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In Vitro Analysis of Anti Inflammatory and Antituberculosis Activity of Calotropis Gigantea Stem Methanolic Extract

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Abstract
Methanol extract of stem of Calotropis gigantea was studied for its anti-inflammatory activity by in vitro methods. The results showed that C. gigantea Stem Methanol Extract (CGSME) at concentrations of 100,200, 400, 600 & 800 µg/ml displayed significant anti-inflammatory activity. CGSME at 800µg/ml showed % inhibition of 62.58 (P<0.01) by Bovine Serum Albumin inhibition of albumin denaturation method. CGSME at concentration of 1000µg/ml showed percentage inhibition of 81.99% and hence it is clearly evident that CGSME demonstrates significant anti tuberculosis activity. Purification of respective bioactive compounds are necessary and the purified form of these compounds can be used, which may show increased activity. This study gives an idea that compounds of CGSME can be used as lead compounds for designing potent anti-inflammatory and anti-tuberculosis drugs, which can be used for treatment of diseases such as inflammation, tumor and tuberculosis.

Keywords: In vitro anti-inflammatory activity, anti-tuberculosis activity, Calotropis gigantea, Phytochemicals, Inhibition of albumin denaturation

1. Introduction

India is endowed with a unique wealth of biota which includes a large number of medicinal and aromatic plants. India is repository of thousands of medicinally important plant species; it is ranked 6 among 12 mega diversity countries of the world. Out of the estimated 18,000 species of vascular plants in India, around 15% are feared to be under threat. Various medicinal plants have been used for years in daily life to treat disease all throughout the world. They have been utilized as a medicinal source.15 More than 50% of all modern clinical drugs are of natural product origin29 and the natural product plays an important role in drug development programs in pharmaceutical industry.3

In the recent past, there has been growing interest in exploiting the biological activities of different ethano-botanical formulations owing to their natural origin, cost effectiveness and lesser side effects. Medicinal plants are an expensive gift from nature to humans. The approval of traditional medicine as an alternative form of health care and the improvement of microbial resistance to the existing antibiotics has lead researchers to scrutinize the antimicrobial compounds.

Calotropis gigantea belonging to Asclepiadaceae is a lactiferous shrub commonly found in tropical and sub-tropical regions around the world23. The plant possesses immense bio potential uses including antimicrobial, anti-inflammatory, anti-cancer, anti-leprosy, anti-tuberculosis, wound healing activity as well as is used against skin diseases also. Considering the significant properties of the plant the present work focuses on the anti-inflammatory efficacy of C. gigantea.

Inflammation is a severe response by living tissue to any kind of injury. There can be four primary indicators of inflammation: pain, redness, heat or warmness and swelling. When there is injury to any part of the human body, the arterioles in the encircling tissue dilate. This gives a raised blood circulation towards the area (redness).4 Inflammation is either acute or chronic inflammation. Acute inflammation may be an initial response of the body to harmful stimuli. In chronic inflammation, the inflammatory response is out of proportion resulting in damage to the body.

Cyclooxygenase (COX) is the key enzyme in the synthesis of prostaglandins, prostacyclins and thromboxanes which are involved in inflammation, pain and platelet aggregation.6 Steroidal and non-steroidal anti-inflammatory drugs (SAIDs and NASIDs respectively) are currently the most widely used drugs in the treatment of acute inflammatory disorders, despite their renal and gastric

negative secondary effects.[18] These drugs block COX-1 and COX-2 enzyme activity. COX enzymes assist prostaglandin production. NSAIDs, steroidal anti-inflammatory drugs are being used till now, As a result of long term use, these drugs cause adverse side effects and damage human biological system such as liver, gastrointestinal tract, etc causing gastric lesions, cardiovascular, renal failure[10] and gastrointestinal damage.[32,25]

In recent times the significant emergence of Multi Drug Resistant and Extensively Drug Resistant strains of Mycobacterium tuberculosis has been causing much complications to problem of Tuberculosis control. The urgent need for newer anti – TB drugs has necessisated this in vitro evaluation of anti –TB activity of CGSME. Mycobacterium smegmatis is fast growing and non-pathogenic when compared to other Mycobacterium species. Another significance is its complementary use of mycothiol biosynthesis for making an essential thiol that is responsible for life. If it is knocked out, the species will be terminated and a treatment will be found

2. Materials and methods

2.1 Sample Preparation

Stem parts of healthy Calotropis gigantea plants were collected as fresh samples from the arid region of Namakkal district in Tamilnadu, India. The samples were identified by Botanical Survey of India (Southern circle), Coimbatore. Shade dry method was followed and later the samples were powdered and ground in mixer grinder.

2.2 Extract Preparation

The powdered samples of C. gigantea stem parts were subjected to Soxhlet extraction. Methanol was used for phytochemical extraction. The extraction was performed overnight to obtain concentrated extract. Further the extract was concentrated using a rotary evaporator and was stored at 4°C for further use.

2.3 Preliminary Phytochemical analysis

Phytochemical evaluation of the aqueous extract was performed using standard procedures. Different phytochemicals, namely alkaloids, flavonoids, phenolics, glycosides, saponins, steroids, tannins and terpenoids were screened.[28]

2.4 In vitro Analysis of Anti-inflammatory Activity

2.4.1 Inhibition of albumin denaturation

The anti-inflammatory activity of the sample extract was studied by using inhibition of albumin denaturation technique which was studied according to Mizushima et al., 1968[13] and Sakat et al.[21], 2010 followed with minor modifications. The reaction mixture consisted of test extracts and 1% aqueous solution of bovine albumin fraction. pH of the reaction mixture was adjusted using small amount of 1N HCl. The sample extracts were incubated at 37ºC for 20 minutes and then heated to 51ºC for 20 minutes. After cooling the samples, the turbidity was measured at 660nm (UV Visible Spectrophotometer Model 371, Elico India Ltd). The experiment was performed in triplicate. The Percentage Inhibition of protein denaturation was calculated as follows

Percentage Inhibition = (Abs Test –Abs control) X 100/ Abs

2.4.2 Antiproteinase action

The reaction mixtures (2 ml) contained 0.06mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml test samples each of different concentrations (100 - 500 µg/ml). The mixtures were incubated at 37°C for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixtures were incubated for an additional 20 min. 2 ml of 70% perchloric acid was added to arrest the reaction. The cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated as follows

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

2.5 Membrane stabilization

2.5.1 Preparation of Red Blood cells (RBCs) suspension

The blood was collected from a healthy human volunteer who had not taken any NSAIDs (Non Steroidal Anti-Inflammatory Drugs) for 2 weeks prior to the experiment and transferred to centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 minutes and were washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline.

2.5.2 Heat Induced Haemolysis

The reaction mixture (2ml) consisted of 1 ml test sample each of different concentrations (100 - 500 µg/ml) and 1 ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Aspirin was used as a standard drug. All the centrifuge tubes containing the reaction mixture were incubated in water bath at 56ºC for 30 minutes. At the end of the incubation, the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 minutes and the absorbance of the supernatant was taken at 560 nm. The experiment was performed in triplicate for all the test samples. The percentage inhibition of haemolysis was calculated as follows

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

2.6 Anti-lipoxygenase activity

Anti-lipoxygenase activity was studied using linoleic acid as substrate and lipoxidase as enzyme. Test samples were dissolved in 0.25ml of 2M borate buffer pH 9.0 and added to 0.25 ml of lipoxidase enzyme solution (20,000U/ml) and incubated for 5 minutes at 250ºC. After which, 1.0 ml of linoleic acid solution (0.6 mM) was added, mixed well and absorbance was measured at 234nm. Indomethacin was used as reference standard. The percentage inhibition was calculated as follows

Percentage inhibition= [{Abs control- Abs sample}/Abs control] x 100

A dose response curve was plotted to determine the IC_{50} values (IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity). All tests and analyses were run in triplicate and averaged.

2.7 In vitro Analysis of anti-tuberculosis activity

2.7.1 Preparation of medium

Lowenstein-Jensen medium (LJ medium) was **used. For its**

preparation 37.24 grams was suspended in 600 ml distilled water containing 12 ml glycerol. Heating was done if necessary to dissolve the medium completely. Sterilization by autoclaving at 15 lb pressure (121°C) was done for 15 minutes. Meanwhile 1000 ml of whole egg emulsion was prepared and the egg emulsion base aseptically added and mixed in the prepared LJ medium. The prepared medium was autoclaved at 85°C for 45 minutes. Then freeze dried culture of Mycobacterium smegmatis (MTCC 6) was added to the LJ medium and the same incubated at 37°C for seven days for the growth of bacteria.

2.7.2 Preparation of bacterial inoculum

One loop full of fresh cultured Mycobacterium smegmatis was added to freshly prepared LJ medium. The prepared medium was incubated with bacteria for 24 hrs at 37°C. The growth of bacteria similar to 0.5 McFarland standards dilution for the In-Vitro assay. If the growth was more than 0.5 McFarland standards dilution then the bacterial culture was again serial diluted. Purity of prepared culture was confirmed by Ziehl-Neelsen Staining specific for confirmation of acid fast bacilli.

2.7.3 MTT assay

MTT assay, which measures the reduction of the tetrazolium salt MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; Roche) into a blue formazan product, mainly by the succinate dehydrogenase was used. Freshly prepared bacterial culture (0.5 McFarland standards) were seeded in a flat-bottomed 96-well plate and incubated in a 5% CO2 humid incubator for 24 hours at 37°C. M. smegmatis was treated with different concentrations of CGSME (1000µg/ml, 500µg/ml and 250µg/ml) and the incubation was continued for 48 hours, followed by adding 20 µl (0.5mg/ml as final concentration, i.e. 20µl/well of stock) of MTT dye solution to each well for 4 hours at 37 ºC. After removal of the MTT dye solution, bacterial suspension was treated with 200µl DMSO and the absorbance at 570nm was quantified using ELISA reader. The cytotoxicity was calculated after comparing with the control (treated with 0.1% DMSO). All tests and analysis were run in triplicate and mean values recorded. Percentage of bacterial viability was determined.

3 Results & Discussion

3.1 Preliminary Phytochemical analysis

It showed the presence of carbohydrates and glycosides, alkaloids, flavanoids, terpenoids, phenols and tannins.

3.2 In vitro Analysis of Anti-inflammatory Activity

3.2.1 Inhibition of albumin denaturation

In the BSA denaturation inhibition assay, concentrations from (100,200, 400, 600 & 800 µg/ml) displayed significant activity.

In vitro anti-inflammatory analysis of C. gigantea Stem Methanol Extract (CGSME) at 800µg/ml showed percentage inhibition of 62.58 ($P<0.01$) by Bovine serum albumin denaturation method The maximum activity was shown at 800µg/ml. Aspirin, a standard anti inflammation drug showed the maximum inhibition 68% at the concentration of 100 µg/ml compared with control (Figure1, Table 1).

3.2.2 Proteinase Inhibitory Action

Neutrophils are known to be a rich source of serine proteinase and are localized at lysosomes. It was previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors[5]. CGSME exhibited significant antiproteinase activity at different concentrations as shown in Table2, Figure 2. It showed maximum inhibition of 71.34% at 800µg/ml. Aspirin showed the maximum inhibition 62% at 100µg/ml (Figure 2, Table 2).

3.2.3 Membrane stabilization

The HRBC membrane stabilization has been used as a method to study the in vitro anti-inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane[6,24] and its stabilization implies that the extract may well stabilize lysosomal membranes. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage upon extra cellular release. The lysosomal enzymes released during inflammation produce various disorders. The extra cellular activity of these enzymes are said to be related to acute or chronic inflammation. The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane.[19]

3.2.4 Heat Induced Haemolysis

The extract was effective in inhibiting the heat induced haemolysis at different concentrations. The results showed that CGSME at concentrations of 600 and 800µg/ml protect significantly ($p<0.05$) the erythrocyte membrane against lysis induced by heat (Figure2, Table2). Aspirin 100µg/ml offered a significant ($p<0.01$) protection against damaging effect of heat solution.

3.3 Anti-lipoxygenase activity

The establishment of new in vitro test systems has stimulated the screening of plants aiming to find leads for the development of new drugs. The plant lipoxygenase pathway is in many respects equivalent to the 'arachidonic acid cascades' in animals[7]. For this reason, the in vitro inhibition of lipoxygenase constitutes a good model for the screening of plants with anti-inflammatory potential[2]. LOXs are sensitive to antioxidants and the most of their action may consist in inhibition of lipid hydroperoxide formation due to scavenging of lipidoxy or lipid peroxy-radical formed in course of enzyme peroxidation. This can limit the availability of lipid hydroperoxide substrate necessary for the catalytic cycle of LOX.

CGSME was analysed at varying concentrations of 100,200,400,600, 800µg/ml for anti lipoxygenase inhibition activity. The strongest inhibition was obtained at concentration 800µg/ml (Figure2, Table2).

3.4 In-Vitr anti-tuberculosis activity of Calotropis gigantea

The activities of the plant extracts against bacteria have been studied for over a century, but work in this area has intensified only in the last 3 decades. The results obtained showed the strong anti-mycobacterial activity of the

extracts. The results were comparable to those of the standard drug (Isoniazid). This may be due to the bioactive constituents, such as alkaloids, tannins and flavonoids, present in the extracts.[9]

The percentage inhibition levels increases with increasing concentrations of the CGSME extract. CGSME at concentration of 1000µg/ml showed percentage inhibition of 81.99% and hence it is clearly evident that CGSME demonstrates significant anti-tuberculosis activity.

Table 1: Inhibition of albumin denaturation

S.No.	Treatments	Conc. (µg/ml)	Triplicate Valu			% Inhibition Mean ± S.E.M(S-I)
			A	B	C	
1	CGSME	100	41.00	42.44	40.28	41.24 ± 1.0
2	CGSME	200	50.35	47.48	47.48	48.43 ± 1.65
3	CGSME	400	56.11	55.39	54.67	55.39 ± 0.72
4	CGSME	600	58.99	58.27	61.87	59.71 ± 1.90
5	CGSME	800	62.58	60.43	64.74	62.58 ± 2.15
6	ASPIRIN	100	-	-	-	62.00 ± 0.00

Table 2: Results of analysis of Proteinase Inhibitory Action (PIA), Heat Induced Haemolysis(HIH) and Anti-lipoxygenase activity(ALA)

S.No.	Conc. (µg/ml)	PIA	HIH	ALA
1	100	32.82±2.97	34.67±2.49	36.71±2.87
2	200	34.54±1.17	37.61±1.01	38.74±1.60
3	400	41.00±0.87	49.21±2.93	49.72±2.35
4	600	53.45±1.19	61.35±1.68	63.69±1.26
5	800	71.34±1.77	72.13±0.56	73.88±1.06

Table 3: Results of analysis of anti-tuberculosis activity

Concentration	$\%CTC_{50}$			Mean ± SD
	Trial 1	Trial 2	Trial 3	
250	43.79	33.57	42.33	39.89 ± 5.52
500	68.61	63.50	67.88	66.66 ± 2.76
1000	83.21	76.64	86.13	81.99 ± 4.86

Pic.1 C. gigantea Plant Pic.2 C. gigantea Stem Pic.3 C. gigantea Stem Powder

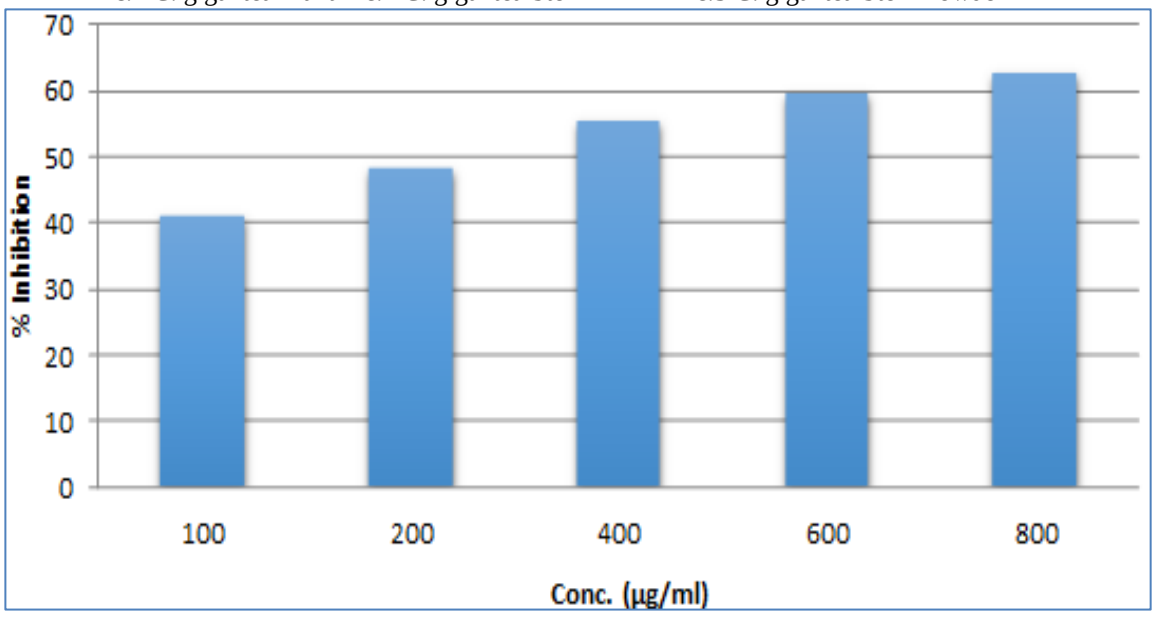

Fig. 1: Inhibition of albumin denaturation

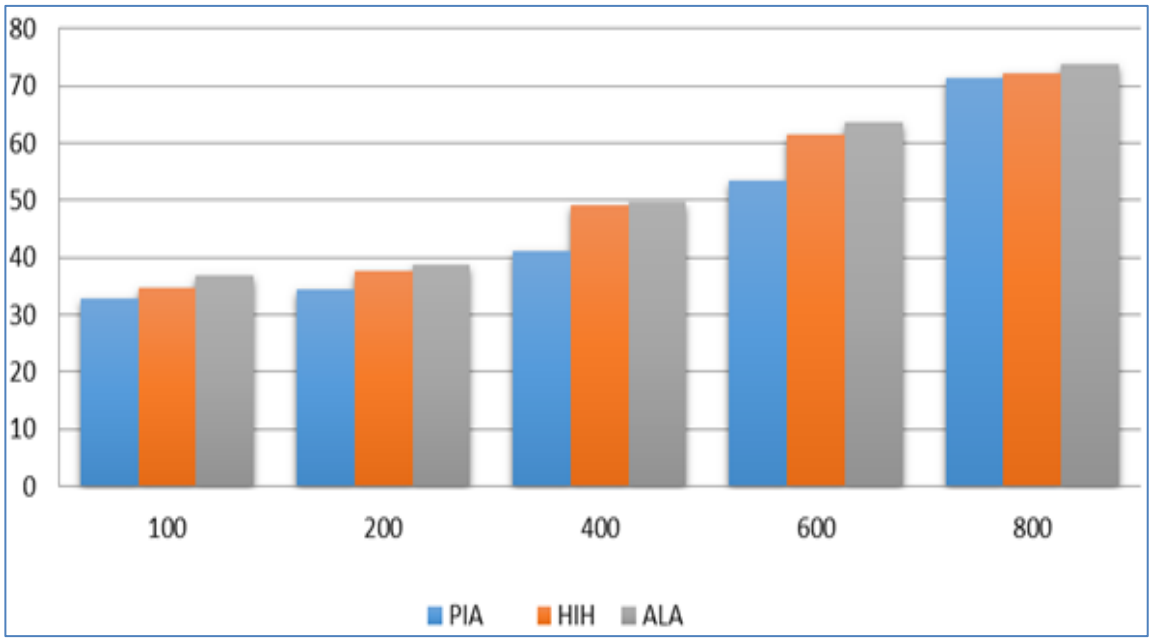

Fig. 2: Results of analysis of Proteinase Inhibitory Action (PIA), Heat Induced Haemolysis (HIH) and Anti-lipoxygenase activity (ALA)

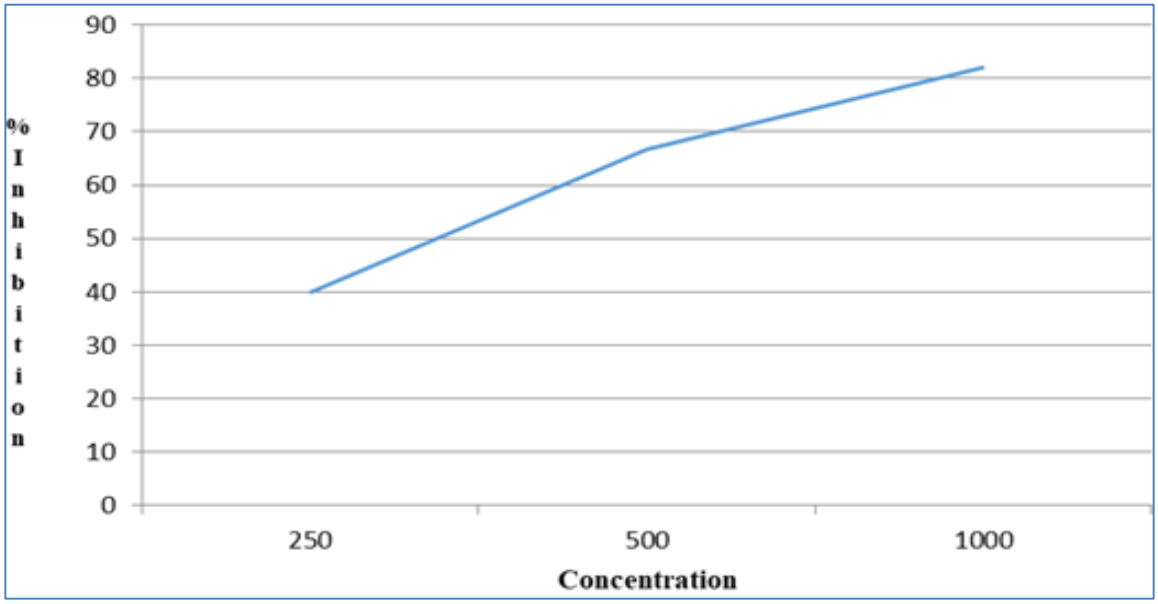

Fig. 3: Results of analysis of anti-tuberculosis activity

Conclusion

The results obtained from our studies on Calotropis gigantea Stem Methanol Extract (CGSME) had shown significant potential for anti-inflammatory and anti-tuberculosis activities.

Purification of each bioactive compound is necessary and this purified form of the compound can be used, which may show increased activity. This study gives an idea that the compound of CGSME can be used as lead compound for designing a potent anti-inflammatory drug, which can be used for treatment of diseases such as inflammation and tumor.[1, 30, 31].

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