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Original Article

EVALUATION OF *IN VITRO* ANTI-INFLAMMATORY ACTIVITY AND HPTLC ANALYSIS OF PLANT *PHYLLANTHUS FRATERNUS*

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ABSTRACT

Objective: The present investigation evaluated *in vitro* anti-inflammatory activity of *Phyllanthus fraternus.* Inhibition of Cyclooxygenase and 5-lipoxygenase was performed along with protein denaturation.

Methods: Alcoholic extract of plant was subjected to *in vitro* anti-inflammatory activity and HPTLC analysis.

Results: The results of anti-inflammatory activity showed significant inhibition in Cyclooxygenase and 5-lipoxygenase assay, extract also showed more than 70 % inhibition in protein denaturation method. HPTLC of plant materials was also performed; spots of alkaloids were recorded.

Conclusion: Different alkaloids were spotted in chromatographic analysis and study suggested that anti-inflammatory activity of *Phyllanthus fraternus* may be due to the presence of alkaloids.

Keywords*:* Anti-inflammatory, *Phyllanthus Fraternus*, HPTLC, Cyclooxygenase, Lipoxygenase

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INTRODUCTION

Inflammation is a process which associated with pain and various biochemical responses such as; protein denaturation, an increase of vascular permeability and membrane alteration. When protein loses its structural integrity due to the external stress then this is called protein denaturation; which involves reduced biological functioning of the protein. Protein denaturation is considered as an important cause of inflammation. The cascade of an inflammatory process initiated from arachidonic acid which forms prostaglandins (PGs) and thromboxane *via* Cyclooxygenase (COX) pathway and hydroperoxy-eicosatetraenoic acids (HPETE's) and leukotrienes (LT's) *via* 5-lipoxygenase pathway; these all mediators leads inflammatory events. The process of 5-LOX and COX inhibition adopted as an anti-inflammatory concept since inhibition of 5-LOX and COX also inhibit production of inflammatory mediators such as; LTs and PGs and thus offer anti-inflammatory responses. Therefore the agent which possesses the ability to inhibit 5-LOX and COX provide anti-inflammatory and analgesic effects with reduced GI side-effects [1-4].

Plants and plant products act as natural sources for various bioactive compounds which possess diversified biological activities such as; anti-hepatotoxic, anti-dysentric, anti-inflammatory, anti-spasmodic, anti-viral, anti-diabetes, wound healing and anti-cancer properties. The primary and secondary metabolites of plants are considered responsible for these properties. The biologically active constituent of plant material may reside in any part of a plant like; leaf, stem, root, seed and in the whole plant. Various researchers investigated and isolated different phytoconstituents as anti-inflammatory agents, the some plant alkaloids known to have anti-inflammatory properties along with other biological activities [2-5]. This article evaluated anti-inflammatory activity along with chromatographic estimations of alkaloids of *Phyllanthus fraternus.*

Phyllanthus fraternus Webster known as *Bhoi amli* is an important medicinal plant, belongs from family *Euphorbiaceae,* chemically it possess constituents such as; Phyllanthin, Hypophyllanthin, Phyllnirurin, Phyllanthenol, Phyllanthol, Rhamnopyrenoside, Phyllanthenone, Lintetralin, Astragalin, Cymene, Niranthin, Niruriside, Nirtetralin, Phyllochrysine, 4-Methoxy-Nirsecurinine, Niruretin, Limonene and Nirurin (Alkaloids). It contains Steroids; β-Sitosterol and Cholesterol, Flavonoids; Quercetin, Quercetol and 3, 4, 5-Trimethoxy flavanone, Saponins; triacontanol and triacontanol. The plant also contains compounds like; Carilagin, Estradiol, Ellagic acid, Rutin, Rutinoside, Gernanine and Methyl salicylate.

The *Phyllanthus fraternus* also known to have diversified biological actions such as; carminative, diuretic and astringent properties. It possesses anti-dysentric, anti-inflammatory, antispasmodic, antiseptic and anti-viral activities. The plant is also used in vertilago, malaria, diabetes, jaundice, indigestion, anemia, gout, cough, dermatosis, urinary disease and vaginitis [5-8].

MATERIALS AND METHODS

Plant materials were collected and subjected for extraction. All the reagents and solvents used were of analytical grade.

Extraction

Powdered plant materials were extracted with methanol then cooled and filtered through filter paper (Whatman No. 1) followed by centrifugation for 10 min, further diluted in ratio of 1:15 with the same solvent, the procedure repeated for several time to obtained concentrated extract and final extract was used for further experiments except for HPTLC analysis [9].

In vitro anti-inflammatory activity [10-16]

Inhibition of protein denaturation

The reaction mixture consisted of 0.45 ml of bovine serum albumin (5% aqueous solution) and 0.05 ml of plant extracts in various concentrations; pH was adjusted at 6.3 using 1N hydrochloric acid. Heated at 57 °C for 3 min after incubating at 37 °C for 20 min., that

after 2.5 ml of phosphate buffer solution was added into each test tube. Turbidity was measured spectrophotometrically at 600 nm. Distilled water (0.05 ml) was used instead of extracts as control tests; while product control tests lacked bovine serum albumin.

The percentage inhibition of protein denaturation was calculated as follows:

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

Assay of cyclooxygenase and 5-lipoxygenase

Lymphocyte culture preparation

Human peripheral lymphocytes were cultured in RPMI 1640 media, supplemented with fetal bovine serum, Penicillin and Streptomycin were used as antibiotics. Phytohaemagglutinin was used as the stimulant for cell proliferation, 0.2 µm sized cellulose acetate filter was used to filter culture in aseptic conditions. Fresh plasma was added to the culture in 1 x 106 cells/ml concentrations; culture was incubated for 72 h. The culture was activated by adding 1 µl lipopolysaccharide, extracts were added at 24 hr of incubation. Ibuprofen was added as standard. After incubation, the cells were pelleted by centrifugation. The isolation was done by spinning at 6000 rpm for 10 min, 50 µl of cell lysis buffer was added after discarding supernatant and the anti-inflammatory assay was done in pellet suspended in a small amount of supernatant.

Assay of cyclooxygenase

Tris-HCl buffer, glutathione, haemoglobin and enzyme was used as assay mixture. The arachidonic acid was added initially and after 20 min. incubated at 37 °C adding 0.2 ml of 10% trichloroacetic acid in 1N HCl and 0.2 ml of thiobarbituric acid, that after contents were heated in 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.

Assay of 5-lipoxygenase

Linoleic acid along with tween 20 was dissolved in oxygen free water avoiding air bubbles, 0.5 N sodium hydroxide was added sufficiently to produce a clear solution and then the volume was made up to 25 ml using oxygen free water. This was flushed with nitrogen gas before closing and kept frozen until needed after dividing into 0.5 ml portions. The assay mixture consisted of 2.75 ml Tris buffer of pH 7.4, 0.2 ml of sodium linoleate and 50 mcl of the enzyme. Optical density was measured at 234 nm.

High-performance thin layer chromatography (HPTLC) [17-20]

Isolation of alkaloids

Dried powdered sample was suspended in methanol, stirred and filtered. The sample was further washed with methanol and filtrate was collected. After repeating process alcoholic filtrates were mixed together and concentrated. The dried content was dissolved in a mixture of ethyl acetate and 1N HCl in equal amount. The acidic aqueous portion was removed while remaining fraction of ethyl acetate was repeatedly washed with 1N HCl. The acidic aqueous fraction was neutralized with sodium bicarbonate and pH was adjusted at 10 using NaOH solution and then partitioned with ethyl acetate. The ethyl acetate fractions were evaporated to dryness and dry residue was triturated with dichloromethane and filtered. The filtrate was used for indole alkaloids.

Chromatographic condition

HPTLC precoated plate, silica gel 60 F254, 10 X 10 cm^2, thickness 250 µm were used. Samples were injected using Linomat injector. Methanol: Ethyl acetate (2:8) was used as mobile phase. 1% Cerric ammonium sulphate was used as spraying reagent. Relative humidity and temperature were 52% and 24 °C respectively. The 80 mm migration distance was allowed for 30 min. Densitometric scanning was performed using UV detector at 254 nm. Ascending separation technique was used in twin-trough glass chamber (10 X 10 cm^2) which was used as development chamber; saturation of chamber was done prior to development.

RESULTS AND DISCUSSION

***In vitro* anti-inflammatory activity**

The extract of *Phyllanthus fraternus* was analyzed for anti inflammatory activity using various *in vitro* models. The results of the study proved that extract possesses ability to inhibit denaturation of proteins and thus may offer significant relief in inflammation. The inhibition of heat induced albumin denaturation by sample extract presented in table 1. The evaluation of cyclooxygenase and 5-lipoxygenase inhibitory activity of plant extract was also performed. The results are tabulated in table 2, the results of COX and 5LOX assay also compared and presented in fig. **1**, as result indicated more COX inhibition was observed as compared to 5-LOX inhibition. The results of the study suggested that the plant extract may reduce productions of inflammatory mediators such as; prostaglandins and leukotriene's since it significantly inhibits cyclooxygenase and 5-lipoxygenase respectively; this anti-inflammatory activity of plant extract may be due to the presence of alkaloids and polyphenols.

It has been reported that the inhibition of prostaglandins and leukotriene leads anti-inflammatory response. The inhibition of COX may cause gastric side effects due to the possible mucosal damage; however inhibition of 5-LOX decreases the production of a compound which may cause gastric damage; thus inhibition of COX along with 5-LOX recommended to achieve maximum anti-inflammatory activity with gastric safety. This dual inhibition prevents the production of prostaglandins and leukotrienes resulting inhibition of migration and activation of inflammatory cells at the sites of inflammation. The inhibition of this inflammatory cascade also reduces tissue damage or necrosis. The results of study suggested that *Phyllanthus fraternus* plant extract may be used as anti-inflammatory agent with gastric safety.

Table 1: Results of protein denaturation assay

S. No.	Concentration (mcg/ml)	% inhibition
1	100	32.21
2	150	45.54
3	200	57.55
4	250	66.55
5	300	72.23

Table 2: Results of cyclooxygenase and 5-lipoxygenase assay

S. No.	Concentration (mcg/ml)	% inhibition	
		COX assay	5-LOX assay
1	50	33.11	22.31
2	100	36.14	35.34
3	150	49.12	46.53
4	200	60.22	59.15
5	250	73.43	70.21

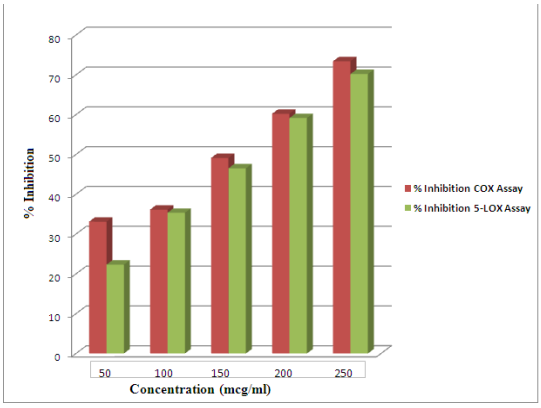

Fig. 1: Comparison of COX and 5-LOX assay

HPTLC analysis was also performed to identify alkaloid of plant extract of P. *fraternus*. HPTLC results identified peaks of alkaloids as mentioned in fig. 2. Densitometric chromatograms of alkaloids were observed with different peak area which proved that various alkaloids found in different concentrations. The correlation coefficient suggested a linear relationship. The presence of alkaloids in *P. fraternus* confirmed by HPTLC analysis and these compounds may be considered responsible for the investigated anti-inflammatory activity.

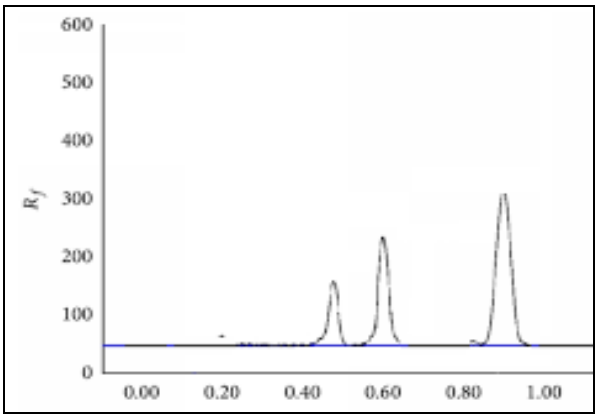

Fig. 2: HPTLC chromatograms of alkaloids present in *Phyllanthus fraternus*

CONCLUSION

Alcoholic extract of plant *Phyllanthus fraternus* was subjected to *in vitro* anti-inflammatory activity and HPTLC analysis. The results of anti-inflammatory activity observed significant inhibition of Cyclooxygenase and 5-lipoxygenase, the present investigation concluded that *Phyllanthus fraternus* may be used as potent anti-inflammatory agents with higher gastric safety profile. The results of protein denaturation assay also proved anti-inflammatory profile of *Phyllanthus fraternus*. HPTLC analysis confirmed presence of alkaloids in plant extract and these alkaloids may be responsible for the anti-inflammatory activity of *Phyllanthus fraternus*.

CONFLICT OF INTERESTS

Declare none

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