

In-vitro Anti-inflammatory Activity Studies on *Syzygium zeylanicum* (L.) DC Leaves

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ABSTRACT

Syzygium zeylanicum (L.) DC is perennial evergreen shrub belongs to the family Myrtaceae having yellow flowers and dark green leaves. Traditionally leaf extracts has been used to treat various ailments such as joint pain, head ache, arthritis and fever. The traditional uses show that the plant is a good candidate for antioxidant related activity such as anti-inflammatory. The present study was to evaluate in-vitro anti-inflammatory activity of *Syzygium zeylanicum* leaves by inhibition of Cyclooxygenase, 5-lipoxygenase and by inhibition of protein denaturation. Successive solvent extraction of shade dried leaves were carried out using solvents of increasing polarity and all the extracts were subjected to estimation of total phenolics and the extract containing maximum of phenolics were subjected to anti-inflammatory studies. Preliminary phytochemical analysis was done on all extracts. Ethyl acetate extract and aqueous extract found to contain maximum of phenolics by Folin-ciocalteu method. Anti-oxidant activity study by using nitric oxide scavenging activity showed the ethyl acetate extract has maximum activity (IC₅₀ = 125 mcg/ml). The evaluation of anti-inflammatory activity by inhibition of Cyclooxygenase (70.71 and 78.79% at 100 and 500 mcg/ml), 5-lipoxygenase (65.95 and 70.26% at 100 and 500 mcg/ml) and by determination of protein denaturation (64.43 and 76.92% at 100 and 250 mcg/ml) showed the ethyl acetate extract possess good anti-inflammatory activity.

Keywords: 5-lipoxygenase, Anti-inflammatory, Cyclooxygenase, Folin-ciocalteu, *Syzygium zeylanicum*

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INTRODUCTION

Inflammation is a complex process, which is frequently associated with pain and involves occurrences such as: the increase of vascular permeability, increase of protein denaturation and membrane alteration. Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when denatured. Denaturation of protein is a well-documented cause of inflammation. As part of the investigation on the mechanism of the anti-inflammatory activity, ability of plant extract to inhibit protein denaturation was studied [1,2]. The mechanisms of inflammation involve a series of events in

which the metabolism of arachidonic acid plays an important role. It can be metabolized by the Cyclooxygenase (COX) pathway to prostaglandins and thromboxane A₂, or by the 5-lipoxygenase (5-LOX) pathway to hydroperoxy-eicosatetraenoic acids (HPETE's) and leukotrienes (LT's), which are important biologically active mediators in a variety of inflammatory events. Upon appropriate stimulation of neutrophils, arachidonic acid is cleaved from membrane phospholipids and can be converted to leukotrienes and prostaglandins through 5-LOX or COX pathways respectively. Inhibition of 5-LOX and COX-leads to decreased production of LTs and PGs, such a drug would have the potential to provide anti-inflammatory and analgesic effects with a reduction in the GI

side-effects. Furthermore, inflammatory processes also involve reactive oxygen species started by leukocyte activation. Therefore, screening of antioxidant properties may provide important information about the potential activity of a drug on inflammatory processes [3].

Syzygium zeylanicum is perennial evergreen shrub having soft wood, yellow flowers and dark green lanceolate leaves. Traditionally leaf extracts has been used to treat various ailments such as joint pain, head ache, arthritis, fever and has got anti microbial and anti fungal properties but very less scientific investigation has been carried out on this plant, literature review indicates the plant is very less explored [4]. The traditional uses show that the plant is a good candidate for antioxidant related activity such as anti-inflammatory. This is leading as to the requirement of anti-inflammatory activity studies.

MATERIALS AND METHODS

Collection and authentication of plant material

Syzygium zeylanicum leaves (**Figure 1**) were collected from Mahatma Gandhi University Campus, Kottayam, Kerala and was authenticated by botanist Mr Joby Paul, School of Environmental Sciences, Mahatma Gandhi University, Kottayam, Kerala and the voucher specimen number 1439 (**Figure 2**) was preserved at School of Environmental Sciences, Mahatma Gandhi University, Kottayam, Kerala for future references.

Extraction

Fully expanded leaves were collected and shade dried. 250 g of dried leaves were taken and size reduced, extracted with solvents of increasing polarity i.e. petroleum ether, chloroform, ethyl acetate, alcohol and water. The extract obtained was collected and concentrated. The concentrated extract was then weighed and was subjected to preliminary phytochemical screening and biological activity studies.

Preliminary phytochemical screening

Petroleum ether extract (PEE), chloroform extract (CHE), ethyl acetate extract (EAE), alcoholic extract (ALE) and aqueous extracts (AQE) of leaves were subjected to various chemical tests for the investigation of presence of various chemical constituents [5].

Estimation of total Phenolic content

The total phenolic content is generally estimated by the Folin-Ciocalteu method using Gallic acid as standard. 10 mg Gallic acid was weighed and made up to 10 ml with methanol in a 10 ml standard flask (stock solution I). From the above solution (1mg/ml), 1ml was pipetted out and made up to 10 ml with methanol to get 100 mcg/ml Gallic acid standard solution (stock solution II). From the stock solution I, 1.25 ml and 1.5 ml were pipetted out and made up to 10 ml to get 125 and 150 mcg/ml solutions. From the stock solution II, 2.5 ml, 5 ml and 7.5 ml were pipetted out and made up to 10 ml to get 25, 50, 75 mcg/ml solutions. To the above solutions, 5 ml of Folin-Ciocalteu reagent was added and 4 ml of 7.5% sodium carbonate solution was added after 5 minutes. It was stirred and incubated at room temperature for 2 hours. After 2 hours, absorbance of the solutions was measured at 750nm using UV-VISIBLE spectrophotometer. The absorbance values were plotted against concentration and standard graph was obtained [6].

Preparation of sample solution

10 mg of the extracts were weighed, dissolved in methanol and made up to 10 ml with methanol. 1ml was pipetted out from each extract solution and 5 ml of Folin-Ciocalteu reagent was added. After 5 minutes, 4 ml of sodium carbonate solution was added and incubated at room temperature for 2 hours. Then, absorbance was measured at 750 nm and the values obtained were interpreted in the standard graph of Gallic acid to get the milligram equivalents of Gallic acid.

In-vitro antioxidant activity

Nitric oxide scavenging assay

Preparation of standard

The standard used here was Ascorbic acid. 50, 100, 150 and 200 mcg/ ml solution of standard in methanol was used for assay.

Preparation of sample

Sample solution was prepared from ethyl acetate and aqueous extracts of *S. zeylanicum* leaves and is dissolved in methanol. 10 mg of each extract were dissolved in methanol and solutions of 50, 100, 150 and 200 mcg/ ml concentration were prepared.

Estimation procedure

In this assay 0.5 ml of Sodium nitro prusside (5 mmolL⁻¹) in phosphate buffered saline pH 7.4, was mixed with different concentration of the extract (50, 100, 150 and 200 mcg/ ml from a stock concentration of 100 mg/ ml methanol) and incubated at 25°C for 180 minutes. A control without the test compound, but an equivalent amount of methanol was taken. After 3 h, 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthyl ethylene diaminedihydrochloride) was

added and incubated for 30 minutes for color development. Absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-1-naphthyl ethylene diaminedihydrochloride was measured at 546 nm and the percentage scavenging activity was measured with reference to the standard. The scavenging activity on the nitric oxide was expressed as inhibition percentage using the following equation:

$$\% \text{ Nitric oxide Scavenging} = \frac{\text{Absorbance of Control} - \text{Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

The tests were carried out in triplicate. The extract concentration providing 50% inhibition (IC₅₀) was calculated from the graph of inhibition percentage plotted against extract concentration [7, 8].

In-vitro anti-inflammatory activity**Inhibition of protein denaturation**

The reaction mixture (0.5 ml) consisted of 0.45 ml of bovine serum albumin (5% aqueous solution) and 0.05 ml of *S. zeylanicum* leaf extracts (100 and 250 mcg/ml of final volume). pH was adjusted at

6.3 using a small amount of 1N Hydrochloric acid. The samples were incubated at 37°C for 20 min and then heated at 57°C for 3 min. After cooling the sample, 2.5 ml of phosphate buffer solution was added into each test tube. Turbidity was measured spectrophotometrically at 600 nm for control tests; 0.05 ml of distilled water was used instead of extracts while product control tests lacked bovine serum albumin. The percentage inhibition of protein denaturation was calculated as follows [9],

$$\text{Percentage inhibition} = 100 - \frac{(\text{O.D. of test} - \text{O.D. of product control}) \times 100}{\text{O.D. of Control}}$$

The Percentage protection from denaturation is calculated by using the formulae and it is tabulated.

Assay of Cyclooxygenase and 5-Lipoxygenase Inhibition**Lymphocyte culture preparation**

Human peripheral lymphocytes was cultured in RPMI 1640 [HIMEDIA] media, supplemented with 20% heat inactivated Fetal bovine serum, antibiotics (Penicillin and Streptomycin). Phytohaemagglutinin (HIMEDIA) was used as the stimulant for cell proliferation. The culture was filtered using 0.2 µm pore sized cellulose acetate filter (Sartorius) in completely aseptic conditions. Fresh plasma was aseptically added to the culture at a concentration of 1 x 10⁶ cells/ml. The culture was then incubated for 72 hours. After 24 hours culture is activated by adding 1 µl lipopolysaccharide. Incubation was done for 24 hours. Ethyl acetate and aqueous extracts were added at 24 hr of incubation in a final concentration of 100, 500 mcg/ml and

incubated for 24 hours. Ibuprofen at concentration of 100 mcg/ml was added and incubated for 24 hours, kept as standard. After incubation the cells were pelleted by centrifugation. The isolation was done by spinning at 6000 rpm for 10 minutes. Supernatant was discarded and 50 µl of cell lysis buffer was added and again centrifugation was done at 6000 rpm for 10 minutes. Supernatant was discarded and anti-inflammatory assay was done in pellet suspended in a small amount of supernatant [10].

Assay of Cyclooxygenase

The assay mixture contained Tris- HCl buffer, glutathione, hemoglobin & enzyme. The assay started by the addition of arachidonic acid and terminated after 20 min incubation at 37°C by addition of 0.2 ml of 10% TCA in 1N HCl, mixed and 0.2 ml of TBA was added and contents heated in a boiling water bath for 20 min, cooled and centrifuged at 1000 rpm for 3 min. The

supernatant was measured at 632 nm for COX activity [10].

Assay of 5- Lipoxygenase

70 mg of linoleic acid and equal weight of tween 20 was dissolved in 4 ml of oxygen free water and mixed back and forth with a pipette avoiding air bubbles. Sufficient amount of 0.5 N sodium hydroxide was added to yield a clear solution and then made up to 25 ml using oxygen free water. This was divided into 0.5 ml portions and flushed with nitrogen gas before closing and kept frozen until needed. The reaction was carried out in a quartz cuvette at 25°C with 1cm light path. The assay mixture contain 2.75 ml Tris buffer of pH 7.4, 0.2 ml of sodium linoleate and 50 mcl of the enzyme. OD was measured in 234 nm [10].

RESULTS AND DISCUSSION

Plant Herbarium



Figure 1: *Syzygium zeylanicum* (L.) DC Leaves

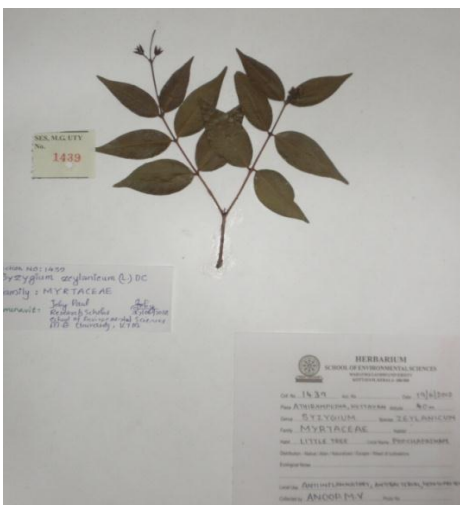


Figure 2: *Syzygium zeylanicum* (L.) DC leaves Herbarium

Preliminary phytochemical screening

The qualitative analysis of extracts of *S. zeylanicum* were carried out and extracts showed the presence of various chemical constituents such as alkaloids, glycosides, phenolics, flavonoids, tannins, saponins, carbohydrates and steroids. This shows high level of its possible medicinal value. The results are shown in (Table 1). Phytochemical screening showed the presence of antioxidant components phenolics and flavonoids, presence of these compounds might be responsible for the use of plant in ameliorating inflammatory ailments [11].

Estimation of total Phenolics

The total phenolic content in the extracts were determined by Folin-Ciocalteu method. The absorbance values obtained for different concentrations of standard Gallic acid are tabulated and the standard graph of Gallic acid is shown in (Figure 3) and the total phenolic content are given in (Table 2). Phenolics are ubiquitous secondary metabolites in plants and possess a wide range of therapeutic uses such as antioxidant, anti-inflammatory, ant mutagenic, ant carcinogenic, free radical scavenging activities and also decrease cardiovascular complications. So the total ethanolic extract was found to a source of wide range of potent photochemical.

Anti-oxidant activity

Nitric oxide scavenging assay

The percentage inhibition obtained in the different concentration of extracts was compared to that of standard ascorbic acid and is shown in (Table 3 and Figure 4). The calculation of IC₅₀ values of standard ascorbic acid and extracts were depicted in (Figure 5).

This assay shows that the ethyl acetate extract has more Nitric oxide scavenging activity than aqueous extract.

Nitric oxide (NO) is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signalling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation and antimicrobial and antitumor activities. Suppression of released

NO may be partially attributed to direct NO scavenging, as the extracts of *S. zeylanicum* decreased the amount of nitrite generated from the decomposition of sodium nitro prusside *in-vitro*. The scavenging of NO by the extracts was increased in dose dependent manner. Results show the

significant decrease in NO radical due to the scavenging ability of extracts and ascorbic acid. The ethyl acetate extract showed maximum activity [8]. The compounds such as phenolics and flavonoids contain hydroxyl functional groups, are responsible for their antioxidant activity [12].

Table 1: Preliminary Phytochemical screening

Sl.no	Phytoconstituents	PEE	CHE	EAE	ALE	AQE
1.	Alkaloids	-	+	-	-	-
2.	Glycosides	-	-	-	+	+
3.	Phenolics	-	-	++	-	+
4.	Flavones and Flavonoids	-	-	++	+	++
5.	Carbohydrates	-	-	-	-	+
6.	Terpenoids	+	-	++	-	-
7.	Sterols	+	-	++	-	-
8.	Proteins and amino acids	-	-	-	-	-
9.	Saponins	-	-	-	-	+

(++) indicate active constituents in high amount, (+) indicate active constituents in low amount, (-) indicates the absence of active constituents.

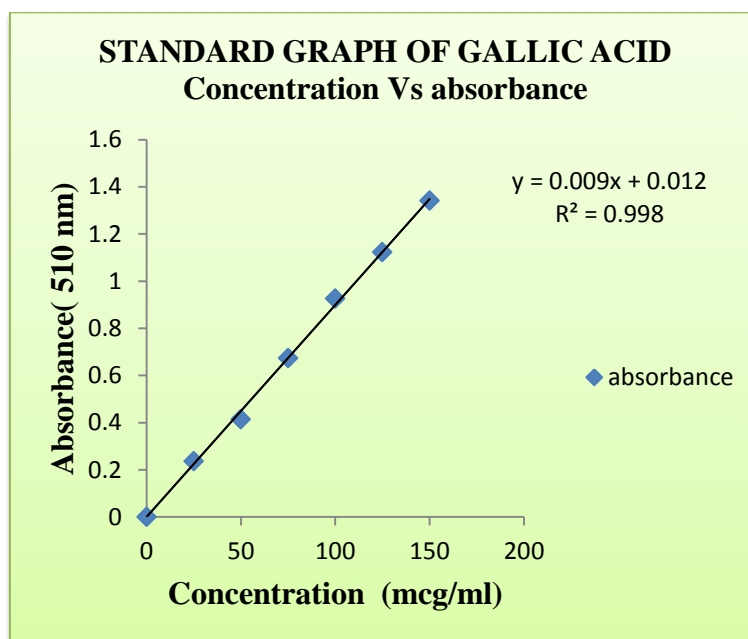


Figure 1: Standard graph of Gallic acid

Table 2: Total phenolics content of different extracts

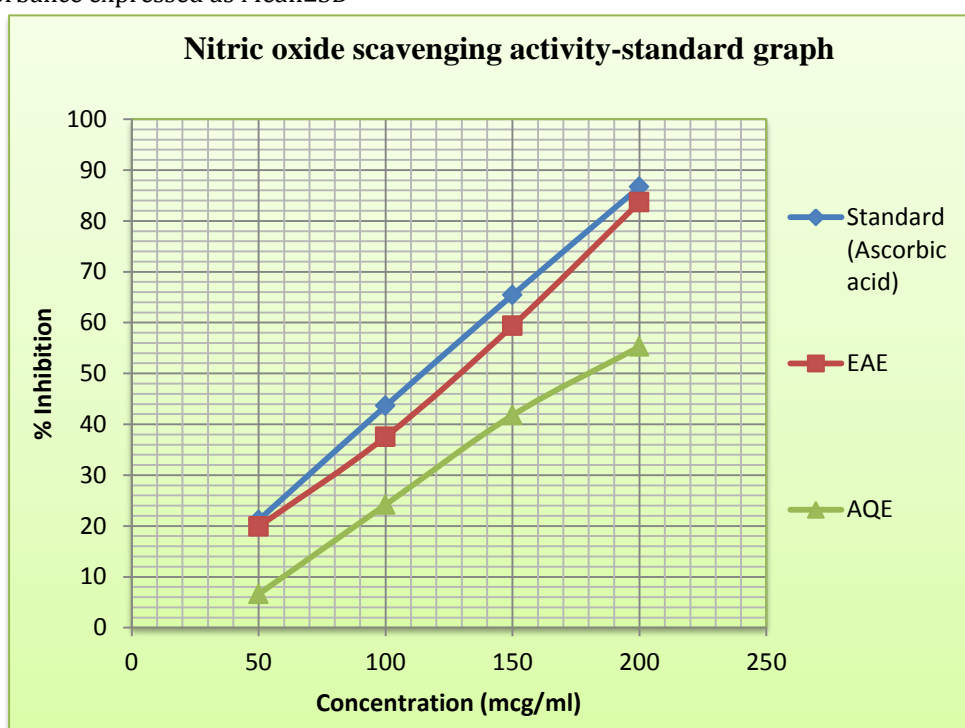
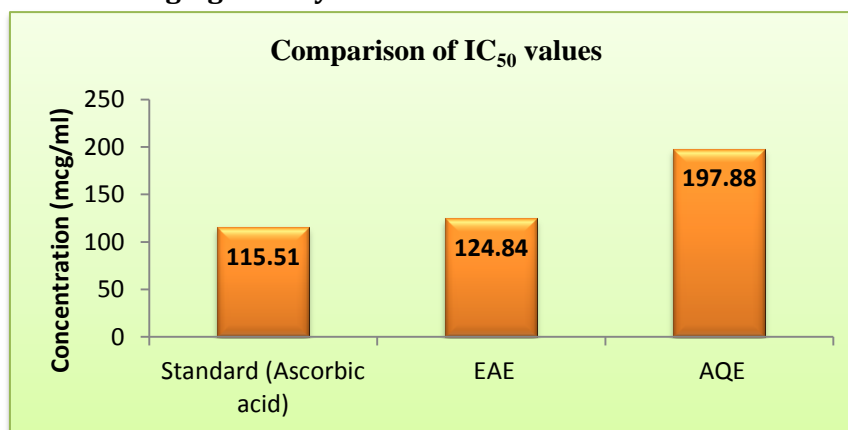
Sl.no	Extracts	Absorbance (750nm)	Phenolic content (mcg/ml)
1	Petroleum ether extract (PEE)	0.087±0.001	8.41
2	Chloroform extract (CHE)	0.099±0.001	9.77
3	Ethyl acetate extract (EAE)	0.646±0.003	70.52
4	Alcoholic extract (ALE)	0.134±0.001	13.51
5	Aqueous extract (AQE)	0.376±0.005	40.41

n=3, absorbance expressed as mean± S.D

Table 3: Nitric oxide scavenging activity

Sl.no	Extracts	Concentration(mcg/ml)	Absorbance (546 nm)	% Inhibition
1.	Control	-	1.649±0.0018	-
2.	Standard (Ascorbic acid)	50	1.30±0.0890	21.16
		100	0.93±0.0314	43.60
		150	0.57±0.0052	65.43
		200	0.22±0.0230	86.66
3.	Ethyl Acetate extract (EAE)	50	1.321±0.005	19.89
		100	1.030±0.005	37.54
		150	0.670±0.002	59.37
		200	0.269±0.001	83.69
4.	Aqueous extract (AQE)	50	1.540±0.005	6.61
		100	1.250±0.009	24.2
		150	0.960±0.014	41.78
		200	0.738±0.008	55.25

n=3 absorbance expressed as Mean±SD

**Figure 2: Nitric oxide scavenging activity****Figure 3: Comparison of IC₅₀ by Nitric oxide scavenging assay**

***In-vitro* anti-inflammatory activity Inhibition of protein denaturation**

The extracts of *Syzygium zeylanicum* were analyzed for its anti-inflammatory activity and it is compared with that of the standard Diclofenac sodium. Denaturation of proteins

is a well documented cause of inflammation. From the results of present study it can be stated that the extracts of *S. zeylanicum* are effective in inhibiting heat induced albumin denaturation. The results are shown in (Figure 6).

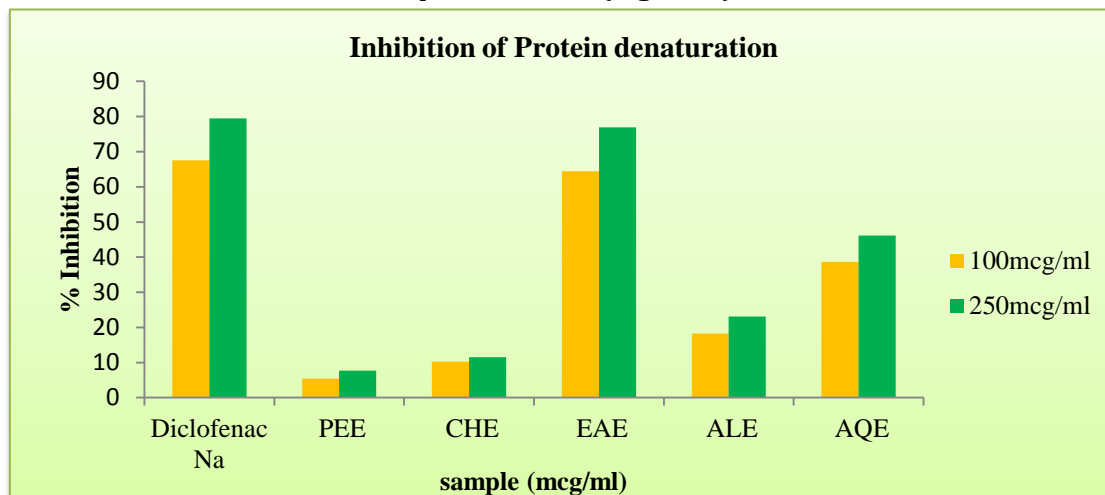


Figure 4: Inhibition of protein denaturation

From the result it is that ethyl acetate extract has the maximum anti-inflammatory activity when compared to the other extracts.

Cyclooxygenase inhibitory assay

The effects of *Syzygium zeylanicum* leaf extracts on production of prostaglandins were determined by the inhibition of Cyclooxygenase activity. The results are tabulated in (Table 4).

5-lipoxygenase inhibitory assay

The effects of *S. zeylanicum* leaf extracts on production of leukotrienes were determined

by the inhibition of 5-lipoxygenase activity and the results are tabulated in (Table 5).

From the above table, we can suggest that ethyl acetate and aqueous extracts has got good 5-lipoxygenase inhibitory activity comparing to standard Ibuprofen. Comparison of Cyclooxygenase and 5-lipoxygenase activity of various extracts are shown in (Figure 7).

Table 4: Cyclooxygenase Inhibitory assay

Sl no	Sample	Concentration (mcg/ml)	Absorbance (632 nm)	% inhibition
1	Control	-	0.099±0.60	-
2	Standard (Ibuprofen)	100	0.012±0.90	87.88
3	Ethyl acetate extract (EAE)	100	0.029±1.32	70.71
		500	0.021±1.08	78.79
4	Aqueous extract (AQE)	100	0.074±0.55	25.25
		500	0.068±0.68	32.33

n=3 absorbance expressed as Mean±SD

Table 5: 5-Lipoxygenase inhibitory assay

Sl.no	Sample	Concentration (mcg/ml)	Absorbance (234 nm)	% inhibition
1	Control	-	0.188±0.85	-
2	Standard (Ibuprofen)	100	0.020±1.13	89.36
3	Ethyl acetate extract (EAE)	100	0.064±1.48	65.95
		500	0.039±0.74	79.26
4	Aqueous extract (AQE)	100	0.11±0.56	41.48
		500	0.042±1.35	77.65

n=3 absorbance expressed as Mean±SD

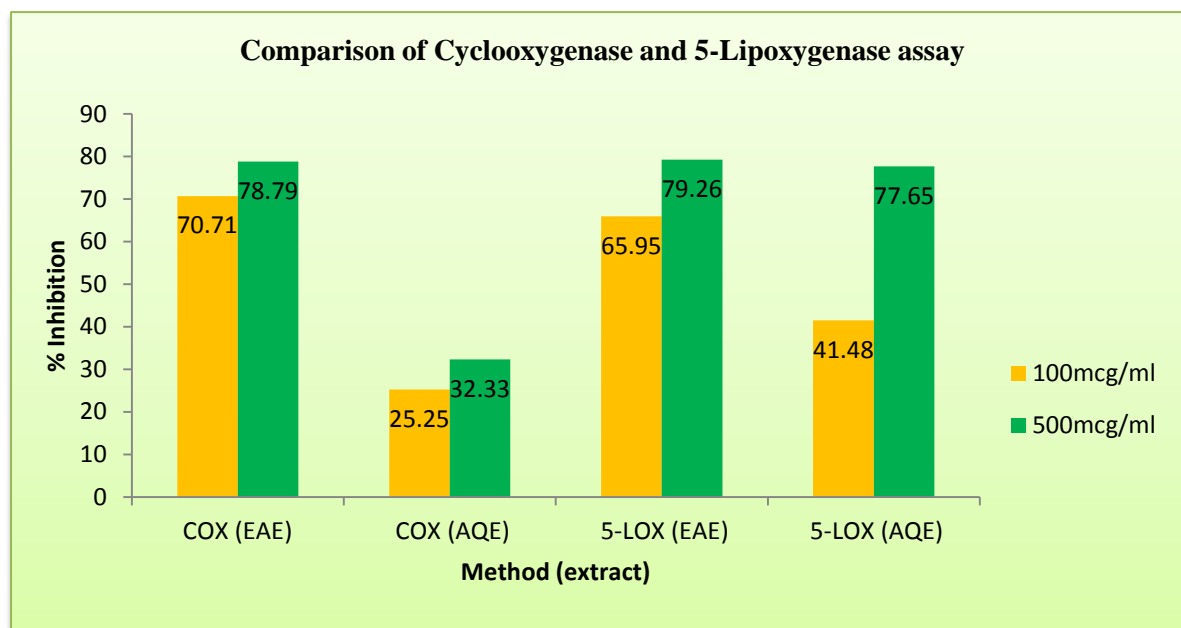


Figure 5: Comparison of COX and 5-LOX assay

In the present study, results indicate that the extracts of *Syzygium zeylanicum* possess good anti-inflammatory properties; however the ethyl acetate extract showed the best anti-inflammatory activity by inhibiting protein denaturation, COX inhibition and 5-LOX inhibition. These activities may be due to the strong occurrence of polyphenolic compounds. The ethyl acetate fraction contains maximum of polyphenolic, flavonoids and terpenoids, serve as free radical inhibitors or scavenger or acting possibly as primary oxidant thereby inhibiting inflammation [1].

Inflammation is a complex physiopathological response to different stimuli. The inflammatory process involves the activity of inflammatory mediators such as neutrophil derived free radical, reactive oxygen species (ROS), nitric oxide (NO), prostaglandins and cytokines [13]. This over production leads to tissue injury by damaging macromolecules, lipid peroxidation of membrane and tissue damage play important role in pathogenesis of many inflammatory diseases. Thus the free radicals are important mediators that provoke or sustain inflammatory processes and consequently, their neutralization by antioxidants and radical scavengers can attenuate inflammation [14].

During the course of inflammatory response, large amount of NO formed by nitric oxide

synthase (iNOS) in activated macrophages surpass the physiological amount of NO, which are usually made by neuronal form of NOS (nNOS) or constitutive form of NOS (eNOS), these NO nitrosylates macromolecules. It also causes increased vascular permeability, vaso dilation, tissue and endothelial damage leads to inflammation, various *in-vivo* studies show that the edema induced by substance-P is mediated through the release of NO. The pro inflammatory role of NO in chronic inflammation has been inferred, based on the observation of elevated levels of nitrite in rheumatoid synovial fluid [15].

Phenolics and Flavonoids are secondary metabolites having several biological activities such as antioxidant, antimutagenic and anti-inflammatory. Flavonoids are capable of inhibiting the expression and activation of iNOS, and therefore could be used additionally during inflammatory therapy. The importance of nitric oxide in inflammation may indicate that drugs which modulate NO production could be successfully used in the management of inflammatory diseases [15].

Arachidonic acid (AA) is metabolized in the body through two main metabolic pathways with the enzymes: Cyclooxygenase (COX) and 5-lipoxygenases (5-LOX) into prostaglandin I₂, prostaglandin E₂, thromboxane A₂ and leukotrienes. It has

been suggested that the inhibition of both prostaglandins and leukotriene production might have synergistic effects and achieve optimal anti-inflammatory activity so the dual inhibition of the COX and 5-LOX pathway could produce a wider spectrum of anti-inflammatory effects. In the past few decades, several compounds have been developed to block both COX and 5-LOX, but their use was abandoned owing to liver toxicity. Prototype experimental dual inhibitors have proved effective in preventing the production of both PGs and LTs and the consequent inhibition of migration and activation of inflammatory cells (mainly PMN, monocytes and macrophages) into inflamed sites. Importantly, the inhibition of migration of inflammatory cells towards the affected sites has translated into a reduction of tissue damaging or necrosis in a model of tissue damage. 5-LOX/COX blockers have an excellent preclinical GI pharmacological safety profile [16].

The ethyl acetate extract showed pronounced NO and DPPH scavenging activity and showed good inhibitory activity against COX and 5-LOX enzyme mediated metabolism of arachidonic acid. Therefore ability of the extract to scavenge free radical during inflammation and inhibition of COX and 5-LOX might be responsible for their inhibitory effect on progression of inflammatory reaction [13].

CONCLUSION

Leaves of *Syzygium zeylanicum* were selected for the study. Phytochemical studies showed the presence of alkaloids, glycosides, phenolics, flavonoids, steroids, terpenoids and saponins. The antioxidant compounds, phenolics were quantitatively estimated and the ethyl acetate extract showed the maximum of these compounds. Antioxidant activity studies using Nitric oxide showed that the ethyl acetate and aqueous extracts could be promising sources of natural antioxidants. Anti-inflammatory studies using Cyclooxygenase, 5-lipoxygenase inhibitory activity, and determination of protein denaturation showed that ethyl acetate extract has good anti-inflammatory activity.

A detailed research on different pharmacological activities can be carried out

such as Hepatoprotective activity, Analgesic activity, and Antipyretic activity. Isolation of more active constituents possessing anti-inflammatory can be assessed

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