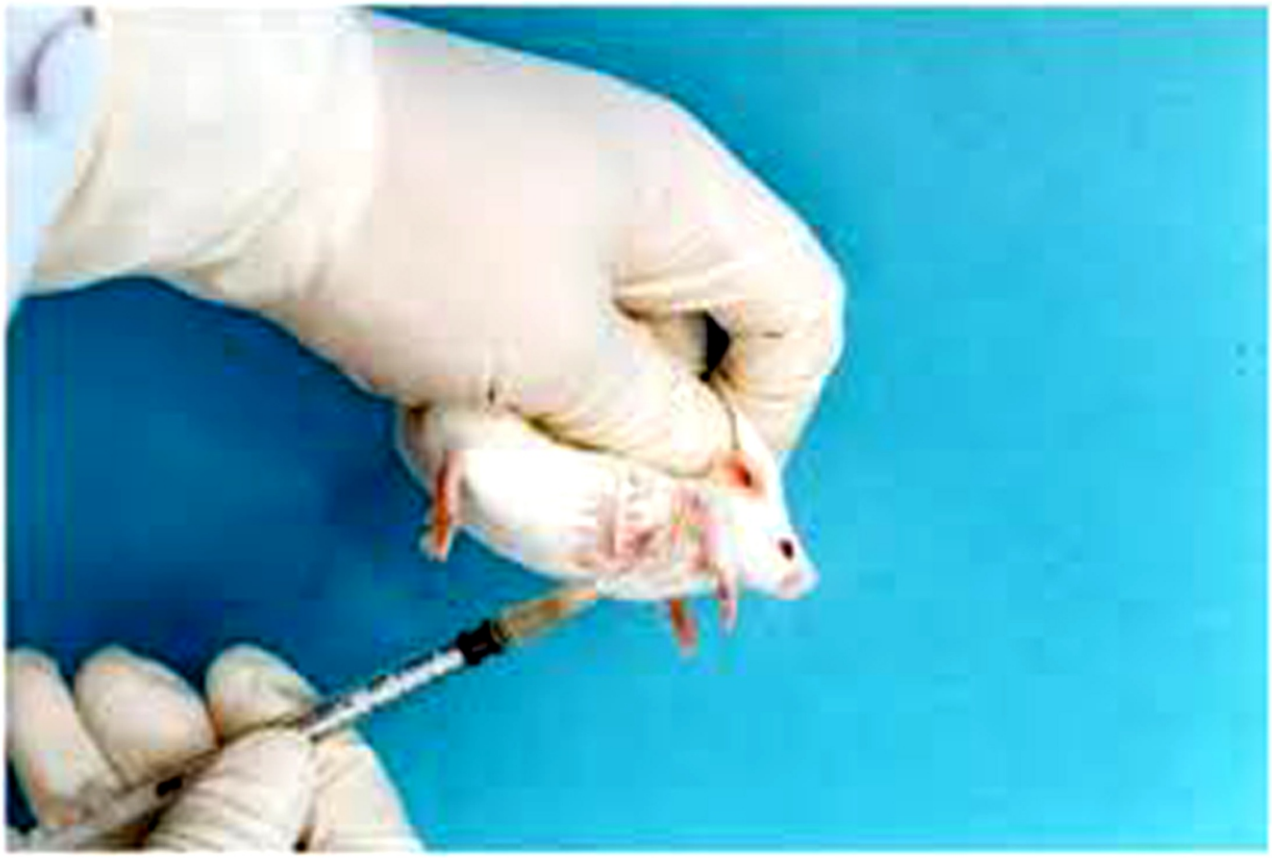
MATERIALS AND METHODS



4. Materials and methods

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 60^0C 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 28th 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°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 60^0C 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 40^0C 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 40^0C 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 40^0C 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 40^0C 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 110^0C 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 110^0C 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°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°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.