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Research Article

INHIBITION OF LIPOXYGENASE BY *ELEPHANTOPUS SCABER* EXTRACT AND DETERMINATION OF ITS INHIBITION PATTERN

Prakash Kumar B*, Anu P.Abhimannue, Mohind C.Mohan, Jenny Jacob, Bashi M.Babu

School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, India

**Corresponding author: prakashkumar@mgu.ac.in*

ABSTRACT

Elephantopus scaber Linn, a medicinal herb indigenous to India, has been widely used in treating a number of inflammatory pathological conditions. In the present study, methanolic extract of *E. scaber* was evaluated for its ability to inhibit 5, 12 and 15 Lipoxygenase enzymes (LO) and the nature of inhibition was determined from Km and Vmax values obtained from Lineweaver Burk plot. The methanolic extract was found to inhibit all three LO enzymes in a mixed competitive non competitive nature. *E. scaber* promises to be a good source of bioactive molecules that could be employed for inflammation research.

Keywords: Lipoxygenase enzyme, Lineweaver Burk plot, Enzyme inhibition, Inflammation.

1. INTRODUCTION

Elephantopus scaber Linn belonging to compositae family is a widely distributed herb throughout the tropical and sub tropical regions like Indian sub continent, Eastern and south East Asia, America, tropical Africa and Australia. *E. scaber* is a heavily exploited plant for treatment of numerous diseases [1, 2]. The plant which is indigenous to India has been in use in folklore medicine since time immemorial [3]. Indian ayurvedic pharmacopeia mentions the role of preparations made of its root, leaves, aerial parts and the whole plant as such in the treatment of several diseases such as leukemia, as an antidote for snake bite, removal of bladder stones, filariasis and infection against virus and bacteria [3]. *E. scaber* is used in the treatment of various inflammatory conditions like bronchitis, arthritis, asthma and rheumatism. Taiwan folk medicine "Teng-Khia-U", a mixture of *E. scaber,* E. *mollis* and *Pseudoelephantopus spicatus* extracts, possess excellent anti inflammatory and hepatoprotective properties. The same has been experimented out in Carrageenan and Complete Freund's Adjuvant induced arthritis in mice, where "Teng-Khia-U" efficiently reduced inflammation [4, 5].

The potential of *E. scaber* has been mentioned in Indian ayurvedic pharmacopeia, where in combination with other medicinal herbs, it has been used for treating vatika grandhi and pittaja arbuda (minor and major neoplasm) [6]. *E. scaber* has an important role in preventing the airway inflammation-asthma, where its ethanolic extract stabilizes the mast cells in albino rats and bronchodilating activity against spasmogens in guinea pigs [7]. The aqueous extract of *E. scaber* possess anti inflammatory activity by inhibiting the Evans blue diffusion to the peritoneal cavity of albino mice which indicates the potential of *E. scaber* extract in preventing the inflammatory exudation of the peritoneal cavity [8].

Inflammation is characterized by the over expression of Lipoxygenase (LO) enzyme which produce leukotrienes, the key mediators of inflammation via activation of NF-κB pathway. Dihydroxy acid leukotrienes and cysteinyl leukotrienes activate PI-3K pathway and NF-κB p65 complex ultimately leading to the activation of NF-κB pathway, which has a greater implication in the inflammatory pathogenesis [9]. Over expression of LO enzyme has been reported in a number of chronic inflammatory conditions like atherosclerosis [10], rheumatoid arthritis [11], inflammatory bowel diseases [12], cardio vascular diseases associated with diabetes [13], acute asthma [14] and various cancers like lung, prostate, breast, colon and pancreatic [15-17].

The present study aims in evaluating the anti inflammatory property of methanolic extract of *E. scaber* through the inhibition of LO enzymes.

2. MATERIALS AND METHODS

2.1. Chemicals

5 and 15 LO pure enzyme and Nordihydroguaiaretic acid (NDGA) were from Cayman chemicals, Ann Arbor, USA. 12 LO was isolated from platelet rich plasma. Sodium linoleate, EDTA, acetylsalicylic acid, heparin and xylenol orange were purchased from Hi Media. Ferrous sulfate and triton x-100 were supplied by Merck. All other reagents used in the experiment were of analytical reagent grade.

2.2. Plant material

Elephantopus scaber was collected from Kasargod district of Kerala, India in the month of September, 2012 and was identified by Dr. Jomy Augustine, Taxonomist, Department of Botany, St. Thomas College, Pala, Kerala.

Voucher specimen of accession number JA5904 was deposited in the herbarium unit of the same department. Fresh sample of plant material was cleaned, chopped into tiny bits and shade dried. It was powdered coarsely and 40g of the plant powder was defatted with hexane and then, extracted with methanol by continuous hot percolation using the Soxhlet extractor. The crude extract was concentrated to a dry mass of 2.5814 g and redissolved in 30 ml of methanol to a concentration of 86.047 mg/ml and stored for further analysis.

2.3. Preparation of plant extract

The crude plant extract was serially diluted to obtain concentrations of 8.604 mg/ml, 0.860 mg/ml, 0 .086mg/ml and 0.008 mg/ml which were checked for interfering compounds according to the protocol provided by Cayman's Lipoxygenase inhibitor screening assay kit. In brief, reactions were carried out in 96 well microplate and consist of blank well which contains 100ul assay buffer, blank plus inhibitor well containing 90 ul buffer and 10 ul *E. scaber* extract (inhibitor), H_2O_2 wells contains 90 ul buffer and 10 ul H_2O_2 and H_2O_2 plus inhibitor wells consist of 80 ul assay buffer, 10 ul *E. scaber* extract and 10 ul H_2O_2. The reaction mixture was incubated for 5 min and the reaction was initiated with the addition of 10 ul of Linoleic acid. After 5 min incubation, the chromogen, provided with the kit was added and absorbance measured at 500 nm. The blank plus inhibitor wells with absorbance less than 0.22 and its corresponding H_2O_2 plus inhibitor wells exhibiting same absorbance as that of H_2O_2 wells were considered to be free of interfering compounds.

2.4. Isolation of 12-LO (crude extract) from human platelets

Fresh platelet-rich plasma (PRP) was collected from blood bank, Government Medical College, Kottayam. 12 LO enzyme was isolated from PRP according to the protocol of Waslidge and Hayes, 1995 [18]. In brief, PRP was centrifuged at 230*g* for 5 min for RBC removal, followed by 30 minutes incubation with 100uM acetylsalicylic acid and a 20 min centrifugation at 500g. The pellet obtained was resuspended in PBS-EDTA (2mM) solution and was further centrifuged at 230g for 5 minutes. The platelet containing supernatant was centrifuged at 500g for 20 min. The resulting pellet was resuspended in calcium free Tyrodes buffer, pH 7.4 and lysed with 0.1% v/v Triton X-100 and centrifuged to remove the cell debris and obtain the 12 LO enzyme which was used as the enzyme source.

The total protein content in the isolate was determined by Bradford's method with bovine serum albumin as the standard. Protein concentration of 100ng/ml was used for the enzyme inhibition studies.

2.5. Inhibition of 5-, 12- and 15-LO

The inhibition studies of 5-, 12- and 15-LO was done according to the protocol of Waslidge and Hayes (1995) with slight modifications in a 96-well microplate. An aliquot of 20 ul of pure enzyme in 50 mM Tris HCl buffer, pH 7.4 and inhibitor, *i.e.*, various concentrations of *E. scaber* extract ranging from 2.083 ng/ul to 16.167 ng/ul was pre-incubated at 25ºC for 10 min. Tris HCl buffer and the Fox reagent were kept as blank. To the pre incubated enzyme inhibitor mixture, 50 ul of 140uM linoleic acid (final concentration in a 120 ul system) in Tris HCl buffer, pH 7.4 was added and incubated in dark for 20 min at 25 ºC.

The assay measures the end product formed during the reaction, by incubating it with the FOX reagent for 30 min at 25ºC. FOX reagent was freshly prepared with 30 mM sulfuric acid, 100uM xylenol orange and 100uM iron (II) sulfate in methanol/water (9:1). After termination, the Fe3+ -dye complex developed was measured at 560nm on BIO RAD iMark Microplate reader. Nordihydroguaiaretic acid (NDGA) was used as standard inhibitor.

Percentage of inhibition of 5, 12 and 15 LO enzyme in the presence of *E. scaber* were calculated from $(A_C\text{-}A_T)/A_C \times 100$, where A_C is the absorbance of the control and A_T is the absorbance of the extract/standard. The graphs were plotted with inhibitor concentration on X-axis and % of inhibition on Y axis. IC_{50} values were determined from the graph.

2.6. Determination of the nature of inhibition by crude *E. scaber* extract

To the cuvette containing Tris HCl buffer, pH 7.4, an aliquot of 20 ul of enzyme was pre incubated with different concentration (0, 0.81ug/ml and 2.43 ug/ml) of the methanolic extract of *E. scaber*. To this enzyme-inhibitor complex, specific concentration of substrate ranging from 50 uM to 250 uM was added to initiate the reaction. The rate of product formation was measured spectrophotometrically at 234 nm. A Lineweaver Burk graph was plotted with the reciprocal of substrate concentration on X axis and the reciprocal of rate of reaction on y axis. The nature of inhibition was identified from this graph.

2.7. Analysis of data

All experiments were done as three independent test and the results were expressed as mean $\pm$ standard deviation. The statistical significance of differences between means was determined using Dunnett's test on sigma plot, Systat Software Inc. **(**SSI), San Jose, California and the values of at least $P<0.05$ were considered as statistically significant.

3. RESULTS

The plant extract diluted to a concentration of 0.086 mg/ml was found to be free of interfering compounds and hence was selected for enzyme inhibition studies. An aliquot of 100 ul of the third dilution was taken and the methanol was vaporized off and reconstituted in Tris HCl, pH 7.4 for 5, 12 and 15 LO inhibition studies.

3.1. Inhibition of LO enzyme by *E. scaber*

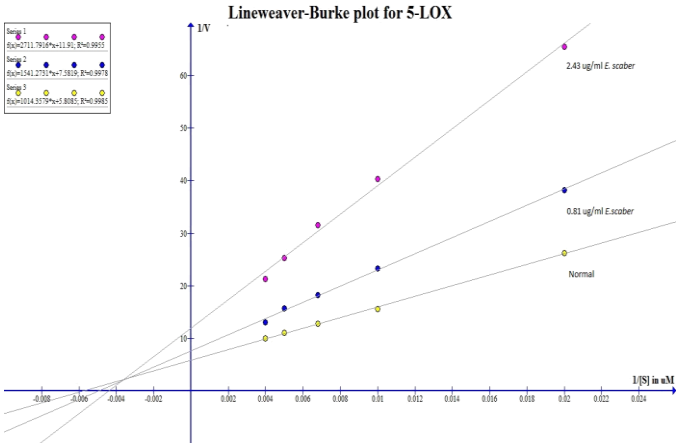
The inhibition of 5, 12 and 15 LO enzyme by the methanolic extract of *E. scaber* was determined by FOX assay with NDGA as the positive control. The plant extract exhibited inhibition of all the three LO enzymes. The *E. scaber* extract had an IC 50 value of 4.234ng/ul, 7.761ng/ul and 3.269ng/ul for 5, 12 and 15 LO respectively (Table 1)

Table 1: IC 50 value of E. scaber and NDGA in 5, 12 and 15 LO inhibition

Enzyme	IC 50 value (ng/ul)	
	E. scaber	NDGA
5 LO	4.234 ± 0.004	2.750 ± 0.050
12 LO	7.761 ± 0.179	0.302 ± 0.002
15 LO	3.269 ± 0.067	8.470 ± 0.150

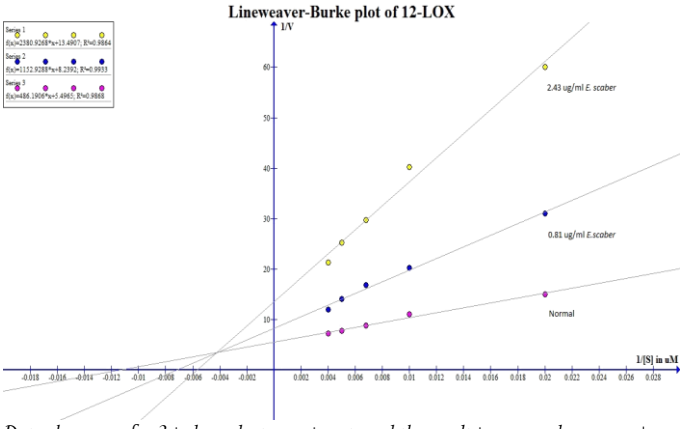
Data shown are for 3 independent experiments and the result is expressed as mean ± standard deviation.

Fig 1: Lineweaver-Burke plot showing mixed inhibition of 5-LO by crude E. scaber methanolic extract



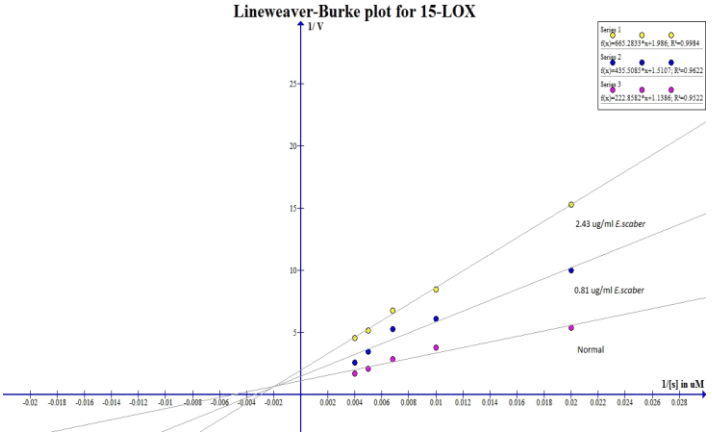
Data shown are for 3 independent experiments and the result is expressed as mean ± standard deviation

Fig 2: Lineweaver-Burke plot showing mixed inhibition of 12-LO by crude E. scaber methanolic extract



Data shown are for 3 independent experiments and the result is expressed as mean ± standard deviation

Fig 3: Lineweaver-Burke plot showing mixed inhibition of 15-LO by crude E. scaber methanolic extract



Data shown are for 3 independent experiments and the result is expressed as mean ± standard deviation

Table 2: Km and V max for mixed inhibition of* 5-, 12- and 15-LO *by crude E. scaber methanolic extract

Sample	5 LO		12 LO		15 LO	
	Km (uM)	Vmax (uMol/min /mg protein)	Km (uM)	Vmax (uMol/min /mg protein)	Km (uM)	Vmax (uMol/min /mg protein)
Free enzyme	172.413 ± 1.603	0.200 ± 0.019	90.909 ± 1.009	0.200 ± 0.051	192.308 ± 3.458	0.909 ± 0.009
Enzyme + [I] at a conc. Of 0.81 ug/ml	200 .000 ± 2.007	0.125 ± 0.037	142.857 ± 4.333	0.125 ± 0.017	263.158 ± 2.179	0.666 ± 0.049
Enzyme + [I] at a conc. Of 2.43 ug/ml	227.272 ± 2.713	0.081 ± 0.002	172.414 ± 3.511	0.071 ± 0.019	333.333 ± 2.413	0.500 ± 0.001

Data shown are for 3 independent experiments and the result is expressed as mean ± standard deviation. The free enzyme without inhibitor serves as the control

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3.2. Determination of nature of inhibition

The double reciprocal plot offers an easy way of determining the nature of inhibition as competitive, non competitive or mixed inhibition. In this case, the crude methanolic extract was found to inhibit all 5, 12 and 15 LO in competitive non competitive mixed fashion (fig 1, 2 & 3 respectively). The Km and V max values were calculated from Lineweaver burke plot and it was found that with increase in the concentration of inhibitor, the km value increased whereas the V max value decreased correspondingly (table 2).

4. DISCUSSION

The methanolic extract of *E. scaber* exhibited efficient inhibition of 5, 12 and 15 LO enzymes with an IC_{50} value of 4.234 ng/ul, 7.761 ng/ul and 3.269 ng/ul respectively. The abundance of LO enzymes and its metabolite products, cysteinyl leukotrienes, have been implicated in a number of pathological conditions. Reports suggest that the early and late phase of asthma is characterized by the over expression of 5 LO activating protein (FLAP). It is evident from the elevated levels of LTE4 in the urine sample of asthmatic patients. Anti leukotriene drugs were found to be very effective in the Early Asthmatic Reaction and Late Asthmatic Reactions, where it can either inhibit leukotriene biosynthesis or act as LT receptor antagonists [19, 20].

A good similarity was observed between 12 and 15 LO enzymes in terms of its reactivity and specificity towards substrate and are often classified as 12/15 LO [21] .These 12/15 LO products were found to have deleterious effects on many inflammatory signaling pathways and plays a significant role in atherosclerosis [22]. Over expression of 12/15 LO has been observed in a number of pathological conditions like hyper tension, restenosis, diabetes etc.. Zhong gao xu *et al*., 2008 [23] have reported that, increased AT1R mRNA and protein expression, via 12 (S) HETE pathway, is responsible for glomerulosclerosis and proteinuria.

Presently, very few drugs are commercially available as Lipoxygenase synthesis inhibitors or cysteinyl leukotrienes receptor antagonists like montelukast, zafirlukast, pranlukast, zileuton and NDGA. NDGA, the non selective inhibitor of Lipoxygenase enzyme inhibit many enzymatic pathway and hence prohibited from using as a food additive. Currently, it is in phase 2 clinical study against prostate cancer [24]. Most of these drugs possess side effects like nausea, syncope, cognitive disturbance, mild headache, flu like illness and severe sinusitis [25]. Though, the IC_{50} values for 5, 12 and 15 LO enzymes by *E. scaber* were lower comparing to NDGA, the concenteration (nanogram levels) at which it had shown inhibition indicate the potency of identifying new chemical entities and hence the kinetics of inhibition was carried out.

A mixed nature of inhibition was observed with methanolic extract of *E. scaber* on 5, 12 and 15 LO enzymes. The components in the *E. scaber* extract can bind to free enzyme or enzyme -substrate complex, in either way the amount of product, cysteinyl leukotrienes, formed is minimal. The ability of bioactive molecules present in methanolic extract of *E.scaber* to bind to both free enzyme as well as to enzyme substrate complex results in a net decrease in the availablity of free enzyme to catalyze the reaction and also from allowing accumulation of substrate which would have otherwise spilled over to other pathways.

5. CONCLUSION

The present study had exhibited the efficacy of *E. scaber* methanolic extract in the inhibition of 5, 12 and 15 LO enzymes in a mixed nature. Further study are being carried out to isolate and purify the bioactive molecules which would be involved in binding to the enzyme and enzyme substrate complex. In the present study, the dual inhibition of 5 and 12/15 LO enzymes by the methanolic extract of *E. scaber* could be assumed to have great therapeutic implications on chronic inflammatory pathological conditions like asthma, atherosclerosis and diabetes.

6. ACKNOWLEDGEMENT

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