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Dual COX/LOX inhibition: screening and evaluation of effect of medicinal plants of Kerala as Anti-inflammatory agents

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Abstract
Screening of 16 medicinal plants for potential anti-inflammatory activity through the dual inhibition of cyclooxygenase and lipoxygenase enzymes (synthesis of eicosanoids) and the evaluation of the phytochemicals in the plants exhibiting dual inhibition were carried out. The plant extracts were preincubated with the enzyme and assayed for LOX and COX activity by ELISA method. Plants with dual inhibition were subject to phytochemical analysis so as to determine the secondary metabolites present in these plants. Seven plants were identified with dual inhibition of LOX and COX enzymes. Saponins and terpenoids were found to be present in all the plant extracts with dual inhibition. The findings of the present study indicate the potential of seven medicinal plants in the use as anti-inflammatory agents. Further studies are being conducted to determine the bioactive molecules involved in the process of dual inhibition of LOX and COX.

Keywords: Lipoxygenase, Cyclooxygenase, Anti-inflammatory, Medicinal plants, Phytochemical analysis.

1. Introduction
Chronic inflammation is characterized by prolonged and persistent infection with massive tissue damage and destruction. The resolution of uncontrolled inflammatory response requires anti-inflammatory agents which includes both steroidal glucocorticoids, which can reduce cytokine induced gene expression [1] and non-steroidal agents that target the cyclooxygenase (COX) enzyme isoforms. Prolonged usage of these drugs result in unwanted side-effects include gastric ulceration [2], renal toxicity [3], joint destruction[4] and cardiovascular disorders [5, 6]. The inhibition of any single pathway in the arachidonic acid metabolism leads to a shunting of the fatty acid to the lipoxygenase (LOX) pathway leading to the formation of harmful leukotrienes and the side effects [7]. The current strategy includes the development of dual LOX/COX inhibitors with a higher safety profile, particularly medicinal plants of folkloric use as pain relievers and anti-inflammatory agents [8]. Pharmacological validations on Indian medicinal plants are limited and the enormous potential of the different plants used in Ayurveda and tribal folklore has not been exploited for their effective use in medicinal therapeutics.

2. Materials and Methods
2.1 Materials
Ultrapure Water, COX Inhibitor Screening Assay Kits and LOX Inhibitor Screening Assay Kits were obtained from Cayman Chemicals, USA. All other reagents and solvents used were of analytical grade from Merck Limited, India and from HiMedia Laboratories, India. 96 well plates were read on the iMark Microplate Absorbance Reader and washed with the Model 1575 Immunowash Microplate Washer from BioRad, India. Spectrophotometric assays were conducted on UV1800 Spectrophotometer from Shimadzu, Japan.

2.2 Collection and Identification of plants
Fresh plant materials were collected from local regions of Kottayam, Kerala. The plant materials were washed repeatedly, cleaned thoroughly and shade dried. The dried materials were then powdered and stored in airtight containers until further use. The plants were authenticated by the Taxonomist at Department of Botany, St. Thomas College, Palai, and Kottayam. The details of the plant, plant parts used, and their vernacular names are given in Table 1.

Table 1: List of plants selected for screening of Dual LOX/COX inhibition

S. No.	Name Of Plant	Family	Vernacular name	Part Used
1	*Tinospora cordifolia*	Menispermaceae	Amruthu	Stem
2	*Pongamia pinnata*	Fabaceae	Ungu	Leaf, Bark
3	*Rubia cordifolia*	Rubiaceae	Manchatti	Stem
4	*Elephantopus scaber*	Asteraceae	Anachuvadi	Whole Plant
5	*Piper longum*	Piperaceae	Thippali	Fruit
6	*Lawsonia inermis*	Lythraceae	Mailanchi	Leaf
7	*Tylophora asthmatica*	Apocynaceae	Vallipala	Leaf
8	*Alpinia calcarata*	Zingiberaceae	Chittarata	Rhizome
9	*Acorus calamus*	Acoraceae	Vayambu	Rhizome
10	*Vernonia cinerea*	Asteraceae	Poovamkurunnila	Whole plant
11	*Hemigraphis colorata*	Acanthaceae	Murikooti	Whole plant
12	*Chromolaena odorata*	Asteraceae	Communist pacha	Whole plant
13	*Vernonia anthelmintica*	Ranunculaceae	Karinjeerakam	Seed
14	*Woodfordia fruticosa*	Lythraceae	Thathiripoove	Flower
15	*Cuminum cyminum*	Apiaceae	Jeerakam	Seed
16	*Foeniculum vulgare*	Asteraceae	Perumjeerakam	Seed

2.3 Extraction of plant materials

The dried powder of the plant materials (40 g) were extracted exhaustively first with hexane in a Soxhlet apparatus to defeat the sample. The supernatant obtained was removed and any traces of the solvent were removed from the residual sample material. The plant material was then extracted with methanol in a Soxhlet apparatus at 40 ˚C. The extraction was considered complete when the solvent in thimble became colourless. The methanol extract was subsequently concentrated to dryness and stored desiccated at 4 ˚C till further use. The yield was recorded.

2.4 Assay for Screening of Lipoxygenase (LOX) Inhibition

The Lipoxygenase Inhibitor Screening Assay Kit detects the hydroperoxides produced in the lipoxygenation reaction [9] using a purified LOX and is used to screen for inhibitors of LOX enzyme. The assay protocol requires the addition of plant extract to the enzyme prior to start of reaction followed by initiation of reaction with the addition of substrate. Blank, Positive control and 100% initial activity reactions were also performed. The 96-well plate was placed on a shaker for at least five minutes, followed by the addition of chromogen to stop enzyme catalysis. The plate was then covered and placed on a shaker for five minutes. The absorbance was measured at 490-500 nm using a plate reader after removing the plate cover. The percent inhibition for each inhibitor can be calculated using the following equation:

Percent Inhibition (%) =

$$\frac{(\text{Activity of Control} - \text{Activity of Test})}{\text{Activity of Control}} \times 100$$

A graph was drawn with the Percent Inhibition as a function of the inhibitor concentration to determine the IC_{50} value (concentration at which there was 50% inhibition).

2.5 Assay for Screening of Cyclooxygenase (COX) Inhibition

The COX Inhibitor Screening Assay directly measures $PGF_{2\alpha}$ by stannous chloride reduction of COX-derived PGH_2 produced in the COX reaction [10]. The reaction system consists of reaction buffer, haem, enzyme and plant extract pre-incubated at 37 ˚C for twenty minutes with background and enzyme controls. The reaction was initiated with the addition of arachidonic acid and incubated for two minutes at 37 °C. The reaction was stopped with addition of saturated stannous chloride solution and five minutes at room temperature. The prostaglandins are quantified by EIA. An aliquot of these reactions were added to the precoated plates in triplicates together with AChE tracer and antiserum and incubated for 18 hours at room temperature on an orbital shaker. The plate was then finally developed with Ellman's Reagent and kept on an orbital shaker in the dark at room temperature for 60 minutes. The absorbance was read at 420 nm. The data was plotted as $\%B/B_0$ (Standard Bound / Maximum Bound) versus log concentration using a 4-parameter logistic curve fit. The concentration of each sample was determined from a standard curve with appropriate dilutions and used to calculate the percent inhibition as per the formula given below:

Percent Inhibition (%) =

$$\frac{(\text{Activity of Control} - \text{Activity of Test})}{\text{Activity of Control}} \times 100$$

The percent inhibition was plotted against the inhibitor concentration to determine the IC_{50} value (concentration at which there was 50% inhibition).

2.6 Qualitative Analysis of Phytochemical Constituents

Preliminary chemical tests were carried out for the methanolic extracts of plants identified as dual inhibitors of COX and LOX enzymes to identify different phytochemical constituents present in the plants as per the protocol described by Harborne[11]. A (+) score was recorded if the reagent produced only a slight positive reaction; a (++) score for a definitive positive reaction and a (+++) score was recorded if heavy reactions were obtained.

Statistical analyses

In all the studies, the values of three independent experiments were expressed as mean ± standard deviation (S.D.) for n determinations where n=3 unless otherwise stated. Data analyses were performed using SigmaPlot version 12.5. The significance of differences from the respective controls was tested using one way ANOVA (Dunnett's test) for each set of experiments.

3. Results

3.1 Yield and dilution of extract for assay

The percentage yield of methanol extracts of plants was as given in Table 2. 10 mg of each extract was further reconstituted in DMSO and serially diluted as per description in ELISA assay kits to a final concentration of 1.25 mg/ml for the screening of LOX and COX inhibition.

Table 2: Percent Yield of Plant Extracts

Sl. No.	Name of Plant	Weight of sample(g)	Percent Yield in hexane (%)	Percent Yield in methanol (%)
1	*Tinospora cordifolia*	20	0.285	6.94
2	*Pongamia pinnata*	20	0.425	4.68
3	*Rubia cordifolia*	20	0.135	9.97
4	*Elephantopus scaber*	20	0.23	5.64
5	*Piper longum*	20	3.97	7.64
6	*Lawsonia inermis*	20	0.39	5.46
7	*Tylophora indica*	20	0.23	6.31
8	*Alpinia calcarata*	20	0.42	5.24
9	*Acorus calamus*	20	0.39	5.97
10	*Vernonia cinerea*	20	0.12	6.43
11	*Hemigraphis colorata*	20	0.065	6.03
12	*Chromolaena odorata*	20	0.08	5.21
13	*Nigella sativa*	20	0.295	5.83
14	*Woodfordia fruticosa*	20	0.23	5.37
15	*Cuminum cyminum*	20	3.23	6.24
16	*Foeniculum vulgare*	20	3.98	5.64

3.2 Screening of plant extracts for effect on LOX activity

The plant extracts reconstituted in DMSO were used as per protocol for evaluation of effect on the activity of LOX and the results of IC_{50} values are as shown in Figure 1. All the plant extracts show a potent inhibition of the 15-LOX enzyme as compared to the positive control, NDGA, and results are expressed as percent of inhibition of LOX activity.

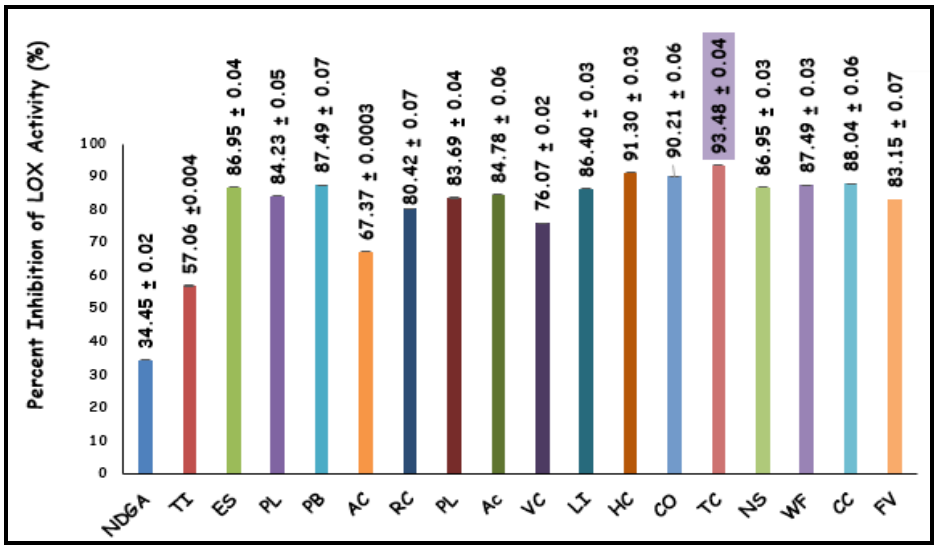

Fig 1: Effect of methanol extracts of medicinal plants on LOX activity

The LOX activity of the plant extracts were compared with the positive control NDGA and all the plants show inhibition of LOX activity. The values are expressed as Mean ± SD of three independent experiments and significance $P<0.001$ as compared to positive control. Abbrev.: NDGA (Nordihydroguaiaretic Acid), TI (*T. indica),* ES (*E. scaber*), PL (*Pongamia* leaf), PB (*Pongamia* bark), AC (*A. calamus*), RC (*R. cordifolia*), Pl (*P. longum*), Ac (*A. calcarata*), VC (*V. cinerea*), LI (*L. inermis*), HC (*H. colorata*), CO (*C. odorata*), TC (*T. cordifolia*), NS (*N. sativa*), WF (*W. fruticosa*), CC (*C. cyminum*), FV (*F. vulgare*).

3.3 Screening of plant extracts for effect on COX activity

All plant extracts were used as per protocol to study the effect on the activity of COX and the results are as shown in Figure 2. Seven plant extracts showed a potent significant inhibition of the COX-2 enzyme as compared to the positive control, Ibuprofen, and results are expressed as percent of inhibition of LOX activity.

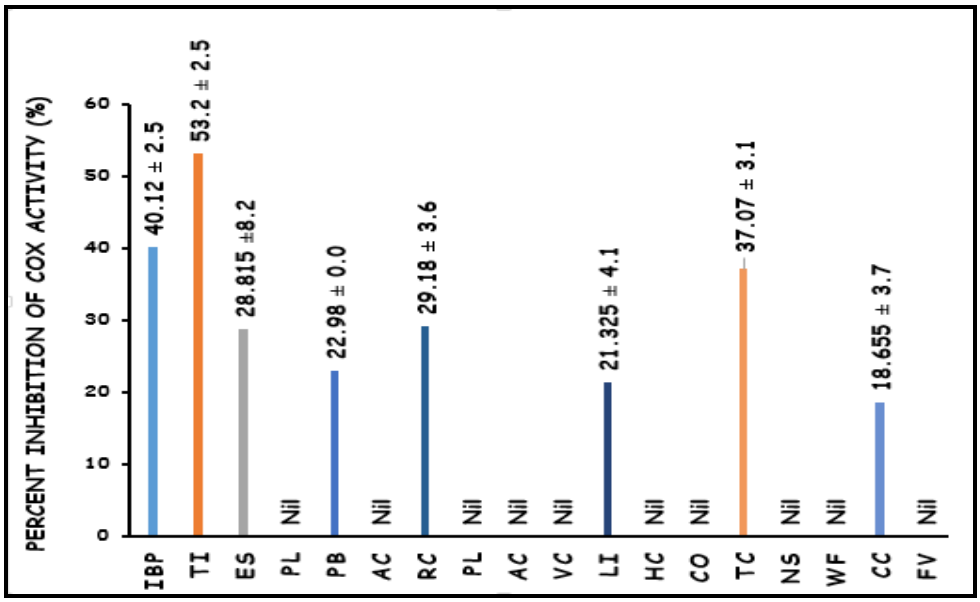

Fig 2: Effect of Methanol Extracts of Medicinal Plants on COX Activity

The plant extracts were screened for COX-2 inhibitory activity and only 7 plants have shown an inhibition for COX. The values are expressed in Mean ± SD of three independent experiments and significance P<0.001 to positive control. Abbreviations: IBP (Ibuprofen),TI (*T. indica),* ES (*E. scaber*), PL (*Pongamia* leaf), PB (*Pongamia* bark), AC (*A. calamus*), RC (*R. cordifolia*), Pl (*P. longum*), Ac (*A. calcarata*), VC (*V. cinerea*), LI (*L. inermis*), HC (*H. colorata*), CO (*C. odorata*), TC (*T. cordifolia*), NS (*N. sativa*), WF (*W. fruticosa*), CC (*C. cyminum*), FV (*F. vulgare*).

3.4 Identification of plants with dual inhibition

The results of the LOX and COX enzyme inhibition screening resulted in the determination and selection of plants with dual inhibition of these enzymes. The plants with dual inhibition are as shown in Table 3.

Table 3: Plants with Dual Inhibition of LOX/COX Enzymes

Sl. No.	Name of Plant (Abbrev.)	Percent of Inhibition (%)	
		LOX	COX
1	*Tylophora indica* (TI)	57.06 ± 0.004	53.2 ± 2.57
2	*Elephantopus scaber* (ES)	86.95 ± 0.043	28.82 ± 8.25
3	*Pongamia pinnata* Bark (PB)	87.49 ± 0.068	22.98 ± 0.08
4	*Rubia cordifolia* (RC)	80.42 ±0.068	29.18 ± 3.61
5	*Lawsonia inermis* (LI)	86.4 ± 0.031	21.33 ± 4.11
6	*Tinospora cordifolia* (TC)	93.48 ± 0.037	37.07 ± 3.08
7	*Cuminum cyminum* (CC)	88.04 ± 0.059	18.66 ± 3.77

Plants with dual inhibition of LOX and COX were selected based on the screening tests for LOX and COX.

3.5 Phytochemical analysis of plants with dual inhibition

The results of the qualitative phytochemical analysis of the methanol extract of plants analysed with dual inhibition of LOX and COX are shown in Table 4. All the plant extracts show the presence of terpenoids and saponins while flavonoid was found only in *P. pinnata* and *R. cordifolia.*

Table 4: Phytochemical Investigations on Methanol Extract of Plants with Dual Inhibition

Name of plant	Alkaloid	Flavans		Cardiac Glycoside	Saponin	Terpenoid	Tannin	Reducing Sugar
		Fld*	Fl#					
P. pinnata	++	++	-	++	+++	+++	-	-
T. cordifolia	+	-	+	+	+	+++	+	+
C. cyminum	+	-	+	-	+++	++	++	+
E. scaber	++	-	-	++	++	++	-	-
L. inermis	-	-	-	+	++	+	+	+
R. cordifolia	++	++	-	+	++	++++	++	++
T. indica	+	-	-	-	+	+	-	-

(-): No presence, (+): Low presence, (++): Moderate presence, (+++): High presence *Fld stands for flavanoids and #Fl stands for flavones

4. Discussion

The importance of the dual inhibition of LOX and COX lies in the effective reduction of chronic inflammatory conditions. COX inhibition has been reported to be associated with loss of the gastrointestinal integrity leading to the development of gastric ulcers [12]. The gastrointestinal integrity of the host remains protected with the dual inhibition of the enzymes as is clinically evidenced in other studies [13]. The incidence of cardiovascular disorders in selective COX-2 inhibition is also reduced in such situations of dual inhibition [14]. The use of medicinal plants for the treatment of chronic inflammatory conditions has existed for a long time but the validation for such use has been of interest in the scientific community only recently. 17 plant extracts were screened for dual inhibition. The plants were selected based on reported anti-inflammatory activities. Seven plants exhibiting COX-2 inhibition was considered to be dual inhibitors. These studies indicate that the plants with dual inhibition have been reported with anti-inflammatory activities but with no studies for correlation of this effect on LOX/COX inhibition. The results presented in this section have conclusively established the presence of bioactive molecules in these plant extracts capable of dual inhibition of LOX/COX enzymes involved in biosynthesis of pro-inflammatory leukotrienes and prostaglandins. The identification of secondary metabolites present in plant extracts could provide lead-like compounds involved in the anti-inflammatory activity reported in these plant extracts. The phytochemical analysis of these plant extracts indicates the presence of terpenoids, saponins and alkaloids. The presence of flavonoids is found in *P. pinnata*, *R. cordifolia, T. cordifolia* and *C. cyminum* plant extracts. The extracts exhibit weak positive results for cardiac glycosides, tannins and reducing sugars.

The medicinal plants used for the present study are routinely used in ayurvedic formulations and folkloric medicines of Kerala. The findings of the present study reveal the presence of potential bioactive molecules capable of dual inhibition of LOX and COX enzymes. The findings of the present study open up new avenues for identification of bioactive molecules which could be used for development of safer anti-inflammatory molecules compared to current anti-inflammatory NSAIDs. The exact nature of activity of the plant extract and the bioactive compounds involved are being investigated.

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