing significantly reduced the minimal inhibitory concentration of antibiotics required to inhibit growth of bacteria, thus making the antibiotic treatment more effective.

*A. galanga* rhizome extract was studied for curing of plasmid - mediated antibiotic resistance in multi-drug resistant pathogens (Shriram et al., 2013).

### 2.1.2.5 Anti-HIV activity

A new halogenated analog was designed for ACA from *A. galanga* for rev-export inhibition action from its mechanism of action. Rev is required for appearance of the majority of HIV-1 proteins (Tamura et al., 2010).

ACA (ACA), a small molecular compound isolated from the rhizomes of *A. galanga*, inhibited Rev transport at a low concentration by binding to chromosomal region maintenance 1 and accumulating full-length HIV-1 RNA in the nucleus, resulting in a block in HIV-1 replication in peripheral blood mononuclear cells (Ying and Baoan, 2006).

### 2.1.2.6 Anti-dibetic activity

In alloxan-induced diabetic rabbits, hypoglycemic effect of *A. galanga* was observed for blood glucose level in rabbits (Akhtar et al., 2002).

Antioxidant and antidiabetic Activity of *A. galanga* was carried out for *in vitro* and *in vivo* models (Srividya et al., 2010).

### 2.1.2.7 Effect on GI tract

1' acetoxyl group of ACA and 1'S-1'-acetoxyeugenol acetate were responsible for gastroprotective effects of phenylpropanoids from the rhizomes of *A. galanga* (Matsuda et al., 2003).

*A. galanga* was studied for gastric antisecretory, antiulcer and cytoprotective effect of ethanolic extract. According to the results the ethanolic extract of *A. galanga* at a dose of 500 mg/kg, significantly reduced the intensity of gastric mucosal damage induced by pyloric ligation and hypothermic restraint stress in rats (Al-Yahya et al., 1990).

### 2.1.2.8 Isolation of actinomycin D

Isolation of actinomycin D from *A. galanga* was evaluated for its antifungal activity against *Colletotrichum musae* and *Candida albicans.* The MIC of actinomycin D was found to be 10 and 20 mg $ml^{-1}$, respectively was carried out (Taechowisan et al., 2006).

### 2.1.2.9 Neuroprotective effect

Anti-amnesic effect was studied for different fractions of *A. galanga*. Chloroform fraction was useful for neuroprotection due to presence of 1'S-1'-acetoxyeugenol acetate. It may be a used in the treatment and management of Alzheimer's type of amnesia (Reddy et al., 2011).

### 2.1.2.10 Antioxidant activity

1´-acetoxycavichol acetate, catechin, and the phenolic compounds; 1´-acetoxycavichol acetate (ACA) are major antioxidants in the ethanolic extract which were shows significant antioxidant activity of galangal (Nopparat and Siree, 2009).

### 2.1.2.11 Hepatoprotective effect

Hepatoprotective effect of *A. galanga* was observed in paracetemol induced hepatotoxicity (Hemabarathy et al., 2009).

### 2.1.2.12 Antiallergic activity

1'S-1'-acetoxychavicol exhibit antiallergic effect on immediate and late phase of the type I allergic reactions. Acetoxybenzhydrols was the stable analogues of 1'S-1'-acetoxychavicol used for treatment of allergy from *A. galanga* (Yasuhara et al., 2009).

## 2.2 *A. officinarum* Hance

*A. officinarum* has also found medicinal properties due to presence of diarylheptanoides which possess anti-inflammatory (Laxmi et al., 2008), anticancer (Ning et al., 2008), antifungal (Jantan et al., 2003) activity. It also possess pancreatic lipase inhibition (Kim et al., 2004), Melanogenesis inhibition in B16 melanoma cells (Yoshikawa et al., 2004), antibacterial (Liao et al., 2010), antitubercular (Bernt et al., 2008) and antiplatelet activity (Doug et al., 1998).

*A. officinarum* have been reported for number of chemical constituent specifically, officinin A, 5-ethoxyl-7-(4-hydroxy-3-methoxy-phenyl)-1-phenyl-3-heptanone (Liang et al., 2010), diarylheptanoids, officinaruminane A, officinaruminane

B, 5 (S)-acetoxy-7-(4-dihydroxyphenyl)-1-phenyl-3-heptanone (Zhong-mei et al., 2010), 1, 8-cineole, methyl cinnamate, a-cadinene, galangin, 3-O-methyl galangin, kaempferide, alpinin, galangol and some diarylheptanoids (Itokawa et al., 1981,Itokawa et al., 1985, Kim et al., 2004).

### 2.2.1 Phytochemistry

Two new diarylheptanoids were isolated from the rhizomes of *A. officinarum* i.e. (5S)-5-hydroxy-7-(3, 4-dihydroxyphenyl)-1-phenyl-3-heptanone (Nadkarni, 1954) and (5R)-5-hydroxy-7-(3-methoxy-4, 5-dihydroxyphenyl)-1-phenyl-3-heptanone (An et al., 2008). The HPLC method was developed for the evaluation of two bioactive flavonoids: galangin and 3-O-methyl galangin (Tao et al., 2006).

The chemical constituents were elucidated on the basis of spectral analysis and identified 5-ethoxyl-7-(4-hydroxy-3-methoxy-phenyl)-1-phenyl-3-heptanone, 5-hydroxy-1,7-diphenyl-3-heptanone,5-hydroxy-7-(4-hydroxyl-3-methoxyphenyl)-1phenyl-3-heptanone, 5- ethoxy-7-(4-hydroxy-3-methoxyphenyl)-1-phenyl-3-heptanone and (E)-7-(4-hydroxy-3 ethoxyphenyl)-1-phenylhept-4-en-3-one. According to the results 5-ethoxyl-7-(4-hydroxy-3-methoxy-phenyl)-1-phenyl-3-heptanone is a new diarylheptanoid (Liang et al., 2010).

Three diarylheptanoids, officinaruminane A, officinaruminane B, 5(S)-acetoxy-7-(4-dihy droxyphenyl)-1-phenyl-3-heptanone were isolated from the rhizomes of *A. officinarum* Hance by column chromatography on silica gel, MPLC and preparative thin-layer chromatography (Zhong-mei., 2010).

A novel diarylheptanoid isolated from the rhizomes of *Alpina officinarum* Hance were officinin A (Liang et al., 2010).

### 2.2.2 Pharmacological activities and clinical trials of *A. officinarum*

#### 2.2.2.1 Anti-inflammatory

Anti-inflammatory property of the rhizomes of 80% ethanolic extract from *A. officinarum* was evaluated on complete Freund's adjuvant-induced arthritis in rats. The 80% ethanolic extract showed acute anti-inflammatory action by reduction in the edema volume in carrageenan-stimulated arthritis and inhibited NO generation in LPS-induced RAW 264.7 cells. Different parameters were studied for anti-rheumatic and analgesic activities by suppressing the swelling volume, by recovering the paw withdrawal latency,

and by inhibiting the flexion scores in CFA-induced arthritis. This study will be helpful for anti-psychiatric effect in CFA-stimulated arthritis (JiSuk et al., 2009).

Isolation of two new compounds was carried out by bioassay-guided purification method from the ether extracts of *A. officinarum*, led to 6-hydroxy-1,7-diphenyl-4-en-3-heptanone and 6-(2-hydroxy-phenyl)-4-methoxy-2-pyrone as well as three known compounds 1,7-diphenyl-4-en-3-heptanone, 1,7-diphenyl-5-methoxy-3-heptanone, and apigenin. These compounds exhibited potent platelet-activating factor (PAF) receptor binding inhibitory activities, with an $IC_{50}$ of 1.3, 5.0, and 1.6 μM, respectively (Fan et al., 2007).

The diarylheptanoid 7-(4-hydroxy-3-methoxyphenyl)-1-phenylhept-4-en-3-one (HMP) from *A. officinarum* suppressed the LPS-induced production of NO, IL-1β, and TNF-α and expression of iNOS and COX-2 gene expression by inhibiting NF-κB activation and phosphorylation of p44/42 MAPK (Yadav et al., 2003).

**2.2.2.2 Antimicrobial**

Diarylheptanoid isolated from *A. officinarum*, a medicinal plant belonging to the Zingiberaceae family, [5-hydroxy-7- (4''-hydroxy-3-methoxyphenyl)-1-phenyl-3-heptanone] was effective against enteropathogenic *Escherichia coli* (EPEC). The diarylheptanoid isolated from *A. officinarum* competently suppressed EPEC lipopolysaccharide-induced inflammation in human peripheral blood mononuclear cells (Laxmi et al., 2008).

Antibacterial activity against *Helicobactor pylori* was studied for the new compounds from *A. officinarum* (Zhang et al., 2010). These compounds were elucidated as 7-(4″, 5″-dihydroxy-3″-methoxyphenyl)-1-phenyl -4-heptene-3-one, 1, 7- diphenyl-5-heptene-3-one and 4-phenethyl-1, 7-diphenyl -1-heptene-3, 5-dione respectively. Galangin, quercetin and baicalein was effective for bacterial resistance to β-lactam antibiotics against penicillin-resistant *S. aureus* (Gasaluck et al., 2006).

**2.2.2.3 Anti-oxidant**

Rhizomes of *A. officinarum* were studied for chemical composition and antioxidant properties for essential oil and methanol extracts. Study was suggested that it will be used as a new potential source of natural antioxidant for the food industry (Borthakur et al., 1999).

The effect of 80% aqueous acetone extract from the rhizomes of *A. officinarum*, from Chinese medicinal herb, was helpful for inhibition of nitric oxide (NO) production in lipopolysaccharide (LPS)-activated mouse peritoneal macrophages (Matsuda et al., 2005).

**2.2.2.4 Melanogenesis inhibitors**

*A. officinarum* was evaluated for diarylheptanoids [5-hydroxy-1, 7-diphenyl-3-heptanone, 7-(4'-hydroxy-3"-methoxyphenyl)-1-phenylhept-4-en-3-one, 5-hydroxy-7-(4"-hydroxy-3"-methoxyphenyl)-1-phenyl-3-heptanone, 3,5-dihydroxy-1,7-diphenylheptane] and for flavonol constituents (kaempferide and galangin). These compounds were beneficial for inhibition of melanogenesis with $IC_{50}$ values of 10–48 µM (Matsuda et al., 2009).

**2.2.2.5 Anticancer activity**

Diarylheptanoids from the rhizomes of *A. officinarum* 7-(3, 4-dihydroxyphenyl)-1-(4-hydroxy-3-methoxyphenyl)-4-en-3-heptanone was useful for cytotoxic effect against human tumor cell lines, HepG2, MCF-7 and SF-268 (Ning et al., 2008).

**2.3 *A. purpurata* (Vieill.) K. Schum**

*A.purpurata* (Vieill.) K. Schum in India is a very popular medicinal garden plant (Sabu M. 2006). The pharmacological activities of extracts of this species have received little attention, rhizomes used for headache, rheumatism, sore throat and renal disease (Prajapathi et al., 2003). Essential oils from *A.purpurata* exhibit a wide variety of biological effects including antifungal, antibacterial, antiviral and antiparasitic. Phytochemical studies on *A. purpurata* discovered that it contains flavonoids *viz* rutin, kaempferol-3-rutinoside and kaempferol-3-oliucronide (Victorio et al., 2009). Phytoconstituents of *A. purpurata* was evaluated for inhibitory activity against mycobacterium tuberculosis (Villaflores et al., 2010).

### 2.3.1 Micropropogation studies

**Table 2.3.1 Micropropogation of *Alpinia* species.**

| Name of the species | Reference | Explants used | Medium | Observation |
|---|---|---|---|---|
| ***A. galanga*** | Direct and Indirect Organogenesis of *A. galanga* (Rao et al., 2010). | Rhizome | 1) MS media supplemented with zeatin at a concentration of 2 mg/l. | 1) Rapid direct regeneration was obtained from the rhizome explants (15.66±0.57 shoots). |
| | | | 2) 2 mg/l each of BAP, 2, 4-D, and NAA. | 2) The callus cultures of *A. galanga* were initiated |
| | | | 3) subsequent transfer of callus to BAP (2 mg/l) containing MS. | 3) The regenerated (indirect) plants have shown 1.6-fold higher ACA content (1.253%) when compared to the control plant (0.783%). |
| ***A. galanga*** | Evaluation of genetic fidelity of *in vitro* propagated greater galangal (*A. galanga*) using DNA based markers (Parida et al., 2011). | Sprouted buds. | 1) MS+ Kinetin (3 mg/l), Benzyladenine (3 mg/l) and Napthalene aceticacid (1.0mg/l). | 1) Maximum number of shoot multiplication as 15.6±0.2.<br>2) After 2 years of culture *in vitro*, plantlets were transplanted to the field and evaluation of phenotypic characteristics was done. |
| ***A. galanga*** | Agrobacterium-Mediated Transformation in *Alpinia galanga* (Linn.) Willd. for enhanced | Leaf sheath, rhizome and root | 1)Five different transgenic hairy root lines have been obtained from *A. galanga*, using varied strains of A. | Elevated levels of ACA were observed in all the transformed lines, with PRTGus showing the highest accumulation at 3.157 % (10.1 fold). |

| | | | | |
|---|---|---|---|---|
| | acetoxychavicol acetate production (Rao et al., 2010). | | rhizogenes (LBA 9402, PRTGus, MTCC 2364, MTCC 532 and A4). | The present study offers a promising system for commercial production of plant metabolites. |
| ***A. galanga*** | A protocol for micropropagation of *A. galanga* (Borthakur et al., 1999). | Emerging buds of the rhizome. | MS medium supplemented with kinetin 3.0 mg1-1. | Each explanted shoot bud produced 8 shoots in average and roots simultaneously within 8 weeks.<br>Protocol can be applied as a part of *in vitro* conservation of Germplasm. |
| ***A. officinarum*** | Rapid *in vitro* Micropropagation of *A. officinarum* Hance, An Important Medicinal Plant (Laxmi et al., 2012) | Rhizome buds | Optimum shoot multiplication was observed on MS medium containing 3% (W/V) sucrose and 3.0 mg L-1 kinetin (Kn) and 1.0 mg L-1 Naphthalene Acetic Acid (NAA). Each rhizome bud gave rise to an average of 11 shoots per explant. Rooting experiments with half-strength Murashige and Skoog medium revealed that 0.5 mg L-1 Indole-3-butyric acid (IBA) was more suitable for root induction when compared to IAA and NAA. | Healthy *in vitro* rooting plantlets were transferred to pots containing a mixture of vermiculite and soil (1:1) for acclimatization for a period of three-four weeks and 93% of plantlets survived under field conditions. |

| | | | | |
|---|---|---|---|---|
| ***A. purpurata*** | Efficient regeneration of *A. purpurata* (Vieill.) K.Schum. plantlets from rhizome bud explant (Kochuthressia et al., 2010). | Rhizome bud explants. | Murashige and Skoog medium supplemented with BA (3.0 mg/l) and Kn (2.0 mg/l) exhibited regeneration rate up to 6.4±0.32 shoots/explants. | Multiple shoot proliferation in *A. purpurata* rhizome buds could not be observed in basal MS medium. MS medium augmented with BA (3.0 mg/l) and Kn (2.0 mg/l) induced shoots (6.4 ± 0.32) per explant within 15 days of culture. |
| ***A. purpurata*** | Influence of sucrose and ascorbic acid on vase life of red ginger (*A. purpurata* Vieill.) (Islam et al.,2013). | The inflorescences with red or pink colored shiny bracts. | | Sucrose and ascorbic acid combinations induce extended vase life in Red ginger (*A. purpurata*). |
| ***A. purpurata*** | *Ex vitro* survival and early growth of *A. purpurata* plantlets inoculated with azotobacter and azospirillum (Ovando et al., 2007). | Plantlets inoculated with azotobacters. | Inoculated with *Azospirilium sp. 11B* and *Azobacter sp PAHAZ 008* at $10^7$, $10^8$, $10^9$ cells $cm^{-3}$. | Inoculation with *Azospirilium sp. 11B* or *Azobacter sp PAHAZ 008* strains induced larger stem diameter, root dry mass, number of shoots and increased survival rate from 77 to 100% compare to plantlets without inoculation. |
| ***A. purpurata*** | Micropropagation of *A. purpurata* from inflorescence buds (Rolf and Faria, 1995). | Inflorescence buds. | Murashige and Skoog medium (MS) containing 10 µM 6-benzyladenine with 5 µM NAA. | Multiple shoot formation with a mean increase of 15 to 20 new shoots each 4 weeks. |

| | | | | |
|---|---|---|---|---|
| ***A. calcarata*** | Micropropagation of selected medicinal plant (Muralidharan, 1997). | Rhizome explants and buds. | Cytokinins viz. BAP and Kn. . | Higher concentrations of cytokinins (both BAP and Kin) gave higher rates of multiple shoot formation whereas at lower levels the elongation of the shoots and root production was enhanced. Results obtained in this study indicate that only the second generation plantlets are suitable as propagules for obtaining a productive crop. |
| ***A. calcarata*** | Improved clonal Propagation of *A. calcarata* Rosc., a commercially important medicinal plant and evaluation of chemical fidelity through comparison of volatile compounds (Sudha et al., 2012). | Rhizome. | Medium with 2.0 mg/L 6-benzylamiopurine (BAP) and 0.2 mg/L indole-3-acetic acid (IAA). | The protocol described herein will have practical applications for the large scale production of phytochemically uniform plants for commercial cultivation of *A. calcarata.* |

# AIMS & OBJECTIVES

## 3. AIMS AND OBJECTIVES

### 3.1 AIMS

The aim of the present work was to carry out pharmacognostical, pharmacological and biotechnological approaches like conservation through micropropagation of some selected endangered or threatened important medicinal plants from Zingiberaceae family. The medicinal plants were selected according to extensive literature survey on endangered or threatened species. Based on the literature survey and current research the most important genus was *Alpinia Roxb* and the three plants were selected from the genus are *A. galanga* L., *A. officinarum* Hance and *A. purpurata* (Vieillard) K. Schumann.

### 3.2 OBJECTIVES

The goal of the present research is to systematically explore the Pharmacognostical, Phytochemical, Pharmacological and Biotechnological characteristics of (1) *A. galanga* (2) *A. officinarum* (3) *A. purpurata*

To achieve this goal, following objectives are set.

- To carry out pharmacognostical and phytochemical study.
- To investigate pharmacological activity of all the extracts.
- Isolation of bioactive fraction by bioassay guided fractionation method.
- To investigate pharmacological activity of isolated bioactive fraction.
- To enhance active compounds by biotechnological strategies.
- To establish analytical protocol for active constituents.

To achieve the set aim and objectives of the research, the work is planned in following systematic manner

#### 3.2.1 Part I: (*A. galanga* L. willd)

- To procure and authenticate plant material of *A. galanga* L.
- To perform preliminary Pharmacognostic studies of of *A. galanga* L.
- To prepare different extracts from the rhizomes of *A. galanga* L.
- To carry out preliminary qualitative phytochemical analysis of extracts of *A. galanga* L.
- To carry out acute oral toxicity studies of the extracts as per Organization for Economic Co-operation and Development (OECD) guidelines (AOT no. 425).

- To investigate anti-inflammatory activity of the extracts using carrageenan induced paw edema.
- To investigate antiarthritic activity of extracts showing superior anti-inflammatory activity using FCA induced arthritis model.
- To isolate important bioactive compounds responsible for significant pharmacological activity.
- To investigate anti-inflammatory activity of bioactive compound using carrageenan induced paw edema.
- To investigate antiarthritic activity of bioactive compound showing superior anti-inflammatory activity using FCA induced arthritis model.
- To characterize important bioactive compounds responsible for significant pharmacological activity
- To establish analytical protocol for active constituents of *A. galanga* L. using chromatographic techniques.

### 3.2.2 Part II: (*A. officinarum* Hance)

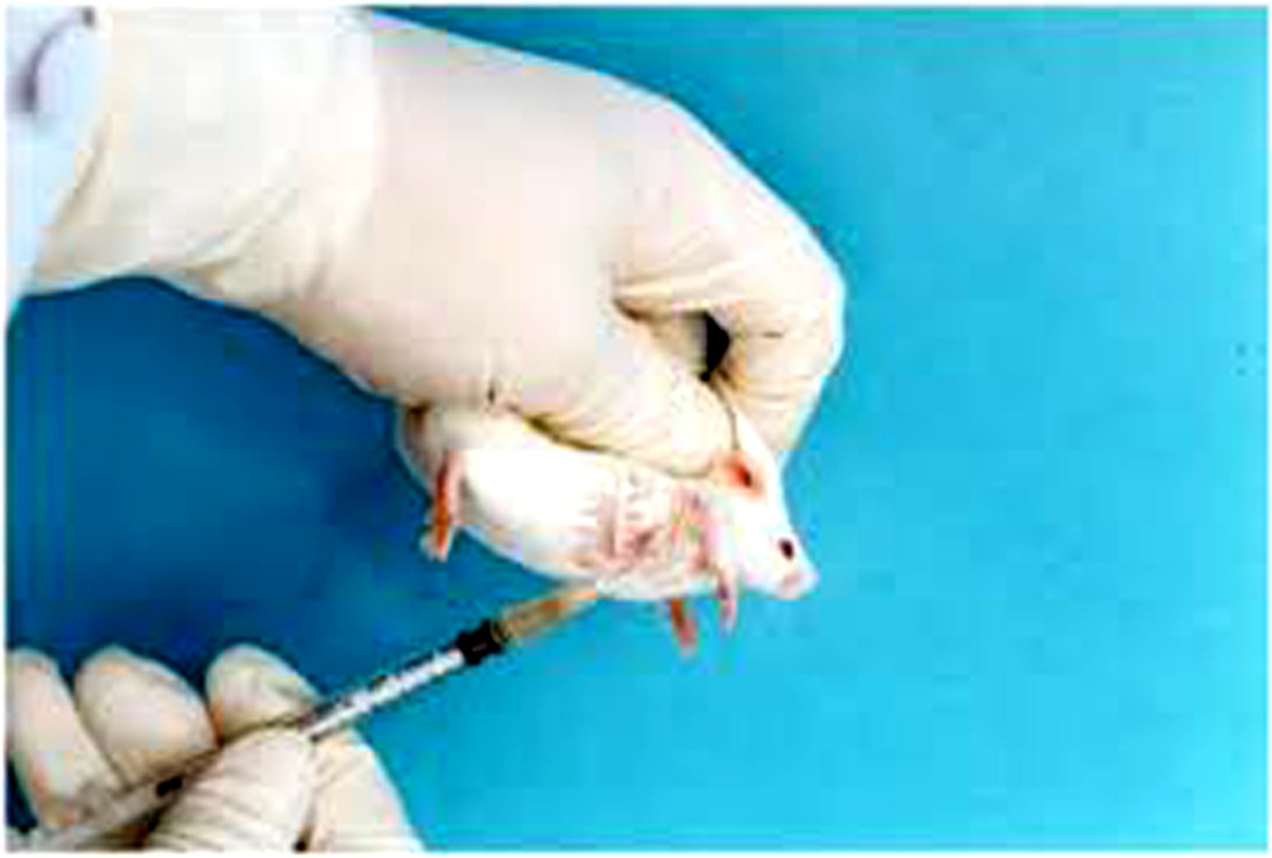
- To procure and authenticate plant material of *A. officinarum* Hance.
- To perform preliminary Pharmacognostic studies of *A. officinarum* Hance.
- To prepare various extracts from the rhizomes of *A. officinarum* Hance.
- To carry out preliminary qualitative phytochemical analysis of extracts of *A. officinarum* Hance.
- To carry out acute oral toxicity studies of the extracts as per Organization for Economic Co-operation and Development (OECD) guidelines (AOT no. 425).
- To investigate anti-inflammatory activity of the extracts using carrageenan induced paw edema.
- To investigate anti-arthritic activity of extracts showing superior anti-inflammatory activity using FCA induced arthritis model.
- To isolate important bioactive compounds responsible for significant pharmacological activity.
- To investigate anti-inflammatory activity of bioactive compound using carrageenan induced paw edema.

- To investigate anti-arthritic activity of bioactive compound showing superior anti-inflammatory activity using FCA induced arthritis model.
- To characterize important bioactive compounds responsible for significant pharmacological activity
- To establish analytical protocol for active constituents of *A. officinarum* Hance using chromatographic techniques.

### 3.2.3 Part III: *A. purpurata* (Vieillard) K. Schumann

- To procure and authenticate plant material of *A. purpurata* (Vieillard) K. Schumann.
- To prepare various extracts from the leaves of *A. purpurata* (Vieillard) K. Schumann.
- To establish analytical protocol for active constituents of *A. purpurata* using various chromatographic techniques.
- To establish callus culture of *A. purpurata* (Vieillard) K. Schumann.
- To enhance active constituent production by various plant biotechnological strategies and micropropagation on *A. purpurata* (Vieillard) K. Schumann.
- To estimate bioactive compounds from *in vitro* developed biomass in culture medium.
- Pre-hardening and hardening of *in vitro* developed plants.

# MATERIALS AND METHODS



## 4. Materials and methods

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|---|
| Part I *A. galanga* (L.) Willd |
| Part II *A. officinarum* Hance |
| Part III *A. purpurata* (Vieillard) K. Schumann |

### Part I

### 4.1 *A. galanga* (L.) Willd

#### 4.1.1 Plant materials

The rhizomes of *A. galanga* (L) Willd and *A. officinarum* Hance were purchased from local market in Pune, and authenticated by Dr. A.S. Upadhye, Head Scientist, Department of Botany, Agharkar Research Institute, Pune, and voucher specimens R-167, R-168 were deposited.

Authenticated fresh plant material of *A. galanga* (L) Willd and *A. purpurata* (Vieillard) K. Schumann were collected from the Jawaharlal Nehru Tropical Botanic and Research Institute, Palode, Thiruvananthapuram in the month of October.

#### 4.1.2 Chemicals and reagents

Analytical grade petroleum ether, ethanol, methanol, ethyl acetate, acetone, toluene (Merck, India) were purchased from commercial source. The reagents for phytochemical identification were obtained from the freshly prepared stock used in pharmacognosy and pharmaceutical chemistry laboratory of the college.

#### 4.1.3 Preliminary pharmacognostic studies

##### 4.1.3.1 Macroscopic and microscopic analysis of *A. galanga*

The macroscopic characters of rhizomes, leaf, root, bark, flowers and seed were determined organoleptically (Anonymous et al., 2003).Observations of shape, size, color, odour, taste and external markings were done in macroscopical studies. Microscopic studies were done according to the method of (Brain and Turner., 1975). For microscopical studies; a few drops of chloral hydrate solution were added to a sample of powdered plant material on a slide and heated gently over a micro Bunsen. The slide was covered with a glass cover slip for examination under the microscope and different cell components were observed. Free hand section of drug material was taken and stained

with safranin and phloroglucinol followed by concentrated hydrochloric acid (Johansen, 1940). The respective figures of reactions were drawn with the help of camera lucida and dissecting microscope.

**4.1.3.2 Physico-chemical analysis of *A. galanga***

Physico-chemical analysis i.e. percentage of ash values and extractive values was performed according to the official methods prescribed by WHO guidelines on quality control methods for medicinal plant materials (WHO/QCMMPM guidelines, 1992). Fluorescence analysis was carried out according to the method prescribed (Chase and Pratt, 1949; Kokoshi et al., 1958). The color and consistency of the extracts were also recorded.

- **Total ash**

Carbon and inorganic matter present in the drug is converted to ash at a temperature of 450ºC. Accurately weighed about 3 grams of air dried powdered drug was taken in a silica crucible and incinerated in Muffle furnace (METALAB enterprises) by gradually increasing the temperature to make it dull red hot until free from carbon. Cooled, weighed and repeated the procedure till constant value. The percentage of total ash was calculated with reference to the air dried drug.

- **Acid insoluble ash**

Total ash was treated with 2N HCL to remove acid insoluble minerals and residue of acid insoluble ash. The half part of total ash obtained, was boiled with 25 ml of 2N hydrochloric acid (HCl) for 5-10 minutes. The insoluble matter was collected on a filter paper, washed with warm water, dried the filter paper, ignited in Muffle furnace, weighed and then the percentage of acid insoluble ash was calculated with the reference to the air dried drug.

- **Water-soluble ash**

It is produced by separating the water soluble material from the total ash. The remaining half part of total ash was boiled with 25 ml of distilled water for 5-10 minutes. The insoluble matter was collected on filter paper, washed with hot water and ignited for 15 minutes at a temperature not exceeding 450°C. The weight of insoluble matter was subtracted from the weight of total ash. The difference in weight represents the water-

soluble ash. The percentage of water-soluble ash was calculated with the reference to the air dried drug.

- **Extractive values**

These values were obtained by measuring the amount of active constituents present in plant material when extracted with water and ethanol. The percentage of water and ethanol soluble extractive with reference to the air dried drug was calculated (Kokate, 1994).

- **Alcohol soluble extractive**

Accurately weighed 4 gm of air-dried powdered drug was macerated with 100 ml of ethyl alcohol in a volumetric flask for 24 hr, firstly shaken for 6 hr and then allowed to keep for 18 hr. It was then filtered and filtrate divided into four parts of 25 ml each. Then 25 ml of the filtrate were evaporated to dryness in a flat-bottomed shallow dish and dried at 100ºC to constant weight. The % w/w of alcohol soluble extractive value was calculated with reference to the air-dried drug.

- **Water soluble extractive**

Accurately weighed 4 gm of air-dried powdered drug was macerated with 100 ml of water in a volumetric flask for 24 hr, firstly shaken for 6 hr and then allowed to keep for 18 hr. It was then filtered and filtrate divided into four parts of 25 ml each. Then 25 ml of the filtrate were evaporated to dryness in a flat-bottom shallow dish and dried at 100°C to constant weight. The % w/w of water soluble extractive value was calculated with reference to the air-dried drug.

- **Loss on drying**

Accurately weigh 1.5 gm of powdered drug into a weighed flat ant thin porcelain dish and dry it in the oven at 100°C until two consecutive weighings do not differ by more than 0.5 mg. Cool in desicators and weigh.

### 4.1.4 Extraction of drug material

The petroleum ether, acetone, methanol and aqueous extracts of rhizomes of *A. galanga* were prepared by following procedure.

#### 4.1.4.1 Petroleum ether extract

The rhizomes of *A. galanga* were shade dried and powdered in hand mixer. About 500 g of powdered material was taken and extracted with cold percolation in a 5L flat bottom

flask at room temperature using petroleum ether (3000 ml) as a solvent at room temperature for 7 days with occasional shaking. Each process was repeated thrice for complete extraction after extraction; extracts were combined and evaporated to dryness in vacuo at 40°C using a rotary evaporator (Eqitron, Roteva). Petroleum ether extract yield was represented as %.

**4.1.4.2 Acetone extract**

The rhizomes of *A. galanga* were shade dried and powdered in hand mixer. About 500 g of powdered material was taken and extracted with cold percolation for 12 h in a 5L flat bottom flask at room temperature using acetone (3000 ml) as a solvent at room temperature for 7 days with occasional shaking. Each process was repeated thrice for complete extraction after extraction; extracts were combined and evaporated to dryness in vacuo at 40°C using a rotary evaporator (Eqitron, Roteva). Acetone extract yield was represented as %.

**4.1.4.3 Methanolic extract**

The rhizomes of *A. galanga* were shade dried and powdered in hand mixer. About 500 g of powdered material was taken and extracted with cold percolation for 12 h in a 5L flat bottom flask at room temperature using methanol (3000 ml) as a solvent at room temperature for 7 days with occasional shaking. Each process was repeated thrice for complete extraction after extraction; extracts were combined and evaporated to dryness in vacuo at 40°C using a rotary evaporator (Eqitron, Roteva). Methanol extract yield was represented as %.

**4.1.4.4 Aqueous extract**

The rhizomes of *A. galanga* were shade dried and powdered in hand mixer. About 500 g of powdered material was taken and extracted with cold percolation for 12 h in a 5L flat bottom flask at room temperature using water (3000 ml) as a solvent at room temperature for 7 days with occasional shaking. Each process was repeated thrice for complete extraction after extraction; extracts were combined and evaporated to dryness in vacuo at 40°C using a rotary evaporator (Eqitron, Roteva). Aqueous extract yield was represented as %.

### 4.1.5 Preliminary phytochemical screening

Preliminary phytochemical screening was carried out for carbohydrates, amino acids, alkaloids, glycosides, resins, tannins, saponins, phenols, terpenoids, coumarins and gums using standard procedures described to identify the constituents (Khandelwal, 2010).

#### 4.1.5.1 Tests for Carbohydrates

Molisch's test: Solution of extract (2 ml) and α-napthol solution (2-3 drops) was mixed in test tube, shaken for few min and concentrated $H_2SO_4$ (1 ml) was added from the side of test tube. A deep violet colored ring at the junction of two layers indicated the presence of sugars.

#### 4.1.5.2 Tests for Proteins

Biuret test: Solution of extract (3 ml) 4% NaOH (1 ml) and 1% $CuSO_4$ (1 ml) was mixed in test tube. The change in color of solution to violet or pink was indicated presence of proteins.

Millon's test: Solution of extract (3 ml) and Millon's reagent (5 ml) were mixed in test tube and observed for the appearance of white precipitate changing to brick red and gave red color to solution on heating indicating presence of proteins.

#### 4.1.5.3 Tests for Steroids

Salkowski reaction: Solution of extract (2 ml) chloroform (2 ml) and $H_2SO_4$ (2 ml) were mixed in test tube, shaken well. The change of chloroform layer to red and acid layer to greenish yellow fluorescence indicated presence of steroids.

Liebermann-Burchard reaction: Solution of extract (2 ml) chloroform (2 ml) acetic anhydride (2 ml) was mixed in test tube. Concentrated $H_2SO_4$ (2 drops) was added from the side of test tube. The change in color first red, then blue and finally green indicated the presence of steroids.

Liebermann's reaction**:** Solution of extract (3 ml) and acetic anhydride (3 ml) were mixed in test tube. Heated the mixture and cooled. Concentrated $H_2SO_4$ (2-3 drops) was added from the side of test tube. Appearance of blue color indicated presence of steroids.

#### 4.1.5.4 Test for Volatile oils

Odour test: Characteristic odour of extract indicates presence of volatile oil.

Solubility test: Solubility in 90% alcohol indicates presence of volatile oil.

#### 4.1.5.5 Tests for Glycosides

Keller-Killani test: Solution of extract (2 ml) glacial acetic acid (1 ml) 5% $FeCl_3$ (3 drops) and concentrated $H_2SO_4$ were mixed in test tube and observed for the appearance of reddish-brown color at the junction of two layers and bluish green in the upper layer indicated presence of cardiac glycosides.

Borntrager's test: Solution of extract (2 ml) and dilute $H_2SO_4$ (2 ml) was mixed in test tube, boiled for 5 min and filtered. In filtrate equal volume of chloroform was added and mixed well. Organic layer was separated and ammonia was added to it. Pink-red color of the ammonia layer indicated presence of anthraquinone glycosides.

#### 4.1.5.6 Tests for Saponins

Foam test: Extract (1 g) was shaken vigorously with water and observed for persistent foam indicating presence of saponins.

#### 4.1.5.7 Test for Tannins and Phenolic compounds

The following reagents were added to the 3 ml of solution of extract.

1. 5% Ferric chloride (3 ml): The blue- black color indicated presence of tannins or phenols.

2. Lead acetate (3 ml): The occurrence of white precipitate indicated presence of tannins or phenols.

3. Potassium permanganate (3 ml): The discoloration of potassium permanganate solution indicated presence of tannins or phenols.

#### 4.1.5.8 Test for presence of flavonoids

Shinoda test: In the solution of extract (5 ml), 95% ethanol (5 ml), few drops of HCl and magnesium tunings (0.5 g) were added. The appearance of pink color indicated presence of flavonoids.

#### 4.1.5.9 Tests for Alkaloids

Extract (10 g) and dilute hydrochloric acid were mixed in test tube, shake well and filtered. With filtrate following tests were performed.

Dragendorff's test: Extract solution filtrate (3 ml) and Dragendorff's reagent (1 ml) were mixed in test tube. The appearance of orange brown precipitate indicated presence of alkaloids.

Mayer's test: Extract solution filtrate (3 ml) and Mayer's reagent (1 ml) were mixed in test tube. The appearance of precipitate indicated presence of alkaloids.

Wagner's test: Extract solution filtrate (3 ml) and Wagner's reagent (1 ml) were mixed in test tube. The appearance of reddish brown precipitate indicated presence of alkaloids.

Hager's test: Extract solution filtrate (3 ml) and Hager's reagent (1 ml) were mixed in test tube. The appearance of yellow precipitate indicated presence of alkaloids.

### 4.1.6 Pharmacological studies

#### 4.1.6.1 Chemicals and drugs

Freund's Complete Adjuvant (Sigma-Aldrich, St. Louis, MO, USA), carrageenan (Sigma-Aldrich, St. Louis, MO, USA), ACA (Merk, India), pentazocin (Ranbaxy, India), acetic acid (Pure Chem. Ltd., India), tween 80 (Research Lab, India) were purchased. Diclofenac was provided as a gift sample from Emcure Pharmaceutical Ltd., Pune. Biochemical diagnostic kits for aspartate aminotransferase, alanine aminotransferase, total protein and alkaline phophatase (Accurex biomedical Pvt. Ltd) were purchased from commercial sources.

#### 4.1.6.2 Apparatus

Microcapillary tubes, centrifuging tubes (Tarson, India), microtips (Tarson, India), test tubes, tissue paper, micropipettes were purchased from authorized vendors.

#### 4.1.6.3 Instruments used

Plethysmometer (Model: 7140, UGO Basile, Italy), plethysmometer (Model: 2888, Almemo, Germany), digital vernier caliper (Model: CD-6 CS, Mitutoyo Corp., Japan), thermal planter tester (Model: 37360, UGO Basile, Italy), Von Frey Hairs (Model: 2888, Almemo, Germany), hot plate (Model: DS-37, UGO Basile, Italy), UV/visible spectrophotometer (Model: Jasco V-530, Japan), eppendorff's cryocentrifuge machine (Model: 5810 R, Germany).

#### 4.1.6.4 Preparation of dosage form

Dosage forms of individual extracts were prepared as per the following procedures.

*Petroleum ether extract* of *A. galanga*:

The petroleum ether extract of *A. galanga* was emulsified with tween 80 (2%) in a glass mortar with gradual addition of distilled water to make up the required volume.

*Aetone extract* of *A. galanga:*

The acetone extract of *A. galanga* was emulsified with tween 80 (2%) in a glass mortar with gradual addition of distilled water to make up the required volume.

*Methanolic extract* of *A. galanga:*

The methanolic extract of *A. galanga* was emulsified with tween 80 (2%) in a glass mortar with gradual addition of distilled water to make up the required volume.

*Aqueous extracts* of *A. galanga:*

Aqueous extract of *A. galanga* was dissolved in distilled water to make up the required volume.

*Drugs:*

Accurately weighed quantity of diclofenac was suspended in distilled water to make volume.

*Vehicles:*

Vehicle was prepared by adding 2% tween 80 into distilled water, without addition of extracts or drugs.

**4.1.6.5 Storage conditions**

All the dosage forms of extracts and drug solutions were prepared freshly on the day of experiment and stored in airtight amber colored vials to protect from exposure to sunlight during the experiments.

**4.1.6.6 Volume of extract solution**

The volume of extract solutions was calculated based upon the body weight of animal.

**4.1.6.7 Route of administration**

The extract solutions were administered orally.

**4.1.6.8 Experimental animals**

Swiss albino female mice (20-25 g) and Wistar rats (170-220 g) of either sex were purchased from National Institute of Biosciences, Pune, India. Animals were housed in an air-conditioned room at a temperature of 22 ± 2 $^{\circ}$C and relative humidity of 45 to 55% under 12-h light: 12-h dark cycle. The animals had free access to food pellets (Pranav agro Pvt. Ltd., Sangli, India). Filtered water was provided *ad libitum*.

**4.1.6.9 Approval of research protocol**

The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) constituted in accordance with the rules and guidelines of the Committee for the

Purpose of Control and Supervision on Experimental Animals (CPCSEA), India (Protocol approval number was CPCSEA/2013/39).

#### 4.1.6.10 Acute oral toxicity study

Healthy female Swiss albino mice of 20-25 g were used in acute toxicity studies as per OECD guidelines-425. The animals were fasted overnight and divided into four groups with 5 mice in each group. Extracts of *A. galanga* (petroleum ether, acetone, methanol and aqueous) were administered orally at one dose level of 2000 mg/kg body weight. The mice were observed continuously for behavioral and autonomic profiles for 2 hrs and for any sign of toxicity or mortality up to 48 hrs (OECD-425, 2001).

### 4.1.7 Anti-inflammatory activity of four extracts of *A. galanga*

#### 4.1.7.1 Carrageenan induced paw edema in rats

Pedal inflammation in rat was produced according to the method of (Winter et al., 1962). Wistar rats of either sex weighing 175 to 200 g were used in this study. The rats were divided in to 15 groups of six rats in each group as follows:

Treatment groups

Group 1 - Vehicle control (2% Tween 80, p.o.)

Group 2 – Diclofenac (10mg/kg, p.o)

Group 3, 4 and 5 – Petroleum ether extract 100, 200 and 400 mg/kg, p.o. respectively

Group 6, 7 and 8 – Acetone extract 100, 200 and 400 mg/kg, p.o. respectively

Group 9, 10 and 11 – Methanolic extract 100, 200 and 400 mg/kg, p.o. respectively

Group 12, 13 and 15 – Aqueous extract 100, 200 and 400 mg/kg, p.o. respectively

Procedure: The extracts and vehicle were administered orally to experimental animals once a day for a period of 7 days. One hour after the last administration, acute paw edema was induced by intradermal injection of 0.1 ml of 1% freshly prepared carrageenan suspension in normal saline into the subplanter region of the right hind paw of each rat. The paw volume was measured before (0 h) and at intervals of 1, 2, 3, 4, 5, 6 and 24 h after carrageenan injection using plethysmometer (Model: 7140, UGO Basile, Italy). Then percentage of inhibition of edema was calculated for each group with respect to the control group as follows,

% Inhibition of paw edema = (VC-VT/VC) 100

VC and VT represent change in paw volume of control and drug treated animals respectively (Ishola et al., 2011; Bose et al., 2007; Wu et al., 2006).

**4.1.7.2 Cotton pellet granuloma in rats**

Subcutaneous implantation of pellets of compressed cotton provokes foreign body granuloma. Wistar rats of either sex weighing 175 to 200 g were used in this study. The rats were divided in to 15 groups of six rats in each group as follows:

Treatment groups

Group 1 - Vehicle control (2% Tween 80, p.o.)

Group 2 – Diclofenac (10mg/kg, p.o)

Group 3, 4 and 5 – Petroleum ether extract 100, 200 and 400 mg/kg, p.o. respectively

Group 6, 7 and 8 – Acetone extract 100, 200 and 400 mg/kg, p.o. respectively

Group 9, 10 and 11 – Methanolic extract 100, 200 and 400 mg/kg, p.o. respectively

Group 12, 13 and 15 – Aqueous extract 100, 200 and 400 mg/kg, p.o. respectively

Procedure: Under aseptic precautions, an incision was made on the back of each rat in each group and sterile cotton pellets (50 ± 1 mg) were implanted subcutaneously in the axilla under ether anesthesia. On the seventh day, animals were sacrificed; pellets were removed along with the granulation tissue and pellets were dried at $60^0$C for 24 hrs. The net dry weight of the granuloma was determined. Stomach was isolated and cut open in greater curvature and subjected to histopathology (Smita et al., 2010; Ilavarasan et al., 2006).

**4.1.8 Anti-arthritic activity of acetone extract of *A. galanga* (AEAG)**

**4.1.8.1 Freund's complete adjuvant induced arthritis**

Arthritis was induced by the indradermal injection of 0.1 ml of Freund's complete adjuvant (FCA, Sigma) in the subplanter region of the right hind paw on day 0. The rats were divided into six groups of six rats each as follows:

Treatment groups

Group 1 - Non-arthritic,vehicle control (2% Tween 80, p.o.)

Group 2 - Arthritic, vehicle control (2% Tween 80, p.o.)

Group 3 - Arthritic animals treated with standard (diclofenac 5 mg/kg, p.o.)

Group 4 - Arthritic animals treated with test drug (AEAG 100 mg/kg, p.o.)

Group 5 - Arthritic animals treated with test drug (AEAG 200 mg/kg, p.o.)

Group 6 - Arthritic animals treated with test drug (AEAG 400 mg/kg, p.o.)

The dosing of all the groups was started from day 12, once day (Ramadan et al., 2010).

The following parameters were measured at regular intervals (Day 0, 1, 4, 8, 12, 16, 20, 24 and 28). (A) Body weight, (B) Paw volume, (c) Joint diameter, (D) Tactile allodynia and (E) Thermal hyperalgesia. On day 28, rats were anaesthetized and blood was withdrawn by retro-orbital puncture and used for biochemical assays. The rats were sacrificed on day 28 to study antioxidant parameters on liver and histology of ankle joint.

**(A) Body weight**

Body weight of each animal was measured at regular time interval during the course of study.

**(B) Paw volume**

The severity of arthritis was quantified by measuring volume of hind paws using plethysmometer (Model: 7140, UGO Basile, Italy). Paw volume (ml) was measured on day 0, 1, 4, 8, 12, 16, 20, 24 and 28 after arthritis induction. Data were expressed as the change in paw volume with respect to day 0 paw volumes (Winter et al., 1962).

**(C) Joint diameter**

Before injection joint diameter were measured using a digital vernier caliper (Model: CD-6 CS, Mitutoyo Corp., Japan) after which adjuvant was administered. The joint diameter was measured again on day 1, 4, 8, 12, 16, 20, 24 and 28. Data were expressed as the change in joint diameter with respect to day 0 joint diameter (Andersen et al., 2004).

**(D) Tactile allodynia**

Tactile allodynia of hind paws was evaluated by Von Frey Hairs (Model: 2888, Almemo, Germany) of increasing gauge. The rats were allowed to acclimatize for 10 min in the Perspex box and Von Frey hairs (0.6 to 12.6g) were applied to planter surface to hind paws. A series of three stimuli were applied to each paw for each hair within a period 2-3 s. the lowest weight of Von Frey hair to evoke a withdrawal from the three consecutive applications was considered to indicate the mechanical withdrawal threshold (MWT) (Chaplan et al., 1994; Tal and Bennett, 1994).

**(E) Thermal hyperalgesia**

Thermal hyperalgesia was tested to evaluate the effect of AEAG and a noxious thermal stimulus was determined using a thermal planter tester (Model: 37360, UGO Basile,

Italy). Briefly, rats were acclimatized to the testing room for at least 10 min prior to start of behavioral testing. Following acclimatization, radiant heat was applied to the planter surface of the hind paw until the rat lifted its paw. A photoelectric cell automatically tuned the heat surface off when the reflected light beam was interrupted (i.e. when the animal withdrew its paw) and the time at which this occurred was recorded as the paw withdrawal latency (PWL). The cut-off time was 15 s (Lee et al., 2009; Mali et al, 2011).

**(F) Biochemical assays**

On day 28, rats were anaesthetized by ether and blood was withdrawn by retro-orbital puncture for biochemical assays. The haematological parameters like haemoglobin (Hb), red blood cell (RBC), white blood cell (WBC) and platelets were determined (Jalapure, 2011). Whereas, separated serum from blood is used to estimate aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phophatase (ALP), total protein (TP) and C-reactive protein (CRP) were also determined (Zhang et al., 2009).

**(G) Antioxidant parameters**

The levels of lipid peroxidation (MDA content), reduced glutathione (GSH) and superoxide dismutase (SOD) in liver were estimated as biomarkers of inflammation.

**1) Removal and processing of tissue for estimation of tissue parameters**

**Reagents**

- **Phosphate Buffered Saline ph (7.4)**

  Disodium ethylene diamine tetra acetic acid (1.38 gm), 0.19 gm of potassium dihydrogen phosphate and 8 gm of sodium chloride was dissolved in 900 ml of distilled water and adjusted pH using dilute hydrochloric acid. The volume was adjusted to 1000 ml using distilled water.

- **Sucrose solution (0.25 M)**

  85.58 gm of sucrose was dissolved in 200 ml of water and diluted to 1000 ml with distilled water.

- **Tris hydrochloric buffer (10mM, pH 7.4)**

  1.21 gm tris was dissolved in 900 ml of distilled water and the pH was adjusted to 7.4 with 1M hydrochloric acid. The resulting solution was diluted to 1000 ml with distilled water.

**Procedure**

The rats were sacrificed, after blood collection; liver was dissected and quickly transferred to ice-cold phosphate buffered saline (pH 7.4). It was blotted free of blood and tissue fluids, weighed on electronic Balance. The liver were cross-chopped with surgical scalpel into fine slices, suspended in chilled 0.25M sucrose solution and quickly blotted on a filter paper. The tissues were then minced and homogenized for 1 min. in chilled tris hydrochloride buffer (10mM, pH 7.4) to a concentration of 10% w/v. Homogenization under hypotonic condition was carried out to disrupt, as far as possible, the structure of the cells so as to release soluble proteins. The homogenate was centrifuged at 7000 rpm at 25 minutes using Remi C-24 high speed cooling centrifuge. The clear supernatant was used for the determination of MDA, GSH, and SOD concentration.

**2) Assay of lipid Peroxidation (MDA content)**

**Reagents**

- **Thiobarbituric acid (0.67% w/v)**

  Thiobarbituric acid 0.67gm was dissolved in 50 ml of distilled water and the final volume was made upto 100 ml with hot distilled water.

- **Trichloroacetic acid (10% w/v)**

  Trichloroacetic acid 10gm was dissolved in 60 ml of distilled water and the final volume was made upto 100 ml with distilled water.

- **Standard Malondialdehyde stock solution (50mM)**

  A standard malondialdehyde stock solution was prepared by mixing 25µ of 1,1,3,3-tetraethoxypropane upto 100 ml with distilled water. 1.0 ml of this stock solution was diluted up to 10 ml to get solution containing 23µ of malondialdehyde/ml. One ml of this stock solution was diluted up to 100 ml to get a working standard solution containing 23ng of malondialdehyde/ml.

**Procedure**

Tissue homogenate (supernatant) 2.0 ml was added to 2.0 ml of freshly prepared 10% w/v trichloroacetic acid (TCA) and the mixture was allowed to stand in an ice bath for 15 minutes. After 15 minutes, the precipitate was separated by centrifugation and 2.0 ml of clear supernatant solution was mixed with 2.0 ml of freshly prepared thiobarbituric acid

(TBA). The resulting solution was heated in a boiling water bath for 10 minutes. It was then immediately cooled in an ice bath for 5 minutes. The color developed was measured at 532nm against reagent blank by U.V spectrophotometer. Different concentrations (0-23nM) of standard malondialdehyde were processed as above for obtaining standard graph. The values were expressed as nM of MDA/mg protein (Slater and Sawyer, 1971).

**3) Assay of endogenous antioxidant (reduced glutathione i.e. GSH)**

**Reagents**

- **Trichloroacetic acid (20% w/v)**

  Trichloroacetic acid 20 gm was dissolved in sufficient quantity of distilled water and the final volume was made up to 100 ml with distilled water.

- **Phosphate Buffer (0.2M, pH 8.0)**

  Sodium phosphate 0.2 M was prepared by dissolving 30.2gm sodium phosphate in 600ml of distilled water, the pH was adjusted to 8.0 with 0.2M sodium hydroxide solution and the final volume was adjusted up to 1000 ml with distilled water.

- **5,5-dithiobis-2-nitrobenzoic acid (DTNB) reagent (0.6mM)**

  DTNB reagent 60 mg was dissolved in 50 ml of buffer and the final volume was adjusted to 100 ml with buffer.

- **Standard glutathione (100 µg/ml)**

  10mg of glutathione was dissolved in 60 ml of distilled water and the final volume was made up to 100 ml with distilled water.

**Procedure**

Equal volumes of tissue homogenate (supernatant) and 20% TCA were mixed. The precipitated fraction was centrifuged at 2500 rpm at 4°C for 15 min and 2.0 ml of DTNB reagent was added to 0.25 ml of supernatant. The final volume was made up to 3.0 ml with phosphate buffer. The color developed was read at 412 nm against reagent blank. Different concentrations (10-50 µg) of standard glutathione were prepared and processed as above for standard graph. The amount of reduced glutathione is expressed as µg of GSH/mg protein (Moron et al., 1979).

**4) Assay of Superoxide Dismutase (SOD)**

**Reagents**

- **Carbonate buffer (0.05 M, pH 10.2)**

  Sodium bicarbonate 16.8 gm and 22 gm of sodium carbonate were dissolved in 500 ml of distilled water and the volume was made up to 1000 ml with distilled water.

- **Ethylene diamine tetra acetic acid (EDTA) solution (0.4 M)**

  EDTA 1.82 gm was dissolved in 200 ml of distilled water and the volume was made up to 1000 ml with distilled water.

- **Hydrochloric acid (0.1 N)**

  Concentrated hydrochloric acid 8.5 ml was mixed with 500 ml of distilled water and the volume was made up to 1000 ml with distilled water.

- **Epinephrine solution (3mM)**

  Epinephrine bitartarate 0.99gm was dissolved in 100 ml of 0.1N hydrochloric acid and the volume was adjusted to 1000 ml with 0.1N hydrochloric acid.

- **Superoxide dismutase standard (10 U/ml)**

  SOD 1 mg (1000 U / mg) from bovine liver was dissolved in 100 ml of carbonate buffer.

**Procedure**

Liver tissue homogenate (0.5 ml) was diluted with (0.5 ml) distilled water, to which 0.25 ml of ice-cold ethanol and 0.15 ml of ice-cold chloroform, were added. The mixture was mixed well using cyclo mixer and centrifuged at 2500 rpm at $4^{\circ}C$ for 15 min. To 0.5 ml of supernatant, 1.5 ml of carbonate buffer and 0.5 ml of EDTA solution were added. The reaction was initiated by the addition of 0.4 ml of epinephrine and the change in optical density/min was measured at 480 nm against reagent blank. Calibration curve was prepared by using 10-125 units of SOD. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme is taken as the enzyme unit. SOD activity is expressed as units/mg protein (Misera and Fridocich., 1972).

**(H) Radiological analysis**

On day 28, rats were anesthetized and radiographs of the adjuvant injected hind paws were taken using X-ray (AGFA CR 30-X unit, Germany). Radiographic analysis of hind paws were taken at 55 kV peak, 50 mA and the exposure time was 5 s.

**(I) Histological analysis of ankle joint**

Rats were sacrificed on 28$^{th}$ day; hind limbs were removed and fixed in 10% buffered formalin. The limbs were decalcified in 5% formic acid, processed for paraffin embedding, sectioned at 5µm thickness, and subsequently stained with haematoxylin-eosin for examination under a light microscope with 10 X magnifications. Sections were examined for the presence of hyperplasia of the synovium, pannus formation and destruction of the joint space (Anderson et al., 1996; Banji et al., 2011; Asquith et al., 2009).

**4.1.9 Isolation of bioactive fraction from AEAG by bioassay guided fractionation method.**

**4.1.9.1 Chemicals and reagents**

Petroleum ether, acetone, methanol, benzene, chloroform, ethanol (Merck, India) of GR grades, column grade Silica (60-120#) (Spectrochem Pvt. Ltd. Mumbai, India) and TLC grade silica gel G (S.D. fine chem., Mumbai, India) were purchased from commercial sources.

**4.1.9.2 Apparatus and instruments**

Borosil glass column (height, 120 cm; diameter, 3 cm) and borosil glass column (height, 30 cm; diameter, 2 cm) was purchased from (Ajay Scientific enterprises), Pune, India. Precoated TLC silica gel plates (Merck, Kieselgel 60, F-254, 0.2 mm) were used for analytical TLC. LC-MS (Model: Micromass, Water U.K.), Infrared spectrophotometer (Model: JASCO, FT/IR-5300), NMR spectra (Model: Bruker, AV-500 MHz for $^{1}$H and 125 MHz for $^{13}$C).

**4.1.9.3 Experimental animals**

Wistar rats (170-200 g) of either sex were purchased from National Institute of Biosciences, Pune, India. Animals were housed in an air-conditioned room at a temperature of 22 ± 2$^{\circ}$C and relative humidity of 45 to 55% under 12-h light: 12-h dark

cycle. The animals had free access to food pellets (Pranav agro Pvt. Ltd., Sangli, India). Filtered water was provided *ad libitum*.

#### 4.1.9.4 Approval of research protocol

The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) constituted in accordance with the rules and guidelines of the Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA), India (Protocol approval number: CPCSEA/2013/40).

#### 4.1.9.5 Column chromatography of acetone extract of *A. galanga* (AEAG)

##### 4.1.9.5.1 Liquid – solid separation chromatographic technique

10 g of acetone extract of *A. galanga* (AEAG) was mixed with 20 ml of acetone and 20 g of silica gel. Sample was mixed thoroughly and dried in oven at 110°C for 10 min. 100 ml of petroleum ether was added into the mixture and shake properly. Then the mixture was allowed to settle, two layers were formed. Upper layer was separated into the separate flask and labeled as Pet. Ether fraction. Same procedure was repeated twice with 100 ml of petroleum ether.

100 ml of (10% acetone in petroleum ether) was added into the residue and shake properly. Then the mixture was allowed to settle, two layers were formed. Upper layer was separated into the separate flask and labeled as 10% acetone fraction. Same procedure was repeated twice with 100 ml of 10% acetone in petroleum ether.

100 ml of (20% acetone in petroleum ether) was added into the residue and shake properly. Then the mixture was allowed to settle, two layers were formed. Upper layer was separated into the separate flask and labeled as 20% acetone fraction. Same procedure was repeated twice with 100 ml of 20% acetone in petroleum ether.

100 ml of (30% acetone in petroleum ether) was added into the residue and shake properly. Then the mixture was allowed to settle, two layers were formed. Upper layer was separated into the separate flask and labeled as 30% acetone fraction. Same procedure was repeated twice with 100 ml of 30% acetone in petroleum ether.

100 ml of (50% acetone in petroleum ether) was added into the residue and shake properly. Then the mixture was allowed to settle, two layers were formed. Upper layer was separated into the separate flask and labeled as 50% acetone fraction. Same procedure was repeated twice with 100 ml of 50% acetone in petroleum ether.

100 ml of acetone was added into the residue and shake properly. Then the mixture was allowed to settle, two layers were formed. Upper layer was separated into the separate flask and labeled as acetone fraction. Same procedure was repeated twice with 100 ml of acetone.

**4.1.9.5.2 Anti-inflammatory activity of 6 fractions from acetone extract of *A. galanga* in carrageenan induced paw edema (Acute study)**

Different fractions from acetone extract of *A. galanga* were labeled as petroleum ether fraction, 10% acetone fraction, 20% acetone fraction, 30% acetone fraction, 50% acetone fraction and 100% acetone fraction. These fractions were further subjected to anti-inflammatory study. Wistar rats of either sex weighing 170-200 g were divided in following groups (n=6) viz;

Group I- Vehicle control (tween 80, 2%),

Group II- Diclofenac (10 mg/kg)

Group III- Petroleum ether fraction (100 mg/kg)

Group IV- 10% acetone fraction (100 mg/kg)

Group V- 20% acetone fraction (100 mg/kg)

Group VI- 30% acetone fraction (100 mg/kg)

Group VII- 50% acetone fraction (100 mg/kg)

Group VIII- acetone fraction (100 mg/kg)

All fractions and diclofenac were administered orally 1 h before carrageenan injection. Paw volume was measured at 0, 1, 2, 3, 4, 5 and 6 h after carrageenan injection by plethysmometer (Model: 2888, Almemo, Germany). The data was presented as change in paw volume (ml).

**4.1.9.5.3 Column chromatography of 10% acetone fraction**

Column was packed with activated silica (60-120#). The 10% acetone extract of *A. galanga* (AEAG) was loaded on to the packed silica gel column. After stabilization column was eluted with the mobile phase. Fractions were collected and analyzed by TLC. Fractions showing similar bands were pooled together.

*Activation of silica*

Column grade silica (60-120#) was placed in oven at 110°C overnight (12 h) to remove the all moisture content present in it. Weighed quantity of activated silica was packed in the column.

*Preparation of mobile phase*

The solvents petroleum ether and acetone were used for the preparation of mobile phase. The composition of mobile phase was petroleum ether: acetone, with successive increase in percentage of acetone.

*Packing of column*

A clean and dry borosil glass column (height, 120 cm; diameter, 3 cm) was aligned in a vertical position with the help of clamps attached to metal stand. A piece of cotton soaked in mobile phase was placed at the bottom of the column and gently tapped down with a glass rod. The column was slowly and evenly filled to about 5/6 capacity with gradual addition of silica. Side of the chromatographic column was gently tapped with a cork during the packing process to compact silica column.

*Application of sample*

Weighed quantity (2.50 g) of 10% acetone extract of *A. galanga* (AEAG) was mixed with 15 ml of acetone and 15 g of activated silica to prepare slurry, acetone was evaporated from the slurry and the dried sample was added to top of the packed silica in column. A thin disc (column diameter) of cotton soaked in mobile phase was placed on top of the bed to prevent disturbing the sample layer after addition of mobile phase. Stopcock was opened to drain excess mobile phase until it reaches top of sample. Column was filled to the top with the mobile phase and allowed to stand overnight (~12 h) to develop a bands.

*Elution*

Elution was carried out by gravity at the flow rate of 2 ml/min. Mobile phase added to top of column was petroleum ether: acetone. Polarity of mobile phase was increased by their polarity order and fractions (25 ml in each 50 ml tube) were collected in tarson tubes. Remaining loaded material in the column which cannot be eluted with mobile phase was eluted with methanol and collected as methanol fraction and completed column chromatography. All fractions were analyzed by TLC and fractions showing similar bands were pooled together and labeled as B1, B2, B3 and B4 respectively.

#### 4.1.9.5.4 Anti-inflammatory activity of 4 pools (B1 to B4) isolated from 10% acetone fraction in carrageenan induced paw edema (Acute study)

Pools from 10% acetone fraction were labeled as B1 to B4 and subjected further for anti-inflammatory study. Wistar rats of either sex weighing 170-200 g were divided in following groups (n=6) viz;

Group I- Vehicle ( tween 80, 2%),

Group II- Diclofenac (10 mg/kg),

Group III to Group VI- B1 to B4 (10 mg/kg) respectively.

All fractions and diclofenac were administered orally 1 h before carrageenan injection. Paw volume was measured at 0, 1, 2, 3, 4, 5 and 6 h after carrageenan injection by plethysmometer (Model: 2888, Almemo, Germany). The data was presented as change in paw volume (ml).

#### 4.1.9.5.5 Statistical analysis

The data of pharmacological experiments were expressed as mean ± standard error mean (SEM). Data analysis was performed using Graph Pad Prism 5.0 software (Graph Pad, San Diego, CA, USA). Data of body weight, paw volume, joint diameter, tactile allodynia and thermal hyperalgesia were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Data of hematological and biochemical parameters were analyzed using one way analysis of variance (ANOVA) followed by Dunnett's test. A value of $P<0.05$ was considered to be statistically significant.

### 4.1.10 Anti-inflammatory activity of bioactive fraction (B2)

#### 4.1.10.1 Acute oral toxicity study

Healthy female Swiss albino mice of 20-25 g were used in acute toxicity studies as per OECD guidelines-425. The animals were fasted overnight and divided into groups with 5 mice in each group. Bioactive fraction (B2) was administered orally at one dose level of 20 mg/kg body weight. The mice were observed continuously for behavioral and autonomic profiles for 2 hrs and for any sign of toxicity or mortality up to 48 hrs (OECD-425, 2001).

#### 4.1.10.2 Carrageenan induced paw edema in rats

Pedal inflammation in rat was produced according to the method of (Winter et al., 1962). Wistar rats of either sex weighing 175 to 200 g were used in this study.

Treatment groups

Group 1 - Vehicle control (2% Tween 80, p.o.)

Group 2 – Diclofenac (10mg/kg, p.o)

Group 3, 4 and 5 – Bioactive fraction (B2) 5, 10 and 20 mg/kg, p.o. respectively

Procedure: The bioactive fraction (B2) and vehicle were administered orally to experimental animals once a day for a period of 7 days. One hour after the last administration, acute paw edema was induced by intradermal injection of 0.1 ml of 1% freshly prepared carrageenan suspension in normal saline into the subplanter region of the right hind paw of each rat. The paw volume was measured before (0 h) and at intervals of 1, 2, 3, 4, 5, 6 and 24 h after carrageenan injection using plethysmometer (Model: 7140, UGO Basile, Italy). Then percentage of inhibition of edema was calculated for each group with respect to the control group as follows,

% Inhibition of paw edema = (VC-VT/VC) 100

VC and VT represent change in paw volume of control and drug treated animals respectively (Ishola et al., 2011; Bose et al., 2007; Wu et al., 2006).

**4.1.10.3 Cotton pellet granuloma in rats**

Subcutaneous implantation of pellets of compressed cotton provokes foreign body granuloma. Wistar rats of either sex weighing 175 to 200 g were used in this study.

Treatment groups

Group 1 - Vehicle control (2% Tween 80, p.o.)

Group 2 – Diclofenac (10mg/kg, p.o)

Group 3, 4 and 5 – Bioactive fraction (B2) 5, 10 and 20 mg/kg, p.o. respectively

Procedure: Under aseptic precautions, an incision was made on the back of each rat in each group and sterile cotton pellets (50 ± 1 mg) were implanted subcutaneously in the axilla under ether anesthesia. On the seventh day, animals were sacrificed; pellets were removed along with the granulation tissue and pellets were dried at $60^0$C for 24 hrs. The net dry weight of the granuloma was determined. Stomach was isolated and cut open in greater curvature and subjected to histopathology (Smita et al., 2010; Ilavarasan et al., 2006).

### 4.1.11 Anti-arthritic studies of isolated bioactive fraction (B2)

#### 4.1.11.1 Freund's complete adjuvant induced arthritis

Arthritis was induced by the indradermal injection of 0.1 ml of Freund's complete adjuvant (FCA, Sigma) in the subplanter region of the right hind paw on day 0. The rats were divided into six groups of six rats each as follows:

Treatment groups

Group 1 - Non-arthritic,vehicle control (2% Tween 80, p.o.)

Group 2 - Arthritic, vehicle control (2% Tween 80, p.o.)

Group 3 - Arthritic animals treated with standard (diclofenac 5 mg/kg, p.o.)

Group 4 - Arthritic animals treated with test drug [Fraction (B2) 5 mg/kg, p.o.]

Group 5 - Arthritic animals treated with test drug [Fraction (B2) 10 mg/kg, p.o.]

Group 6 - Arthritic animals treated with test drug [Fraction (B2) 20 mg/kg, p.o.]

The dosing of all the groups was started from day 12, once day (Ramadan et al., 2010).

The following parameters were measured at regular intervals (Day 0, 1, 4, 8, 12, 16, 20, 24 and 28). (A) Body weight, (B) Paw volume, (c) Joint diameter, (D) Tactile allodynia and (E) Thermal hyperalgesia. On day 28, rats were anaesthetized and blood was withdrawn by retro-orbital puncture and used for biochemical assays. The rats were sacrificed on day 28 to study antioxidant parameters on liver and histology of ankle joint.

### 4.1.12 Preparative TLC of pool P 2 (B2)

*Application of sample*

The fraction P2 (B2) was applied by streaking across the full length of the plate by glass capillary.

*Mobile phase*

The solvents n-hexane and ethyl acetate were used for the preparation of mobile phase. The composition of mobile phase was n-hexane:ethylacetate (8:2).

*Preconditioning of chamber (saturation)*

Chromatogram was developed in a saturated twin trough chambers. To achieve saturation, at least half of the total area of the inside the wall of the chamber was lined with filters paper. A sufficient quantity (approximately 10 ml) of mobile phase was poured along the side of filter paper into the chamber to saturate the chamber and form a

layer about 5 mm deep. Chamber was then closed and allowed to stand for at least 30 min at room temperature.

*Development of chromatogram*

The plate was marked 10 mm below the upper edge. Plate was placed vertically into the chamber ensuring that the points of application were above the surface of the mobile phase. Chamber was closed and mobile phase was allowed to ascend to the specific distance at the room temperature. Plate was removed; the position of mobile phase front was marked. Mobile phase was allowed to evaporate at room temperature and dried under hot air.

*Observation and elution of compound*

Chromatogram was observed in daylight, under ultra violate (UV) light at 254 and 366 nm wavelength. Area was marked and scrapped off with sharp blade, about 10% additional area was then marked to compensate for three-dimensional development of band in the layer. With minimum volume of mobile phase the components from scrapped material was eluted. Scrapped material and mobile phase were homogenized in vortex mixer to ensure complete elution, centrifuged; supernatant was collected and allowed to evaporate.

### 4.1.13 Characterization of bioactive fraction (B2)

The chemical structure of isolated compound was elucidated by LC-MS, IR, $^{1}$H-NMR, $^{13}$C-NMR, and DEPT spectroscopy.

**Part II**

## 4.2 *A. officinarum* Hance

### 4.2.1 Plant materials

The rhizomes of *A. officinarum* were purchased from local market in Pune, Maharashtra, India. The rhizomes of *A. officinarum* were identified and authenticated by Dr. A.S. Upadhye, Department of Botany, Agharkar Research Institute, Pune, India and voucher specimens R-168 were deposited at that Institute.

### 4.2.2 Chemicals and reagents

Analytical grade petroleum ether, ethanol, methanol, ethyl acetate, acetone, toluene (Merck, India) were purchased from commercial source. The reagents for phytochemical identification were obtained from the freshly prepared stock used in pharmacognosy and pharmaceutical chemistry laboratory of the college.

### 4.2.3 Preliminary pharmacognostic studies of *A. officinarum*

#### 4.2.3.1 Macroscopic and microscopic analysis of *A. officinarum*

The macroscopic characters of rhizomes, leaf, root, bark, flowers and seed were determined organoleptically (Anonymous et al., 2003).Observations of shape, size, color, odour, taste and external markings were done in macroscopical studies. Microscopic studies were done according to the method of (Brain and Turner., 1975). For microscopical studies; a few drops of chloral hydrate solution were added to a sample of powdered plant material on a slide and heated gently over a micro Bunsen. The slide was covered with a glass cover slip for examination under the microscope and different cell components were observed. Free hand section of drug material was taken and stained with safranin and phloroglucinol followed by concentrated hydrochloric acid (Johansen, 1940). The respective figures of reactions were drawn with the help of camera lucida and dissecting microscope.

#### 4.2.3.2 Physico-chemical analysis of *A. officinarum*

Physico-chemical analysis i.e. percentage of ash values and extractive values was performed according to the official methods prescribed by WHO guidelines on quality control methods for medicinal plant materials (WHO/QCMMPM guidelines, 1992). Fluorescence analysis was carried out according to the method prescribed (Chase and

Pratt, 1949; Kokoshi et al., 1958). The color and consistency of the extracts were also recorded.

### 4.2.4 Extraction of drug material

The petroleum ether, acetone, methanol and aqueous extracts of rhizomes of *A. officinarum* were prepared by following procedure.

#### 4.2.4.1 Petroleum ether extract

The rhizomes of *A. officinarum* were shade dried and powdered in hand mixer. About 500 g of powdered material was taken and extracted with cold percolation for 12 h in a 5L flat bottom flask at room temperature using petroleum ether (3000 ml) as a solvent at room temperature for 7 days with occasional shaking. Each process was repeated thrice for complete extraction after extraction; extracts were combined and evaporated to dryness in vacuo at $40^0$C using a rotary evaporator (Eqitron, Roteva). Petroleum ether extract yield was represented as %.

#### 4.2.4.2 Acetone extract

The rhizomes of *A. officinarum* were shade dried and powdered in hand mixer. About 500 g of powdered material was taken and extracted with cold percolation for 12 h in a 5L flat bottom flask at room temperature using acetone (3000 ml) as a solvent at room temperature for 7 days with occasional shaking. Each process was repeated thrice for complete extraction after extraction; extracts were combined and evaporated to dryness in vacuo at $40^0$C using a rotary evaporator (Eqitron, Roteva). Acetone extract yield was represented as %.

#### 4.2.4.3 Methanolic extract

The rhizomes of *A. officinarum* were shade dried and powdered in hand mixer. About 500 g of powdered material was taken and extracted with cold percolation for 12 h in a 5L flat bottom flask at room temperature using methanol (3000 ml) as a solvent at room temperature for 7 days with occasional shaking. Each process was repeated thrice for complete extraction after extraction; extracts were combined and evaporated to dryness in vacuo at $40^0$C using a rotary evaporator (Eqitron, Roteva). Methanol extract yield was represented as %.

#### 4.2.4.4 Aqueous extract

The rhizomes of *A. officinarum* were shade dried and powdered in hand mixer. About 500 g of powdered material was taken and extracted with cold percolation for 12 h in a 5L flat bottom flask at room temperature using water (3000 ml) as a solvent at room temperature for 7 days with occasional shaking. Each process was repeated thrice for complete extraction after extraction; extracts were combined and evaporated to dryness in vacuo at $40^0$C using a rotary evaporator (Eqitron, Roteva). Aqueous extract yield was represented as %.

### 4.2.5 Preliminary phytochemical screening of *A. officinarum*

Preliminary phytochemical screening was carried out for alkaloids, glycosides, resins, tannins, saponins, carbohydrates, amino acids, phenols, terpenoids, coumarins and gums using standard procedures described to identify the constituents (Khandelwal, 2010).

### 4.2.6 Pharmacological studies of *A. officinarum*

#### 4.2.6.1 Chemicals and drugs

Freund's Complete Adjuvant (Sigma-Aldrich, St. Louis, MO, USA), carrageenan (Sigma-Aldrich, St. Louis, MO, USA), galangin (Merk, India), pentazocin (Ranbaxy, India), acetic acid (Pure Chem. Ltd., India), tween 80 (Research Lab, India) were purchased from respective vendors. Diclofenac was provided as a gift sample from Emcure Pharmaceutical Ltd., Pune. Biochemical diagnostic kits for aspartate aminotransferase, alanine aminotransferase, total protein and alkaline phophatase (Accurex biomedical Pvt. Ltd) were purchased from commercial sources.

#### 4.2.6.2 Apparatus

Microcapillary tubes, centrifuging tubes (Tarson, India), microtips (Tarson, India), test tubes, tissue paper, micropipettes were purchased from commercial source.

#### 4.2.6.3 Instruments used

Plethysmometer (Model: 7140, UGO Basile, Italy), plethysmometer (Model: 2888, Almemo, Germany), digital vernier caliper (Model: CD-6 CS, Mitutoyo Corp., Japan), thermal planter tester (Model: 37360, UGO Basile, Italy), Von Frey Hairs (Model: 2888, Almemo, Germany), hot plate (Model: DS-37, UGO Basile, Italy), UV/visible spectrophotometer (Model: Jasco V-530, Japan), eppendorff's cryocentrifuge machine (Model: 5810 R, Germany).

#### 4.2.6.4 Preparation of dosage form

Dosage forms of individual extracts were prepared as per the following procedures.

*Petroleum ether extract*:

The petroleum ether extract of *A. officinarum* was emulsified with tween 80 (2%) in a glass mortar with gradual addition of distilled water to make up the required volume.

*Aetone extract:*

The acetone extract of *A. officinarum* was emulsified with tween 80 (2%) in a glass mortar with gradual addition of distilled water to make up the required volume.

*Methanolic extract:*

The methanolic extract of *A. officinarum* was emulsified with tween 80 (2%) in a glass mortar with gradual addition of distilled water to make up the required volume.

*Aqueous extracts:*

Aqueous extract of *A. officinarum* was dissolved in distilled water to make up the required volume.

*Drugs:*

Accurately weighed quantity of diclofenac was suspended in distilled water to make volume.

*Vehicles:*

Vehicle was prepared by adding 2% tween 80 into distilled water, without addition of extracts or drugs.

#### 4.2.6.5 Storage conditions

All the dosage forms of extracts and drug solutions were prepared freshly on the day of experiment and stored in airtight amber colored vials to protect from exposure to sunlight during the experiments.

#### 4.2.6.6 Volume of extract solution

The volume of extract solutions was calculated based upon the body weight of animal.

#### 4.2.6.7 Route of administration

The extract solutions were administered orally.

#### 4.2.6.8 Experimental animals

Swiss albino female mice (20-25 g) and Wistar rats (170-220 g) of either sex were purchased from National Institute of Biosciences, Pune, India. Animals were housed in

an air-conditioned room at a temperature of 22 ± 2 °C and relative humidity of 45 to 55% under 12-h light: 12-h dark cycle. The animals had free access to food pellets (Pranav agro Pvt. Ltd., Sangli, India). Filtered water was provided *ad libitum*.

**4.2.6.9 Approval of research protocol**

The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) constituted in accordance with the rules and guidelines of the Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA), India (Protocol approval number was CPCSEA/2013/41).

**4.2.6.10 Acute oral toxicity study**

Healthy female Swiss albino mice of 20-25 g were used in acute toxicity studies as per OECD guidelines-425. The animals were fasted overnight and divided into groups with 5 mice in each group. Extracts of *A. officinarum* (petroleum ether, acetone, methanol and aqueous) were administered orally at one dose level of 2000 mg/kg body weight. The mice were observed continuously for behavioral and autonomic profiles for 2 hrs and for any sign of toxicity or mortality up to 48 hrs (OECD-425, 2001).

**4.2.7 Anti-inflammatory activity of four extracts of *A. officinarum***

**4.2.7.1 Carrageenan induced paw edema in rats**

Pedal inflammation in rat was produced according to the method of (Winter et al., 1962). Wistar rats of either sex weighing 175 to 200 g were used in this study. The rats were divided in 15 groups of six rats in each group as follows:

Treatment groups

Group 1 - Vehicle control (2% Tween 80, p.o.)

Group 2 – Diclofenac (10mg/kg, p.o)

Group 3, 4 and 5 – Petroleum ether extract 100, 200 and 400 mg/kg, p.o. respectively

Group 6, 7 and 8 – Acetone extract 100, 200 and 400 mg/kg, p.o. respectively

Group 9, 10 and 11 – Methanolic extract 100, 200 and 400 mg/kg, p.o. respectively

Group 12, 13 and 15 – Aqueous extract 100, 200 and 400 mg/kg, p.o. respectively

Procedure: The extracts and vehicle were administered orally to experimental animals once a day for a period of 7 days. One hour after the last administration, acute paw edema was induced by intradermal injection of 0.1 ml of 1% freshly prepared carrageenan suspension in normal saline into the subplanter region of the right hind paw of each rat.

The paw volume was measured before (0 h) and at intervals of 1, 2, 3, 4, 5, 6 and 24 h after carrageenan injection using plethysmometer (Model: 7140, UGO Basile, Italy). Then percentage of inhibition of edema was calculated for each group with respect to the control group as follows,

% Inhibition of paw edema = (VC-VT/VC) 100

VC and VT represent change in paw volume of control and drug treated animals respectively (Ishola et al., 2011; Bose et al., 2007; Wu et al., 2006).

**4.2.7.2 Cotton pellet granuloma in rats**

Subcutaneous implantation of pellets of compressed cotton provokes foreign body granuloma. Wistar rats of either sex weighing 175 to 200 g were used in this study. The rats were divided in 15 groups of six rats in each group as follows:

Treatment groups

Group 1 - Vehicle control (2% Tween 80, p.o.)

Group 2 – Diclofenac (10mg/kg, p.o)

Group 3, 4 and 5 – Petroleum ether extract 100, 200 and 400 mg/kg, p.o. respectively

Group 6, 7 and 8 – Acetone extract 100, 200 and 400 mg/kg, p.o. respectively

Group 9, 10 and 11 – Methanolic extract 100, 200 and 400 mg/kg, p.o. respectively

Group 12, 13 and 15 – Aqueous extract 100, 200 and 400 mg/kg, p.o. respectively

Procedure: Under aseptic precautions, an incision was made on the back of each rat in each group and sterile cotton pellets (50 ± 1 mg) were implanted subcutaneously in the axilla under ether anesthesia. On the seventh day, animals were sacrificed; pellets were removed along with the granulation tissue and pellets were dried at 60°C for 24 hrs. The net dry weight of the granuloma was determined. Stomach was isolated and cut open in greater curvature and subjected to histopathology (Smita et al., 2010; Ilavarasan et al., 2006).

**4.2.8 Anti-arthritic activity of methanolic extract of *A. galanga* (MEAO)**

**4.2.8.1 Freund's complete adjuvant induced arthritis**

Arthritis was induced by the indradermal injection of 0.1 ml of Freund's complete adjuvant (FCA, Sigma) in the subplanter region of the right hind paw on day 0. The rats were divided into six groups of six rats each as follows:

Treatment groups

Group 1 - Non-arthritic,vehicle control (2% Tween 80, p.o.)

Group 2 - Arthritic, vehicle control (2% Tween 80, p.o.)

Group 3 - Arthritic animals treated with standard (diclofenac 5 mg/kg, p.o.)

Group 4 - Arthritic animals treated with test drug (MEAO 100 mg/kg, p.o.)

Group 5 - Arthritic animals treated with test drug (MEAO 200 mg/kg, p.o.)

Group 6 - Arthritic animals treated with test drug (MEAO 400 mg/kg, p.o.)

The dosing of all the groups was started from day 12, once day (Ramadan et al., 2010).

The following parameters were measured at regular intervals (Day 0, 1, 4, 8, 12, 16, 20, 24 and 28). (A) Body weight, (B) paw volume, (C) Joint diameter, (D) Tactile allodynia and (E) Thermal hyperalgesia. On day 28, rats were anaesthetized and blood was withdrawn by retro-orbital puncture and used for biochemical assays. The rats were sacrificed on day 28 to study antioxidant parameters on liver and histology of ankle joint.

**4.2.9 Isolation of bioactive fraction from MEAO by bioassay guided fractionation method.**

**4.2.9.1 Chemicals and reagents**

Petroleum ether, acetone, methanol, benzene, chloroform, ethanol (Merck, India) of GR grades, column grade Silica (60-120#) (Spectrochem Pvt. Ltd. Mumbai, India) and TLC grade silica gel G (S.D. fine chem., Mumbai, India) were purchased from commercial sources.

**4.2.9.2 Apparatus and instruments**

Borosil glass column (height, 120 cm; diameter, 3 cm) and borosil glass column (height, 30 cm; diameter, 2 cm) was purchased from (Ajay Scientific enterprises), Pune, India. Precoated TLC silica gel plates (Merck, Kieselgel 60, F-254, 0.2 mm) were used for analytical TLC. LC-MS (Model: Micromass, Water U.K.), Infrared spectrophotometer (Model: JASCO, FT/IR-5300), NMR spectra (Model: Bruker, AV-500 MHz for $^{1}H$ and 125 MHz for $^{13}C$).

**4.2.9.3 Experimental animals**

Wistar rats (170-200 g) of either sex were purchased from National Institute of Biosciences, Pune, India. Animals were housed in an air-conditioned room at a temperature of 22 ± 2 $^{\circ}C$ and relative humidity of 45 to 55% under 12-h light: 12-h dark

cycle. The animals had free access to food pellets (Pranav agro Pvt. Ltd., Sangli, India). Filtered water was provided *ad libitum*.

#### 4.2.9.4 Approval of research protocol

The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) constituted in accordance with the rules and guidelines of the Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA), India (Protocol approval number: CPCSEA/2013/42).

#### 4.2.9.5 Column chromatography of methanolic extract of *A. officinarum* (MEAO)

##### 4.2.9.5.1 Liquid – solid separation chromatographic technique

20 g of methanolic extract of *A. officinarum* (MEAO) was mixed with 40 ml of methanol and 40 g of silica gel. Sample was mixed thoroughly and dried in oven at $110^0$C for 10 min. 100 ml of petroleum ether was added into the mixture and shake properly. Then the mixture was allowed to settle, two layers were formed. Upper layer was separated into the separate flask and labeled as Pet. Ether fraction. Same procedure was repeated twice with 100 ml of petroleum ether.

100 ml of (10% ethyl acetate in petroleum ether) was added into the residue and shake properly. Then the mixture was allowed to settle, two layers were formed. Upper layer was separated into the separate flask and labeled as 10% ethyl acetate fraction. Same procedure was repeated twice with 100 ml of 10% ethyl acetate in petroleum ether.

100 ml of (20% ethyl acetate in petroleum ether) was added into the residue and shake properly. Then the mixture was allowed to settle, two layers were formed. Upper layer was separated into the separate flask and labeled as 20% ethyl acetate fraction. Same procedure was repeated twice with 100 ml of 20% ethyl acetate in petroleum ether.

100 ml of (30% ethyl acetate in petroleum ether) was added into the residue and shake properly. Then the mixture was allowed to settle, two layers were formed. Upper layer was separated into the separate flask and labeled as 30% ethyl acetate fraction. Same procedure was repeated twice with 100 ml of 30% ethyl acetate in petroleum ether.

100 ml of (50% ethyl acetate in petroleum ether) was added into the residue and shake properly. Then the mixture was allowed to settle, two layers were formed. Upper layer was separated into the separate flask and labeled as 50% ethyl acetate fraction. Same procedure was repeated twice with 100 ml of 50% ethyl acetate in petroleum ether.

100 ml of ethyl acetate was added into the residue and shake properly. Then the mixture was allowed to settle, two layers were formed. Upper layer was separated into the separate flask and labeled as ethyl acetate fraction. Same procedure was repeated twice with 100 ml of ethyl acetate.

**4.2.9.5.2 Anti-inflammatory activity of 6 fractions from methanolic extract of *Alpinia officinarum* in carrageenan induced paw edema (Acute study)**

Different fractions from methanolic extract of *Alpinia officinarum* were labeled as n-hexane fraction, 10% ethyl acetate fraction, 20% ethyl acetate fraction, 30% ethyl acetate fraction, 50% ethyl acetate fraction and 100% ethyl acetate fraction. These fractions were further subjected to anti-inflammatory study. Wistar rats of either sex weighing 170-200 g were divided in following groups (n=6) viz;

Group I- Vehicle control (tween 80, 2%),

Group II- Diclofenac (10 mg/kg)

Group III- Petroleum ether fraction (100 mg/kg)

Group IV- 10% ethyl acetate fraction (100 mg/kg)

Group V- 20% ethyl acetate fraction (100 mg/kg)

Group VI- 30% ethyl acetate fraction (100 mg/kg)

Group VII- 50% ethyl acetate fraction (100 mg/kg)

Group VIII- 100% ethyl acetate fraction (100 mg/kg)

All fractions and diclofenac were administered orally 1 h before carrageenan injection. Paw volume was measured at 0, 1, 2, 3, 4, 5 and 6 h after carrageenan injection by plethysmometer (Model: 2888, Almemo, Germany). The data was presented as change in paw volume (ml).

**4.2.9.5.3 Column chromatography of 100% ethyl acetate fraction**

Column was packed with activated silica (60-120#). The 100% ethyl acetate fraction was loaded onto the packed silica gel column. After stabilization column was eluted with mobile phase. Fractions were collected and analyzed by TLC. Fractions showing similar bands were pooled together.

*Activation of silica*

Column grade silica (60-120#) was placed in oven at $110^0$C overnight (12 h) to remove the all moisture content present in it. Weighed quantity of activated silica was packed in the column.

*Preparation of mobile phase*

The solvents n-hexane and ethyl acetate were used for the preparation of mobile phase. The composition of mobile phase was n-hexane: ethyl acetate, with successive increase in percentage of ethyl acetate.

*Packing of column*

A clean and dry borosil glass column (height, 120 cm; diameter, 3 cm) was aligned in a vertical position with the help of clamps attached to metal stand. A piece of cotton soaked in mobile phase was placed at the bottom of the column and gently tapped down with a glass rod. The column was slowly and evenly filled to about 5/6 capacity with gradual addition of silica. Side of the chromatographic column was gently tapped with a cork during the packing process to compact silica column.

*Application of sample*

Weighed quantity (9.0 g) of the 100% ethyl acetate fraction was mixed with 20 ml of ethyl acetate and 20 g of activated silica to prepare slurry, ethyl acetate was evaporated from the slurry and the dried sample was added to top of the packed silica in column. A thin disc (column diameter) of cotton soaked in mobile phase was placed on top of the bed to prevent disturbing the sample layer after addition of mobile phase. Stopcock was opened to drain excess mobile phase until it reaches top of sample. Column was filled to the top with the mobile phase and allowed to stand overnight (~12 h) to develop a bands.

*Elution*

Elution was carried out by gravity at the flow rate of 2 ml/min. Mobile phase added to top of column was n-hexane: ethyl acetate. Polarity of mobile phase was increased by their polarity order and fractions (25 ml in each 50 ml tube) were collected in tarson tubes. Remaining loaded material in the column which cannot be eluted with mobile phase was eluted with methanol and collected as methanol fraction and completed column chromatography. All fractions were analyzed by TLC and fractions showing similar bands were pooled together and labeled as fraction I, II, III and IV respectively.

#### 4.2.9.5.4 Anti-inflammatory activity of 4 pools (I to IV) isolated from 100% ethyl acetate fraction in carrageenan induced paw edema (Acute study)

Pools from 100% ethyl acetate fraction were labeled as I to IV and subjected further for anti-inflammatory study. Wistar rats of either sex weighing 170-200 g were divided in following groups (n=6) viz;

Group I- Vehicle (tween 80, 2%)

Group II- Diclofenac (10 mg/kg)

Group III to Group VI- I to IV (10 mg/kg) respectively.

All fractions and diclofenac were administered orally 1 h before carrageenan injection. Paw volume was measured at 0, 1, 2, 3, 4, 5 and 6 h after carrageenan injection by plethysmometer (Model: 2888, Almemo, Germany). The data was presented as change in paw volume (ml).

#### 4.2.9.5.5 Statistical analysis

The data of pharmacological experiments were expressed as mean ± standard error mean (SEM). Data analysis was performed using Graph Pad Prism 5.0 software (Graph Pad, San Diego, CA, USA). Data of body weight, paw volume, joint diameter, tactile allodynia and thermal hyperalgesia were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Data of hematological and biochemical parameters were analyzed using one way analysis of variance (ANOVA) followed by Dunnett's test. A value of $P<0.05$ was considered to be statistically significant.

### 4.2.10 Anti-inflammatory activity of bioactive fraction (III)

#### 4.2.10.1 Acute oral toxicity study

Healthy female Swiss albino mice of 20-25 g were used in acute toxicity studies as per OECD guidelines-425. The animals were fasted overnight and divided into groups with 5 mice in each group. Bioactive fraction (III) was administered orally at one dose level of 20 mg/kg body weight. The mice were observed continuously for behavioral and autonomic profiles for 2 hrs and for any sign of toxicity or mortality up to 48 hrs (OECD-425, 2001).

#### 4.2.10.2 Carrageenan induced paw edema in rats

Pedal inflammation in rat was produced according to the method of (Winter et al., 1962). Wistar rats of either sex weighing 175 to 200 g were used in this study.

Treatment groups

Group 1 - Vehicle control (2% Tween 80, p.o.)

Group 2 – Diclofenac (10mg/kg, p.o)

Group 3, 4 and 5 – Bioactive fraction (III) 5, 10 and 20 mg/kg, p.o. respectively

Procedure: The bioactive fraction (III) and vehicle were administered orally to experimental animals once a day for a period of 7 days. One hour after the last administration, acute paw edema was induced by intradermal injection of 0.1 ml of 1% freshly prepared carrageenan suspension in normal saline into the subplanter region of the right hind paw of each rat. The paw volume was measured before (0 h) and at intervals of 1, 2, 3, 4, 5, 6 and 24 h after carrageenan injection using plethysmometer (Model: 7140, UGO Basile, Italy). Then percentage of inhibition of edema was calculated for each group with respect to the control group as follows,

% Inhibition of paw edema = (VC-VT/VC) 100

VC and VT represent change in paw volume of control and drug treated animals respectively (Ishola et al., 2011; Bose et al., 2007; Wu et al., 2006).

**4.2.10.3 Cotton pellet granuloma in rats**

Subcutaneous implantation of pellets of compressed cotton provokes foreign body granuloma. Wistar rats of either sex weighing 175 to 200 g were used in this study.

Treatment groups

Group 1 - Vehicle control (2% Tween 80, p.o.)

Group 2 – Diclofenac (10mg/kg, p.o)

Group 3, 4 and 5 – Bioactive fraction (III) 5, 10 and 20 mg/kg, p.o. respectively

Procedure: Under aseptic precautions, an incision was made on the back of each rat in each group and sterile cotton pellets (50 ± 1 mg) were implanted subcutaneously in the axilla under ether anesthesia. On the seventh day, animals were sacrificed; pellets were removed along with the granulation tissue and pellets were dried at 60°C for 24 hrs. The net dry weight of the granuloma was determined. Stomach was isolated and cut open in greater curvature and subjected to histopathology (Smita et al., 2010; Ilavarasan et al., 2006).

### 4.2.11 Anti-arthritic studies of isolated bioactive fraction (III)

#### 4.2.11.1 Freund's complete adjuvant induced arthritis

Arthritis was induced by the indradermal injection of 0.1 ml of Freund's complete adjuvant (FCA, Sigma) in the subplanter region of the right hind paw on day 0. The rats were divided into six groups of six rats each as follows:

Treatment groups

Group 1 - Non-arthritic,vehicle control (2% Tween 80, p.o.)

Group 2 - Arthritic, vehicle control (2% Tween 80, p.o.)

Group 3 - Arthritic animals treated with standard (diclofenac 5 mg/kg, p.o.)

Group 4 - Arthritic animals treated with test drug [Fraction (III) 5 mg/kg, p.o.]

Group 5 - Arthritic animals treated with test drug [Fraction (III) 10 mg/kg, p.o.]

Group 6 - Arthritic animals treated with test drug [Fraction (III) 20 mg/kg, p.o.]

The dosing of all the groups was started from day 12, once day (Ramadan et al., 2010).

The following parameters were measured at regular intervals (Day 0, 1, 4, 8, 12, 16, 20, 24 and 28). (A) Body weight, (B) paw volume, (C) Joint diameter, (D) Tactile allodynia and (E) Thermal hyperalgesia. On day 28, rats were anaesthetized and blood was withdrawn by retro-orbital puncture and used for biochemical assays. The rats were sacrificed on day 28 to study antioxidant parameters on liver and histology of ankle joint.

### 4.2.12 Preparative TLC of pool P 3 (III)

*Application of sample*

The sample P3 (III) was applied by streaking across the full length of the plate by glass capillary.

*Mobile phase*

The solvents hexane, ethyl acetate and acetic acid were used for the preparation of mobile phase. The composition of mobile phase was hexane:ethylacetate:acetic acid (7.5:2:0.5).

*Preconditioning of chamber (saturation)*

Chromatogram was developed in a saturated twin trough chambers. To achieve saturation, at least half of the total area of the inside the wall of the chamber was lined with filter paper. A sufficient quantity (approximately 10 ml) of mobile phase was poured along the side of filter paper into the chamber to saturate the chamber and form a layer

about 5 mm deep. Chamber was then closed and allowed to stand for at least 30 min at room temperature.

*Development of chromatogram*

The plate was marked 10 mm below the upper edge. Plate was placed vertically into the chamber ensuring that the points of application were above the surface of the mobile phase. Chamber was closed and mobile phase was allowed to ascend to the specific distance at the room temperature. Plate was removed; the position of mobile phase front was marked. Mobile phase was allowed to evaporate at room temperature and dried under hot air.

*Observation and elution of compound*

Chromatogram was observed in daylight, under ultra violate (UV) light at 254 and 366 nm wavelength. Area was marked and scrapped off with sharp blade, about 10% additional area was then marked to compensate for three-dimensional development of band in the layer. With minimum volume of mobile phase the components from scrapped material was eluted. Scrapped material and mobile phase were homogenized in vortex mixer to ensure complete elution, centrifuged; supernatant was collected and allowed to evaporate.

### 4.2.13 Characterization of bioactive fraction (III)

The chemical structure of isolated compound was elucidated by LC-MS, IR, $^{1}$H-NMR, $^{13}$C-NMR, and DEPT spectroscopy.

**Part III**

**4.3 *A. purpurata***

**4.3.1 Plant material**

Authenticated healthy plant material of *Alpinia purpurata* was collected from the Jawaharlal Nehru Tropical Botanic & Research Institute, Palode, Thiruvananthapuram.

**4.3.2 Biotechnological studies**

**4.3.2.1 Surface sterilization of explants**

Rhizomes of *Alpinia purpurata* were washed thoroughly under running tap water for 30 min, followed by dettol soap solution (10%) for 2 min, then treated with dettol (10% liquid) for 2 min. Explants were rinsed thoroughly with distilled water to remove the traces of soap and disinfectant. Under Laminar airflow, these explants were treated with 70% alcohol for 3-5 min, rinsed with 3-4 times with sterile distill water, then washed with freshly prepared 0.1% mercuric chloride solution in sterile distilled water 2-4 min. Finally, the explants were washed 4-5 times with sterile distilled water to give surface sterilized explants.

**4.3.2.2 Preparation of explants**

The surface sterilized rhizomes were transferred on sterile petri-dish and the upper part of rhizomes was removed with the help of sterile forceps and scalpel. The rhizomes were then excised (approx. 10 mm x 10 mm) sections under laminar air flow. These small pieces were ready to be transferred in pre-sterilized culture medium.

**4.3.2.3 Inoculation and media preparation**

The explants were inoculated on Murashige and Skoog's (MS) medium supplemented with different concentrations of NAA, IAA, BA, 2, 4-D, coconut water, marketed sugar, cornflower and kinetin. The pH of media was adjusted to 5.6-5.8 with the help of 0.1N NaOH and 0.1N HCl prior to gelling with 0.8% agar, dispensed (15 ml) into culture tubes and sterilized by autoclaving (121$^{\circ}$C at 15 psi for 15 min).

**4.3.2.4 Incubation of cultures**

The cultures were maintained in culture room under a regime of 16 hr photoperiod (intensity 40μE cm2 /min/sec) at 25 ± 1$^{\circ}$C

#### 4.3.2.5 Subculture

The callus cultures were maintained on medium of same composition and same culture conditions by sub-culturing every three weeks for the development of friable callus.

### 4.3.3 Micropropogation

#### 4.3.3.1 Shoot multiplication

The callus was transferred in solid and liquid basal MS medium supplemented with different concentration of (NAA) and (BA) was used to establish shoot cultures. Media was adjusted to pH 5.7 before autoclaving. Cultures were incubated under the same culture conditions as used for callus initiation. After 42 days, the shoots were measured for growth and subsequently subculture. For each treatment 20 replicates were used and each experiment was repeated at least three times.

#### 4.3.3.2 Root induction

Isolated shoots derived from media containing 3 ppm of (NAA) and 0.1 ppm of (NAA) was then placed on rooting medium, $M_1$ and $M_2$. Media $M_1$ and $M_2$ contain different concentration of IAA. The effect of different concentration of IAA (0-4 ppm) on root proliferation was also studied.

#### 4.3.3.3 Prehardening

At the end of $42^{nd}$ day, the rooted plantlets were removed from culture media and thoroughly washed with luke warm tap water to remove adhered medium. The plantlets were subjected for prehardening in various substrates like autoclaved soil, sand and vermiculite mixture (1:1:1) in 4"× 6" polythene bags and incubated at 25±1°C under 90-100% relative humidity for 7 weeks under aseptic conditions at 16 h light and 8 h dark photoperiod. Plantlets were irrigated with ¼ MS medium (10-15 ml) without sucrose and vitamin every day. Moisture was maintained with tap water spraying three times after every 2 h and plantlets were covered with transparent thin polythene bags. Uncovering the poly bags gradually increased exposure time to external atmosphere with 30 minutes for first eight days and 2 h at the end of $22^{nd}$ day. On $23^{rd}$ day onwards, plantlets were exposed to outside culture room, with temperature of 25±1°C and changed photoperiod (14 h light and10 h dark). Plantlets were irrigated with tap water and relative humidity (90-100%) was maintained with tap water spraying three times after every 2 h for $42^{nd}$ day.

**Part IV**

## 4.4 Analytical studies

### 4.4.1 Development of HPTLC protocol for the active constituents from *A. galanga* and *A. officinarum*

#### 4.4.1.1 Apparatus

The spotting device used was Linomat IV automatic sample spotter (Camag, Muttenz, Switzerland); the syringe was 100 µL (Hamilton Bonaduz, Switzerland); The TLC chamber was a glass twin trough chamber 20 × 10 × 4 cm (Camag, Switzerland); the densitometer was a TLC Scanner 3 linked to WINCATS software (Camag, Switzerland); The HPTLC plates of 20 × 10 cm, 0.2 mm thickness, precoated with silica gel 60F254 (E. Merck Kga A, Cat. no. 1.05548, Darmstadt, Germany) were used.

#### 4.4.1.2 Preparation of standard solution of galangin

Stock solution of galangin was prepared by dissolving 10 mg of accurately weighed galangin in 10 mL methanol. From this stock solution, standard solutions of 100 ppm/mL were prepared in 10 mL volumetric flasks and adjusting the volume with methanol.

#### 4.4.1.3 Preparation of sample solution

**(A) *Alpinia galanga***

The dried extracts of *A. galanga* (10 mg) were transferred to 10 mL volumetric flask and the volume was made upto 10 mL with methanol to furnish the final concentration 100 µg/mL.

**(B) *Alpinia officinarum***

The dried extracts of *Alpinia officinarum* (10 mg) were transferred to 10 mL volumetric flask and the volume was made upto 10 mL with methanol to furnish the final concentration 100 µg/mL.

#### 4.4.1.4 Calibration curve for galangin

The standard solution of galangin was applied in triplicate on a HPTLC plate (20×10 mm) in 2,4,8,12,16 and 20 µL quantity. The plate was developed in a solvent system hexane-ethylacetate-acetic acid (7.5:2:0.5 v/v) at 25±2ºC temperature and 40% relative humidity up to a distance of 8 cm. After development, the plate was dried in air and scanned at 254 nm wavelength. The peak area was recorded. Calibration curve was prepared by plotting peak area vs. concentration.

#### 4.4.1.5 Validation of the method

The proposed method was validated as per the recommendations laid down by International Conference on Harmonization (ICH) guidelines (ICH Q2A, 1994; ICH Q2B, 1996).

##### 4.4.1.5.1 Precision

Instrumental precision was checked by repeated scanning (n = 6) of the same spot of galangin (240 ng/spot) and was expressed as coefficient of variance (% CV) of the peak areas. Variability of the method was studied by analyzing aliquots of standard solution of galangin (240, 320, 400 ng/spot) on the same day (intraday precision) and on different days (interday precision) and the results were expressed as % CV.

##### 4.4.1.5.2 Repeatability

The repeatability of the method was affirmed by analyzing 240 ng/spot of standard solution of galangin after application on the HPTLC plate (n = 6) and analyzing them as described in the preparation of calibration plot, which was expressed as % CV.

##### 4.4.1.5.3 Robustness

Mobile phases having different composition like hexane-ethyl acetate-acetic acid (7.5:2:0.5 v/v), (7.0:2.5:0.5 v/v), (8.0:1.5:0.5 v/v) etc., were tried and chromatograms were run. The amount of mobile phase, temperature and relative humidity was varied in the range of ± 5%. The plates were pre washed by methanol and activated at 60ºC for 5, 10 and 15 min respectively. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 30, 60 and 90 min. Robustness of the method was studied at three different concentration levels: 240, 320, 400 ng/spot for galangin.

##### 4.4.1.5.4 Specificity

The specificity of the method was ascertain by analysis of standard and samples. The identities of the bands of galangin in the chromatogram obtained from the samples were confirmed by comparison of Rf values and spectra of the band with standard. The peak purity was assessed by comparing the standard and sample spectra acquired at the peak start (S), peak apex (M) and peak end (E) of the bands.

#### 4.4.1.5.5 Accuracy

Accuracy of the method was tested by performing recovery studies at three levels (80%, 100% and 120%). The percent recovery as well as average percent recovery was calculated.

#### 4.4.1.5.6 Limit of detection and Limit of quantification

LOD represents the lowest concentrations of galangin that can be detected, whereas the LOQ represents the lowest concentrations of galangin that can be determined with acceptable precision and accuracy. LOD and LOQ were determined on the basis of signal-to-noise (S/N) ratio. The known concentrations of standard solution of galangin were diluted and applied along with methanol as blank until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

### 4.4.2 High performance thin layer chromatography (HPTLC) for *A. purpurata* (Vieillard) K. Schumann

#### 4.4.2.1 Apparatus

The spotting device used was Linomat IV automatic sample spotter (Camag, Muttenz, Switzerland); the syringe was 100 µL (Hamilton Bonaduz, Switzerland); The TLC chamber was a glass twin trough chamber 20 × 10 × 4 cm (Camag, Switzerland); the densitometer was a TLC Scanner 3 linked to WINCATS software (Camag, Switzerland); The HPTLC plates of 20 × 10 cm, 0.2 mm thickness, precoated with silica gel 60F254 (E. Merck Kga A, Cat. no. 1.05548, Darmstadt, Germany) were used.

#### 4.4.2.2 Preparation of standard solution of rutin and quercetin

Stock solution of rutin and quercetin was prepared by dissolving 10 mg of accurately weighed rutin and quercetin in 10 mL methanol. From this stock solution, standard solutions of 100 ppm/mL were prepared in 10 mL volumetric flasks and adjusting the volume with methanol.

#### 4.4.2.3 Preparation of sample solution of *Alpinia purpurata*

The hexane, ethyl acetate and methanolic leaves extracts of *Alpinia purpurata* (1 mg) were transferred in1 mL methanol to furnish the final concentration 100 µg/mL.

#### 4.4.2.4 Calibration curve for rutin and quercetin

The standard solution of rutin and quercetin was applied in triplicate on a HPTLC plate (20×10 mm) in 2, 4, 8, 12, 16 and 20 µL quantity. The plate was developed in a solvent system tolune-acetone-formic acid (4.5:4.5:1 v/v) at 25±2ºC temperature and 40% relative humidity up to a distance of 8 cm. After development, the plate was dried in air and scanned at 254 nm wavelength. The peak area was recorded. Calibration curve was prepared by plotting peak area vs. concentration.

#### 4.4.2.5 Validation of the method

The proposed method was validated as per the recommendations laid down by International Conference on Harmonization (ICH) guidelines (ICH Q2A, 1994; ICH Q2B, 1996).

##### 4.4.2.5.1 Precision

Instrumental precision was checked by repeated scanning (n = 6) of the same spot of rutin and quercetin (240 ng/spot) and was expressed as coefficient of variance (% CV) of the peak areas. Variability of the method was studied by analyzing aliquots of standard solution of rutin and quercetin (240, 320, 400 ng/spot) on the same day (intraday precision) and on different days (interday precision) and the results were expressed as % CV.

##### 4.4.2.5.2 Repeatability

The repeatability of the method was affirmed by analyzing 240 ng/spot of standard solution of rutin and quercetin after application on the HPTLC plate (n = 6) and analyzing them as described in the preparation of calibration plot, which was expressed as % CV.

##### 4.4.2.5.3 Robustness

Mobile phases having different composition like tolune-acetone-formic acid (4.5:4.5:1 v/v), (4:5:1 v/v), (4.5:4:0.5 v/v) etc., were tried and chromatograms were run. The amount of mobile phase, temperature and relative humidity was varied in the range of ± 5%. The plates were pre washed by methanol and activated at 60ºC for 5, 10 and 15 min respectively. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 30, 60 and 90 min. Robustness of the method was studied at three different concentration levels: 240, 320, 400 ng/spot for rutin and quercetin.

#### 4.4.2.5.4 Specificity

The specificity of the method was ascertained by analysis of standard and samples. The identities of the bands of rutin and quercetin in the chromatogram obtained from the samples were confirmed by comparison of Rf values and spectra of the band with standard. The peak purity was assessed by comparing the standard and sample spectra acquired at the peak start (S), peak apex (M) and peak end (E) of the bands.

#### 4.4.2.5.5 Accuracy

Accuracy of the method was tested by performing recovery studies at three levels (80%, 100% and 120%). The percent recovery as well as average percent recovery was calculated.

#### 4.4.2.5.6 Limit of detection and Limit of quantification

LOD represents the lowest concentrations of rutin and quercetin that can be detected, whereas the LOQ represents the lowest concentrations of rutin and quercetin that can be determined with acceptable precision and accuracy. LOD and LOQ were determined on the basis of signal-to-noise (S/N) ratio. The known concentrations of standard solution of rutin and quercetin were diluted and applied along with methanol as blank until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

#### 4.4.2.5.7 Quantitation of rutin and quercetin from methanolic extract of *in vitro* grown plant leaves of *Alpinia purpurata*.

Culture grown material of *Alpinia purpurata* leaves sample solution (10 µL of 100 µg/mL concentrations) was applied in triplicate on a precoated silica gel 60F254 HPTLC plate (0.2 mm thickness). The plates were developed and scanned at 254 nm wavelength. The peak area and absorption spectra were recorded. Overlaying the absorption spectra at the start, middle and end positions of the bands checked the purity of the bands in the sample extract. The amount of rutin and quercetin in the samples were calculated using the linear regression equation derived from the calibration curves.

# RESULTS

# 5. RESULTS

## PART-I

## 5.1 *A. galanga*

### 5.1.1 Pharmacognostic studies

#### 5.1.1.1 Macroscopic studies

Rhizomes of *A. galanga* are branched, cylindrical, 2 to 8 cm in diameter, longitudinally grooved with prominent rounded warts marked with fine annulations; scaly leaves set circularly. Externally reddish- brown and internally orange yellow in color, fracture hard and fibrous, fractured surface coarse; odour pleasant and aromatic, taste spicy and sweet.

#### 5.1.1.2 Microscopic studies

##### 5.1.1.2.1 TS of rhizome

Transverse section of rhizome in *A. galanga* shows an external cortical region and an inner stelar region. The outer cortex is 1 to 1.1 cm thick and the central stele is 1.2 cm in diameter. Epidermis is made up of a single row of tangentially elongated cells. In the cortex region residual part of the outer zone consists of several scattered vascular bundles and every bundle is surrounded by lignified layer. Vascular bundles are almost circular in shape and consist of groups of xylem elements and small patch of phloem. Xylem consists of parenchyma, vessels, tracheids & fibers. Phloem is seen over the xylem and consists of sieve elements, companion cells. The cells of the cortex are thin walled, multilateral and arranged with small intercellular spaces. Cortical and stellar region are demarcated by a continuous ring of endodermoidal layer. The stellar region is made up of thin walled parenchymatous cells with abundant vascular bundles. Small vascular bundles are radially arranged, xylem elements are present below this layer. These parenchymatous cells are slightly smaller than that of the cortical region and polygonal to circular in shape and closely arranged with small intercellular spaces. Smaller bundles are towards the peripheral region and bigger ones occupy the central part. Oleoresin cells are present close to each bundle. Sclerenchymatous sheath moderately encircling the bundle is less thickened. Starch grains are simple, elongated and are additional in number. Numerous oleoresin cells are scattered all over the rhizome.

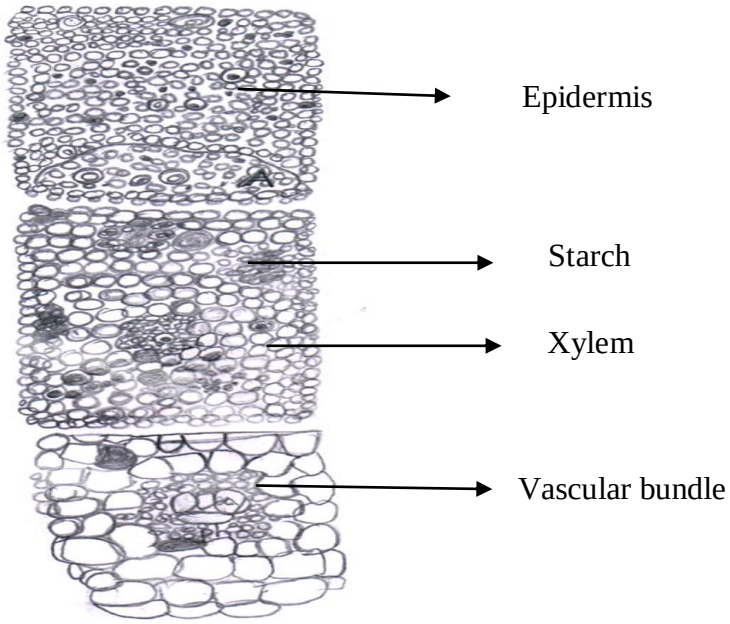



**Fig. 5.1.1.2.1: T. S. of rhizome of *A. galanga***

### 5.1.1.3 Powder microscopy

Powdered microscopy of the rhizomes revealed the presence of fragments of epidermal cells in exterior view, parenchymatous cells with yellow colouring matter and starch grains. Vessels are wide walled, elongated having scalariform or reticulate thickening with simple pits. Starch grains show different in size and shape, few are circular. The majority are elongated rod shaped with rounded wide end. Tracheids are also observed.

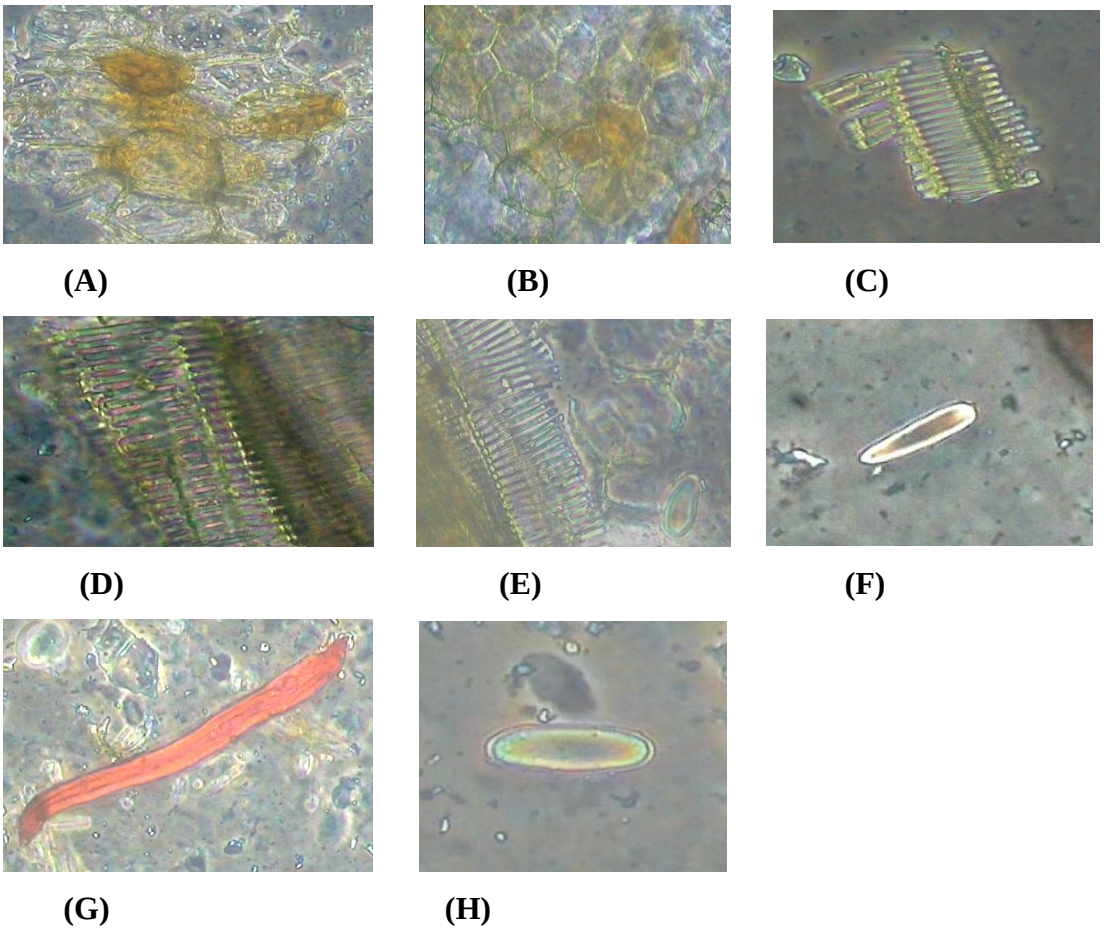



**Fig. 5.1.1.3: Powder microscopy of *A. galanga*: (A) Epidermal cells, (B) Parenchyma cells with starch grains, (C and D) Vessels with scalariform thickening, (E) Vessels, (F) Starch grains, (G) Trachied, (H) Starch grains**

### 5.1.1.4 Physicochemical analysis

#### 5.1.1.4.1 Ash value

The residue left behind after incineration of plant material is the ash content or ash value, which basically represents inorganic salts, naturally occurring in crude drug or adhering to it or purposely added to it as a form of adulteration. The percentage of active chemical constituents in crude drugs is mentioned on air-dried basis. Therefore, the loss on drying of plant materials should be determined and the water content should also be controlled. This is especially important for materials that take up moisture easily or deteriorate quickly in presence of water. The test for loss on drying determines both water and volatile matter. The acid insoluble ash consist mainly silica and indicate the presence of contaminate with

earthy material. The water soluble ash is used to determine the amount of inorganic elements present in drugs.

**Table 5.1.1.4.1: Physicochemical parameters of *A. galanga* rhizome.**

| Sr. no. | Parameters | Values (%) |
|---|---|---|
| 1. | Total Ash | 10.2 |
| 2. | Acid insoluble ash | 4.1 |
| 3. | Water soluble ash | 5.3 |
| 4. | Loss on drying at110°C | 11 |

### 5.1.1.4.2 Extractive values

The extractive values are helpful to evaluate the chemical constituents present in the crude drug and also help in estimation of particular constituents soluble in a particulars solvent.

**Table 5.1.1.4.2: Extractive values of *A. galanga* rhizome.**

| S. No. | Solvents | Extractive values |
|---|---|---|
| 1. | Pet.-ether | 3.47 |
| 2. | Acetone | 1.48 |
| 3. | Methanol | 4.7 |
| 4. | Water | 12.50 |

### 5.1.1.5 Preliminary phytochemical studies

Preliminary phytochemical screening was useful in prediction of nature of drugs and also useful for the finding of different constituents present in different polarity solvent. So it could be helpful to remove out particular constituents by solvent. The qualitative phytochemical analysis indicated that the petroleum ether extract contained fats and oils, volatile oil and alkaloids. The alcoholic extract contained steroids, volatile oil, saponins, glycosides, flavonoids, tannins, phenolic compounds and triterpenoids. The aqueous extract contained carbohydrates, proteins, amino acids, glycosides, alkaloids, tannins and phenolic compounds.

**Table 5.1.1.5: Phytochemical analysis of various extracts of *A. galanga* rhizome.**

| Sr. no. | Chemical Test | Observations of different extracts of *A. galanga* | | | |
|---|---|---|---|---|---|
| | | **Pet. ether** | **Acetone** | **Methanol** | **Aqueous** |
| Tests for Carbohydrates | | | | | |
| 1. | Molisch's test | - | - | - | - |
| Tests for Proteins | | | | | |
| 2. | Biuret test | - | - | + | + |
| 3. | Millon's test | - | - | - | + |
| Tests for Steroids | | | | | |
| 4. | Salkowski reaction | - | - | + | - |
| 5. | Liebermann-Burchard reaction | - | - | - | - |
| Test for Volatile oils | | | | | |
| 6. | Solubility test | + | - | - | - |
| Tests for Glycosides | | | | | |
| 7. | Keller-Killani test | - | - | - | - |
| 8. | Borntrager's test | - | - | - | - |
| Tests for Saponins | | | | | |
| 9. | Foam test | - | - | - | - |
| Test for Tannins and Phenolic compounds | | | | | |
| 10. | 5%Ferric chloride (3 ml) | - | - | - | + |
| 11. | Lead acetate | - | + | + | + |
| 12. | Potassium permanganate | - | + | + | + |
| Test for presence of Flavonoids | | | | | |
| 13. | Shinoda test | - | - | - | + |
| Tests for Alkaloids | | | | | |
| 14. | Dragendorff's test | + | + | + | + |
| 15. | Mayer's test | - | - | - | + |
| 16. | Wagner's test | - | + | - | + |
| 17. | Hager's test | - | - | - | + |

(+Present;-Absent)

### 5.1.1.6 Morphological characteristics

All the extracts are brown to brownish yellow in colour and showed semisolid consistency while aqueous extract is brown in colour and showed solid consistency.

**Table 5.1.1.6: Table shows morphological characterization of rhizome extracts of *A. galanga*.**

| Sr. No. | Extracts | Colour | Consistency |
|---|---|---|---|
| 1. | Pet.ether | Brownish yellow | Semisolid |
| 2. | Acetone | Brownish yellow | Semisolid |
| 3. | Methanol | Brownish yellow | Semisolid |
| 4. | Water | Brown | Solid |

### 5.1.1.7 Fluorescence analysis of rhizome extracts of *A. galanga*.

**Table 5.1.1.7: Table shows fluorescence analysis of rhizome extracts of *A. galanga*.**

| Sr. No. | Extracts | Visible light | Short UV light | Long UV light |
|---|---|---|---|---|
| **1.** | Pet.ether | Yellowish Brown | Emerald Green | Brown |
| **2.** | Acetone | Brownish yellow | Yellowish green | Pale Green |
| **3.** | Methanol | Brownish yellow | Yellowish Brown | Pale Green |
| **4.** | Water | Brown | Pale Green | Reddish Orange |

### 5.1.2 PHARMACOLOGICAL STUDY

#### 5.1.2.1 Acute oral toxicity test (AOT) of *A. galanga*

Administration of 2000 mg/kg of the four extracts of *A. galanga* did not produce any behavioral abnormalities and mortality (Table 5.1.2.1). So the dose selected for further study was 100, 200 and 400 mg/kg for each extracts.

**Table 5.1.2.1: Acute toxicity test of *A. galanga***

| Sr. No. | Extracts<br>2000 mg/kg p.o. | No. of animals dead/survived |
|---|---|---|
| 1. | Pet.ether extract of *A. galanga* | 0/5 |
| 2. | Acetone extract of *A. galanga* | 0/5 |
| 3. | Methanolic extract of *A. galanga* | 0/5 |
| 4. | Aqueous extract of *A. galanga* | 0/5 |

#### 5.1.2.2 Anti-inflammatory activity

##### 5.1.2.2.1 Carrageenan induced inflammation in rats

Intradermal injection of carrageenan into subplantar region of right hind paw caused significant increase in the paw edema, which was recorded as change in paw volume at $3^{rd}$ h 3.00± 0.087 ml and at $5^{th}$ h 3.84± 0.060 ml. Diclofenac (10 mg/kg) was administered 1 h before the injection of carrageenan caused significant ($P<0.01$ and $P<0.001$) inhibition of increase in paw edema at $3^{rd}$ and $5^{th}$ h respectively. The inhibitory effect of the diclofenac was recorded (5.32%) at $3^{rd}$ h and (26.01%) at $5^{th}$ h. All four extracts of *A. galanga* was administered for 7 days before the injection of carrageenan. The inhibitory effect of the pet.ether extract of *A. galanga* was recorded with a dose of 200 and 400 mg/kg at $5^{th}$ h (0.80%) and (6.90%). The inhibitory effect of the acetone extract of *A. galanga* was recorded with a dose of 200 and 400 mg/kg at $3^{rd}$ h (4.91%), (5.49%) and at $5^{th}$ h (15.21%), (24.84%) respectively. The inhibitory effect of the methanolic extract of *A. galanga* was recorded with a dose of 200 and 400 mg/kg at $5^{th}$ h (2.75%) and (2.75%). The inhibitory effect of the aqueous extract of *A. galanga* was recorded with a dose of 200 and 400 mg/kg at $5^{th}$ h (6.82%) and (10.27%). The inhibition elicited by the acetone extract of *A. galanga* (AEAG) was comparable to that of diclofenac. (Table 5.1.2.2.1)

**Table 5.1.2.2.1: Effect of oral administration of four extract of *A. galanga* on inhibition of right hind paws edema on carrageenan induced inflammation in rats**

| Treatment Groups | Change in paw edema volume (ml) | | | % Inhibition at | | |
|---|---|---|---|---|---|---|
| | 1 h | 3 h | 5 h | 1 h | 3 h | 5 h |
| Carrageenan control | 1.04 ± 0.148 | 3.00± 0.087 | 3.84± 0.060 | -- | -- | -- |
| Diclofenac 10 mg/kg | 1.01 ± 0.049 | 2.84± 0.088 | 2.84± 0.094*** | 2.88 | 5.32 | 26.01 |
| Pet.ether ext. of *A. galanga* 100 mg/kg | 1.10 ±0.005 | 3.09 ± 0.035 | 4.06 ± 0.113 | 0.90 | -1.09 | -3.09 |
| Pet.ether ext. of *A. galanga* 200 mg/kg | 1.09 ± 0.015 | 3.09 ± 0.044 | 3.91 ± 0.048 | 1.06 | -0.98 | 0.80 |
| Pet.ether ext. of *A. galanga* 400 mg/kg | 1.08 ± 0.010 | 3.05 ± 0.045 | 3.67 ± 0.071* | 1.96 | 0.27 | 6.90 |
| Acetone ext. of *A. galanga* 100 mg/kg | 1.04 ± 0.144 | 2.92 ± 0.081 | 3.36 ± 0.080 | 0.24 | 2.58 | 12.42 |
| Acetone ext. of *A. galanga* 200 mg/kg | 1.03± 0.162 | 2.85± 0.085 | 3.26± 0.246** | 0.72 | 4.91 | 15.21 |
| Acetone ext. of *A. galanga* 400 mg/kg | 1.02± 0.062 | 2.84±0. 116 | 2.89± 0.071*** | 2.16 | 5.49 | 24.84 |
| Methanolic ext. of *A. galanga* 100 mg/kg | 1.08 ±0.013 | 3.18 ± 0.042 | 3.83 ± 0.076 | 2.71 | -3.98 | 2.75 |
| Methanolic ext. of *A. galanga* 200 mg/kg | 1.08 ± 0.011 | 3.09 ± 0.047 | 3.83 ± 0.023 | 2.26 | -1.25 | 2.75 |
| Methanolic ext. of *A. galanga* 400 mg/kg | 1.07 ± 0.018 | 3.08 ± 0.051 | 3.83 ± 0.055 | 3.32 | -0.87 | 2.75 |
| Aqueous ext. of *A. galanga* 100 mg/kg | 1.08 ± 0.079 | 2.92 ± 0.081 | 3.75 ± 0.011 | 3.35 | 2.57 | 2.47 |
| Aqueous ext. of *A. galanga* 200 mg/kg | 1.03 ± 0.16 | 2.89 ± 0.093 | 3.71 ± 0.10 | 0.71 | 3.57 | 3.51 |
| Aqueous ext. of *A. galanga* 400 mg/kg | 1.02 ± 0.062 | 2.80 ± 0.093 | 3.45 ± 0.091 | 2.15 | 6.82 | 10.27 |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with carrageenan control $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

### 5.1.2.2.2 Cotton pellet granuloma in rats

Subcutaneous implantation of 50 mg of cotton pellet in rats caused significant increase in weight of cotton pellet, recorded as 88.5 ± 2.93 mg which was due to granuloma formation. Diclofenac (10 mg/kg) and acetone extract of *A. galanga* (400 mg/kg) significantly (P<0.001) inhibited the granuloma formation by 62.71 and 54.80.

**Table 5.1.2.2.2: Effect of oral administration of four extract of *A. galanga* on cotton pellet granuloma in rats**

| Sr. No. | Treatment Groups | Dose mg/kg p.o. | Dry granuloma weight (mg) (Mean ± SEM) | % Inhibition |
|---|---|---|---|---|
| 1. | Vehicle control | | 88.5 ± 2.93 | |
| 2. | Diclofenac | 10 | 33 ± 2.86*** | 62.71186 |
| 3. | Pet.ether extract of *A. galanga* | 100 | 92 ± 3.3 | 2.21 |
| 4. | Pet.ether extract of *A. galanga* | 200 | 87 ± 2.32 | 5.43 |
| 5. | Pet.ether extract of *A. galanga* | 400 | 82 ± 2.99* | 10.87 |
| 6. | Acetone extract of *A. galanga* | 100 | 82 ± 1.86 | 7.344633 |
| 7. | Acetone extract of *A. galanga* | 200 | 78.3 ± 1.9* | 11.49153 |
| 8. | Acetone extract of *A. galanga* | 400 | 40 ± 1.93*** | 54.80226 |
| 9. | Methanolic extract of *A. galanga* | 100 | 96 ± 2.5 | -4.35 |
| 10. | Methanolic extract of *A. galanga* | 200 | 87.2 ± 1.11 | 5.43 |
| 11. | Methanolic extract of *A.galanga* | 400 | 84.8 ± 2.29 | 7.61 |
| 12. | Aqueous extract of *A. galanga* | 100 | 81.66 ± 2.11 | 6.31 |
| 13. | Aqueous extract of *A. galanga* | 200 | 85.23± 2.31 | 8.65 |
| 14. | Aqueous extract of *A. galanga* | 400 | 79.43±1.90 | 3.32 |

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test when compared with vehicle control *P<0.05, **P<0.01, ***P<0.001.

### 5.1.2.3 Anti-arthritic activity (AEAG)

#### 5.1.2.3.1 Body weight

- **Effect of oral administration of acetone extract of *A. galanga* (L.) Willd (AEAG) on body weights in arthritic rats**

The body weight of rats was recorded at regular intervals during the course of the study. The body weight of CFA induced arthritic control (group II) rats was less significantly reduced compared to non arthritic rats (group I) on 28$^{th}$ day. The body weight reduction in AEAG (400 mg) treated rats was non-significant compared that of arthritic rats (Table 5.1.2.3.1).

**Table 5.1.2.3.1: Effect of oral administration of acetone extract of *A. galanga* (L.) Willd (AEAG) on body weights in arthritic rats**

| Sr. No. | Treatment Groups | Body weight (gm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 192.25 ± 6.63 | 206.5 ± 7.36 | 210.75 ±7.12 | 214.75 ±7.12 | 219.25 ±7.27 | 223.75 ±7.39 |
| 2. | Arthritic control | 187 ± 15.64 | 178.25 ±15.42 | 174.5 ± 15.47 | 170.5 ± 15.12 | 166.5 ± 15.0 | 163.0 ± 14.97 |
| 3. | Diclofenac 5 mg/kg | 188.5 ± 8.49 | 178.0 ± 9.06 | 179.25 ±8.87 | 181.25 ±6.95 | 183.25 ±5.10 | 186.0 ± 3.41 |
| 4. | AEAG 100 mg/kg | 191.5 ± 6.51 | 180.5 ± 6.03 | 176.5 ± 6.03 | 172.25 ±6.20 | 169.0 ± 6.12 | 171.5 ± 5.95 |
| 5. | AEAG 200 mg/kg | 193 ± 9.38 | 182.75 ±9.60 | 178.5 ± 9.50 | 174.5 ± 9.50 | 176.75 ±9.36 | 181.75 ±9.69 |
| 6. | AEAG 400 mg/kg | 195.25 ± 5.80 | 185.75± 5.34 | 181.5 ± 5.57 | 184 ± 5.71 | 188 ± 5.71 | 192 ± 5.71 |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

**5.1.2.3.2 Paw volume**

- **Effect of oral administration of acetone extract of *A. galanga* (L.) Willd (AEAG) on right hind paw volume in arthritic rats**

One day after the CFA injection, primary arthritis of the right hind paw was induced, and inflammation was maintained up to 28 days (Table 5.1.2.3.2). Oral administration of AEAG (100, 200 and 400 mg/kg) and diclofenac (5 mg/kg) from 12$^{th}$ day of arthritis induction significantly (P<0.001) suppressed the increased paw edema up to 28$^{th}$ day.

**Table 5.1.2.3.2: Effect of oral administration of acetone extract of *A. galanga* (L.) Willd (AEAG) on right hind paw volume in arthritic rats.**

| Sr. No. | Treatment Groups | Change in Paw volume (ml) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 0.022 ± 0.006 | 0.052± 0.011 | 0.062 ± 0.017 | 0.070 ± 0.012 | 0.082 ± 0.007 | 0.075 ± 0.010 |
| 2. | Arthritic control | 1.15± 0.017 | 1.71 ± 0.010 | 1.74 ± 0.004 | 1.76 ± 0.007 | 0.78 ± 0.008 | 1.82 ± 0.001 |
| 3. | Diclofenac 5 mg/kg | 1.15 ± 0.014 | 1.70 ± 0.017 | 1.67 ± 0.020 | 1.46 ± 0.014 | 1.25 ± 0.014 | 0.98 ± 0.01*** |
| 4. | AEAG 100 mg/kg | 1.15 ± 0.011 | 1.70 ± 0.01 | 1.73 ± 0.01 | 1.74 ± 0.03 | 1.68 ± 0.03 | 1.50 ± 0.03*** |
| 5. | AEAG 200 mg/kg | 1.15± 0.008 | 1.70 ± 0.011 | 1.73 ± 0.01 | 1.69 ± 0.013 | 1.63 ± 0.013 | 1.47 ± 0.008*** |
| 6. | AEAG 400 mg/kg | 1.15 ± 0.028 | 1.70 ± 0.075 | 1.70 ± 0.06 | 1.64 ± 0.07 | 1.49 ± 0.08 | 1.33 ± 0.07*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001.

**5.1.2.3.3 Joint diameter**

- **Effect of oral administration of acetone extract of *A. galanga* (L.) Willd (AEAG) on right hind joint diameter in arthritic rats**

Administration of CFA in the right hind paw resulted in significant (P<0.001) increase in the joint diameter of hind paw compared to that of non arthritic (control) rats. Diclofenac (5 mg/kg) and AEAG (100, 200 and 400 mg/kg) showed significant (P<0.001) reduction of the right hind limb joint diameter compared with that of arthritic rat on 28$^{th}$ day.

**Table 5.1.2.3.3: Effect of oral administration of acetone extract of *A. galanga* (L.) Willd (AEAG) on right hind joint diameter in arthritic rats.**

| Sr. No. | Treatment Groups | Change in Joint diameter (mm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 0.022 ± 0.011 | 0.057± 0.011 | 0.077 ± 0.014 | 0.092 ± 0.015 | 0.10 ± 0.011 | 0.11 ± 0.010 |
| 2. | Arthritic control | 3.13± 0.17 | 3.27 ± 0.14 | 3.30 ± 0.15 | 3.32 ± 0.14 | 3.60 ± 0.12 | 3.62 ± 0.12 |
| 3. | Diclofenac 5 mg/kg | 3.12 ± 0.18 | 3.23 ± 0.19 | 3.17 ± 0.19 | 2.92 ± 0.17 | 2.85 ± 0.18 | 2.53 ± 0.21*** |
| 4. | AEAG 100 mg/kg | 3.11 ± 0.22 | 3.23 ± 0.22 | 3.19 ± 0.22 | 3.13 ± 0.22 | 3.08 ± 0.22 | 2.88 ± 0.22*** |
| 5. | AEAG 200 mg/kg | 3.11± 0.07 | 3.22 ± 0.06 | 3.19 ± 0.05 | 3.11 ± 0.06 | 2.91 ± 0.07 | 2.68 ± 0.08*** |
| 6. | AEAG 400 mg/kg | 3.11 ± 0.14 | 3.25 ± 0.15 | 3.20 ± 0.16 | 3.08 ± 0.16 | 2.77 ± 0.15 | 2.49 ± 0.16*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001.

### 5.1.2.3.4 Mechanical hyperalgesia

- **Effect of oral administration of acetone extract of *A. galanga* (L.) Willd (AEAG) on right hind paw mechanical withdrawal threshold in arthritic rats (Tactile allodynia)**

The dose-dependent effects of AEAG are presented in (Table 5.1.2.3.4). The mechanical withdrawal threshold (MWT) of the right hind paw was decreased in arthritic rat compared to the basal level 1 after the CFA injection. The oral treatment of diclofenac (5 mg/kg) and AEAG (200 and 400 mg/kg) significantly ($P<0.001$) suppressed the MWT on $28^{th}$ day, while the lower doses of AEAG showed lesser suppression of the MWT $28^{th}$ day.

**Table 5.1.2.3.4: Effect of oral administration of acetone extract of *A. galanga* (L.) Willd (AEAG) on right hind paw mechanical withdrawal threshold in arthritic rats.**

| Sr. No. | Treatment Groups | Mechanical Withdrawal Threshold (gm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 69.32 ± 2.94 | 73.95± 5.09 | 69.90 ± 3.53 | 72.62 ± 3.57 | 72.15 ± 4.40 | 66.70 ± 3.41 |
| 2. | Arthritic control | 73.87± 4.96 | 30.42 ± 1.48 | 30.90 ± 3.16 | 31.40 ± 1.88 | 30.32 ± 1.95 | 30.52 ± 2.40 |
| 3. | Diclofenac 5 mg/kg | 71.92 ± 5.89 | 28.35 ± 3.21 | 30.92 ± 2.49 | 50.95 ± 4.08 | 70.72 ± 3.38 | 75.60 ± 3.09*** |
| 4. | AEAG 100 mg/kg | 70.70 ± 3.96 | 29.10 ± 2.80 | 28.47 ± 2.82 | 30.85 ± 2.49 | 31.37 ± 3.43 | 45.62 ± 2.29** |
| 5. | AEAG 200 mg/kg | 70.72± 5.14 | 31.40 ± 2.16 | 32.17 ± 2.03 | 30.57 ± 2.34 | 46.57 ± 3.36 | 68.60 ± 2.70*** |
| 6. | AEAG 400 mg/kg | 71.90 ± 4.09 | 30.52 ± 1.32 | 30.50 ± 2.15 | 47.47 ± 3.17 | 64.50 ± 2.15 | 71.85 ± 3.47*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001.

### 5.1.2.3.5 Thermal hyperalgesia

- **Effect of oral administration of acetone extract of *A. galanga* (L.) Willd (AEAG) right hind paw withdrawal latency in arthritic rats (Thermal hyperalgesia)**

The dose-dependent effects of AEAG are presented in (Table 5.1.2.3.5). The paw withdrawal latency (PWL) of the right hind paw of arthritic rats was decreased compared to non arthritic rats. Treatment with diclofenac (5 mg/kg) and AEAG (200 and 400 mg/kg) showed significant ($P<0.001$) increase in PWL on 28$^{th}$ day, while the lower doses of AEAG showed less significant ($P<0.01$) reduction in the PWL.

**Table 5.1.2.3.5: Effect of oral administration of acetone extract of *A. galangal* (L.) Willd (AEAG) on right hind paw withdrawal latency in arthritic rats.**

| Sr. No. | Treatment Groups | Paw Withdrawal Latency (sec) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 11.45 ± 0.36 | 11.35± 0.63 | 11.15 ± 0.70 | 10.90 ± 0.48 | 11.90 ± 0.67 | 11.42 ± 0.49 |
| 2. | Arthritic control | 10.17 ± 0.48 | 5.17 ± 0.31 | 5.10 ± 0.29 | 5.32 ± 0.28 | 5.15 ± 0.25 | 5.00 ± 0.28 |
| 3. | Diclofenac 5 mg/kg | 10.02 ± 0.32 | 5.52 ± 0.24 | 5.40 ± 0.37 | 8.30 ± 0.28 | 10.55 ± 0.18 | 11.35 ± 0.27*** |
| 4. | AEAG 100 mg/kg | 10.5 ± 0.35 | 5.07 ± 0.22 | 4.87 ± 0.20 | 5.32 ± 0.25 | 5.07 ± 0.16 | 6.75 ± 0.27** |
| 5. | AEAG 200 mg/kg | 10.87 ± 0.39 | 5.40 ± 0.24 | 5.37 ± 0.20 | 5.20 ± 0.33 | 6.60 ± 0.10 | 11.40 ± 0.32*** |
| 6. | AEAG 400 mg/kg | 10.67 ± 0.26 | 5.40 ± 0.19 | 5.20 ± 0.36 | 5.02 ± 0.57 | 10.20 ± 0.35 | 10.95 ± 0.30*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001.

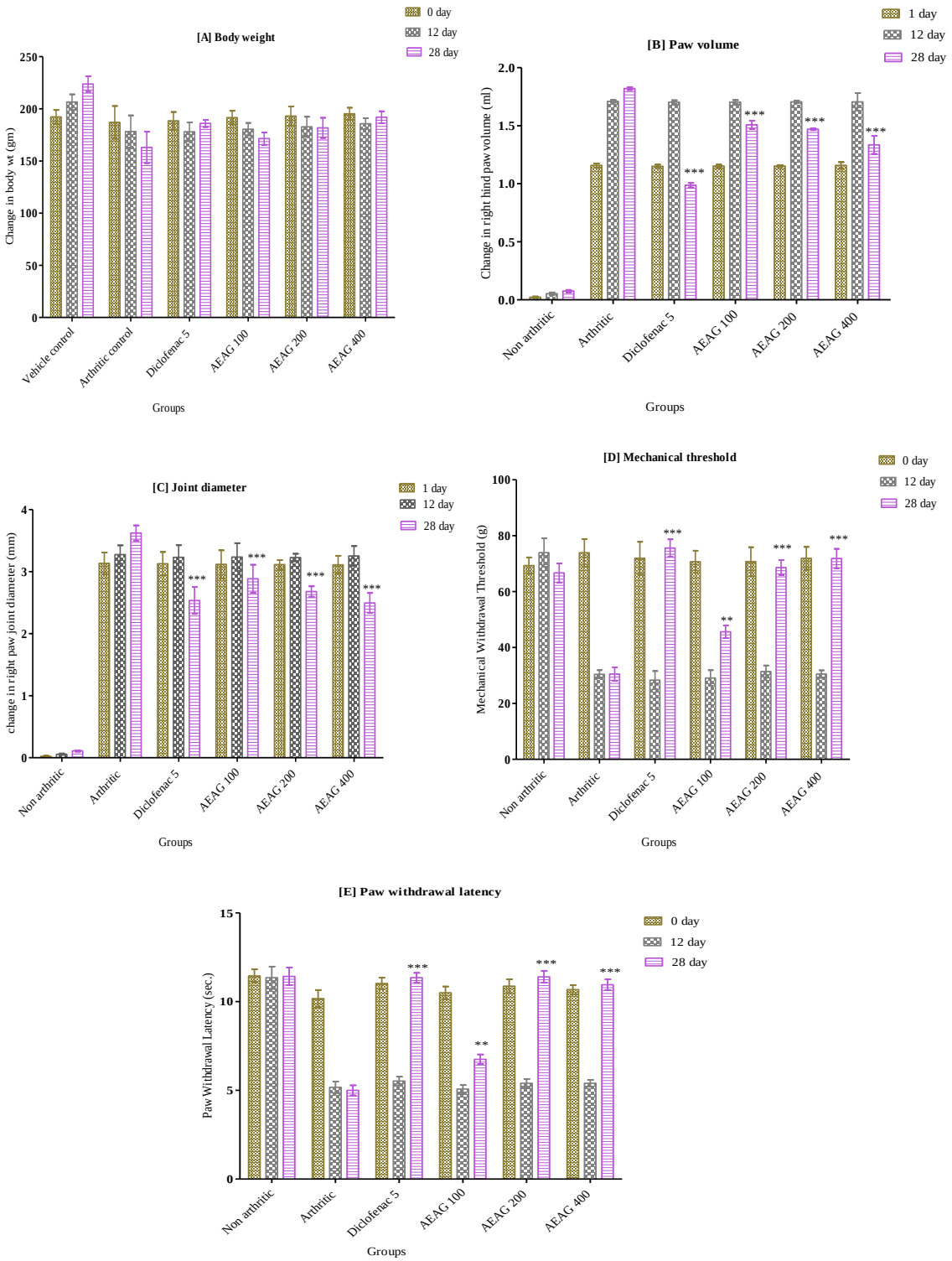



**Fig 5.1.2.3 Effect of AEAG on [A] Body weight, [B] Paw volume, [C] Joint diameter, [D] Mechanical hyperalgesia, [E] Thermal hyperalgesia**

### 5.1.2.4 Isolation of bioactive Fraction from AEAG by bioassay guided fractionation method.

The rhizomes were shade dried; cut in small pieces and powdered in hand mixer. The dried powder of the rhizomes of *A. galanga* (500 g) was extracted with 1.5L acetone as a solvent by cold percolation for 12 h in a 5L flat bottom flask at room temperature. The process of extraction was repeated three times with acetone. Each time the filtrate was concentrated *in vacuo* at 40°C using a rotary evaporator (Eqitron, Roteva) and pooled together to obtain 18.60 g of reddish extract. The crude acetone extract (10.0 g) was packed in silica-gel column with (60-120 mesh). The material was eluted stepwise with a gradient of n-hexane-acetone. The Fractions were collected separately and concentrated *in vacuo* at 40°C. These Fractions in the TLC were pooled together to obtain 6 Fractions: A (3.23 g), B (2.88 g), C (1.92g), D (0.55 g) E (0.25 g), and F (2.32). The Fraction B (2.50 g) showed significant ($P<0.001$) anti-inflammatory activity in carrageenan induced paw edema therefore it was repacked in silica gel column to separate active constituent. The column was eluted with gradient of hexane and acetone. The Fractions obtained were concentrated and identified by TLC and similar Fractions were pooled together to obtain sub-Fraction $B_1$ (0.23 g), $B_2$ (1.65 g), $B_3$ (0.45 g) and $B_4$ (0.25 g). The sub-Fraction $B_2$ (1.60 g) showed significant ($P<0.001$) anti-inflammatory activity in carrageenan induced paw edema therefore it was repacked in silica gel column to separate active constituent. The sub-Fraction $B_2$ was separated by preparative TLC [n-hexane:ethylacetate (8:2)] to yield compound, as a yellow oil (yield 1.20 g), identification of which was performed by comparison of the spectral data of mass and nuclear magnetic resonance with that of reported spectral data in literature (Latha et al., 2009). All spectral data were obtained on the following instruments; IR was recorded on a Perkin-Elmer model (683 B) and absorption is expressed in $cm^{-1}$ NMR on (Brucker AC-200) spectrometer. GC- MS on a (Clarus 500) spectrometer. Optical rotation was measured using sodium D line on a (JASCO-181) digital polarimeter.

#### 5.1.2.4.1 Anti-inflammatory activity

##### 5.1.2.4.1.1 Anti-inflammatory activity of 6 Fractions from acetone extract of *A. galanga* in carrageenan induced paw edema (Acute study)

The rats were pretreated with all the 6 Fractions collected from AEAG and diclofenac for 1 h before the injection of carrageenan caused inhibition of increase in paw edema from 1 h to 5 h. Diclofenac (10 mg/kg) caused significant ($P<0.001$) inhibition of increase in paw edema at $5^{th}$ h. The inhibitory effect of the diclofenac at 10 mg/kg was recorded (12.29%) at $3^{rd}$ h and (25.97%) at $5^{th}$ h. The inhibitory effect of the 10% acetone Fraction was recorded at $3^{rd}$ h (11.04%) and at $5^{th}$ h (20.85%) respectively. The inhibitory effect of the 20%, 30% acetone and 50% acetone Fractions were recorded at $3^{rd}$ h (4.52, 4.60 and 2.83%) and at $5^{th}$ h (17.55, 11.20 and 2.92%) respectively. The inhibition elicited by the 10% acetone Fraction was comparable to that of diclofenac. However there was no significant inhibition of increase in paw edema on treatment with 100% acetone Fractions observed.

**5.1.2.4.1.1: Effect of 6 Fractions from *A. galanga* on inhibition of right hind paws edema on carrageenan induced inflammation in rats.**

| Treatment Groups | Change in paw edema volume (ml) | | | % Inhibition at | | |
|---|---|---|---|---|---|---|
| | 1 h | 3 h | 5 h | 1 h | 3 h | 5 h |
| Carrageenan control | 1.025 ± 0.135 | 2.99 ± 0.065 | 3.86 ± 0.049 | - | - | - |
| Diclofenac 10 mg/kg | 0.92 ± 0.043 | 2.62 ± 0.076$^{ns}$ | 2.85 ± .081$^{***}$ | 10 | 12.29 | 25.97 |
| N-hexane Fraction 100 mg/kg (A) | 1.03 ± 0.161 | 2.85 ± 0.078 | 3.27 ± 0.025$^{**}$ | -0.73 | 4.52 | 15.09 |
| 10 % acetone Fraction 100 mg/kg (B) | 0.92 ± 0.074 | 2.66 ± 0.098$^{ns}$ | 3.05 ± .064$^{***}$ | 9.76 | 11.04 | 20.85 |
| 20 % acetone Fraction 100 mg/kg (C) | 1.04 ± 0.054 | 2.85 ± 0.052 | 3.42 ± 0.082$^{**}$ | 0.73 | 4.52 | 17.55 |
| 30 % acetone Fraction 100 mg/kg (D) | 1.04 ± 0.054 | 2.85 ± 0.052 | 3.29 ± 0.06$^{**}$ | -2.20 | 4.60 | 11.20 |
| 50 % acetone Fraction 100 mg/kg (E) | 1.02 ± 0.049 | 2.83 ± 0.065 | 3.32 ± 0.160$^{*}$ | 1.02 | 2.83 | 2.92 |
| Acetone Fraction 100 mg/kg (F) | 1.02 ± 0.042 | 2.81 ± 0.087 | 3.48 ± 0.086 | 0.24 | 5.77 | 9.78 |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with carrageenan control $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

**5.1.2.4.1.2 Anti-inflammatory activity of 4 pools from 10% acetone Fraction from *A. galanga* in carrageenan induced paw edema (acute study)**

The rats were pretreated with all the 4 pools from 10% acetone extract of *A. galanga* and diclofenac for 1 h before the injection of carrageenan caused inhibition of increase in paw edema from 1 h to 5 h. Diclofenac (10 mg/kg) caused significant ($P<0.001$) inhibition of increase in paw edema at 5$^{th}$ h. The inhibitory effect of the diclofenac at 10 mg/kg was recorded (13.69%) at 3$^{rd}$ h and (28.32%) at 5$^{th}$ h. Pool 2 (10 mg/kg) caused significant

(P<0.001) inhibition of increase in paw edema at 5th h. The inhibitory effect of the pool 2 was recorded at 3rd h (16.98%) and at 5th h (23.86%) respectively. The inhibitory effect of the pool 3 was recorded at 3rd h (12.60%) and at 5th h (17.96%) respectively. The inhibition elicited by the pool 2 was comparable to that of diclofenac. However there was no significant inhibition in increase in paw edema on treatment with pool 1 and 4 (Table 5.1.2.4.1.2).

**Table 5.1.2.4.1.2: Effect of 4 pools from 10% acetone Fraction from *A. galanga* on inhibition of right hind paws edema on carrageenan induced inflammation in rats.**

| Treatment Groups | Change in paw edema volume (ml) | | | % Inhibition at | | |
|---|---|---|---|---|---|---|
| | 1 h | 3 h | 5 h | 1 h | 3 h | 5 h |
| Carrageenan control | 1.02 ± 0.123 | 2.73 ± 0.302 | 3.81 ± 0.060 | - | - | - |
| Diclofenac 10 mg/kg | 0.91 ± 0.13 | 2.36 ± 0.137$^{ns}$ | 2.73 ± 0.086$^{***}$ | 10.29 | 13.69 | 28.32 |
| p 1 10 mg/kg (B1) | 0.99 ± 0.16 | 2.83 ± 0.14 | 3.14 ± 0.18 | 2.20 | -3.47 | 17.50 |
| p 2 10 mg/kg (B2) | 0.92 ± 0.015 | 2.27 ± 0.073$^{ns}$ | 2.90 ± 0.070$^{***}$ | 9.80 | 16.98 | 23.86 |
| p 3 10 mg/kg (B3) | 0.92 ± 0.129 | 2.39 ± 0.34 | 3.12 ± 0.051$^{**}$ | 9.55 | 12.60 | 17.96 |
| p 4 10 mg/kg (B4) | 1.04 ± 0.081 | 2.86 ± 0.068 | 3.22 ± 0.077 | -1.96 | -4.74 | 15.41 |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with carrageenan control $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001.

**5.1.2.4.2 Anti-inflammatory activity of bioactive Fraction (B2)**

**5.1.2.4.2.1 Acute oral toxicity study**

Administration of 20 mg/kg of Fraction (B2) did not produce any behavioral abnormalities and mortality. So the dose selected for further study was 5, 10 and 20 mg/kg for isolated Fraction (B2).

### 5.1.2.4.2.2 Carrageenan induced paw edema in rats

Intradermal injection of carrageenan into subplantar region of right hind paw caused significant increase in the paw edema, which was recorded as change in paw volume at 3rd h 3.00± 0.087 ml and at 5th h 3.84± 0.060 ml. Diclofenac (10 mg/kg) were administered 1 h before the injection of carrageenan caused significant (P<0.01 and P<0.001) inhibition of increase in paw edema at 3rd and 5th h respectively. The inhibitory effect of the diclofenac was recorded (5.32%) at 3rd h and (26.01%) at 5th h. The Fraction (B2) was administered for 7 days before the injection of carrageenan caused dose dependent inhibition of increase in paw edema from 1 h to 5 h. The inhibitory effect of the Fraction (B2) was recorded with a dose of 20 mg/kg at 3rd h (5.99%) and at 5th h (25.94%) respectively.

**Table 5.1.2.4.2.2: Effect of oral administration of Fraction (B2) from AEAG on inhibition of right hind paws edema on carrageenan induced inflammation in rats.**

| Treatment Groups | Change in paw edema volume (ml) | | | % Inhibition at | | |
|---|---|---|---|---|---|---|
| | 1 h | 3 h | 5 h | 1 h | 3 h | 5 h |
| Carrageenan control | 1.04 ± 0.148 | 3.00± 0.087 | 3.84± 0.060 | -- | -- | -- |
| Diclofenac 10 mg/kg | 1.01 ± 0.049 | 2.84±0.088 | 2.84± 0.094*** | 2.88 | 5.32 | 26.01 |
| Fraction (B2) 5 mg/kg | 1.03 ±0.045 | 2.84 ± 0.045 | 3.25 ± 0.043** | 0.96 | 5.24 | 15.34 |
| Fraction (B2) 10 mg/kg | 1.02± 0.040 | 2.83± 0.063 | 2.85± .071*** | 1.44 | 5.74 | 25.68 |
| Fraction (B2) 20 mg/kg | 1.02 ± 0.037 | 2.82 ± 0.090 | 2.84 ± 0.121*** | 1.92 | 5.99 | 25.94 |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with carrageenan control *P<0.05, **P<0.01, ***P<0.001.

### 5.1.2.4.2.3 Cotton pellet granuloma in rats

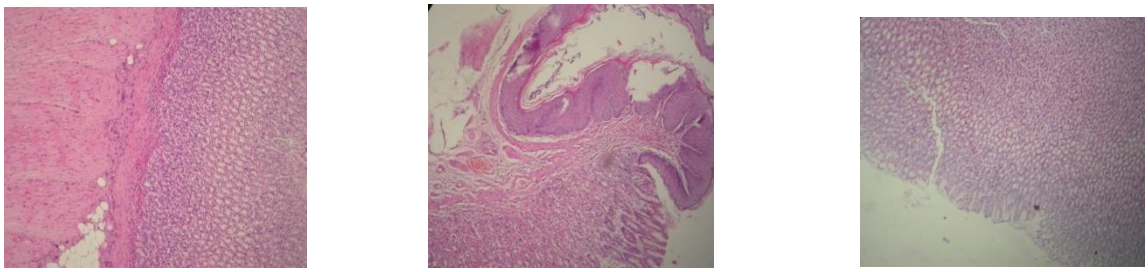
Subcutaneous implantation of 50 mg of cotton pellet in rats caused significant increase in weight of cotton pellet, recorded as 88.5 ± 2.93 mg which was due to granuloma formation. Diclofenac (10 mg/kg) and Fraction B2 (20 mg/kg) significantly (P<0.001) inhibited the granuloma formation by 62.71 and 17.14 % respectively, where as Fraction B2 (10 mg/kg) significantly (P<0.01 and P<0.05, respectively) inhibited the granuloma formation compared to vehicle control group.

**Table 5.1.2.4.2.3: Effect of oral administration of Fraction (B2) from AEAG on cotton pellet granuloma in rats**

| Sr. No. | Treatment Groups | Dose mg/kg p.o. | Dry granuloma weight (mg) (Mean ± SEM) | % Inhibition |
|---|---|---|---|---|
| 1. | Vehicle control | | 88.5 ± 2.93 | |
| 2. | Diclofenac | 10 | 33 ± 2.86*** | 62.71186 |
| 3. | Fraction (B2) | 5 | 88.33 ± 2.45 | 0.19209 |
| 4. | Fraction (B2) | 10 | 76 ± 1.86** | 14.12429 |
| 5. | Fraction (B2) | 20 | 73.33 ± 2.14*** | 17.14124 |

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test when compared with vehicle control *P<0.05, **P<0.01, ***P<0.001.

### 5.1.2.4.2.4 Gastric ulcerogenic effect of oral administration of Fraction (B2) on cotton pellet granuloma in rats



**A** **B** **C**

**Fig 5.1.2.4.1 Histopathology of stomach (A) Vehicle control (B) Diclofenac 10 mg / kg treated (C) Fraction (B2) 20 mg/kg treated.**

### 5.1.2.4.3 Anti-arthritic studies of isolated bioactive Fraction (B2)

**[A] Body weight**

- **5.1.2.4.3.1 Effect of oral administration of isolated bioactive Fraction (B2) on body weights in arthritic rats**

The body weight of rats was recorded at regular intervals during the course of the study. The body weight of CFA induced arthritic control (group II) rats was less significantly reduced compared to non arthritic rats (group I) on 28$^{th}$ day. The body weight reduction in Fraction B2 (20 mg) treated rats was non-significant compared that of arthritic rats (Table 5.1.2.4.3.1).

**Table 5.1.2.4.3.1: Effect of oral administration of isolated bioactive Fraction (B2) on body weights in arthritic rats.**

| Sr. No. | Treatment Groups | Body weight (gm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 192.25 ±6.63 | 206.5 ±7.36 | 210.75 ±7.12 | 214.75 ±7.12 | 219.25 ±7.27 | 223.75 ±7.39 |
| 2. | Arthritic control | 187 ± 15.64 | 178.25 ± 15.42 | 174.5 ± 15.47 | 170.5 ± 15.12 | 166.5 ± 15.0 | 163.0 ± 14.97 |
| 3. | Diclofenac 5 mg/kg | 188.5 ± 8.49 | 178.0 ± 9.06 | 179.25 ± 8.87 | 181.25 ± 6.95 | 183.25 ± 5.10 | 186.0 ± 3.41 |
| 4. | Fraction (B2) 5 mg/kg | 189.25 ± 8.13 | 178.5± 8.39 | 174.5 ± 8.39 | 170.5 ± 8.39 | 172.5± 8.46 | 177.75 ± 8.37 |
| 5. | Fraction (B2) 10 mg/kg | 195.25 ± 7.36 | 185.75± 7.0 | 181.5 ± 6.95 | 178.75 ± 6.86 | 183.75 ± 8.86 | 184.25 ± 5.20 |
| 6. | Fraction (B2) 20 mg/kg | 192.75 ± 9.51 | 183± 9.21 | 182.5 ± 9.22 | 186.5 ± 9.22 | 191 ± 9.19 | 195.5 ± 9.18 |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

**[B] Paw volume**

**5.1.2.4.3.2 Effect of oral administration of isolated bioactive Fraction (B2) on right hind paw volume in arthritic rats**

One day after the CFA injection, primary arthritis of the right hind paw was induced, and inflammation was maintained up to 28 days (Table 5.1.2.4.3.2). Oral administration of Fraction B2 (5, 10 and 20 mg/kg) and diclofenac (5 mg/kg) from 12$^{th}$ day of arthritis induction significantly (P<0.001) suppressed the increased paw edema up to 28$^{th}$ day.

**Table 5.1.2.4.3.2: Effect of oral administration of isolated bioactive Fraction (B2) on right hind paws volume in arthritic rats**

| Sr. No. | Treatment Groups | Change in Paw volume (ml) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 0.022± 0.006 | 0.052± 0.011 | 0.062± 0.017 | 0.070± 0.012 | 0.082± 0.007 | 0.075 ± 0.010 |
| 2. | Arthritic control | 1.15± 0.017 | 1.71 ± 0.010 | 1.74 ± 0.004 | 1.76 ± 0.007 | 0.78 ± 0.008 | 1.82 ± 0.001 |
| 3. | Diclofenac 5 mg/kg | 1.15 ± 0.014 | 1.70 ± 0.017 | 1.67 ± 0.020 | 1.46 ± 0.014 | 1.25 ± 0.014 | 0.98 ± 0.01*** |
| 4. | Fraction (B2) 5 mg/kg | 1.19 ± 0.02 | 1.73± 0.02 | 1.72 ± 0.03 | 1.69 ± 0.03 | 1.65± 0.03 | 1.30 ± 0.02*** |
| 5. | Fraction (B2) 10 mg/kg | 1.14 ± 0.006 | 1.71± 0.006 | 1.68 ± 0.006 | 1.62 ± 0.010 | 1.43 ± 0.01 | 1.19 ± 0.008*** |
| 6. | Fraction (B2) 20 mg/kg | 1.15 ± 0.004 | 1.73± 0.013 | 1.69 ± 0.011 | 1.53 ± 0.022 | 1.32± 0.029 | 1.11 ± 0.032*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001.

**[C] Joint diameter**

**5.1.2.4.3.3 Effect of oral administration of isolated bioactive Fraction (B2) on right hind joint diameter in arthritic rats**

Administration of CFA in the right hind paw resulted in significant (P<0.001) increase in the joint diameter of hind paw compared to that of non arthritic (control) rats. Diclofenac (5 mg/kg) and Fraction B2 (5, 10 and 20 mg/kg) showed significant (P<0.001) reduction of the right hind limb joint diameter compared with that of arthritic rat on 28$^{th}$ day.

**Table 5.1.2.4.3.3: Effect of oral administration of isolated bioactive Fraction (B2) on right hind joint diameter in arthritic rats**

| Sr. No. | Treatment Groups | Change in Joint diameter (mm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 0.022 ± 0.011 | 0.057± 0.011 | 0.077 ± 0.014 | 0.092 ± 0.015 | 0.10 ± 0.011 | 0.11 ± 0.010 |
| 2. | Arthritic control | 3.13± 0.17 | 3.27 ± 0.14 | 3.30 ± 0.15 | 3.32 ± 0.14 | 3.60 ± 0.12 | 3.62 ± 0.12 |
| 3. | Diclofenac 5 mg/kg | 3.12 ± 0.18 | 3.23 ± 0.19 | 3.17 ± 0.19 | 2.92 ± 0.17 | 2.85 ± 0.18 | 2.53 ± 0.21*** |
| 4. | Fraction (B2) 5 mg/kg | 3.04 ± 0.08 | 3.21 ± 0.02 | 3.18 ± 0.024 | 3.13 ± 0.017 | 3.00 ± 0.04 | 2.77 ± 0.06*** |
| 5. | Fraction (B2) 10 mg/kg | 3.31 ± 0.25 | 3.22± 0.013 | 3.16 ± 0.013 | 3.67 ± 0.014 | 2.90± 0.020 | 2.61 ± 0.027*** |
| 6. | Fraction (B2) 20 mg/kg | 3.05 ± 0.004 | 3.23± 0.007 | 3.15 ± 0.010 | 3.05 ± 0.010 | 2.74 ± 0.051 | 2.43 ± 0.08*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001.

**[D] Mechanical hyperalgesia**

**5.1.2.4.3.4 Effect of oral administration of isolated bioactive Fraction (B2) on right hind paw mechanical withdrawal threshold in arthritic rats (Tactile allodynia)**

The dose-dependent effects of Fraction (B2) are presented in (Table 5.1.2.4.3.4). The mechanical withdrawal threshold (MWT) of the right hind paw was decreased in arthritic rat compared to the basal level 1 after the CFA injection. The oral treatment of diclofenac (5 mg/kg) and Fraction B2 (10 and 20 mg/kg) significantly ($P<0.001$) suppressed the MWT on 28$^{th}$ day, while the lower doses of Fraction (B2) showed lesser suppression of the MWT 28$^{th}$ day.

**Table 5.1.2.4.3.4: Effect of oral administration of isolated bioactive Fraction (B2) on right hind paw mechanical withdrawal threshold in arthritic rats (Tactile allodynia)**

| Sr. No. | Treatment Groups | Mechanical Withdrawal Threshold (gm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 69.32± 2.94 | 73.95± 5.09 | 69.90± 3.53 | 72.62 ± 3.57 | 72.15 ± 4.40 | 66.70 ± 3.41 |
| 2. | Arthritic control | 73.87± 4.96 | 30.42 ± 1.48 | 30.90 ± 3.16 | 31.40 ± 1.88 | 30.32 ± 1.95 | 30.52 ± 2.40 |
| 3. | Diclofenac 5 mg/kg | 71.92 ± 5.89 | 28.35 ± 3.21 | 30.92 ± 2.49 | 50.95 ± 4.08 | 70.72 ± 3.38 | 75.60 ± 3.09*** |
| 4. | Fraction (B2) 5 mg/kg | 72.05 ± 4.15 | 27.97 ± 2.43 | 28.05 ± 2.94 | 29.60 ± 1.97 | 41.60 ± 3.06 | 44.27 ± 2.70** |
| 5. | Fraction (B2) 10 mg/kg | 72.07 ± 3.72 | 31.22± 1.52 | 27.80 ± 2.11 | 35.87 ± 1.50 | 46.45± 2.83 | 70.77 ± 3.38*** |
| 6. | Fraction (B2) 20 mg/kg | 73.12 ± 3.35 | 28.37± 1.67 | 28.85 ± 2.38 | 47.47 ± 3.34 | 72.72 ± 3.38 | 73.85 ± 2.81*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001.

**[E] Thermal hyperalgesia**

**5.1.2.4.3.5 Effect of oral administration of isolated bioactive Fraction (B2) on right hind paw withdrawal latency in arthritic rats (Thermal hyperalgesia)**

The dose-dependent effects of Fraction (B2) are presented in (Table. 5.1.2.4.3.5). The paw withdrawal latency (PWL) of the right hind paw of arthritic rats was decreased compared to non arthritic rats. Treatment with diclofenac (5 mg/kg) and Fraction B2 (10 and 20 mg/kg) showed significant ($P<0.001$) increase in PWL on 28$^{th}$ day, while the lower doses of Fraction (B2) showed less significant ($P<0.01$) reduction in the PWL.

**Table 5.1.2.4.3.5: Effect of oral administration of isolated bioactive Fraction (B2) on right hind paw withdrawal latency in arthritic rats (Thermal hyperalgesia)**

| Sr. No. | Treatment Groups | Paw Withdrawal Latency (sec) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 11.45 ± 0.36 | 11.35± 0.63 | 11.15 ± 0.70 | 10.90 ± 0.48 | 11.90 ± 0.67 | 11.42 ± 0.49 |
| 2. | Arthritic control | 10.17 ± 0.48 | 5.17 ± 0.31 | 5.10 ± 0.29 | 5.32 ± 0.28 | 5.15 ± 0.25 | 5.00 ± 0.28 |
| 3. | Diclofenac 5 mg/kg | 10.02 ± 0.32 | 5.52 ± 0.24 | 5.40 ± 0.37 | 8.30 ± 0.28 | 10.55 ± 0.18 | 11.35 ± 0.27*** |
| 4. | Fraction (B2) 5 mg/kg | 10.60 ± 0.38 | 5.12 ± 0.30 | 5.22 ± 0.17 | 5.25 ± 0.15 | 6.75 ± 0.15 | 6.97 ± 0.16** |
| 5. | Fraction (B2) 10 mg/kg | 10.92 ± 0.38 | 5.17± 0.11 | 5.17 ± 0.17 | 5.62 ± 0.26 | 7.00± 0.34 | 11.02 ± 0.19*** |
| 6. | Fraction (B2) mg/kg | 10.62 ± 0.33 | 5.20± 0.31 | 5.30 ± 0.16 | 6.77 ± 0.14 | 10.97 ± 0.13 | 11.52 ± 0.37*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001.

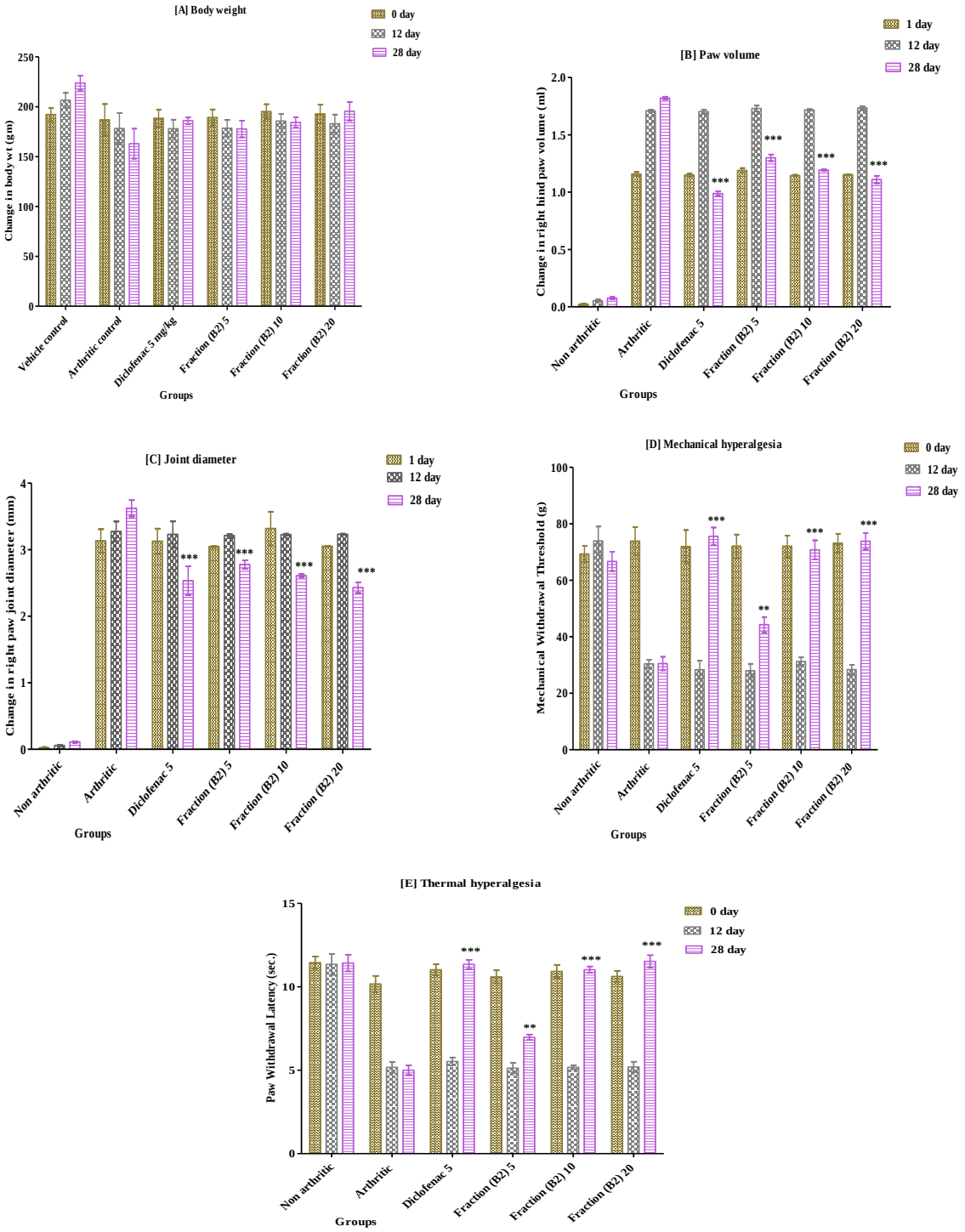



**Fig. 5.1.2.4.3 Effects of Fraction (B2) on [A] Body weight [B] Paw volume [C] Joint diameter [D] Thermal hyperalgesia [E] Mechanical hyperalgesia**

### 5.1.2.4.3.6 Haematological parameters

Arthritic rats showed significant reduction in hemoglobin and RBC, on other hand significantly increased in WBC and platelets count compare to non arthritic rats. Diclofenac (5 mg/kg) and Fraction B2 (20 mg/kg) significantly increased the hemoglobin and RBC count. The WBC and platelet counts were significantly reduced by high doses of Fraction B2 (20 mg/kg). Results were shown in (Table 5.1.2.4.3.6).

**Table 5.1.2.4.3.6 Effect of oral administration of isolated bioactive Fraction (B2) on blood haematological parameters in arthritic rats**

| Treatment Groups | Dose mg/kg | Hb (gm/100ml) | WBC (thousands/µl) | RBC (million/µl) | Platelet (lacks/µL) |
|---|---|---|---|---|---|
| Healthy control | | 14.5 ± 0.32 | 7.71 ± 0.19 | 6.89 ± 0.11 | 9.30 ± 0.13 |
| Arthritic control | | 9.0 ± 0.18$^{\#}$ | 15.4 ± 0.25$^{\#}$ | 3.32 ± 0.07$^{\#}$ | 18 ± 0.13$^{\#}$ |
| Diclofenac | 5 | 13.3 ± .15*** | 14.4 ± 0.20** | 6.08 ± .12*** | 17 ± 0.29** |
| Fraction (B2) | 5 | 9.02 ± 0.14 | 14.6 ± 0.23* | 3.70 ± 0.10 | 17.4 ± 0.06 |
| | 10 | 10.5 ± 0.24** | 14.3 ± 0.18** | 4.30 ± 0.24* | 17.3 ± 0.21 |
| | 20 | 10.9±0.34*** | 13.7 ±0.08*** | 4.69±0.18*** | 16.5±0.10*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test when compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and when arthritic control group compared with vehicle control group $^{\#}P<0.001$.

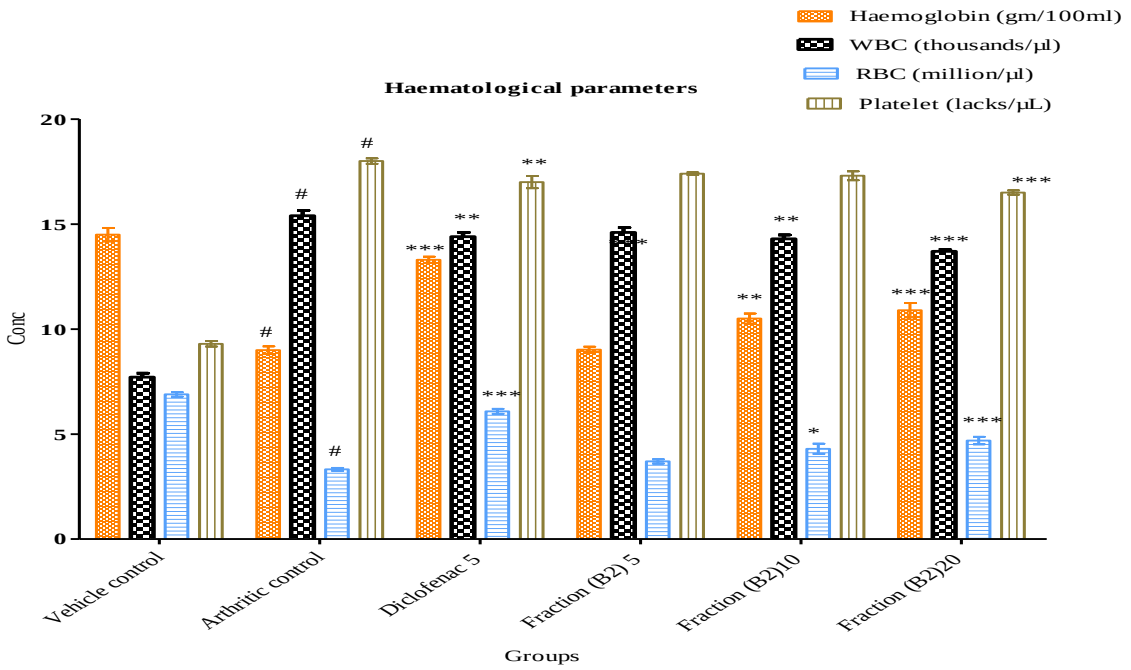



**Fig. 5.1.2.4.3.6 Effect of oral administration of isolated bioactive Fraction (B2) on blood haematological parameters in arthritic rats**

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test when compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and when arthritic control group compared with vehicle control group $^{\#}P<0.001$.

**5.1.2.4.3.7 Biochemical parameters of Fraction (B2)**

The markers for cellular toxicity (serum ALT, AST, and ALP level) were significantly increased (P<0.001), while TP level significantly (P<0.001) decreased in arthritic rats compared with the non arthritic rats. All of these changes were completely modulated in arthritic rats that received Fraction (B2) from 12$^{th}$ day onwards. The treatment with Fraction B2 (20 mg/kg) showed significant (P<0.001) decrease in the serum AST, ALT, and ALP levels; whereas the TP level was less significantly (P<0.01) increased as compared to arthritis control. Diclofenac (5mg/kg) showed insignificant reduction in the serum AST, ALT and ALP levels; whereas TP level showed insignificant increase compared to arthritis control, (Table 5.1.2.4.3.7).

**Table 5.1.2.4.3.7: Effect of oral administration of isolated bioactive Fraction (B2) on serum biochemical parameters in arthritic rats**

| Treatment Groups | Dose mg/kg | AST (U/L) | ALT (U/L) | ALP (U/L) | TP (gm/dl) |
|---|---|---|---|---|---|
| Healthy control | | 42.2 ± 1.88 | 52.7 ± 1.77 | 75.7 ± 1.85 | 7.45 ± 0.22 |
| Arthritic control | | 125 ± 2.83# | 186 ± 1.94# | 449 ± 2.99# | 5.83 ± 0.23# |
| Diclofenac | 5 | 118 ± 3.33 | 180 ± 2.17 | 438 ± 3.57 | 6.35 ± 0.26 |
| Fraction (B2) | 5 | 115 ± 2.08* | 179 ± 0.47* | 438 ± 5.25* | 5.70 ± 0.25 |
| | 10 | 109 ± 1.87*** | 177 ± 1.25** | 433 ± 2.03** | 6.08 ± 0.14 |
| | 20 | 98.3 ± 2.42*** | 160 ± 1.66*** | 404 ±2.80*** | 6.90 ± .13** |

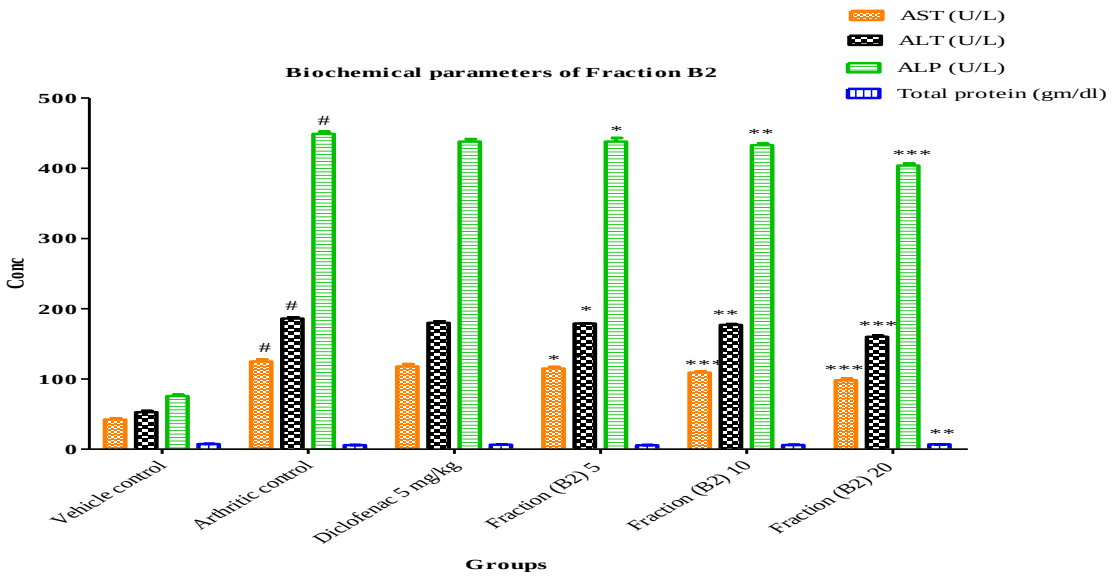



**Fig. 5.1.2.4.3.7: Effect of oral administration of isolated bioactive Fraction (B2) on serum biochemical parameters in arthritic rats**

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test when compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and when arthritic control group compared with vehicle control group $^{\#}P<0.001$.

### 5.1.2.4.3.8 Antioxidant parameters

CFA induced a significant reduction in SOD and GSH level in the liver of arthritic rats accompanied by an increased of MDA level. Oral administration of Fraction (B2) 20 mg/kg showed significant (P<0.001 and P<0.01) increased in SOD and GSH level respectively, while MDA level was significantly (P<0.001) decreased in the liver of arthritic rats. Treatment of 10 mg/kg of Fraction (B2) showed significant (P<0.01 and P<0.05) increased in SOD and GSH level respectively, while MDA level was significantly (P<0.01) decreased in the liver of arthritic rats. Diclofenac 5 mg/kg also induced a significant (P<0.001) increase of SOD activity and a reduction of MDA level in the liver of arthritic rats. (Table 5.1.2.4.3.8)

**Table 5.1.2.4.3.8: Effect of oral administration of isolated bioactive Fraction (B2) on liver antioxidant parameters level in arthritic rats**

| Treatment Groups | Dose mg/ kg | MDA(nmole MDA/mg protein) | SOD (mU/mg protein) | GSH (µmole/mg protein) |
|---|---|---|---|---|
| Healthy control | | 1.97 ± 0.019 | 4.40 ± 0.021 | 71.5 ± 0.90 |
| Arthritic control | | 3.42 ± 0.021$^{\#}$ | 2.38 ± 0.016$^{\#}$ | 46 ± 1.41$^{\#}$ |
| Diclofenac | 5 | 2.95 ± 0.028*** | 2.90 ± 0.017*** | 52.3 ± 2.17 |
| Fraction (B2) | 5 | 3.37 ± 0.041 | 2.50 ± 0.041 | 42.3 ± 0.85 |
| | 10 | 3.28 ± 0.030** | 2.54 ± 0.039** | 54.1 ± 2.36* |
| | 20 | 2.86 ± 0.018*** | 2.98 ± 0.020*** | 56.3 ± 2.69** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test when compared with arthritic control group $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001 and when arthritic control group compared with vehicle control group $^{\#}$P<0.001.

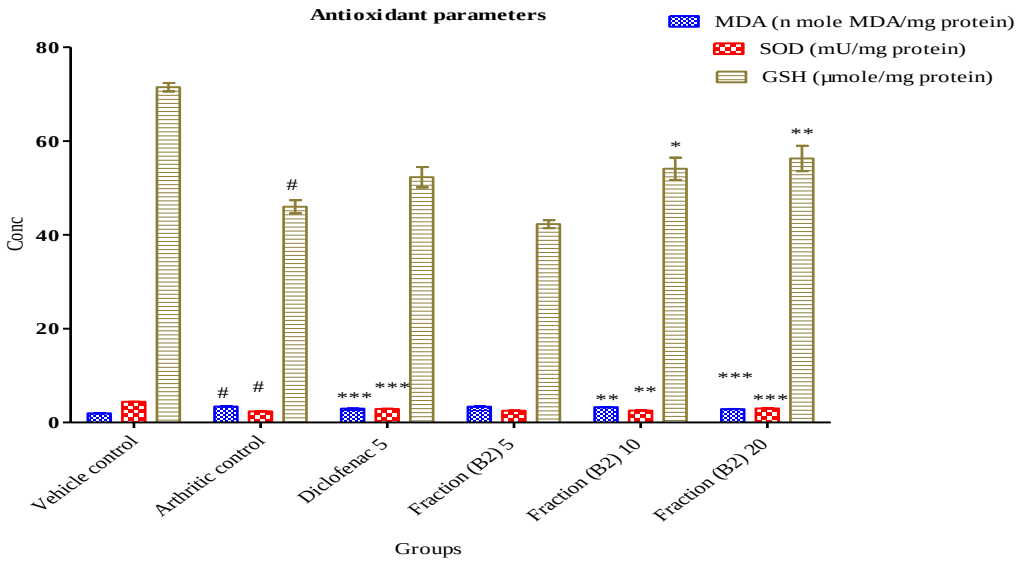



**Fig. 5.1.2.4.3.8: Effect of oral administration of isolated bioactive Fraction (B2) on liver antioxidant parameters level in arthritic rats**

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test when compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and when arthritic control group compared with vehicle control group $^{\#}P<0.001$.

**5.1.2.4.3.9 Radiological analysis**

X-ray radiography of the different treatment group animal paws taken on 28$^{th}$ day. Adjuvant treated rats had developed definite joint space narrowing of the intertarsal joints, diffuse soft tissue swelling that included the digits, marked periosteal thickening, cystic enlargement of bone and extensive erosions produced narrowing of all joint spaces. Despite a similar clinical course of arthritis, CFA control rats suffered from more pronounced bone destruction than AEAG and Fraction (B2) treated groups (Fig. 5.1.2.4.3.9)

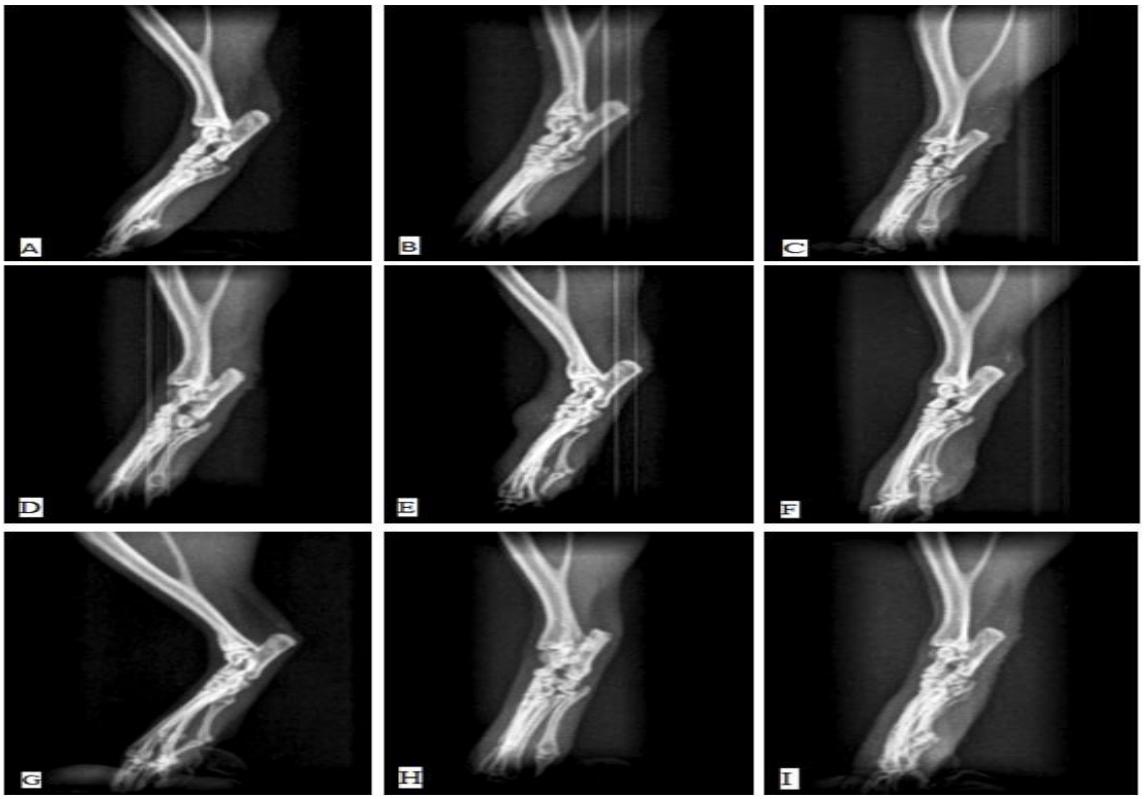



**Fig. 5.1.2.4.3.9: Radiological analysis of synovial joint. (A) Normal non-arthritic (B) Arthritic control, (C) Diclofenac 5 mg/kg treated, (D) AEAG 100 mg/kg treated, (E) AEAG 200 mg/kg treated, (F) AEAG 400 mg/kg treated. (G) Fraction (B2) 5 mg/kg (H) Fraction (B2) 10 mg/kg (I) Fraction (B2) 20 mg/kg.**

**5.1.2.4.3.10 Histopathology of synovial joint:**

Histopathology of synovial joint of normal rats showed intact morphology of synovium. No inflammation and influx of inflammatory cells was observed. CFA treated rats showed cartilage destruction, influx of inflammatory cells, pannus formation, fibrin deposition, synovitis and chronic inflammation. Diclofenac treated rats showed significant protection against cartilage destruction, vascular proliferation and synovial space thickening, low influx of inflammatory cells and no pannus formation. AEAG 400 mg/kg treated rats showed significant lesser cartilage destruction, synovial space thickening, vascular proliferation, low influx of inflammatory cells and no pannus formation. AEAG 200 mg/kg treated rats showed moderate cartilage destruction and synovial space thickening, influx of few inflammatory cells. AEAG 100 mg/kg treated

rats showed minimal inflammation, influx of few inflammatory cells in synovium with evidence of disturbed synovial lining or pannus formation. Fraction (B2) 5 mg/kg treated rats showed moderate cartilage destruction and synovial space thickening, influx of few inflammatory cells. Fraction (B2) 10 mg/kg treated rats showed significant lesser cartilage destruction, synovial space thickening, vascular proliferation, low influx of inflammatory cells and no pannus formation. Fraction (B2) 20 mg/kg treated rats showed significant protection against cartilage destruction, vascular proliferation and synovial space thickening, low influx of inflammatory cells and no pannus formation. (Fig. 5.1.2.4.3.10)

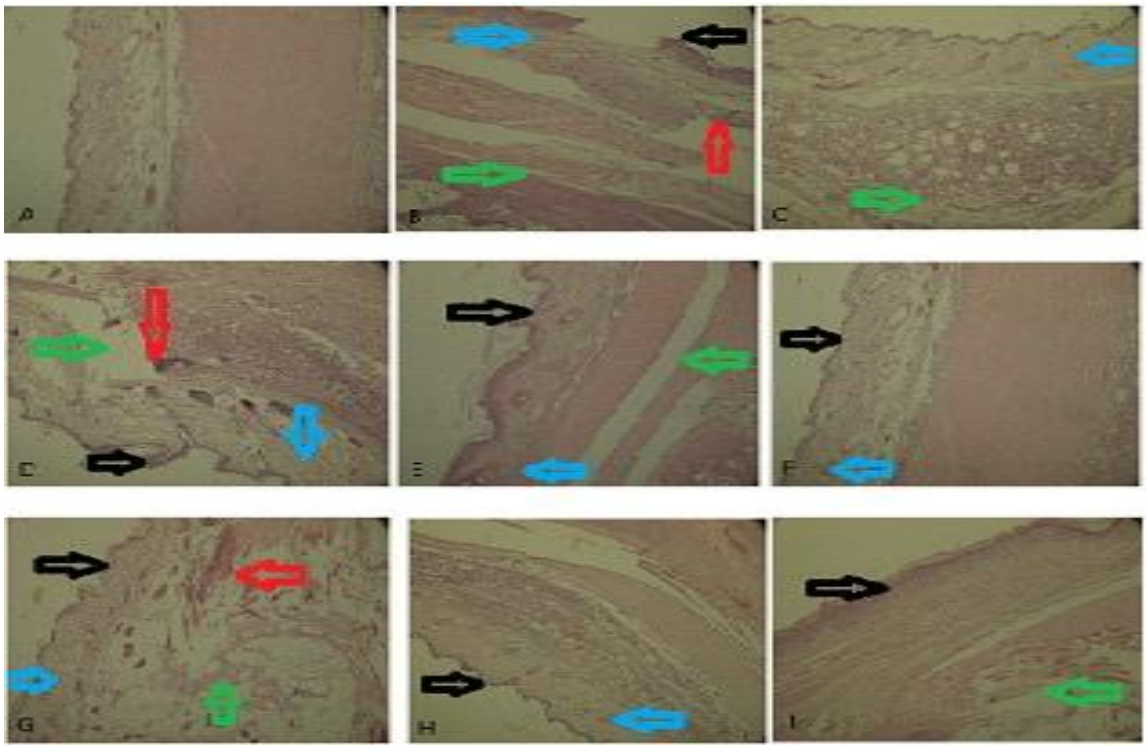


**Fig. 5.1.2.4.3.10 Histopathology of synovial joint. (A) Normal non-arthritic (B) Arthritic control, (C) Diclofenac 5 mg/kg treated, (D) AEAG 100 mg/kg treated, (E) AEAG 200 mg/kg treated, (F) AEAG 400 mg/kg treated. (G) Fraction (B2) 5 mg/kg (H) Fraction (B2) 10 mg/kg (I) Fraction (B2) 20 mg/kg.(Black arrow – synovial lining, Blue arrow – influx of inflammatory cells, Red arrow – pannus formation, Green arrow – cartilage destruction).**

### 5.1.2.4.4 Characterization of isolated Fraction (B2) from *A. galanga*

The characterization of the structures of the isolated compounds was on the basis of NMR spectra by comparison of their NMR spectral data with literature values. The chemical structure of isolated compound was elucidated by LC-MS, IR, $^{1}$H-NMR, $^{13}$C-NMR and DEPT.

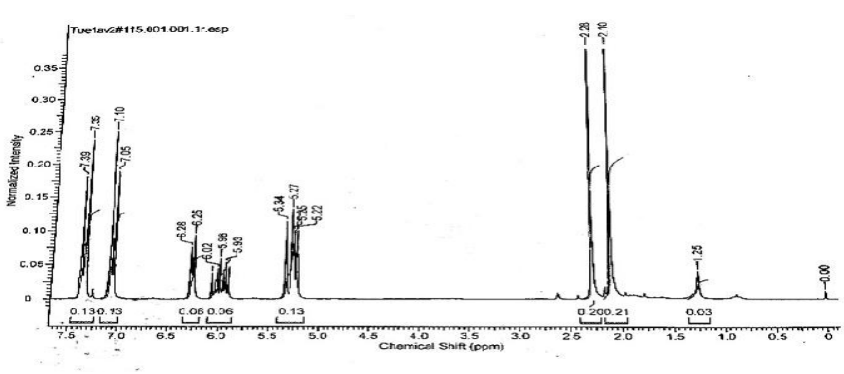



**Fig 5.1.2.4.4 (A): $^{1}$H-NMR spectrum of isolated compound (B2) from *A. galanga***

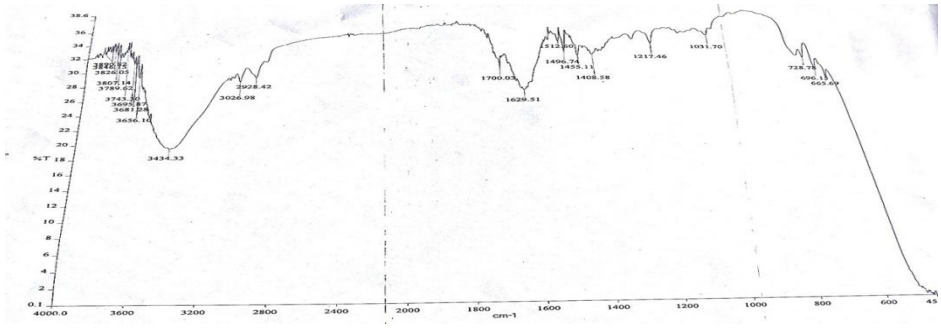



**Fig 5.1.2.4.4 (B): Infra-Red spectrum of isolated compound (B2) from *A. galanga***

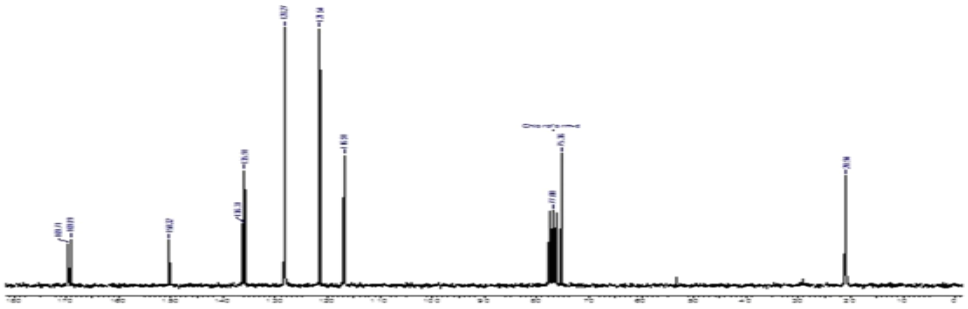


**Fig 5.1.2.4.4 (C): $^{13}$C-NMR spectrum of isolated compound (B2) from *A. galanga***

**Fig 5.1.2.4.4 (D): DEPT spectrum of isolated compound (B2) from *A. galanga***

### 5.1.2.4.5 Structure assigned to isolated compound (B2)

Compound (B2), yellow oil, (α) $D^{25}$:- 52.80°(C 0.2, ethanol); IR (film) $\lambda_{max}$ ($cm^{-1}$): 1741, 1607, 1508, 1424, 1371, 1215;$^{1}$H-NMR ($CDCl_3$, 200$MH_z$) δ: 7.35(2H, d, J=8.5$H_z$, H-2 and H-6), 7.05 (2H, d, J=8.5$H_z$, H-3 and H-5), 6.25 (1H, d, 8.0$H_z$, H-1δ), 5.98 (1H ddd, J=17.0, 10.4, 6.0$H_z$, H-2δ), 5.27 (1H, dt, J=17.0, 1.2$H_z$, H-3δ), 5.22 (1H, dt, J=10.4, 1.2$H_z$,H-3δ), 2.28 (3H, s, OCO$\underline{CH_3}$) and 2.10 (3H, s, OCO$\underline{CH_3}$); $^{13}$C-NMR ($CDCl_3$, 50$MH_z$) δ: 169.71(S, $\underline{OCO}CH_3$), 169.19 (S, $\underline{OCO}CH_3$), 20.94 (S, 2× OCO$\underline{CH_3}$), 135.91 (S, C-1), 128.27 (d, C-2, C-6), 121.54(d, C-3, C-5), 150.32 (S, C-4), 75.36 (d, C-1δ), 136.31 (d, C-2δ) and 116.91 (t, C-3δ); GCMS m/z (% rel.int.) 234 ($[M]^+$, 3), 192 (25), 174 (5), 150 (42), 132 (50) and 43(100).

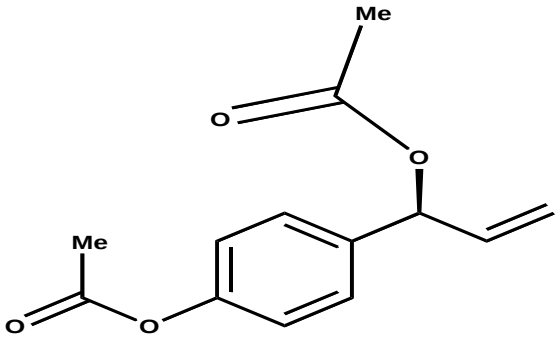



**Fig 5.1.2.4.5: Structure of isolated molecule from *A. galanga* (L.) willd**

**PART-II**

**5.2 *A. officinarum***

**5.2.1 PHARMACOGNOSTIC STUDIES**

**5.2.1.1 Macroscopic studies**

*A. officinarum* rhizome is a somewhat curved and cylindrical rhizome, sometimes branched; 2.8 cm in length, 6. 15 mm in length; externally red-brown to dark brown with fine striped lines, grayish white nodes and a number of traces of rootlet; hard to break; fracture surface, light brown in color and thickness of cortex is just about the same as that of stele. Odour is characteristic, taste is extremely pungent.

**5.2.1.2 Microscopic studies**

**5.2.1.2.1 TS of rhizome**

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

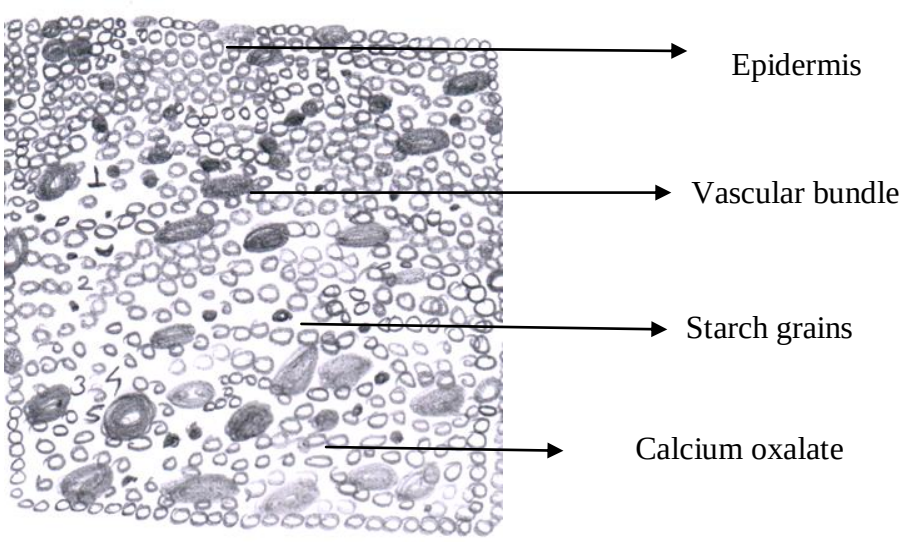



**Fig. 5.2.1.2.1: T. S. of rhizome of *A. officinarum***

### 5.2.1.3 Powder microscopy

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

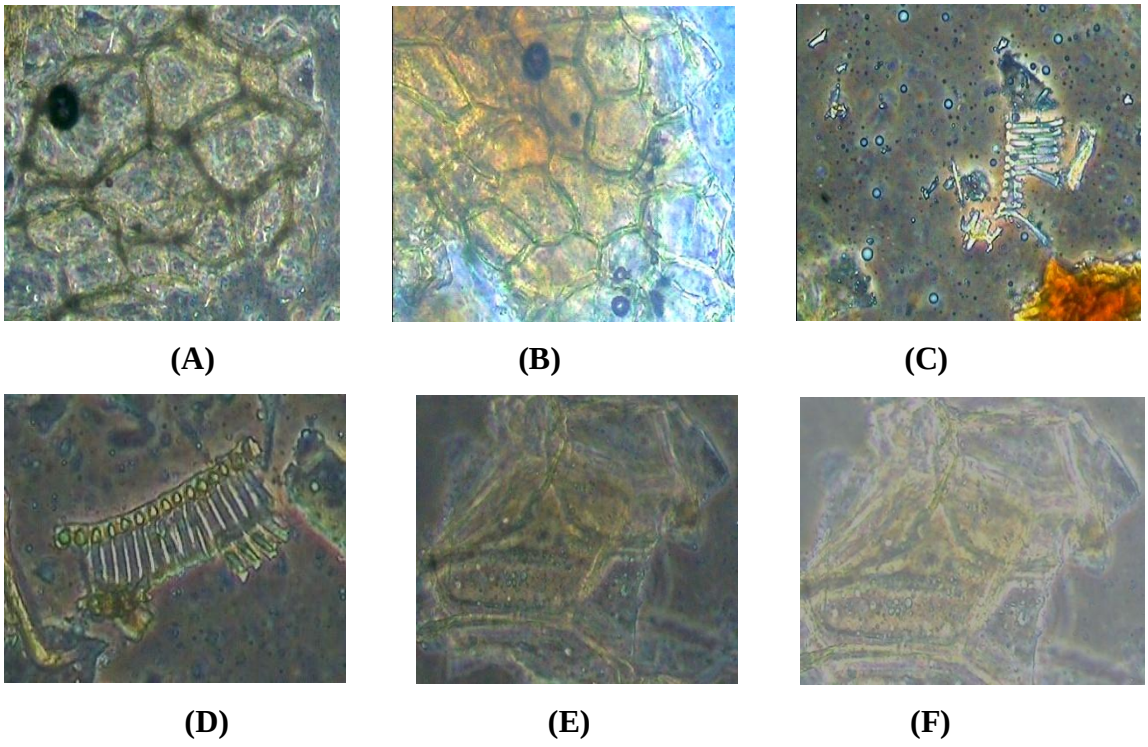



**Fig. 5.2.1.3: Powder microscopy of *A. officinarum*: (A) Parenchymatous cells, (B) Epidermal cells, (C) Fragments of vessels, (D) Vessels with scalariform thicknings, (E and F) Parenchyma cells containing starch grains.**

### 5.2.1.4 Physicochemical analysis

#### 5.2.1.4.1 Ash value

The residue left behind after incineration of plant material is the ash content or ash value, which basically represents inorganic salts, naturally occurring in crude drug or adhering to it or purposely added to it as a form of adulteration. The percentage of active chemical constituents in crude drugs is mentioned on air-dried basis. Therefore, the loss on drying of plant materials should be determined and the water content should also be controlled. This is especially important for materials that take up moisture easily or deteriorate quickly in presence of water. The test for loss on drying determines both water and volatile matter.

The acid insoluble ash consist mainly silica and indicate the presence of contaminate with earthy material. The water soluble ash is used to determine the amount of inorganic elements present in drugs

**Table 5.2.1.4.1: Physicochemical parameters of *A. officinarum* rhizome.**

| Sr. No. | Parameters | Values (%) |
|---|---|---|
| 1. | Total ash | 7.5 |
| 2. | Acid in soluble ash | 1.5 |
| 3. | Water soluble ash | 1.6 |
| 4. | Loss on drying at110°C | 1.5 |

### 5.2.1.4.2 Extractive values of *A. officinarum* rhizome

The extractive values are useful to evaluate the chemical constituents present in the crude drug and also assist in estimation of specific constituents soluble in a particulars solvent.

**Table 5.2.1.4.2: Extractive values of *A. officinarum* rhizome**.

| Sr. No. | Solvents | Extractive values (%w/w) |
|---|---|---|
| 1. | Pet-ether | 0.60 |
| 2. | Acetone | 1.8 |
| 3. | Methanol | 2.7 |
| 4. | Water | 1.6 |

### 5.2.1.5 Preliminary phytochemical studies

Preliminary phytochemical screening was useful in prediction of nature of drugs and also useful for the detection of several constituents present in different polarity solvent. So it could be useful to extract out particular constituents by solvent. The alcoholic extract revealed the presence of alkaloids, tannins, coumarins, terpenoids and phenolics while aqueous revealed the presence of carbohydrates, tannins, glycosides, aminoacids, phenols, gums and saponins.

**Table 5.2.1.5: Phytochemical analysis of different extracts of *A. officinarum* rhizome**

| Sr. no. | Chemical Test | Observations of different extracts of *Alpinia officinarum* | | | |
|---|---|---|---|---|---|
| | | **Pet. ether** | **Acetone** | **Methanol** | **Aqueous** |
| Tests for Carbohydrates | | | | | |
| 1. | Molisch's test | - | - | - | - |
| Tests for Proteins | | | | | |
| 2. | Biuret test | - | - | + | - |
| 3. | Millon's test | - | - | - | - |
| Tests for Steroids | | | | | |
| 4. | Salkowski reaction | - | - | - | - |
| 5. | Liebermann-Burchard reaction | - | - | - | - |
| Test for Volatile oils | | | | | |
| 6. | Solubility test | - | - | - | - |
| Tests for Glycosides | | | | | |
| 7. | Keller-Killani test | + | + | + | + |
| 8. | Borntrager's test | + | + | + | + |
| Tests for Saponins | | | | | |
| 9. | Foam test | - | - | - | - |
| Test for Tannins and Phenolic compounds | | | | | |
| 10. | 5%Ferric chloride (3 ml) | - | - | - | + |
| 11. | Lead acetate | + | + | + | + |
| 12. | Potassium permanganate | + | + | + | + |
| Test for presence of Flavonoids | | | | | |
| 13. | Shinoda test | - | + | + | + |
| Tests for Alkaloids | | | | | |
| 14. | Dragendorff's test | + | + | + | + |
| 15. | Mayer's test | + | + | - | + |
| 16. | Wagner's test | + | + | - | + |
| 17. | Hager's test | - | - | - | + |

(+Present;-Absent)

### 5.2.1.6 Morphological characterization of extracts of *A. officinarum* rhizome

All the extracts are brown to brownish yellow in colour and showed semisolid consistency while aqueous extract is brown in colour and showed solid consistency.

**Table 5.2.1.6: Table shows morphological characterization of extracts of *Alpinia officinarum* rhizome.**

| Sr. No. | Extracts | Colour | Consistency |
|---|---|---|---|
| 1. | Pet.ether | Brownish yellow | Semisolid |
| 2. | Acetone | Brownish yellow | Semisolid |
| 3. | Methanol | Brownish yellow | Semisolid |
| 4. | Water | Brown | Powder |

### 5.2.1.7 Fluorescence analysis of rhizome extracts of *A. officinarum*.

**Table 5.2.1.7: Table shows fluorescence analysis of extracts of *A. officinarum* rhizome.**

| Sr. No. | Extracts | Visible light | Short UV light | Long UV light |
|---|---|---|---|---|
| **1.** | Pet.ether | Yellowish Brown | Emerald Green | Brown |
| **2.** | Acetone | Brownish yellow | Yellowish green | Pale Green |
| **3.** | Methanol | Emerald Green | Yellowish Brown | Pale Green |
| **4.** | Water | Brown | Pale Green | Reddish Orange |

## 5.2.2 PHARMACOLOGICAL STUDY

### 5.2.2.1 Acute oral toxicity test (AOT) of *A. officinarum*

Administration of 2000 mg/kg of the four extracts of *A. officinarum* did not produce any behavioral abnormalities and mortality (Table 5.2.2.1). So the dose selected for further study was 100, 200 and 400 mg/kg for each extracts.

**Table 5.2.2.1: Acute toxicity test of *A. officinarum***

| Sr. No. | Extracts 2000 mg/kg p.o. | No. of animals dead/survived |
|---|---|---|
| 1. | Pet.ether extract of *A. officinarum* | 0/5 |
| 2. | Acetone extract of *A. officinarum* | 0/5 |
| 3. | Methanolic extract of *A. officinarum* | 0/5 |
| 4. | Aqueous extract of *A. officinarum* | 0/5 |

### 5.2.2.2 Anti-inflammatory activity

#### 5.2.2.2.1 Carrageenan induced inflammation in rats

Intradermal injection of carrageenan into subplantar region of right hind paw caused significant increase in the paw edema, which was recorded as change in paw volume at 3rd h 2.87± 0.063ml and at 5th h 3.80± 0.061ml. Diclofenac (10 mg/kg) was administered 1 h before the injection of carrageenan caused significant ($P<0.01$ and $P<0.001$) inhibition of increase in paw edema at 3rd and 5th h respectively. The inhibitory effect of the diclofenac was recorded (10.86%) at 3rd h and (26.12%) at 5th h. All four extracts of *A. officinarum* was administered for 7 days before the injection of carrageenan. The inhibitory effect of the pet.ether extract of *A. officinarum* was recorded with a dose of 200 and 400 mg/kg at 5th h (4.95%) and (12.34%). The inhibitory effect of the acetone extract of *A. officinarum* was recorded with a dose of 200 and 400 mg/kg at at 5th h (2.97%) and (7.00%) respectively. The inhibitory effect of the methanolic extract of *A. officinarum* was recorded with a dose of 200 and 400 mg/kg at 3rd h (9.47%), (10.51%) and 5th h (15.53%) and (24.74%). The inhibitory effect of the aqueous extract of *A. officinarum* was recorded with a dose of 200 and 400 mg/kg at 5th h (4.35%) and (10.72%). The inhibition elicited by the methanolic extract of *A. officinarum* (MEAO) was comparable to that of diclofenac. (Table 5.2.2.2.1)

**Table 5.2.2.2.1: Effect of oral administration of four extract of *A. officinarum* on inhibition of right hind paws edema on carrageenan induced inflammation in rats**

| Treatment Groups | Change in paw edema volume (ml) | | | % Inhibition at | | |
|---|---|---|---|---|---|---|
| | 1 h | 3 h | 5 h | 1 h | 3 h | 5 h |
| Carrageenan control | 0.99±0.143 | 2.87± 0.063 | 3.80± 0.061 | | | |
| Diclofenac 10 mg/kg | 0.97 ± 0.060 | 2.56± 0.098$^{*}$ | 2.80± 0.105$^{***}$ | 2.26 | 10.86 | 26.12 |
| Pet.ether extract of *A. officinarum* 100 mg/kg | 0.74 ± 0.027 | 1.24 ± 0.028 | 1.32 ± 0.030 | -0.23 | 1.36 | 6.29 |
| Pet.ether ext. of *A. officinarum* 200 mg/kg | 0.74 ± 0.023 | 1.22 ± 0.010 | 1.30 ± 0.015 | 0.00 | 2.96 | 7.59 |
| Pet.ether ext. of *A. officinarum* 400 mg/kg | 0.74 ± 0.033 | 1.19 ± 0.029 | 1.23 ± 0.027$^{**}$ | 0.45 | 4.95 | 12.34 |
| Acetone ext. of *A. officinarum* 100 mg/kg | 0.74 ± 0.041 | 1.25 ± 0.039 | 1.40 ± 0.041 | 0.00 | 0.03 | 0.59 |
| Acetone ext. of *A. officinarum* 200 mg/kg | 0.74 ± 0.068 | 1.23 ± 0.072 | 1.36 ± 0.071 | 0.23 | 2.16 | 2.97 |
| Acetone ext. of *A. officinarum* 400 mg/kg | 0.74 ± 0.035 | 1.20 ± 0.043 | 1.31 ± 0.035 | 0.45 | 4.42 | 7.00 |
| Methanolic ext. of *A. officinarum* 100 mg/kg | 1.00 ± 0.134 | 2.69± 0.087 | 3.33± 0.087 | -0.50 | 6.52 | 12.37 |
| Methanolic ext. of *A. officinarum* 200 mg/kg | 0.98± 0.162 | 2.60± 0.085 | 3.21± 0.246 | 1.25 | 9.47 | 15.53 |
| Methanolic ext. of *A. officinarum* 400 mg/kg | 0.99± 0.070 | 2.57±0.158 | 2.86± 0.068$^{***}$ | 0.75 | 10.51 | 24.74 |
| Aqueous ext. of *A. officinarum* 100 mg/kg | 1.00 ± 0.07 | 2.87± 0.09 | 3.69± 0.10 | 3.35 | 4.24 | 3.83 |
| Aqueous ext. of *A. officinarum* 200 mg/kg | 1.03± 0.16 | 2.89± 0.093 | 3.67± 0.11 | 1.19 | 3.57 | 4.35 |
| Aqueous ext. of *A. officinarum* 400 mg/kg | 1.03± 0.08 | 2.77± 0.10 | 3.43± 0.11 | 0.71 | 7.73 | 10.72 |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with carrageenan control $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

**5.2.2.2.2 Cotton pellet granuloma in rats**

Subcutaneous implantation of 50 mg of cotton pellet in rats caused significant increase in weight of cotton pellet, recorded as 96.17 ± 2.67 mg which was due to granuloma formation. Diclofenac (10 mg/kg) and methanolic extract of *A. officinarum* (400 mg/kg) significantly (P<0.001) inhibited the granuloma formation by 73.66 and 63.78.

**5.2.2.2.2: Effect of oral administration of four extract of *A. officinarum* on cotton pellet granuloma in rats**

| Sr. No. | Treatment Groups | Dose mg/kg p.o. | Dry granuloma weight (mg) (Mean ± SEM) | % Inhibition |
|---|---|---|---|---|
| 1. | Vehicle control | | 96.17 ± 2.67 | |
| 2. | Diclofenac | 10 | 25.33 ± 2.29*** | 73.66122 |
| 3. | Pet.ether ext. of *A. officinarum* | 100 | 100 ± 1.90 | -5.26 |
| 4. | Pet.ether ext. of *A. officinarum* | 200 | 95 ± 2.90 | --- |
| 5. | Pet.ether ext. of *A. officinarum* | 400 | 95 ± 3.50 | --- |
| 6. | Acetone ext. of *A. officinarum* | 100 | 93 ± 3.50 | 2.11 |
| 7. | Acetone ext. of *A. officinarum* | 200 | 91 ± 3.70 | 4.21 |
| 8. | Acetone ext. of *A. officinarum* | 400 | 93 ± 2.70 | 2.11 |
| 9. | Methanolic ext. of *A. officinarum* | 100 | 89.67 ± 2.51 | 6.758865 |
| 10. | Methanolic ext. of *A. officinarum* | 200 | 84 ± 2.23** | 12.65467 |
| 11. | Methanolic ext. of *A. officinarum* | 400 | 34.83 ± 2.25*** | 63.78288 |
| 12. | Aqueous ext. of *A. officinarum* | 100 | 90 ± 2.34 | 5.43 |
| 13. | Aqueous ext. of *A. officinarum* | 200 | 92.23 ± 2.11 | 3.21 |
| 14. | Aqueous extract of *A. officinarum* | 400 | 94.53 ± 2.76 | 1.73 |

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test when compared with vehicle control *P<0.05, **P<0.01, ***P<0.001.

### 5.2.2.3 Anti-arthritic activity (MEAO)

**[A] Body weight**

**5.2.2.3.1 Effect of oral administration of methanolic extract of *A. officinarum* Hance (MEAO) on body weights in arthritic rats**

Body weight was recorded at every four days up to 28 days. The body weight of rats was recorded at regular intervals during the course of the study. The body weight of CFA induced arthritic control (group II) rats was less significantly reduced compared to non arthritic rats (group I) on 28$^{th}$ day. The body weight reduction MEAO (400 mg) treated rats was non-significant compared that of arthritic rats (Table 5.2.2.3.1).

**Table 5.2.2.3.1: Effect of oral administration of methanolic extract of *A. officinarum* Hance (MEAO) on body weights in arthritic rats**

| Sr. No. | Treatment Groups | Body weight (gm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 190.25± 7.42 | 203.5± 6.98 | 208.5± 6.98 | 213.0± 6.89 | 216.7± 6.98 | 220.7± 6.86 |
| 2. | Arthritic control | 188.2± 13.30 | 182.5± 13.76 | 180.2± 13.39 | 177.7± 13.66 | 175.7± 13.30 | 172.5± 13.46 |
| 3. | Diclofenac 5 mg/kg | 187.5± 8.13 | 181.0 ± 8.02 | 177 ± 7.62 | 179.75 ± 7.43 | 184 ± 7.77 | 188 ± 7.64 |
| 4. | MEAO100 mg/kg | 190 ± 10.26 | 183± 10.34 | 179.7 ± 10.57 | 176.7 ± 10.49 | 174.2 ± 10.64 | 178.2 ± 10.96 |
| 5. | MEAO 200 mg/kg | 191 ± 10.57 | 184.2 ± 10.08 | 179.7 ± 10.33 | 177 ± 10.18 | 181 ± 10.55 | 185 ± 10.55 |
| 6. | MEAO 400 mg/kg | 199.7 ± 9.18 | 193± 9.48 | 188.2 ± 9.75 | 192.2 ± 9.75 | 196.2 ± 9.75 | 200.7 ± 9.56 |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

**[B] Paw volume**

**5.2.2.3.2 Effect of oral administration of methanolic extract of *A. officinarum* Hance (MEAO) on right hind paw volume in arthritic rats**

One day after the CFA injection, primary arthritis of the right hind paw was induced, and inflammation was maintained up to 28 days (Table. 5.2.2.3.2). Oral administration of MEAO (100, 200 and 400 mg/kg) and diclofenac (5 mg/kg) from 12$^{th}$ day of arthritis induction significantly (P<0.001) suppressed the increased paw edema up to 28$^{th}$ day.

**Table 5.2.2.3.2: Effect of oral administration of methanolic extract of *A. officinarum* Hance (MEAO) on right hind paw volume in arthritic rats**

| Sr. No. | Treatment Groups | Change in Paw volume (ml) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 0.022 ± 0.006 | 0.052± 0.011 | 0.062 ± 0.017 | 0.070 ± 0.012 | 0.082 ± 0.007 | 0.075 ± 0.010 |
| 2. | Arthritic control | 1.15± 0.017 | 1.70 ± 0.010 | 1.73 ± 0.004 | 1.75 ± 0.007 | 1.78 ± 0.010 | 1.81 ± 0.013 |
| 3. | Diclofenac 5 mg/kg | 1.14 ± 0.022 | 1.69 ± 0.021 | 1.61 ± 0.024 | 1.47 ± 0.024 | 1.37 ± 0.021 | 1.30 ± 0.034*** |
| 4. | MEAO100 mg/kg | 1.15 ± 0.011 | 1.69 ± 0.03 | 1.72 ± 0.027 | 1.73 ± 0.033 | 1.62 ± 0.029 | 1.51 ± 0.04*** |
| 5. | MEAO 200 mg/kg | 1.14± 0.020 | 1.69 ± 0.024 | 1.71 ± 0.025 | 1.64 ± 0.014 | 1.57 ± 0.027 | 1.48 ± 0.034*** |
| 6. | MEAO 400 mg/kg | 1.13 ± 0.04 | 1.68 ± 0.086 | 1.70 ± 0.082 | 1.61 ± 0.086 | 1.51 ± 0.067 | 1.47± 0.081*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001.

**[C] Joint diameter**

**5.2.2.3.3 Effect of oral administration of methanolic extract of *A. officinarum* Hance (MEAO) on right hind joint diameter in arthritic rats**

The joint diameter was measured again on day (1, 4, 8, 12, 16, 20, 24 and 28). MEAO (100, 200 and 400 mg/kg) and diclofenac (5 mg/kg) from 12$^{th}$ day of arthritis induction significantly (P<0.001) reduce the right hind joint diameter up to 28$^{th}$ day (Table 5.2.2.3.3).

**Table 5.2.2.3.3: Effect of oral administration of methanolic extract of *A. officinarum* Hance (MEAO) on right hind joint diameter in arthritic rats**

| Sr. No. | Treatment Groups | Change in Joint diameter (mm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 0.022 ± 0.011 | 0.057± 0.011 | 0.077 ± 0.014 | 0.092 ± 0.015 | 0.10 ± 0.011 | 0.11 ± 0.010 |
| 2. | Arthritic control | 3.13± 0.17 | 3.25 ± 0.15 | 3.27 ± 0.15 | 3.30 ± 0.15 | 3.33 ± 0.15 | 3.36 ± 0.14 |
| 3. | Diclofenac 5 mg/kg | 2.92 ± 0.18 | 3.02 ± 0.19 | 2.65 ± 0.20 | 2.24 ± 0.19 | 2.08 ± 0.19 | 1.76 ± 0.19*** |
| 4. | MEAO100 mg/kg | 3.09 ± 0.20 | 3.15 ± 0.22 | 2.80 ± 0.31 | 2.65 ± 0.31 | 2.47 ± 0.31 | 2.25 ± 0.31*** |
| 5. | MEAO 200 mg/kg | 3.03± 0.07 | 3.14 ± 0.05 | 2.78 ± 0.05 | 2.60 ± 0.05 | 2.37 ± 0.06 | 2.13 ± 0.07*** |
| 6. | MEAO 400 mg/kg | 3.01 ± 0.14 | 3.15 ± 0.15 | 2.72 ± 0.27 | 2.41 ± 0.26 | 2.13 ± 0.28 | 1.88± 0.31*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001.

**[D] Mechanical hyperalgesia**

**5.2.2.3.4 Effect of oral administration of methanolic extract of *A. officinarum* Hance (MEAO) on right hind paw mechanical withdrawal threshold in arthritic rats (Tactile allodynia)**

The dose-dependent effects of MEAO are presented in (Table. 5.2.2.3.4). The mechanical withdrawal threshold (MWT) of the right hind paw was decreased in arthritic rat compared to the basal level 1 after the CFA injection. The oral treatment of diclofenac (5 mg/kg) and MEAO (200 and 400 mg/kg) significantly ($P<0.001$) suppressed the MWT on 28$^{th}$ day, while the lower doses of MEAO showed lesser suppression of the MWT 28$^{th}$ day.

**Table 5.2.2.3.4: Effect of oral administration of methanolic extract of *A. officinarum* Hance (MEAO) on right hind paw mechanical withdrawal threshold in arthritic rats (Tactile allodynia)**

| Sr. No. | Treatment Groups | Mechanical Withdrawal Threshold (gm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 69.02 ± 2.94 | 73.65± 5.09 | 69.60 ± 3.53 | 72.32 ± 3.57 | 71.85 ± 4.40 | 66.39 ± 3.41 |
| 2. | Arthritic control | 73.57± 4.96 | 30.12 ± 1.48 | 30.60 ± 3.16 | 31.10 ± 1.88 | 30.02 ± 1.95 | 30.22 ± 2.40 |
| 3. | Diclofenac 5 mg/kg | 71.62 ± 5.89 | 28.05 ± 3.21 | 30.62 ± 2.49 | 50.65 ± 4.08 | 70.42 ± 3.38 | 75.03 ± 3.09*** |
| 4. | MEAO100 mg/kg | 70.40 ± 3.96 | 28.80 ± 2.80 | 28.17 ± 2.82 | 30.55 ± 2.49 | 31.07 ± 3.43 | 45.32 ± 2.29** |
| 5. | MEAO 200 mg/kg | 70.42 ± 5.14 | 31.10 ± 2.16 | 31.87 ± 2.03 | 30.27 ± 2.34 | 46.27 ± 3.36 | 68.30 ± 2.70*** |
| 6. | MEAO 400 mg/kg | 71.60 ± 4.09 | 30.22 ± 1.32 | 30.20 ± 2.15 | 47.17 ± 3.17 | 64.20 ± 2.15 | 71.55 ± 3.47*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001.

**[E] Thermal hyperalgesia**

**5.2.2.3.5 Effect of oral administration of methanolic extract of *A. officinarum* Hance (MEAO) right hind paw withdrawal latency in arthritic rats (Thermal hyperalgesia)**

The dose-dependent effects of MEAO are presented in (Table 5.2.2.3.5). The paw withdrawal latency (PWL) of the right hind paw of arthritic rats was decreased compared to non arthritic rats. Treatment with diclofenac (5 mg/kg) and MEAO (200 and 400 mg/kg) showed significant ($P<0.001$) increase in PWL on 28$^{th}$ day, while the lower doses of MEAO showed less significant ($P<0.01$) reduction in the PWL.

**Table 5.2.2.3.5: Effect of oral administration of methanolic extract of *A. officinarum* Hance (MEAO) right hind paw withdrawal latency in arthritic rats (Thermal hyperalgesia)**

| Sr. No. | Treatment Groups | Paw Withdrawal Latency (sec) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 11.25 ± 0.36 | 11.15± 0.63 | 10.95 ± 0.70 | 10.70 ± 0.48 | 11.70 ± 0.67 | 11.22 ± 0.49 |
| 2. | Arthritic control | 9.97± 0.48 | 4.97 ± 0.31 | 4.90 ± 0.29 | 5.12 ± 0.28 | 4.95 ± 0.25 | 4.80 ± 0.28 |
| 3. | Diclofenac 5 mg/kg | 10.82 ± 0.32 | 5.32 ± 0.24 | 5.20 ± 0.37 | 8.10 ± 0.28 | 10.35 ± 0.18 | 11.15 ± 0.27*** |
| 4. | MEAO100 mg/kg | 10.30 ± 0.35 | 4.87 ± 0.22 | 4.67 ± 0.20 | 5.12 ± 0.25 | 4.87 ± 0.16 | 6.55 ± 0.27** |
| 5. | MEAO 200 mg/kg | 10.67 ± 0.39 | 5.20 ± 0.24 | 5.17 ± 0.20 | 5.00 ± 0.33 | 6.40 ± 0.10 | 11.20 ± 0.32*** |
| 6. | MEAO 400 mg/kg | 10.47 ± 0.26 | 5.20 ± 0.19 | 5.00 ± 0.36 | 4.82 ± 0.57 | 10.0 ± 0.35 | 10.75 ± 0.30*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001.

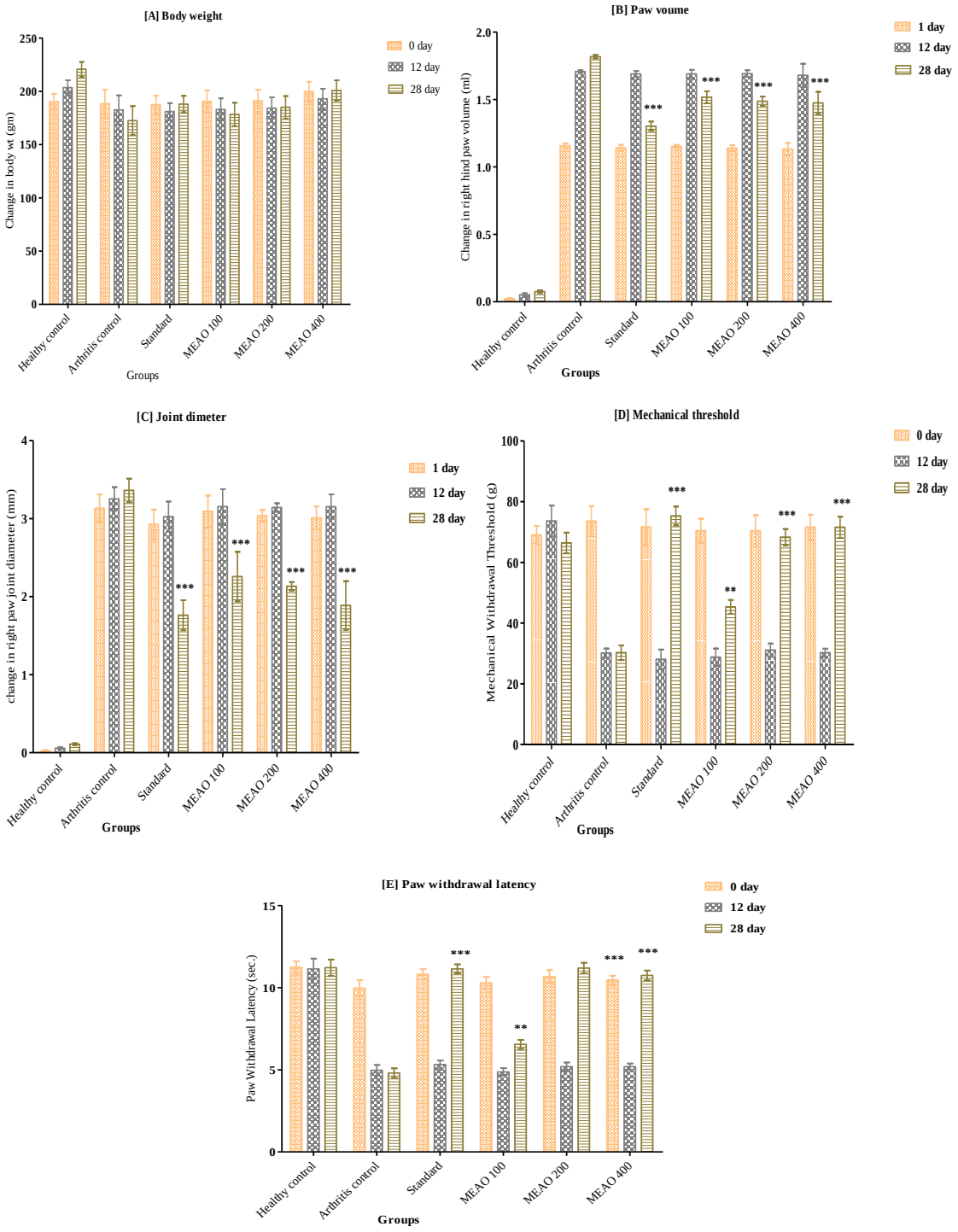



**Fig 5.1.2.3 Effect of MEAO on [A] Body weight, [B] Paw volume, [C] Joint diameter, [D] Mechanical hyperalgesia, [E] Thermal hyperalgesia**

### 5.2.2.4 Isolation of bioactive Fraction from MEAO by bioassay guided fractionation method.

The rhizomes were shade dried and powdered in hand mixer. The dried powder of the rhizomes of *A. officinarum* (500 g) was extracted with 1.5L methanol as a solvent by cold percolation for 12h in a 5L flat bottom flask at room temperature. The process of extraction was repeated three times with methanol. Each time the filtrate was concentrated *in vacuo* at 40°C using a rotary evaporator (Eqitron, Roteva), and pooled together to obtain 70 g of extract. The methanol extract (20.0 g) was redissolved in methanol: water (80:20) in 1 lit and partitioned with n-hexane followed by ethyl acetate gradient (0-100%) as mobile phase with the increasing polarity of ethyl acetate. The material was eluted stepwise to obtain six Fractions (Fraction A to F). The 100 % ethyl acetate Fraction B (9.5 g) shows significant ($P<0.001$) anti-inflammatory activity in carrageenan induced paw edema therefore it was subjected to column chromatography over silica gel (100-200 mesh). Four Fractions were obtained (Fraction I, II, III, and IV). Fraction III shows significant ($P<0.001$) anti-inflammatory activity in carrageenan induced paw edema therefore Fraction III was further purification by repeated column chromatography yield compound as a yellow crystal, identification of which was performed by comparison of the spectral data of mass and nuclear magnetic resonance to that of reported compound.

#### 5.2.2.4.1 Anti-inflammatory activity

##### 5.2.2.4.1.1 Anti-inflammatory activity of 6 Fractions from *A. officinarum* in carrageenan induced paw edema (Acute study)

The rats were pretreated with all the 6 Fractions from *A. officinarum* and diclofenac for 1 h before the injection of carrageenan caused inhibition of increase in paw edema from 1 h to 5 h. Diclofenac (10 mg/kg) caused significant ($P<0.001$) inhibition of increase in paw edema at $5^{th}$ h. The inhibitory effect of the diclofenac at 10 mg/kg was recorded (14.89%) at $3^{rd}$ h and (26.01%) at $5^{th}$ h. The inhibitory effect of the 100% ethyl acetate Fraction was recorded at $3^{rd}$ h (12.56%) and at $5^{th}$ h (24.32%) respectively. The inhibition elicited by the 100% ethyl acetate Fraction was comparable to that of diclofenac. However there was less significant inhibition of increase in paw edema on treatment with n-hexane, 10%, 20%, 30%, %, and 50% ethyl acetate Fractions observed. (Table 5.2.2.4.1.1)

**Table 5.2.2.4.1.1: Effect of 6 Fractions from *A. officinarum* on inhibition of right hind paws edema on carrageenan induced inflammation in rats.**

| Treatment Groups | Change in paw edema volume (ml) | | | % Inhibition at | | |
|---|---|---|---|---|---|---|
| | 1 h | 3 h | 5 h | 1 h | 3 h | 5 h |
| Carrageenan control | 1.04 ± 0.148 | 3.00 ± 0.087 | 3.84 ± 0.060 | - | - | - |
| Diclofenac 10 mg/kg | 0.92 ± 0.076 | 2.55 ± 0.084$^{ns}$ | 2.84 ± 0.106$^{***}$ | 11.03 | 14.89 | 26.01 |
| N-hexane Fraction 100 mg/kg (A) | 1.05 ± 0.036 | 2.86 ± 0.10 | 3.26 ± 0.074 | -1.20 | 4.83 | 15.21 |
| 100 mg/kg (B) 10 % Ethyl acetate Fraction | 1.06 ± 0.15 | 2.89 ± 0.072 | 3.29 ± 0.24 | -2.16 | 3.83 | 14.43 |
| 20 % Ethyl acetate Fraction 100 mg/kg (C) | 1.07 ± 0.058 | 2.82 ± 0.134 | 3.17 ± 0.131 | -2.64 | 5.91 | 17.36 |
| 30 % Ethyl acetate Fraction 100 mg/kg (D) | 1.08 ± 0.034 | 2.91 ± 0.023 | 3.29 ± 0.032 | -4.08 | 2.91 | 14.30 |
| 50 % Ethyl acetate Fraction 100 mg/kg (E) | 1.03 ± 0.029 | 2.88 ± 0.056 | 3.17 ± 0.21 | 0.48 | 3.99 | 17.36 |
| Ethyl acetate Fraction 100 mg/kg (F) | 0.93 ± 0.13 | 2.62 ± 0.062$^{ns}$ | 2.91 ± 0.045$^{***}$ | 10.31 | 12.56 | 24.32 |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with carrageenan control $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

**5.2.2.4.1.2 Anti-inflammatory activity of 4 Fraction from 100% ethyl acetate Fraction from *A. officinarum* in carrageenan induced paw edema (Acute study)**

The rats were pretreated with all the 4 Fraction and diclofenac for 1 h before the injection of carrageenan caused inhibition of increase in paw edema from 1 h to 5 h. Diclofenac (10 mg/kg) caused significant (P<0.001) inhibition of increase in paw edema at 5$^{th}$ h. The inhibitory effect of the diclofenac at 10 mg/kg was recorded (13.53%) at 3$^{rd}$ h and

(25.72%) at 5th h. Fraction III (10 mg/kg) caused significant (P<0.001) inhibition of increase in paw edema at 5th h. The inhibitory effect of the Fraction III was recorded at 3rd h (12.19%) and at 5th h (24.87%) respectively. The inhibition elicited by the Fraction III was comparable to that of diclofenac. However there was no significant inhibition in increase in paw edema on treatment with Fraction I, II and IV. (Table 5.2.2.4.1.2)

**Table 5.2.2.4.1.2: Effect of 4 Fraction from 100% ethyl acetate Fraction from *A. officinarum* on inhibition of right hind paws edema on carrageenan induced inflammation in rats**

| Treatment Groups | Change in paw edema volume (ml) | | | % Inhibition at | | |
|---|---|---|---|---|---|---|
| | 1 h | 3 h | 5 h | 1 h | 3 h | 5 h |
| Carrageenan control | 1.01 ± 0.13 | 2.79 ± 0.15 | 3.82 ± 0.06 | - | - | - |
| Diclofenac 10 mg/kg | 0.89 ± 0.123 | 2.41 ± 0.28$^{ns}$ | 2.83 ± 0.066$^{***}$ | 11.85 | 13.53 | 25.72 |
| Fraction (I) 10 mg/kg | 1.04 ± 0.158 | 2.88 ± 0.081 | 3.58 ± 0.127 | -2.22 | -2.42 | 14.66 |
| Fraction (II) 10 mg/kg | 1.03 ± 0.061 | 2.88 ± 0.104 | 3.36 ± 0.24 | -0.74 | -1.79 | 24.35 |
| Fraction (III) 10 mg/kg | 0.91 ± 0.129 | 2.45 ± 0.047$^{ns}$ | 2.87 ± 0.117$^{***}$ | 10.12 | 12.19 | 24.87 |
| Fraction (IV) 10 mg/kg | 1.04 ± 0.049 | 2.87 ± 0.047 | 3.26 ± 0.057 | -1.98 | -2.06 | 9.39 |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with carrageenan control $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001.

### 5.2.2.4.2 Anti-inflammatory activity of bioactive Fraction (III)

#### 5.2.2.4.2.1 Acute oral toxicity study

Administration of 20 mg/kg of Fraction (III) did not produce any behavioral abnormalities and mortality. So the dose selected for further study was 5, 10 and 20 mg/kg for isolated Fraction (III).

### 5.2.2.4.2.2 Carrageenan induced paw edema in rats

Intradermal injection of carrageenan into subplantar region of right hind paw caused significant increase in the paw edema, which was recorded as change in paw volume at 3rd h 2.87± 0.063 and at 5th h 2.80± 0.061 ml. Diclofenac (10 mg/kg) were administered 1 h before the injection of carrageenan caused significant (P<0.001) inhibition of increase in paw edema at 5th h. The inhibitory effect of the diclofenac was recorded (10.86%) at 3rd h and (26.12%) at 5th h. The Fraction (III) was administered for 7 days before the injection of carrageenan caused dose dependent inhibition of increase in paw edema from 1 h to 5 h. The inhibitory effect of the galangin was recorded with a dose of 20 mg/kg at 5th h (26.32%). The inhibition elicited by the Fraction (III) was comparable to that of diclofenac.

**Table 5.2.2.4.2.2: Effect of oral administration of Fraction (III) from MEAO on inhibition of right hind paws edema on carrageenan induced inflammation in rats.**

| Treatment Groups | Change in paw edema volume (ml) | | | % Inhibition at | | |
|---|---|---|---|---|---|---|
| | 1 h | 3 h | 5 h | 1 h | 3 h | 5 h |
| Carrageenan control | 0.99± 0.143 | 2.87± 0.063 | 3.80± 0.061 | | | |
| Diclofenac 10 mg/kg | 0.97 ± 0.060 | 2.56± 0.098* | 2.80 ± 0.105*** | 2.26 | 10.86 | 26.12 |
| Fraction (III) 5 mg/kg | 0.98 ±0.045 | 2.59 ± 0.045 | 3.20 ± 0.043 | 1.50 | 9.73 | 15.66 |
| Fraction (III) 10 mg/kg | 0.97± 0.040 | 2.58± 0.066 | 2.80± 0.071 | 2.01 | 10.34 | 26.12 |
| Fraction (III) 20 mg/kg | 0.97 ± 0.035 | 2.57 ± 0.091 | 2.80 ± 0.121** | 2.26 | 10.43 | 26.32 |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with carrageenan control *P<0.05, **P<0.01, ***P<0.001.

### 5.2.2.4.2.3 Cotton pellet granuloma in rats

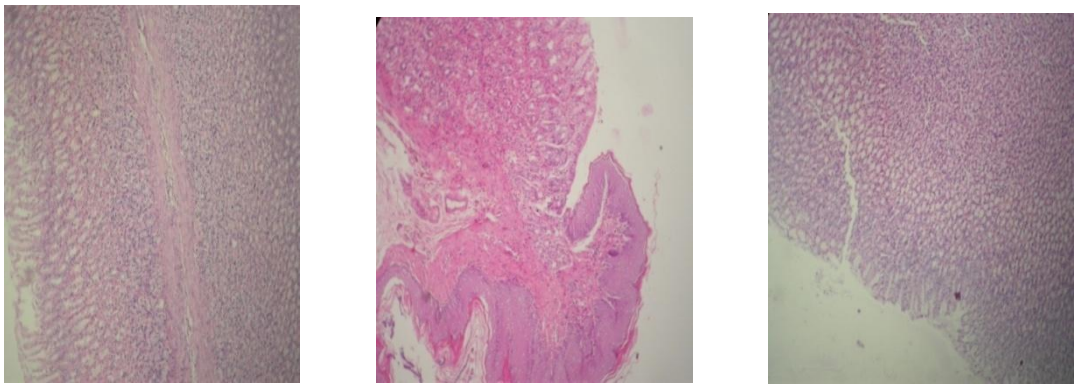
Subcutaneous implantation of 50 mg of cotton pellet in rats caused significant increase in weight of cotton pellet, recorded as 96.17 ± 2.67mg which was due to granuloma formation. Diclofenac (10 mg/kg) significantly (P<0.001) inhibited the granuloma formation by 73.66, whereas Fraction III (20 mg/kg) significantly (P<0.01) inhibited the granuloma formation by 64.30%. However there was no significant inhibition in granuloma formation on treatment with (galangin 10 and 20 mg/kg).

**Table 5.2.2.4.2.3: Effect of oral administration of Fraction (III) from MEAO on cotton pellet granuloma in rats**

| Sr. No. | Treatment Groups | Dose mg/kg p.o. | Dry granuloma weight (mg) (Mean ± SEM) | % Inhibition |
|---|---|---|---|---|
| 1. | Vehicle control | | 96.17 ± 2.67 | |
| 2. | Diclofenac | 10 | 25.33 ± 2.29*** | 73.66122 |
| 3. | Fraction (III) | 5 | 87.17 ± 1.10* | 9.358428 |
| 4. | Fraction (III) | 10 | 84.83 ± 2.28** | 11.79162 |
| 5. | Fraction (III) | 20 | 34.33 ± 1.99*** | 64.3028 |

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test when compared with vehicle control *P<0.05, **P<0.01, ***P<0.001.

### 5.2.2.4.2.4 Gastric ulcerogenic effect of oral administration of Fraction (III) on cotton pellet granuloma in rats



**A** **B** **C**

**Fig 5.2.2.4.2.4: Histopathology of stomach (A) Vehicle control (B) Diclofenac 10 mg / kg treated (C) Fraction (III) 20 mg/kg treated.**

### 5.2.2.4.3 Anti-arthritic studies of isolated bioactive Fraction (III)

### [A] Body weight

#### 5.2.2.4.3.1 Effect of oral administration of isolated bioactive Fraction (III) on body weights in arthritic rats

The body weight of rats was recorded at regular intervals during the course of the study. The body weight of CFA induced arthritic control (group II) rats was less significantly reduced compared to non arthritic rats (group I) on 28$^{th}$ day. The body weight reduction in Fraction III (20 mg) treated rats was non-significant compared that of arthritic rats (Table 5.2.2.4.3.1).

**Table 5.2.2.4.3.1: Effect of oral administration of isolated bioactive Fraction (III) on body weights in arthritic rats**

| Sr. No. | Treatment Groups | Body weight (gm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 190.25 ±7.42 | 203.5 ±6.98 | 208.5 ±6.98 | 213.0 ±6.89 | 216.7 ±6.98 | 220.7 ±6.86 |
| 2. | Arthritic control | 188.2± 13.30 | 182.5± 13.76 | 180.2± 13.39 | 177.7± 13.66 | 175.7± 13.30 | 172.5± 13.46 |
| 3. | Diclofenac 5 mg/kg | 187.5± 8.13 | 181.0± 8.02 | 177 ± 7.62 | 179.75 ±7.43 | 184 ± 7.77 | 188 ± 7.64 |
| 4. | Fraction (III) 5 mg/kg | 193.25 ±11.52 | 186.7± 11.2 | 183.2 ± 11.5 | 179.2 ±11.8 | 176.2± 11.9 | 181.7 ±12.2 |
| 5. | Fraction (III) 10 mg/kg | 192 ± 15.63 | 184.2± 14.2 | 180 ± 14.07 | 176 ± 14.07 | 180.5 ± 3.52 | 185.2 ±13.70 |
| 6. | Fraction (III) 20 mg/kg | 193.5 ± 11.58 | 186± 11.03 | 183 ± 11.23 | 187 ± 11.15 | 191.2 ±11.18 | 195.5 ±11.32 |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

**[B] Paw volume**

**5.2.2.4.3.2 Effect of oral administration of isolated bioactive Fraction (III) on right hind paw volume in arthritic rats**

One day after the CFA injection, primary arthritis of the right hind paw was induced, and inflammation was maintained up to 28 days (Table 5.2.2.4.3.2). Oral administration of Fraction III (5, 10 and 20 mg/kg) and diclofenac (5 mg/kg) from 12$^{th}$ day of arthritis induction significantly (P<0.001) suppressed the increased paw edema up to 28$^{th}$ day.

**Table 5.2.2.4.3.2 Effect of oral administration of isolated bioactive Fraction (III) on right hind paws volume in arthritic rats**

| Sr. No. | Treatment Groups | Change in Paw volume (ml) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 0.022 ± 0.006 | 0.052± 0.011 | 0.062 ± 0.017 | 0.070 ± 0.012 | 0.082 ± 0.007 | 0.075 ± 0.010 |
| 2. | Arthritic control | 1.15± 0.017 | 1.70 ± 0.010 | 1.73 ± 0.004 | 1.75 ± 0.007 | 1.78 ± 0.010 | 1.81 ± 0.013 |
| 3. | Diclofenac 5 mg/kg | 1.14 ± 0.022 | 1.69 ± 0.021 | 1.61 ± 0.024 | 1.47 ± 0.024 | 1.37 ± 0.021 | 1.30 ± 0.034*** |
| 4. | Fraction (III) 5 mg/kg | 1.15 ± 0.02 | 1.71± 0.069 | 1.73 ± 0.070 | 1.73 ± 0.068 | 1.60± 0.056 | 1.51 ± 0.049*** |
| 5. | Fraction (III) 10 mg/kg | 1.13 ± 0.034 | 1.68± 0.041 | 1.70 ± 0.045 | 1.63 ± 0.054 | 1.56 ± 0.058 | 1.47 ± 0.056*** |
| 6. | Fraction (III) 20 mg/kg | 1.11 ± 0.023 | 1.66± 0.041 | 1.66 ± 0.056 | 1.62 ± 0.065 | 1.45± 0.059 | 1.42 ± 0.052*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001.

**[C] Joint diameter**

**5.2.2.4.3.3 Effect of oral administration of isolated bioactive Fraction (III) on right hind joint diameter in arthritic rats**

Administration of CFA in the right hind paw resulted in significant (P<0.001) increase in the joint diameter of hind paw compared to that of non arthritic (control) rats. Fraction III (5, 10 and 20 mg/kg) and diclofenac (5 mg/kg) from 12$^{th}$ day of arthritis induction significantly (P<0.001) reduce the right hind joint diameter up to 28$^{th}$ day

**Table 5.2.2.4.3.3 Effect of oral administration of isolated bioactive Fraction (III) on right hind joint diameter in arthritic rats**

| Sr. No. | Treatment Groups | Change in Joint diameter (mm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 0.022 ± 0.011 | 0.057± 0.011 | 0.077 ± 0.014 | 0.092 ± 0.015 | 0.10 ± 0.011 | 0.11 ± 0.010 |
| 2. | Arthritic control | 3.13± 0.17 | 3.25 ± 0.15 | 3.27 ± 0.15 | 3.30 ± 0.15 | 3.33 ± 0.15 | 3.36 ± 0.14 |
| 3. | Diclofenac 5 mg/kg | 2.92 ± 0.18 | 3.02 ± 0.19 | 2.65 ± 0.20 | 2.24 ± 0.19 | 2.08 ± 0.19 | 1.76 ± 0.19*** |
| 4. | Fraction (III) 5 mg/kg | 3.07 ± 0.09 | 3.16 ± 0.10 | 2.78 ± 0.11 | 2.63± 0.12 | 2.37 ± 0.12 | 2.04 ± 0.09*** |
| 5. | Fraction (III) 10 mg/kg | 3.02 ± 0.16 | 3.10 ± 0.16 | 2.77 ± 0.13 | 2.57 ± 0.13 | 2.35± 0.07 | 2.01± 0.05*** |
| 6. | Fraction (III) 20 mg/kg | 2.99 ± 0.13 | 3.11 ± 0.13 | 2.70 ± 0.15 | 2.39 ± 0.14 | 2.12 ± 0.15 | 1.87 ± 0.18*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001.

**[D] Mechanical hyperalgesia**

**5.2.2.4.3.4 Effect of oral administration of isolated bioactive Fraction (III) on right hind paw mechanical withdrawal threshold in arthritic rats (Tactile allodynia)**

The dose-dependent effects of Fraction (III) are presented in (Table 5.2.2.4.3.4). The mechanical withdrawal threshold (MWT) of the right hind paw was decreased in arthritic rat compared to the basal level 1 after the CFA injection. The oral treatment of diclofenac (5 mg/kg) and Fraction III (10 and 20 mg/kg) significantly ($P<0.001$) suppressed the MWT on 28$^{th}$ day, while the lower doses of Fraction (III) showed lesser suppression of the MWT 28$^{th}$ day.

**Table 5.2.2.4.3.4: Effect of oral administration of isolated bioactive Fraction (III) on right hind paw mechanical withdrawal threshold in arthritic rats (Tactile allodynia)**

| Sr. No. | Treatment Groups | Mechanical Withdrawal Threshold (gm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 69.02 ±2.94 | 73.65± 5.09 | 69.60 ±3.53 | 72.32 ±3.57 | 71.85 ±4.40 | 66.39 ±3.41 |
| 2. | Arthritic control | 73.57± 4.96 | 30.12 ± 1.48 | 30.60 ± 3.16 | 31.10± 1.88 | 30.0± 1.95 | 30.22± 2.40 |
| 3. | Diclofenac 5 mg/kg | 71.62 ± 5.89 | 28.05 ± 3.21 | 30.62 ± 2.49 | 50.65± 4.08 | 70.4± 3.38 | 75.03± 3.09*** |
| 4. | Fraction (III) 5 mg/kg | 71.75 ± 4.15 | 27.67 ± 2.43 | 27.75 ± 2.94 | 29.30± 1.97 | 41.3± 3.06 | 43.97± 2.70* |
| 5. | Fraction (III) 10 mg/kg | 71.77 ± 3.72 | 30.92± 1.52 | 27.50 ± 2.11 | 35.57± 1.50 | 46.1± 2.83 | 70.47± 3.38*** |
| 6. | Fraction (III) 20 mg/kg | 72.82 ± 3.35 | 28.07 ± 1.67 | 28.55 ± 2.38 | 47.17± 3.34 | 72.4± 3.38 | 73.55± 2.81*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *$P<0.05$, **$P<0.01$, ***$P<0.001$.

**[E] Thermal hyperalgesia**

**5.2.2.4.3.5 Effect of oral administration of isolated bioactive Fraction (III) on right hind paw withdrawal latency in arthritic rats (Thermal hyperalgesia)**

The dose-dependent effects of Fraction (III) are presented in (Table 5.2.2.4.3.5). The paw withdrawal latency (PWL) of the right hind paw of arthritic rats was decreased compared to non arthritic rats. Treatment with diclofenac (5 mg/kg) and Fraction III (10 and 20 mg/kg) showed significant ($P<0.001$) increase in PWL on 28$^{th}$ day, while the lower doses of Fraction (III) showed less significant ($P<0.01$) reduction in the PWL.

**Table 5.2.2.4.3.5: Effect of oral administration of isolated bioactive Fraction (III) on right hind paw withdrawal latency in arthritic rats (Thermal hyperalgesia)**

| Sr. No. | Treatment Groups | Paw Withdrawal Latency (sec) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 day | 12 day | 16 day | 20 day | 24 day | 28 day |
| 1. | Vehicle control | 11.25 ± 0.36 | 11.15± 0.63 | 10.95 ± 0.70 | 10.70 ± 0.48 | 11.70 ± 0.67 | 11.22 ± 0.49 |
| 2. | Arthritic control | 9.97± 0.48 | 4.97 ± 0.31 | 4.90 ± 0.29 | 5.12 ± 0.28 | 4.95 ± 0.25 | 4.80 ± 0.28 |
| 3. | Diclofenac 5 mg/kg | 10.82 ± 0.32 | 5.32 ± 0.24 | 5.20 ± 0.37 | 8.10 ± 0.28 | 10.35 ± 0.18 | 11.15 ± 0.27*** |
| 4. | Fraction (III) 5 mg/kg | 10.40 ± 0.38 | 4.92 ± 0.30 | 5.02 ± 0.17 | 5.05 ± 0.15 | 6.55 ± 0.15 | 6.77 ± 0.16 |
| 5. | Fraction (III) 10 mg/kg | 10.72 ± 0.38 | 4.97 ± 0.11 | 4.97 ± 0.17 | 5.42 ± 0.26 | 6.80 ± 0.34 | 10.82 ± 0.19*** |
| 6. | Fraction (III) 20 mg/kg | 10.42 ± 0.35 | 5.00± 0.31 | 5.10 ± 0.16 | 6.57 ± 0.14 | 10.77 ± 0.13 | 11.32 ± 0.37*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001.

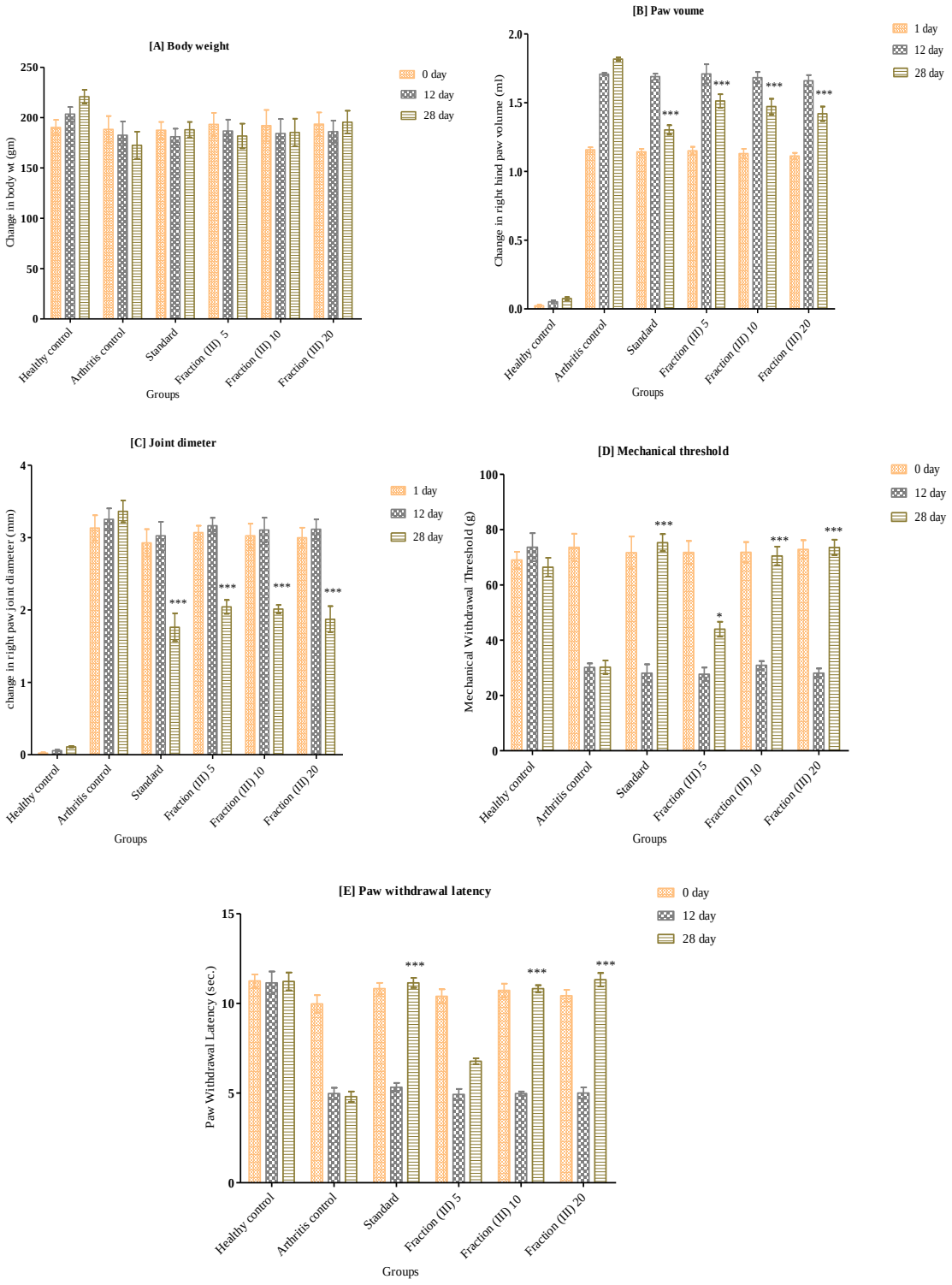



**Fig. 5.1.2.4.3 Effects of Fraction (III) [A] Body weight [B] Paw volume [C] Joint diameter [D] Thermal hyperalgesia [E] Mechanical hyperalgesia**

### 5.2.2.4.3.6 Haematological parameters

Arthritic rats showed significant (P<0.001) reduction in hemoglobin and RBC, on the other hand WBC and platelets count were significantly (P<0.01) increased compared to nonarthritic rats. Diclofenac (5 mg/kg) and Fraction III (20 mg/kg) significantly (P<0.001) increased the hemoglobin and RBC count. The WBC and platelet counts were significantly (P<0.001) reduced by high doses of Fraction III (20 mg/kg). Results were shown in (Table 5.2.2.4.3.6).

**Table 5.2.2.4.3.6 Effect of oral administration of isolated bioactive Fraction (III) on blood haematological parameters in arthritic rats**

| Treatment groups | Dose mg/kg | HB (gm/100 ml) | WBC (Thousands/µL) | RBC (Million/ µL) | Platelet (Lacks/µ L) |
|---|---|---|---|---|---|
| Healthy control | | 14.4 ± 0.322 | 7.63 ± 0.212 | 6.85 ± 0.113 | 9.24 ± 0.132 |
| Arthritic control | | 8.90 ± 0.182$^{\#}$ | 15.4 ± 0.255$^{\#}$ | 3.27 ± 0.074$^{\#}$ | 18 ± 0.127$^{\#}$ |
| Diclofenac | 5 | 13.2± 0.152*** | 14.3 ± 0.196** | 6.03 ± 0.119*** | 16.9 ± 0.287* |
| Fraction (III) | 5 | 9.43 ± 0.206 | 14.5 ± 0.244* | 3.66 ± 0.189 | 17.4 ± 0.274 |
| | 10 | 10.3 ± 0.146** | 14.3 ± 0.195** | 4.36 ± 0.242** | 17.1 ± 0.164 |
| | 20 | 11.5± .246*** | 13 ± 0.129*** | 5.30 ± 0.147*** | 16.1 ± .089*** |

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test when compared with arthritic control group $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001 and when arthritic control group compared with vehicle control group $^{\#}$P<0.001.

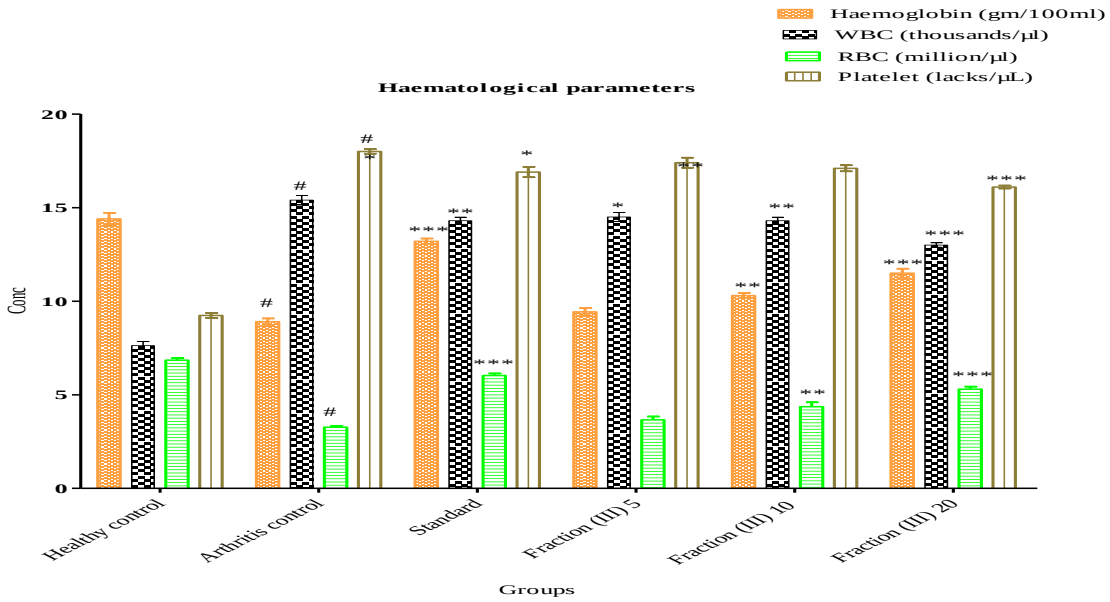



**Fig 5.2.2.4.3.6 Effect of oral administration of isolated bioactive Fraction (B2) on blood haematological parameters in arthritic rats**

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test when compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and when arthritic control group compared with vehicle control group $^{\#}P<0.001$.

**5.2.2.4.3.7 Biochemical parameters of Fraction (III)**

The markers for cellular toxicity (serum ALT, AST, and ALP level) were significantly increased (P<0.001), while TP level significantly (P<0.001) decreased in arthritic rats compared with the non arthritic rats. All of these changes were completely modulated in arthritic rats that received Fraction III from day 12$^{th}$ onwards. Fraction III (20 mg/kg) showed significant (P<0.001) decrease in the serum AST, ALT, and ALP levels; whereas the TP level was less significantly (P<0.001) increased as compared to arthritis control. Diclofenac (5mg/kg) showed insignificant reduction in the serum AST, ALT and ALP levels; whereas TP level showed insignificant increase compared to arthritis control.

**Table 5.2.2.4.3.7: Effect of oral administration of isolated bioactive Fraction (III) on serum biochemical parameters in arthritic rats**

| Treatment groups | Dose mg/kg | AST (U/L) | ALT (U/L) | ALP (U/L) | TP (gm/dl) |
|---|---|---|---|---|---|
| Healthy control | | 41.1 ± 1.89 | 53.5 ± 1.76 | 74.7 ± 1.88 | 7.25 ± 0.225 |
| Arthritic control | | 124 ± 2.83$^{\#}$ | 187 ± 1.99$^{\#}$ | 448 ± 2.99$^{\#}$ | 5.63 ± 0.229$^{\#}$ |
| Diclofenac | 5 | 117 ± 3.33 | 181 ± 2.21 | 438 ± 3.68 | 6.15 ± 0.263 |
| Fraction (III) | 5 | 113 ± 1.52* | 177 ± 2.11* | 436 ± 3.02* | 5.85 ± 0.144 |
| | 10 | 110 ± 4.09** | 173 ± .51*** | 434 ± 4.01* | 6.33 ± 0.170 |
| | 20 | 87 ± 2.73*** | 157 ± .85*** | 400 ± 1.86*** | 7.20 ± .129*** |

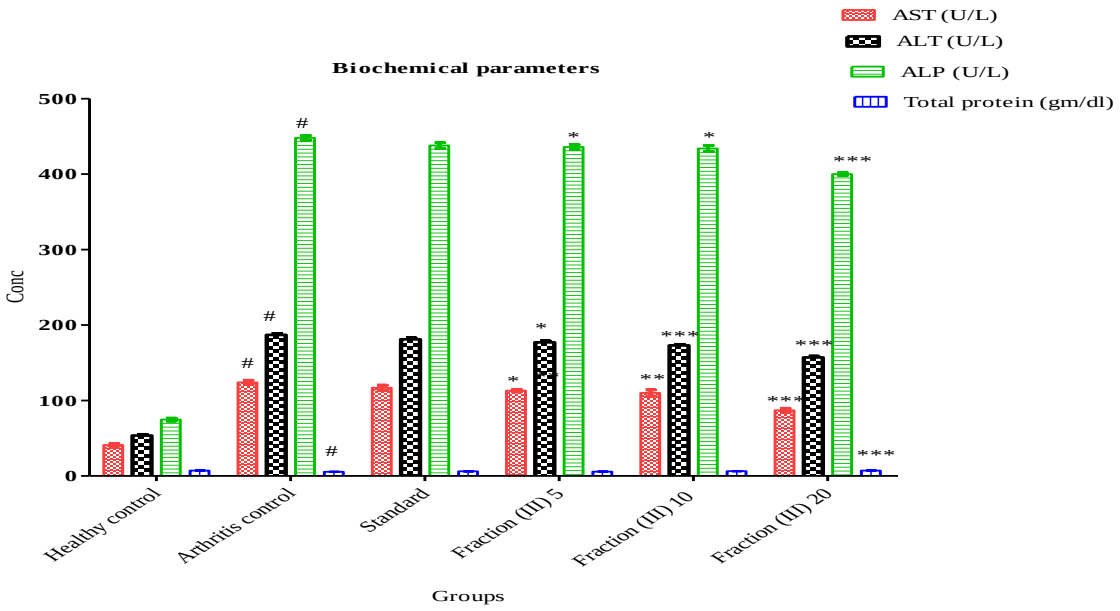



**Fig 5.2.2.4.3.7: Effect of oral administration of isolated bioactive Fraction (III) on serum biochemical parameters in arthritic rats**

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test when compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and when arthritic control group compared with vehicle control group $^{\#}P<0.001$.

### 5.2.2.4.3.8 Antioxidant parameters

Arthritic rats showed significant (P<0.001) reduction in liver SOD and GSH level and increased MDA level compared to that in liver of healthy control. Oral administration of Fraction III (20 mg/kg) showed significant (P<0.001 and P<0.01) increase in SOD and GSH level respectively, while MDA level was significantly (P<0.001) decreased in the liver of arthritic rats. Diclofenac 5 mg/kg also produced a significant (P<0.001) increase in SOD activity and a reduction of MDA level in the liver of arthritic rats. (Table 5.2.2.4.3.8)

**Table 5.2.2.4.3.8: Effect of oral administration of isolated bioactive Fraction (III) on liver antioxidant parameters level in arthritic rats**

| Treatment groups | Dose mg/kg | MDA (nmole/mg protein) | SOD (mu/mg protein) | GSH (µMole/mg protein) |
|---|---|---|---|---|
| Healthy control | | 1.99 ± 0.0187 | 4.43 ± 0.0208 | 70.6 ± 0.946 |
| Arthritic control | | 3.44 ± 0.0208$^{\#}$ | 2.41 ± 0.0165$^{\#}$ | 44.9 ± 1.45$^{\#}$ |
| Diclofenac | 5 | 2.97 ± 0.0284*** | 2.93 ± 0.0171*** | 56.2 ± 0.928*** |
| Fraction (III) | 5 | 3.35 ± 0.0132** | 2.49 ± 0.0229 | 45.8 ± 0.888 |
| | 10 | 3.32 ± 0.0165** | 2.54 ± 0.0314* | 50.1 ± 1.18 |
| | 20 | 3.31 ± 0.0189*** | 2.83 ± 0.0384*** | 52.9 ± 1.89** |

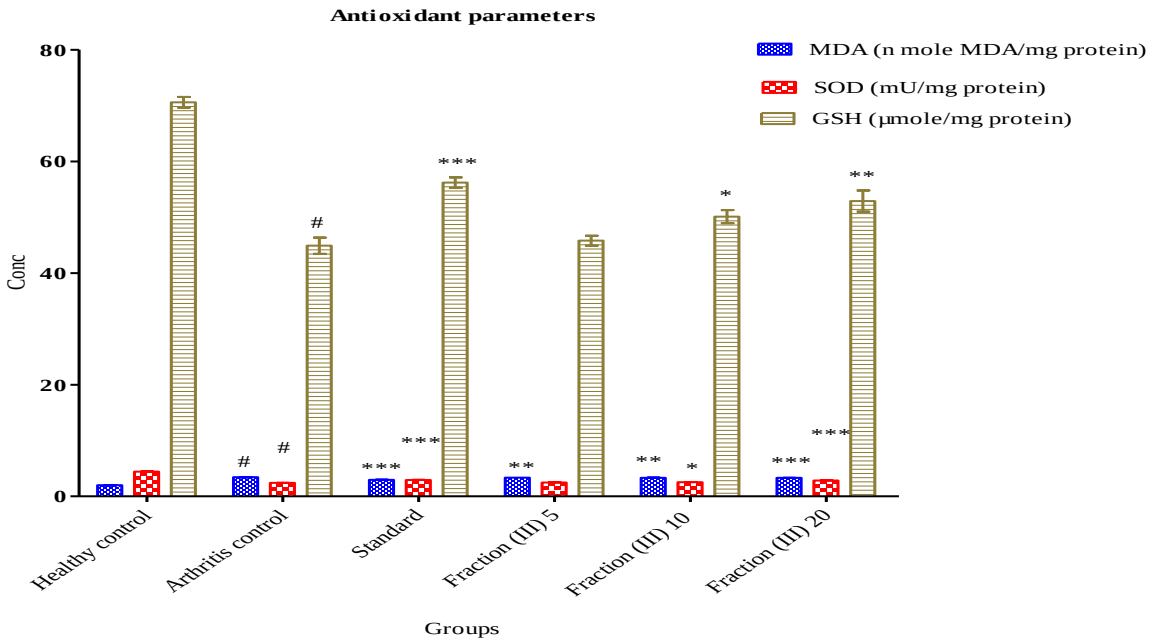



**Fig. 5.2.2.4.3.8: Effect of oral administration of isolated bioactive Fraction (III) on liver antioxidant parameters level in arthritic rats**

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test when compared with arthritic control group $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and when arthritic control group compared with vehicle control group $^{\#}P<0.001$.

**5.2.2.4.3.9 Histopathology of synovial joint:**

Histopathology of synovial joint of normal rats has intact morphology of synovium. No inflammation and influx of inflammatory cells was observed. Arthritic rats showed cartilage destruction, influx of inflammatory cells, pannus formation, fibrin deposition, synovitis and chronic inflammation. Diclofenac treated rats showed protection against cartilage destruction, vascular proliferation and synovial space thickening, low influx of inflammatory cells and no pannus formation. MEAO (400 mg/kg) treated rats showed lesser cartilage destruction, synovial space thickening, vascular proliferation, low influx of inflammatory cells and no pannus formation. MEAO (200 mg/kg) treated rats showed moderate cartilage destruction and synovial space thickening and influx of few inflammatory cells. MEAO (100 mg/kg) treated rats showed minimal inflammation, influx of few inflammatory cells in synovium with evidence of disturbed synovial lining or pannus formation. Fraction III (5 mg/kg) treated rats showed lesser cartilage destruction and synovial space thickening, influx of few inflammatory cells. Fraction III

(10 mg/kg) treated rats showed moderate cartilage destruction, synovial space thickening, vascular proliferation, low influx of inflammatory cells and no pannus formation. Fraction III (20 mg/kg) treated rats showed protection against cartilage destruction, vascular proliferation and synovial space thickening, low influx of inflammatory cells and no pannus formation.

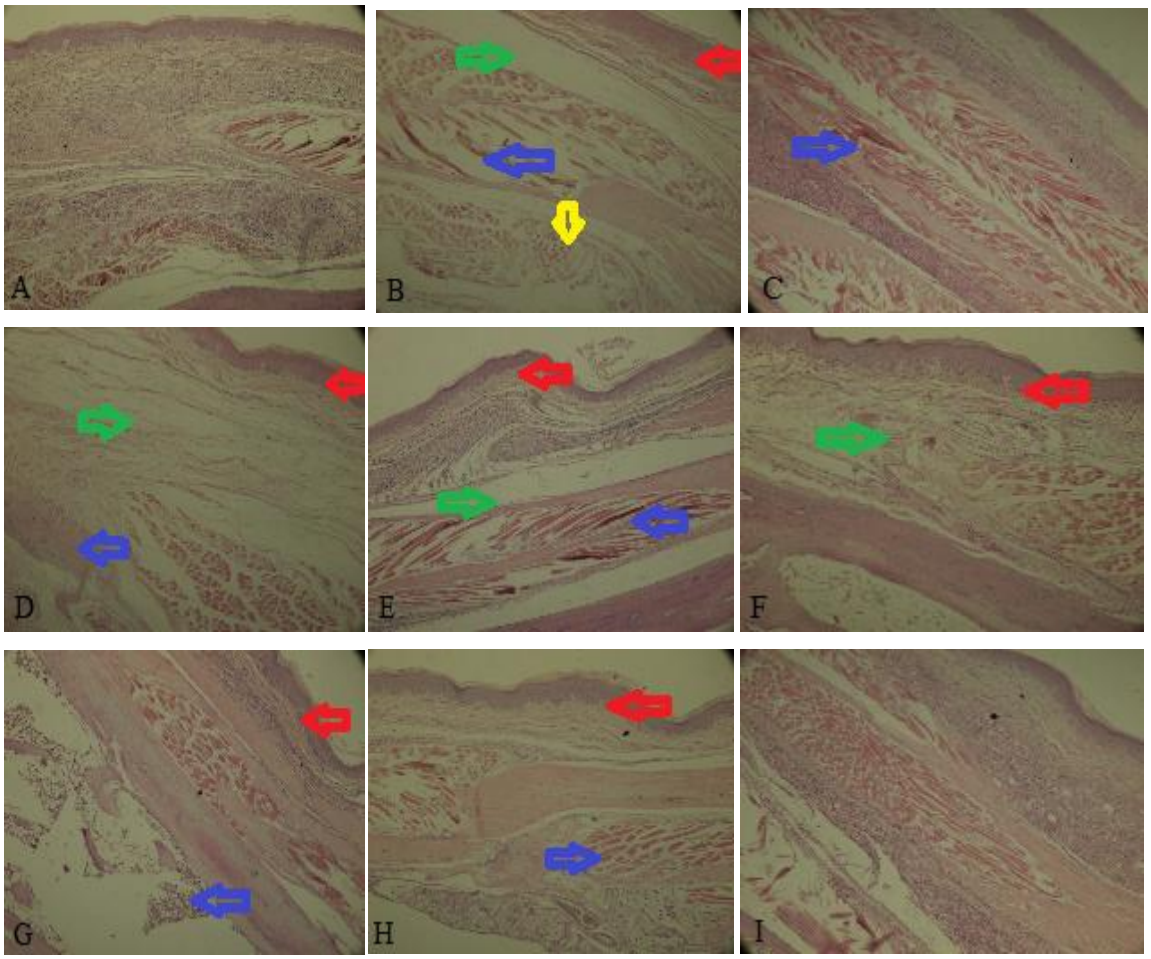



**Fig. 5.2.2.4.3.9: Histopathology of synovial joint. (A) Normal non-arthritic, (B) Arthritic control, (C) Diclofenac 5 mg/kg, (D) MEAO 100 mg/kg, (E) MEAO 200 mg/kg, (F) MEAO 400 mg/kg, (G) Fraction (III) 5 mg/kg, (H) Fraction (III) 10 mg/kg, (I) Fraction (III) 20 mg/kg. (Red colour – synovitis, Green colour – synovial space, Blue colour – pannus, Yellow colour – cartiladge destruction).**

### 5.2.2.4.4 Characterization of isolated Fraction (III) from *A. officinarum*

The characterization of the structures of the isolated compounds was on the basis of NMR spectra by comparison of their NMR spectral data with literature values. The chemical structure of isolated compound was elucidated by LC-MS, IR, $^{1}$H-NMR, $^{13}$C-NMR and DEPT.

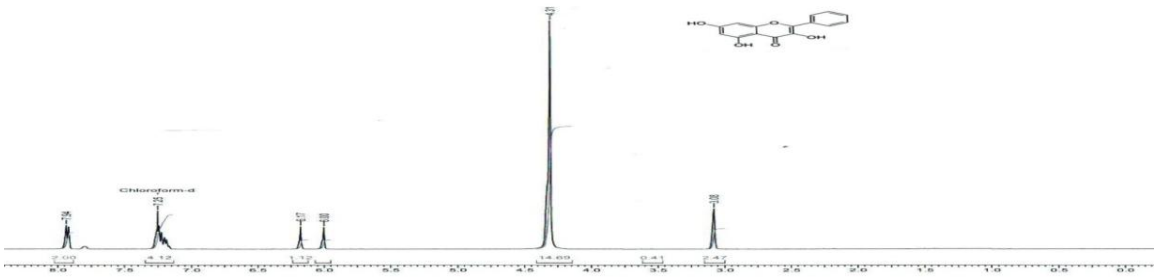


**Fig 5.2.2.4.4 (A): $^{1}$H-NMR spectrum of isolated compound** P3 **(III) from *A. officinarum***

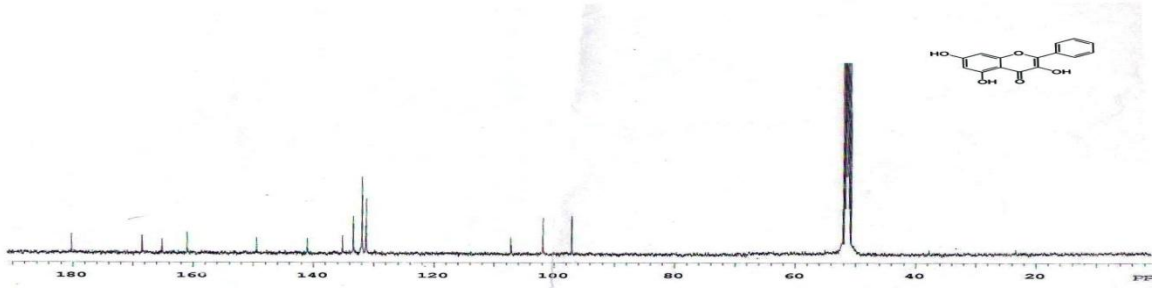


**Fig 5.2.2.4.4 (B): $^{13}$ C-NMR spectrum of isolated compound** P3 **(III) from *A. officinarum***

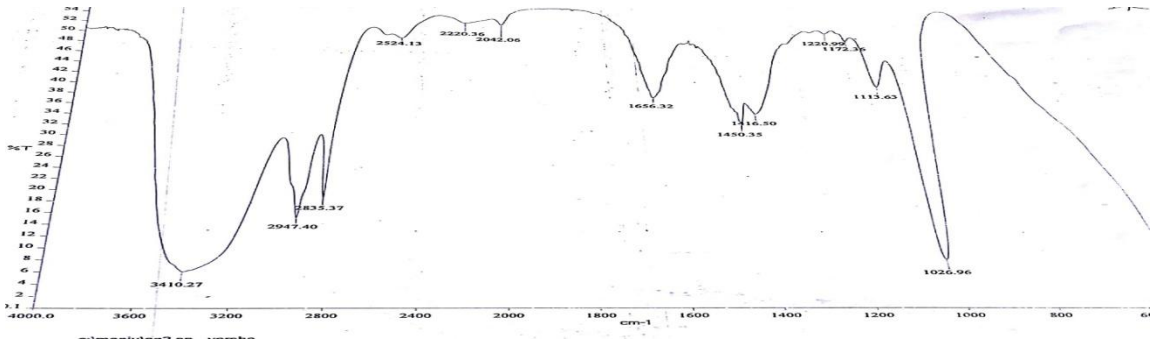



**Fig 5.2.2.4.4 (C): IR spectrum of isolated compound** P3 **(III) from *A. officinarum***

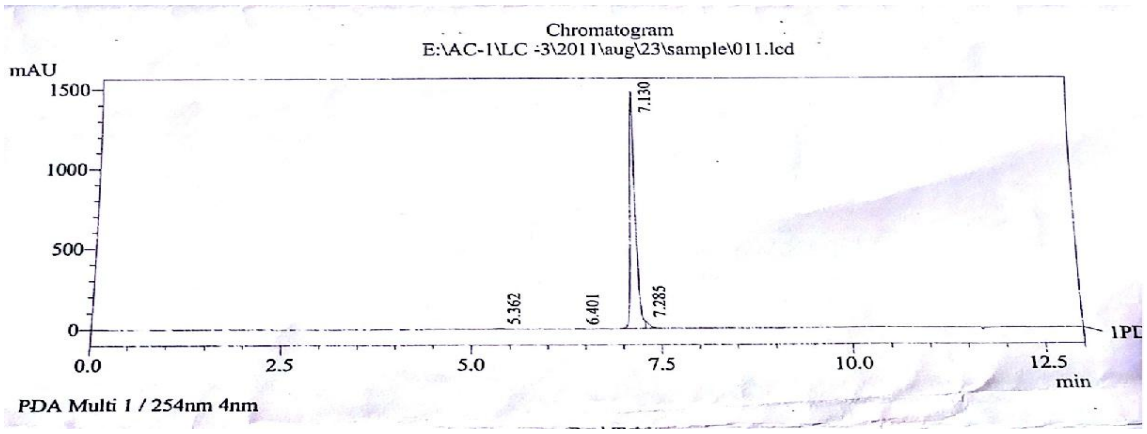



**Fig 5.2.2.4.4 (D): Chromatogram of isolated compound P3 (III) rebelled as AG-2 from *A. officinarum***

**5.2.2.4.5 Structure assigned to isolated compound** P3 **(III)**

Yellow solid, mp: 213-2150C [Lit.mp: 213-2140C] FT-IR (KBr) cm-1:3410 (s), 1656 (m), 1450 (m), 1026 (s). 1H-NMR (400 MHz), inCD3OD: δ (ppm) 8.20 (2H, dd, H-2', 6'), 7.62 (2H, dd, H-3', 5'), 7.53(1H, m, H-4'), 6.43 (JH8/H6= 1.5 Hz, d, 1H, H-8), 6.21 (1H, d, H-6). 13C-NMR (100 MHz), in CD3OD : δ (ppm) 149.5 (C-2), 141 (C-3),180.2 (C-4), 168.5 (C-5), 101.9 (C-6), 165.2 (C-7), 97 (C-8), 160.9 (C-9), 107.2 (C-10), 135.2 (C-1'), 131.3 (C-2', 6'), 133.4 (C-3', 5'), 132 (C-4'). MS: [M+1+] peak = 271.

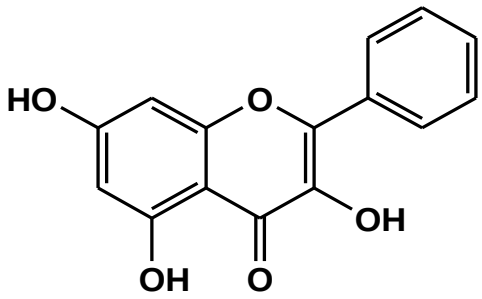



**Fig 5.2.2.4.5: Structure of isolated compound [13, 5, 7-Trihydroxy flavones (galangin)**

## PART III *A. purpurata*

## 5.3 BIOTECHNOLOGICAL STUDIES:

### 5.3.1. Callus initiation

Healthy and friable callus is the requirement of suspension culture for mass cultivation of plant cells for any biotechnological approach like precursor feeding and elicitation. Callus consists of undifferentiated mass of cells developed on a semi-solid medium that can be initiated from any viable explants of whole plant. The maintenance of callus cultures depends on adequate supply of nutrients, growth hormones and controlled sterile environment. The cells, under *in vitro* conditions, contained all the genetic information present in parent plant. MS media containing different combination of growth hormones were used for callus initiation from *A. purpurata*. Different media compositions were tried for callus initiation from rhizomes explant of *A. purpurata* on MS medium supplemented with growth hormones and other low cost additives. Though the callus initiation was observed in different media, the maximum callus biomass was observed in MS medium supplemented with 2, 4-D (2ppm), Kn (2ppm) and 10% coconut water as compared to other media. The composition was given healthy, green, friable callus within a period of 28 days. The growth of callus was observed as fresh weight (FW) and dry weight (DW) measured every week. Both fresh weight (FW) and dry weight (DW) increased over the period of incubation. Effect of phyto hormones on callus growth is presented in (Table 5.3.1). Maximum dry weight of callus (540 mg) was obtained on MS medium supplemented with 2, 4-D + Kn (2:2) after 6 months of incubation.

**Table 5.3.1: Effect of phytohormones on callus growth**

| Media | Hormones | Conc. (ppm) | FW (mg) | DW (mg) |
|---|---|---|---|---|
| MS | NAA + BAP +Kn | 1:2:1 | 1540 | 120 |
| MS | NAA + BAP | 2:2 | 240 | 15 |
| MS | NAA + BAP + Kn | 1:2:2 | 1625 | 80 |
| MS | BA + Kn | 2:2 | 550 | 20 |
| MS | NAA + BA | 1:2 | 650 | 22 |
| MS | 2,4-D +Kn | 2:2 | 2260 | 540 |
| MS | 2,4-D +BAP | 1:2 | 840 | 45 |
| MS | NAA + BAP+NAA | 2:2:2 | 75 | 10 |

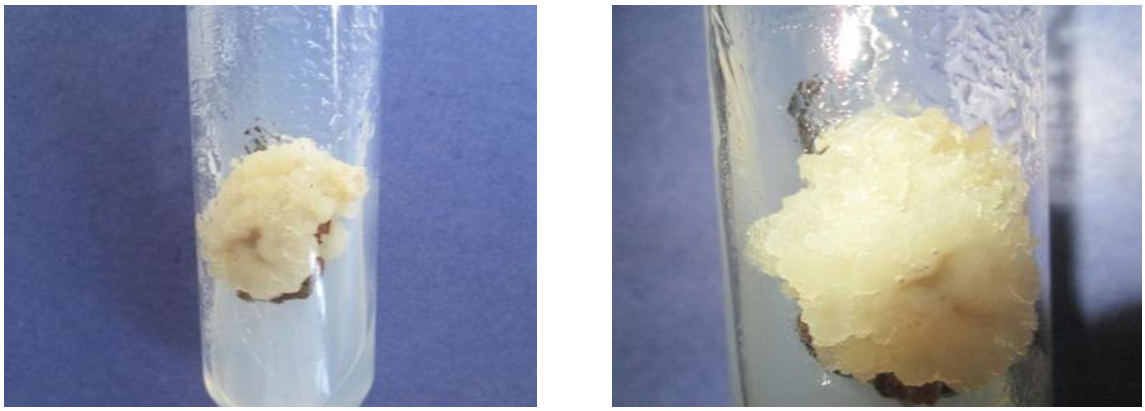


**Fig 5.3.1 Initiation of callus on MS media supplemented with 2, 4-D +Kn (2:2).**

### 5.3.2 Multiplication and elongation of shoot:

#### 5.3.2.1 Initiation of shoots

For the initiation of shoots, two week old callus obtained in MS medium supplemented with 2, 4-D (2ppm) + Kn (2ppm) was transferred in various shooting medium. Table 5.3.2.1 indicates different growth hormones and their combinations for the initiation of shoots from *A. purpurata* callus. Shoot initiation was observed after one month of incubation. Maximum number (9-11) of shoots was observed in medium with NAA (0.1ppm) combination with BA (3.0ppm) after incubation. Shoots were allowed to grow in this medium for one month and then separated and transferred individually in culture flask and bottles for rooting. (Fig.5.3.2.1) shows maximum shoot multiplication in MS medium containing NAA (0.1ppm) combination with BA (3.0ppm), after two successive sub culturing we get elongated shoot with mature leaves.

**Table 5.3.2.1: Effect of NAA and BA on shoot proliferation of *A. purpurata*.**

| Sr. No. | Hormones | Conc. (ppm) | Observation |
|---|---|---|---|
| 1 | NAA+BA | 0.1+1 | Cell mass was less, less granular callus, no rooting formation. |
| 2 | NAA+BA | 0.1+2 | After sub culturing the callus in same media 2-3 small shoots arises from some test tubes, shorter, thicker shoots about 0.5 mm-2 cm heights. |
| 3 | NAA+BA | 0.1+3 | Superficial callus granular, core compact, more callus, rooting was observed with some test tube. |

**Fig. 5.3.2.1 Effect of NAA and BA on shoot proliferation of *A. purpurata*.**

### 5.3.3 Initiation of roots

The shoots with gradual decline in cytokinin and gibberellins and subsequent sub culturing to basal media were exposed to various auxins for root initiation. IAA, IBA and NAA at concentration ranging from 1-4 ppm were used in both MS medium full strength ($M_1$) and MS medium half strength ($M_2$). The observations were reported at the end of $15^{th}$ day and $30^{th}$ day for number of roots per shoot, frequency of rooting and nature of rooting.

The effect of IAA on root induction from *A. purpurata* shoots using MS rooting media ($M_1$ and $M_2$) is presented in (Table 5.3.3). Fast rooting was observed in all concentration of IAA and the rapid initiation of roots was observed after 2 weeks incubation. However, shoots incubated in MS medium with IBA showed delayed root initiation (3-4 weeks). Fig 5.4.3 shows initiation of root from *A. purpurata* shoots in MS medium containing different growth hormone and their combinations. However, the use of IAA ($M_1$ medium) greatly improved rooting on all shoots with maximum rooting percentage (89.32%) found

in MS media containing 3 ppm of Indole acetic acid (IAA). At higher concentration of IAA (8 ppm and 10 ppm) the percentage of rooting decreased slightly due to negative effect.

**Table 5.3.3: Effect of IAA on rooting in MS solid medium.**

| S. No. | Medium | IAA (PPM) | Number of roots/shoot | % rooting | |
|---|---|---|---|---|---|
| | | | | After 15 days | After 30 days |
| 1 | MS ($M_1$) | 1 | 2-1,white, long, slender | 21 | 43 |
| 2 | MS ($M_1$) | 2 | 3-4, white, long, slender | 13.48 | 76.19 |
| 3 | MS ($M_1$) | 3 | 9-11, white, long, slender, green white. | 32.34 | 89.32 |
| 4 | MS ($M_1$) | 4 | 7-8 white, long, slender | 23.8 | 71.42 |
| 5 | ½ MS ($M_2$) | 1 | 2-1, roots long, medium, White | ---- | 18.18 |
| 6 | ½ MS ($M_2$) | 2 | 4-5, shorter thicker, longer, green white | 17.64 | 34.20 |
| 7 | ½ MS (M2) | 3 | 3-4 | 32.21 | 45.30 |
| 8 | ½ MS (M2) | 4 | 3-5 | 21.30 | 44.57 |

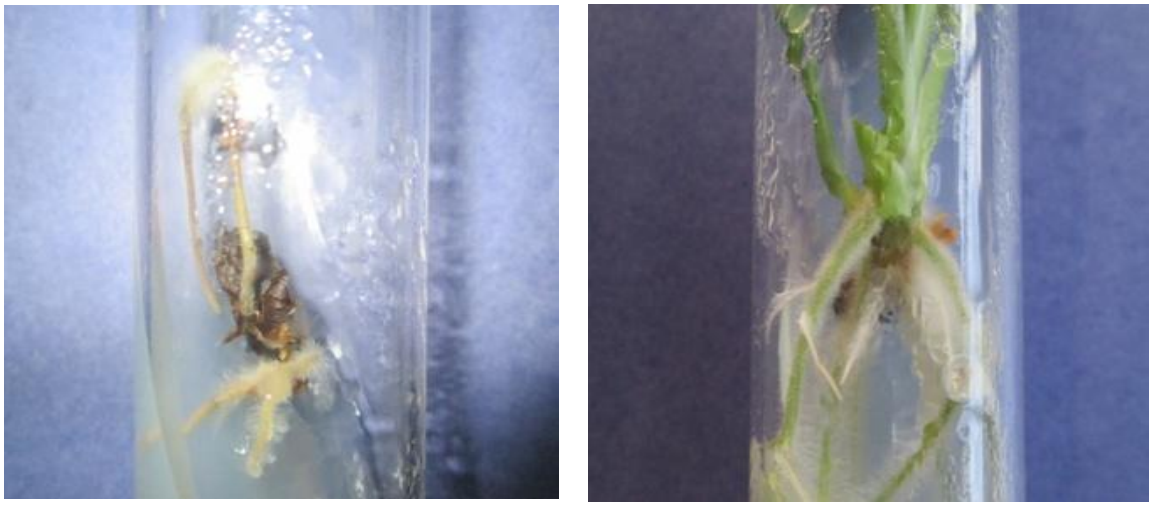


**Fig. 5.4.3: Effect of IAA on rooting in MS solid medium.**

### 5.3.4 Hardening

For the acclimatization of the plantlets, micro-cuttings were transferred to a sterile jars containing soil, irrigated with sterile water and incubated at 25±1ºC under 90-100% relative humidity for 7 weeks under aseptic conditions. The survival rate of rooted plantlets after transferring to soil under high humidity conditions was 90%. After that the plantlets were transferred to a similar mixture of soil in trays and maintained in green house at 25±1ºC under 16 h light and 8 h dark conditions (50 µmoL M-2S-1, Philips white fluorescent light) at relative humidity 60%. Later, plants were individually transplanted to earthen pots and placed in a room with a day time temperature of 25±1ºC and night time temperature of 15±1ºC at relative humidity 30%. Micropropagated plants were morphologically and phytochemically similar to the mother plant. With this method, about 40 plants could be obtained from 10 explants every 90 days. In present work economical pre-hardening were developed using garden soil: sand: vermiculite at 1:1:1 with highest survival rate 97.5%.

### 5.3.5 Rutin and phenolic compound (quercetin) content analysis by HPTLC at different stages of *in vitro* grown plants of *A. purpurata*.

Marker compound, rutin and quercetin, quantified by HPTLC from methanolic extract of tissue culture grown callus of *A. purpurata* is presented in (Table 5.3.5). It was observed that MS medium supplemented with 2, 4 D+ Kn (2:2) contain maximum content of rutin and quercetin.

**Table 5.3.5: Rutin and phenolic compound (quercetin) content analysis by HPTLC at callus level of *in vitro* grown plants of *A. purpurata*.**

| Media | Hormones | Conc. (ppm) | Rutin (% w/w) | phenolic (% w/w) |
|---|---|---|---|---|
| MS | NAA + BAP +Kn | 1:2:1 | 1.2 | ---- |
| MS | NAA + BAP | 2:2 | 2.7 | 0.34 |
| MS | NAA + BAP + Kn | 1:2:2 | 2.8 | 0.28 |
| MS | BA + Kn | 2:2 | 4.7 | 0.55 |
| MS | NAA + BA | 1:2 | 3.8 | 0.32 |
| MS | 2,4-D +Kn | 2:2 | 24.4 | 13.7 |
| MS | 2,4-D +BAP | 1:2 | 3.4 | 0.41 |
| MS | NAA + BAP+NAA | 2:2:2 | 3.2 | 0.745 |

### 5.3.6 Quantification of rutin in natural grown and tissue culture medium

The rutin content increases with the percentage of coconut water in both, conventional and modified MS medium. Conventional MS medium showed less accumulation of rutin while at modified MS medium with 15% v/v percentage of coconut water, tap water in place of distilled water and 3 % marketed sugar in place of sucrose showed best results for rutin.

**Table 5.3.6a: Quantification of rutin in the leaves of natural grown plant of *A. purpurata***

| Extract used | Rutin (%W/W) |
|---|---|
| Leaves | 14.93±0.36 |

Results are mean ± SEM of three parallel measurements (n=3)

**Table 5.3.6b: Quantification of rutin in conventional MS medium and modified medium**

| Extract used | Conventional MS medium (distilled water + sucrose + Agar) | Modified MS medium (Tap water + marketed sugar + coconut water) |
|---|---|---|
| | Rutin (%W/W) | Rutin (%W/W) |
| Callus | 10.16±0.24 | 13.13±0.25 |
| Shoots | 11.06±0.14 | 15.11±0.24 |
| Leaves | 20.79±0.43 | 24.93±0.32 |

Results are mean ± SEM of three parallel measurements (n=3)

### 5.3.7 Determination of concentration of rutin in natural grown and tissue culture grown plant of *A. purpurata*

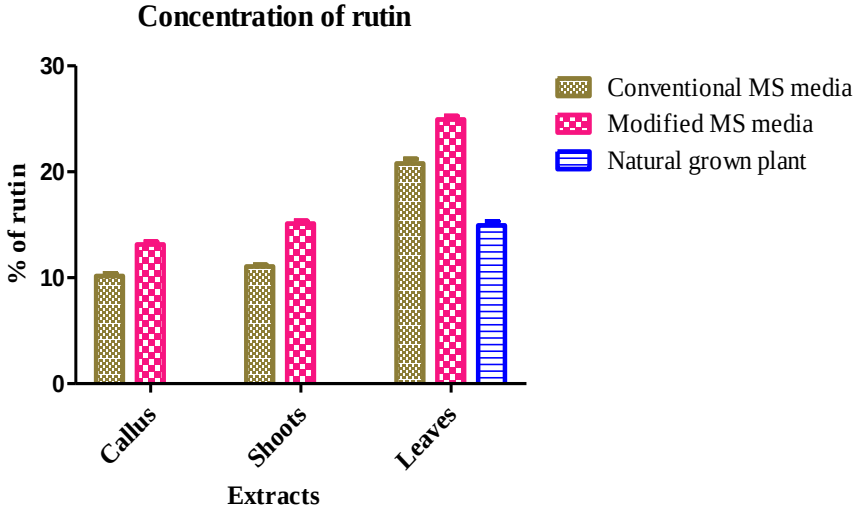



**Fig. 5.3.7 Concentration of rutin in natural grown and tissue culture grown plant of *A. purpurata***

## 5.4 PART IV (ANALYTICAL STUDIES):

### 5.4.1 HPTLC studies of galangin for *A. galanga* and *A. officinarum*

For the analysis of raw herbal materials and herbal preparations, HPTLC is superior to other instrumental analytical techniques because it is simple, economical and requires minimum sample clean up. The time required for sample analysis in HPTLC is much less as compare to HPLC, liquid chromatography and electro-spray mass spectrometry. In HPLC, one sample is injected at a time and after every injection there is a washing period. On the other hand, in HPTLC more than one sample is applied on a plate and quantified in a single run. The HPTLC densitometric technique was therefore, selected for the quantitative and qualitative determination of galangin in *A. galanga* and *A. officinarum* respectively. Out of number of solvent systems tried, the one containing hexane-ethylacetate-acetic acid (7.5:2:0.5 v/v) gave the best resolution of galangin with the retention factor (Rf) of 0.46. HPTLC profile of galangin in *A. galanga* and *A. officinarum* at wavelength of 254 nm are illustrated in (Fig. 5.4.1)

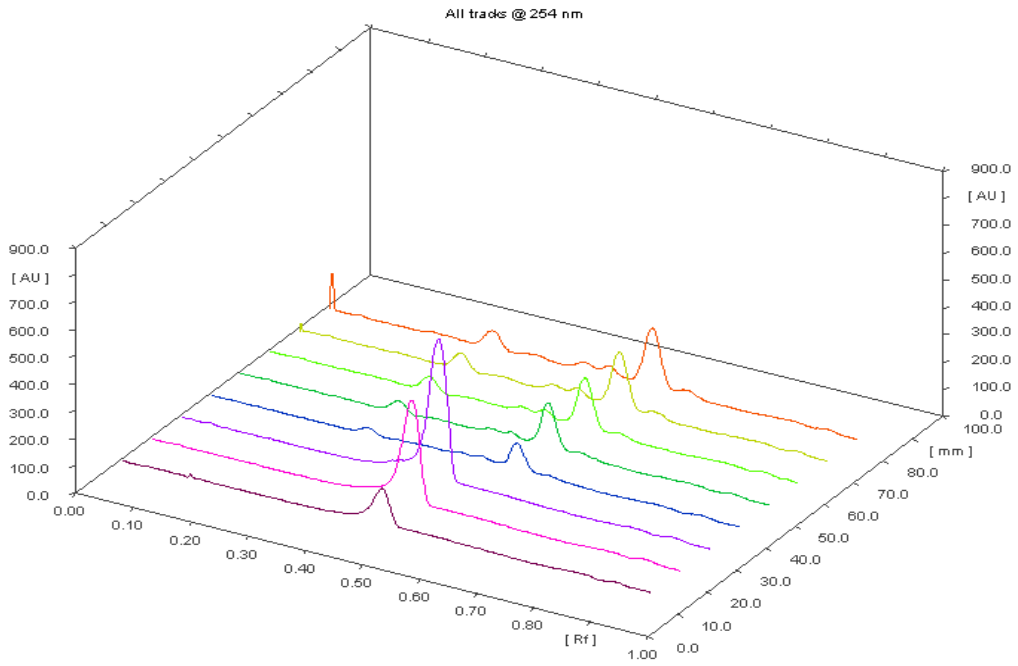



**Fig. 5.4.1a: HPTLC Chromatogram of galangin in *A. galanga* and *A. officinarum***

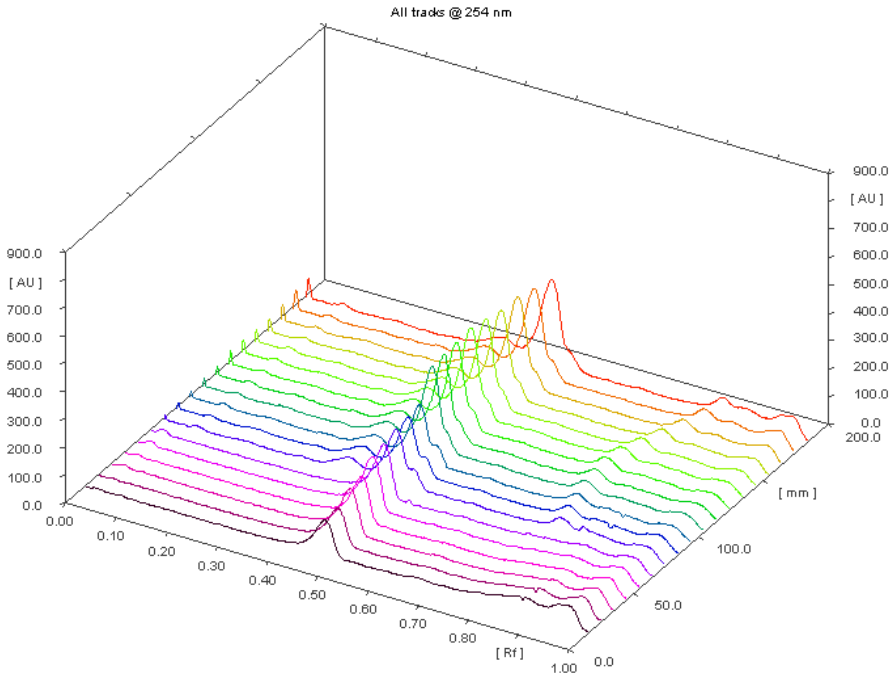



**Fig. 5.4.1b: HPTLC Chromatogram of galangin in *A. officinarum***

### 5.4.2 Linearity of galangin

Linear regression revealed good relationship between the concentration of standard solutions and the peak response within the concentration range of 200 to 2000 ng/spot

with a correlation coefficient ($r^2$) of 0.999 (y=7.765x + 3739). Fig 5.4.2 shows linearity and densitogram of galangin.

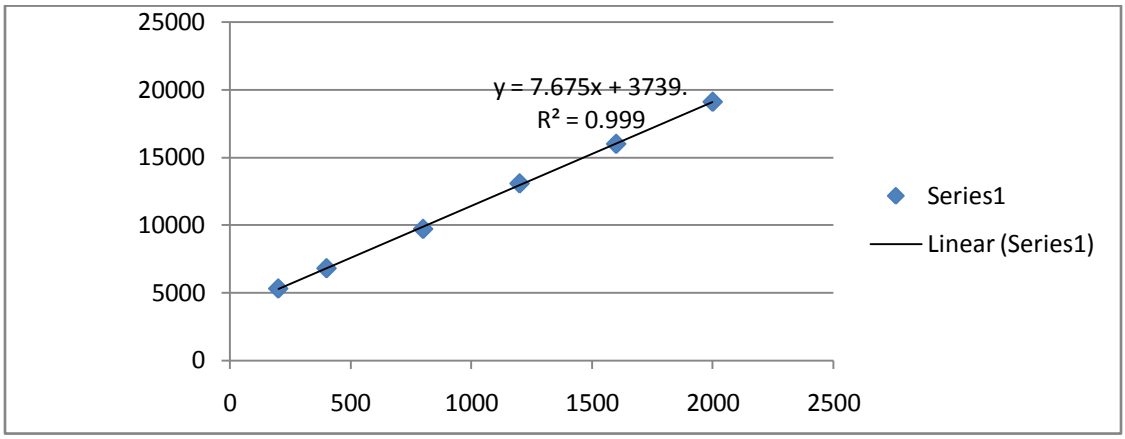



**Fig. 5.4.2a: Linearity of galangin**

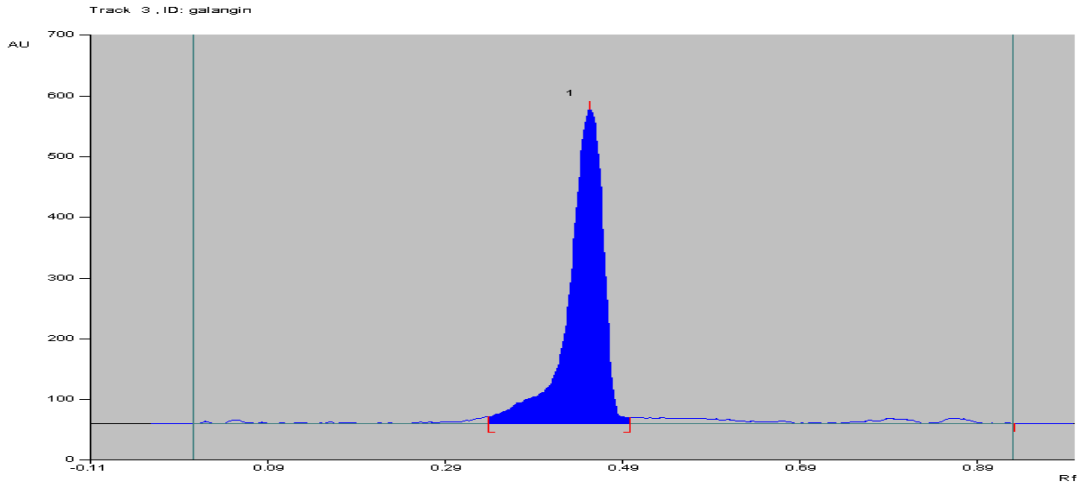



**Fig. 5.4.2b: Densitogram of galangin**

### 5.4.3 Limit of detection (LOD) and Limit of quantification (LOQ) for galangin

Limit of detection (LOD) and limit of quantification were calculated to determine sensitivity as 3.3 σ/s and 10 σ/s, respectively. Where σ is the standard deviation of the response (y- intercept) and S is the slope of linearity plot. The LOD and LOQ were obtained with the signal-to-noise ratio of 3.3 and 10. The LOD and LOQ were found to be 45.14 and 136.80 ng/spot for galangin. This indicated that the new method exhibited a good sensitivity for the quantification of galangin.

### 5.4.4 Precision

The precision and the repeatability at three different concentration levels reflect the robustness of the method. The intraday and interday precision results are presented in (Table 5.4.4.)

**Table 5.4.4: Intraday and interday precision of galangin**

| Standard drug | Nominal concentration | Concentration obtained | | Precision obtained | |
|---|---|---|---|---|---|
| | | Intra day | Inter day | Intra day | Inter day |
| Galangin | 400 | 403.87 | 399.78 | 0.99 | 0.92 |
| | 800 | 797.39 | 797.22 | 0.20 | 0.57 |
| | 1200 | 1200.52 | 1198.87 | 0.37 | 0.15 |

### 5.4.5 Robustness

The standard deviation of peak areas was calculated for each condition and percentage Relative Standard Deviations (% RSD) was found to be less than 2%. These low values of % RSD was indicative of the robustness of the method.

**Table 5.4.5: Robustness of galangin**

| Parameters | Galangin | |
|---|---|---|
| | Concentration found | % RSD |
| Mobile phase (Ethyal Acetate) composition (± 0.1 mL) | 1.73 | 0.02 |
| Amount of mobile phase (± 5 %) | 15.53 | 0.16 |
| Time from band application to chromatography (+ 10 min) | 27.40 | 0.28 |
| Time from chromatography to scanning (+ 15 min) | 5.86 | 0.06 |

### 5.4.6 Specificity

The peak purity for galangin was assessed by comparing visible spectra acquired at the peak start (S), peak apex (M) and peak end (E) of the peaks obtained from scanning of bands. The results obtained were *r* (S, M) = 0.999 and *r* (M, E) = 0.998 respectively. Peak purity data showed that peak obtained for galangin was pure.

### 5.4.7 Accuracy

The accuracy of the method was evaluated by the recovery study. Both the percent recovery and average percent recovery was calculated. After the addition of standard galangin to same amount of the sample solution at three different concentration levels, the percentage recovery of galangin was found to be 100.04%, 100.39% and 100.25% with an average of 100.22%. The results are presented in (Table 5.4.7).

**Table 5.4.7: Accuracy of galangin**

| Amount Taken | Amount added | Amount found | | % Recovery ± % R.S.D. | |
|---|---|---|---|---|---|
| Galangin | Galangin | Galangin | SD | Galangin | RSD |
| 300 | 240 | 540.24 | 1.012046054 | 100.04 | 0.187333069 |
| 300 | 300 | 602.35 | 1.585084698 | 100.39 | 0.263152175 |
| 300 | 360 | 661.63 | 3.113434044 | 100.25 | 0.470571215 |

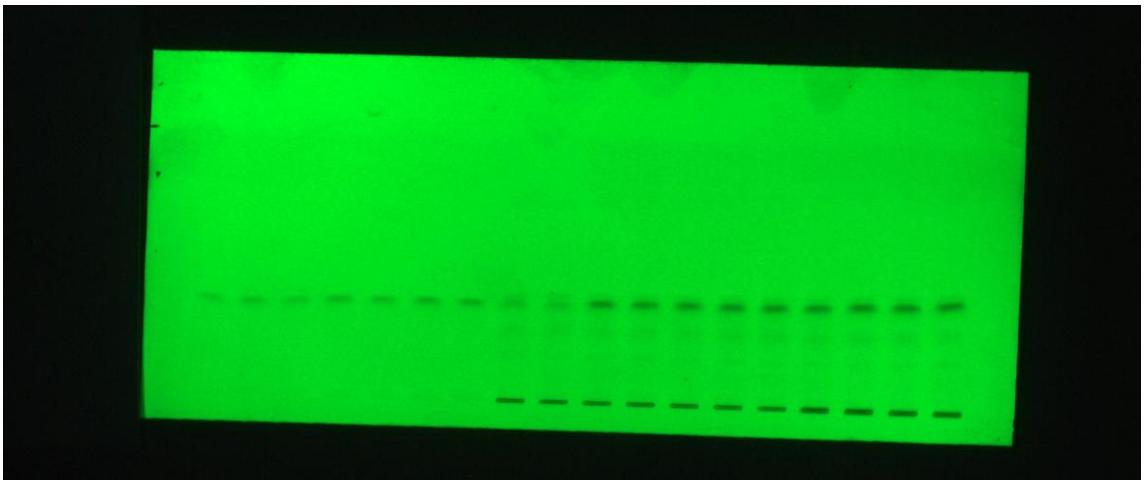


**Fig. 5.4.7: Accuracy of galangin**

### 5.4.8 Method validation parameters

**Table 5.4.8: Method validation parameters for the quantitation of galangin by proposed HPTLC method.**

| Parameters | Galangin |
|---|---|
| Linearity range | 45.14 and 136.80 ng/spot |
| Correlation coefficient | 0.999 |
| Limit of detection | 45.14 |
| Limit of quantitation | 136.80 |
| Specificity | Specific |
| Robustness | Robust |

### 5.4.9. Linearity of rutin and quercetin

Linear regression revealed good relationship between the concentration of standard solutions and the peak response within the concentration range of 100 to 2000 ng/spot with a correlation coefficient ($r^2$) of 0.999 (y=2.544x + 676.4) for rutin and correlation coefficient ($r^2$) of 0.999 (y=4.739x + 1759) for quercetin (Fig. 5.4.9) shows linearity and densitogram of rutin and quercetin.

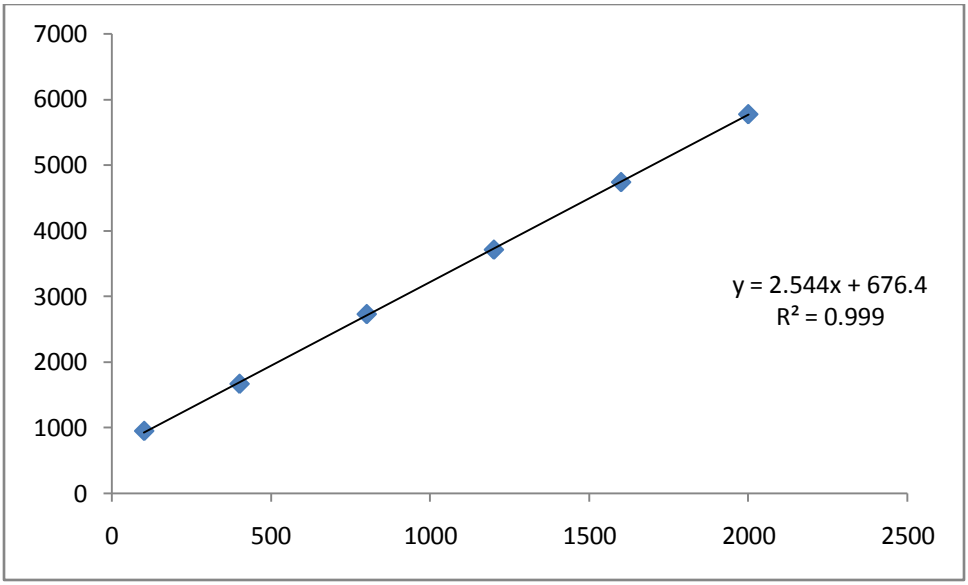



**Fig.5.4.9a: linearity of rutin**

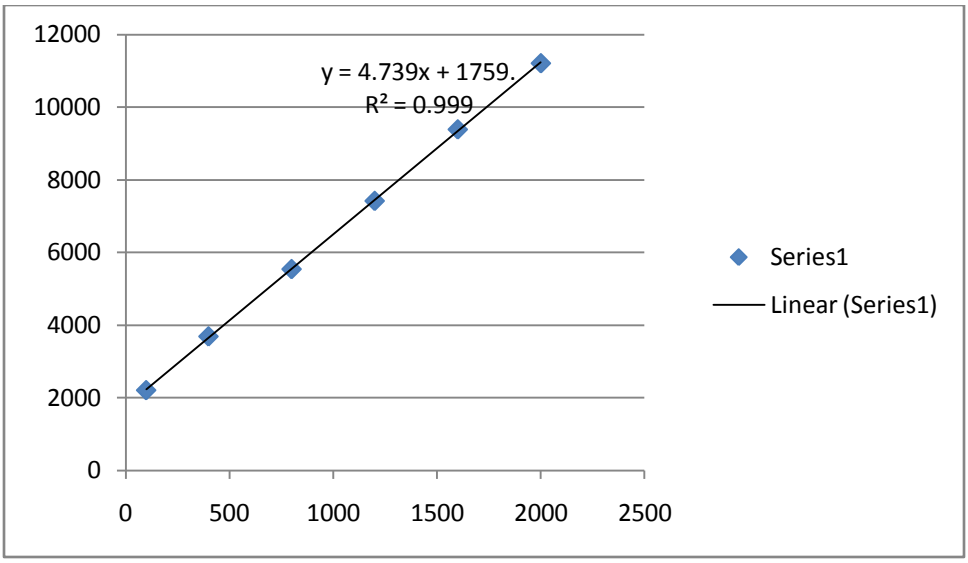



**Fig.5.4.9b: linearity of quercetin**

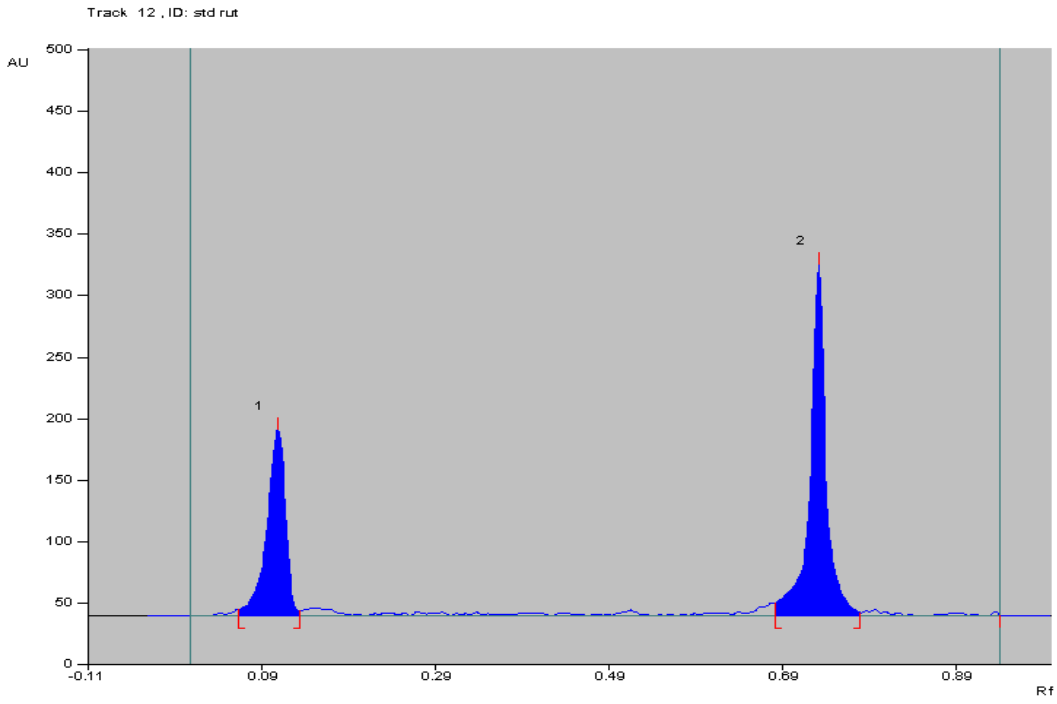



**Fig.5.4.9c: Densitogram of rutin and quercetin**

### 5.4.10 Limit of detection (LOD) and Limit of quantification (LOQ) for rutin and quercetin

Limit of detection (LOD) and limit of quantification were calculated to determine sensitivity as 3.3 σ/s and 10 σ/s, respectively. Where σ is the standard deviation of the

response (y- intercept) and S is the slope of linearity plot. The LOD and LOQ were obtained with the signal-to-noise ratio of 3.3 and 10. The LOD and LOQ were found to be 28.08 and 85.10 ng/spot for rutin and the LOD and LOQ were found to be 25.89 and 78.45 ng/spot for quercetin. This indicated that the new method exhibited a good sensitivity for the quantitation of rutin and quercetin.

### 5.4.11 Precision

The precision and the repeatability at three different concentration levels reflect the robustness of the method. The intraday and interday precision results are presented in Table 5.4.11a and b.

**Table 5.4.11a: Intraday and interday precision of rutin**

| Standard drug | Nominal concentration | Concentration obtained | | Precision obtained | |
|---|---|---|---|---|---|
| | | Intra day | Inter day | Intra day | Inter day |
| Rutin | 400 | 391.35 | 399.34 | 0.92 | 0.86 |
| | 800 | 799.24 | 814.57 | 0.94 | 0.82 |
| | 1200 | 1207.26 | 1216.30 | 0.57 | 0.77 |

**Table 5.4.11b: Intraday and interday precision of quercetin**

| Standard drug | Nominal concentration | Concentration obtained | | Precision obtained | |
|---|---|---|---|---|---|
| | | Intra day | Inter day | Intra day | Inter day |
| Quercetin | 400 | 202.93 | 208.62 | 1.38 | 1.54 |
| | 800 | 395.65 | 396.57 | 0.98 | 0.78 |
| | 1200 | 795.81 | 804.25 | 0.52 | 0.21 |

### 5.4.12 Robustness

The standard deviation of peak areas was calculated for each condition and percentage Relative Standard Deviations (% RSD) was found to be less than 2%. These low values of % RSD was indicative of the robustness of the method.

**Table 5.4.12a: Robustness for rutin**

| Parameter | Rutin | |
|---|---|---|
| | Concentration found | % RSD |
| Mobile phase (Ethyal Acetate) composition (± 0.1 mL) | 11.53 | 0.42 |
| Amount of mobile phase (± 5 %) | 20.50 | 0.75 |
| Time from band application to chromatography (+ 10 min) | 25.63 | 0.94 |
| Time from chromatography to scanning (+ 15 min) | 18.52 | 0.67 |

**Table 5.4.12b: Robustness for quercetin**

| Parameter | Quercetin | |
|---|---|---|
| | Concentration found | % RSD |
| Mobile phase (Ethyal Acetate) composition (± 0.1 mL) | 19.08 | 0.34 |
| Amount of mobile phase (± 5 %) | 19.50 | 0.35 |
| Time from band application to chromatography (+ 10 min) | 12.12 | 0.22 |
| Time from chromatography to scanning (+ 15 min) | 20.81 | 0.37 |

### 5.4.13 Specificity

The peak purity for rutin and quercetin was assessed by comparing visible spectra acquired at the peak start (S), peak apex (M) and peak end (E) of the peaks obtained from scanning of bands. The results obtained were *r* (S, M) = 0.999. 0.998 and *r* (M, E) = 0.999, 0.998 respectively. Peaks purity data showed that peaks obtained for rutin and quercetin were pure.

### 5.4.14 Accuracy

The accuracy of the method was evaluated by the recovery study. Both the percent recovery and average percent recovery was calculated. After the addition of standard rutin and quercetin to same amount of the sample solution at three different concentration levels, the percentage recovery of rutin was found to be 100.79%, 99.90% and 98.66% with an average of 99.78% and the percentage recovery of quercetin was found to be 99.96%, 100.48% and 100.13% with an average of 100.19%. The results are presented in (Table 5.4.14).

**Table 5.4.14a: Accuracy study of rutin by HPTLC method.**

| Amount Taken | Amount added | Amount found | | % Recovery ± % R.S.D. | |
|---|---|---|---|---|---|
| Rutin | Rutin | Rutin | SD | Rutin | RSD |
| 300 | 240 | 544.26 | 4.141373331 | 100.79 | 0.760916781 |
| 300 | 300 | 599.42 | 3.545007667 | 99.90 | 0.59140287 |
| 300 | 360 | 651.18 | 8.45811116 | 98.66 | 1.298891391 |

*Results are mean ± SEM of three parallel measurements (n=3).

**Table 5.4.14b : Accuracy study of quercetin by HPTLC method.**

| Amount Taken | Amount added | Amount found | | % Recovery ± % R.S.D. | |
|---|---|---|---|---|---|
| Quercetin | Quercetin | Quercetin | SD | Quercetin | RSD |
| 300 | 240 | 539.78 | 3.908052158 | 99.96 | 0.724013259 |
| 300 | 300 | 602.87 | 3.454464137 | 100.48 | 0.573003344 |
| 300 | 360 | 660.83 | 3.687231462 | 100.13 | 0.557970939 |

*Results are mean ± SEM of three parallel measurements (n=3).

### 5.4.15 HPTLC studies of rutin and phenolic compound (quercetin) in *in vitro* grown plant material (*A. purpurata*)

The HPTLC densitometric technique was selected for the quantitative and qualitative determination rutin and quercetin in *A. purpurata*. Out of number of solvent systems tried, the one containing tolune-acetone-formic acid (4.5:4.5:1 v/v) gave the best resolution of rutin and quercetin with the retention factor (Rf) of 0.17 and 0.67

respectevely. HPTLC profile of rutin and quercetin in different extracts of *A. purpurata* at wavelength of 254 nm are illustrated in Fig 5.4.15.

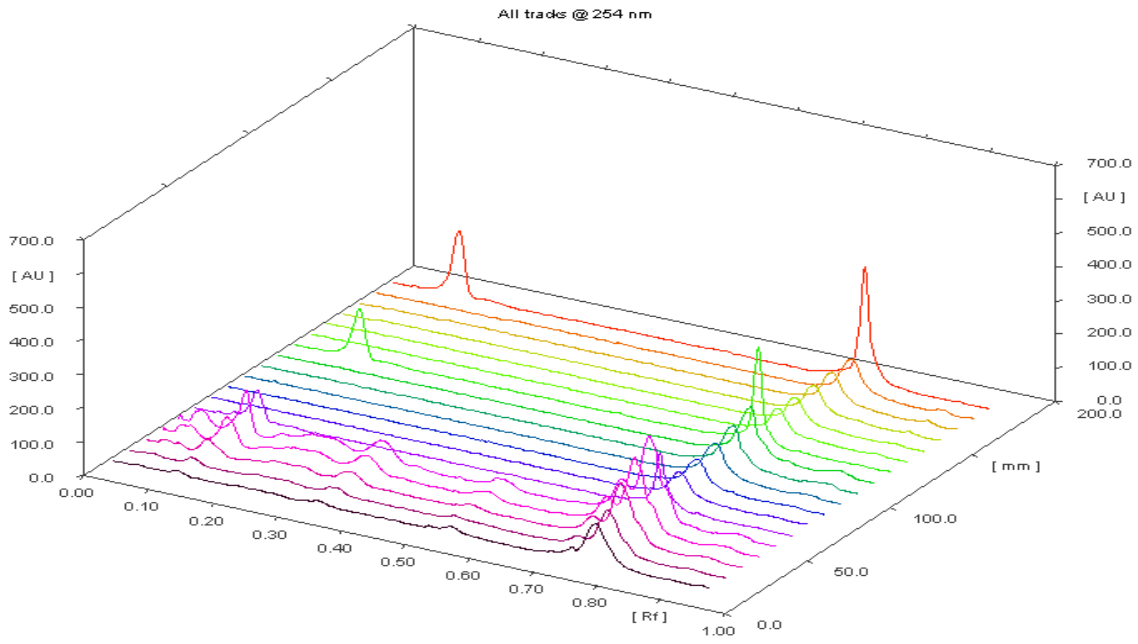



**Fig. 5.4.15 HPTLC profile of rutin and quercetin in hexane, ethyl acetate and methanolic extracts of *A. purpurata***

# DISCUSSION

## DISCUSSION:

Medicinal plants plays very fundamental role to create a large number of various organic chemicals, which are of pharmaceutical and industrial interest. A huge number of pharmaceutically essential chemicals are extracted from plants. For thousands of year's natural products have played a vital role in health care system. Throughout the evolutions, the value of natural products for medicine and health has been enormous. About 25% of drugs prescribed worldwide are basically come from plants, 121 such an active compounds are being in current use. About 252 drugs as basic and essential by the World Health Organization (WHO), 11% are exclusive of plant origin and significant number of synthetic drug obtained from natural products. Moreover, natural products have also been an invaluable source of inspiration for organic chemists to synthesize novel drug candidates (Beghyn et al., 2008).

Zingiberaceae family consist of large number of rhizomatous medicinal and aromatic plants characterized by the existence of volatile oils and oleoresins, generally, the rhizomes and fruits are aromatic, tonic and stimulant in nature; occasionally these are nutritive. Some of the plants are used as a food due to presence of starch in large quantities while others yield an astringent and diaphoretic juice (Joyet al., 1998). Zingiberaceae family is an important natural resource that provides many useful products for food, spices, medicines, dyes, perfume and aesthetics (Jantanet al., 2003). Rhizomes of certain ginger species like *A. officinarum, A. galanga, A. calcarata, Kaempfera galangal* have high medicinal values (Indranyl et al., 2009) and the ethnomedical uses of Zingiberaceous plants of Northeast India have been extensively reviewed by (Lath et al., 2010). Many *Alpinia* species are valued for the medicinal properties and are also utilized in traditional system of medicines as a spasmolytic, anti-oxidant, anti-inflammatory, bacteriastatic, hypotensive, anti-emetic, fungistatic property in India, China and other regions (Parida et al., 2011).

Standardization is an important tool used for herbal drugs in order to found their identity, purity, safety and quality (Mukherjee, 2002). In order to standardize a drug, various macroscopic, fluorescence analysis, physicochemical analyses and phytochemical analysis were done. The quantitative estimation of some pharmacognostical parameters is useful for setting standards for crude drugs. (Ravichandra et al., 2011). Raw drugs create

a problem of identification and to establish their genuineness when they not have any external diagnostic features or any organoleptic clues, during such situations, the microscopic analyses of the specimen will suggest a helping hand to set up the identity of the phytodrugs (Mukherjee, 2002).

Macroscopic studies revealed that *A. galanga* are cylindrical rhizomes and about 2 to 8 cm in diameter. Externally reddish brown color, odor is pleasant and aromatic, taste spicy and sweet, while *A. officinarum* rhizome is a slightly curved and cylindrical rhizome, 2.8 cm in length, 6. 15 mm in diameter; externally red-brown to dark brown, odor is characteristic, taste is extremely pungent. Microscopic studies of *A. galanga* revealed an outer cortical region and an inner stelar region. Cortex consists of xylem which showed presence of parenchyma, vessels, tracheids, fibers, oleoresin cells and starch grains. Transverse section of *A. officinarum* reveals the presence of epidermal cells, cortex, endodermis; vascular bundles and fibers. Calcium oxalate and starch grains were also observed.

Powdered *A. galanga* showed the presence of epidermal cells, parenchyma cells with starch grains, vessels with scalariform thickening, parenchyma contain trachied and starch grains. Powdered *A. officinarum* showed the presence of epidermal cells, parenchymatous cells, fragments of vessels, vessels with scalariform thicknings, parenchyma cells containing starch grains.

The total ash, acid insoluble ash, water soluble ash and loss on drying of *A. galanga* was found to be 10.2%, 4.1%, 5.3%, 11% respectively, and *A. officinarum* 7.5%, 1.5%, 1.6%, 1.5 % respectively. The ether soluble extractive value, chloroform soluble extractive value, ethanol soluble extractive value, methanol soluble extractive value and water soluble extractive value of *A. galanga* extract was found to be 3.47%, 1.48%, 9.04%, 4.7% and 12.50% respectively, while for *A. officinarum* extract it was found to be 0.60%, 1.8%, 14%, 2.7%, 1.6% respectively.

Preliminary phytochemical screening was useful in prediction of nature of drugs and also useful for the recognition of different constituents present in different polarity solvent. So it could be helpful to extract out particular constituents by solvent (Harborne et al., 1998). The phytochemical study of *A. galanga* revealed the presence of alkaloids, tannins, terpenoids and phenolics, alkaloids, carbohydrates, tannins, aminoacids, and

saponins, while the phytochemical study of *A. officinarum* revealed the presence of alkaloids, tannins, coumarins, terpenoids and phenolics, carbohydrates, tannins, glycosides, amino acids, phenols, gums and saponins. All the extracts of *A. galanga* and *A. officinarum* are brown to brownish yellow in color and showed semisolid consistency while aqueous extract is brown in color and showed solid consistency.

Phytochemical analysis of the *A. galanga* and *A. officinarum* has mainly demonstrated the presence of phenylpropanoides, saponins, flavonoids, terpenoids and steroids. Steroids can decrease inflammation and reduce the action of the immune system, while triterpenoids impairs histamine release from mast cells and exerts anti-inflammatory effects (Mehta et al., 2012). Flavonoids are often used for their antioxidant effect against free radicals. There are also strong indications that they have antiviral, anti-inflammatory and anti-hypertensive properties (Ibrahim et al., 2012). We suggest that the anti-inflammatory activity of the *A. galanga* and *A. officinarum* could be due to combined effect of phenylpropanoides, flavonoids, saponins, steroids and triterpenoids, which are the major components of the extract *A. galanga* and *A. officinarum*.

*A. galanga* and *A. officinarum* was fractionated by chromatographic technique and it was screened by carrageenan induced paw edema in rat. Taking into consideration the result obtained in above study it was further fractionated by column chromatography and evaluated for anti-inflammatory potential at the dose of 10 mg/kg using carrageenan induced paw edema model.

The present study demonstrated that ACA had showed anti-inflammatory activity in carrageenan induced paw edema in rats. These results with isolated ACA and previous results with AEAG confirm that ACA is beneficial in the treatment of pain and inflammation.

The present study also demonstrated that galangin had showed anti-inflammatory activity in carrageenan induced paw edema in rats. These results with isolated galangin and previous results with MEAO confirm that galangin is beneficial in the treatment of pain and inflammation.

Health diseases are the major problem towards the advancement of human civilization. In order to overcome this problem, a worldwide approach has been made through scientific research against most important health disorder like cancer, AIDS,

heart disease and arthritis. WHO pointed out that musculoskeletal situation are a major burden on individuals, health systems and social care system? It has been predicted that arthritis particularly rheumatoid arthritis would rank fourth for the primary cause of disability by 2020. Arthritis is a global crisis that will increase in significance with the rising elderly population. The condition affects both sexes and all races. This disease is characterized by inflammation of one or more joints, pain, wear and tear of joint and muscle strains.

The traditional therapy suggested for the treatment of arthritis includes non-steroidal anti-inflammatory drugs like diclofenac, aceclophenac, glucocorticoid therapy and disease modifying anti rheumatic drugs like anti TNF-α blockers, methotreaxate, cyclosporine A and stem cell therapy. Therapeutic managements of arthritis is well known for its several side effects as a result of which the past decades there is dramatic increase and growing interest in the use of alternative treatments and herbal therapies in arthritis.

Animal models for the assessment of novel anti-rheumatic or anti-inflammatory drugs are widely used in pharmacological research (Greenwald and Diamond, 1988, Seed et al., 1991). Various agents when injected into a joint of rabbits or rats can cause arthritis via different pathogenetic mechanisms. FCA induced arthritis model have been extensively used to study the pathogenesis of rheumatoid arthritis for therapeutics testing (Mizushimaet al., 1972). FCA induced arthritis is an experimental model pioneered by (Pearson and Wood 1959) that shares several human clinical and pathological states of rheumatoid arthritis.

In experimental arthritis animal model wistar rats were used because animal model provides more uniform experimental data and allow for extensive testing of potential therapies. It has been observed that this animal model share features with human arthritis. It has also been found that a rat model has similar pathological features to human pathological features and has the capacity to predict the efficacy of a given therapeutic agent in humans (Hegen et al., 2008).

Complete Freund’s adjuvant induced arthritis is one of the most widely used models for chronic arthritis (Chillingworth et al., 2003). Freund's adjuvant (a mixture of heat killed *Mycobacterium tuberculosis* with liquid paraffin) produced inflamed lesions

in areas of the body remote from the injection site after a delay of 10 to 15 days (Newbould, 1963). In adjuvant-induced arthritis model rats developed a chronic swelling in multiple joints with influence of inflammatory cells, erosion of joint cartilage and bone destruction and remodeling, it is commonly used for preclinical studies of NSAIDs and anti-rheumatic drugs and this model is most suitable as like human arthritis (Sofia et al., 1973).

In arthritis different inflamantory mediators were involved which are the products of arachadonic acid metabolism, histamine, 5-HT, bradykinin, cytokines, and nitric acid. CFA produces a characteristic inflammation and associated hyperalgesia, which can be used to quantify the anti-inflammatory or anti-hyperalgesic actions of drugs (Sammons, 2000). Mediators, like bradykinin, which are released from injured tissue directly stimulate nociceptors, and stimulate tumour necrosis factor-alpha (TNF-α) release. The TNF-α in turn, stimulates the release of interleukin-1beta (IL-1β) and interleukin-6 (IL-6), promoting the initiation of cycloxygenase enzymes, which convert arachidonic acid to prostaglandins (Wim and Berg 1999). Tumour necrosis factor-alpha (TNF-α) also stimulates the release of cytokine-induced neutrophil chemoattractant (CINC-1) in rats or interleukin-8 (IL-8) in humans. Cytokines, like IL-1β, TNF-α and IL-6, plays imp role in rhumatoid arthritis (Carteron NL 2000), these cytokines plays imp role in hyperalgesia by sensitizing peripheral nociceptors, decreasing the peripheral nociceptor threshold (Loram et al, 2007). As per the results galangin and MEAO were significant to treat CFA induced arthritis.

As per the results of our study ACA and AEAG exhibited a significant anti-arthritic activity by inhibition of paw volume and reduced joint diameter in arthritic treated rats. ACA and AEAG also show significant anti- hyperalgesic activity. The actual mechanism may be due to the suppression of inflammatory mediators like IL-1β, TNF-α and IL-6 released due to induction of Freund's adjuvant in arthritic rats.

Radiographic changes in RA conditions are useful diagnostic measures which indicate the severity of the disease. Soft tissue swelling is the earlier radiographic sign, whereas prominent radiographic changes like bony erosions and narrowing of joint spaces can be observed only in the developed stages (final stages) of arthritis (Harris 1991). The standard drug diclofenac prevented the bony destruction and also there was no

swelling of the joint. Treatment with AEAG (400 mg/kg) and ACA (20 mg/kg) for 28 days prevented bony destruction as there was less soft tissue swelling and narrowing of joint spaces than that observed on $14^{th}$ day.

Free radicals production that occurs during development of arthritis in the articular cartilage leads to decreased GSH and SOD levels, increased ROS levels in rhumatoid arthritis may result in a pro-oxidation environment, which in turn could result in increased MDA levels. As a result, lipid peroxidation may have a role in the pathogenesis of the rhumatoid arthritis (Bhowmicket al., 2008). Pathogenesis of arthritis is associated predominantly with the formation of free radicals at the site of inflammation. In rheumatic condition oxidative injury and inflammatory status was confirmed by increased levels of prostaglandins in serum and synovial fluid compared to controls. T cells isolated from the synovial fluid of patients with rheumatoid arthritis showed signs of decreased intracellular GSH level (Valko et al., 2007). In the present study, the levels of SOD and GSH were increased, while the level of MDA was reduced by AEAG, MEAO, ACA and galangin.

From the results it is clear that reduction in RBC count and hemoglobin level represents the anemic condition in arthritic rats. More significant causes are the irregular storage of iron in the reticulo endothelial system and synovial tissue and the breakdown of bone marrow to respond to anemia (Mowat, 1971). Anaemia is the most common haematological deformity seen in patients with rheumatoid arthritis (Weiss and Goodnough, 2005). Inflammation causes increase in the WBC (Castro and Gourley, 2010). The increase in both WBC and platelet counts might be due to the stimulation of immune system against the invading pathogenic microorganism (Maria et al., 1983). It is clear by the infiltration of inflammatory mononuclear cells in the joints of arthritic rats. In the present study, the level of Hb and RBC was significantly increased, while the level of WBC and platelets was significantly reduced by AEAG, MEAO, ACA and galangin.

Lysosomal enzymes play an important role in the physiology and pathology of the joint tissues in arthritis (Dingle, 1973). Measurement of their level provide an excellent tool for anti-arthritic activity of drugs, the activities of aminotransferases and ALP were significantly increased in arthritic rats, since these are excellent indices of liver impairment, which are also measured as the features of adjuvant arthritis (Vijayalakshmi

et al., 1997, Mythilypriya et al., 2008). Treatment with AEAG, MEAO, ACA and galangin significantly ($P<0.001$) decreased the levels of ALT, AST, and ALP in arthritic cases.

Presently the secondary metabolites were obtained by conventional method of cultivation, collection, extraction and isolation, which has its limitations like unforeseen environmental conditions, time consuming, desired quality, and a gap between the demand and supply. Therefore micropropagation through plant tissue culture can be an attractive alternative method. By suitable manipulation of hormones and contents of the medium, it is possible to initiate the developments of roots, shoots and complete plants from callus cultures (Evans, 2001).

On the cellular level, auxin is essential for cell growth, affecting both cell division and cellular expansion (Mcsteen and Yunde, 2008). *A. purpurata* on MS medium supplemented with 2, 4-D + kinetin (2:2) give better results for callus. Maximum numbers (9-11) of shoots were observed in medium with NAA (0.1ppm) combination with BA (3.0ppm) after incubation. Maximum roots were found in MS media containing 3 ppm of Indole acetic acid (IAA).

# CONCLUSION

## CONCLUSION:

- In the present investigation rhizomes of *A. galanga* (L.) Willd, *A. officinarum* Hance and *A. purpurata* (Vieillard) K. Schumann were selected for evaluation pharmacognostical, pharmacological, bioanalytical and analytical properties.
- Detailed macroscopic and microscopic evaluations were done on the selected important medicinal plants.
- Extractions were carried out by different solvent system and the most pharamacological active extract was selected for isolation of active molecule. The extracts were labeled as AEAG for acetone extract of *A. galanga* and MEAO for methanolic extract of *A. officinarum*.
- Phytochemical analysis of all the extracts revealed for presence of alkaloids, flavonoids, tannins and phenolic compounds.
- Acute oral toxicity studies performed according to OECD guideline-425 revealed that all the extracts were safe up to a dose of 2000 mg/kg of body weight.
- Anti-inflammatory activity of all the extracts were investigated using carrageenan induced paw edema and cotton pellet granuloma model in rats.
- In carrageenan induced paw edema model, pretreatment with AEAG and MEAO for 7 days before carrageenan injection showed significant inhibition of increase in paw edema and the results were comparable to that of the standard diclofenac.
- In cotton pellet induced granuloma model, treatment of AEAG and MEAO for 7 days showed significant inhibition of granuloma formation and the results were comparable to that of diclofenac.
- Histopathology of stomach was also performed to assess ulcerogenic property of all the extracts and standard diclofenac. Diclofenac showed ulceration and congestion in stomach. In comparison to that all the extracts showed lesser ulceration and congestion.
- Results of anti-inflammatory activity suggested that, AEAG and MEAO had anti-inflammatory potential in both carrageenan-induced paw edema as well as cotton pellet granuloma, so it was assumed that it is effective in the later phase by reducing the release of prostaglandins, proteases and lysosome.

- AEAG and MEAO showed superior anti-inflammatory activity so both were selected for further determination of its antiarthritic potential in FCA induced arthritis model.
- Antiarthritic activity of AEAG and MEAO were investigated using FCA induced arthritis in rats.
- Antiarthritic activity of AEAG and MEAO were assessed by various parameters such as, body weight, paw volume, joint diameter, tectile allodynia and thermal hyperalgesia. On the last day, haematological, biochemical, antioxidant parameters and histology of ankle joint were also assessed.
- AEAG and MEAO were showed significant antiarthritic potential in FCA induced arthritis in rats, comparable to that of the standard diclofenac.
- Isolations were carried out on AEAG and MEAO for searching active molecule responsible for arthritic activity.
- Fractionations of both extracts were evaluated for anti-inflammatory activity using carrageenan induced paw edema.
- Results of anti-inflammatory activity suggested that, fraction (B2) from AEAG and fraction P3 (III) from MEAO had anti-inflammatory potential in carrageenan-induced paw edema, so it was assumed that it is effective in the later phase by reducing the release of prostaglandins, proteases and lysosome.
- Characterization of fraction (B2) from AEAG was done by using NMR, IR. The isolated compound of fraction (B2) from AEAG was 1'-Acetoxychavicol acetate (ACA).
- Characterization of fraction P3 (III) from MEAO was done by using NMR, IR. The isolated compound of fraction P3 (III) from MEAO was galangin.
- Antiarthritic activity of AEAG and ACA were investigated using FCA induced arthritis in rats.
- Antiarthritic activity of AEAG and ACA were assessed by various parameters such as, body weight, paw volume, joint diameter, tectile allodynia and thermal hyperalgesia. On the last day, haematological, biochemical, antioxidant parameters and histology of ankle joint were also assessed.

- AEAG and ACA were showed significant antiarthritic potential in FCA induced arthritis in rats, comparable to that of the standard diclofenac.
- In case of body weight, AEAG and ACA treated group showed non-significant improvement as compared to arthritic group.
- In case of paw volume and joint diameter, AEAG and ACA at 400 and 20 mg/kg treated group showed significant inhibition of increase in paw volume and joint diameter as compared to arthritic group.
- In case of tectile allodynia and thermal hyperalgesia, AEAG and ACA at 400 and 20 mg/kg treated group showed significant increase in paw withdrawal latency as compared to arthritic group.
- In case of biochemical parameters, AEAG and ACA at 400 and 20 mg/kg treated group showed significant decrease in AST, ALT and ALP level, whereas TP level was non-significantly increased as compared to arthritic group.
- In case of haematological parameters, AEAG and ACA at 400 and 20 mg/kg treated group showed significant decrease in WBC, platelet and CRP level as compared to arthritic group. AEAG and ACA at 400 and 20 mg/kg treated group showed significant increase in Hb and RBC level as compared to arthritic group.
- In case of antioxidant parameters, AEAG and ACA at 400 and 20 mg/kg treated group showed significant increase in SOD and GSH level as compared to arthritic group. AEAG and ACA at 400 and 20 mg/kg mg/kg treated group showed significant decrease in MDA level as compared to arthritic group.
- In histopathological studies, arthritic group showed severe synovitis, influx of inflammatory cells, pannus formation and cartilage destruction. Treatment with AEAG and ACA at 400 and 20 mg/kg showed partial protection such as mild pannus formation and cartilage destruction.
- Antiarthritic activity of MEAO and galangin were investigated using FCA induced arthritis in rats.
- Antiarthritic activity of MEAO and galangin were assessed by various parameters such as, body weight, paw volume, joint diameter, tectile allodynia and thermal hyperalgesia. On the last day, haematological, biochemical, antioxidant parameters and histology of ankle joint were also assessed.

- MEAO and galangin were showed significant antiarthritic potential in FCA induced arthritis in rats, comparable to that of the standard diclofenac.
- In case of body weight, MEAO and galangin treated group showed non-significant improvement as compared to arthritic group.
- In case of paw volume and joint diameter, MEAO and galangin at 400 and 20 mg/kg treated group showed significant inhibition of increase in paw volume and joint diameter as compared to arthritic group.
- In case of tectile allodynia and thermal hyperalgesia, MEAO and galangin at 400 and 20 mg/kg treated group showed significant increase in paw withdrawal latency as compared to arthritic group.
- In case of biochemical parameters, MEAO and galangin at 400 and 20 mg/kg treated group showed significant decrease in AST, ALT and ALP level, whereas TP level was non-significantly increased as compared to arthritic group.
- In case of haematological parameters, MEAO and galangin at 400 and 20 mg/kg treated group showed significant decrease in WBC, platelet and CRP level as compared to arthritic group. MEAO and galangin at 400 and 20 mg/kg treated group showed significant increase in Hb and RBC level as compared to arthritic group.
- In case of antioxidant parameters, MEAO and galangin at 400 and 20 mg/kg treated group showed significant increase in SOD and GSH level as compared to arthritic group. MEAO and galangin at 400 and 20 mg/kg mg/kg treated group showed significant decrease in MDA level as compared to arthritic group.
- In histopathological studies, arthritic group showed severe synovitis, influx of inflammatory cells, pannus formation and cartilage destruction. Treatment with MEAO and galangin at 400 and 20 mg/kg showed partial protection such as mild pannus formation and cartilage destruction.
- Based upon these pharmacological data, we concluded that the acetone extract of *A. galanga* (L.) Willd, methanolic extract of *A. officinarum* Hance, isolated ACA and galangin possess significant anti-inflammatory and antiarthritic activity activities in animal models.

- It is thus concluded that, acetone extract of *A. galanga* (L.) Willd, methanolic extract of *A. officinarum* Hance, isolated ACA and galangin possessed anti-inflammatory and antiarthritic activity. The probable mechanism of action appears to be due to inhibition of the later phase of carrageenan induced paw edema model by restraining the release of kinin-like substances and prostaglandins productions.
- Present study showed competitive response of *in vitro* grown callus when compared to naturally grown plant material of *A. purpurata*.
- The work was started with the initiation of callus from different explants of *A. purpurata* on growth medium supplemented with various combinations of growth hormones.
- The best result for callus initiation of *A. purpurata* was found in MS media with a combination of 2, 4-D (2 ppm) +kinetin (2ppm).
- The best result for roots initiation of *A. purpurata* was found in MS media with a combination of IAA 3ppm.
- Optimization of biomass production and enhanced accumulation of active compounds by different biotechnological strategies like elicitation and precursor feeding of this important medicinal plant will be matter of further research.
- The present study describes HPTLC method for the qualitative and quantitative estimation of rutin and quercetin (phenolic compound)
- Both the methods were found to be simple, precise, specific, reproducible, sensitive and accurate and can be used for the quantitation of rutin and quercetin (phenolic compound) in the plant materials, tissue culture extracts, routine quality control of raw materials and formulations containing rutin and quercetin.
- The results of HPTLC methods revealed that rutin and quercetin (phenolic compound) content was more in tissue culture grown material than naturally grown plants.

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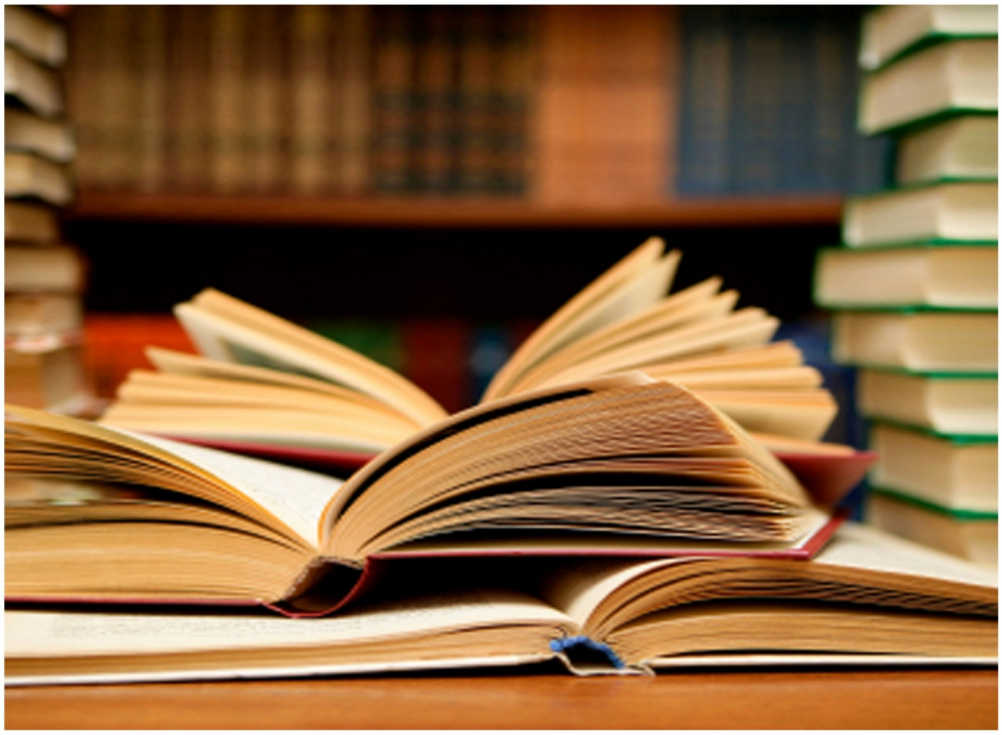

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(A) Authentication certificate of *A. galanga*

महाराष्ट्र विज्ञान वर्धिनी

आघारकर अनुसंधान संस्था

Maharashtra Association for the Cultivation of Science

**AGHARKAR RESEARCH INSTITUTE**

*(An Autonomous Grant-in-Aid Institute under the Department of Science and Technology, Govt. of India)*

May 10, 2013

AUTHENTICATION CERTIFICATE

Name of the party: - Mr. Vijaykumar M Kale

Address: Bharati Vidyapeeth Deemed University, Poona College of Pharmacy, Erandwane, Paud Road, Pune-38, India

Reference: - Letter no: Nil, dated: 25/4/2013

Name of the sample: - *Alpinia galanga*

Sample size: - Dried rhizome

Date of the receipt: - April 26, 2013

Report: -

The sample has been critically studied with macroscopic, organoleptic characters and fluorescence test. We hereby authenticate that the sample belongs to the rhizome of *Alpinia galanga* (L.)Willd. (Family- Zingiberaceae)

As per your request, the sample is deposited and voucher number is allotted as below:

| Scientific name | Family | Deposited on | Voucher number allotted |
|---|---|---|---|
| *Alpinia galanga* (L.)Willd. | Zingiberaceae | 7/5/2013 | R-167 |

This certificate is issued at his request and is given only for the academic use.

Upadhye

(A.S. Upadhye)

Auth.13-085

Scientist
Plant Drug Authentication Service
Botany Group
Plant Sciences Division

आगरकर पथ, पुणे - ४११ ००४, भारत, दूरभाष : (०२०) २५६७-८९१६/१७/१८,२५६५-३६८०/४३५७/४१०६/४०९७/४१६७ फॅक्स : (०२०) २५६५ १५४२
Agarkar Road, Pune - 411 004, India, Phone : (020) 2567-8916/17/18, 2565 - 3680/4357/4106/4097/4167 Fax : (020)2565 1542
Web : www.aripune.org E-mail : arimacs@pn2.vsnl.net.in

(B) Authentication certificate of *A. officinarum*

महाराष्ट्र विज्ञान वर्धिनी

आघारकर अनुसंधान संस्था

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| Scientific name | Family | Deposited on | Voucher number allotted |
|---|---|---|---|
| *Alpinia officinarum* Hance | Zingiberaceae | 7/5/2013 | R-168 |

This certificate is issued at his request and is given only for the academic use.

(A.S. Upadhye)

Auth 13-086

Scientist
Plant Drug Authentication Service
Botany Group
Plant Sciences Division

आगरकर पथ, पुणे – ४११ ००४, भारत, दूरभाष : (०२०) २५६७-८९१६/१७/१८,२५६५-३६८०/४३५७/४१०६/४०९७/४१६७ फॅक्स : (०२०) २५६५ १५४२
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(C) Authentication certificate of *A. purpurata*

**MATERIAL TRANSFER AGREEMENT**

I/We Rahul Pavan Kumar Jain, Poona college of pharmacy, Pune-38

requested for and received the rhizomes of the following authenticated MATERIAL and their corresponding accessions specified below from **Jawaharlal Nehru Tropical Botanic Garden & Research Institute, Palode, Thiruvananthapuram – 695 562**.

1. Alpinia galanga
2. Alpna calcarata
3. Alpinia purpurata
4. Alpinia zerumbet

I/we agree to abide by all the terms and conditions of this IN-PGRS MTA

- Not to claim ownership over the MATERIAL nor to seek intellectual property right over the germplasm MATERIAL received/ accessed and its related information.
- Not to use the MATERIAL or its derivatives for commercial purposes or profit making, whatsoever, without Written Prior Approval from JNTBGRI.
- Not to distribute or transfer samples of the MATERIAL to any other party except those directly engaged in research under my/our supervision Without Written Approval from JNTBGRI.
- To supply feedback information to JNTBGRI.

**List of Publications:**

1) Vijaykumar M. Kale and Ajay G. Namdeo. Anti-arthritic effect of galangin isolated from rhizomes of *Alpinia officinarum* in Complete Freund's Adjuvant-induced arthritis in rats. Int J Pharm Pharm Sci 2014; (6) 4: 499-505.
2) Vijaykumar M. Kale and Ajay G. Namdeo. Anti-arthritic effect of 1'-acetoxychavicol acetate in Complete Freunds Adjuvant-induced arthritis in rats. Int J Pharm Bio Sci 2014; 5(4): 9-19.
3) Ajay G. Namdeo and Vijaykumar M. Kale. Comparative pharmacognostic and phytochemical investigation of two *Alpinia* species from zingiberaceae family. World Journal of Pharmaceutical Research 2015; 4(5): 1417-1432.
4) Vijaykumar M. Kale and Ajay G. Namdeo. Micropropogation of *Alpinia purpurata* using low cost media for quantification of rutin. Der pharmacia letter 2015; 7 (5):50-57.
5) Vijaykumar M. Kale and Ajay G. Namdeo. HPTLC densitometric evaluation by simultaneous estimation of galangin in *Alpinia galanga* and *Alpinia officinarum*. Der pharmacia letter 2015; 7 (7):158-164.