Materials and Methods
4.1 MATERIALS

4.1.1 Plant material

*Cyathocline purpurea* (Buch-Ham ex D. Don.) Kuntze Fam. Asteraceae was collected in the month of January 2013 from Mulshi, Pune, Maharashtra.

4.1.2 Identification and authentication of plant material

*Cyathocline purpurea* (Buch-Ham ex D. Don.) Kuntze was identified and authenticated by J. Jayanthi, Scientist C, Botanical Survey of India, Pune and voucher specimen (No. BSI/WRC/Tech/2013/1094) was deposited at that institute.
Materials and Methods

4.1.2a Authentication certificate of *Cyathocline purpurea* (Buch-Ham ex D. Don.) Kuntze

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No. BSI/WRC/Tech./2013/1054

Date 08-02-2013

**CERTIFICATE**

This is to certify that the plant specimen brought by Mr. Gopal V. Bihani, student of Ph.D. from Poona College of Pharmacy, Pune is identified as:

<table>
<thead>
<tr>
<th>Number/locality</th>
<th>Name</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mulshi, Pune</td>
<td><em>Cyathocline purpurea</em> (Buch.-Ham. ex D. Don.) Kuntze</td>
<td>Astereaceae</td>
</tr>
</tbody>
</table>

(J.Jayanthi)

Scientist 'C' & I.I.O.O

Botanical Survey of India

Western Regional Centre, Pune

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Study of Analgesic, Anti-inflammatory and Antiarthritic activity of Indian medicinal plant in laboratory animals
4.2 METHODS: Part A

(Analgesic, anti-inflammatory and antiarthritic activity of *Cyathocline purpurea* extracts)

### 4.2.1 Drugs and Chemicals

Petroleum ether (Merck) and Methanol (Molychem) were purchased from authorized vendor. The reagents for phytochemical identification were obtained from the freshly prepared stock used in pharmacognosy and pharmaceutical chemistry laboratories of the college.

### 4.2.2 Preparation of extracts

The whole plant was shade dried and powdered. Dried powder (500 g) was subjected to successive extractions by maceration using petroleum ether followed by methanol and then distilled water. The extracts were filtered and concentrated on a rotary evaporator (Medica Instrument, India) and stored in desiccator. The percentage yields of petroleum ether extract of *Cyathocline purpurea* (PECP), methanol extract of *Cyathocline purpurea* (MECP) and aqueous extract of *Cyathocline purpurea* (AECP) were 3.3 %, 6.7 % and 7.7 % respectively.

### 4.2.3 Storage of extracts

The PECP, MECP and AECP were stored in tightly closed amber color glass bottles in refrigerator.

### 4.2.4 Phytochemical analysis of PECP, MECP and AECP

Phytochemical analysis of PECP, MECP and AECP were performed using standard procedures to identify the constituents present in them (Khandelwal, 2010).

*Preparation of extract solutions:* The extracts PECP, MECP and AECP were dissolved in petroleum ether, methanol and water, respectively to obtained solution of respective extract (100 mg/ml) for phytochemical analysis.

#### 4.2.4.1 Test for Carbohydrates

*Molisch’s test:* Solution of extract (2 ml) and α-napthol solution (2-3 drops) were mixed in test tube, shaken for few min and concentrated H$_2$SO$_4$ (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.

4.2.4.2 Test for Proteins

**Biuret test:** Solution of extract (3 ml), (sodium hydroxide) 4% NaOH (1 ml) and (copper sulphate) 1% CuSO₄ (1 ml) were mixed in test tube. The change in color of solution to violet or pink indicates 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 appearance of red color to solution on heating indicates presence of proteins.

4.2.4.3 Test for Steroids

**Salkowski reaction:** Solution of extract (2 ml), chloroform (2 ml) and H₂SO₄ (2 ml) were mixed in test tube, shaken well. The change of chloroform layer to red and acid layer to greenish yellow fluorescence indicates presence of steroids.

**Liebermann-Burchard reaction:** Solution of extract (2 ml), chloroform (2 ml), acetic anhydride (2 ml) were mixed in test tube. Concentrated H₂SO₄ (2 drops) was added from the side of test tube. The change in color first red, then blue and finally green indicates 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 H₂SO₄ (2-3 drops) was added from the side of test tube. Appearance of blue color indicates presence of steroids.

4.2.4.4 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.

4.2.4.5 Test for Glycosides

**Keller-Killani test:** Solution of extract (2 ml), glacial acetic acid (1 ml), 5% FeCl₃ (3 drops) and concentrated H₂SO₄ 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 indicates presence of cardiac glycosides.
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Borntrager’s test: Solution of extract (2 ml) and dilute H₂SO₄ (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 indicates presence of anthraquinone glycosides.

4.2.4.6 Test for Saponins

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

4.2.4.7 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 indicates presence of tannins or phenols.
2. Lead acetate (3 ml): The occurrence of white precipitate indicates presence of tannins or phenols.
3. Potassium permanganate (3 ml): The discoloration of potassium permanganate solution indicates presence of tannins or phenols.
4. Acetic acid solution (3 ml): The red color indicates presence of tannins or phenols.

4.2.4.8 Test for presence of Flavonoids

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

4.2.4.9 Test for Alkaloids

Extract (10 g) and dilute hydrochloric acid were mixed in test tube, shaken 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 indicates 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 indicates presence of alkaloids.
Materials and Methods

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 indicates 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 indicates presence of alkaloids.

4.2.5 Pharmacological study

4.2.5.1 Chemicals and drugs
Carrageenan (Sigma-Aldrich, St. Louis, USA), Freund’s Complete Adjuvant (FCA) (Sigma-Aldrich, St. Louis, USA), Acetic acid (Pure Chem. Ltd., India), Acetylsalicylic acid (Cipla Pharmaceuticals, India), Pentazocine (Fortwin, Ranbaxy), Tween 80 (Research Lab, India), ELISA kits for determination of serum TNF-α, IL-1β, and IL-6 (Raybiotech), Biochemical diagnostic kits for determination of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Total protein and Alkaline phophatase (ALP) (Accurex biomedical Pvt. Ltd) were purchased from authorized vendors. Diclofenac was obtained as a gift sample from Emcure pharmaceuticals Ltd., Pune. All other chemicals and solvents used were of analytical grade purchased from authorized vendors.

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

4.2.5.3 Instruments used
Plethysmometer (Model: 7140, UGO Basile, Italy), 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).

4.2.5.4 Preparation of dosage form
Dosage forms of individual extracts were prepared as per the following procedures.
**Petroleum ether extract:**
The petroleum ether extract of *Cyathocline purpurea* 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 *Cyathocline purpurea* was suspended 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 *Cyathocline purpurea* was dissolved in distilled water to make up the required volume.

**Drugs**
Accurately weighed quantity of acetylsalicylic acid and diclofenac were 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.

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

### 4.2.5.6 Volume of extract solution
The volume of extract solution was calculated based upon the body weight of animal.

### 4.2.5.7 Route of administration
The extract solution was administered per orally.

### 4.2.5.8 Experimental animals
Female Swiss albino mice (25-30 g) and female Wistar rats (180-220 g) were purchased from National Institute of Biosciences, Pune, India. Animals were housed in an air-conditioned room at a temperature of 25 ± 1 °C and relative humidity of 45
to 55% under 12-h light: 12-h dark cycle. The animals had free access to food pellets (Manufactured by Pranav Agro Industries Ltd., Sangli, India) and water *ad libitum*.

### 4.2.5.9 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/28/2014 and CPCSEA/29/2014).

### 4.2.5.10 Acute oral toxicity (AOT) study

Healthy female Swiss albino mice of 25-30 g were used in acute toxicity studies as per OECD guidelines-425. The animals were fasted overnight and divided into 3 groups with 5 mice in each group. Extracts (PECP, MECP and AECP) were administered at dose of 2000 mg/kg, p.o. body weight. The mice were observed continuously for behavioral and autonomic profiles for 2 hrs and for any signs of toxicity or mortality up to 48 hrs (OECD-425, 2001).

### 4.2.5.11 Analgesic activity

#### 4.2.5.11.1 Hot plate test in mice

Female Swiss albino mice (25 – 30 g) were treated according to the method described by Eddy and Leimbach, 1953. Mice were screened by placing them on hot plate (UGO Basile, Italy. Model No. DS-37) maintained at 55 ± 1 °C and the reaction time was recorded in seconds. The time for paw licking or jumping on the hot plate was considered as a reaction time. The responses were recorded before and after 30, 60, 90, 120, 150 and 180 min of the administration of PECP, MECP, AECP and pentazocine. A cut-off time of 15s was used to avoid injury to the animals.

The mice were divided into eleven groups with six mice in each group.

Group 1: - Vehicle control (2% Tween 80).

Group 2: - Standard (Pentazocine 5 mg/kg, s.c.).

Group 3, 4 and 5: - PECP (100, 200 and 400 mg/kg, p.o.), respectively.

Group 6, 7 and 8: - MECP (100, 200 and 400 mg/kg, p.o.), respectively.
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Group 9, 10 and 11: - AECP (100, 200 and 400 mg/kg, p.o.), respectively.

4.2.5.11.2 Acetic acid induced writhing in mice
Female Swiss albino mice (25 – 30 g) were treated according to the method described by Collier et al, 1963. Mice were pretreated orally with PECP, MECP, AECP and acetylsalicylic acid, 60 min before administration of acetic acid solution at a dose of 10 ml/kg (0.6%, i.p.). The number of abdominal constrictions (full extension of both hind paws) was cumulatively counted over a period of 15 min.

The mice were divided into eleven groups of six mice each.

Group 1: - Vehicle control (2% Tween 80).
Group 2: - Standard (Acetylsalicylic acid 100 mg/kg p.o.).
Group 3, 4 and 5: - PECP (100, 200 and 400 mg/kg, p.o.), respectively.
Group 6, 7 and 8: - MECP (100, 200 and 400 mg/kg, p.o.), respectively.
Group 9, 10 and 11: - AECP (100, 200 and 400 mg/kg, p.o.), respectively.

The percent inhibition of writhing was calculated as follows:

% Inhibition = (VC-VT/VC) * 100

Where, VT, number of writhes in drug treated mice.

VC, number of writhes in control group mice.

4.2.5.12 Anti-inflammatory activity
4.2.5.12.1 Carrageenan induced paw edema in rats
Female Wistar rats (180 – 220 g) were treated according to the method described by Winter et al, 1962. Inflammation was produced by injecting 0.1ml of 1% lambda carrageenan in sterile normal saline into the sub plantar region of the right hind paw of the rat. Rats were pretreated orally with PECP, MECP, AECP and diclofenac 1h before the carrageenan injection. The paw volume was measured from 0-6 h, at an hourly interval using plethysmometer (Ugo Basile, Italy, Model No. 7140). The mean changes in injected paw volume with respect to initial paw volume were calculated.

Female Wistar rats were divided into eleven groups of six rats each.

Group 1: - Carrageenan control (2% Tween 80).
Group 2: - Standard (Diclofenac 10 mg/kg p.o.).
Group 3, 4 and 5: - PECP (100, 200 and 400 mg/kg, p.o.), respectively.
Group 6, 7 and 8: - MECP (100, 200 and 400 mg/kg, p.o.), respectively.
Group 9, 10 and 11: - AECP (100, 200 and 400 mg/kg, p.o.), respectively.
Percentage inhibition of paw volume between treated and control group was calculated by the following formula,

\[ \% \text{ Inhibition} = \left( \frac{VC - VT}{VC} \right) \times 100 \]

Where, VT and VC are the mean increase in paw volume in treated and control groups, respectively.

4.2.5.12.2 Cotton pellet induced granuloma in rats
Method described by D’Arcy et al, 1960 was followed.
Chronic inflammation was produced by implanting the pre-weighed sterile cotton pellets (50 mg) in the axilla region of the each rat through a small incision. PECP, MECP, AECP and diclofenac were administered orally for seven consecutive days after the cotton pellet implantation. Before implanting the cotton pellets, rats were anaesthetized with anesthetic ether. On the eight day animals were sacrificed by cervical dislocation and stomach was removed for histopathology study and cotton pellets were removed from animal’s body, freed from the extraneous tissues, dried in oven at 60 °C for 24 h and weighed.
Female Wistar rats weighing (180 – 220 g) were divided into eleven groups of six rats each.
Group 1: - Vehicle control (2% Tween 80).
Group 2: - Standard (Diclofenac 10 mg/kg p.o.).
Group 3, 4 and 5: - PECP (100, 200 and 400 mg/kg, p.o.), respectively.
Group 6, 7 and 8: - MECP (100, 200 and 400 mg/kg, p.o.), respectively.
Group 9, 10 and 11: - AECP (100, 200 and 400 mg/kg, p.o.), respectively.

4.2.5.13 Antiarthritic activity
Freund’s complete adjuvant induced arthritis in rats
Arthritis was induced by single intra-dermal injection of 0.1 ml of Freund’s complete adjuvant into a foot pad of the left hind paw of female Wistar rats (180-220 gm) rats. Each ml contains 1 mg of *Mycobacterium tuberculosis* (Strain H37Ra, ATCC-25177) (Patel et al., 2012).
The female Wistar rats weighing (180 – 220 g) were divided into six groups consisting of six animals per group:
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Study of Analgesic, Anti-inflammatory and Antiarthritic activity of Indian medicinal plant in laboratory animals

Group I – Healthy control
Group II – Arthritic control
Group III – Standard, Diclofenac 5 mg/kg, p.o.
Group IV – MECP 100 mg/kg, p.o.
Group V – MECP 200 mg/kg, p.o.
Group VI – MECP 400 mg/kg, p.o.

Anti-arthritic activity of MECP on the injected paw was evaluated on the following parameters change in paw volume, change in joint diameter, pain threshold, paw withdrawal latency, mechanical nociceptive threshold and body weight on day 0, 1, 4, 8, 12, 16, 20, 24, and day 28 (Kumar et al., 2006). On day 28 the animals were anaesthetized with anesthetic ether and the blood was withdrawn by retro-orbital puncture. Serum was used for the estimation of biochemical parameters (AST, ALT, Alkaline phosphatase, and Total protein), CRP and RF value. Whole blood was used for estimation of hematological parameters (WBC, RBC, Hb and Platelets) and isolated liver was used for estimation of antioxidant parameters (SOD, MDA and GSH). Radiological and histopathological analyses of ankle joints were also done.

4.2.5.13.1 Measurement of change in paw volume
Change in paw volume was measured using a Plethysmometer (UGO Basile, Italy) on day 0 before FCA injections and thereafter on day 1, 4, 8, 12, 16, 20, 24, and day 28 (Lee et al., 2009). The change in paw volume was calculated as the difference between the final and initial paw volume.

4.2.5.13.2 Measurement of change in joint diameter
Change in joint diameter was measured using a digital Vernier caliper (Mitutoyo, Japan) on day 0 before FCA injections and thereafter on day 1, 4, 8, 12, 16, 20, 24, and day 28 (Banchet et al., 2009). The change in joint diameter was calculated as the difference between the final and initial joint diameter.

4.2.5.13.3 Measurement of pain threshold (Randall Selitto)
Pain threshold was measured using Randall-Selitto analgesiometer (UGO Basile, Italy) on day 0 just before FCA injections and thereafter on day 1, 4, 8, 12, 16, 20, 24, and day 28. The hind paw was placed between the flat surface and blunt pointer and
Materials and Methods

was applied with increasing pressure. The cut-off pressure was 450 g. The pain threshold was determined when rat attempted to remove the hind paw from the apparatus (Authier et al., 2003).

4.2.5.13.4 Measurement of paw withdrawal latency (Thermal hyperalgesia)

Paw withdrawal latency was measured using a radiant heat apparatus (UGO Basile, Italy) on day 0 just before FCA injections and thereafter on day 1, 4, 8, 12, 16, 20, 24, and day 28. The paw was placed on the heat radiator with infrared intensity of lamp was set at 40. A cut of latency of 15 s was used to avoid tissue damage (Ramteke et al., 2009).

4.2.5.13.5 Measurement of mechanical nociceptive threshold (Tactile allodynia)

Mechanical nociceptive threshold was determined by measuring paw withdrawal following probing of the plantar surface with a series of calibrated fine filaments (Von Frey hairs, Almemo, Germany) of increasing gauge (Jalalpure et al., 2011; Pepys and Hirschfield, 2003). The rats were allowed to acclimatize for 10 min in the Perspex box and Von Frey hairs (0.6 to 12.6 g) were applied to plantar surface of left hind paw. A series of three stimuli were applied to 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 threshold. Lifting of the paw was recorded as a positive response (Mali et al., 2011).

4.2.5.13.6 Body weight recording

Body weight was recorded on day 0 just before FCA injections and thereafter on day 1, 4, 8, 12, 16, 20, 24, and day 28 (Asquith et al., 2009).

4.2.5.13.7 Radiological analysis of ankle joints

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.
4.2.5.13.8 Haematological and serum parameters
On day 28, haematological parameters like red blood cell (RBC) count, white blood cell (WBC) count, haemoglobin (Hb), and platelets (PLT) were determined by usual standardized laboratory method (Mythilypriya et al., 2008). Serum C-reactive protein (CRP) and Rheumatoid factor (RF) level was also measured (Mehta et al., 2012).

4.2.5.13.9 Biochemical parameters
On day 28, blood of the rats was withdrawn by retro-orbital puncture and centrifuged at 7000 rpm at 4°C for 15 minutes. Serum was used for the estimation of AST, ALT, alkaline phosphatase and total protein levels (Mythilypriya et al., 2008).

4.2.5.13.10 Antioxidant parameters
The rats were sacrificed on day 28 by cervical dislocation, the levels of malondialdehyde (MDA), reduced glutathione (GSH) and superoxide dismutase (SOD) in liver were estimated as biomarkers of inflammation.

4.2.5.13.10.1 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 were dissolved in 900 ml of distilled water and pH was adjusted 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 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.

4.2.5.13.10.2 Assay of lipid Peroxidation (MDA content)

**Reagents**

*Thiobarbituric acid (0.67% w/v)*

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

*Trichloroacetic acid (10% w/v)*

Trichloroacetic acid 10 gm was dissolved in 60 ml of distilled water and the final volume was made up to 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 up to 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 23 ng 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).

4.2.5.13.10.3 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 30.2 gm sodium phosphate in 600 ml 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)  
10 mg 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 (Morgon et al., 1979).

4.2.5.13.10.4 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 0.99 gm 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°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 (Misra and Fridovich., 1972).

4.2.5.13.11 Histopathological analysis of ankle joints
On day 28, ankle joints were separated from the hind paw and immersed in 10% buffered formalin for 24 h followed by decalcification in 5% formic acid, processed for paraffin embedding sectioned at 5µ thickness. The sections were stained with haematoxylin-eosin and evaluated under light microscope with 10X magnifications for the presence of inflammatory cells, hyperplasia of synovium, pannus formation and destruction of joint space (Patil et al., 2012).
4.3 METHODS: Part B
(Isolation and characterization)

4.3.1 Chemicals and reagents
Petroleum ether, ethyl acetate, acetone, methanol, ethanol (Merck) of GR grades, column grade silica (60-120 mesh) (Merck Specialties Private Limited, Mumbai) and thin layer chromatography (TLC) silica gel plates 60 F_{254} (Merck, Germany) were purchased from respective vendors.

4.3.2 Apparatus and instruments
Borosil glass column (height: 60 cm; diameter: 3 cm) was purchased from Ajay scientific enterprises, Pune, India. Precoated TLC silica gel plates (Merck, Kieselgel 60, F-254, 0.2 mm, Germany) were used for analytical TLC. IR spectra were recorded using KBr pellets on JASCO FT-IR 5300 spectrophotometer. $^1$H-NMR and $^{13}$C-NMR spectra were recorded on 200 MHz and 50 MHz spectrometer, respectively (Bruker, Germany). Deuterated chloroform (CDCl$_3$) was used for recording NMR and tetramethylsilane (TMS) was used as an internal standard. Chemical shifts were reported as $\delta$ (ppm). The coupling constants ($J$) were reported as Hz. Mass spectrum was obtained on a Thermo Finigen Surveyor MSQ spectrometer.

4.3.3 Experimental animals
Female Wistar rats (180-220 g) were purchased from National Institute of Biosciences, Pune, India. Animals were housed in an air-conditioned room at a temperature of 25 ± 1 °C and relative humidity of 45 to 55% under 12-h light: 12-h dark cycle. The animals had free access to food pellets (Manufactured by Pranav Agro Industries Ltd., Sangli India) and water ad libitum.

4.3.4 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 was CPCSEA/PCL/07/2014-2015).
4.3.5 Liquid – solid separation chromatographic technique

15 g of MECP (most active extract) was mixed with 30 ml of acetone and 30 g of silica gel. Sample were mixed thoroughly and dried in oven at 110°C for 10 min and following fractions were prepared.

4.3.5.1 Petroleum ether fraction (F – 1)

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

4.3.5.2 10% acetone in petroleum ether fraction (F – 2)

100 ml of 10% acetone in petroleum ether was added into the residue and shaken 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 in petroleum ether fraction. Same procedure was repeated twice with 100 ml of 10% acetone in petroleum ether.

4.3.5.3 20% acetone in petroleum ether fraction (F – 3)

100 ml of 20% acetone in petroleum ether was added into the residue and shaken 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 in petroleum ether fraction. Same procedure was repeated twice with 100 ml of 20% acetone in petroleum ether.

4.3.5.4 30% acetone in petroleum ether fraction (F – 4)

100 ml of 30% acetone in petroleum ether was added into the residue and shaken 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 in petroleum ether fraction. Same procedure was repeated twice with 100 ml of 30% acetone in petroleum ether.
4.3.5.5 50% acetone in petroleum ether fraction (F – 5)
100 ml of 50% acetone in petroleum ether was added into the residue and shaken 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 in petroleum ether fraction. Same procedure was repeated twice with 100 ml of 50% acetone in petroleum ether.

4.3.5.6 Methanol fraction (F – 6)
100 ml of methanol was added into the residue and shaken properly. Then the mixture was allowed to settle, two layers were formed. Upper layer was separated into the separate flask and labeled as methanol fraction. Same procedure was repeated twice with 100 ml of methanol.

4.3.6 Anti-inflammatory activity of fractions (F – 1 to F – 6) in carrageenan induced paw edema in rats.
Inflammation was produced by injecting 0.1ml of 1% lambda carrageenan (Sigma Chemical Co., USA) in sterile normal saline into the sub plantar region of the right hind paw of the rat (Winter et al., 1962). Rats were pretreated by test substance orally 1h before the carrageenan injection. The paw volume was measured from 0-6 h, at an hourly interval using plethysmometer (Ugo Basile, Italy, Model No. 7140). The mean changes in injected paw volume with respect to initial paw volume were calculated. Percentage inhibition of paw volume between treated and control group was calculated by the following formula,
% Inhibition = (VC-VT / VC *100)
Where, VT and VC are the mean increase in paw volume in treated and control groups, respectively.
Female Wistar rats weighing 180-220 g were divided in following groups (n=6) viz;
Group 1: - Carrageenan control.
Group 2: - Standard, diclofenac 10 mg/kg, p.o.
Group 3: - Petroleum ether fraction (F – 1), 100 mg/kg, p.o.
Group 4: - 10% acetone in petroleum ether fraction (F – 2), 100 mg/kg, p.o.
Group 5: - 20% acetone in petroleum ether fraction (F – 3), 100 mg/kg, p.o.
Group 6: - 30% acetone in petroleum fraction (F – 4), 100 mg/kg, p.o.
Materials and Methods

Group 7: - 50% acetone in petroleum fraction (F – 5), 100 mg/kg, p.o.
Group 8: - Methanol fraction (F – 6), 100 mg/kg, p.o.

4.3.7 Column chromatography of most active anti-inflammatory fraction i.e. 30% acetone in petroleum ether fraction (F – 4)

Figure 9- Column chromatography of 30% acetone in petroleum ether fraction (F – 4) (A) Silica gel adsorbed with 30% acetone in petroleum ether fraction (F – 4) (B) Column loaded with 30% acetone in petroleum ether fraction (F – 4) (C) 30% acetone in petroleum ether fraction (F – 4) uniformly spread in column after addition of mobile phase (D) 30% acetone in petroleum ether fraction uniformly spread in column continued (E) Elution collection started.

Column was packed with activated silica (60-120#). The 30% acetone in petroleum ether fraction (F – 4) was adsorbed on silica gel and the dried silica was added to top of the column. The mobile phase was then added and the elution collection started.
of the packed silica in column (Figure 9). After stabilization column was eluted with mobile phase (petroleum ether: ethyl acetate, initially starting with lower proportion of ethyl acetate and then increasing the proportion of ethyl acetate). Fractions were collected and analyzed by TLC. Fractions showing similar bands were pooled together and labeled as pool P – 1 to P – 10.

**Activation of silica:**
Column grade silica (60 – 120 mesh) was placed in oven at 110°C overnight (12 h) to remove all moisture content present in it. This activated silica was packed in the column.

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

**Packing of column:**
A clean and dry borosil glass column (height: 60 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 column was gently tapped with a cork during the packing process to compact silica column.

**Application of sample:**
Weighed quantity (2.5 g) of the 30% acetone in petroleum ether fraction (F – 4) was dissolved in 5 ml of acetone and was adsorbed on 5 gm of silica gel (particle size: 60-120 mesh) 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 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: ethyl acetate. Polarity of mobile phase was
increased by increasing the proportion of ethyl acetate and fractions (25 ml in each 50 ml tube) were collected in tubes. Remaining loaded material in the column which could not be eluted with mobile phase was eluted with methanol and collected as methanol fraction and completed the column chromatography. All fractions were analyzed by TLC and fractions showing similar bands were pooled together and labeled as pool P – 1 to P – 10 (Figure 10).

![Figure 10- TLC of pools (P – 1 to P – 10) collected from 30% acetone in petroleum ether fraction (F – 4). (A) TLC of pool P – 1 to P – 6 (B) TLC of pool P – 7 to P – 10](image)

4.3.8 Anti-inflammatory activity of pools (P – 1 to P – 10) collected from 30% acetone in petroleum ether fraction (F – 4) in carrageenan induced paw edema

Pools (P – 1 to P – 10) collected from 30% acetone in petroleum ether fraction (F – 4) were subjected further for anti-inflammatory activity. Female Wistar rats weighing 180-220 g were divided in following groups (n=6) viz;

Group 1: - Carrageenan control.
Group 2: - Standard, diclofenac (10 mg/kg, p.o)
Group 3 to 12: - P – 1 to P – 10, respectively (10 mg/kg, p.o)
Materials and Methods

Inflammation was produced by injecting 0.1 ml of 1% lambda carrageenan (Sigma Chemical Co., USA) in sterile normal saline into the sub plantar region of the right hind paw of the rat (Winter et al., 1962). Rats were pretreated by test substance orally 1h before the carrageenan injection. The paw volume was measured from 0-6 h, at an hourly interval using plethysmometer (Ugo Basile, Italy, Model No. 7140). The mean changes in injected paw volume with respect to initial paw volume were calculated. Percentage inhibition of paw volume between treated and control group was calculated by the following formula,

\[
% \text{Inhibition} = \left( \frac{VC - VT}{VC} \right) \times 100
\]

Where, VT and VC are the mean increase in paw volume in treated and control groups, respectively.

4.3.9 Preparative TLC of most active anti-inflammatory pool i.e. P – 8

Application of sample:
The sample of pool P – 8 was applied by streaking across the full length of the plate by glass capillary.

Mobile phase:
The solvents petroleum ether and ethyl acetate were used for the preparation of mobile phase. The composition of mobile phase was petroleum ether: ethyl acetate (70:30).

Preconditioning of chamber (saturation):
Chromatogram was developed in a saturated twin trough chambers. A sufficient quantity (approximately 10 ml) of mobile phase was poured along the side into the chamber to saturate the chamber. Chamber was then closed and allowed to stand for at least 30 min at room temperature (RT).

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 violet (UV) light at 254 and 366 nm wavelength. Area was marked and scrapped off with sharp blade. 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 then filtered by Whatman filter paper and filtrate was allowed to evaporate.

4.3.10 Spectral characterization of isolated compound (P – 8)
The chemical structure of isolated compound was elucidated by IR, $^1$H-NMR, $^{13}$C-NMR, DEPT and MS spectroscopy.

4.3.11 Docking study
Glide (Glide, 2009) was used for docking study to examine the binding mode of isolated compound with TNF-alpha converting enzyme (TACE) (PDB: 1ZXC). The ligands were prepared using LigPrep (LigPrep, 2009). The protein was refined using the protein preparation wizard present in Maestro 9.0 (Maestro, 2009). All the water molecules were deleted. Hydrogen atoms were added to the protein, including the protons necessary to define the correct ionization and tautomeric states of the amino acid residues. Prime interface module incorporated in Maestro 9.0 was used to add the missing residues of the side chain. Each structure minimization was carried out with the impact refinement module to alleviate steric clashes potentially existing in the structures. Minimization was terminated when the energy converged or the root mean square deviation reached a maximum cutoff of 0.30 Å. To find out active site grid was prepared using grid generation panel of glide with the default settings. Grid is prepared for defining the binding site of native ligand on the receptor. The ligand was selected to define the position and size of the active site (Friesner et al., 2004; Halgren et al., 2004; Bhansali and Kulkarni, 2014). Glide XP docking was used for docking purposes.
4.4. METHODS: Part C
(Antiarthritic activity of isolated compound, Isoivangustin)

**Freund’s complete adjuvant induced arthritis in rats**
Arthritis was induced by single intra-dermal injection of 0.1 ml of Freund’s complete adjuvant into a foot pad of the left hind paw of female Wistar rats (180-220 gm) rats. Each ml contains 1 mg of *Mycobacterium tuberculosis* (Strain H37Ra, ATCC-25177). The rats were anesthetized with ether inhalation during adjuvant injection as the viscous nature of adjuvant exerts difficulty while injecting (Patel et al., 2012). The animals were divided into six groups consisting of six animals per group:
- Group I – Healthy control
- Group II – Arthritic control
- Group III – Standard, diclofenac 5 mg/kg, p.o.
- Group IV – Isoivangustin 2.5 mg/kg, p.o.
- Group V – Isoivangustin 5 mg/kg, p.o.
- Group VI – Isoivangustin 10 mg/kg, p.o.

Anti-arthritic activity of Isoivangustin was evaluated on the following parameters: change in paw volume, change in joint diameter, pain threshold, paw withdrawal latency, mechanical nociceptive threshold and body weight on day 0, 1, 4, 8, 12, 16, 20, 24, and day 28 (Kumar et al., 2006). On day 28 the animals were anaesthetized with anesthetic ether and the blood was withdrawn by retro-orbital puncture and centrifuged. Serum was used for the estimation of cytokines (TNF-α, IL-1β, and IL-6), biochemical parameters (AST, ALT, Alkaline phosphatase, and Total protein), CRP and RF value. Whole blood was used for estimation of hematological parameters (WBC, RBC, ESR, Hb and Platelets) and isolated liver was used for estimation of antioxidant parameters (SOD, MDA and GSH). Radiological and histopathological analyses of ankle joints were also done on last day.

**4.4.1 Measurement of change in paw volume**
Change in paw volume was measured using a Plethysmometer (UGO Basile, Italy) on day 0 before FCA injections and thereafter on day 1, 4, 8, 12, 16, 20, 24, and day 28 (Lee et al., 2009). The change in paw volume was calculated as the difference between the final and initial paw volume.
4.4.2 Measurement of change in joint diameter
Change in joint diameter was measured using a digital Vernier caliper (Mitutoyo, Japan) on day 0 before FCA injections and thereafter on day 1, 4, 8, 12, 16, 20, 24, and day 28 (Banchet et al., 2009). The change in joint diameter was calculated as the difference between the final and initial joint diameter.

4.4.3 Measurement of pain threshold (Randall Selitto)
Pain threshold was measured using Randall-Selitto analgesiometer (UGO Basile, Italy) on day 0 just before FCA injections and thereafter on day 1, 4, 8, 12, 16, 20, 24, and day 28. The hind paw was placed between the flat surface and blunt pointer and was applied with increasing pressure. The cut-off pressure was 450 g. The pain threshold was determined when rat attempted to remove the hind paw from the apparatus (Authier et al., 2003).

4.4.4 Measurement of paw withdrawal latency (Thermal hyperalgesia)
Paw withdrawal latency was measured using a radiant heat apparatus (UGO Basile, Italy) on day 0 just before FCA injections and thereafter on day 1, 4, 8, 12, 16, 20, 24, and day 28. The paw was placed on the heat radiator with infrared intensity of lamp was set at 40. A cut of latency of 15 s was used to avoid tissue damage (Ramteke et al., 2009).

4.4.5 Measurement of mechanical nociceptive threshold (Tactile allodynia)
Mechanical nociceptive threshold was determined by measuring paw withdrawal following probing of the plantar surface with a series of calibrated fine filaments (Von Frey hairs, Almemo, Germany) of increasing gauge (Jalalpure et al., 2011; Pepys and Hirschfield, 2003). The rats were allowed to acclimatize for 10 min in the Perspex box and Von Frey hairs (0.6 to 12.6 g) were applied to plantar surface of left hind paw. A series of three stimuli were applied to 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 threshold. Lifting of the paw was recorded as a positive response (Mali et al., 2011).
4.4.6 **Body weight recording**

Body weight was recorded on day 0 just before FCA injections and thereafter on day 1, 4, 8, 12, 16, 20, 24, and day 28 (Asquith et al., 2009).

4.4.7 **Radiological analysis of ankle joints**

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 was performed at 55 kV peak, 50 mA and the exposure time was 5 s.

4.4.8 **Haematological and serum parameters**

On day 28, haematological parameters like red blood cell (RBC) count, white blood cell (WBC) count, haemoglobin (Hb), platelets (PLT) and erythrocyte sedimentation rate (ESR) were determined by usual standardized laboratory method (Mythilypriya et al., 2008). Serum C-reactive protein (CRP) and Rheumatoid factor (RF) level was also measured (Mehta et al., 2012).

4.4.9 **Biochemical parameters**

On day 28, blood of the rats was withdrawn by retro-orbital puncture and centrifuged at 7000 rpm at 4°C for 15 minutes. Serum was used for the estimation of serum AST, ALT, ALP and total protein levels (Mythilypriya et al., 2008).

4.4.10 **Cytokine measurement by ELISA**

On day 28, serum was used for estimation of levels of TNF-α, IL-1β and IL-6.

4.4.10.1 **Measurement of serum TNF-α**

Reagent preparation:

Sample dilution: 100 µl serum was diluted with 200 µl of Assay Diluent A

Standard preparation: The vial of Item C (Recombinant Rat TNF-α) was briefly spin and 400 µl of Assay Diluent A was added to it with gentle mixing. Then 100 µl of TNF-α standard from the vial of Item C was added into a tube with 400 µl Assay Diluent A to prepare a 20,000 pg/ml stock standard solution. 200 µl of these prepared solution was diluted serially into more six tubes containing 400 µl Assay Diluent A to prepare different concentrations.
Wash buffer solution preparation: 20 ml of wash buffer concentrate was diluted with 400 ml of distilled water.

Biotin antibody solution preparation: Detection Antibody TNF-α (Item F) was briefly spin before use. 100 µl of 1x Assay Diluent B was added into the vial to prepare a detection antibody concentrate. The prepared detection antibody concentrate was again diluted 80 fold with 1x Assay Diluent B.

Streptavidin solution preparation: 50 µl of HRP-Streptavidin concentrate was added into a tube with 10 ml 1x Assay Diluent B.

Procedure:
100 µl of standard and samples were added to each well and incubated for 2.5 hr at room temperature and then washed 4 times with wash buffer solution.

Then 100 µl of prepared biotin antibody was added to each well and incubated for 1 hr at room temperature and then washed 4 times with wash buffer solution.

Then added 100 µl of prepared Streptavidin solution and incubated for 45 minutes at room temperature and then washed 4 times with wash buffer solution.

Then added 100 µl of TMB (tetramethylbenzidine) to each well, the blue color was developed in proportion to the amount of TNF-α bound and incubated for 30 minutes.

Finally 50 µl of Stop solution was added to each well, the color changed from blue to yellow and reading was noted at 450 nm immediately.

Calculations:
The TNF-α protein was quantified by comparing the sample to the standard curve generated. The results were expressed as cytokine concentrations (e.g., pg/ml protein).

4.4.10.2 Measurement of serum IL-1β

Reagent preparation:
Sample dilution: 100 µl serum was diluted with 200 µl of Assay Diluent A
Standard preparation: The vial of Item C (Recombinant Rat IL-1β) was briefly spin and 400 µl of Assay Diluent A was added to prepare a 50,000 pg/ml stock standard
solution. 130 µl of these prepared solution was diluted serially into more seven tubes containing 260 µl Assay Diluent A to prepare different concentrations.

Wash buffer solution preparation: 20 ml of wash buffer concentrate was diluted with 400 ml of distilled water.

Biotin antibody solution preparation: Detection Antibody IL-1β (Item F) was briefly spin before use. 100 µl of 1x Assay Diluent B was added into the vial to prepare a detection antibody concentrate. The prepared detection antibody concentrate was again diluted 80 fold with 1x Assay Diluent B.

Streptavidin solution preparation: 50 µl of HRP-Streptavidin concentrate was added into a tube with 10 ml 1x Assay Diluent B.

Procedure:

100 µl of standard and samples were added to each well and incubated for 2.5 hr at room temperature and then washed 4 times with wash buffer solution.

Then 100 µl of prepared biotin antibody was added to each well and incubated for 1 hr at room temperature and then washed 4 times with wash buffer solution.

Then added 100 µl of prepared Streptavidin solution and incubated for 45 minutes at room temperature and then washed 4 times with wash buffer solution.

Then added 100 µl of TMB to each well, the blue color was developed in proportion to the amount of IL-1β bound and incubated for 30 minutes.

Finally 50 µl of Stop solution was added to each well, the color changed from blue to yellow and reading was noted at 450 nm immediately.

Calculations:
The IL-1β protein was quantified by comparing the sample to the standard curve generated. The results were expressed as cytokine concentrations (e.g., pg/ml protein).

4.4.10.3 Measurement of serum IL-6

Reagent preparation:
Sample dilution: 100 µl serum was diluted with 200 µl of Assay Diluent C
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Standard preparation: The vial of Item C (Recombinant Rat IL-6) was briefly spin and 500 µl of Assay Diluent C was added to prepare a 10,000 pg/ml stock standard solution. 200 µl of these prepared solution was diluted serially into more seven tubes containing 300 µl Assay Diluent C to prepare different concentrations.

Wash buffer solution preparation: 20 ml of wash buffer concentrate was diluted with 400 ml of distilled water.

Biotin antibody solution preparation: Detection Antibody IL-6 (Item F) was briefly spin before use. 100 µl of 1x Assay Diluent B was added into the vial to prepare a detection antibody concentrate. The prepared detection antibody concentrate was again diluted 80 fold with 1x Assay Diluent B.

Streptavidin solution preparation: 30 µl of HRP-Streptavidin concentrate was added into a tube with 12 ml 1x Assay Diluent B.

Procedure:
100 µl of standard and samples were added to each well and incubated for 2.5 hr at room temperature and then washed 4 times with wash buffer solution.

Then 100 µl of prepared biotin antibody was added to each well and incubated for 1 hr at room temperature and then washed 4 times with wash buffer solution.

Then added 100 µl of prepared Streptavidin solution and incubated for 45 minutes at room temperature and then washed 4 times with wash buffer solution.

Then added 100 µl of TMB to each well, the blue color was developed in proportion to the amount of IL-1β bound and incubated for 30 minutes.

Finally 50 µl of Stop solution was added to each well, the color changed from blue to yellow and reading was noted at 450 nm immediately.

Calculations:
The IL-6 protein was quantified by comparing the sample to the standard curve generated. The results were expressed as cytokine concentrations (e.g., pg/ml protein).
4.4.11 Antioxidant parameters

The rats were sacrificed on day 28 by cervical dislocation, the levels of malondialdehyde (MDA), reduced glutathione (GSH) and superoxide dismutase (SOD) in liver were estimated as biomarkers of inflammation.

4.4.11.1 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 were dissolved in 900 ml of distilled water and pH was adjusted 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 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.
4.4.11.2 Assay of lipid Peroxidation (MDA content)

Reagents

Thiobarbituric acid (0.67% w/v)
Thiobarbituric acid 0.67 gm was dissolved in 50 ml of distilled water and the final volume was made up to 100 ml with distilled water.

Trichloroacetic acid (10% w/v)
Trichloroacetic acid 10 gm was dissolved in 60 ml of distilled water and the final volume was made up to 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 up to 100 ml with distilled water. 1 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).

4.4.11.3 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.
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Study of Analgesic, Anti-inflammatory and Antiarthritic activity of Indian medicinal plant in laboratory animals

Phosphate Buffer (0.2M, pH 8.0)
Sodium phosphate 0.2 M was prepared by dissolving 30.2 gm sodium phosphate in 600 ml 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)
10 mg 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 (Morgon et al., 1979).

4.4.11.4 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 0.99 gm was dissolved in 100 ml of 0.1N hydrochloric acid and the volume was adjusted to 1000 ml with 0.1N hydrochloric acid.
**Materials and Methods**

*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°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 was expressed as units/mg protein (Misra and Fridovich., 1972).

4.4.12 **Histopathological analysis of ankle joints**

On day 28, ankle joints were separated from the hind paw and immersed in 10% buffered formalin for 24 h followed by decalcification in 5% formic acid, processed for paraffin embedding sectioned at 5µ thickness. The sections were stained with haematoxylin-eosin and evaluated under light microscope with 10X magnifications for the presence of inflammatory cells, hyperplasia of synovium, pannus formation and destruction of joint space (Patil et al., 2012).

4.5 **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 change in paw volume, change in joint diameter, pain threshold, mechanical nociceptive threshold, paw withdrawal latency and body weight were analyzed by Two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Data of hematological, serum, biochemical and antioxidant 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.