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Research Paper

Inhibition of lipoxygenase enzymes by extracts of *Tinospora cordifolia*: A study of enzyme kinetics

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ABSTRACT

Lipoxygenase (LOX) enzymes are significant in the inflammatory pathways in humans and are implicated in various hypersensitivity reactions as well as several cancers. The present study aims to comparatively evaluate the effect of methanol and aqueous extracts of *Tinospora cordifolia (T. cordifolia)*, an ayurvedic herb, on LOX enzymes. LOX assay was conducted by the ferrous oxidation-xylenol orange method with linoleic acid as substrate and IC_{50} values were calculated. The methanol extract was fractionated and the bioactive fraction was selected for comparison with methanol and aqueous extracts. The results showed that the *T. cordifolia* extracts exhibit a potent inhibition of the 5-LOX, 12-LOX and 15-LOX enzymes. Further studies were conducted into the mode of inhibition of the enzymes and the data analysed using Lineweaver-Burk plots which demonstrated a mixed inhibition of the LOX enzymes by the plant extracts. The presence of plant extracts decrease the affinity of the enzyme for the substrate as indicated by Km and Vmax values. The Ki values were also calculated for an insight into the binding affinity of inhibition.

Keywords: Km value; Anti-inflammatory; Lineweaver-Burk Plot; *T. cordifolia*; Lipoxygenase.

INTRODUCTION

LOXs are dioxygenase enzymes which give rise to inflammatory mediators called leukotrienes (LTs) predominantly involved in triggering asthmatic and allergic conditions (Berger, 1999). These leukotrienes are synthesized from arachidonic acid with the help of three major isoenzymes namely, 5-LOX, 12-LOX, and 15-LOX, initially forming the hydroperoxyeicosatetraenoic (HPETE) acids. The HPETE in turn gives rise to the different classes of LTs and can be induced under inflammatory conditions. LTs have important physiological roles in inflammation like vascular permeability, smooth muscle contraction and chemotaxis (Crooke, 1991). The expression of LOX enzymes are also found elevated in various chronic inflammatory conditions like lung tumours (Avis, et al., 1996), colon tumours (Rao, et al., 1995) and prostate cancer (Herrmann, et al., 1997). A study conducted by Ghosh and Myers (1998) has demonstrated the production of 5-HETE in human prostate cancers without stimulation and the inhibition of which can lead to massive apoptosis in these cells. The 15-LOX pathway has also been implicated in the pathogenesis of atopic

asthmatics by Serhan et al. (2008). The role of LOXs in several chronic inflammatory conditions underline the requirement for further studies that can explore the possibilities of new and efficient inhibitors of LOXs.

A study in murine colon adeno-carcinoma cell lines indicates that the inhibition of 5-, and 12-LOX enzymes by LOX inhibitors like zileuton can simultaneously cause the inhibition of tumour cells proliferation (Hussey and Tisdale, 1996) while a study on 12-LOX enzyme inhibition can reportedly lead to reduction in platelet aggregation (Holm, et al., 2014). Chen et al. (2002) reported the expression of a natural LOX inhibitor in the human epidermoid carcinoma cells. Detailed study into the mechanism of action of such inhibitors also reveal that the LOX inhibitors can either bind to iron in the haem group of LOX enzyme or form complexes that block substrate access to this iron (Berger, 1999). The studies conducted on ebselen showed that the same compound can bind to LOXs in a non-competitive and competitive manner and is influenced by the ionization state of iron in the enzyme (Walther, et al., 1999). In the absence of substrate the ebselen binds covalently to the catalytically silent ground-state LOXs while in the presence of substrate it binds competitively to the catalytically active enzyme (Skrzypczak, et al., 2002). The conversion of the curcumin and quercetin into its metabolites was reported in soyabean LOX-3 where enzyme degrades these compounds into active inhibitors (Borbulevych, et al., 2004).

Numerous studies have been conducted with phytoherbals involved in ayurvedic formulations as effective LOX inhibitors and are considered safe alternatives to conventional drugs. Such investigations require detailed studies as to the mode of action and the bioactive constituents involved. *T. cordifolia* has traditionally been used in ayurvedic formulations in the treatment for acute and chronic rheumatoid arthritis (Chopra, et al., 2012) and as a general adaptogenic immunomodulant (Sharma, et al., 2012; Sudhakaran, et al., 2006). A standardized extract from *T. cordifolia* known as Tinofend has been used in a clinical study by Badar, et.al. (2005) which includes 75 patients with allergic rhinitis (hay fever) and showed statistically significant reduction of symptoms compared to placebo. We have earlier reported that the methanol extract of *T. cordifolia* demonstrates potent inhibition of LOX enzymes (Kumar, et al., 2011; Jacob and Kumar, 2013). Here, we evaluate the efficacy of aqueous extract of *T. cordifolia* as compared to the methanol extract. We have attempted to investigate the exact mechanism of action of the inhibition of LOX enzymes with varying substrate concentrations and is being reported for the first time here.

MATERIALS AND METHODS

Materials: 5-, 12-, and 15-LOXs were purchased from Cayman chemicals, USA. HPLC grade solvents (hexane, MeOH, $CHCl_3$), Silica gel (60-120mesh) and other chemicals used in fractionation were purchased from Merck Specialities, India. Linoleic acid, NDGA and xylenol orange were obtained from HiMedia Laboratories, India. Ultra-pure water was prepared by PURELAB Option-Q ultra-pure water purification system (ELGA LabWaters, India). Spectrophotometric assays performed on UV-Visible Spectrophotometer (Model UV1800) from Shimadzu, Japan.

Plant Preparation and Extraction: *T. cordifolia* was collected locally in March, 2010 and identified by taxonomist. Voucher specimen with Voucher No: JA 17151 was deposited at the Kerala Forest Research Institute herbarium for future reference. The stem of collected plant material was cleaned, shade dried and powdered. The dry powder (30g) was initially defatted by extraction with 400ml of hexane and solvent

allowed to evaporate completely. The plant material was then extracted exhaustively with 400ml methanol in soxhlet apparatus at 40°C. The extract (TCM) was concentrated to dryness, weighed and reconstituted in methanol giving a yield of 7.3%. Freshly ground stem (30g) was allowed to stand for 24 hours in ultrapure water at 37°C. Precipitate formed was filtered, filtrate was concentrated and lyophilised (WSF) with a yield of 13.5%.

Lipoxygenase assay (Ferrous Oxidation-Xylenol Orange (FOX) Assay: was carried out with modifications to Gay and Gebicki (2003) method where hydroperoxides formed by enzymatic reaction of LOXs oxidize ferrous sulphate in reagent to ferric sulphate forming a blue coloured complex measured at 560nm.. Phosphates, EDTA, thiols etc. can interfere in this FOX assay and give large background values. The extracts were thereby checked for interference as per standard protocol (Gay and Gebicki, 2003) and diluted as required to remove interference. The 5-, 12-, or 15-LOX enzyme was pre incubated with the plant fraction at different concentrations for 5minutes at 37°C. Typical reaction mixture contains 0.1M Tris-HCl buffer, pH 7.4, enzyme-plant extract mixture (50µl) and 150µM sodium linoleate (substrate concentration as determined by earlier studies) in a total volume of 300ul. The reaction was initiated by the addition of sodium linoleate and incubated for 20 minutes at room temperature in the dark. FOX reagent was then added and followed by 20minutes of incubation at room temperature also in the dark. The absorbance of the colour developed was measured at 560nm. In all experiments a reagent blank and an enzyme control without plant extract was carried out. Nordihydroguaiaretic acid (NDGA), a standard LOX inhibitor was used as positive control.

Fractionation of Methanol Extract of T. cordifolia: The methanol extracts of *T. cordifolia* was adsorbed on silica gel and subjected to column chromatography on a silica gel (60-120 mesh) column (3.0 x 50cm). The flow-rate was fixed at 2ml per minute with chloroform-methanol gradients (100:0, 80:20, 60:40, 40:60, 20:80, 0:100) and followed by methanol-water gradients (100:0, 50:50, 0:100). The fractions collected (10ml) were pooled on the basis of spectral analysis data and then concentrated to dryness, lyophilized and weighed. The reconstituted fractions were checked for lipoxygenase inhibition as described earlier. Fraction with maximal bioactivity (TBF) was considered for kinetic studies.

Kinetics studies of lipoxygenase assay: The mode of inhibition was determined by using Lineweaver–Burk equations. The LOX assay was carried out as per protocol given earlier. Different substrate concentrations (based on earlier analysis) 5µM, 10µM, 25µM, 50µM, 150µM, 200µM & 250µM were prepared for the study of kinetic data in presence and absence of plant extract. The values of kinetic parameters of the enzymes (Km, Vmax, Km*app* and Vmax*app*) were determined by analysis of Lineweaver-Burk plots and the apparent inhibition constant (Ki) values were calculated as per the equation by Garrett and Grisham (2010).

$$V_I = \frac{V_{MAX}[S]}{K_M\left(1 + \frac{[I]}{K_I}\right) + S}$$

Statistical Analysis: All samples and experiments were conducted in triplicate with results expressed as mean±SD or Percentage±SD as required by the assay. Care was taken to ensure that the correlation (R^2) values were above 0.98 for every experiment. All statistical analyses were performed by using Dunnett's test, which is a multiple comparison procedure to compare each of a number of treatments with a single

control. These calculations were done using SigmaPlot (Systat Software, San Jose, CA). Differences were considered to be significant when the $P < 0.05$.

RESULTS

The studies conducted earlier on the effect of the methanol extract of *T. cordifolia* have demonstrated the efficiency of the methanol extract (TCM) as comparable to and better than the action of NDGA standard for LOX inhibitory activity (Jacob, 2013). The present work performs a comparative analysis of the effect of TCM and WSF on inhibition of lipoxygenase isoenzymes. The IC_{50} values for LOX inhibition by the TBF and WSF extract is given in table 1. Comparing the IC_{50} values, we can conclude that the TCM was found to be more potent as compared to WSF extract. The selectivity ratio for the IC_{50} of 5-LOX to 12-LOX and 5-LOX to 15-LOX were also compared to evaluate the affinity of the bioactive molecules to each enzyme. The kinetic type and parameters of inhibition of *T. cordifolia* by TCM, TBF, and WSF was evaluated using the Lineweaver-Burk plot (Fig 1: (a, b, c). The Lineweaver-Burk plot showed that the steady-state experiments performed in the presence of *T. cordifolia* bioactive fractions on the activity of 5-LOX, 12-LOX and 15-LOX has revealed a mixed type of inhibition. The apparent Vmax was decreased in all cases and apparent Km values increased compared to uninhibited enzyme. The results are indicated in table 2. The Ki values were calculated and compared using the enzyme kinetics module of Sigma Plot software and are as given in table 3.

DISCUSSIONS

T. cordifolia has been extensively used in ayurvedic formulations and has a safety profile as evidenced by its continual use as an anti-inflammatory agent in the ayurvedic systems of medicine prevalent in India. Ayurveda practices the use of aqueous extracts and decoctions of its plant formulations for bioactivity. Validation of the use of aqueous extracts of *T. cordifolia* has been attempted in this study with the inhibition of the LOX enzymes and comparison with the methanol extract. The activity of WSF aqueous extract was comparable to NDGA standard which successfully validates the use of aqueous extracts in Ayurveda. The methanol extracts of *T. cordifolia* show an increased potency for LOX inhibition when compared to the aqueous extract and could probably be due to the choice of the extracting solvent, methanol. Plant phenolics and other bioactive principles are better extracted in methanol. The increased potency of the TCM extract justifies the selection of the methanol extract of *T. cordifolia* for the bioactivity guided fractionation studies. The selectivity ratios for the LOX enzymes by the plant extracts show a potent inhibition of all three isoenzymes with different affinities. The aqueous extract (WSF) and bioactive fraction (TBF) show a greater selectivity for 5-LOX than 12-LOX while TCM has greater selectivity for 15-LOX enzyme.

The kinetics of biological processes appears to differ only quantitatively from those of *in vitro* enzyme-catalysed reactions as reported by Nangia and Anderson (2012). This underlines the relevance of conducting such studies on the *in vitro* systems of enzyme catalysis. The LOX enzymes show an increase in Km values and a simultaneous decrease in Vmax values in the presence of the plant extracts leading to a mixed inhibition. The mixed mode of inhibition could suggest the presence of one or more inhibitors in the extracts which may act synergistically with each other (Rasoanaivo, et al., 2011) and bind to sites proximal or distal from the active site of the enzyme (Collom, et al., 2008). This further suggests that the decrease in the

affinity of the enzymes for the substrate, linoleic acid in presence of the plant extracts causes a simultaneous decrease in the rate of reaction and product formation. Mixed inhibitors can bind to both the free enzyme and the enzyme-substrate complex forming the EI and the ESI complexes. The formation of the EI complex decreases the availability of free enzyme by distorting the active site to a non-optimal conformation for catalysis and the ESI complex hinders the release of final product from the active site. The biological significance of such a mixed inhibition is the non-accumulation of substrate. When the substrate accumulates there is an increase in the flux of the substrate to other parallel metabolic pathways in a biological system. LOX inhibition and subsequent accumulation of substrate could result in the activation of the cyclooxygenase (COX) pathway leading to further augmentation of chronic inflammatory conditions (Yang, et al., 2007).

Ki values indicate the binding affinity of the inhibitor for the enzyme, where lower values indicate a better binding to the enzyme (Segel, 1993). Values of Ki indicate that the methanol extract had a higher affinity to enzyme followed by the aqueous extract as compared with the bioactive fraction. Lower Ki values of TCM and WSF could be due to synergistic effects of various bioactive molecules present in these extracts. Further studies as to the purification of lead compounds from the extract and effect on kinetic parameters are being conducted for thorough knowledge as to the mechanism of activity of *T. cordifolia* in inflammation.

CONCLUSION

T. cordifolia, an important medicinal plant used in ayurveda was evaluated for its anti-inflammatory activity by the inhibition of the LOX enzymes. The methanol (TCM) extract was a better inhibitor of the LOXs as demonstrated by the IC_{50} values and Ki values when compared to the aqueous (WSF) extract and to the standard drug, NDGA. Kinetic studies conducted in the presence of different substrate concentrations reveal a mixed mode of inhibition of the LOX enzymes in presence of plant extracts. The decrease in Vmax values and increase in the Km values indicate a decrease in affinity of enzyme for substrate. The Ki values also reflect the affinity of *T. cordifolia* methanol and aqueous extracts for the LOX enzymes.

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Table- 1: IC50 Values for LOX Inhibition.

Name of Fraction	5-LOX	12-LOX	15-LOX	5LOX/ 12LOX	5LOX/ 15LOX
TCM	9.34e-14 ± 0.19	9.01e-14 ± 0.09	2.5e-11 ± 0.09	1.04	0.004
WSF	0.021e-3 ± 0.003	0.034e-3 ± 0.002	0.014e-3± 0.0007	0.62	1.5
TBF	0.041 ± 0.0003*	0.058±0.003	15.94 ± 0.009	0.71	0.003
NDGA	2.75 ± 0.05	0.302 ± 0.68	8.47 ± 0.15	9.11	0.32

- All values are represented in ng/µl as mean ± standard deviation of data in triplicate unless otherwise specified. NDGA represents positive control. $P<0.001$ $^*P<0.05$

Table- 2: Km and Vmax values for the LOX enzymes.

Name of Extract	K_M (µM)			V_{MAX} (µMol/min/mg protein)		
	5-LOX	12-LOX	15-LOX	5-LOX	12-LOX	15-LOX
Control	19.86±1.8	11.06±0.01	4.08±0.05	11.5±0.13	1.47±0.002	0.5±0.004
TCM	109.8±3.7	20.59±0.04	91.83±0.56	6.8±0.22	1.33±0.0003	0.38±0.002
TBF	65.28±1.66	29.97±1.19	109.47±6.2	10.08±0.35	1.009±0.041	0.195±0.01
WSF	31.57±2.7#	40.4±4.3	62.57±0.93	2.6±0.15	1.418±0.009	0.301±0.009

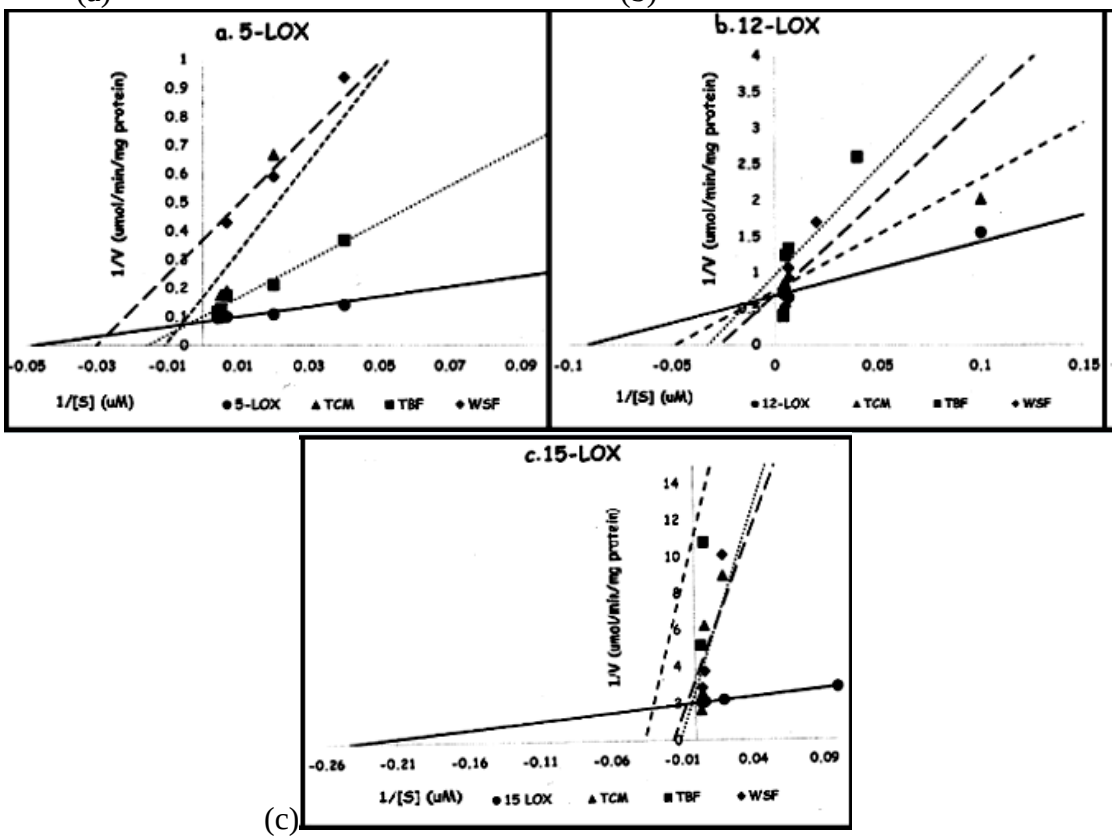
- All values are represented as mean ± standard deviation of data in triplicate unless otherwise specified. Control represents assay without plant extract. $P<0.05$ $\#P=0.25$

Table -3: Ki values for the LOX enzymes

Name of Extract	K_i (pg/µl)		
	5-LOX	12-LOX	15-LOX
TCM	6.61e-12 ± 0.025e-4	1.53e-10 ± 0.0001	2.32e-11 ± 0.01e-3
TBF	11.8 ± 0.004	92 ± 0.18	11 ± 0.006
WSF	0.00161 ± 0.0005	0.011 ± 0.005	0.0066 ± 0.003 •

- Values expressed as mean ± standard deviation, unless otherwise stated.

(a) (b)



(c)

Figure-1: The Lineweaver-Burk plots for kinetic studies on LOX activity in presence of *T. cordifolia* extracts.

- (a) 5-LOX (b) 12-LOX (c) 15-LOX.

