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Extraction of Phytochemical Constituents from the Leaves of *Vitex trifolia* in the Inhibition of Lipoxygenase, Cyclooxygenase, Tyrosinase and Xanthine Oxidase

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ABSTRACT

Background: Herbal medicines are often used to stimulate the immune system in an attempt to prevent disease, as well as to induce specific cures. The use of phyto medicines is becoming more scientifically based, with increasing emphasis placed on proven product safety and efficacy.

Methods: In the present study, the leaves of *Vitex trifolia* were extracted with distilled water, 1M Na_2CO_3 and 70% methanol. The extracted contents were screened to identify the presence of various phytochemicals. The contents of Na_2CO_3 and methanol were filtered, centrifuged and the supernatant was collected. The supernatant of Na_2CO_3 extract was collected and the alkaloid layer was separated in a separating funnel using alcohol. The contents were analysed for thin layer chromatography and phytochemical constituents.

Result: Thin layer chromatography was performed at 360 nm the Rf value were 0.953, 0.230, 0.184, 0.153, 0.123 and in 240 nm Rf values 0.953, 0.338, 0.184, 0.153, 0.123 were obtained. The methanol extract was condensed at 40°C and dissolved in chloroform. Rf at 360 nm of 0.714, 0.396, 0.158 and at 240 nm with Rf values 0.714, 0.492, 0.349. The activity of extracts on Xanthine oxidase, tyrosinase and cyclooxygenase were estimated and the inhibition percentage of all the three was calculated as 59.34, 61.23, 71.02 and 51 respectively. The values obtained with flavanoid extracts for the same set of enzymes were 70.22, 81.48, 94.8 and 74.36 respectively. In this study, inhibition properties of flavanoid extracts were greater than that of alkaloid extracts.

Key words: Enzymes, Inhibition, Phytochemicals, Rf values, *Vitex trifolia*.

INTRODUCTION

Enzymes are the sparks that start the essential chemical reactions in our bodies need to live. They are necessary for digesting food, for stimulating the brain, for providing cellular energy and for repairing all tissues, organs and cells. The use of plant-based medications has become extremely popular in the United States and Europe, with the botanical industry in the US earning $1.5 billion per annum and the European market nearly three times as much (Gupta *et al*., 2006). Pure compounds are used when the activity is strong and specific and has a small therapeutic index. Natural products, such as plant extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched chemical diversity they can provide (Cos *et al*., 2006). According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. This has captured the interest of many researchers to explore local medicinal plants for valuable medicinal traits. Several studies indicate that medicinal plants contain compounds like peptides, unsaturated long chain fatty acids, aldehydes, alkaloids, essential oils, phenols and water or ethanol soluble compounds (Zerihun, 2022). These compounds are significant in therapeutic applications against human and animal pathogens, including bacteria, fungi and viruses (Pavrez *et al*., 2005).

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Alkaloids primarily act as feeding deterrents and toxins to insects and other herbivores, in many cases by directly interacting with molecular targets within the nervous system (Wink, 2000). Flavonoids are a secondary metabolite of plants and the most abundant polyphenols in the human diet (Burda and Oleszek, 2001). Cyclo oxygenase (COX) are lipid metabolising enzymes that catalyse the oxygenation of polyunsaturated fatty acids (PUFA), preferably

arachidonicacid (AA), to form the prostanoids, which are potent cell-signalling molecules associated with the initiation, maintenance and resolution of inflammatory processes (Charlier and Michaux, 2003). Lipoxygenases (LOXs) is involved in terminal differentiation of keratinocytes and has a major role in skin barrier function. The subsequent dopaquinone is converted to dopa and dopachrome through auto-oxidation (Roh *et al*., 2004). The plant *Vitex trifolia*Linn (Verbaneaceae) is stout aromatic shrubor a small tree, found from the foot of Himalayas southwards throughout greater part of India, Western Ghat and in Andamans (Agrawal, 1997). The leaves are simple or 3-foliolate. *V. negundo* closely resembles. The flowers are numerous and borne in terminal, oblong panicles 5 to 10 centimeters in length. The fruit is rounded and 4 to 5 millimeters in diameter (Padmalatha *et al*., 2009). Hence, the further studies were decided to be carried over on the coastal plant *Vitex trifolia* and also to check how well it can inhibit the above mentioned inflammatory enzymes.

MATERIALS AND METHODS

Collection of plant material

Vitex trifolia leaves were collected early in the morning from Medicinal Plant Garden at Sri Sairam Siddha Medical College and Research Centre, West Tambaram, Chennai, a recognized institution of Government of Tamil Nadu and the Department of AYUSH, Government of India. The sample was collected during the winter session (November-December, 2020) and experiments were carried out in Department of Biotechnology, Prathyusha Engineering College, Tiruvallur, India.

Phytochemical analysis of plant material

The collected plant samples were shadow dried and powdered with help of mixer grinders. The powder samples were extracted with aqueous and methanol solvents using Soxlet apparatus. Freshly prepared extracts of aqueous and methanol were divided into different test tubes. Phyto constituents such as alkaloids, terpenoids, glycosides, tannins, saponin, flavonoids were analysed in test extracts by methods of Harborne and Williams (2000).

Partial characterizations of thin layer chromatography (TLC) in *Vitex trifolia* anthocyanin extract

The Methanol extract of *Vitex trifolia* were loaded on to pre coated TLC (60 F2 54) and it was developed using solvent system in the ratio of 1:0.5:0.1 (Hexane, Chloroform and Methanol) visible and the non visible spot given and it is fluorescent with UV light at 360 nm.

Cyclooxygenase inhibition assay

The cyclooxygenase inhibition assay was performed according to amodified method of Lin and Kao (2001). Leuco-2,7-dichlorofluorescein diacetate (5 mg) was hydrolysed at room temperature in 1 MNaOH (50 µL) for 10 min, then 1 M HCl (30 µL) was added to neutralise excess NaOH before the resulting 1-DCF was diluted in 0.1 M Tris-buffer, pH 8. Cyclooxygenase enzyme (COX-1 or COX-2) was diluted in 0.1 M Tris-buffer, pH 8, so that a known aliquot gave an absorbance change of 0.05/min in the testreaction. Test samples were pre-incubated with enzyme at room temperature for 5 min in the presence of hematin. Premixed phenol, 1-DCF and arachidonic acid were added to the enzyme mixture to begin the reaction and to give a final reaction mixture of arachidonic acid (50 µM), phenol (500 µM), 1-DCF (20 µM) and hematin (1 µM) in 1 mL final volume of 0.1 M Tris-buffer, pH 8. The reaction was recorded. Lyprinol was investigated at multiple concentrations and exhibited 50% inhibition of the enzyme reaction at approximately 1 µg/mL (final concentration), therefore the other lipid samples were tested at this final concentration for comparison.

Lipoxygenase inhibition assay

A spectrophotometric assay for determination of LOX activity was used as reported (Khan *et al*., 2006) with slight modification. Inhibition experiments were run by measuring the loss of soybean 15-LOX activity (5 µg) with 0.2 µM linoleic acid (Sigma) as the substrate prepared in solubilized state in 0.2 M borate buffer (pH 9.0). Inhibition studies in presence of various concentrations of extracts (5, 10, 15, 20 µg/mL) and reference compound *viz*., quercetin was recorded at 234 nm using UV-Vis spectrophotometer. The inhibitory effect of the extracts was also expressed as percentage of enzyme activity inhibition. IC_{50} indicating the concentration required to inhibit 50% LOX activity was also calculated (Patel *et al*., 2022).

Tyrosinase inhibition assay

Tyrosinase inhibition activity is measured by the dopachrome method (Agarwal, 1997).

Enzymatic Assay of Xanthine oxidase

Xanthine combines with hydrogen peroxide and enzyme xanthine oxidase in the presence of oxygen to form uric acid, at pH 7.5, 25°C. The reaction is investigated in the lab conditions using a continuous spectrophotometric rate determination method, absorbance of 290 nm, light path of 1 cm (Cotran *et al*., 1994).

RESULTS AND DISCUSSION

The leaves of *Vitex trifolia* were collected, dried and powdered and was subjected to various phytochemical screening, extraction of crude phytochemicals and estimation of its response to various enzymes like lipoxygenase, cyclooxygenase, xanthine oxidase and tyrosinase. The preliminary phytochemical screening with the various qualitative chemical tests of various leaf extracts revealed the presence of carbohydrates, flavonoids, protein and amino acids, tannins, phytosterols and saponins phytoconstituents. The phytochemical screening of the *Vitex trifolia* studied presently showed the presence of alkaloids, flavonoids and Glycosides (Table 1).

The alkaloid and flavonoid extract of *Vitex trifolia* were loaded on Precoated TLC plates (60 F2 54 Merck) and developed with a solvent system of hexane, ethyl acetate and acetic acid in the ratio of 10:5:0.5. The developed plate was viewed under UV 240 nm and 360 nm (Table 2). The concentration of flavonoids in various concentration extracts of the *Vitex trifolia* was determined using spectrophotometric method with aluminum chloride. The content of flavonoids was expressed in terms of quercetin equivalent. The concentration of flavonoids in plant extract from *Vitex trifolia* ranged from 20.72 to 59.26 mg/g. Methanolic extract contains the flavonoid concentration. The inhibitory effects of COX-mediated TMPD oxidation activity were examined using purified COX as enzyme sources COX-2 activity was strongly inhibited by used in this study alkaloid and flavonoid extract from *vitex trifolia* inhibited COX-2 the inhibition potency quite different based on the results obtained in Graph 1. The following experiments focused on the inhibition potential of CBDA for COX-2 activity. Whereas the standard drug Celecoxib inhibited the COX2 enzyme with an IC_{50} of 52 nM. The results are shown in Graph 1.

The anti-inflammatory activity of the alkaloid and flavonoid extract of *vitex trifolia* was evaluated by measuring the inhibition of LOX using linoleicacid as substrate. The results were reported in Graph 2 flavonid extract showed inhibition percentage above 70.22% at 20 µg/mL. The standard n-propylgallate showed 72% inhibition at 20 µg/mL. The alkaloid and flavonoid extract of *Vitex trifolia* were tested for the effects on the oxidation of DOPA by mushroom tyrosinase. With increasing the concentrations of the alkaloid and flavonoid extract of *Vitex trifolia*, the diphenolase activity of mushroom tyrosinase markedly decreased concentration dependently. The values of IC50, the inhibitor concentration leading to 50% activity lost, of *Vitex trifolia* were estimated to be 5, 10, 15 and 20 µg/ml, respectively (Graph 3). The four different concentrations of *Vitex trifolia* alkaloid and flavonoid extract were tested for the inhibition activity of xanthine oxidase. *Vitex trifolia* extracts in different concentrations inhibited the xanthine oxidase activity. The maximum inhibition was found at 20 µg/ml concentration (Graph 4).

In many inflammatory disorders there is excessive activation of phagocytes, production of 0_2-, OH radicals as well as non free radicals species (H_20_2), which can harm severely tissues either by powerful direct oxidizing action or indirect with hydrogen peroxide and -OH radical formed from O_2- which initiates lipid peroxidation resulting in membrane destruction. Tissue damage then provokes inflammatory response by production of mediators and chemotactic factors. The reactive oxygen species are also known to activate matrix metello proteinase damage seen in various arthritic tissues (Cotran *et al.*, 1994).

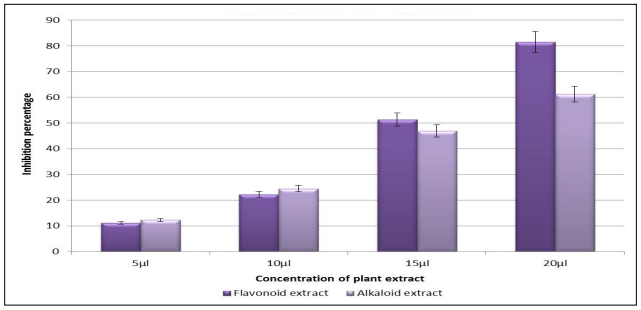
Phytochemicals from medicinal plants showing anti-inflammatory activities have the potential of filling this need because of structures are different from those of the more studied and their those of the more action may too very likely differ (Fabricant and Fanworth, 2001 and Prachayasittikul *et al.*, 2008). Some studies have demonstrated that flavonoid possessanti-inflammatory activities by inhibition of cyclooxygenase-2 (COX-2) expression in lipopolysaccharide (LPS)-activated RAW 264 cells or

Table 1: Phytochemical screenings of aqueous methanol leaf extract *Vitex trifolia*.

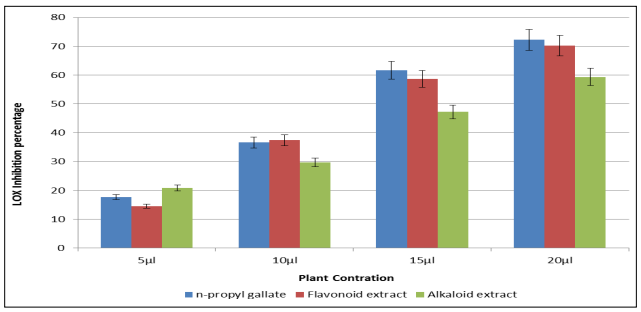
Phytochemicals	Result
Alkaloids	Positive
Flavonoids	Positive
Glycosides	Positive
Tannin	Negative
Saponin	Negative
Terpenoids	Negative
Polyphenols	Negative

Table 2: Partial characterization of different phytochemical extract from the *Vitex trifolia*leaves by TLC.

Extracts	Rf Value at UV 240 nm	Rf Value at UV 360 nm	Rf Value at Visible nm
Alkaloid	0.953	0.963	-
	0.338	0.230	-
	0.184	0.184	-
	0.153	0.153	-
	0.123	0.123	-
Flavonoid	0.714	0.714	-
	0.492	0.396	-
	0.349	0.158	-



Graph 1: Cycloxygenase inhibiton activity of flavonoid and alkaloid extract from *Vitex trifolla.*

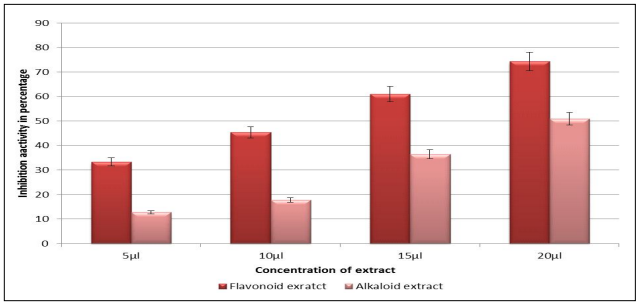


Graph 2: Lipoxygenase inhibiton activity of alkaloid and flavonoid extract of *Vitex trifolla.*

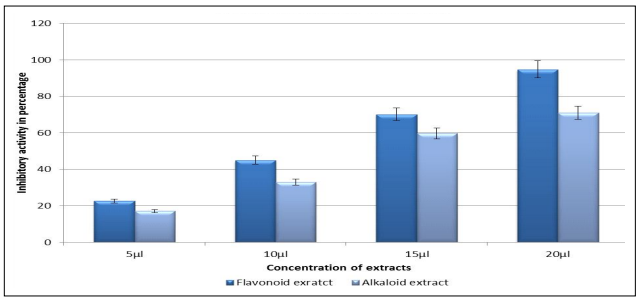
inhibiting inducible nitric oxide (iNOS) protein and mRNA expression in LPS-activated murine J774 macrophages (Hou *et al*., 2005) and such activities appear to be structure dependent. COX-2 seems to be involved in many inflammatory processes. Some antioxidants inhibit the expression of COX-2 by interfering with the signalling mechanisms that regulate the COX-2 gene (Hou *et al*., 2005). From the result, it is clear that caragennan induced paw oedema for the administered dose (500 mg/kg, p.o.) is comparable with reference standard indomethacine, which is a cycloxygenase inhibitor. But anti-inflammatory activity against caragennan-induced paw oedema is also shown by lipoxygenase inhibitor. Hence inhibition of caragennan-induced paw oedema by crude extract may be due to inhibitory activity of lipoxygenase enzymes (Gupta *et al*., 2006). Therefore, from the present study, we can conclude that alkaloid and flavonoid extracts of *Vitex trifolia* for the dose of 20 µl/ml shows anti-inflammatory activity in the early stage as well as in the late stage (up to 180 min) and after that, the effect becomes similar to that of negative control.

Lipoxygenases (LOXs) (LOX; EC 1.13.11.12) are a family of non-hemeiron-containing dioxygenases catalyzing the biosynthesis of leukotrienes. Leukotrienes function as initiators of inflammation and their inhibition is considered to be partly responsible for the anti-inflammatory activity. In the present study alkaloid and flavonoid extracts of *Vitex trifolia* showed good anti-LOX activity with an IC_{50} value of 29.87 µg/ml (Graph 2). LOX inhibition was used to evaluate anti-inflammatory activity of a few medicinal plants used in Limousin country. *Filipendula ulmaria* (Meadow sweet) recorded LOX inhibition with IC_{50} of 60 µg/ml and *Urtica dioica* (Nettle) methanolic extract inhibited LOX with IC_{50} of 348 µg/ml (Trouillas *et al*., 2003). In another study, eight methanolic extract out of 18 undomesticated plants of South Africa showed significant inhibition of 5-lipoxygenase (5-LOX) activity. *Bidenspilosa* extract exhibited IC_{50} of 21.8 µg/ml and *Emexaus trails* extract recorded IC_{50} of 81.4 µg/ml for LOX inhibition (Wang *et al*., 2006). LOXs are sensitive to antioxidants as antioxidants are involved in inhibition of lipid hydroperoxide formation due to scavenging of lipidoxy orlipidperoxy-radicals. This could lead to less availability of lipid hydro peroxide substrate required for LOX catalysis (Rackova *et al*., 2007 and Gentallan *et al*., 2019).

The effects of alkaloid and flavonoid extract of *Vitex trifolia*on the monophenolase and the diphenolase activities of tyrosinase were studied. The results showed that alkaloid and flavonoid extracts of *Vitex trifolia* could lengthen the lag phase of the monophenolase and decrease the steady-state rate of both monophenolase and diphenolase activity. The inhibition was displayed as reversible and the inhibition type was found to be mixed type. The inhibition constants for alkaloid and flavonoid extracts of *Vitex trifolia* binding with the free enzyme (E), KI, were obtained to be 0.507 and 1.543 mM, respectively, indicating that alkaloid and flavonoid extracts of *Vitex trifolia* was three times as potent for inhibiting the free enzyme. However, the inhibition constants for these two inhibitors



Graph 3: The inhibitions of flavonoid alkaloid extract of *Vitex trifolla on* the diphenolase activity of mushroom tyrosinase for the catalysis of DOPA.



Graph 4: *In vitro* xanthine inhibitory activity of flavoniod and alkaliod extract from *Vitex trifolla.*

binding with the enzyme substrate complex (ES), KIS, were obtained to be 1.354 and 1.321 mM, respectively. The values are almost the same, indicating alkaloid and flavonoid extracts of *Vitex trifolia* inhibited ES complex as potently as 4-cyanobenzoicacid. In addition, anthocynin extracts of *Eclipta alba* inhibited the free enzyme more potently than it did the ES complex. However, the inhibitory effect of alkaloid and flavonoid extracts of *Vitex trifolia*was the contrary.

CONCLUSION

Alkaloid and flavonoid extracts of *Vitex trifolia* hold great promise for natural treatments of arthritis that are safe and effective and can be provided as dietary supplements, added to multiple vitamins and incorporated into food products to create functional foods. In addition, the novel bioactives identified in the alkaloid and flavonoid extracts of *Vitex trifolia* extracts, when fully characterized, could prove to be promising new drug leads for Cycloxygenase, Lipoxygenases, Tyrosinase and Xanthine oxidase inhibition as well as triple inflammatory enzyme inhibitors for treatment of a range of inflammatory diseases that are safe and efficacious.

Conflict of interest

All authors declare that they have no conflict of interest.

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