CHAPTER 4: MATERIALS AND METHODS

4.1 Chemicals, Drugs and Instruments

FCA, Evan blue, carrageenan and histamine were purchased from Sigma Aldrich and Indomethacin was obtained from GMH Pharmaceutical Karnal, Haryana, India. Anti-arthritis herbal drug Rumalaya forte tablets were obtained from Himalayan drug store local market Belgaum, India. Standard curcuminoid purchased from Euca company bombay. All other chemicals used were analytical grade and were obtained from Qualigen Fine Chemicals, Mumbai, India.

Instruments that were used in the present study are ESR stand and Westergren pipettes, Sahil’s haemometer, Plethysmometer (7141), X-ray machine (Siemens Multipho 10), Spencer type wax microtome, Metzer biomedical research microscope, TLC chamber, hot air oven, Rotary flash evaporator, High performance liquid chromatography, High performance thin layer liquid chromatography, U/V spectrophotometer, Infra-Red spectrum, Proton nuclear magnetic resonance, Liquid chromatography- mass chromatography and Mechanical shaker.

4.2 Pharmacognostic Investigations

The roots of C. zedoaria Rosc were collected in the month of February, 2009 from Cochin, Kerala, India. Roots were identified by Dr. A. K. S. Rawat, Scientist-E, National Botanical Research Institute, Lucknow, India (Specification no NBRI-SOP-202). A voucher specimen of the root is deposited in the department for future reference.

Morphological / Organoleptic evaluation

Morphology is the study of the form of an object whilst morphography is the description of that form where the material is known to occur in a particular form.
Morphological features and organoleptic features viz. colour, odor, taste, shape and size were observed.

4.3 Standardization of Selected Plant Materials

The roots of *C. zedoaria* were washed in tap water, cut into small pieces and then shade dried. The dried pieces were then pulverized with an electric blender, and a yellow powder was obtained (25-45 mesh size) and stored in well-closed container. Then uniform powder was subjected to standardization with different parameters.  

1) **Determination of alcohol-soluble extractive:**

5 g of shade-dried *C. zedoaria* root powder was macerated with 100 ml of 95% ethanol in a closed flask, shaking frequently during the first 6 hours and allowed to stand for 18 hrs. Thereafter it was filtered rapidly taking precaution against loss of ethanol. 25 ml of filtrate was evaporated to dryness in a tared flat bottom shallow dish, dried at 105°C and weighed. Percentage ethanol soluble extractive was calculated with reference to the shade-dried plant powder.

2) **Determination of water-soluble extractive:**

5 g of shade-dried *C. zedoaria* root powder was macerated with 100 ml of water in a closed flask, shaking frequently during the first 6 hrs and allowed to stand for 18 hrs. Thereafter it was filtered rapidly. 25 ml of filtrate was evaporated to dryness in a tared flat bottom shallow dish, dried at 105°C and weighed. Percentages of extractive values were calculated with reference to the shade-dried leaf powder.

3) **Determination of petroleum-ether soluble extractive:**

5 g of shade-dried *C. zedoaria* root powder was macerated with 100 ml of water in a closed flask, shaking frequently during the first 6 hrs and allowed to stand for 18 hrs. Thereafter it was filtered rapidly. 25 ml of filtrate was evaporated to dryness in a tared
flat bottom shallow dish, dried at 105°C and weighed. Percentages of extractive values were calculated with reference to the shade-dried leaf powder.

4) **Loss on drying:**

An accurately weighed quantity of the shade-dried coarsely powdered *Curcuma zedoaria* root powder was taken in a tared glass bottle and the initial weight was taken. The crude drug was heated at 105°C in an oven and weighed. This procedure was repeated till a constant weight was obtained. The moisture content of the sample was calculated as percentage with reference to the shade-dried material

\[
\text{Loss on drying (\%) } = \frac{\text{Loss in weight}}{\text{Weight of drug in g.}} \times 100
\]

5) **Determination of total ash:**

About 2 to 3 g, accurately weighed, the ground drug was taken in a tared platinum silica dish previously ignited and weighed. The ground drug was scatter on the bottom of the dish. It was incinerated by gradually increasing the heat not exceeding dull red heat until free from carbon, cool and weigh. When a carbon free ash cannot be obtained in this way, was added the charred mass with hot water, the residue was collected on an ash less filter paper, was added filtrate, evaporate to dryness and ignited at a low temperature. the percentages of ash were calculated with reference to air dried drug.

\[
\text{Total ash value of sample } \% = \left(\frac{Z-X}{Y}\right) \times 100
\]

Where,  
Z = weight of dish + ash (after complete incineration)  
X = weight of empty dish  
Y = weight of drug taken

6) **Determination of acid-insoluble ash:**

The ash obtained by total ash was boiled for 10 minutes with 25 ml of dilute hydrochloric acid. The insoluble matter was collected in a Gooch crucible or on an ash
less filter paper. It was washed with hot water. Ignited and weighed. The percentage of acid insoluble ash was collected with reference to the air dried drug.

Acid insoluble ash value of the sample % = \( \frac{a}{y} \times 100 \)

Where, 
- \( a \) = weight of residue
- \( y \) = weight of air dried drug

7) Determination of water-soluble ash:

The ash obtained by total ash was boiled for 10 minutes with 25 ml of water. The insoluble matter was collected in a Gooch crucible or on an ash less filter paper. It was washed with hot water. Ignited and weighed. The percentage of water insoluble ash was collected with reference to the air dried drug.

Percentage soluble ash = \( \frac{a}{b} \times 100 \)

Where, 
- \( a \) = water soluble ash
- \( b \) = air dried drug

4.4 Extraction of *C. zedoaria* Root:

4.4.1. Preparation of petroleum ether, chloroform, methanol and ethanol extracts

The powdered material was subjected to successive extractions in an increasing order of polarity using petroleum ether (40-60), chloroform, methanol and ethanol in a soxhlet apparatus for 72 hrs. The extracts were concentrated under reduced pressure using a rotary flash evaporator and the residues were dried in a dessicator over sodium sulfite. After drying, the respective extracts were weighed and percentage yield was determined.

4.4.2. Aqueous extract by cold maceration

100 g. ethanol marc powder was subjected to cold maceration with water in a one litter conical flask for about 7 days at room temperature. The flask was securely plugged with absorbent cotton and was shaken periodically till complete maceration.
The marc was placed in a muslin cloth and the filtrate was concentrated to residue at low temperature. The extracts were subjected to phytochemical investigations by qualitative chemical tests.

4.5 Preliminary Phytochemical Investigation of Extracts

Qualitative chemical test of petroleum ether, chloroform, methanol, ethanol and aqueous extracts of *C. zedoaria* were subjected to tests detect the presence of various phytoconstituents.

**Tests for carbohydrates:**

- **Molish’s test (General test):** To 2–3 ml test solution (T.S.) and a few drops of α-napthol solution in alcohol was added. The mixture was shaken and concentrated KOH was added from the sides of the test tube. A violet ring was observed at the junction of two liquids.

- **For reducing Sugars:**
  - **Fehling’s test:** 1 ml Fehling’s A and 1ml Fehling’s B were mixed and boiled for 1 minute. Added equal volume of TS was added and heated in boiling water bath for 5 – 10 min, first it emitted a yellow and then brick red precipitate.
  - **Benedict’s test:** Equal volume of Benedict’s reagent and T.S. were mixed in a test tube. Heated on a boiling water bath for 5 min. solution appears green, yellow or red depending on amount of reducing sugar present in the test solution.

- **Test for Monosaccharides:**
  - **Barfoed’s test:** Equal volumes of Barfoed’s reagent and T.S. were added. Heated for 1–2 mins, in boiling water and cooled, the red precipitate was formed.

- **Tests for Hexose Sugars:**
  - **Cobalt – Chloride test:** 3 ml of test solution was mixed with 2 ml cobalt chloride, boiled and cooled. Few drops of FeCl₃ on NaOH solution were added. The solution
showed greenish blue (glucose), purplish (fructose) or upper layer greenish blue and lower layer purplish (Mixture of glucose and fructose).

**Tests for non –reducing sugars:**

- T.S. does not give response to Fehling’s and Benedict’s test.

**Tannic acid test for starch:**

- With 20% tannic acid, test solution white precipitate formed.

**Tests for Proteins:**

- **Biuret test (General test):** To 2 -3 ml T.S. 4% NaOH and a few drops of 1% CuSO₄ solution was added. A violet or pink colour was observed.

- **Millon’s test (for proteins):** To 5 ml millon’s reagent, 3 ml T.S. was added. It formed white precipitate. When warmed the precipitate dissolves giving brick red or precipitate red colour.

- **Xanthoprotein test (For protein containing tyrosine or tryptophan):** 3 ml test solution was mixed with 1ml concentrated H₂SO₄. White precipitate was formed.

- **Test for protein containing sulphar:** 5 ml test solution was mixed with 2 ml 40% NaOH and 2 drops 10% of the lead acetate solution. When boiled the solution turned black or brownish due to PbS formation.

**Precipitation test:**

The test solution gave white colloidal precipitate with following reagents:

- Absolute alcohol
- 5% HgCl₂ solution
- 5% CaSO₄ solution
- 5% Lead acetate
- 5% Ammonium sulphate
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Tests for steroid:

- **Salkowski reaction**: To 2 ml of T.S. 2 ml chloroform and 2 ml conc. H₂SO₄ was added. It was shaken well until, chloroform layer appeared red and acid layer showed greenish yellow fluorescence.

- **Liebermann – Burchard reaction**: 2 ml T.S. with was mixed with chloroform. 1-2 ml acetic anhydride and 2 drops conc. H₂SO₄ were added from the side of the test tube. First red, then blue and finally green colour was observed.

- **Liebermann’s reaction**: 3 ml T.S. was mixed with 3 ml acetic anhydride. The mixture was heated and cooled. A few drops concentrated H₂SO₄ were added and observed for blue colour.

Tests for Amino acids:

- **Ninhydrin test (General test)**: 3 ml T.S. and 3 drops 5% Ninhydrin solution were heated in boiling water bath for 10 minutes. A bluish purple colour was observed.

- **Test for Tyrosine**: 3 ml T.S. was heated and 3 drops millon’s reagent were added to it. The solution turned dark red colour was observed.

- **Test for Tryptophan**:

  To 3 ml T.S. A few drops glycoxalic acid and concentrated H₂SO₄ were added. Observed for reddish violet ring at junction of the 2 layers.

Tests for Glycosides:

Tests for Cardiac Glycosides:

- **Baljet’s test**: A T.S. changed from for yellow to orange colour with sodium picrate.

- **Legal’s test (for Cardenolides)**: To aqueous or alcoholic test solution, 1ml pyridine and 1 ml sodium nitroprusside were added the colour turned pink and changed red.
Test for deoxy sugars (Keller Killiani test): To 2 ml T.S. glacial acetic acid, one drop of 5% FeC₃ and conc. H₂SO₄ were added. It gave rise to reddish brown colour at the junction of the two liquids and the upper layers appeared bluish green.

Libermann’s test (for Bufadienolides): 3 ml T.S. was mixed with 3 ml acetic anhydride. The mixture was heated and cooled. A few drops conc. H₂SO₄ were added it showed blue colour.

Tests for Saponin Glycosides:

Foam test: The drug extract or dry powder was shaken vigorously with water. Persistent foam was observed.

Haemolytic test: Test solution was added to one drop of blood placed on a glass slide. It was observed whether haemolytic zone appeared.

Tests for coumarin glycosides: Test solution when made alkaline, showed for blue or green fluorescence.

Grignard’s test: Strips of sodium picrate filter paper were inserted between split cork stoppers which were fitted into the neck of the test tube containing a small amount of powdered drug in the water. Care was exercised to see that the paper did not touch the inner side of the test tube. The content was warmed for half an hour. The red colour of the strips indicated the presence of cyanogenetic glycosides.

Tests for Flavanoids:

Shinoda test:

To dried powder or T.S., 5 ml 95% ethanol, few drops concentrated HCl and 0.5 g Mg turnings were added. Pink colour was observed.

To a small quantity of residue, a lead acetate solution was added. It gave rise to yellow coloured precipitate.
Addition of the increasing amount of NaOH to the residue exhibited yellow colouration, which was decolourised after addition of acid.

**Ferric chloride test:**

To T.S., few drops of ferric chloride solution were added. Intense green colour was observed.

**Tests for Alkaloids:**

- **Dragendroff’s test:** To 2–3 ml T.S. few drops Dragendroff’s reagent were added an orange brown precipitate was observed.
- **Mayer’s test:** 2 – 3 ml T.S. mixed with a few drops Mayer’s reagent gave rise to precipitate.
- **Hager’s test:** 2–3 ml T.S. with Hager’s reagent showed yellow precipitate.
- **Wagner’s test:** 2 – 3 ml T.S. with a few drops of Wagner’s reagent gave reddish brown precipitate observed.

**Tests for Tannins and Phenolic compounds:** To 2 – 3 ml T.S., few drops of the below mentioned solution was added. Tend the following observation were made

- 5% FeCl$_3$ solution: Deep blue-black colour.
- Lead acetate solution: White precipitate.
- Gelatin solution: White precipitate.
- Bromine water: Decoloration of bromine water.
- Acetic acid solution: Red colour solution.
- Potassium dichromate: Red precipitate.
- Dilute iodine solution: Transient red colour.
- Dilute HNO$_3$: Reddish to yellow colour.
Test for gums and mucilages:

The test solution was hydrolyze using dilute HCl and Fehling’s or Bendict’s test was performed as mentioned above. The red colour confirms the presence of gums and mucilage.

Test for Triterpenoids:

Salkowski test: Few drops of concentrated H\textsubscript{2}SO\textsubscript{4} were added to the test solution, shaken and on standing the lower layer turned golden yellow.

Liebermann–Burchard test: To the test solution of the extract, few drops of acetic anhydride were added and mixed well. Then 1 ml of concentrated H\textsubscript{2}SO\textsubscript{4} added from the sides of the test tube, a red colour was produced in the lower layer indicating presence of triterpenes.

4.6 Animal Selection:

Female Wistar rats between 2 and 3 months of age weighing 160 ± 40 g were used which were obtained from the central animal house of Jawaharlal Nehru Medical College, Belgaum, India. All animals were housed in an animal room under normal condition of 24±1°C, 12-h light and dark cycle and 55±5% humidity. The animals were allowed free access to commercial rat pallet diet (Lipton India Ltd, Mumbai, India) and water *ad libitum*. The bedding material of the cages was changed every day. All the experimental procedures were carried out in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. The study designs were approved by the Institutional Animal Ethical Committee of K.L.E.’S College of Pharmacy, Belgaum, India. (Resolution No. 31/7/2010-13).
4.7 Acute Toxicity Studies\textsuperscript{118-120}

The acute oral toxicity studies were carried out according to the guidelines set by the Organization for Economic Co-operation and Development (OECD), revised draft guideline 425, received from CPCSEA. The acute oral toxicity study was carried out by using female Wistar rats (150-200 gm). The temperature in the experimental room was around 25°C. Lighting was natural sequence that is, being 12 hours darkness, 12 hrs light. The conventional laboratory diet was fed with adequate supply of drinking water. The animals were randomly selected, marked to permit individual identification and kept in propylene cages for one week prior to dosing to allow their acclimatization to laboratory condition. The principal was based on stepwise procedure with the use of a minimum number of animals per step to obtain sufficient information on the acute toxicity of the test substance to enable its classification. The substance was tested using a stepwise procedure, each step using five animals of each dose. The method enabled judgment with respect to classifying the test substance to one of the series of toxicity classics defined by the fixed LD\textsubscript{50} cut of values.

All extracts were prepared as a suspension by triturating with water and 1% tween 60. Female Wistar rats (160-200 g) were used for acute toxicity study to determine the LD\textsubscript{50} of extracts of C. zedoaria root. The text substance was administered in a single dose by gauge into the stomach tube. Prior to dosing, animals were kept for 12 h of fasting. Then animals were weighed and test substances were administered. Each extracts of C. zedoaria root, at different doses in an increasing order (175, 550, 1500 and 2000, mg/kg), were administered orally to albino rats. In first step each dose was tested on single rat and then administered to other four rats. Observation was made during the first four hours after the drug administration to notice change in skin and fur,
eye, mucus membrane, hyperactivity, grooming, convulsions, sedation, hypothermia, tremor, salivation, coma, lethargy, body weight, and mortality up to 14 days. 1/10th of the lethal dose was taken as effective dose (therapeutic dose) LD50 cut off value is 200 and 1/5th 400 were selected to evaluate the dose dependent action for the evaluation of anti-inflammatory and anti-arthritic activity.

4.8 Evaluation of Anti-Arthritic Activity

4.8.1 Preparation of drug and animal groups for anti-arthritic and anti-inflammatory activity

The animals were divided into 14 groups of six animals in each. 0.1 ml FCA and mineral oil were injected through intra-articular injection in left ankle joint of rats on 0 day. Mineral oil injected in left ankle joint of rats kept as normal group received normal saline and FCA injected group as control group also received normal saline, (10 mg/kg.i.p. Indomethacin) was used as standard drug, (200 mg/kg. p.o.) Rumalaya forte was used as herbal standard drug (Marketed preparation for arthritic disease, (200 and 400 mg/kg p.o.) petroleum ether, chloroform, methanol, ethanol and aqueous extracts of C. Zedoaria were used as test drugs. Dose calculation was based on w/w of each extract. Each extract was dissolved in normal saline and triturated with 2% tween 60 to form an emulsion and the dose calculation was based on w/w for each extract.

The animal groups are as follows.

Normal Group

Group-I: Treated with (5 ml/kg.p.o) normal saline) + mineral oil

Control Group

Group-II: Treated with (5 ml/kg.p.o normal saline) + FCA

Standard groups:

Group-III: Treated with (10 mg/kg.i.p Indomethacin) + FCA considered as [standard-I]
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Group-IV: treated with (200 mg/kg.p.o Rumalaya forte) + FCA considered as [standard-II]

**Test groups:**

Group-V: Treated with (200 mg/kg.p.o. petroleum ether extract) +FCA considered as [Pet. ether-I]

Group-VI: Treated with (400 mg/kg.p.o petroleum ether extract) +FCA considered as [Pet. Ether-II]

Group-VII: Treated with (200 mg/kg.p.o chloroform extract) + FCA considered as [CHCl₃-I]

Group-VIII: Treated with (400 mg/kg.p.o chloroform extract) + FCA considered as [CHCl₃-II]

Group-IX: Treated with (200 mg/kg.p.o methanol extract) + FCA considered as [MetOH-I]

Group-X: Treated with (400 mg/kg.p.o methanol extract) + FCA considered as [MetOH-II]

Group-XI: Treated with (200 mg/kg.p.o ethanol extract) + FCA considered as [EtOH-I]

Group-XII: Treated with (400 mg/kg.p.o ethanol extract) + FCA considered as [EtOH-II]

Group-XIII: Treated with (200 mg/kg.p.o aqueous extract) + FCA considered as [Aq-I]

Group-XIV: treated with (400 mg/kg.p.o aqueous extract) + FCA considered as [Aq-II]

4.8.2 Induction of arthritis

Pre-induction baseline was taken prior to the injection of Freund's Complete Adjuvant (FCA) measured by left paw volume of each animal at 0 day for the induction of arthritis in female Wistar rats. All the rats were anesthetized with 40 mg/kg thiopentone sodium intraperitoneal. Mineral oil was injected in left ankle joint of normal group of animals. FCA was injected into the left ankle joint of control and drug-
treated groups. The tarsal area of hind paw was grasped and the fossa distal and medial to the “lateral malleolus” of the fibula was palpated. A 26 G needle was introduced into the capsule of the tibiotarsal joint percutaneously by directing it to word cephalad, mesiad, and superiorly from the midpoint of the “inframalleolar fossa,” until a distinct loss of resistance was felt. Approximately 4 mm and complete adjuvant or vehicle was injected. With a true intracapsular injection, a firm resistance to injection was characteristically felt after the injection of 0.1 ml of FCA$^{121-124}$.

4.8.3 Measurement of rat paw edema

The severity of adjuvant arthritis was quantified by measuring the volume of the hind paw using Plethysmometer. Paw volume (ml) was measured at 0 days and thereafter 3, 7, 14, 21, 28, 35, and 42 days of FCA post-inoculation. Data were expressed as the increase in paw volume with respect to day 0 paw volume$^{125}$.

4.8.4 Measurement of hematological profile

Body weights were observed at 0, 3, 7, 14, 21, 28, 35, and 42 days by using a single pan weighing balance. All animals were anaesthetized and blood was collected from the retro-orbital plexus of the entire arthritic and non-arthritic animals in plain EDTA containing tubes respectively. Samples were subjected to physiological examinations such as hemoglobin level measured using Sahli’s Hellige hemometer and the result are expressed in g.% unit and Erythrocyte sedimentation rate (ESR) was measured by Westergren pipettes having 2.5 mm internal diameter, 300 mm length and 1 ml capacity and was expressed in terms of mm/hrs, red blood cell (RBC), white blood cell (WBC)$^{126-127}$.

4.8.5 Measurement of biochemistry profile

At 42 days blood was withdrawn from arthritic and non arthritic rats from retro orbital plexus on the last day of the experiment. Serum samples were collected after
blood centrifugation at 3000 RPM for 10 minutes. These serum samples were used to determine by using a commercial kit with the help of auto analyser. To detect any effects on liver and kidney functions.

1) **Estimations of aspartate amino transferase (AST)**

**Clinical significance:**

AST: Also known as serum glutamic oxaloacetic transaminase (SGOT), AST is an enzyme that is normally present in liver and heart cells. AST is released into the blood when the liver or heart is damaged. The blood AST levels are thus elevated with liver damage (for example, from viral hepatitis) or with an insult to the heart (for example, from a heart attack). Some medications can also raise AST levels.

**Increases:** Increased levels are associated with liver disease or damage, myocardial infarction, cholecystitis, heart attack, alcohol abuse and liver toxicity. Long treatment with NSAIDs cause liver toxicity.

**Decreases:** Decreased levels are observed in patient undergoing renal dialysis and those with vitamin B₆ deficiency.

**Methodology:** International federation of clinical chemistry (IFCC).  

**Principle:** Aspartate amino transferase in serum catalyse the following reaction

\[
\text{L-aspartate} + 2\text{-Oxoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L-Glutamate}
\]

\[
\text{Oxaloacetate} + \text{NAD} \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}
\]

\[
\text{Sample pyruvate} + \text{NAD} \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}
\]

**Procedure**

Pipette into a clean dry test tube labeled as a test (T) the following

Working reagent: 1 ml and sample: 0.1 ml
Mix well and take the readings from the spectrophotometer. The procedure is linear up to 450 IU/L at 37°C. Sample having very high activity show a very low initial absorbance as most of the NADH is consumed prior to start of measurement. If this is suspected then dilute the sample and repeat the assay.

**Calculation:** Calculate the mean absorbance change per minute (ΔA/ min).

\[
\text{AST in IU/L} = \frac{\Delta A/ \text{min} \times T.V \times 10^3}{S.V \times \text{Absortivity} \times P}
\]

T.V= Total reaction volume in µl
S.V= Sample volume in µl
P = Cuvette length path= 1 cm

2) Estimations of alkaline amino transferase (ALT)\textsuperscript{128-132}

Alanine aminotransferase (ALT) also referred to as serum glutamate pyruvate transferase (SGPT) is an enzyme involved in amino acid metabolism. ALT is found in liver, but a little amount also occurs in the kidneys, heart, muscles and pancreas. Low levels of ALT are normally found in the blood. However, higher concentration of ALT in the blood is observed in the liver disease, viral hepatitis, circulatory failure with shock, infectious mono-nucleosis and myopathy.

**Clinical significance:** ALT (formerly called Serum Glutamate Pyruvate Transaminase; SGPT), is mainly in the cytosol of liver cell, but also in smaller amounts in kidneys, heart, muscles and pancreas.

<table>
<thead>
<tr>
<th>Pipette</th>
<th>Volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Test</td>
<td>100 µl</td>
</tr>
</tbody>
</table>
Increases: Its level increases in primary liver diseases such as cirrhosis, carcinoma, viral or toxic hepatitis and myocardial infarction attack.

Decreases: Decreased levels may be observed in renal dialysis patients and those with vitamin B₆ deficiency.

Methodology: International federation of clinical chemistry.

Principle:

\[
\text{L - Alanine + 2 - oxoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate + L-Glutamate}
\]

\[
\text{Pyruvate + NADH} \xrightarrow{\text{LDH}} \text{Lactate + NAD}
\]

ALT: Alanine aminotransferase

LDH: Lactate dehydrogenase

Assay procedure: Set the Auto-analyzer instrument

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Pipette</th>
<th>Volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Working reagent</td>
<td>1000 µl</td>
</tr>
<tr>
<td>2</td>
<td>Test</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

Procedure:

1. Add 1000 µl of reagent 1 to 100 µl of sample 2. Mix and incubate for approximately 1 min.
2. Add 250 µl of reagent 2.
3. Mix The samples well and measure change in absorbance at 340 nm.

Calculation: Calculate the mean absorbance change per minute (ΔA/ min) at 340 nm.

\[
\text{ALT in IU/L} = \frac{\Delta A/ \text{min} \times T.V. \times 10^3}{S.V. \times \text{Absorptivity} \times P}
\]

T.V = total reaction volume in µl

S.V = sample volume in µl

P = cuvette length path = 1cm
3) **Estimations of blood urea nitrogen (BUN)**

**Clinical significance:** A blood urea nitrogen (BUN) test measures the amount of nitrogen in the blood that comes from the waste product urea. Urea is formed when protein is broken down in the body. It is made in the liver and removed from the body through urine. A BUN test is done to conform the working of the kidneys.

**Increase:** When the kidneys can to remove urea from the blood normally, the BUN level increases. Heart failure, dehydration, certain medicines, intestinal bleeding, or a diet high in protein also can increase your BUN level. Liver disease or damage can decrease the BUN level, because urea is made in the liver.

**Decrease:** A decreased BUN level can occur normally in the second or third trimester of pregnancy.

**Methodology:** GLDH-Urease method, Talke and Schubert, Tiffany et al

**Principle:** Urea is the principle waste product of protein catabolism. It is synthesized in the liver from ammonia which is produced as a result of the deamination of amino acids. Normally, urea nitrogen in the blood comprises only about 45% of the non-protein nitrogen. The importance of urea nitrogen determination is its value as an indicator of liver and kidney functions. Urea is catalytically converted to ammonium carbonate by the use of urease. The reaction rate is dependent upon the concentration of the influence of glutamic dehydrogenase. The rate of this second reaction is dependent upon the first and can be measured by the rate of conversion of NADH to NAD by the change of absorbency at 340 nm.

The estimation of Urea in serum involves the following enzyme catalyzed reactions:

\[
\begin{align*}
\text{Urea} + \text{H}_2\text{O} & \xrightarrow{\text{Urease}} \text{NH}_3 + \text{CO}_2 \\
\text{NH}_3 + \alpha - \text{KG} + \text{NADH} & \xrightarrow{\text{GLDH}} \text{Glutamate} + \text{NAD}
\end{align*}
\]
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Alpha-KG: α- Ketoglutarate

GLDH: Glutamate dehydrogenase

The rate of decrease in absorbance is monitored at 340 nm and is directly proportional to the urea concentration in the sample.

Procedure: The autoanalyser instrument was set to the specifications mentioned in the protocol supplied with the kit. The samples were prepared as per the protocol. The samples were mixed well and absorbance read at 340 nm, 20 seconds after mixing ($A_1$) and finally at 80 seconds ($A_2$).

Calculation: $\Delta A = A_1 - A_2$

BUN (mg/dl) = $\frac{\Delta A \text{ of test}}{\Delta A \text{ of standard}} \times \text{Concentration of Standard} \times 2.14$

4) Estimation of uric acid$^{138-142}$

Clinical significance: Uric acid is a catabolic product of purine metabolism. The determination of uric acid in serum is most commonly performed for the diagnosis of gout.

Increased: Increased uric acid levels are also found in leukemia, polycythemia, familial idiopathic hyperuricemia, and conditions associated with decreased renal function.

Methodology: Trivedi and Kabasakalian with a modified Trinder peroxidise method using TBHB.

Principle:

Uricase

Uric acid + O$_2$ + H$_2$O $\rightarrow$ Allantoin + CO$_2$ + H$_2$O$_2$

Peroxidase

H$_2$O$_2$ + 4-AAP + TBHB $\rightarrow$ Quinoneimine$+$H$_2$O 4-AAP : 4-Aminoantipyrine

TBHB : 2,4,6- Tribromo-3-hydroxy benzoic acid
Uric acid is oxidized by uricase to allantoin and hydrogen peroxide. The hydrogen peroxide is reacts with the trinder reagent, catalyzed by peroxidase, to form a quinoneimine dye. The intensity of the colored complex formed is directly proportional to the Uric acid concentration of the sample, when measured at 505 nm (500-540 nm).

**Procedure:** The autoanalyser instrument was set to the specifications mentioned in the protocol supplied with the kit. The samples were prepared as per the protocol. The samples were mixed well and incubated for 5 mins at 37°C. The absorbance of standard and each test was read at 505nm (505-540nm) against the reagent blank.

**Calculation:**

\[
\text{Uric Acid (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of Standard}
\]

5) **Estimation of creatinine (CRE)**

**Clinical significance:** There is impaired formation or elimination of urine, there is an increase in several compounds present in the blood plasma. These compounds are relatively small nitrogen containing molecules that are collectively called the “non-protein nitrogen constituents” of plasma or serum. The compounds whose serum concentrations are of great significance in kidney disease are creatinine and urea and, to a lesser degree, uric acid. Circulating levels of creatinine are used primarily as an index of renal function. High plasma creatinine concentrations are encountered in nephritis and renal obstruction, reflecting the degree of impairment.

**Methodology:** Modified Jaffe’s reaction creatinine reacts with picric acid under alkaline conditions to form a yellow-orange complex. The color is derived from
creatinine as well as certain other non-specific substances. Upon the addition of acid, the color contributed by creatinine is destroyed, while that produced by non-specific substances remains. The difference in color intensity measured at 500 nm before and after acidification is proportional to the creatinine concentration. The samples were prepared as per the protocol. The samples were mixed well and absorbance read at 505 nm, 20 seconds after mixing (A₁) and finally at 80 seconds (A₂).

**Principle:** Creatinine reacts with alkaline picrate to produce an orange yellow colour (the Jaffe’s reaction) specificity of the assay has been improved by the introduction of an initial rate method. The absorbance of the orange-yellow colour formed is directly proportional to creatinine concentration.

\[
\text{Calculation : } \Delta A = A_1 - A_2
\]

\[
\text{CRE (mg/dl) } = \frac{\Delta A_{\text{of test}}}{\Delta A_{\text{of standard}}} \times \text{Concentration of Standard}
\]

6) **Estimations of total protein:**

**Clinical Significance**

The total protein concentration of serum is usually increased in patients with dehydration, monoclonal disease (multiple myeloma, macroglobulinemia, and cryoglobulinemia) and in some chronic polyclonal disease (liver cirrhosis, sarcoidosis, SLE, and chronic infections). The serum total protein concentration is decreased in inadvertent over-hydration, in conditions involving protein loss through the kidneys (nephrotic syndrome), from skin severe burns), or gut (protein-losing enteropathies), or in failure of protein synthesis (starvation, protein malnutrition, severe nonviral liver cell damage). Total protein is useful for monitoring gross changes in protein levels caused by various disease states. It is usually performed in conjugation with other tests such as serum albumin, protein electrophoresis.
Methodology

The peptide bonds of protein react with copper II ions alkaline solution to form blue violet complex, (biuret reaction). Each copper ions complexion with 5 or 6 peptide bonds. Tartarate is added as a stabilizer whilst iodide is used to prevent auto oxidation of the alkaline copper complex. The colour is proportional to the protein concentration and is measured at 546 nm (520-560 nm).

Assay Procedure

<table>
<thead>
<tr>
<th>Pipette</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>20 µl</td>
<td>......</td>
<td>......</td>
</tr>
<tr>
<td>Standard</td>
<td>......</td>
<td>20 µl</td>
<td>......</td>
</tr>
<tr>
<td>Test</td>
<td>......</td>
<td>......</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

Incubate for 10 min. at 37°C. Read absorbance of the standard and each test at 546 nm (520-560 nm) against reagent blank.

Formula

\[
\text{Total protein} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times \text{concentration of standard (g/dl)}
\]

4.8.6 Nitric oxide synthesis

Serum was separated from each group of animals. Sodium nitroprusside (5 Mm) in standard phosphate buffer solution with different serum samples dissolved in standard phosphate buffer (0.025 M, pH 7.4) solutions were incubated in equal amount at 25°C for 5 h. After 5 h, 0.5 ml of the incubation solution was removed and diluted with 0.5 ml of Griess reagent. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthaethylene diamine was read at 546 nm using Shimadzu UV-visible spectrophotometer.\textsuperscript{149-152}. 
4.8.7 Assessment of vascular permeability

Evan’s blue (50 mg/kg) was administered via the jugular vein into the anaesthetized rat. After 4 h, the anterior and posterior synovial capsules and fat pad were dissected from each ankle joint, which were small. Thus, tissues obtained from four ankles were grouped to form one sample. The samples were then weighed, and the amount of Evans blue in the sample was estimated using dye extraction technique. This entailed cutting the capsule in to smaller pieces and mixing them with acetone in 1% NaSO₄ in the ratio of 7:3. The samples were shaken gently and continuously for 24 h at room temperature. Each preparation was centrifuged for 10 min at 2000 rpm and 2 ml of the supernatant was separated for measurement of absorbance at 620 nm using shimadzu UV/Visible spectrophotometer. The amount of dye recovered was calculated by comparing the absorption of the fluid with that of standard curve prepared with known concentration of Evan’s blue solution¹⁵³-¹⁵⁵.

4.8.8 Behavioral observations

Open-field test

For behavioral observations, all the animals were subjected to open-field test before the induction of arthritis and thereafter 3, 14, 21, 28, 35, and 42 days of post-inoculation of FCA injection. The Rat was placed in an open field in the sound-attenuated room. The floor was white polyvinyl with a black grid dividing open field into 84 squares (10 × 10). Illumination was provided by a bulb (60 W) placed above the center of the field, while the rest of the room was darkened³⁴. The rat was initially placed in the center of the open field and all the behavior test were observed for 5 minutes. After each animal observations test, the open field was cleaned with wet sponge and tissue paper and all observations were made between 18.00 and 20.00 hours¹⁰,¹⁵⁶-¹⁵⁸.
Based on the previous scientific study on behavioral observations of normal rats, the following behaviors were quantified:

I. Latency time to explore: It is the time taken “to start explore (second)” from insertion time

II. Ambulatory behavior: This indicates that the rat “the crossed grid line” (horizontal locomotor activity)

III. Rearing: Means that the “look for” some time in the air. For this it elevates its head and forepaws, almost standing up, (vertical locomotor activity)

IV. Grooming behavior: This means licking, rubbing the ears, nose, and head or the snout with forepaws and preening

V. Urination: (Number of urine passes) considered as anxiety behavior of rat

VI. Defecation: (Number of bolus passes) also considered as anxiety behavior of rat

4.8.9 Radiography examination

At the end of the experiments, all rats were anesthetized with 40 mg/kg sodium thiopental intraperitoneal injection. Once anesthetized, the animals were kept on X-ray plates, the projections of the left ankle joint were taken at day 42. The following parameters were evaluated blindly using the tarsometatarsal region: erosion, a destruction of bony structure resulting in irregular bone surface; periosteal reaction, a fine ossified line, paralleling normal bone producing bone thickening; increase in soft tissue which was manifested as an increase in width of the soft tissue and calcification. The parameters used score as follows: 0, no sign; 1, mild; 2, moderate; and 3, severe.

4.8.10 Histopathology examination

Animals were scarified at 43 day after the induction of arthritis. Left ankle joints were removed and post fixed with 10% formalin for 10 days and then decalcified in 5%
formic acid. Left hind paws were removed from all groups of animals and post fixed with normal saline and then decalcified in 5% formic acid. Joints were then trimmed, embedded in paraffin and sectioned at 6 microns by using a microtome. Sections were then stained with haematoxyline and eosin. All the slides were observed for changes in joint histopathological characteristics and photographs were taken. Pathology lesions of rats ankle joint were graded on a blind scale under light. Microscopic 100 X Histological evaluation was carried out according to the following scale Nil 0, Mild 1, moderate 2 and marked 3 and the following parameters were considered skin congestion, skin odema, skin inflammatory infiltration, synovial ulceration, synovial neutrophilic infiltration, synovial lymphocytic infiltration, synovial macrophages, synovial granulation tissue, synovial granulations tissue, synovial cellular degeneration, cartilage destruction and bone destruction\textsuperscript{161-164}.

4.8.11 Organ to body weight ratio\textsuperscript{165-167}

All groups were sacrificed after 42 days of last dose for calculating organ to body weight ratio. All vital organs, viz spleen, thymus, adrenals, isolated and weighed. This relationship assumes that:

The ratio of organ weight (Y) to body weight (X) within each treatment group is some constant $\mu$, i.e., $Y/X = \mu$.

\[
\text{% Change in organ body weight ratio} = \frac{C-C_1}{C} \times 100
\]

$\Diamond$ C = Represent normal animals organ weight

$\Diamond$ C\textsubscript{1} = Represent control and drug treated animals +FCA organ weight.

4.9 Evaluation of Anti-inflammatory Activity of C. zedoaria Extracts

4.9.1 Carrageenan induced paw edema in rat:\textsuperscript{160, 168-169}

Before the experiment, food was withdrawn overnight but adequate water was given to the rats. The selected doses were 200 and 400 mg/kg for each extract. The
animals were divided into eight groups of 6 animals each. All the doses were given orally half an hour before the administration of carrageenan (Sigma chemical co, St. Louis MO, USA) into the plantar side of the left hind paw. The paw was marked with ink at the level of the lateral malleolus and immersed in mercury up to the mark in the plethysmometer. The paw volume was measured after (1h) injection carrageenan and then every hour till 6 h of each group. The difference between the initial and subsequent reading gave the actual edema volume. The average paw swelling was calculated by comparing to normal group with control. Standards and all treated groups compared with the control and percent inhibition of inflammation was calculated by using the formula,

\[
\% \text{ Inhibition paw edema} = \frac{V_c-V_t}{V_c} \times 100
\]

- \( V_c \) = Represent paw edema in control group rats
- \( V_t \) = Represent paw edema in drug treated group

**4.9.2 Histamine–induced paw edema in rat:**

For the study of histamine–induced paw edema in the animals, they were treated exactly with the same method as carrageenan induced model. But instead of carrageenan, here 0.1 ml of 1% w/w histamine in normal saline was used. All carrageenan-induced inflammation methodology was adopted for histamine-induced inflammation.

**4.10 Statistical Analysis:**

The values are expressed as mean ± S.E.M. Statistical difference between normal to control and control to drug treatments were analyzed by one-way ANOVA followed by Dunnett’s multiple comparison tests by using Graph pad prism version. The
difference was considered as significant P value less than 0.05, when $^a p<0.001$, $^b p<0.01$, $^c p<0.05$.

**Scheme-1 Separation of Curcuminoid from Petroleum Ether Extract of C. zedoaria root**

- **Root of CZ**
- **Petroleum ether extract**
- **Soxhlet**
- **TLC with solvent system Chloroform, acetone, and ethanol (45: 45: 10)**
- **Separated Curcuminoid**
- **Separated by Column chromatography with solvent system ratio of chloroform and methanol**
- **Column Fractions**
  - Total Fractions 150
  - 1-12: No spot
  - 13-22: 1 Spot
  - 23-82: 2 Spot
  - 83-105: 1 Spot
  - 106-114: No Spot
  - 115-150: No Spot

Comparison with standard curcuminoid on preparative thin layer chromatography with solvent system Chloroform, acetone, and ethanol (45: 45: 10)

**Recrystallized and Dried**

- **HPLC**
- **HPTLC**

**Spectroscopy**

- $^1$HNMR
- LCMS
- IR
- UV
4.11. Separation and Purification of Crude Curcuminoid by Preparative TLC and Column Chromatographic Studies

4.11.1 Crude curcuminoid obtained from pet-ether extract of CZ root by preparative TLC

Preliminary phytochemical analysis showed steroids, terpenoids, glycosides, alkaloids, and curcuminoid test positive for petroleum ether extract and pet ether extract showed more potent than other extracts. Hence, curcuminoid were separated from the pet ether extract.

**Stationary phase:** Silica gel G for TLC.

**Mobile phase:** Different solvent system such as chloroform, benzene, ethyl acetate and acetone in different ratio has been tried for optimization of better resolution. Better resolution was observed in Chloroform: Benzene: Ethanol (45: 45: 10 v/v)

**Reference standard:** Standard curcuminoids

**Spraying reagent:** 2% Boric acid in methanol

Petroleum ether extract was evaluated by TLC for the presence of curcuminoid, using a specific solvent system & detecting reagent, to substantiate the presence of curcuminoid detected in qualitative chemical tests & to know how many phytoconstituents are present in that extract.

TLC is a mode of chromatography in which sample is applied as a small spot on to the origin of a thin sorbent layer supported on a glass, plastic or metal plate. The mobile phase moves through the stationary phase by capillary action, sometimes assisted by gravity or pressure. TLC separation takes place in the open layers with each component having the same total migration time but different migration distances. Mobile phase consists of a single solvent or mixture of solvents. Numerous fixed sorbents have been
used including silica gel, cellulose, polyamide, alumina, ion exchanger & chemically bonded silica gel \(^{175-177}\).

The stationary phase of the TLC is prepared using various techniques such as pouring, dipping and spraying. However, ready-made stationary phases (TLC plates) are also available in the market. The prepared plates allow set (air drying). This is done to avoid cracks on the surface of an adsorbent. After setting, the plates are activated by keeping in an oven at 100 - 120\(^{0}\)C for 1 hour. Activation of TLC plates is the process of removing water / moisture and other adsorbed substances from the surface of any adsorbent, by heating at high temperature so that adsorbent activity is retained \(^{177}\).

\(R_f\) values were determined using formula:

\[
\text{Resolution Factor (R}_f\text{)} = \frac{\text{Distance traveled by the solute from the origin}}{\text{Distance traveled by the solvent front from the origin}}
\]

### 4.11.2 Column chromatography of crude curcuminoids obtained from petroleum ether extract of \(C. zedoaria\) root

Separation of curcumin from petroleum ether extract was carried out, as this particular extract showed four (yellowish red, yellow, light yellow and light yellowish red) spots respectively. From these, curcumin was isolated by column chromatography.

Different chemical constituents were from the petroleum ether extract of \(C. zedoaria\). Column chromatography is generally used as a purification technique, in which it is possible to isolate chemical constituent from a mixture of curcuminoid.

This requires further fractionation \(^{178-179}\). This can be identified by preparative TLC.
Chapter 4

Materials and Methods

- Adsorbent: Silica gel 60-120#
- Mobile Phase: Methanol Chloroform (1:9 v/v)
- Rate of elution: 5 ml/min. (Till Methanol (10):Chloroform (90)) 25
- Volume of elute collected: 50 ml (Till 10 :90) Afterwards 5ml
- Fraction collected: 165
- Type of elution: Gradient elution.

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids. Column chromatography is another solid-liquid technique in which the two phases are a solid (stationary phase) and a liquid (moving phase). The theory of column chromatography is analogous to that of thin-layer chromatography. The most common adsorbents are silica gel and alumina. The sample is dissolved in a small quantity of solvent (the eluent) and applied to the top of the column. The eluent, instead of rising by capillary action up as in TLC, flows down through the column filled with the adsorbent. There is an equilibrium established between the solute adsorbed on the silica gel or alumina and the eluting solvent flowing down through the column. Column chromatography is generally used as a purification technique, in which it is possible to isolate desired compounds from a mixture.

1) Preparation of sample

- 2.5 g. of the successive petroleum ether extract was dissolved in 15 ml of methanol and then washed 3-4 times with petroleum ether (40-60).

- Both the fractions were separated and methanolic fraction was further concentrated.

- It was dissolved in 10 ml of methanol and mixed with 2.5 g. of silica gel (60-120 mesh size) and dried in a vacuum oven at 45°C. The adsorbed material obtained was transferred to the column.
2) **Column packing**

- 100 g. of silica gel was activated in hot air oven at 110°C for 1 h.
- The glass wool was fixed at the bottom of the column.
- The slurry of activated silica was made in pet. ether and charged in the column in small portions with keeping the knob open with gentle taping after each addition, in order to ensure uniform packing. A small quantity of solvent was allowed to remain at the top of the column in order to avoid the drying or cracking of the column. Tapping is necessary to avoid the air bubble formation in the column during packing which otherwise may interfere in the separation. The packed column was kept undisturbed overnight.

The column was run fast for some time with pet ether in order to remove any impurities. Prepared sample was then charged in the column and was allowed to settle. A small cotton pad was placed above the sample to prevent the mixing of dust particles with the sample. Then it was eluted with the mobile phase to collect fractions and is concentrated. Each fraction was evaluated by TLC to know how many different phytoconstituents are there in it.

Fractions showing the same number of compounds and Rf values were combined, concentrated and evaporated to dryness.

3) **TLC of Column Elutes**

- Adsorbent : Silica gel G
- Solvent System : Benzene: Chloroform: Ethanol
- Proportion : 45: 45: 10
- Spraying agent : 2% Boric acid in methanol

4.11.3 **High performance thin layer liquid chromatography (HPTLC)**

The results of the qualitative TLC analysis were confirmed by subjecting the standard curcuminoid, isolated curcuminoid, curcumin and petroleum ether extract to...
qualitative HPTLC analysis. The HPTLC screening was carried out using “CAMAG” Linomat sample applicator, “CAMAG TLC 3” densitometry scanner and “CAMAG Win CATs” software (CAMAG, Switzerland, Version 1.2.3) on pre – coated HPTLC plates.

**Various steps involved in HPTLC are:**

1) **Selection of HPTLC plate**
   
   Pre–coated TLC layer: Silica gel GF254 plates
   
   Size of the plate: 10 x 10 cm
   
   Support material: Aluminum sheet (0.1mm thickness).

2) **Sample preparation**
   
   Separated compound by preparative TLC was dissolved in methanol.

3) **Application of sample**
   
   The purified isolated sample was applied in the form of a band using CAMAG LINOMAT an automatic sample application device, maintaining a bandwidth–6 mm, space – 3 mm, sec/µl-5. Quantity of sample applied was 10 µl.

4) **HPTLC development**
   
   Mobile –Phase: Chloroform: benzene: Ethanol  (45: 45 10).

   The plates were developed by placing in pre–saturated or pre – conditioned tank (12cm height) with mobile phase until it reached to 8 cm height. The plates were dried by evaporating the solvent either at room temperature or by spraying hot air by the drier.

5) **Detection/scanning**
   
   Instrument: CAMAG TLC Scanner IV, a densitometric evaluation system with WINCAT software was used for scanning of thin layer chromatogram objects in reflectance mode at 425 nm.
6) Evaluation

Rf value of various samples were evaluated using the following formula.

\[
Rf = \frac{\text{Distance travelled by the sample from the base line}}{\text{Distance travelled by the solvent from the baseline}}
\]

It’s were performed for the identification of active constituents at high resolution.

4.11.4 High performance liquid chromatography (HPLC)\textsuperscript{183}

4.12 Characterization of Phytoconstituents Using Spectroscopy Techniques:

All the separated compounds from petroleum ether root extract of \textit{C. zedoaria} have been characterized using physical, chemicals and spectroscopy technique such as

4.12.1 Physical and chemical methods: Include determination of description, solubility, melting point, boiling point, and Rf value.

4.12.2 UV Spectrum: The isolated compound was dissolved in methanol. The UV absorption spectrum of isolated compound was recorded on 1601 UV-Visible spectrophotometer (Schimadzu). Methanol was used as blank.

4.12.3 FTIR Spectrum: The isolated compound was dissolved in methanol and the FTIR absorption spectrum was recorded on FTIR 8400\textsuperscript{s}\textsuperscript{179} (Shimadzu) at Maratha Mandal College of Pharmacy, Belgaum.

4.12.4 \textsuperscript{1}HNMR: The isolated compound was dissolved in CDCl\textsubscript{3} and chemical shifts were measured as the relative distance from the peak of TMS. Spectra are given in proton nuclear magnetic resonance was recorded on a varian-400 MHz NMR spectrometer\textsuperscript{198-199} (Shimadzu) at Hyderabad, India. Tetramethylsilane (TMS) was as an internal standard.

4.12.5 LCMS: Liquid chromatography mass spectrum was recorded at higher resolution on a mass spectrometer\textsuperscript{179-184} (Perkin Elmer Autosystem XL with Turbomass) at Hyderabad, India. The separated compound was inserted directly into the ionization chamber using a probe. The molecular ion and fragments were produced by electron
impact of 10–15 eV in ionization chamber. The mass spectrum was recorded and plotted as m/z vs relative abundance.

4.13 Development of Single Herb Formulation\textsuperscript{185}

In the present study, it was thought worthwhile to formulate the potent extracts of \textit{C. zedoaria} in the form of suspension. The formulation of all bio-active extracts were prepared by applying probability for that all the bio-active extracts were mixed in equal proportion as per the probable applied and LD\textsubscript{50} cut off value was calculated and according to the cut off value formulation was done.

1. **Single Herb Formulation-A**

Formulation contains pet ether + chloroform and ethanol extracts of \textit{C. zedoaria} root.

2. **Single Herb Formulation-B**

Formulation contains pet ether and chloroform extract of \textit{C. zedoaria} root.

3. **Single Herb Formulation-C**

Formulation contains pet ether and ethanol extract of \textit{C. zedoaria} root.

4. **Single Herb Formulation-D**

Formulation contains chloroform and ethanol extract of \textit{C. zedoaria} root.

**Procedure\textsuperscript{186}**

The bioactive extracts will be mixed with light kaolin in a mortar and add compound powder of tragacanth than orange syrup is added and triturate so as to form a smooth cream. The foreign particles are removed with the tip of the glass rod than benzoic acid solution was incorporated and amaranth solution previously diluted with chloroform water was added and stir thoroughly so as to form a uniform mix. Add more of orange syrup up to the required volume.
4.13 Acute Toxicity Studies

The acute oral toxicity studies were carried out according to the guidelines set by the Organization for Economic Co-operation and Development (OECD), revised draft guideline 425.

1/10th of the lethal dose was taken as effective dose (therapeutic dose) LD₅₀ cut off value is 200 mg/kg were selected to evaluate the dose dependent action for the evaluation of anti-inflammatory and anti-arthritic activity.

4.14 Evaluation of Anti-arthritic and Anti-inflammatory Activity of Single herb formulations of C. zedoaria Root Extracts

4.14.1 Preparation of test sample and animals groups for anti-arthritis activity, carrageenan and histamine induced–inflammation in rat paw
Chapter 4

Materials and Methods

The rats were divided into eight groups of six animals in each group normal, control and standard-I and standard-II groups were selected from our previous studies. “Potential effect of *C. zedoaria* root extracts on arthritic and acute inflammation in rats.

**Normal Group**

Group-I: treated with (5 ml/kg.p.o normal saline) + mineral oil

**Control Group**

Group-II: treated with (5 ml/kg.p.o normal saline) + FCA

**Standard groups:**

Group-III: treated with (10 mg/kg.i.p. Indomethacin) + FCA consider as [standard-I]

Group-IV: treated with (200 mg/kg.p.o Rumalaya forte) +FCA consider as [standard-II]

**Formulation test groups**

Group-V: Treated with (200 mg/kg, p.o formulation-A) +FCA consider as [SHF-A]

Group-VI: Treated with (200 mg/kg, p.o formulation-B) + FCA+consider as [SHF-B]

Group-VII: Treated with (200 mg/kg, p.o formulation-C) + FCA+ consider as [SHF-C]

Group- VIII: Treated with (200 mg/kg,p.o formulation-D) +FCA consider as [SHF-D]

4.15 Evaluation of Antiarthritic Activity of Single Herbal Formulations of *C. zedoaria*

4.15.1 Induction of arthritis

4.15.2 Evaluation Parameters

1) Paw edema

2) Behavioral observation

 проведен "Open-field test"

- Latency time to explore
- Ambulatory
- Gearing
- Grooming
- Urination
- Defecation

3) Measurement of physiology profile
4) Measurement of biochemistry profile

- Aspartate amino transferase (AST)
- Alkaline amino transferase (ALT)
- Blood urea nitrogen (BUN)
- Uric acid
- Creatinine (Cr)
- Total protein (TP)

5) Nitric oxide synthesis

6) Assessment of vascular permeability

7) Radiography examination

8) Histopathology examination

9) Organ to body weight ratio

Evaluation of anti-arthritis activity was followed as per the methodology. The details are mentioned in 4.8.2 to 4.8.11.

4.14 Evaluation of Anti-Inflammatory Activity of Single Herb Formulations

4.14.1 Carrageenan-and Histamin induced paw edema

Carrageenan-induced inflammation was followed as per the study which is mentioned in 4.9.1 and 4.9.2 respectively.

4.15 Statistical Analysis:

The values are expressed as mean ± S.E.M. Statistical difference were analyzed by one-way ANOVA followed by Dunnett’s multiple comparison tests. The difference was considered as significant P value less than 0.05, when $p<0.05$.

4.16 Accelerated Stability Studies of Single Herb Formulations of C. zedoaria

The accelerated stability studies were carried out for single herb formulations of bio-active constituents at Temperature $40^\circ C \pm 2^\circ C$ at 80% humidity. The stability was studied for the period of three months. The different parameters such as colour, odour,
viscosity, pH, and sedimentation volume and redispersibility test were studied for all the formulations at 1<sup>st</sup>, 2<sup>nd</sup> and at 3<sup>rd</sup> months.