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Polyphenolic content correlates with anti-inflammatory activity of root bark extract from *Clerodendrum infortunatum* L. and inhibit carrageenan induced paw edema

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Abstract
Clerodendrum infortunatum is a traditional Indian medicinal plant reported to be used for the treatment of inflammation, skin diseases, tumor, bronchitis etc. The anti-inflammatory activity of various extracts of the root bark of *Clerodendrum infortunatum* were screened by proteinase inhibitory assay and nitric oxide scavenging assay. The total phenolic content was evaluated by Folin-Ciocalteu method. The correlation between anti-inflammatory activity and phenolic content was also carried out. The active extract was tested against carrageenan induced paw edema. Aqueous acetone extract was found to possess maximum anti-inflammatory activity in the *in vitro* screening study and the phenolic content correlates with the antiinflammatory activities. In the carrageenan induced acute inflammatory study, administration of the aqueous acetone extract significantly reduced the paw volume in carrageenan treated rats. The haematological, biochemical and histopathological findings also points to the antiinflammatory activity of the extract. Thus the phenolic compounds present in the root bark of the plant may be responsible for the activities exhibited by the plant and hence it can be used against inflammatory diseases.

Keywords: *Clerodendrum infortunatum*, inflammation, nitric oxide scavenging assay, polyphenolics, proteinase inhibitory assay

1. Introduction
Inflammation is a normal protective response mediated by activated immune cells to tissue injury. It is a multistep process which includes enzyme activation, release of inflammatory mediators like cytokines and tumor necrosis factors, fluid extravasations, cell migration, tissue breakdown and repair [1]. This complex process is initiated by factors such as bacterial infection, chemical injury and environmental pollution which results in cell injury or death [2]. Inflammation is associated with pain and occurrences such as increased vascular permeability, protein denaturation and membrane alteration, which are mediated by inflammatory mediators [3, 4]. Although it is recognized as a process of remission of diseases, the persistence of inflammation may lead to diseases like arthritis, atherosclerosis and cancer [5]. Inflammatory processes involve the excessive activation of phagocytes and production of free radicals and non-free radicals [6]. These reactive species can harm tissues either by direct oxidizing action or indirectly with H_2O_2 and OH^- radical formed from O_2^- and initiates lipid peroxidation resulting in membrane destruction and tissue damage. Drugs commonly used for the management of inflammation are non-steroidal anti-inflammatory drugs (NSAIDS), which acts by inhibiting the prostaglandin synthesis by blocking cyclooxygenase activity [7]. These are associated with side effects like gastric irritation leading to the formation of gastric ulcers, toxicity to liver and kidney and adverse cardiovascular effects [8]. Inorder to counteract the side effects, cost and multidrug resistance associated with such drugs, there is an urgent need for the development of new anti-inflammatory drugs from medicinal plants.
Medicinal plants, valuable sources of bioactive secondary metabolites are a promising choice for the anti-inflammatory drugs. Polyphenolics are the most widely distributed group of chemicals in the plant kingdom. They are hydroxylated phenolic compounds that possess one or more aromatic rings derived from L-phenylalanine with one or more hydroxyl groups. They are synthesized from the precursors derived from the phenylpropanoid pathway [9]. Polyphenols are widely classified into phenolic acids, flavonoids, tannins, stilbenes and lignans [10]. Dietary polyphenols have been reported to exhibit antioxidant, anti-inflammatory, antimicrobial and antiproliferative activity, regulation of cell cycle arrest etc [11]. Phenolic compounds may interact with molecular targets in the cell signalling machinery and modulate cellular action [12].

They are reported to possess lipoxygenase inhibitory and antioxidant properties that have been exploited for the treatment of inflammatory diseases [13].

Clerodendrum infortunatum is a traditional medicinal plant which has been used by the people of various tribes. Different parts of the plant has been used in medicinal systems like Ayurveda, Unani, and Homeopathy for the treatment of bronchitis, asthma, epilepsy, skin diseases, inflammation, small pox, tumor and as vermifuge, anthelminthic etc [14]. The plant is used in the tribal preparation known as Shuktani, which is a common medication for many ailments [15]. Phytochemical analysis of the root revealed the presence of alkaloids, flavonoids, saponins, tannins and phenolics [16]. Antioxidant activity of the root has also been reported [17]. Our previous studies have reported the presence of phenolics and flavonoids in different extracts of the root bark of *Clerodendrum infortunatum* [18]. The preliminary *in vitro* antioxidant screening of various extracts of the root bark by DPPH radical scavenging assay, FRAP assay and total antioxidant assay revealed that aqueous acetone extract possess the greatest activity. The aim of the present study was to screen different extracts of the root bark of *Clerodendrum infortunatum* for anti-inflammatory activity by proteinase inhibitory assay and nitric oxide scavenging assay and to quantify the total phenolic content of the extracts. The possible correlation between phenolic content and anti-inflammatory activity was also evaluated. *In vivo* anti-inflammatory activity of the active extract was then tested in carrageenan induced paw edema model.

2. Materials and Methods

2.1 Plant material

The root bark of *Clerodendrum infortunatum* was collected from the Idukki District of Kerala, India, during the month of April, 2015. The plant was authenticated and the voucher specimen (SBS BRL 21) was kept at School of Biosciences, M.G. University, Kottayam, Kerala, India. The plant material was washed properly, shade dried, powdered and was stored in air tight containers until further analysis.

2.2 Preparation of extracts

About 100g powdered root bark was extracted with 300 ml of each solvents, viz; petroleum ether, chloroform, acetone, ethanol, methanol, aqueous acetone (70% acetone), aqueous ethanol (70% ethanol), aqueous methanol (70% methanol) and water for 72 hours in a soxhlet apparatus. The extracts were filtered through Whatman No.1 filter paper, concentrated to dryness in a rotary evaporator and stored at 4ºC in sterile vials for further studies.

2.3 *In vitro* anti-inflammatory assays

2.3.1 Proteinase inhibitory assay

The trypsin inhibitory assay was performed by the method adopted by Jyothilakshmi et al [19]. 100 µL of trypsin (1mg/mL in 1mM HCl) was added to 200 µL of test samples at different concentrations (100-600 µg) and made upto 1mL with 50mM Tris – HCl buffer (pH 7.8) containing 1mM $CaCl_2$. The mixture was incubated at room temperature for 10 minutes and 1 mL of bovine serum albumin (BSA) was added to all tubes and incubated at 37ºC in a water bath for 20 minutes. 3mL of 5% TCA was added to stop the reaction, centrifuged at 2500 rpm for 10 minutes and absorbance of the supernatant was read at 280 nm. Appropriate controls and blanks were taken. Diclofenac sodium (10 mg/mL) was taken as the positive control. The percentage of inhibition was calculated as,

$$\% \text{ Inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

2.3.2 Nitric oxide scavenging assay

The nitric oxide scavenging activity of extracts was determined by the method adopted by Sreejayan and Rao [20]. 1 mL of 10 mM sodium nitroprusside was mixed with 1 mL of the test samples at various concentrations (100-600 µg/mL) and incubated at 25ºC for 150 min. The control was run as above but the sample was replaced with same amount of distilled water. After incubation, the reaction mixture was mixed with 1 mL of Griess reagent (1% Sulphanilamide, 0.1% naphthyl ethylene diamine dihydrochloride and 2% orthophosphoric acid) and the absorbance of the resulting mixture was measured at 540 nm. Ascorbic acid (10-60 µg/mL) was used as the positive control. The nitric oxide scavenging activity was expressed as the inhibition percentage and can be calculated using the formula

$$\% \text{ inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

The IC_{50} value was calculated from the graph by plotting inhibition percentage against extract concentration.

2.4 Determination of total phenolic content

The total phenolic content of the plant extracts were determined by the modified method of Singleton and Rossi.[21] To 100 µL of the standard gallic acid and different extracts of *Clerodendrum infortunatum*, 500 µL of Folin-Ciocalteu reagent and 1mL of distilled water were added. It was kept at room temperature for 5 minutes and then added 1.5 mL of 20% sodium carbonate. The resulting mixture was incubated in the dark for 2 h at room temperature and the absorbance value was read at 760 nm. The total phenolic content was expressed as mg gallic acid equivalents (GAE)/ g plant extract.

2.5 *In vivo* anti-inflammatory assay

2.5.1 Carrageenan induced paw edema

In vivo anti-inflammatory activity of the aqueous acetone extract from the root bark of *Clerodendrum infortunatum* (AACI) was assessed by carrageenan induced paw edema model in male Wistar albino rats [23]. The animals were maintained under standard conditions of light and temperature (12 h light/dark cycle and 25 ± 2ºC) and fed with food and water *ad libitum*. The experiments were carried out according to CPCSEA guidelines after obtaining permission from Institutional Animal Ethics Committee (Reg No. B21032014-06).

Animals were grouped into 6 with 6 animals in each group. Group 1 served as normal control, group 2 as toxic control (0.1 mL 1% carrageenan in normal saline), group 3 as vehicle control (0.1 mL 1% carrageenan + 0.5% tween 20), group 4 and 5 as treatment groups and received 250 and 500 mg/ kg aqueous acetone extract respectively and group 6 as standard control (Diclofenac sodium- 10 mg/kg b.w). All the drugs were administered orally. One hour after drug administration, acute inflammation was produced in all groups except group 1 by subplantar injection of 0.1 mL of freshly prepared 1% carrageenan in normal saline into the right hind paw of the rats and the paw volume was measured by paw edema meter at 1 h interval for 5 hours and then 24 hour after carrageenan

injection. Mean increase in paw volume was measured and the percentage inhibition of edema formation was calculated for 1st, 3rd and 5th hours. Blood and tissue samples were collected and used for the biochemical, haematological and histopathological analyses.

Increase in paw volume = Vt-Vc,

Where Vt is the paw volume at time t after carrageenan administration and Vc is the paw volume before carrageenan administration

% inhibition = [(volume of control group- volume of treated group)/volume of control group] × 100

2.5.2 Analysis of haematological and serum biochemical parameters

After 5th hour half of the animals were sacrificed and blood was collected in tubes containing EDTA and the estimation of various haematological parameters such as erythrocyte sedimentation rate (ESR), erythrocyte, leucocyte and platelet count were performed with an automatic cell counter (Cell Dyn 3700, Abott, USA).

The blood collected in clot activating tubes was kept at room temperature for 30 minutes to separate the serum. The serum samples were used for the estimation of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and total protein using standard diagnostic kits (Span Diagnostics Limited, Surat, India) according to manufactures procedure and the absorbance was read in a UV-Vis spectrophotometer (Hitachi U-5100).

2.5.3 Analysis of antioxidant status

The ether anaesthetized rats were sacrificed 5 h after carrageenan administration and the paw tissue was removed and washed in ice cold saline. Paw tissue was homogenized in phosphate buffer (100mM) containing EDTA (1mM) and centrifuged at 12,000 g for 10 minutes at 4°C. The clear supernatant obtained was used for the estimation of lipid peroxidation (MDA), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase [23].

2.5.4 Nitric oxide assay

Nitric oxide (NO) levels were measured as its breakdown product of nitrite by using the Griess method. [24] NO is rapidly converted into nitrite and nitrate in presence of H_2O. Therefore the total production of NO can be determined by measuring the nitrite level (NO_2^-), the stable metabolite of NO. Paw tissue was rinsed in ice cold normal saline and homogenized in normal saline (four times its volume) at 4°C. The homogenate was centrifuged at 12,000 rpm for 5 min and the supernatant was used for the assay. Equal volume of serum and Griess reagent (1% Sulphanilamide, 0.1% naphthyl ethylene diamine dihydrochloride and 2% orthophosphoric acid) was mixed and absorbance was measured at 550 nm. The amount of nitrite was calculated from a standard curve of sodium nitrite.

2.5.5 Cyclooxygenase assay

The paw tissue was collected at 5th h and rinsed in ice cold normal saline. Cyclooxygenase (COX) activity in the paw tissue was carried out according to the method of Shimizu *et al*, [25]. The paw tissue was incubated with Tris-HCl buffer (pH 8) containing 5 mM glutathione and 5 mM hemoglobin at 25°C for 1 min. The reaction was started by the addition of 200 µM arachidonic acid followed by the incubation at 37°C for 20 min. 0.2 mL 10 % trichloroacetic acid (TCA) (prepared in 1N HCl) was added to terminate the reaction. 0.2 mL 1 % thiobarbiturate was added to the mixture, centrifuged at 120g and the COX activity was determined by measuring the absorbance of the supernatant at 530 nm.

2.5.6 Reactive oxygen species assay

The paw tissue was washed in ice cold normal saline and homogenized in 50 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 500g for 10 minutes at 4°C and the supernatant was used to assess the reactive oxygen species (ROS) content. Quantification of ROS in the carrageenan treated paw tissue was carried out according to Vrablic *et al*, [26]. To the supernatant, 200 µL nitro blue tetrazolium (NBT) (1mg/mL) was added and incubated for 1 h at 37°C. The solutions were then treated with 100 µL KOH (2M). The absorbance was measured at 570 nm and expressed as µmol NBT reduced/ g tissue.

2.5.7 Histopathological evaluation of paw tissue

5 h after carrageenan injection, the paw tissue was removed, washed in normal saline and fixed in 10% neutral buffered formaldehyde solution. For histopathological examinations, paraffin-embedded paw tissue sections were stained with hematoxylin–eosin (H&E) followed by examination and photographed under a light microscope for observation of structural abnormality. The severity of paw tissue inflammation was judged by two independent observers blinded to the experimental protocol [27].

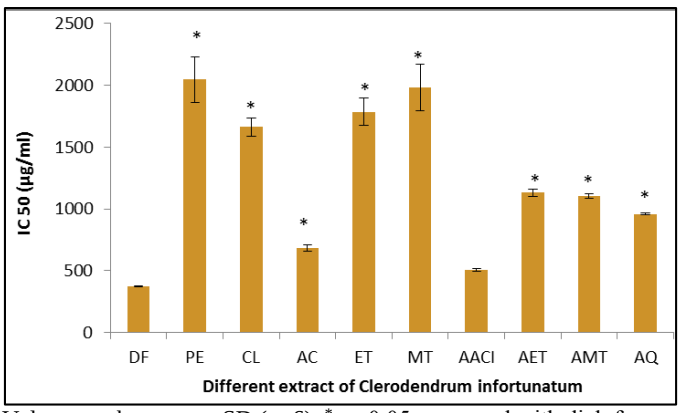
2.6 Correlation analysis and Statistics

The correlation analysis was carried out using IBM SPSS Statistics 20 software. All the results are expressed as the mean ± standard deviation (n=6). The statistical differences between different groups were analysed using One way Analysis of Variance (ANOVA) followed by Tukey's post hoc test and the difference between the means were regarded significant at $p<0.05$.

3. Results

3.1 Proteinase inhibitory assay

Proteinases are associated with the initiation of tissue damage during inflammatory processes. The ability of extracts to inhibit proteinase action was determined to evaluate its anti-inflammatory potential. All the extracts showed proteinase inhibition in a dose dependent manner. The IC 50 values of various extracts were presented in Fig. 1. The aqueous acetone extract was found to exhibit the greatest percentage of scavenging at all concentrations. The IC 50 value was found to be 505.6±13.33µg/mL, which was comparable to that of the standard drug diclofenac sodium (370.7±3.404µg/mL).



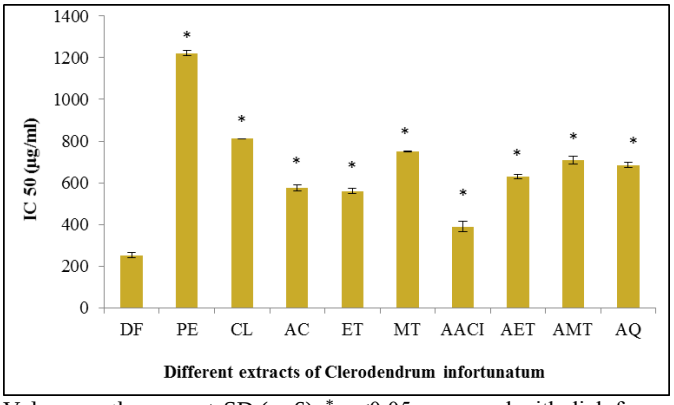
Values are the mean ± SD (n=6). * p <0.05 compared with diclofenac sodium

Fig 1: Proteinase inhibitory activity of various extracts of the root bark of *Clerodendrum infortunatum*

DF- Diclofenac sodium, PE-petroleum ether, CL- chloroform, AC- acetone, ET- ethanol, MT-methanol, AACI- aqueous acetone, AET- aqueous ethanol, AMT- aqueous methanol and AQ- aqueous extracts

3.2 Nitric oxide scavenging activity

The amount of nitrite ions formed was assessed to evaluate the nitric oxide scavenging activity of the extracts. The decrease in absorbance at 546 nm indicated an increase in scavenging activity. All the extracts showed dose dependant scavenging activity at the tested concentrations. The IC 50 values of the extract were shown in Fig.2. Aqueous acetone extract exhibited lowest IC 50 value (389.3±24.92 µg/mL) compared to other extracts. Diclofenac sodium also exhibited significant scavenging activity (IC 50 is 252.8.±11.89 µg/mL).



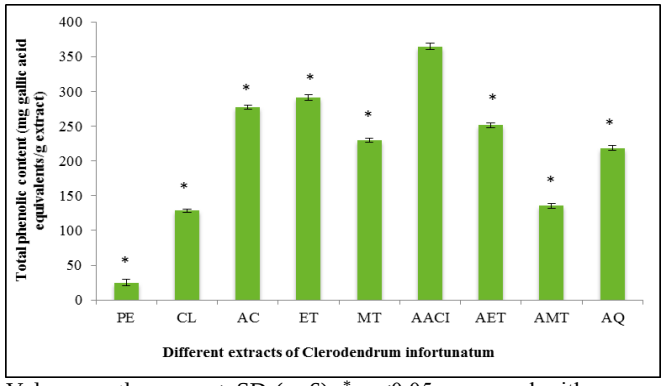
Values are the mean ± SD (n=6). * p <0.05 compared with diclofenac sodium

Fig 2: Nitric oxide scavenging activity of various extracts of *Clerodendrum infortunatum*

DF- Diclofenac sodium, PE-petroleum ether, CL- chloroform, AC- acetone, ET- ethanol, MT-methanol, AACI- aqueous acetone, AET- aqueous ethanol, AMT- aqueous methanol and AQ- aqueous extracts

3.3 Total phenolic content

The total phenolic contents of various extracts were determined by Folin-Ciocalteu method and are expressed as mg gallic acid equivalence/ g plant extract (Fig.3). The total phenolic content was higher for the aqueous acetone extract followed by ethanol, acetone, aqueous ethanol extracts. The phenolic content in the petroleum ether extract was almost insignificant compared to other extracts.



Values are the mean ± SD (n=6). * p <0.05 compared with aqueous acetone extract

Fig 3: Total phenolic content of various extracts of the root bark of *Clerodendrum infortunatum*

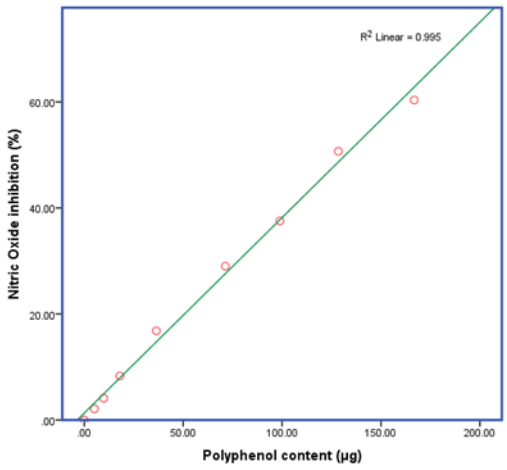
PE-petroleum ether, CL- chloroform, AC- acetone, ET-ethanol, MT-methanol, AACI- aqueous acetone, AET-aqueous ethanol, AMT- aqueous methanol and AQ- aqueous extracts

3.4 Correlation analysis

The Pearson correlation coefficient between the anti-inflammatory activities of the aqueous acetone extract and polyphenol content was shown in table 1. The results obtained showed a strong positive correlation (Fig.4) between the polyphenolic content and the anti-inflammatory assays; trypsin inhibition assay (r^2= 0.971, p<0.01) and nitric oxide scavenging assay (r^2= 0.997, p<0.01).

Table 1: Pearson correlation coefficients between anti-inflammatory assays and polyphenolic content

		Polyphenol	Trypsin	NO_2
Polyphenol	Pearson Correlation	1	.971**	.997**
	Sig. (2-tailed)		.000	.000
	N	9	9	9
Trypsin	Pearson Correlation	.971**	1	.983**
	Sig. (2-tailed)	.000		.000
	N	9	9	9
NO_2	Pearson Correlation	.997**	.983**	1
	Sig. (2-tailed)	.000	.000	
	N	9	9	9
**. Correlation is significant at the 0.01 level (2-tailed).				



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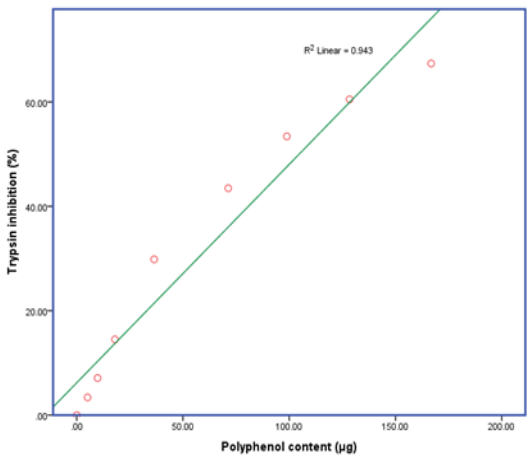


B

Fig 4: Linear regression between (A) polyphenolic content and nitric oxide scavenging assay (B) and trypsin inhibition assay

3.5 *In vivo* anti-inflammatory activity

3.5.1 Paw volume changes

The anti-inflammatory potential of the aqueous acetone extract was tested against carrageenan induced rat paw edema. The subplantar injection of carrageenan on the right hind paw of the rats produced a rapid inflammation with a maximum volume noted at the 5th h (Table 2). The vehicle treated group did not showed any decrease in paw volume when compared to the carrageenan induced rats. The rate of increase in paw volume of the extract treated rats was found to be minimal until 3rd h. The paw volume starts decreasing form the 4th h compared to the control. Diclofenac sodium also significantly reduced the formation of edema.

The percentage inhibition of paw edema induced by carrageenan injection was calculated for normal and experimental rats (Table 3). Aqueous acetone extract at 250 and 500 mg/kg exhibited dose dependant percentage of inhibition at the 1st hour (28.50 and 32.88 % respectively). The inhibition exhibited during vehicle treatment was negligible and it was insignificant when compared to the carrageenan induced rats. The extract exhibited maximum inhibition of edema formation at the 5th hour. The results were compared with the reference drug diclofenac sodium (DF) which showed 84.11 % inhibition at the 5th hour. Aqueous acetone extract of *Clerodendrum infortunatum* at the dose of 250 and 500 mg/kg showed 56.88 and 69.7 % inhibition respectively at the 5th hour.

Table 2: Effect of aqueous acetone extract of *Clerodendrum infortunatum* (AACI) on carrageenan induced paw edema represented as mean difference in paw volume

Time	Normal	Toxic	Vehicle	AACI (250 mg/kg)	AACI (500 mg/kg)	DF
1 h	0.002 ± 0.003*	1.028 ± 0.138	1.054 ± 0.048	0.735 ± 0.076*	0.69 ± 0.053*	0.525 ± 0.050*
2 h	0.004 ± 0.002*	1.493 ± 0.084	1.467 ± 0.029	1.005 ± 0.050*	0.878 ± 0.054*	0.923 ± 0.106*
3 h	0.004 ± 0.002*	2.058 ± 0.107	2.036 ± 0.024	1.273 ± 0.062*	1.093 ± 0.066*	0.858 ± 0.096*
4 h	0.004 ± 0.001*	2.148 ± 0.144	2.132 ± 0.032	1.14 ± 0.042*	0.938 ± 0.044*	0.575 ± 0.096*
5 h	0.003 ± 0.001*	2.203 ± 0.092	2.195 ± 0.029	0.95 ± 0.048*	0.668 ± 0.049*	0.35 ± 0.058*
24 h	0.003 ± 0.001*	1.89 ± 0.063	1.875 ± 0.025	0.595 ± 0.076*	0.305 ± 0.058*	0.1 ± 0.016*

Values are the mean ± SD (n=6). * $p < 0.5$ when compared with toxic control

Table 3: Effect of aqueous acetone extract of *Clerodendrum infortunatum* (AACI) on percentage inhibition of carrageenan induced paw edema

Treatment	% inhibition at 1st h	% inhibition at 3rd h	% inhibition at 5th h
Normal	99.81	99.80	99.86
Vehicle	-2.53	1.044	0.363
AACI (250 mg/kg)	28.50	38.13	56.88
AACI 500 mg/kg)	32.88	46.88	69.70
Diclofenac	48.93	58.30	84.11

3.5.2 Hematological changes

Table 4 represents the hematological changes associated with carrageenan induced inflammation. Level of platelet and erythrocyte count was found to be decreased in the carrageenan induced rats while ESR and leucocyte count was increased. The aqueous acetone extract treated rats showed dose dependent increase in the platelet and erythrocyte levels and decrease in the ESR and leucocyte levels as compared to the carrageenan control. Diclofenac sodium also showed significant change in the haematological levels.

Table 4: Effect of AACI on haematological parameters during carrageenan induced inflammation

Parameters	Normal	Toxic	Vehicle	AACI (250mg/kg)	AACI (500mg/kg)	DF
ESR (mm/h)	4.5 ± 0.082#	8.875 ± 0.096*	8.65 ± 0.208*	7.123 ± 0.093*#	6.75 ± 0.129*#	5.158 ± 0.043*#
Leucocyte (X 10^3/µL)	5.965 ± 0.039#	7.1 ± 0.016*	7.168 ± 0.043*	6.555 ± 0.044*#	6.443 ± 0.030*#	6.095 ± 0.070*#
Platelet (X 10^3 mm^3)	817.5 ± 4.435#	422.3 ± 4.193*	425 ± 3.559*	533 ± 3.559*#	685.8 ± 4.787*#	719.5 ± 2.646*#
Erythrocytes (X 10^6/µL)	5.513 ± 0.059#	4.158 ± 0.043*	4.17 ± 0.032*	4.85± 0.054*#	5.252 ± 0.026*#	5.312 ± 0.049*#

Values are the mean ± SD (n = 6). * denotes $p < 0.05$ compared with normal control group; # denotes $p < 0.05$ compared with toxic control group

3.5.3 Serum biochemical parameters

There was a significant increase in the liver marker enzymes in the carrageenan treated rats compared to the normal control (Table 5). Group treated with aqueous acetone extract at 250 and 500 mg/kg showed significant dose dependent decrease in the marker enzymes AST, ALT and ALP. Total protein levels were found to be decreased in the carrageenan treated rats compared to the normal control, whereas treatment with the aqueous acetone extract was found to regain the protein levels. Rats treated with diclofenac sodium also showed marked improvement in the protein levels and decreased the liver marker enzymes.

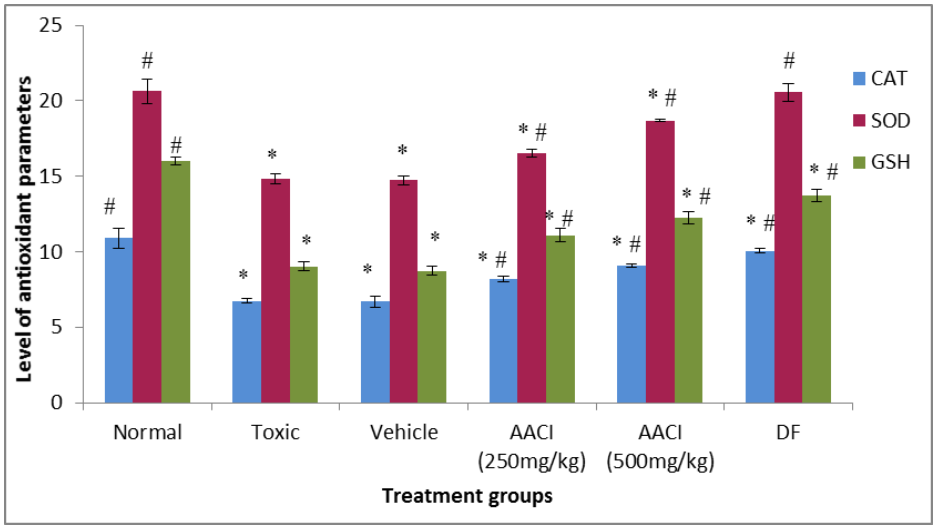
Table 5: Effect of AACI on serum biochemical parameters during carrageenan induced inflammation

Parameters	Normal	Toxic	Vehicle	AACI (250mg/kg)	AACI (500mg/kg)	DF
ALT (U/L)	79 ± 2.944#	102.5 ± 1.291*	101.3 ± 1.5*	91 ± 1.826*#	86.5 ±1.291*#	80 ± 3.367#
AST (U/L)	223.8 ± 4.113#	241.5 ± 2.38*	240.8 ± 1.708*	235 ± 1.826*#	227 ± 1.826#	229.8 ±1.893*#
ALP (U/I)	635.5 ± 2.082#	645.3 ± 2.217*	644.3 ± 2.63*	639.5 ± 1.291#	635.8 ± 1.708#	638.3 ± 2.5#
TP (g/dl)	7.8 ± 0.082#	6.825 ± 0.097*	6.8 ± 0.082*	7.125 ± 0.096*#	7.275 ±0.09#	7.7 ± 1.41#

Values are the mean ± SD (n=6). * denotes $p < 0.5$ when compared with normal control, # denotes $p < 0.5$ when compared with toxic control

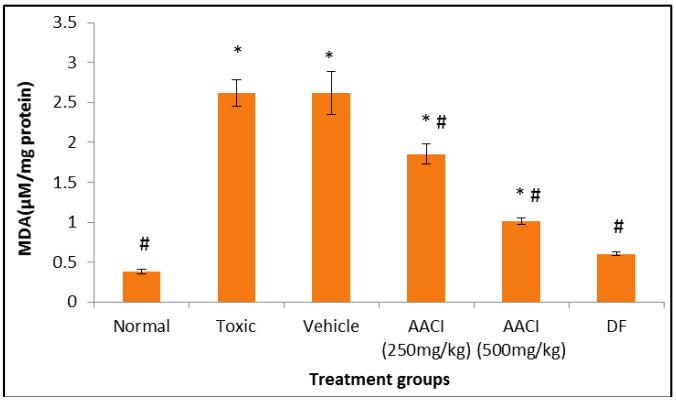
3.5.4 Antioxidant enzyme levels

The levels of antioxidant enzymes catalase, superoxide dismutase (SOD) and reduced glutathione (GSH) following sub plantar injection of carrageenan were presented in fig.5. The levels of catalase, SOD and glutathione were found to be significantly reduced at the 5th hour compared to the normal control. Treatment with the aqueous acetone extract at the dose of 250 and 500 mg/kg significantly increased the levels of catalase, SOD and GSH in a dose dependant manner. Treatment with diclofenac sodium also showed significant improvement in the antioxidant status. The concentration of malondialdehyde was significantly increased in the carrageenan treated group compared to the normal control (Fig.6). Treatment with both the doses (250 and 500 mg/kg) of aqueous acetone extract significantly decreased the MDA levels in the paw tissue when compared to the carrageenan control. Diclofenac sodium also significantly reduced the MDA levels.



Values are the mean ± SD (n=6). * denotes p < 0.5 when compared with normal control, # denotes p < 0.5 when compared with toxic control. Values are expressed as units/mg protein for catalase and SOD and µM GSH/mg protein for GSH

Fig 5: Effect of AACI on antioxidant parameters during carrageenan induced inflammation



Values are the mean ± SD (n=6). * denotes p < 0.5 when compared with normal control, # denotes p < 0.5 when compared with toxic control.

Fig 6: Effect of AACI on MDA levels during carrageenan induced inflammation

3.5.5 NO levels

Subplantar injection of carrageenan resulted in significant increase in the NO levels compared to the normal group (Table 6). Administration of aqueous acetone extract of *Clerodendrum infortunatum* reduced the NO levels significantly (p < 0.05).

3.5.6 COX levels

The COX activity was significantly increased in the carrageenan treated rats (8.72 ± 0.187 U/mg protein) compared to the normal rats. However treatment with the aqueous acetone extract at the dose 250 and 500 mg/kg and standard drug significantly (p< 0.05) reduced the COX activity compared to the toxic control (Table 6).

3.5.7 ROS levels

The level of ROS during carrageenan induced inflammation when treated with the aqueous acetone extract was presented in table 6. Subplantar injection of carrageenan resulted in the increased production of reactive oxygen species as evident from the increased ROS levels. Treatment with the aqueous acetone extract resulted in significant reduction of ROS levels.

Table 6: Effect of AACI on NO, COX and ROS during carrageenan induced inflammation

Parameters	Normal	Toxic	Vehicle	AACI (250mg/kg)	AACI (500mg/kg)	DF
NO (µmol/g tissue)	19.42 ± 0.554$^{\#}$	39.10 ± 0.540*	38.26 ±0.404*	33.40 ±0.275$^{*\#}$	26.11 ±0.583$^{*\#}$	23.11 ± 0.369$^{*\#}$
COX (U/mg protein)	4.188 ± 0.059$^{\#}$	8.827 ± 0.077*	8.648 ± 0.054*	6.635 ± 0.081$^{*\#}$	5.143 ± 0.108$^{*\#}$	4.665 ± 0.066$^{*\#}$
ROS (µmol NBT/g tissue)	306.2 ± 6.641$^{\#}$	786.3 ± 4.841*	778.8 ± 4.6*	635.2 ± 3.581$^{*\#}$	451.3 ± 4.078$^{*\#}$	434 ± 2.729$^{*\#}$

Values are the mean ± SD (n=6). * denotes p < 0.5 when compared with normal control, # denotes p < 0.5 when compared with toxic control.

3.5.8 Histopathological analysis

The paw tissue of the normal rats showed no signs of inflammation (Fig.7a) with normal keratin, sub epidermal layer and sub cutaneous layer. In the rats treated with carrageenan shows massive influx of inflammatory cell infiltration, Proliferated collagen, Hyper keratotic skin, Sub

epidermal edema (Fig.7b). Histological examination of the rats treated with vehicle also showed inflammatory cell infiltration and edema (Fig.7c). Treatment with the aqueous acetone extract showed marked improvement in the inflammatory signs. Keratinization was decreased and dermis and hypodermis appeared to be normal. Mild sub epidermal edema, mild epithelial hyper plasia and mild inflammation were also observed in aqueous acetone extract treated group (Fig.7d-e).

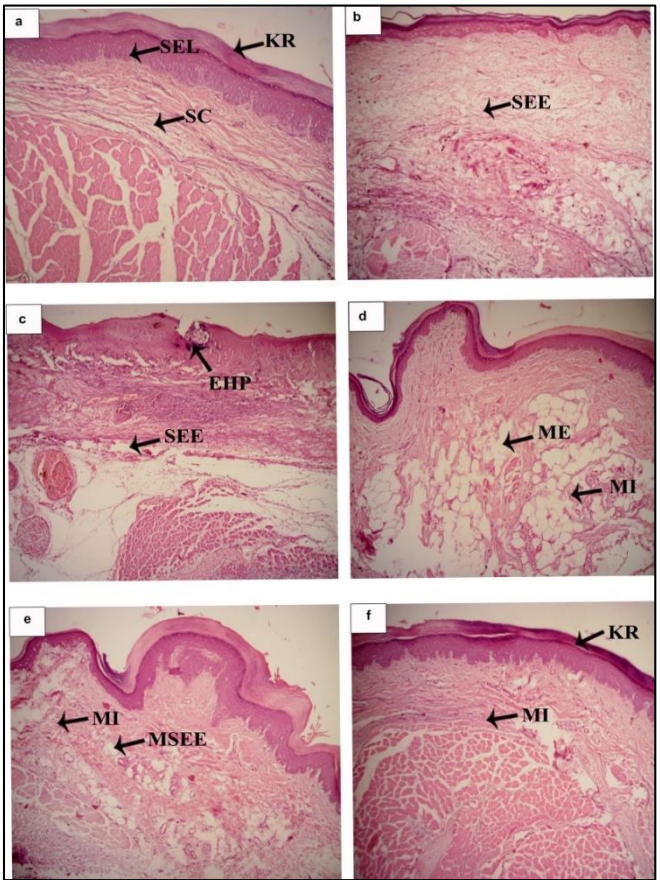


Fig 7: Histopathology of paw tissue of rat treated with aqueous acetone extract and diclofenac sodium following carrageenan injection

Keratin- KR, Sub epidermal layer - SEL, Sub cutaneous layer - SC, inflammatory cell infiltration - ICI, Proliferated collagen - CP, Hyper keratotic skin - HKS, Sub epidermal edema - SEE, mild epithelial hyper plasia - EHP, Mild edema - ME and Mild inflammation -MI, Mild sub epidermal edema - MSEE

4. Discussion

Various medicinal plants are reported to contain bioactive compounds that possess anti-inflammatory activities. Phytomedicines generally exert their action by four mechanisms: immunoprotective property, inhibition of inflammatory mediators and ROS generation, inhibition of enzymes like trypsin and prevention of microbial entry by membrane stabilization property [2]. Compounds with antioxidant property interact with the generation of reactive oxygen and nitrogen species by activated macrophages and may exerts its anti-inflammatory activity [28].

Proteolytic enzymes like bromelain, papain, trypsin and chymotrypsin are essential regulators and modulators of the inflammatory processes [29]. The aqueous acetone extract were found to exhibit the greatest percentage inhibition of the proteolytic enzyme trypsin compared to other extracts and it was found to be dose dependent. Proteases, particularly serine proteases are reported to be key factors in mediating the initiation and progression of inflammatory processes [30]. Neutrophils in the lysosomal granules are rich source of serine proteases. Previous reports demonstrated the role of leukocyte proteinases in tissue damage during inflammatory reactions [31]. Thus proteinase inhibitors can provide protection against inflammatory responses. Protease activated receptors (PAR) are seven transmembrane G protein coupled receptors stimulated by serine proteases [32]. By the activation of PAR 2 family, trypsin was shown to induce *in vivo* epidermal proliferation, vasodilation and inflammatory infiltration in the upper epidermis. The expression of PAR2 on inflammatory cells and endothelial cells including neutrophils and macrophages determines their involvement in pro-inflammatory and anti-inflammatory responses [33]. Thus inhibition of trypsin action points to the anti-inflammatory activity of the plant.

Nitric oxide has implication in cancer, inflammation and other pathological conditions [34]. It is a potent inhibitor of physiological processes such as smooth muscle relaxation, neuronal signalling, inhibition of platelet aggregation and regulation of cell mediated cytotoxicity [35]. Oxygen reacts with nitric oxide to form nitrate and peroxynitrate anions which act as free radicals [36]. Thus the scavengers of nitric oxide compete with oxygen and leads to the reduced production of nitrite ion [37]. Aqueous acetone extract was found to possess the greatest ability to counteract the effects of nitric oxide and thus it can protect the ill effects of nitric oxide in biological system and it was dose dependent. Nitric oxide is a proinflammatory mediator of inflammation. Studies have shown that nitric oxide inhibitors have beneficial effects on inflammation and tissue damage [38]. The results obtained proved aqueous acetone extract to be potent scavengers of nitric oxide and hence it may possess anti-inflammatory activity. The toxicity study conducted previously revealed that the extract was safe upto the dose of 2000 mg/kg body weight [39] and hence it can be used as an alternative for conventional anti-inflammatory drugs.

Polyphenolics are strong antioxidants as they contains phenyl ring that are oxidized to quinones by reactive oxygen species and this accounts for their free radical scavenging property [13]. In the present study the aqueous acetone extract showed the highest phenolic content and it also revealed positive correlation with the anti-inflammatory activities. Many authors have revealed the correlation between polyphenolics and anti-inflammatory activities [40, 41]. Phenolic compounds can up/down regulate transcriptional factors like NFκB, Nrf-2 in inflammatory and antioxidant pathways [42]. The present study revealed a positive correlation between total phenolic content and the trypsin inhibitory assay and nitric oxide scavenging assay. The positive r value points to the role of phenolics in the prevention of inflammation.

Carrageenan induced paw edema is a commonly employed animal model for acute inflammation studies. It is the widely used to test the efficacy of new anti-inflammatory drugs and its mechanism of action [43]. Acute phase inflammation is characterized by increased vascular permeability and plasma extravasation which caused accumulation of fluid, leukocytes and mediators at the site of inflammation [44]. Inflammation induced by carrageenan is biphasic. The first phase is mediated by the release of histamine and serotonin and the second stage is by the release of bradykinins, polymorphonuclear cells and prostaglandins by the induction

of inducible cyclooxygenase (COX-2) in the paw [45]. Prostaglandins and nitric oxide are two important mediators of inflammation and thus inhibition of their production may be beneficial in treating inflammatory diseases. Prostaglandins may contribute to the cardinal signs of acute inflammation such as redness, heat, swelling and pain [46]. Compounds with anti-inflammatory activity may suppress the release of these mediators and thus reduce the inflammatory processes. The search for effective anti-inflammatory drugs gains more significance recognising the role of inflammation in the onset of cancer, arthritis, diabetes, multiple sclerosis, cardiovascular diseases etc.

During carrageenan induced inflammation, oral treatment of the aqueous acetone extract of *Clerodendrum infortunatum* significantly inhibit edema formation at both the concentrations. Diclofenac sodium also showed significant inhibition of edema. It was a proven inhibitor of prostaglandin formation and thus reduces pain and inflammation [47]. At the 5th hour, aqueous acetone extract exhibit 56.88% and 69.70% inhibition at 250 and 500 mg/kg respectively. These results suggest that the extract may act on the mediators and suppress inflammation. The haematological indices also points to the protective efficacy of the aqueous acetone extract during carrageenan induced inflammation. The level of erythrocytes, leucocytes, platelets and ESR reaches to the normal on treatment with the extract compared to the toxic control. The extract may exert its effects through suppression of leucocytes towards inflamed areas, stabilisation of reticulo endothelial system etc [48].

During inflammation, lysosomal enzymes may leak into the extracellular components and cause damage to the surrounding organelles and cause a variety of disorders [49]. The anti-inflammatory drugs may inhibit either the release of lysosomal enzymes or stabilize the membrane which is one of the major events during the inflammatory processes [50]. Serum transaminases and phosphatase levels returned to the normal range on treatment with the aqueous acetone extract which may be due to its membrane stabilising ability.

Carrageenan induced inflammation is associated with the generation of free radicals such as nitric oxide, superoxide and peroxy nitrite and they further contributes to the edema formation [51]. Excessive production of ROS leads to oxidative damage of macromolecules such as proteins, nucleic acids and carbohydrates and causes peroxidation of membrane lipids. Our body is equipped with an effective system of enzymatic (SOD, catalase, peroxidase) and non-enzymatic (GSH, ascorbic acid, ∞-tocopherol) antioxidant mechanism to prevent or neutralize the damages caused by ROS. When the antioxidant system fails to scavenge the free radicals and ROS produced, oxidative stress develops. The extend of lipid peroxidation is assessed by measuring the MDA levels, which is the final product of lipid peroxidation [52]. The present study revealed that carrageenan induced inflammation resulted in the increased levels of ROS along with higher MDA levels in the paw tissues and this indicates increased lipid peroxidation during inflammation which further contributes to edema formation. The levels of antioxidants (SOD, catalase, GSH) were also decreased in the carrageenan treated rats. The results showed that treatment with the aqueous acetone extract reduced the ROS levels and improves the antioxidant defence systems. A significant decrease in the level of MDA was observed in the aqueous acetone treated rats that points to the efficiency of the extract in preventing lipid peroxidation.

Nitric oxide reacts with superoxide anion to form peroxynitrite and induces peroxidation of lipids and cellular damage [53]. During carrageenan induced inflammation the NO levels were found to be increased suggesting of inflammation induced oxidative stress. NO causes changes in the local blood flow and increases vascular permeability and edema formation [51]. It increases the production of pro-inflammatory prostaglandins by modification of the catalytic domain of cyclooxygenase [54]. Aqueous acetone administration significantly decreased the levels of NO and thus protects from cellular damage and edema formation.

Cyclooxygenase (COX) is the first enzyme involved in arachidonic acid metabolism responsible for the synthesis of prostaglandins. Prostaglandin production is increased in inflamed tisuues and they contribute to the four cardinal signs of acute inflammation [46]. COX exists in two isoforms; COX-1 and COX-2. COX-1 is constitutive whereas COX-2 is inducible and is expressed during tissue damage or inflammation mediated by proinflammatory cytokines.[55] Effective COX-2 inhibitors were considered superior to conventional anti-inflammatory drugs considering the toxicity associated with them [56]. COX activity which was increased during carrageenan treatment was found to be significantly reduced on treatment with the aqueous acetone extract and it suggests that the anti-inflammatory activity exhibited by the extract may be due to the suppression of cyclooxygenase enzyme.

The histopathological findings also substantiate with the haematological and biochemical analyses. Histopathological studies indicated that inflammatory cell infiltration and hyperkeratinisation were markedly suppressed in the rats treated with the aqueous acetone extract. This points to the potent anti-inflammatory activity of the aqueous acetone extract from the root bark of *Clerodendrum infortunatum*.

5. Conclusion

The *in vitro* screening study of various extracts from the root bark revealed that the aqueous acetone extract of *Clerodendrum infortunatum* exhibited the greatest anti-inflammatory activity. All the extracts showed the presence of phenolics with the greatest concentration in the aqueous acetone extract. A positive correlation was observed in between the total phenolic content and anti-inflammatory activities exhibited by the aqueous acetone extract. So the activity manifested by the extract may be due to the polyhenolic compounds present. *In vivo* anti-inflammatory activity was tested in carrageenan induced inflammatory model. Administration of the aqueous acetone extract showed significant percentage inhibition of edema formation, reduces oxidative stress and lipid peroxidation and the levels of COX and NO. These findings suggest that the aqueous acetone extract from the root bark of *Clerodendrum infortunatum* may be developed into a potent pharmacological agent for the management of inflammatory diseases. Further studies are in progress to identify the active principle and also to study its mechanism of action.

6. Conflicts of interest

The authors declare that they have no conflict of interest.

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