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Extraction, characterization and pharmacological activity and evaluation of various extracts of *calotropis procera* leaves

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***Abstract*---**All over Saudi Arabia, the medicinal plant Calotropis procera (C. procera) is a common wild shrub. We looked into the phytochemical makeup and several extracts of C. procera in this work. The presence of different ingredients was checked in all extracts. Table displays the findings of this exploratory phytochemical investigation. Following a phytochemical analysis, it was discovered that Calotropis procera contains alkaloids, steroids, flavonoids, tannins, and terpenoids. To separate the active compounds that might have pharmacologically active ingredients, a TLC chromatographic research was conducted. Ethanol and chloroform extract of Calotropis procera leaves demonstrate the presence of flavonoids and phenolic substances in preliminary phytochemical testing. Additionally, the TLC of both extracts reveals the presence of several phytometabolites. After collection of all fractions of both ethanol and chloroform extract, these fractions then screened for anti-rheumatic arthritis activity. Results depict that 150 mg/kg of crude extract and ethanolic fraction on 10th day showed more superior repression of paw edema i.e., 80.26% and 81.25% ($p < 0.001$) as compared to 79.56% reduction in paw edema by 100 mg/kg aspirin on 10th day.

***Keywords*---**Calotropis procera, crude extract, anti-rheumatic arthritis activity.

Introduction

Plants are the most beautiful creation of nature which forms the basis of almost all life on the Earth, providing protection and substance for organisms ranging from bacteria to large mammals.Various societies across the world have shown great interest in curing diseases using plants or plant based drugs. Calotropis procera Linn. Family Asclepiadaecae is an Ayurvedic plant with important medicinal properties. It is known by various vernacular names like Swallow wort in English, madar in Hindi, and Alarka in Sanskrit. It is found in most parts of the world with a warm climate in dry, sandy and alkaline soils. Calotropis is primarily harvested because of its distinctive medicinal properties. The decoction of the aerial parts of Cp is commonly used in Saudi Arabian traditional medicine for the treatment of a variety of diseases including fever, tumors, joint pain, ulcer, muscular spasm, and constipation [1–3]. Different extracts of the plant showed significant antipyretic, analgesic, anticancer, antibacterial, nematocidal [4], larvicidal [5], and neuromuscular blocking activities [2]. The whole parts of Cp have been investigated previously for its phytoconstituents, which revealed the presence of cardenolides, anthocyanins, and triterpenoids.

Rheumatoid arthritis (RA) is one of the most common chronic inflammatory disorders, which is major cause of disability. The word arthritis means inflammation of the joint (‘artho” means joint and “itis”means inflammation). Arthritis is a musculoskeletal system disorder following mechanical and biological events that destabilize normal coupling between degradation and synthesis within articular cartilage. [6-8] There are more than 100 different types of arthritis and related conditions. Out of which rheumatoid arthritis and osteoarthritis are the major ones. Symptoms of one type arthritis are unlike other type. [9]Some people may show mild but some are with strong symptoms. The common symptoms of arthritis are: Pain, Edema of Joints, Rigidity, Tenderness, Redness, Warmth, Loss of Flexibility, Limping, Bone Spurs, Discomfort when Standing or Walking, Fatigue (feeling tired).[10]

Material and Methods

Plant Collection and authentication

Fresh sample of leaves of Calotropis procera were collected from Pune district (Maharashtra) and dried in the shade at room temperature. Dried plant material was coarsely powdered in grinder and powder material was passed through 120mesh to remove fine powders and coarse powder was used for extractions. The plant was authenticated by C.R. Jadhav, Botanist, Botanical Survey of India, Pune by comparing morphological features. The herbarium of the plant specimen was deposited at Botanical Survey of India, Pune; with the Voucher specimen number SVK-01 (Ref. No. BSI/WRC/IDEN. CER. /CRC/2019/H7 Dated 14/04/2019).

Chemicals and Reagents

Pharmacognostic investigations of selected plant material

Organoleptic, Morphological and Microscopic evaluation

1. Macroscopy

Organoleptic characters, extra feature and macroscopical details of all parts of plant were carried out.

2. Microscopy

Microscopical study was done as per the method described by Khandelwal, (2005). Transverse section of stem and leaf was taken, stained with phloroglucinol: Hydrochloric acid (1:1) and observed under microscope at 10X, 45X.

Physical evaluation

a. Determination of foreign organic matter

5 gm of air dried coarsely powdered drug was spreaded in a thin layer. The sample was inspected with the unaided eye or with the use of 6X lens. The foreign organic matter was separated manually as completely as possible. Sample was weighed and percentage of foreign organic matter was determined from the weight of the drug taken.

b. Determination of moisture content

Accurately weighed glass-stopper, shallow weighing bottle, was dried. 2gm of sample was transferred to the bottle and covered, the weight was taken, and sample was distributed evenly and poured to a depth not exceeding 10 mm. Then loaded bottle was kept in an oven and was removed. The sample was dried to constant weight. After drying it was collected to room temperature in a desiccator. Weighed and the loss on drying was calculated in terms of percent w/w.

c. Ash value

Ash value is used to determine quality and purity of crude drug. Ash value contains inorganic radicals like phosphates carbonates and silicates of sodium, potassium, magnesium, calcium etc. sometimes inorganic variables like calcium oxalate, silica, carbonate content of the crude drug affects 'Total Ash Value'. Such variables are then removed by treating with acid and then acid insoluble ash value is determined.

d. Determination of Total ash

Accurately weighed 2gm of air-dried crude drug was taken in a tared silica dish and incinerated at a temperature not exceeding 450°C until free from carbon, cooled and weight was taken. The percentage of ash was calculated with reference to the air-dried drug.

e. Determination of Water- soluble ash

The ash was obtained as per method described above and boiled for 5 minutes with 25 ml of water, filtered and collected the insoluble matter on an ash less filter paper, washed with hot water and ignited for 15 minutes at a temperature not exceeding 450°C and weight was taken. Subtracted the weight of the insoluble matter from the weight of the ash; the difference in weight represents the water-soluble ash. The percentage of water –soluble ash was calculated with reference to the air-dried drug.

f. Determination of Acid -insoluble ash

The ash was obtained as per method described above and boiled for 5 minutes with 25 ml of 2M hydrochloric acid, filtered and collected the insoluble matter on an ash less filter paper, washed with hot water and ignited cooled in a desiccator and weighed. The percentage of acid –insoluble ash was calculated with reference to the air-dried drug.

g. Extractive values

Different extractive values like alcohol soluble extractive, water soluble extractive values were performed by standard method.

h. Determination of water-soluble extractive value

Five gm of air dried coarsely powdered drug was macerated with 100 ml of chloroform water in a closed flask for 24 hours, and it was shaken frequently during first 6 hours and allowed to stand for 18 hours. Then it was filtered, 25 ml of the filtrate was evaporated in a flat shallow dish, and dried at 105^0c and weighed. Percentage of water-soluble extractive value was calculated with reference to air-dried drugs.

i. Determination of Alcohol-soluble extractive value

Five gm of air-dried coarsely powdered drug was macerated with 100 ml of ethanol of specified strength in a closed flask for 24 hours, and it was shaken frequently during first 6 hours and allows standing for 18 hours. Then it was filtered, during filtration precaution was taken against loss of ethanol, 25 ml of the filtrate was evaporated in a flat shallow dish, and dried at 105^0c and weighed. Percentage of ethanol soluble extractive value was calculated with reference to air-dried drugs.

Extraction

The extraction was carried out in several batches using Petroleum ether (60-80), ethanol and water as solvent. The extraction was carried out in Soxhlet extractor till all the constituents were extracted. The completion of extraction was indicated by taking sample of siphon tube on TLC plate and placing it in iodine chamber. Absence of colored spot on plate indicated complete extraction. After completion of extraction, solvent was distilled off and concentrated extract was air-dried. The extract was stored in airtight container. The same procedure was followed during extraction with other solvents. After petroleum ether, Ethanol (95 %) extraction the exhausted marc was kept in oven to remove the solvent completely. Finally, the new dried powdered material was refluxed for about 3 hours with distilled water to obtain aqueous extract.

Liquid-liquid fractionation with chloroform and ethyl acetate

1. 22.8 gm Dried ethanolic extract mix with 100 ml of ethanol: water (1: 1) in a separating funnel.
2. 5 times successive extraction with chloroform (50 ml) was carried out.
3. Chloroform layer was combined and collected.
4. Concentrate it up to thick paste was obtained.
5. Alcoholic layer placed in a separating funnel and 5 times successive extraction with ethyl acetate (50 ml) was carried out.
6. Collect and combine ethyl acetate layers and concentrate it up to thick paste. Remaining alcoholic extract collected and concentrated.

Preliminary Phytochemical Screening for Various Extracts

1) Test for Carbohydrates

a) Molisch test (General test)

Two ml of extract solution was added with few drops of 15 % ethanolic alpha naphthol solution in a test tube and 2ml of concentrated sulphuric acid was added carefully along the side of the test tube. The formation of reddish violet ring at the junction of two layers indicates the presence of carbohydrates.

b) Test for reducing sugar

i) Benedict's test

Mix equal volume of Benedict's regent and extract solution in test tube. Heat in a boiling water bath for 5 min. Solution appears green, yellow or red depending on amount of reducing sugar present.

ii) Fehling's test

Five ml of extract solution was mixed with 5 ml Fehling's solution (equal mixture of Fehling's solution A and B) and boiled. Development of brick red precipitate indicates the presence of reducing sugars.

c) Test for monosaccharides

Barford's test

Mix equal volume of barford's reagent and extract solution. Heat for 1-2 min. in boiling water bath and cool. Development of red precipitate indicates presence of monosaccharides.

2) Test for Proteins

i) Biuret test

The extract was treated with 1 ml of 10 percent sodium hydroxide solution and heated. A drop of 0.7 percent copper sulphate solution was added to the above mixture. The formation of purple violet color indicates the presence of proteins.

ii) Millon's test

The extract was treated with 2 ml of Millon's reagent. Formation of white precipitate indicates the presence of proteins and amino acids.

3) Test for Amino acids

Ninhydrin test

The extract was treated with Ninhydrin reagent at pH range of 4-8 and boiled. Formation of purple color indicates the presence of amino acids.

4) Test for Steroids

i) Salkowski test

One ml of concentrated sulphuric acid was added to 10 mg of extract dissolved in 1 ml of chloroform. A reddish brown color exhibited by chloroform layer and green fluorescence by the acid layer suggests the presence of steroids.

ii) Liebermann-burchard test

10 mg extract was dissolved in 1 ml of chloroform and 1 ml of acetic anhydride was added following the addition of 2 ml of concentrated sulphuric acid from the side of the test tube. Formation of reddish violet color at the junction indicates the presence of steroids.

iii) Liebermann's test

To 2 ml of the residue a few ml of acetic anhydride was added and gentle heated. The content of the test tube was cooled and 2 ml of concentrated sulphuric acid was added from the side of the test tube. Development of blue color gave the evidence for presence of steroids.

5) Test for Terpenoids

One ml of extract added with one ml of Vanillin sulfuric acid. Development of violet color gave the evidence for presence of Terpenoids.

6) Test for Glycosides

i) Cardiac glycoside

Keller-Killiani test (Test for deoxysugars)

To 2 ml of extract, glacial acetic acid, one drop 5 % Ferric chloride and conc. Sulphuric acid was added. Presence of cardiac glycosides is indicated by formation of reddish brown color at junction of the two liquid layers and upper layer appeared bluish green.

ii) Anthraquinone glycosides

Borntrager's test

To 3 ml extract add dilute sulphuric acid, boil and filter. To the cold filtrate, add equal volume benzene or chloroform. Shake well. Separate organic solvent. Add ammonia, the ammonia layer turns pink or red color.

7) Test for Saponins

Foam formation test

One ml solution of the extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. The development of stable foam indicates the presence of Saponins.

8) Test for Alkaloids

Evaporate all extracts separately. To residue, add dilute HCL. Shake well and filter. Use filtered solution for test.

i) Dragendroff's test

2-3 ml test solution and 0.1 ml Dragendroff's reagent was added in test tube. Formation of orange brown precipitate indicates the presence of alkaloids.

ii) Mayer's test

2-3 ml test solution and 0.1 ml of Mayer's reagent were added. Formation of yellowish buff precipitate indicates the presence of alkaloids.

iii) Hager's test

2-3 ml test solution and 0.1 ml of Hager's reagent. Formation of yellowish precipitate indicates the presence of alkaloids.

iv) Wagner's test

2-3 ml test solution and 0.1 ml of Wagner's reagent. Formation of reddish brown precipitate indicates the presence of alkaloids.

9) Test for Tannins and Phenolic compounds

i) 5 %Ferric chloride

Five ml of extract solution was allowed to react with 1 ml of 5 percent ferric chloride solution. Deep blue-black coloration indicates the presence of tannins.

ii) Dilute nitric acid test

Two ml of extract solution was allowed to react with few drops of dilute HNO_3 solution. Formation of reddish to yellow color indicates the presence of tannins.

iii) Bromine water test

Two ml of extract solution mix with 2 ml of bromine water. Discoloration of bromine water indicates presence of tannins.

iv) Potassium dichromate test

2-3 ml of extract solution and mix with 2 ml of Potassium dichromate. The formation of red precipitate indicates presence of tannins.

10) Test for Flavonoids

i) Shinoda test

To the extract 5 ml (95%) ethanol and few drops of con. HCl and 0.5 g of magnesium turnings was added gives pink color.

ii) Lead acetate test

Few drops of 10 percent lead acetate are added to the extract. Development of yellow colored precipitate confirms the presence of flavonoids.

iii) Sodium hydroxide test

Increasing amount of sodium hydroxide add in a extract solution which shows yellow coloration, which disappears after addition of acid.

Thin Layer Chromatography

After pharmacological evaluation of ethanol and chloroform fractions the only active fraction was evaluated by thin layer chromatography for determination of phytocomponents by following ways.

Table 1. TLC-Characterization of active Fractions

Sr. no.	Chemical constituent	Mobile Phase	Detection
1.	Alkaloids	n- butanol : Ethyl acetate: Formic acid : Water (30:50:10:10)	UV -365nm
2.	Glycoside	Ethyl acetate : Methanol : Water (100 : 16.5 : 13.5)	UV -365nm
3.	Flavonoid	Toluene : Ethyl acetate : Glacial acetic acid :Water (100:11:11:26)	Anisaldehyde – Sulfuric acid. UV -365nm
4.	Tannins	Ethyl acetate: Formic acid : Acetic acid : Water (100:11:11:26)	5% $FeCl_3$ in 0.1N HCl
5.	Steroids	Ethyl acetate : Methanol : Water (70 : 20 : 10)	Vanillin – Sulfuric acid.
6.	Terpenoids and Carotenoids	Cyclohexane : Ethyl acetate (75 : 25)	UV- 268nm
		Petroleum ether : Benzene (9 : 1)	UV- 254nm
7.	Triterpenoids	Chloroform : Glacial acetic acid Methanol :Water (60 : 32 : 12 : 8)	--
		Ethyl acetate : Glacial acetic acid : Formic acid : Water (100 : 11: 11 : 26)	Anisaldehyde – Sulfuric acid. UV – 254nm, 365nm

Detection of Steroids (Wagner 2004)

Solvent system used
Toluene: Ethyl acetate (9: 1) **(Ayurvedic Pharmacopoeia)**
Ethyl acetate: Methanol: Acetic acid (70:20:10) **(Stahl, 2005)**
Spray reagents
(i) Vanillin-Sulphuric acid reagent:
0.5 g vanillin is dissolved in 100 ml sulphuric acid- ethanol (40+10). Heated at 120^0c until maximum spot color intensity is reached.
Color observed - blue, blue-violet or pink colored spots.
(ii) Anisaldehyde-Sulphuric acid reagent:
0.5 ml of anisaldehyde was mixed with 10 ml glacial acetic acid, followed by 85 ml of methanol and 5 ml of concentrated sulphuric acid, in that order. The developed TLC plate was sprayed with reagent, heated at 100^0c for 5-10 minutes.
Color observed: blue, blue-violet or pink colored spots.

Detection of Alkaloids

Solvent system used
Toluene: Ethyl acetate: Formic acid (50:40:10) **(Wagner, 1996)**
Spray reagents
Sulphuric acid reagent
1% solution of concentrated sulphuric acid in ethanol. The developed TLC plate was sprayed with reagent, heated at 100^0c for 3-5 minutes.
Color observed: red-violet or brown colored spots.

Detection of Flavonoids
Solvent system used
Toluene: Ethyl acetate: Glacial acetic acid: Water (100:11:11:26)
N-Butanol: Acetic acid: Water (4:1:5)
Toluene: Ethyl acetate (9: 1) **(Ayurvedic Pharmacopoeia)**
Ethyl acetate: Formic acid: Acetic acid: Water (100:11:11:26) **(Wagner, 1996)**
Spray reagents
Anisaldehyde-Sulphuric acid reagent:
0.5 ml of anisaldehyde was mixed with 10 ml glacial acetic acid, followed by 85 ml of methanol and 5 ml of concentrated sulphuric acid, in that order. The developed TLC plate was sprayed with reagent, heated at 100^0c for 5-10 minutes.
Color observed: yellow-green spots.

Detection of Saponin
Solvent system used
Toluene: Ethyl acetate (9:1) **(Ayurvedic Pharmacopoeia)**
Ethyl acetate: Formic acid: Acetic acid: Water (100:11:11:26)
Spray reagents
Anisaldehyde-Sulphuric acid reagent
0.5 ml of anisaldehyde was mixed with 10 ml glacial acetic acid, followed by 85ml of methanol and 5 ml of concentrated sulphuric acid, in that order. The developed TLC plate was sprayed with reagent, heated at 100^0c for 5-10 minutes.
Color observed: green spots.

Detection of Tannins
Solvent system used
Toluene: Acetone: Ethyl acetate (3:1:2)
Ethyl acetate: Formic acid: Acetic acid: Water (100:11:11:26)
Spray reagents
5% Ferric chloride reagent:
5% $FeCl_3$ in 0.1N HCl. The developed TLC plate was sprayed with reagent, heated at 100^0c for 5-10 minutes. Color observed: bluish black spots.

Detection of Glycoside
Solvent system used
Ethyl acetate: Methanol: Water (100: 16.5: 13.5)
Methanol: water: chloroform (35:10:65) **(Stahl, 2005)**
Visualization: Under UV -365, Violet –blue color observed
Spray reagents
Sodium nitropruside reagent
1.5gm of Sodium nitroprusside is dissolved in 5 ml of 2N HCl, 95 ml of methanol and 10 ml of 25% ammonium hydroxide solution are added and solution is filtered.
Color observed: Orange-red.

Column chromatography of active extract
Phytocomponents from ethanol and chloroform were needed to isolate using column chromatography. About 10 gm. of extract was subjected to column chromatography to obtain further separated fractions.
A] Ethanol extract:
Height of column : 40 cm.
Diameter of column : 3.5 cm.
Stationary phase : Silica gel for column chromatography (60-120#)
Mobile phase : 1. n-Hexane, 2. Ethyl acetate 3. Methanol
Flow rate : 6-8 drops per minute.
Number of fractions Collected: 5
Volume of each fraction : 200 ml

B] Chloroform extract:
Height of column : 40 cm.
Diameter of column : 3.5 cm.
Stationary phase : Silica gel for column chromatography (60-120#)
Mobile phase : 1. Pet-ether, 2. Ethyl acetate 3. Methanol
Flow rate : 6-8 drops per minute.
Number of fractions Collected: 5
Volume of each fraction : 200 ml

In-Vitro Methods of Evaluation of Arthritic Activity
In the present study investigation of anti-arthritic activity of extracts of herbal plant parts was determined by the in-vitro models viz-. protein denaturation, membrane stabilization and proteinase (trypsin) inhibition.

Method 1 : Inhibition of protein Denaturation Assay

The reaction mixture consisted of the 100 µl test extracts (final concentration 100-1000 µg/ml) and 100 µl of 5 % aqueous solution of bovine serum albumin (BSA); pH was adjusted adding a small volume of glacial acetic acid. The sample extracts were incubated at 37 °C for 20 min and then heated to 70 °C for 10 min. The mixture was allowed to cool for 10 min after which turbidity was measured at 660 nm. The blank comprised the sample and distilled water. Distilled water was used as the negative control. The positive control was methotrexate (final concentration 10-100 µg/ml. Percentage inhibition was calculated.

Method 2: Effect on Membrane Stabilization Assay

The reaction mixtures 4.5ml consists of 2ml hypotonic saline (0.25% NaCl) + 1ml 0.15M phosphate buffer (pH 7.4) + 1ml test solution (100-500µg/ml) in normal saline + 0.5ml of 10% Sheep RBC in normal saline. The mixture was incubated at 56oC for 30 minutes. The tubes were cooled under running tap water for 20 minutes. The mixture was centrifuged for 3000rpm for 10min and the absorbance of the supernatant was measured at 560nm.For control sample 1 ml of isotonic saline was used instead of test solution

Method 3: Proteinase inhibition assay

Procedure:

The reaction mixture (2.0ml) contains 0.06 mg trypsin,1.0 ml of 25 mM Tris -HCL buffer (pH 7.4) and 1.0 ml of aqueous solution of test sample and were incubated at 37C for 5 minutes. Then 1.0 ml of 0.8% w/v casein was added and incubated for 20 minutes.2.0 ml of 70% V/V of perchloric acid was added to terminate the reaction. The cloudy suspension was centrifuged.

Optical density of the supernatant was read at 280 nm against buffer as blank. The percentage of inhibition was calculated using the following formula

In-Vivo Pharmacological Study

Animals

Sprague Dawley rats (either sex), weighing 200–300 g were selected from animal house of In-vivo Biotech Ltd., Hyderabad. The animals were maintained in house at room temperature, humidity and light. They were feed with standard pellet feed and water ad libitum. Protocol was approved by the Institutional Animal Ethics Committee (IAEC) Reg. No.

Drugs and chemicals

Formaldehyde (VWR, International Ltd), Aspirin (UNI-CHEM). All the other chemicals used were of analytical grade.

Evaluation of anti-arthritic activity of *Calotropis procera* against formaldehyde induced arthritis in rats

The rats were distributed into 4 groups (n = 5).

Group I: Arthritic control rats (3 ml/kg distilled water).

Group II: 100 mg/kg aspirin.

Group III: Ethanolic crude extract (150 mg/kg, p.o. respectively).

On day 1, 30 min subsequent to drug administration, arthritis was induced by sub plantar injection of 2% formaldehyde solution (0.1 ml) and recurrent induction on day 3. Drug treatment was sustained for 10 days. Arthritis was evaluated by checking mean increase in paw diameter for 10 days via digital

vernier calliper. Percentage inhibition of paw edema was worked out as described previously.

Result and Discussion

Macroscopy

Calotropis is a large, bushy shrub with decussate, obovate, coriaceous, auriculate, leaves with acute, subsessile apices extraaxillary, umbellate, panicale inflorescene with purple corolla and erect lobes. The morphological studies revealed the leaves to be subsessile, 6-15 cm by 4.5-8 cm, broadly ovate, ovate-oblong, elliptical, or obovate, acute, pubescent when young and glabrous on both sides on maturity.

Microscopy

Transverse sections through the midrib showed an upper and lower, single- layered epidermis that was externally covered with a thick, striated cuticle, a few epidermal cells on both lower and upper surfaces, parenchymatous cells that were thin-walled and isodiametric to circular. Intracellular spaces were present in ground tissue and the stele was crescent-shaped and composed of bicollateral and open vascular bundles. The xylem consisted mostly of vessels and tracheids, and a strip of cambium was present between the xylem and phloem tissues. Laticifers were also present along with the phloem and parenchymatous zone. The lamina which was dorsiventral with the mesophyll, was seen to be differentiated into a palisade and spongy tissue. The upper and lower epidermise were covered externally with a thick, striated cuticle. Below the upper epidermis were three rows of elongated, closely arranged, palisade parenchyma. Spongy parenchyma tissues were almost radially elongated with intracellular spaces. Central cells were irregular in shape; laticifers and vascular bundles were also present scattered in this region; the details are shown in Figure 2.

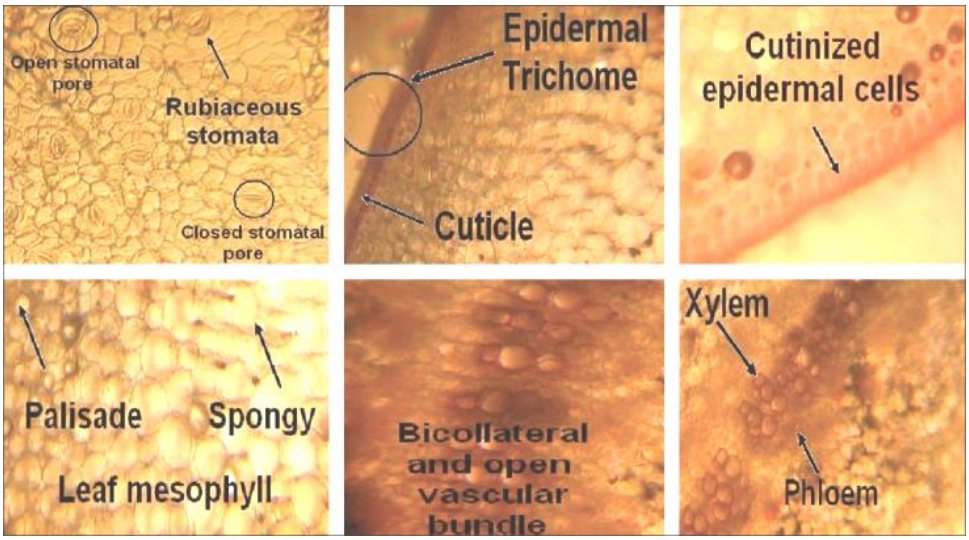

Figure 1: Transverse Section of leaf showing single enlarged portions (X400)

Microscopy: Powder characteristics

Fragments of cork occur with thin walled cells and appear reddish brown in colour.

b) Lignified stone cells occur in groups with rectangular to elongated shape.
c) Phloem fibres appear in groups, individual fibre is thick and can appear either entire or in fragments.
d) Starch grains are simple, round and rarely compound.

Table 2. Fluorescence of powder with various reagents

Reagent Added	Visible light	Short wave U.V. light	Long wave U.V. light
None	Light Green	Dark Green	-
Water	Light Green	Dark Green	-
Conc. H2SO4	Light Green	Dark Green	Green
Conc. HNO3	Light Brown	Dark Green	Green
Conc. HCl	Light Green	Dark Green	Green
10% HCl	Light Green	Dark Green	Yellow
10% H2SO4	Light Brown	Dark Green	Yellow
10% HNO3	Light Green	Dark Green	Yellow
Glacial Acetic Acid	Light Brown	-	Yellow
Aq. NaOH	Light Green	Dark Green	-
Alc. NaOH	Light Green	Dark Green	Reddish Yellow

Physical evaluation

1. Determination of foreign organic matter

Foreign organic matter in leaves of *Calotropis procera* powder was found to be 0.5% w/w when observed under 6X lens.

2. Determination of moisture content

Moisture content was measured and it was observed that, the results were complying as per standard guidelines.

Table 3. Observation of loss on drying.

Time (hrs.)	Loss of moisture (%w/w)
0	0.00
01	0.321
02	0.358
03	0.369
04	0.369

3. Determination of Ash value

The total ash value, acid insoluble ash, water soluble ash was found to be 5%, 2% and 2%. This percentage clearly indicates that the root is best for drug action and effects. The water-soluble extractive value proved to be lesser than alcohol soluble extractive value. It was found to be 0.3%. This shows that the constituents of the drug are more extracted and soluble in alcohol as compared to water.

Table 4. Ash value of *Calotropis procera* leaves

Sr. No.	Evaluation Parameters	Value (%w/w)
1.	Total ash value	5 %
2	Acid insoluble ash value	2 %
3	Water soluble ash value	2%

4. Determination of Extractive values:

Ethanol-soluble extractive value was found to be greater than other extractive value; it indicates that compounds present in the leaves are soluble in alcohol in high amount. This might guide us for the isolation of maximum active components form plant.

Table 5. Extractive Values (%w/w) of *Calotropis procera* leaves

Sr. No	Extractive values	Extractive value (%w/w)
1	Ethanol soluble extractive values	14
2	Water soluble extractive values	8.9

Extraction

Table 6. Yield of various extracts obtained from the leaves of *Calotropis procera*

Sr. No.	Evaluation Parameters	Color	Nature	Percentage Yield (% W/W)
1.	Petroleum ether	Green	Semisolid and sticky	21.20 %
2.	Ethanol	Dark Green	Semisolid	38.56 %
3.	Chloroform	Dark Green	Jelly like	16.20%
4.	Ethyl acetate	Green	Semisolid	8.45 %
5.	Aqueous	Dark brown	Sticky powder	6.23%

Preliminary Phytochemical Screening

Table 7. Preliminary Phytochemical Screening of Various Extracts of *Calotropis procera*

Extracts	Petroleum ether	Ethanol	Chloroform	Ethyl acetate	Aqueous
Tests for carbohydrates					
Molish Test	-	+	-	-	-
Fehling Test	-	+	-	+	-
Benedict Test	-	+	-	+	-
Test for Monosaccharide					
Barfoed's Test	+	-	-	-	-
Test for Non-reducing polysaccharides					
Iodine Test	-	-	-	-	-
Test for Proteins					
Biuret test	-	-	-	-	-

Millions test	-	-	-	-	-
Tests for Steroids					
Salkowaski reaction	-	+	+	+	-
Libermann Burchard reaction	+	-	-	-	-
Libermann reaction	+	-	-	-	-
Tests for Terpenoids	+	+	-	-	-
Test for Glycosides					
Borntrager's Test	+	-	-	+	-
Killer- Killani Test	-	+	+	-	-
Test for Saponin					
Foam test	+	-	-	-	+
Tests for Flavonoids					
Shinoda test	-	-	+	-	-
Lead acetate Test	-	+	+	-	-
Sod-hydroxide Test	-	+	+	-	-
Tests for Alkaloids					
Meyers Test	**-**	**+**	**-**	**-**	**-**
Wagner's Test	**-**	**-**	**-**	**-**	**+**
Hager's Test	**-**	**-**	**-**	**-**	**-**
Dragendorff Test	**-**	**+**	**+**	**+**	**-**
Test for Tannins & Phenolic compounds					
$FeCl_3$	**-**	**+**	-	**-**	**-**
Lead acetate	**-**	**+**	-	**+**	**-**

+ Indicates presence of phytoconstituents, - Indicates absence of phytoconstituents

The all extracts were screened for the presence of various constituents. The result of this preliminary phytochemical examination is shown in Table 7. The result of phytochemical study on *Calotropis procera* revealed presence of alkaloids, **steroids,** flavonoids, tannins and terpenoids.

Thin Layer Chromatography of Extract

TLC chromatographic study were carried out to separate the active constituents which may having pharmacological active constituents.

Table 8. Thin Layer Chromatography of all extracts

Sr. no.	Chemical constituent	Mobile Phase	Visualization Spraying reagent	Color of spot	Rf- value
1.	Alkaloids	Toluene : Ethyl acetate: Formic acid (50:40:10)	10% H_2SO_4 in ethanol	Violet - blue	Petroleum ether :0.72 Ethanol : 0.85 Chloroform : 0.81
2.	Glycoside	Ethyl acetate: Methanol : Water (100 : 16.5 : 13.5)	Under UV - 365	Violet - blue	Ethanol : 0.73 Chloroform : 0.63
3.	Flavonoid	Toluene : Ethyl acetate : Glacial acetic acid : Water	Anisaldehyde – Sulfuric	Yellowish green	Petroleum ether: 0.75 Ethanol : 0.81

		(100:11:11:26)	acid.		Chloroform : 0.87
4.	Tannin	Ethyl acetate: Formic acid : Acetic acid : Water (100:11:11:26)	5 % $FeCl_3$ in 0.1N HCl	Black	Ethanol : 0.65
5.	Steroids	Ethyl acetate : Methanol : Acetic acid (70 : 20 : 10)	Vanillin – Sulfuric acid.	Pink	Petroleum ether: 0.82 Ethanol : 0.72 Chloroform : 0.52 Ethyl acetate : 0.79
6.	Saponin	Ethyl acetate: Formic acid : Acetic acid : Water (100:11:11:26)	Anisaldehyde – Sulfuric acid.	Green	Petroleum ether: 0.85

Column Chromatography of Active Extract of *Calotropis Procera*

In preliminary phytochemical test of leaves of *Calotropis procera* ethanol and chloroform extract show presence of flavonoids and phenolic compounds. Also the TLC of both extract of shows presence of different phytometabolites.

A] ETHANOL EXTRACT COLUMN CHROMATOGRAPHY

Table 9. Appearance and percent yield of all fractions

Sr. no.	Mobile phase	Weight of fraction (gm.)	% Yield w/w	Appearance
1	n-Hexane	0.75	7.5	Dark Brown (Semisolid and sticky)
2	n-Hexane: Ethyl acetate (5: 5)	1.22	12.2	Green
3	Ethyl acetate	0.83	8.3	Reddish brown
4	Ethyl acetate: Methanol (5: 5)	0.31	3.1	Faint Brown
5	Methanol	0.82	8.2	Yellow

Note: EF-Ethanol Fraction

B] CHLOROFORM EXTRACT COLUMN CHROMATOGRAPHY

Table 10. Appearance and % yield of all fractions

Sr. No.	Mobile phase	Weight of fraction (gm)	% Yield w/w	Appearance
1	Petroleum ether	0.68	6.8	Faint Brown
2	Petroleum ether: ethyl acetate (5: 5)	1.21	12.1	Brown
3	Ethyl acetate	1.35	13.5	Reddish brown
4	Ethyl acetate: Methanol (5: 5)	0.86	8.6	Dark Brown
5	Methanol	0.35	3.5	Brown

Note: CF-Chloroform Fraction

Anti-rheumatic arthritis activity

A. Ethanol Fraction

The inhibition % of protein denaturation of aqueous extracts of leaves of *Calotropis procera* Linn was within the range from 48.12 ±1.02 to 72.14 ±0.58 at the concentration range of 100-1000 µg/ml. Maximum inhibition of 72.14% was observed at 1000 µg/ml compared to, methotrexate showed the maximum inhibition of 88.12% of at the concentration of 100 µg/ml. The IC50 was observed as 324.68. The protein denaturation inhibitory activity of ethanol and Chloroform extracts of leaves of *Calotropis procera* Linn is shown in Table and Graph.

Table 11. The protein denaturation inhibitory activity of ethanol extracts of leaves of *Calotropis procera* Linn

Sample	Conc. (µg/ml)	OD	% Inhibition	IC50
Blank (Control)	-	0.35	-	314.25
Standard	100	0.64	88.12±0.69	
Calotropis Procera ***Linn Ethanol Fraction***	100	0.37	48.12 ±1.02	
	200	0.34	52.15 ±1.13	
	400	0.31	53.14 ±0.85	
	800	0.21	65.23 ±1.06	
	1000	0.19	72.14 ±0.58	

Values represent in the results are mean±SD of three replicates; linear regression analysis was used to calculate IC50 value.

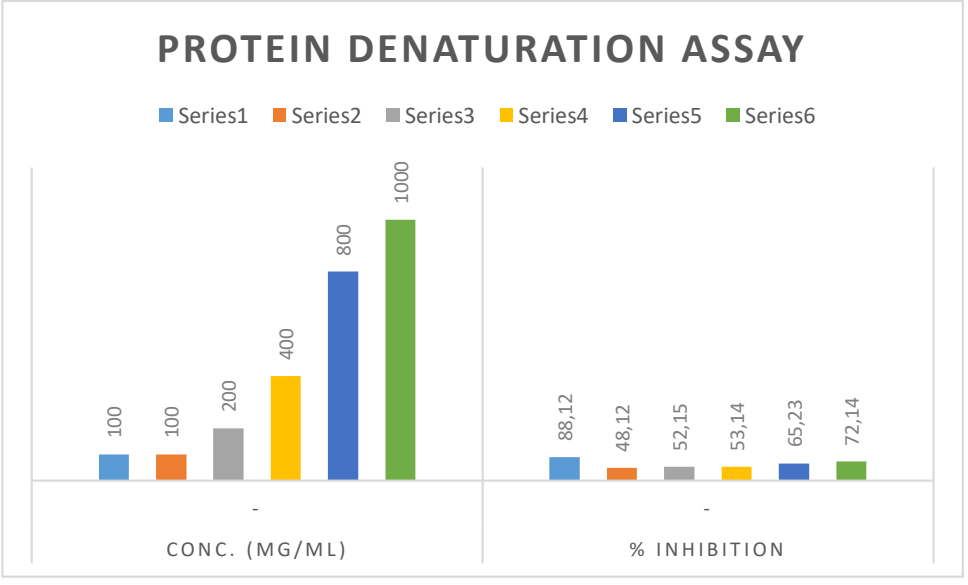

Figure 2- Protein Denaturation Assay

Table 12. The protein denaturation inhibitory activity of chloroform extracts of leaves of *Calotropis procera* Linn

Sample	Conc. (µg/ml)	OD	% Inhibition	IC50
Blank (Control)	-	0.35	-	294.56
Standard	100	0.64	88.12±0.78	
Calotropis Procera ***Linn Chloroform Fraction***	100	0.31	41.23 ±0.36	
	200	0.30	48.56 ±1.89	
	400	0.29	47.12 ±0.56	
	800	0.19	49.63 ±1.44	
	1000	0.17	65.42 ±0.89	

Values represent in the results are mean±SD of three replicates; linear regression analysis was used to calculate IC50 value.

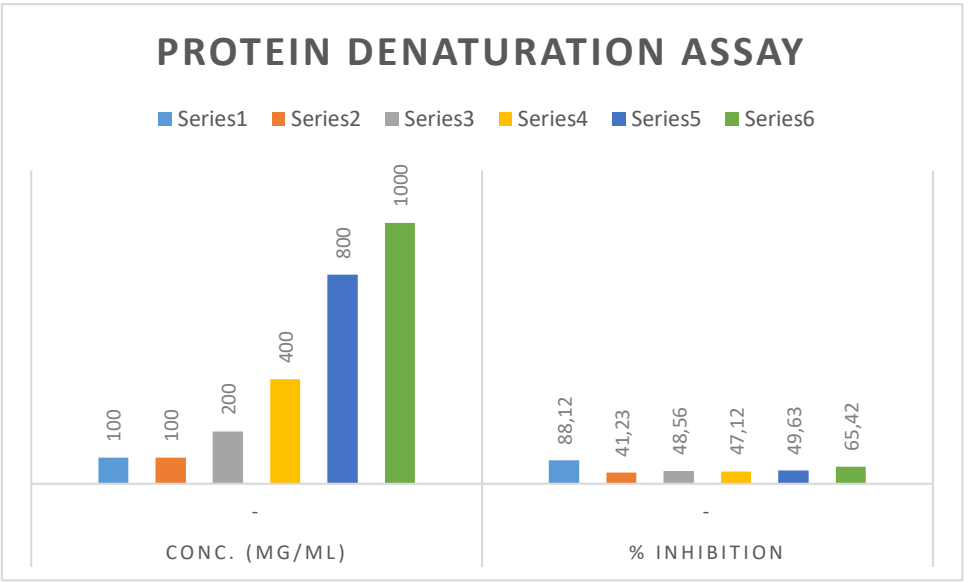

Figure 3- Protein Denaturation Assay

Effect on Membrane Stabilization

The percentage membrane stabilization of ethanolic and chloroform extracts of leaves of *Calotropis procera* Linn was within the range from 33.20 ±0.67 to 66.58 ±3.38 at the concentration range of 100-1000 µg/ml. The IC50 was observed as 591.37. The percentage membrane stabilization activity of ethanolic extracts of leaves of *Calotropis procera* Linn is shown in Table and Graph.

Table 13. The Membrane Stabilsation Assay of ethanol extracts of leaves of *Calotropis procera* Linn

Plant name	Conc (µg/ml)	OD	% Inhibition	IC50
Blank	-	0.32	-	591.77
STD	100	0.61	72.15±0.85	
Calotropis Procera Linn Ethanolic extract	100	0.21	33.20 ±0.67	
	200	0.20	36.52 ±0.71	
	400	0.17	47.48 ±0.62	
	800	0.15	52.15 ±1.29	
	1000	0.11	66.58 ±3.38	

Values represent in the results are mean±SD of three replicates; linear regression analysis was used to calculate IC50 value.

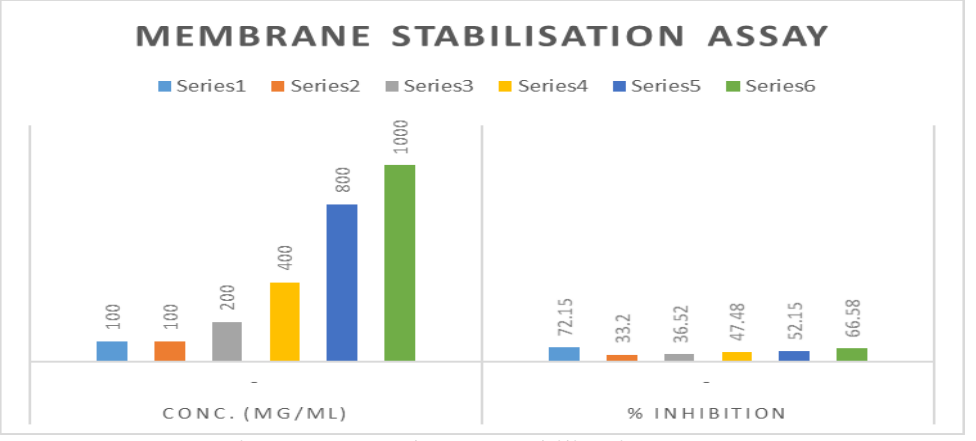

Figure 4- Membrane Stabilization Assay

Table 14. The Membrane Stabilsation Assay of chloroform extracts of leaves of *Calotropis procera* Linn

Plant name	Conc (µg/ml)	OD	% Inhibition	IC50
Blank	-	0.32	-	587.56
STD	100	0.61	68.45±0.85	
Calotropis Procera Linn Chloroform extract	100	0.22	29.51 ±0.56	
	200	0.23	31.36 ±0.89	
	400	0.19	41.47 ±0.45	
	800	0.18	48.56 ±1.23	
	1000	0.12	61.26 ±1.87	

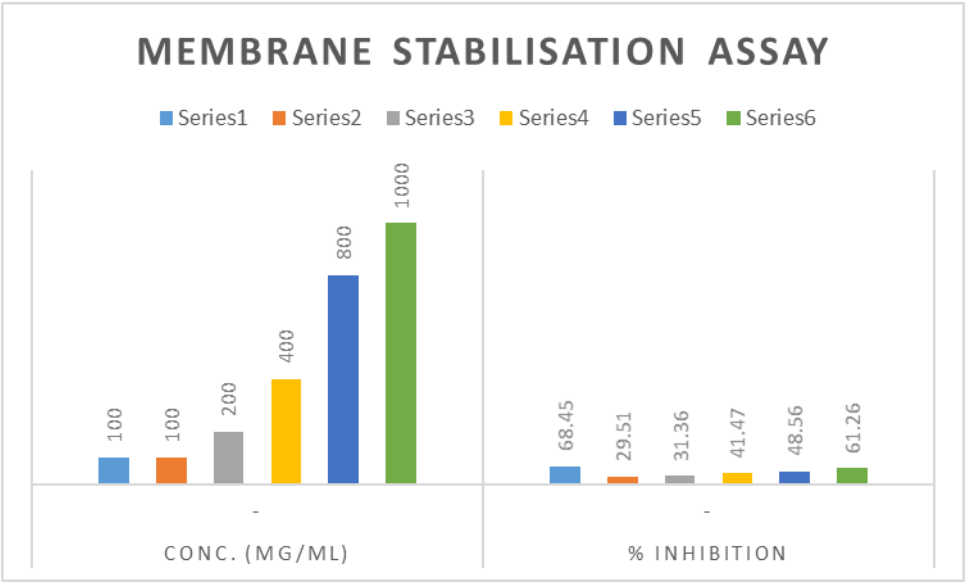

Figure 5. Membrane Stabilization Assay

Proteinase Inhibition Assay

The percentage proteinase inhibition of ethanolic and chloroform extracts of leaves of *Calotropis Procera* Linn was within the range from 45.12 ±2.02 to 68.45 ±1.17 at the concentration range of 100-1000 µg/ml. Maximum inhibition of 68% was observed at 1000 µg/ml. The IC50 was observed as 171.56.

Table 15. The Proteinase Inhibition Assay of ethanolic extracts of leaves of *Calotropis procera* Linn

Plant name	Conc (µg/ml)	OD	% Inhibition	IC50
Blank	-	0.75		171.56
STD	100	0.69	78.25±2.25	
Calotropis Procera Linn Ethanolic extract	100	0.89	44.25 ±2.02	
	200	0.95	56.36 ±1.06	
	400	0.89	59.47 ±1.48	
	800	0.72	65.12 ±1.26	
	1000	0.68	68.45 ±1.17	

Values represent in the results are mean±SD of three replicates; linear regression analysis was used to calculate IC50 value.

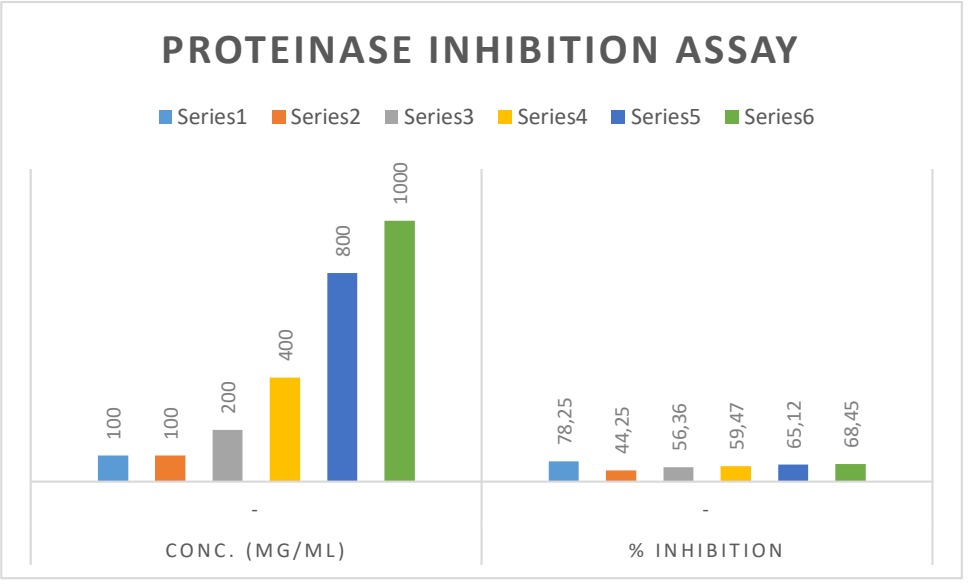

Figure 6- Proteinase Inhibition Assay

Table 16. The Proteinase Inhibition Assay of chloroform extracts of leaves of *Calotropis procera* Linn

Plant name	Conc (µg/ml)	OD	% Inhibition	IC50
Blank	-	0.69	-	165.12
STD	100	0.64	74.56±2.25	
Calotropis Procera Linn Chloroform extract	100	0.78	41.25 ±1.15	
	200	0.89	51.58 ±1.22	
	400	0.84	56.89 ±1.09	
	800	0.69	61.15 ±1.56	
	1000	0.59	65.23 ±1.22	

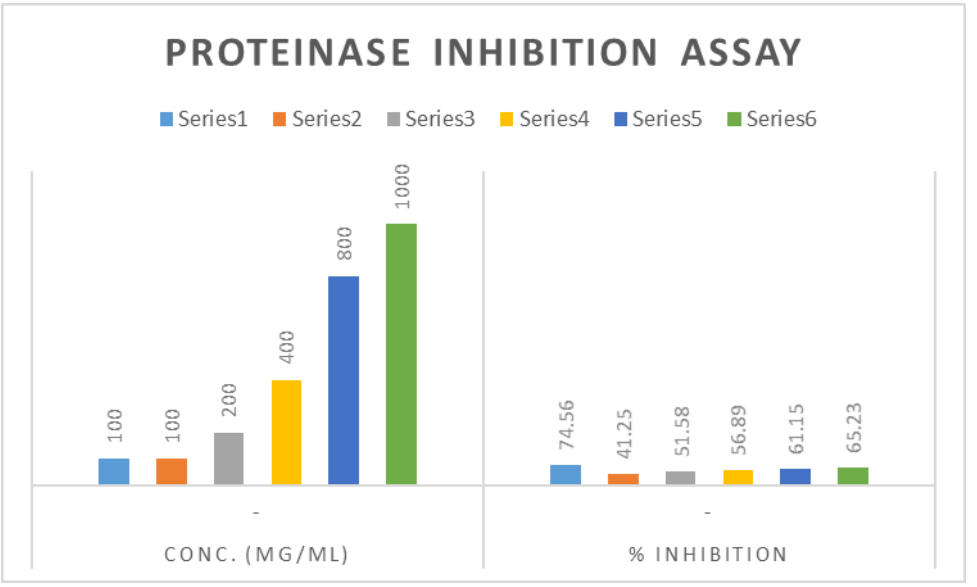

Figure 7- Membrane Stabilization Assay

From the results obtained after the study, it can conclude that, ethanolic extract shows good anti-rheumatic arthritic activity. So, to carry out the in-vivo study only ethanolic extract was considered.

Anti-arthritic activity of *Calotropis procera* **against formaldehyde induced arthritis in rats**

Results displayed in Table depict that 150 mg/kg of crude extract and *ethanolic* fraction on 10th day showed more superior repression of paw edema i.e., 80.26% and 81.25% ($p < 0.001$) as compared to 79.56% reduction in paw edema by 100 mg/kg aspirin on 10th day.

Table 19. Effect of ***Calotropis procera*** on formaldehyde induced arthritis in rats (n = 5, Mean ± SEM)

Treatment groups	Increase in Paw Diameter (mm)				
	Day 2	Day 4	Day 6	Day 8	Day 10
Arthritic control (3 ml/kg)	7.41 ± 0.05	9.56 ± 0.59	11.02 ± 0.26	13.20 ± 0.89	15.45 ± 0.56
Standard Aspirin (100 mg/kg)	4.58 ± 0.020***	4.65 ± 0.029***	4.12 ± 0.014***	3.88 ± 0.045***	3.53± 0.013***
Ethanolic fraction (100 mg/kg)	5.23 ± 0.020***	5.26± 0.029***	5.15 ± 0.021***	5.09 ± 0.023***	4.95± 0.029***

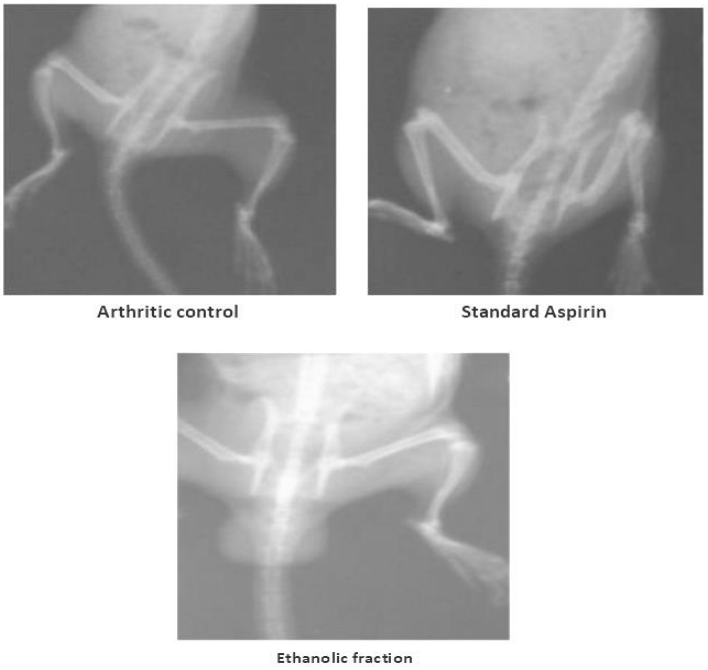

Figure 8: Radiography of the rat joints in formaldehyde induced arthritic model

Conclusion

Fresh leaves of *Calotropis procera* Linn. (Moraceae) were collected from Pune district (Maharashtra). Dried leaves material was coarsely powdered in grinder and powder material was passed through 120 mesh to remove fine powders and coarse powder was used for extractions. Using different solvents in increasing order of polarity. Petroleum ether (60-80), ethanol and water. The extraction was carried out in several batches. The leaves of plant shade dried were powdered and extracted with various solvents. The Extracts were tested for the presence of active principles such as Triterpenoids, Steroids, Glycosides, Saponins, Alkaloids, Flavonoids, Tannins, Proteins, Free Amino Acids, Carbohydrate and Vitamin C. The most of drug contain definite chemical constituents to which their pharmacological and Biological activity depended. Qualitative chemical test used to identify drug quality and purity. The identification, isolation and purification of active chemical constituents is depending chemical methods of evaluation. The all extracts were screened for the presence of various constituents. The result of phytochemical study on *Calotropis procera* revealed presence of alkaloids, steroids, flavonoids, tannins and terpenoids. TLC chromatographic study were carried out to separate the active constituents which may having pharmacological active constituents. In preliminary phytochemical test of leaves of *Calotropis procera* ethanol and chloroform extract show presence of flavonoids and phenolic compounds. Also the TLC of both extract of shows presence of different phytometabolites. After collection of all fractions of both ethanol and chloroform extract, these fractions then screened for anti-rheumatic arthritis activity. Results

depict that 150 mg/kg of crude extract and ethanolic fraction on 10th day showed more superior repression of paw edema i.e., 80.26% and 81.25% ($p < 0.001$) as compared to 79.56% reduction in paw edema by 100 mg/kg aspirin on 10th day.

Conflict of Interest
None declared by authors.

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