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ANTI INFLAMMATORY ACTIVITY OF *BAUHINIA TOMENTOSA* (STEM BARK AND ROOTS) AND *BAUHINIA VAHLII* ROOTS

D.Swarnalatha*,T.Satyanarayana

Department of Pharmacognosy and Phytochemistry,

A.U College of Pharmaceutical Sciences, Andhra University,

Visakapatnam, A.P, INDIA

*Corresponding Author E-mail:reddy_latha9@yahoo.co.in

ABSTRACT

The Ayurvedic system of medicine suggests large number of plants has been used as anti-inflammatory agents since Vedic era**.** The genus *Bauhinia* (Caesealpinaceae) has numerous traditional uses. From the genus selected two plants were subjected for the *In vitro* anti inflammatory activity against 5-lipoxygenase enzyme**.** The activity exhibited by ethyl acetate and methanol extracts of *B. tomentosa* (stem bark and roots) and *B. vahlii* (roots) was one of a concentration dependant effect such that increasing concentrations of extract produced a greater inhibition of the 5-LOX enzyme. Of the extracts the most promising activity was observed for the ethyl acetate extract of *B. tomentosa* roots (yielding complete inhibition at 100 µg/ml and an IC_{50} value of 18.47 ± 0.67 µg/ml). the results obtained was compared with the 5-LOX inhibitor, NDGA.

Key words: *Bauhinia tomentosa, Bauhinia vahlii*, 5-LOX, NDGA.

INTRODUCTION

It is a body defense reaction in order to eliminate or limit the spread of injurious agents. In the acute stages of the inflammatory process, inflammation serves a vital role in the healing process of the

body. Chronic inflammation, involves the release of a number of mediators, resulting in the proliferation of fibroblasts, vascular endothelium, as well as lymphocytes, plasma cells and macrophages (Brooks *et al*., 1998). The release of all these mediators can contribute to chronic degenerative diseases such as arthritis, cancer, heart disease, Alzheimer's disease, diabetes and asthma, which may increase disease-associated morbidity.

Prostaglandins are involved in the complex process of inflammation and are responsible for pain (Jagar *et al*., 1996).Upon appropriate stimulation of neutrophils, arachidonic acid (20-carbon fatty acid) derived from the breakdown of cell membrane phospholipids by any number of phospholipase A2 (PLA_2) isoforms. By separate action of enzymatic pathways, viz. the cyclo-oxygenase (COX) and lipoxygenase (LOX) pathways, on arachidonic acid produces Prostaglandins, thromboxane A_2 and hydroperoxy-eicosatetraenoic acids (HPETE's) and a hydroperoxide intermediate, further converted to leukotrienes (LT's), which are biologically active mediators in a variety of inflammatory events (Alitonou *et al*., 2006; Piper *et al* ., 1994; Bouriche *et al*., 2005). The metabolism of arachidonic acid produces leukotrienes and lipoxins via the LOX pathway (Werz and Steinhilber, 2006).

Leukotrienes have been identified as mediators of a number of inflammatory and allergic reactions. These include rheumatoid arthritis, inflammatory bowel disease, atopic dermatitis, psoriasis, chronic urticaria, asthma (Claesson *et al*., 1999) and allergic rhinitis (Samuelsson *et al.*, 1987; Lewis *et al*., 1990).

The 5-LOX pathway has also recently been linked to the development of atherosclerosis, osteoporosis and certain types of cancers (Werz and Steinhilber, 2006).

As a result of the pathophysiological implications of 5-LOX products and the potential benefits of anti-leukotriene therapy, different strategies have been employed (targeting PLA_2, 5-LOX, FLAP, LTA_4 hydrolase and leukotriene C_4 (LTC_4) synthase) with 5-LOX being the ideal and most promising target (Werz and Steinhilber, 2006).

A range of therapies are available for the treatment of inflammation. In most cases, these therapies have undesirable side effects. The greatest problem with the presently available synthetic drugs is (both steroidal and non-steroidal drugs) lie in their toxicity and reappearance of symptoms after discontinuation of treatment. An urgent need therefore exists for the research and development of additional or alternative therapies. The research on screening and development of drugs for their activity is therefore, an unending process and there is hope of finding out anti-inflammatory drugs from indigenous plants. Various plant extracts and their isolated compounds have been proven to be good anti-inflammatory agents (Iracema *et al* ., 2005). The traditional remedies are important, not only for their active principles, but also for the synergistic effects of a number of active constituents, especially when such a large proportion of traditional medicines are used as remedies for the treatment of skin conditions and wound healing (Bodeker *et al*., 1999).

5-Lipoxygenase is inhibited by quinones, hydroxyquinones and a variety of phenolic compounds, including certain flavonoids such as, quercetin, kaempferol, morin, myricetin and cirsiliol (Kim *et al*., 2004).

Flavonoids and sesquiterpene lactones, isolated from plants, have been

shown to possess both anti-inflammatory and anti-ulcerogenic activity (Abad *et al.*, 1994).

Brief review on anti-inflammatory activity of *Bauhinia* species

flavonoids and tannins, flavonoid quercitrin makes a significant contribution to the antinociceptive properties of this plant (Gadotti *et al.*, 2005). Both triterpenic acids have been recognized as having antiinflammatory activity, as well as other important biological properties (Liu, 1995; Deepak and Handa, 2000; Cipak *et al.*, 2006). This effect may be related to the presence of several types of natural products including flavonoids, coumarins, triterpenoids, stilbenes, steroids and tannins (Prakash and Khosa, 1976; Balasooriya *et al.*, 1982; Anjaneyulu *et al.*, 1984, 1986). A new flavonol glycoside, 5,7,3′,4′-tetrahydroxy-3-methoxy-7-O-alpha-L-rhamnopyranosyl(1→3)-O-betagalactopyranoside was isolated from *B. variegata*, which showed anti inflammatory potential (Yadava and Reddy *et al.,* 2003). Recently, several known flavonoids together with a triterpene caffeate were isolated from the aerial parts of this plant (Rao *et al.,* 2008). Some of them, particularly kaempferol, ombuin and the triterpene caffeate caused significant inhibition of lipopolysaccharide (LPS) and interferon (INF)- gamma induced nitric oxide (NO) and cytokines [tumor necrosis factor (TNF)-alpha and interleukin (IL)-12]. racemosol and 10-O-demethylracemosol, which exhibits potent *in vitro* anti -inflammatory activity against cyclooxygenase-1 and -2 (COX-1 and 2) enzymes (Kittakoop *et al.*, 2000; Songarsa *et al.*, 2005). especially bibenzyls and dihydrodibenzoxepins, some with anti-inflammatory activity were isolated from the roots of this plant (Boonphong *et al.,* 2007) are the isolated phytoconstituents with anti inflammatory activity from the following *Bauhinia* genus . stem extract of *B. splendens* (Cechinel Filho *et al.*, 1995),leaves of *B. microstachya* (Meyre Silva *et al.*, 2001). Another species collected and studied in Brazil is *B. guianensis*, in mice (Carvalho *et al.*, 1999). The leaves of *B. tarapotensis* are traditionally used in Ecuador as an antiinflammatory agent (Sosa *et al.,* 2002). Stem bark extract of *B. racemosa* (Rao *et al.,* 2008).

As plants of *Bauhinia* genus have been reported to have antiinflamatory activity traditionally and several *Bauhinia* species were scientifically reported to exhibit this activity, the anti-inflammatory activity of the *B. tomentosa* (stem bark and roots) and *B. vahlii* (roots) were selected for the study.

The anti-inflammatory activity of the plant extracts were determined using the *in vitro* 5- LOX assay. This assay measures the inhibitory activity against the 5-LOX enzyme, which is a key enzyme in the metabolism of arachidonic acid that is responsible for the formation of leukotrienes (which play a pivotal role in the pathophysiology of chronic inflammatory and allergic diseases) as first determined (Sircar *et al*., 1983) and later modified (Evans, 1987).

Materials and methods

DMSO and Tween 20 – Merck Co., Mumbai, India, Linoleic acid (purity 99%) and **5-** Lipoxygenase (5-LOX), Nordihydroguaiaretic acid (NDGA) (1,4-Bis(3,4-dihydroxyphenyl)-2,3-dimethylbutane, 4,4′-(2,3-Dimethyltetramethylene) dipyrocatechol, Masoprocol,)-Sigma-Aldrich Co.St. Louis, MO. UV-visible spectrophotometer-Shimadzu Corporation, Japan. Other

chemicals used were of analytical grade and were purchased from Desai Chemicals.

Preparation of plant samples

Stock solution of extracts (hexane, ethyl acetate and methanol) of *B. tomentosa* (stem bark and roots) and *B. vahlii* (roots) were prepared by using DMSO and Tween 20 mixture (1:29, w/w) at a concentration of 50 mg/ml. From stock solution 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml and 6.25 µg/ml were prepared.

Preparation of potassium phosphate buffer

0.1 M potassium phosphate buffer (pH 6.3) was prepared with analytical grade reagents, which was maintained at 25°C in a thermostated waterbath

Principle of the assay

The anti-inflammatory activity was determined using the 5-Lipoxygenase assay (LOX) (Baylac and Racine, 2003; Kamatou *et al*., 2009; Vieira *et al.*, 2001). 5-Lipoxygenases (LOX) are dioxygenases that catalyse the addition of molecular oxygen to polyunsaturated fatty acids containing a 1, 4-pentadiene group. The 5-LOX enzyme, converts its substrate, arachidonic acid, to the conjugated diene product 5-hydroxy-6,8,11,1-eicosatetraenoic acid (5-HETE) which in turn, converted to LTA_4 and then to LTB_4, by LTA_4 hydrolase. Linoleic acid was used as the substrate in the experiment, because it shares a high degree of structural similarity to arachidonic acid (containing the 1,4-pentadiene group) and it is easy to handle as well as having a stronger affinity for the 5-LOX enzyme and resulting in greater UV absorbance readings The experiment specifically determines increases in absorbance at 234 nm as a result of the formation of conjugate double bonds in linoleic acid hydroperoxide (from a 1,4-diene to a 1,3-diene), as used in the biochemical evaluation of the LOX pathway of soybean plants submitted to wounding.

Experimental procedure

5-Lipoxygenase assay

The standard assay mixture contained 10 µl of the plant extract dissolved in a solution of DMSO and Tween 20 with the concentration of 100 µg/ml was placed in a 3 ml cuvette. The addition of 2.95 ml of 0.1 M potassium phosphate buffer (pH 6.3) and 45 µl of 100 µM linoleic acid (purity 99%) followed. The enzymatic initiation of the reaction occurred upon addition of 100 units of the isolated 5-LOX enzyme, the latter diluted with 12 µl of 0.1 M potassium phosphate buffer (pH 6.3) and maintained at 4°C until required.

Increases in absorbance were recorded at 234 nm for 10 min, using a UV-VIS spectrophotometer. Further dilutions of the extracts were prepared for those species that exhibited anti-inflammatory activity by atleast 50% and these were assayed in a similar way. The results were plotted and initial reaction rates were determined from the slope of the straight-line portion of the curve. The control was prepared only with DMSO and Tween 20 mixture with out enzyme inhibition.

Nordihydroguaiaretic acid (NDGA), an inhibitor of the 5-LOX enzyme was used as a positive control in the assay.

The percentage of enzyme inhibition of each of the extracts were determined by comparison with the negative control, the latter comprising DMSO and Tween 20 in the absence of plant extract. The percentage enzyme inhibition was calculated using following equation. Which denotes the anti-inflammatory activity and graph was plotted against the concentration of plant extract (µg/ml). The IC_{50} (the concentration at which 50% inhibition is achieved) values

were determined from the dose-response curves.

Equation

$$\% \text{ 5-lipoxygenase inhibition} = \frac{100 - (\text{5-HETE with inhibitor})}{(\text{5-HETE without inhibitor})} \times 100$$

Results and Discussion

The *in vitro* anti-inflammatory activity for each of the species of *Bauhinia* were performed and indicated as the percentage 5-LOX enzyme inhibition, together with their corresponding IC_{50} values, as shown in Table 5.01.

5-LOX inhibition of the hexane, ethyl acetate and methanol extracts of the *B. tomentosa* stem bark at 100µg/ml were found to be 24.7%, 87.5% and 64.9%. 5-LOX inhibition of the hexane, ethyl acetate and methanol extracts of the *B. tomentosa* roots at 100µg/ml were found to be 35.5%, 100% and 73.49%. 5-LOX inhibition of the hexane, ethyl acetate and methanol extracts of the *B. vahlii* roots were found to be 8.3%, 74.9% and 56.7% and 5-LOX inhibition of the positive control NDGA was found to be 100%. By observing the percentage inhibition of 5-LOX of all the extracts of the plants , further dilution of the ethyl acetate and methanol extracts of the *B. tomentosa* (stem bark and roots) and *B. vahlii* roots were prepared for observation of anti-inflammatory activity and from these results IC_{50} values (concentration of the extracts required for 50% inhibition of LOX) was calculated. The hexane extracts of the *B. tomentosa* (stem bark and roots) and *B. vahlii* roots were not used for the preparation of dilutions, due to less than 50% inhibition of the 5-LOX at a concentration of 100 µg/ml.

The IC_{50} values of ethyl acetate and methanol extracts of the *B. tomentosa* stem bark were found to be 24.09±0.97, 59.61±0.62. The IC_{50} values of ethyl acetate and methanol extracts of the *B. tomentosa* roots were found to be 18.47±0.67, 46.39±0.29. The IC_{50} values of ethyl acetate and methanol extracts of the *B. vahlii* roots were found to be 45.96±0.14, 89.67±0.39 and the IC_{50} of NDGA is 5.47±0.04. It was observed that ethyl acetate extracts of the *B. tomentosa* (stem bark and roots) and *B.vahlii* roots showed better activity than the methanol extracts of the *B. tomentosa* (stem bark and roots) and *B.vahlii* roots. The order of the activity of plants were in the following order.

B. tomentosa roots > *B. tomentosa* stem bark > *B.vahlii* roots.

The measure of the 5-LOX inhibitory activity (Baylac and Racine, 2003) was defined according to an arbitrary scale of IC_{50} values, where an IC_{50} value above 100 µg/ml was inactive, between 51 µg/ml and100 µg/ml was poorly active (+), between 31 µg/ml and 50 µg/ml was moderately active (++), between 10 µg/ml and 30 µg/ml showed good activity (+++) and an IC_{50} value of less than 10 µg/ml showed excellent activity (++++). By comparing the IC_{50} values of the ethyl acetate and methanol extracts of *B. tomentosa* (stem bark and roots) and *B. vahlii* (roots) with the arbitrary scale of IC_{50} value (Baylac and Racine, 2003) , it was found that ethyl acetate extract of the *B. tomentosa* (stem bark and roots) showed good activity. Methanol extract of *B. tomentosa* (roots) showed moderately good activity. Methanol extract of *B. tomentosa* (stem bark) found to be poorly active. The ethyl acetate extract of the *B. vahlii* roots showed moderate activity and the methanol extract of the *B. vahlii* roots showed poor activity. Hexane extracts of *B. tomentosa* (stem bark and roots) and *B. vahlii* (roots) were found to be inactive. Since the hexane extracts of *B. tomentosa* (stem bark and roots) and *B. vahlii* (roots) displayed

minimal inhibition of the 5-LOX enzyme. Thus serial dilution assays were not performed on these extracts.

Nordihydroguaiaretic acid (NDGA), an inhibitor of the 5-LOX enzyme (positive control), was determined to exert excellent (++++) inhibitory effects on the 5-LOX enzyme. Fig. 5.05 depicts the percentage 5-LOX enzyme inhibition for each of the extracts at a concentration of 100 µg/ml, revealing the extensive activity of the ethyl acetate extract of *B. tomentosa* roots as compared to other extracts.

The results obtained for the extracts of *B. tomentosa* and *B. vahlii* indicate that anti-inflammatory activity varies widely amongst the extracts. It is clear that, through inhibition of the enzyme, ethyl acetate extract of *B. tomentosa* roots (IC_{50} = 18.47 ± 0.67) completely inhibited the formation of the 5-LOX products at 100 µg/ml.

The activity exhibited by ethyl acetate and methanol extracts of *B. tomentosa* (stem bark and roots) and *B. vahlii* (roots) was one of a concentration dependant effect such that increasing concentrations of extract produced a greater inhibition of the 5-LOX enzyme. Of the extracts the most promising activity was observed for the ethyl acetate extract of *B. tomentosa* roots (yielding complete inhibition at 100 µg/ml and an IC_{50} value of 18.47 ± 0.67 µg/ml).

The absence of an inhibitory effect on the 5-LOX enzyme by hexane extracts is not necessarily indicative of a total lack of anti-inflammatory activity by these extracts. The 5- LOX pathway is by far not the only pathway involved in the inflammatory process. Certain compounds present within each of the extracts may act at other sites or may follow other models of action such as 5-LOX activating protein (FLAP), 8, 12 or 15- LOX, COX-1 or COX-2.

In a review study flavonols such as quercetin, morin, myricetin and kaempferol, were found to be 5-LOX inhibitors that were less active against 12-LOX, but were stronger inhibitors than flavones. Exceptions to this finding were the flavone derivatives including cirsiliol and its analogues, being strong inhibitors of 5-LOX (Kim *et al.* (2004).

In a study it was reported that certain flavonoids were relatively selective inhibitors of the 5-LOX enzyme and that certain structural characteristics are required to produce this anti-inflammatory activity. The minimal requirement for inhibition of 5-LOX is the presence of the keto group at C-4 with the absence of substitution at C2' (Yoshimoto *et al.* 1983; Abad *et al*., 1994). A catechol structure in ring B (a vicinal diol at R_5 and R_6), as mentioned previously appeared necessary to inhibit 5-LOX. The 4'-hydroxyl in the B-ring, C2-C3 double bond in the C-ring and the 5,7-hydroxyl groups on the A-ring are all characteristic of kaempferol.

Quercetin, a flavonoid sharing structural similarities with kaempferol, is a potent inhibitor of 5-LOX isolated from rat basophilic leukaemia cells. Quercetin also bears some structural resemblance with NDGA (Hope *et al*., 1983). Nordihydroguaiaretic acid putatively blocks the formation of 5-LOX products, exerting a significant effect with a determined IC_{50} value of 5.47 ± 0.04 µg/ml. This suggests that the structural resemblance may also apply to kaempferol and that there is a probable similarity in the mechanism of action.

A novel flavonol glycoside 5,7,3',4'-tetrahydroxy-3-methoxy-7-O-alpha –L-rhamnopyranosyl(1--)3)-O -beta –galactopyranoside isolated from *B.variegata* showed a significant anti-inflammatory activity (Yadava *et al*., 2003).

From the phytochemical study, kaempferol was isolated from *B.tomentosa* roots, Quercetin was isolated from *B.tomentosa* stem bark and *B. vahlii* roots, which may be responsible for above said activity.

Further investigation involving other inflammatory routes is required in order to determine whether the solvent extracts do exert anti-inflammatory activity in other steps of the complex anti-inflammatory.

Table - 1 The percentage 5-lipoxygenase enzyme inhibitory activity of *B. tomentosa* (stem bark and roots) and *B. vahlii* (roots) extracts *in vitro* at 100 µg/ml and their corresponding IC_{50} values.

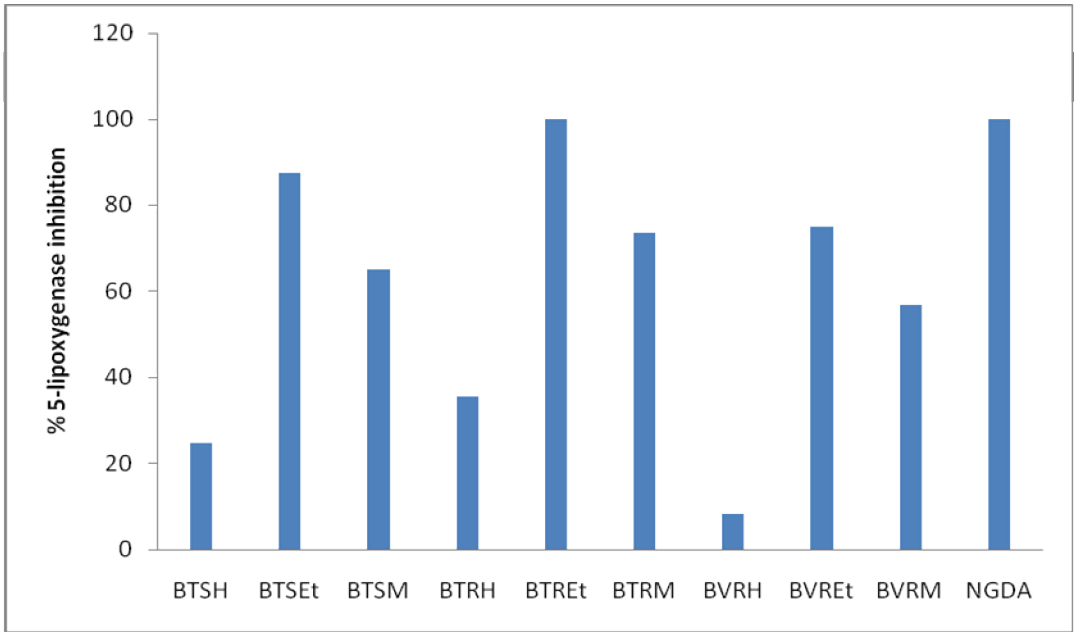
Plant/Positive control	Extract	5-lipoxygenase inhibition at 100 µg/ml	IC_{50} (µg/ml)
B. tomentosa stem bark	Hexane	24.7	n.d.
	Ethyl acetate	87.5	24.09 ± 0.97
	Methanol	64.9	59.61 ± 0.62
B. tomentosa roots	Hexane	35.5	n.d.
	Ethyl acetate	100	18.47 ± 0.67
	Methanol	73.4	46.39 ± 0.29
B. vahlii roots	Hexane	8.3	n.d.
	Ethyl acetate	74.9	45.96 ± 0.14
	Methanol	56.7	89.67 ± 0.39
Control NDGA		100	5.47 ± 0.04

Results are given as mean ± s.d, n=3.

NDGA= Nordihydroguaiaretic acid.

n.d. = not determined, as serial dilutions were prepared for the extracts exhibiting 5-lipoxygenase inhibition at 100 µg/ml over 50%.

Figure -01 The percentage 5-lipoxygenase enzyme inhibition by *B. tomentosa* (stem bark and roots) and *B. vahlii* (roots) extracts at a concentration of 100 µg/ml



BTSH = Hexane extract of *B. tomentosa* stem bark

BTSEt = Ethylacetate extract of *B. tomentosa* stem bark

BTSM = Methanol extract of *B. tomentosa* stem bark

BTRH = Hexane extract of *B. tomentosa* roots

BTREt = Ethylacetate extract of *B. tomentosa* roots

BTRM = Methanol extract of *B. tomentosa* roots

BVRH = Hexane extract of *B. vahlii* roots

BVREt = Ethylacetate extract of *B. vahlii* roots

BVRM = Methanol extract of *B. vahlii* roots

NGDA = Nordihydroguaiaretic acid

CONCLUSION:

It can be concluded that extract of *Bauhinia variegate and vahilli* has anti-inflammatory activity Induced paw edema in rats. These activities may be due to their content of tannins, steroids, flavonoids and carbohydrates. This study demonstrates the efficacy of *Bauhinia variegata and vahlii h*as an anti-inflammatory agent and also scientifically justifies the use of this plant.

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