RESEARCH ARTICLE

INHIBITION OF COX-2 ENZYME BY THE VARIOUS EXTRACTS OF *LEUCAS ASPERA* FOR ANTI-INFLAMMATORY ACTIVITY IN ALBINO RAT

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ABSTRACT

The present investigation was aimed at evaluating the ethanol extract of leaves of *Leucas aspera* has been found to possess antibacterial activity and Anti-inflammatory Activity, Cyclooxygenase (COX), also called Prostaglandin H Synthase or (PGHS) is a bifunctional enzyme exhibiting both cyclooxygenase and peroxidase activities. COX-2 products are responsible for modulating dynamic processes such as inflammation. COX-2 is the true molecular target for developing anti-inflammatory and analgesic agents. Pathogen free, Wistar strain albino rats were used in the present study, In order to study the anti-inflammatory activity of four extracts of *Leucas aspera* viz., petroleum ether, chloroform, ethanol, water extracts .whereas the purified fraction F_5 of ethanol extract were screened for their *in vivo* COX-2 enzyme inhibition assay, And the purified fraction (F_5) of ethanol extract it is essential to carry out the COX enzyme inhibition assay. The fraction F_5 of ethanol extract of *L. aspera* has shown significant COX-2 enzyme inhibition activity. The chemical molecule present in the fraction F_5 is responsible for the inhibition of COX-2 enzyme present in the blood plasma of experimental rats. The present study reveals that ethanolic extract of *leucas aspera* spreng showed significant anti-inflammatory activity and COX- II enzyme inhibition studies.

INTRODUCTION

Since thousands of years, humans have relied almost entirely on plants to treat all manners of illness; from minor problems to life-threatening diseases (Chevallier, 2001). About 80% of the people still depend largely on traditional plant derived drugs for their primary health care (Akerele, 1993). In the developing countries, the herbal medicine is deep rooted even today. Traditional practitioners play important role in their communities particularly with regard to common ailments and chronic disorders (Bannerman, 1982). The discovery of the penicillin from microbes and isolation of antimalarial alkaloids, cardiotonic glycosides, anticancer agents etc., from plants are the significant evidences for importance of natural products for the benefit of mankind. *Leucas aspera* Spreng (Labiatae) is an annual herb found throughout India as a weed in cultivated fields, waste lands and roadsides. Its leaves, roots, stalks, young shoots and flowers have been used for wide range of medicinal properties (Kirtikar and Basu, 1991). Literature survey revealed that ethanol extract of leaves of

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Leucas aspera has been found to possess antibacterial activity and Anti-inflammatory Activity (Shirazi, 1947), Anti-inflammatory agents may thus act by interfering with any one of several mechanisms including immunologic mechanisms such as antibody production, antigen-antibody complexation, activation of complement, cellular activities such as phagocytosis, formation and release of chemical mediators of inflammation and stabilization of lysosomal membranes. For many years COX was thought to be a single enzyme. The l990s saw a new dawn in this area with biological studies demonstrating increased COX activity in a variety of cells after exposure to endotoxin (Fuj *et al.,* 1990; Masferrer *et al.,* 1990), proinflammatory cytokines (Raz *et al.,* 1989; Risti Maci *et al.,* 1994) growth factors hormones (Sirois and Richard, 1993); tumor promoters (Kujubu *et al.,* 1991). This activity required new protein synthesis and was inhibitable by corticosteroids. These data suggested the existence of a second novel COX isoenzyme synthesised *de novo* in the presence of inflammatory stimuli and the concept of constitutive COX-1 and inducible COX-2. COX-l products are responsible for normal homeostasis i.e. the house keeping functions whereas COX-2 products are responsible for modulating dynamic processes such as inflammation. NSAIDs inhibit the two
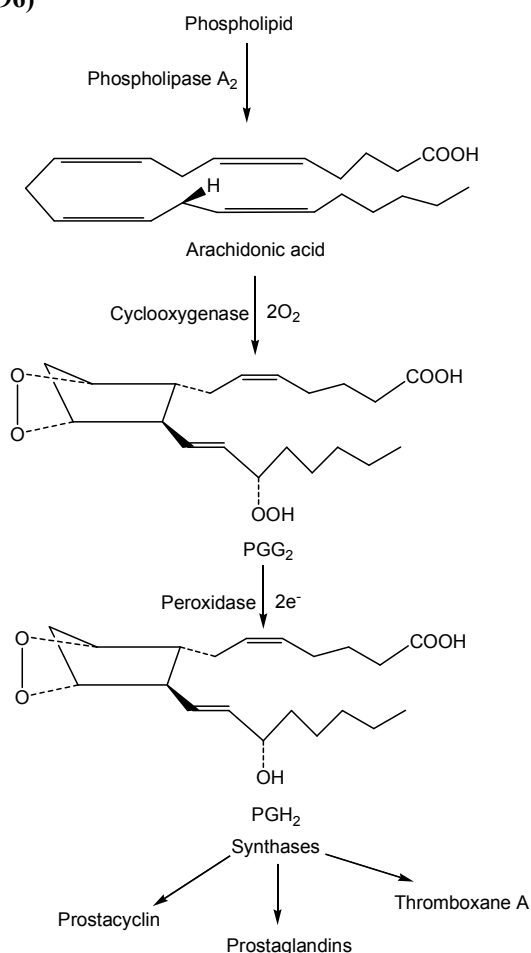
isoforms to different extents and this feature accounts for their shared therapeutic properties and side effects (Vane *et al.,* 1998).

Cyclooxygenase isoenzymes

Cyclooxygenase enzyme also known as prostaglandin endoperoxide H synthase. (PGHS) are bifunctional heme containing proteins with both cyclooxygenase and peroxidase activities (Needleman *et al.,* 1986; Dewitt,DL 1991). Because the cyclooxygenase activity catalyzes the first step in the biosynthesis of prostaglandin endoperoxides the protein is frequently referred to by the name of this activity (COX). COX catalyzes the conversion of arachidonic acid to prostaglandin PGH$_2$ (Chart 1), the later being the essential precursor of prostaglandins, thromboxane and prostacyclin. In both COX-1 and COX-2, Tyr 385 is the catalytic active site. Arachidonic acid lies in a bent conformation with its C-13 in the vicinity of Tyr 385 and the carboxylate group interacting with Arg 120. Initially the heme group of the enzyme is oxidized at the peroxidase active site. The oxidized heme group of the enzyme is oxidized at the peroxidase active site. The oxidized heme group then oxidizes the Try 385 and forms the tyrosyl radical, which abstracts the 13S-hydrogen from the arachidonic acid and allows the subsequent insertion of molecular oxygen at C-11. The resulting 11-peroxyl radical initiates cyclization events that produces, 9,11-endoperoxide into which another molecule of oxygen inserts to form 15-peroxyl radical. The donation of a hydrogen atom back to the substrate creates PGG$_2$ (Picot *et al.,* 1994). PGG$_2$ undergoes a two electron reduction at the peroxidase active site to form PGH$_2$ (Rowlinson *et al.,* 2000).

Chart-1:   The prostanoid biosynthetic pathway (Smith *et al.,* 1996)

Phospholipid

Phospholipase A$_2$ ↓

Arachidonic acid

Cyclooxygenase | 2O$_2$ ↓

PGG$_2$

Peroxidase | 2e⁻ ↓

PGH$_2$

Synthases

Prostacyclin   Prostaglandins   Thromboxane A

NSAIDS compete directly with arachidonate for binding to the cyclooxygenase active site and inhibit COX activity by excluding arachidonate from the active site.

Physicochemical properties

Cyclooxygenase-l and 2 are homodimeric, heme containing, glycosylated integral membrane proteins with two catalytic sites (Jouzeau *et al.,* 1997). Both COX isoforms are seen in most mammals. In humans, the COX- 1 gene is on chromosome 9 and the COX-2 gene is on chromosome 1 (Bjorkman, 1998). Genetic analysis shows that for a given COX isoform there is approximately 90% identity between species, but there is only 60% homology between the two COXs at the nucleic acid and amino acid levels. The signal peptide for COX-1 is seven residues longer than that of COX-2. COX-1 contains a sequence of 17 amino acids in its amino terminus that is not present in COX-2, while COX-2 has an additional insert of 18 amino acids in its carboxyl terminus (Jouzeau *et al.,* 1997).

Amino acid residue	COX-1	COX-2
434	Ile	Val
513	His	Arg
516	Ser	Ala
523	Ile	Val

Source: The amino acids thought to be important for catalysis are conserved. However, there are some differences in the active site (De Witt *et al.,* 1993).

The presence of Val 523 in COX-2 instead of Ile in COX- 1 plays a crucial role in selectivity of NSAIDs for COX-2 enzyme (Kurumbail *et al.,* 1996). The two isoenzymes have about same affinity (K_m) and capacity (V_{max}) to convert arachidonic acid to PGH$_2$ (Percival *et al.,* 1994). COX-2 is also able to metabolize C18 and C20:3 fatty acids as substrates, while COX-1 is mainly restricted to arachidonic acid (C20:4).

Regulation and localization

The two COX isoenzymes are distinctly regulated at both transcriptional and post transcriptional level (Smith and Dewitt, 1996; Herschmann, 1996; Williams and Dusois, 1996). The transcripts of COX-1 and COX-2 differ significantly in size. The transcript of COX-2 gene is prone to degrade quickly. COX-2 is undetectable in most tissue but can be rapidly induced in fibroblasts, ovarian follicles, endothelial cells, monocytes in response to growth factors, tumor promoters, hormones, bacterial endotoxins and cytokines and hence called inducible. Anti-inflammatory steroids inhibit COX-2 expression. COX-1 on the other hand is present in most of the tissues and hence is called constitutive. This concept is however an oversimplification, as COX-2 is constitutively expressed in brain, testes, tracheal epithelia, and macula densa in kidney, while COX-1 level changes during development and its expression can be down regulated in endothelial cells and upregulated in mast cells (Smith and Dewit, 1996).

MATERIALS AND METHODS

Collection of the Plant Material

The fresh whole plant of *Leucas aspera* (3 kg) was collected in the month of July and August in and around Sri Venkateswara University campus and authenticated at the Herbarium (Voucher No. 210), Department of Botany, S.V.University,

Tirupati. Collected plants were immediately sprayed with alcohol to cease the enzymatic degradation of secondary metabolites. They were chopped into small fragments, thick stem, roots, twigs and other woody parts were chopped into 1" to 2" pieces and split longitudinally into several sections to enable early drying under shade. Chopping fresh plant materials at once after collection hastens drying and advantages on reduction in bulk and drying speed. The plant was kept under shade for drying for about 7 days.

Procurement of chemicals

Lipopolysaccharide (LPS) and N,N,N,N-tetramethyl p-phenylenediamine (TMPD) were purchased from Sigma chemical Co. Ltd. Enzyme inhibition assay kit was obtained from Assay Designs, Inc. (Ann Arbor, MI, USA). Recombinant human COX-2 was a generous gift from Shozo Yamamoto (School of Medicine, University of Tokushima, Japan).

Animals

Pathogen free, Healthy wistar strain albino rats were selected in the present study, the usage of animals was approved by the Institutional Animal Ethics Committee. The rats were housed in clean polypropylene cages under hygienic conditions with photoperiod of 12 hours light and 12 hours dark. The rats were fed with standard laboratory chow (Hindustan lever Ltd, Mumbai) and water *ad libitum*.

Present experimental work

Cyclooxygenase is the key enzyme in the biosynthesis of prostanoids, biologically active substances involved in several physiological processes and also in pathological conditions such as inflammation. It has been well known for 10 years that this enzyme exists under two forms, a constitutive (COX-1) and an inducible form (COX-2). Both enzymes are sensitive to inhibition by conventional non-steroidal anti-inflammatory drugs NSAIDs. Observations were made that COX-1 was mainly involved in homeostatic processes, while the COX-2 expression was associated with pathological conditions leading to the development of COX-2 selective inhibitors. Several methods have been reported for the evaluation of the COX-l and COX-2 inhibitory potency and selectivity of conventional or COX-2 selective NSAIDs. In this study, we evaluated the COXs inhibitory profile of both conventional NSAIDs and COX-2 selective inhibitors using two different *in vitro* methods; the first test was performed using purified enzymes while the second method consisted of a whole blood assay (Fuj *et al.,* 1990). Cyclooxygenase (COX), also called Prostaglandin H Synthase or (PGHS) is a bifunctional enzyme exhibiting both cyclooxygenase and peroxidase activities. The cyclooxygenase component converts arachidonic acid to a hydroperoxy endoperoxide (Prostaglandin G PGG and the perxodiase component reduces the endoperoxide to the corresponding alcohol (Prostaglandin H PGH the precursor of prostaglandins, thromboxanes, and prostacyclins (Hamberg and Samuelsson, 1973). It is now well established that there are two distinct isoforms of cyclooxygenase. Cyclooxygenase-1 (COX-1) is constitutively expressed in a variety of cell types and is involved in normal cellular homeostasis. A variety of mitogenic stimuli such as phorbolesters, lipopolysaccharides and cytokines, lead to the induced expression of a second isoform of COX, cyclooxygenase-2 (COX). COX-2 is responsible for the biosynthesis of prostaglandins under acute inflammatory conditions (Xie *et al.,* 1991). The inducible COX-2 is believed to be the target enzyme for the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs. The Colorimetric COX (ovine) Inhibitor Screening Assay utilizes the peroxidase component of cyclooxygenases. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm (Kulmacz and Lands, 1983). Inhibition of COX activity by a variety of selective and non-selective inhibitors, showed potencies similar to those observed with other *in vitro* methods. The Colorimetric COX (ovine) inhibitor screening assay includes both oven COX-1 and COX-2 enzymes in order to screen isoenzyme specific inhibitors. This COX assay is a time saving tool for screening vast numbers of inhibitors. In the present work, we have screened the various extracts of *Leucas aspera* and semipurified fraction (F_5) of ethanol extract of *L. aspera*.

Treatment schedule

The assay was carried out as previously described (Brideau *et al.,* 1996) with little modification. Albino rats of either sex weighing between 150-200 g maintained on standard pellet diet and water were used for the study and they were fasted overnight. These animals were separated into seven groups of five animals each. Group first received 5% gum acacia solution and served as a control. Group second received celecoxib at a dose of 50 mg/kg body weight and used as reference standard. Groups three, four, five and six received petroleum ether, chloroform, ethanol and water extracts of *Leucas aspera,* respectively at a dose of 400 mg/kg body weight. Group seven received fraction F_5 of ethanol extract of *Leucas aspera* at a dose of 400 mg/kg body weight. All the treatments were given orally one hour before their sacrifice. All the experimental animals were sacrificed and whole blood was collected by venipuncture into heparinised tubes. 1 ml aliquot of whole blood was incubated both in presence and absence of Lipopolysaccharide (LPS) obtained from *E. coli* in a concentration of 10 μl-l00 μl. The contribution of platelet COX was suppressed by the addition of 200 μm of aspirin. After incubation, the plasma from the samples was separated by centrifugation of the whole blood samples at 1000 x g for 10-15 min. The optical density of the resulting plasma was recorded by using spectrophotometer at a wavelength of 611 nm. The UV-visible spectrum of phycocyanobilin was recorded between 280-500 nm in methanol 2% HC1 and the concentration was estimated at 374 nm using extinction coefficient of 47.900 $M^{-1}\ cm^{-1}$. The results obtained were calculated as A611/0.00826 μm^{-1} x 0.21 / 0.01 / 2* n mole/ml (U/ml) (*It takes 2 molecules of TMPD to reduce PGG_2 to PGH_2. The data so obtained was subjected to statistical analysis and are listed in Table 1.

Statistical analysis

Statistical analysis has been carried out using ANOVA followed by Dunnets (t) test. The data was analyzed for the significance and the results were presented with the P-value.

RESULTS AND DISCUSSION

Previous report (Saundane, 2000) on antiinflammatory activity of *L. aspera* showed good antiinflammatory activity of crude ethanolic extract. Srinivas *et al*. also reported that the dried

Table 1. Evaluation of cyclooxygenase-2 activity in whole blood (LPS method) UNITS/ML

	Control	Celecoxib	Pet. Ether extract	Chloroform extract	Alcohol extract	Water extract	Fraction F_5
	10.58	65.05	20.59	33.3	16.27	21.35	58.72
	14.3	54.25	15.7	28.25	35.84	30.5	36.35
	16.2	56.25	15.3	29.5	40.67	26.69	54.4
	21.33	57.25	21.06	28.95	21.58	27.12	42.25
	14.28	50.09	17.44	32.47	31.29	28.01	58.84
	11.38	61.15	14.05	29.98	39.92	29.41	48.24
Mean	15.33	56.18	17.24	30.59	30.93	26.18	49.82
±SEM	1.734	2.613*	1.220	1.026	4.091*	1.700	3.754*

Data are expressed in mean ± SEM (Units/ml).
$P < 0.01$ when compared with control.
ANOVA followed by Dunnets (t) test.
Drugs are prepared in suspension with 5% gum acacia given by per orally.

leaves of *L. aspera* possessed significant antiinflammatory activity respectively. The four extracts of *Leucas aspera* viz., petroleum ether, chloroform, ethanol, water extracts and the purified fraction F_5 of ethanol extract were screened for their *in vivo* COX-2 enzyme inhibition assay by whole blood plasma method (Brideau *et al.,* 1996) using albino rats. Fraction F_5 of ethanol extract showed significant inhibition activity compared to control. The inhibition activity of chloroform, ethanol and water extracts is moderate and that of petroleum ether extract is nearly as that of control group (Table.1) As described earlier COX enzyme heads complex biosynthetic cascade that results in the conversion of polyunsaturated fatty acids to prostaglandins and thromboxane which are responsible for many physiological effects and pathophysiological responses. The synthesis of prostaglandins during the inflammation is more and the inhibition of COX enzyme for the benefit of management of inflammation and pain is well established and many agents are already in the clinical practice for the same purpose (X de Leval *et al.,* 2001). In order to study the anti-inflammatory activity of four extracts of *Leucas aspera* and the purified fraction (F_5) (Table.1) of ethanol extract it is essential to carryout the COX enzyme inhibition assay. The fraction F_5 of ethanol extract of *L. aspera* has shown significant COX-2 enzyme inhibition activity. The chemical molecule present in the fraction F_5 is responsible for the inhibition of COX-2 enzyme present in the blood plasma of experimental rats. Similarly the extracts of *L. aspera* may contain chemical moieties which show moderate or least activity. In the present study the anti-inflammatoryactivity four extracts of *Leucas aspera*, it is essential to carryout the COX enzyme inhibition assayasshown in TABLE 1. The chemical molecule(s) present in them are responsible for the inhibition of COX-2 enzyme that present in the blood plasma of experimental rats (Kalaskar *et al.,* 2010).

Conclusion

Cyclooxygenase is the key enzyme in the biosynthesis of prostanoids, biologically active substances involved in several physiological processes and also in pathological conditions such as inflammation. Hence, the present investigation is taken up to study the anti-inflammatory activity of various solvent extracts of *leucas aspera* spreng. The present study reveals that ethanolic extract of *leucas aspera* spreng showed significant anti-inflammatory activity and COX- II enzyme inhibition studies.

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