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Research Article

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In Vitro Anti-Inflammatory Activity of the Root of *Thottea siliquosa* (Lam.) Rottb., a Medicinal Undershrub in Western Ghats, India

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Abstract: Inflammation can be considered as an essential immune response that helps the body to survive during the conditions of infection and injury. Drugs for curing inflammation had their origin in the accidental discovery of certain plants and their extracts being used for relief of pain, fever and anti-inflammation. Since the plants are considered as the first source of remedies for ailments in human history, a large number of plants were used as drugs against all kinds of ailments through all ages. *Thottea siliquosa*, commonly known as 'Alpam' belongs to the family Aristolochiaceae is a medicinal undershrub, the crude root extracts of which are widely used in traditional tribal healthcare systems in the slopes of the Western Ghats, India, for treating inflammatory responses. The present study is an attempt to scientifically validate this ethnic practice. Root extracts of *T. siliquosa* were prepared using solvents like chloroform, ethyl acetate, 70% ethanol, methanol, and distilled water by soxhlet mode of extraction. Anti-inflammatory responses in different solvent extracts were analyzed by albumin denaturation assay, anti-protease assay, Human red blood cell membrane stabilization assay (HRBC method), Cyclooxygenase (COX), and Lipoxygenase (LOX) assays. RAW 264.7 cell lines were used for the COX and LOX study. The results illustrate the anti-inflammatory potential of the root extracts. The studies on cell lines clearly indicated that the higher anti-inflammatory potential of chloroform extract over other solvent extracts. In tune with the ethno-botanic information, the aqueous extract offered a dose-dependent anti-inflammation property. Since most of the bio activity of plant extracts are contributed by the phytochemicals present in it, further efforts shall be made for the isolation and purification of bioactive phyto components responsible for the anti-inflammation property of the *T. siliquosa* root.

Keywords: Anti-inflammation, Membrane stabilization, Cyclooxygenase, Lipoxygenase, *T. siliquosa*

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1. INTRODUCTION

Inflammation is defined as a localized physical condition in which part of the body becomes reddened, swollen, hot and often painful, especially as a reaction to injury or infection.[1]It also enables the body to maintain tissue homeostasis when a noxious stimulus that threatens the host and its responses may vary from localized to a generalized one. The cardinal symptoms of inflammation include increased rate of blood circulation, enhanced cellular metabolism, widening of blood vessels and release of soluble mediators, diapedesis, and cellular influx. This may lead to a pathologic condition ranging from rheumatism, diabetes, and cardiovascular accident, etc.[2] Inflammation is, therefore, a major health issue for the world population and has invoked serious responses among the scientific community for developing drugs to combat the crisis. At present, there are different groups of medicines discovered and in practice for medically treating the inflammation cases such as steroidal and non-steroidal anti-inflammatory drugs, immunosuppressant drugs, glucocorticoids. But most anti-inflammatory drugs are not effective and with intolerable side effects. This in turn demands natural anti-inflammatory drugs producing increased pharmacological response with minimal side effects.[3] Plants are the major reservoirs of most of the drugs, and those used to combat anti-inflammatory responses are not an exception.[4]The leads of most of these drugs were from the traditional curative system prevalent throughout the world. The present study was designed and undertaken based on such a lead on *T. siliquosa*, an undershrub in the slopes of Western Ghats, India. The study focused on the evaluation of anti-inflammatory potentials of the *T. siliquosa*, traditionally well-known for its medicinal uses among the *Kani* and *Malampandaram* tribes, residing in deep forests of Western Ghats. There are many reports regarding the medicinal properties of *T. siliquosa*,[5, 6] including ethnobotanical information with regard to the anti-inflammatory property of *T. siliquosa*. [7]The present study is focused on this lead.

2. MATERIALS AND METHODS

2.1. Plant Materials

Plant materials of *T. siliquosa* were collected from Palode (geographical coordinates: 8.7033^0N77.0264^0E), Thiruvanthapuram, Kerala, India, and the voucher specimens were deposited at JNTBGRI herbarium, Palode (voucher no. TBGT95930).

2.2. Preparation of Plant Extracts

The roots were shade dried, powdered and soxhlet extraction was performed using solvents such as distilled water, ethyl acetate, methanol, ethanol (70%), and chloroform. The extracts were dried and concentrated using a rotary evaporator and calculated the yield using the formula.[8]

$$\%\ Yield\ of\ extract = \frac{Weight\ of\ dried\ extract}{Weight\ of\ plant\ material} \times 100$$

2.3. *In Vitro* Anti-inflammatory Assays

2.3.1. Inhibition of Albumin Denaturation Assay

The reaction mixture was prepared using an aqueous solution of BSA (1%) and plant extract. The pH of the mixture was adjusted to 6.3 using 1 N HCl and incubated at 37 ^{0}C for 20 minutes. The whole mixture was heated at 57^0C for 20 minutes. The turbidity of the cooled solution was measured at 660 nm against aspirin standard. [9, 10] Percentage of inhibition was calculated by the following formula.

$$\%\ Denaturation\ inhibition = \frac{Absorbance\ of\ the\ control - Absorbance\ of\ the\ test}{Absorbance\ of\ the\ control} \times 100$$

2.3.2. Anti-proteinase Action

2 ml of the reaction mixture was prepared using 0.06 mg trypsin, 1ml 20 mM Tris-HCl buffer (pH 7.4), and 1 ml of the test sample at different concentrations and incubated at 37^0C for 5 minutes. One ml each of 0.8% (w/v) casein was added into this solution and incubated for an additional period of 20 minutes. The reaction was arrested by adding 2ml of 70% perchloric acid. The cloudy suspension was centrifuged at 3000 rpm for 10 minutes and the OD was read at 210 nm against blank. [10, 11] Aspirin was used as the standard. Percentage of inhibition was calculated by the following formula.

$$\%\ Proteinase\ inhibition = \frac{Absorbance\ of\ the\ control - Absorbance\ of\ the\ test}{Absorbance\ of\ the\ control} \times 100$$

2.3.3. Preparation of RBC Suspension

Blood collected from a healthy person who has not taken any NSAIDS for 2 weeks was centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and washed thrice with normal saline. Final blood volume was measured and reconstituted as 10% (v/v) suspension. [12]

2.3.4. Heat-Induced Haemolysis

The reaction mixture was prepared by adding 1 ml of the plant extract and 1 ml of 10 % RBC suspension. The mixture was heated in a water bath at 56 ^{0}C for 30 minutes and then cooled. Then the reaction mixture was centrifuged at 2500 rpm for 5 minutes. The absorbance of the suspension was read at 560 nm. Aspirin was used as a standard for comparison. [10] Percentage of haemolysis was calculated by the following formula.

$$\% \ Protection = \frac{Absorbance\ of\ the\ control - Absorbance\ of\ the\ test}{Absorbance\ of\ the\ control} \times 100$$

2.3.5. Hypotonicity Induced Haemolysis

Inhibition of hypotonicity induced haemolysis by plant extract was carried out as per the methods described by Azeem *et al.* [13]Diclofenac Sodium was used as the standard.

2.4. Anti-inflammatory Studies on Cell lines

2.4.1. Culturing and Maintenance of Cell line

RAW 264.7 cell was initially procured from National Centre for Cell Sciences (NCCS), Pune, India, and maintained in Dulbecco's modified Eagle's medium. [14]

2.4.2. Cyclooxygenase (COX) Inhibition Assay

Cyclooxygenase assay was carried out as described by Walker& Gierse. [15]

2.4.3. 5-Lipoxygenase (LOX) Inhibition Assay

The reaction mixture (2 ml final volume) contained a Tris-HCl buffer (pH 7.4), 50 µL of cell lysate, sodium linoleate (200 µl) and plant extract in different aliquots. The LOX activity was monitored as an increase of absorbance at 234 nm (Shimadzu Cary 60 UV-Vis Spectroscopy, Agilent technology, UAS). [16]

3. STATISTICAL ANALYSIS

The data were analyzed by one way ANOVA using SPSS software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp).

4. RESULTS AND DISCUSSION

4.1. Extractive Value

The yield of plant extract was in the order of Aqueous>70% Ethanol>Methanol>Ethyl acetate> Chloroform and it is found to be 12.375±0.377679, 9.2925±0.280576, 3.350±.2081270, 1.685±.111692, 0.8875±0.065876% respectively (Fig. 1). The extractive yield of water was higher when compared with the other solvents. This indicates that major phytochemicals in *T. siliquosa* root mostly higher in polarity and soluble in water. Pin *et al.*,[17] reported a similar extraction pattern when tried with betel leaves.

4.2. In vitro Anti-inflammatory Assays

4.2.1. Inhibition of Albumin Denaturation Assay

Protein denaturation is considered an important factor behind inflammatory conditions like rheumatoid arthritis. [18] Most of the anti-inflammatory drugs are working based on their dose-dependent inhibition of protein denaturation. In the present study, the ethyl acetate fraction of the extracts showed the least IC 50 value (Fig. 6), and the maximum percentage of inhibition was observed at 500 µg/ml of ethyl acetate extract (Fig. 2). Inflammatory drugs like salicylic acid, Flufenamic acid, Phenylbutazone and Ibufenac were reported to have dose-dependent inhibition on heat mediated coagulation of serum proteins.[9]Several other anti-inflammatory drugs also known for their inhibition on thermally induced protein denaturation.[19]Similar results were also reported from many plant extracts. [10]

4.2.2. Anti-proteinase Assay

Proteinases are abundantly seen in the lysosomal granules of neutrophils which implicate tissue damage during inflammation. It is already reported that leukocyte proteinases play an important role in causing tissue damages during inflammation conditions.[12] Therefore inhibition of proteinase offers a significant level of protection from inflammation. [20] Trypsin is a proteinase, which hydrolyzes the casein into fragments and each plant extract in the present study; dose-dependently inhibited the action of proteinases. 70% Ethanol fraction possessed the least IC 50 value (Fig. 6) and maximum anti-proteinase action was observed at 500 µg/ml concentration of methanol extract (Fig.3). A Similar kind of result was also reported from the endophytic extracts from Loranthus species.[21]

4.2.3. Heat Induced Haemolysis

Membrane stabilization is a process in which the integrity of the membranes is stabilized by using anti-inflammatory drugs. [22]During inflammation, lysosomal membrane lysis happens which results in the release of enzymes that causes numerous disorders such as cardiovascular diseases and cancer. Since the human RBC membrane is similar to the lysosomal membrane, its membrane stabilization action can account for the anti-inflammatory action. [23] The lysis of the erythrocyte membrane will occur when the RBC cells are exposed to heat. If the plant extract possesses the anti-inflammation property, it will protect the cell from membrane damage. [24] In the present study, each plant extract dose-dependently stabilized the membrane, and in this way stimulated the anti-inflammatory responses. In this assay, aqueous extract possessed the lowest IC_{50} value (Fig. 6) and the maximum percentage of protection was observed at 500 µg/ml concentration of aqueous extract (Fig. 4) Several other plants were also reported to this kind of inhibition on heat-induced haemolysis. [10, 25]

4.2.4. Hypotonicity Induced Haemolysis

Hypotonicity induces membrane damage by facilitating the accumulation of liquids and it will lead to rupturing of the cell membrane. [18] In this study, each plant extracts exhibited dose-dependent membrane stabilizing action by protecting the RBC membrane from hypotonicity-induced lysis. 70% ethanol fraction possessed the highest membrane-stabilization action (Fig. 5) with the least IC_{50} value (Fig.6). There are many reports on the anti-inflammation property of many herbal preparations exerted by stabilizing red blood cell membrane. [11, 26-27]

4.3. Anti-inflammatory Studies on Cell lines

4.3.1. Cyclooxygenase (COX) Activity

Each plant extract exhibited a dose-dependent COX inhibition activity. Among the plant extracts, the least IC_{50} value was observed in chloroform extract (Fig. 9) and its

maximum activity was observed at a concentration of 100µg/ml (Fig. 7). Cyclooxygenase inhibition activity was also reported by other plant extracts as well.[28, 29] Cyclooxygenase is one of the key enzymes which are involved in the synthesis of inflammatory mediators like prostaglandins, prostacyclins, and thromboxanes. [30] The inhibition of the enzyme cyclooxygenase (COX) is a suitable target in inflammatory therapies. [31]Prostanoids are the end products of fatty acid metabolism, which is produced via COX pathway. These prostanoids have long been known to behave as important pathological and physiological mediators implicated in a range of therapeutic areas of interest, including inflammation, pain, cancer, etc. [32] So targeting the COX may inhibit the formation of prostanoids, thus, can help in the inhibition of inflammation.

4.3.2. Lipoxygenase (LOX) Activity

Lipoxygenase is a family of iron-containing enzymes present in eukaryotes most of which catalyzes the dioxygenation of polyunsaturated fatty acids in lipids. In the present study, every plant extract inhibited the lipoxygenase activity to a certain extent. The maximum inhibition was found at 100µg/ml concentration of chloroform extract (Fig. 8) and which also possessed the least IC 50 value (Fig. 9). Arachidonic acid is the precursor for prostanoids. In response to a wide variety of stimuli, free arachidonic acid is converted into various lipid mediators via catalyzation reactions of COX, LOX Cytochrome P450, etc. [33] Inhibition of 5-LOX lead to decreases the production of mediators of inflammation. So the inhibition of lipoxygenase can protect the cell from inflammation. [28] There are reports on the anti-inflammatory activity of methanolic leaf extract of *T. siliquosa* by inhibiting lipoxygenase action. [34]

5. STATISTICAL ANALYSIS

The results were presented as mean ± Standard error (SE), and the data was found significant at P = 0.01.

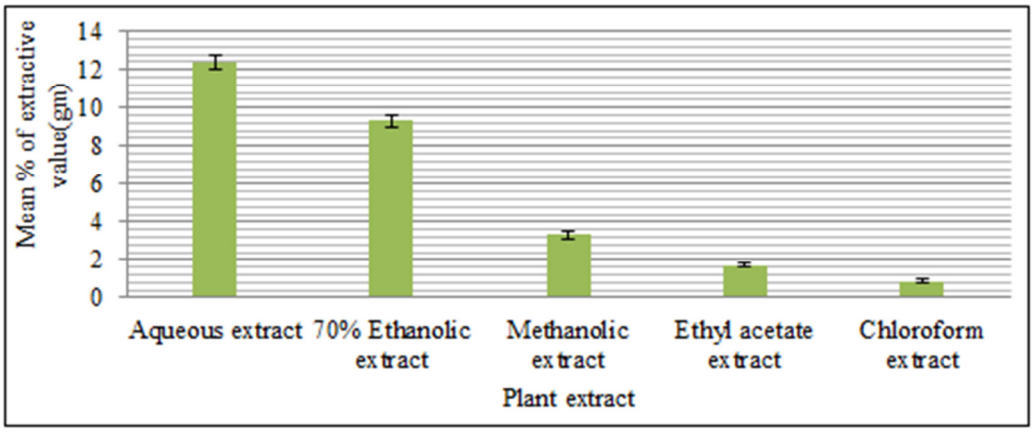

Fig 1. *Mean percentage extractive value of T. siliquosa in different solvent*

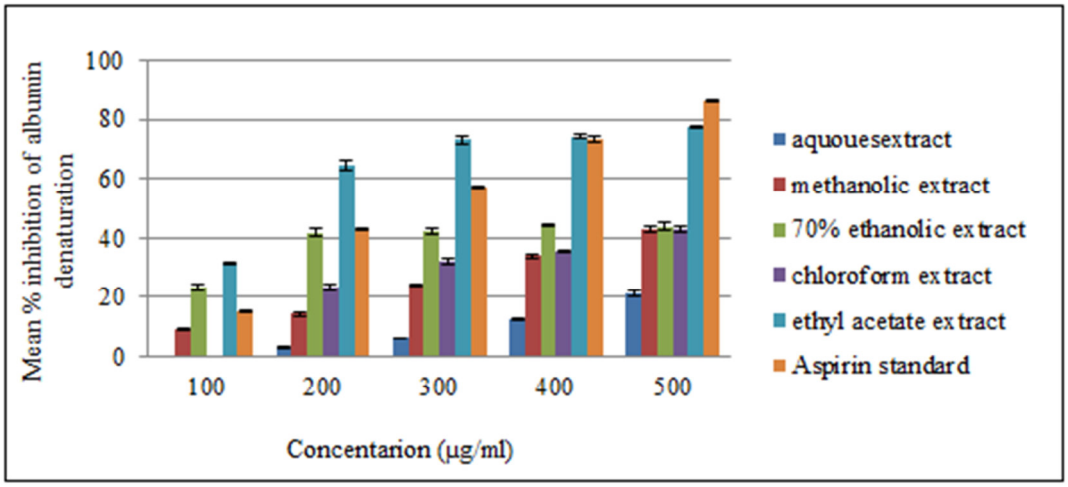

Fig 2.*Mean percentage of protein denaturation inhibition exhibited by T. siliquosa plant extract in Albumin denaturation inhibition assay. Aspirin was used as the standard.*

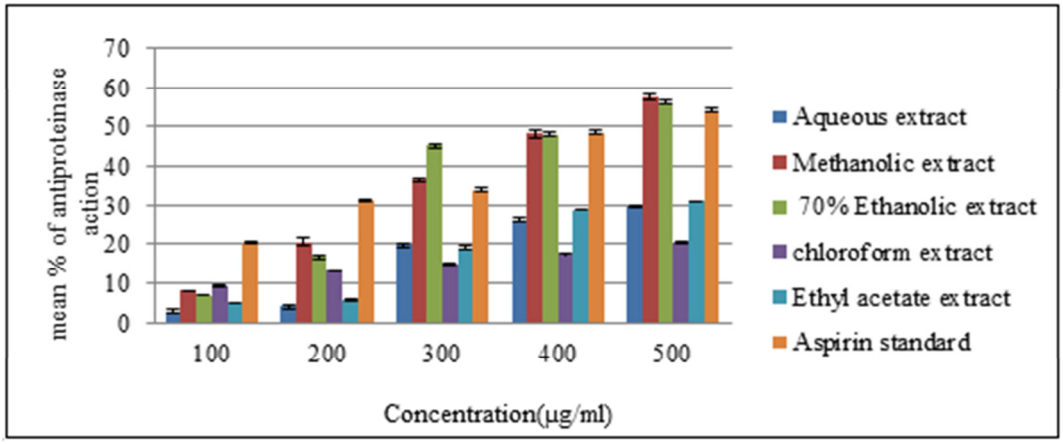

Fig 3. *Mean percentage of anti-proteinase action exhibited by T. siliquosa plant extract in Anti-proteinase assay. Aspirin was used as the standard.*

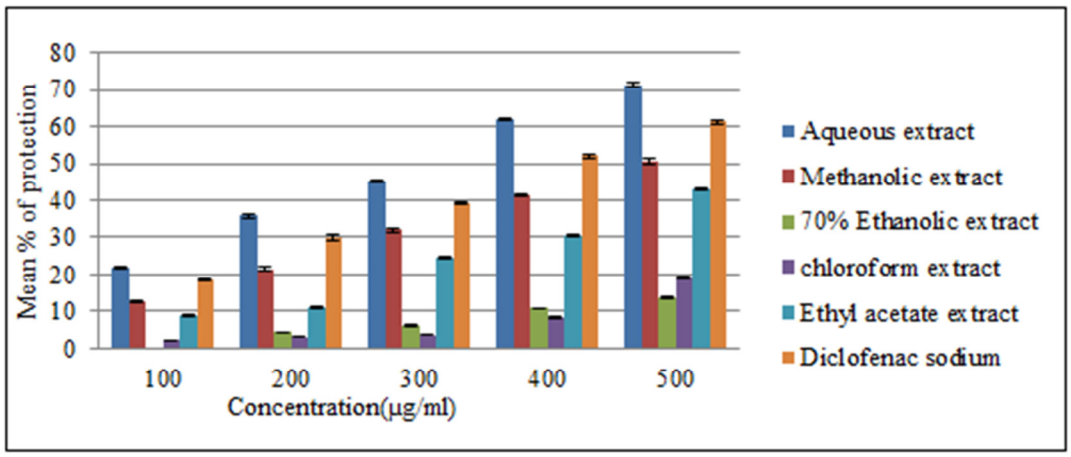

Fig 4. *Mean percentage of membrane stabilization offered by T. siliquosa plant extracts in Heat induced haemolysis assay. Diclofenac sodium was used as the standard.*

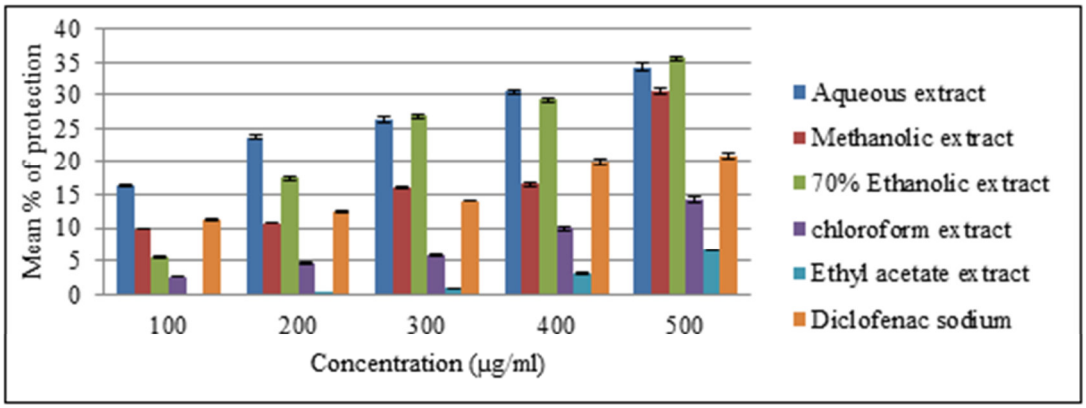

Fig 5. *Mean percentage of membrane stabilization offered by T. siliquosa plant extracts in Hypotonicity Induced haemolysis. Diclofenac sodium was used as the standard.*

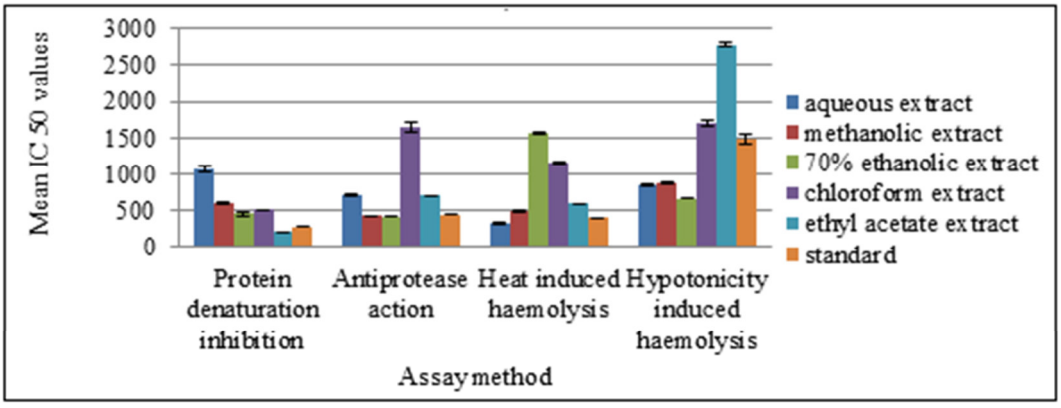

Fig 6. *Mean IC 50 value of plant extracts and standard in different in vitro anti-inflammatory assays*

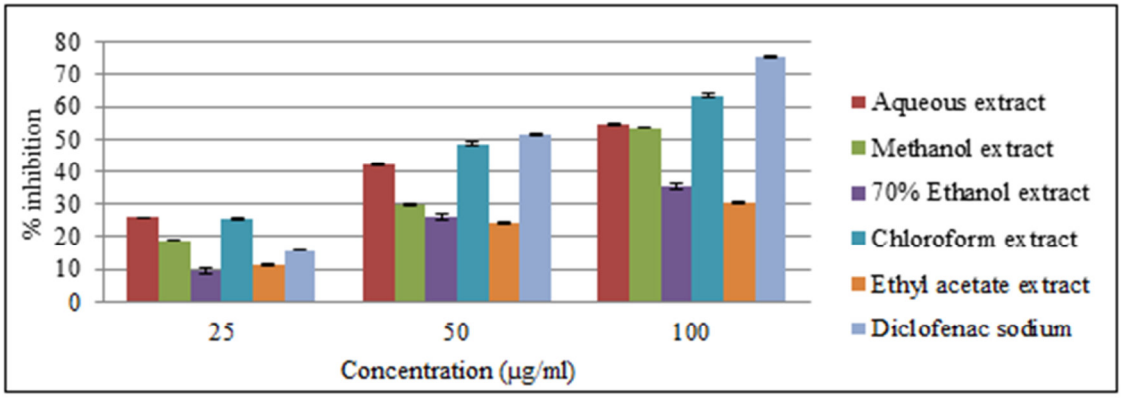

Fig 7. *Mean percentage inhibition of COX activity by plant extract. Diclofenac sodium was used as the standard*

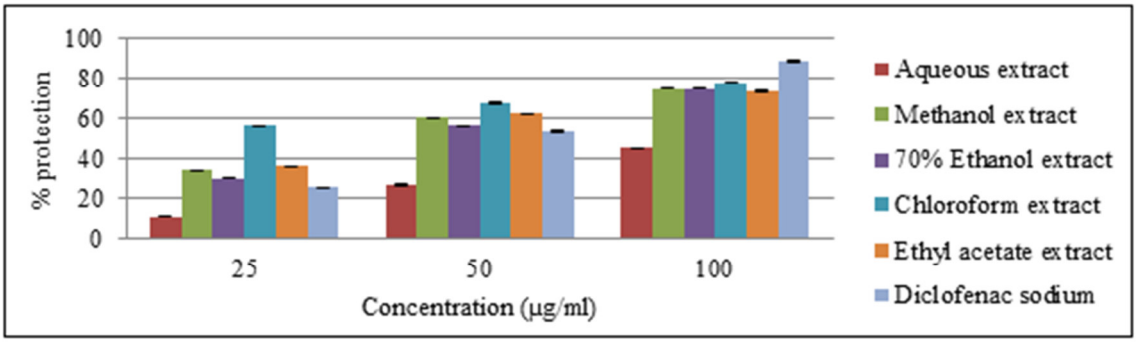

Fig 8. *Mean percentage inhibition of LOX activity by plant extract. Diclofenac sodium was used as the standard*

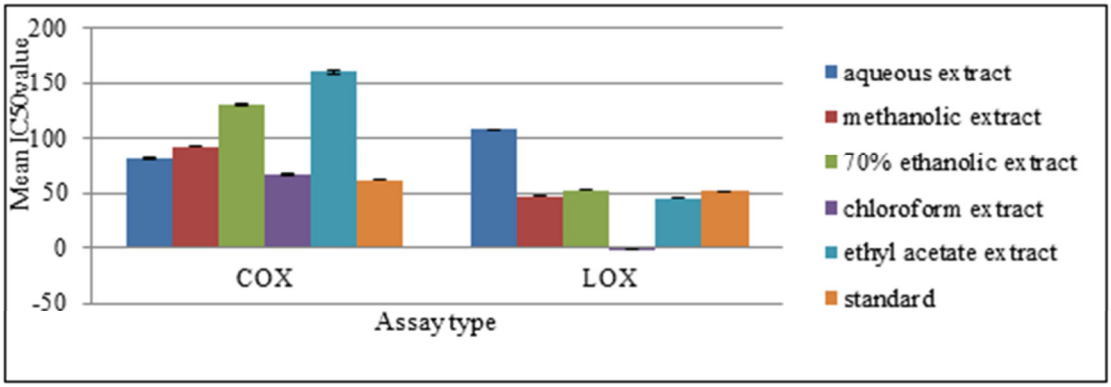

Fig 9. *Mean IC 50 values of the plant extract and standard in COX and LOX assay*

6. CONCLUSION

The dose-dependent activity of the root extracts of *T. siliquosa* with regard to different anti-inflammatory parameters unveils the pharmaceutical prospects of the plant in therapies associated with inflammatory responses. Irrespective of their polarity, the different solvent fractions contributed significantly eliciting anti-inflammatory responses. While comparing the anti-inflammatory activity with regard to different plant extracts, it was clear that ethyl acetate fraction excelled in inhibition of protein denaturation assay; 70% ethanol fraction offered maximum protection in anti-protease and hypotonicity assays; aqueous extract offered maximum protection in heat induced haemolysis. COX and LOX studies revealed the chloroform extract as the leading solvent fraction promoting anti-inflammatory responses. Further research shall be made for streamlining the bio active phytochemicals from the roots of *T. siliquosa* for the development of a novel therapeutic drug to treat different inflammatory responses.

7. ACKNOWLEDGEMENT

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8. AUTHORS CONTRIBUTION STATEMENT

Mrs. Athira M carried out the experiment under the guidance of Dr. Shaiju P.N. and Dr. S.R. Suja. Analysis and interpretation was done by Mrs. Athira M. and verified by both Dr. Shaiju P.N. and Dr. S.R. Suja. All three authors helped to shape this manuscript.

9. FUNDING ACKNOWLEDGEMENT

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10. CONFLICT OF INTEREST

Conflict of interest declared none.

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