Journal of Medicinal and Aromatic Plant Sciences 39(1) (2017) 18-23

In Vitro Anti-inflammatory Activity of *Bidens biternata*(Lour.) Merr. & Sheriff – a Wild Medicinal Plant of Waynadu District of Kerala

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Article History

Received: 14th July, 2016
Accepted: 30th November, 2016

Key words

5-LOX
Anti-inflammatory activity
Bidens biternata
COX-2
Western Ghats

ABSTRACT

Bidens biternata (Lour.) Merr. & Sheriff, of the family Asteraceae, is an erect annual herb, commonly found in cultivated areas in Western Ghats regions of Kerala state. It is used as a leafy vegetable by Paniya, Chetti, Kani and Kattunaayika tribes of Waynadu Districts in Kerala and also to cure hepatitis, inflammation, cold, cough, dysentery, asthma etc. In the present study, crude methanol extract and isolated bioactive compound of B. biternata leaves was analyzed for its in vitro anti-inflammatory activity. The anti-inflammatory activity was evaluated by cyclooxygenase (COX-2) and 5-lipoxygenase (5-LOX) inhibitory assays. In COX-2 inhibitory assay of crude methanolic leaf extract, the percentage of inhibition was found to be 36.71%, 63.92% and 87.35% for sample concentration 100 μgml^{-1}, 500 μgml^{-1} and 1000 μgml^{-1} respectively. In the 5-LOX percentage of inhibition was found to be 35.64%, 61.76% and 85.95%.Column chromatography and Thin Layer Chromatography were used for separation of compounds followed by UV, IR, mass and NMR spectroscopic analysis for the characterization of phytochemical from methanolic leaf extract of B.biternata. The compound isolated from methanolic leaf extract of B. biternata, which is identified as, quercetin showed higher inhibitory effects in both COX (40.36%, 72.61% and 90.73%) and LOX (39.25%, 70.14% and 89.68%) than crude extract. COX-2 inhibition assay of isolated compound quercetin showed high activity than 5-LOX assay. The results revealed that the isolated compound quercetin (3, 3', 4', 5, 7-pentahydroxy flavones) of B. biternata possessed high anti-inflammatory activity, so could be used as an effective therapeutic agent against inflammatory disorders.

INTRODUCTION

India is one of the richest countries in the world with regard to genetic resources for medicinal flora. The revival of interest in herbal medicines is due to increased awareness of the limited horizon of synthetic pharmaceutical products to control major diseases and to the current wide spread belief that ''green medicine'' is safe, more accessible and more affordable [14] than costly synthetic drugs, many of which have adverse side effects. Since

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Doi: https://doi.org/10.62029/jmaps.v39i1.Pradeesh

time immemorial, man has used various parts of plants in the treatment and prevention of various ailments [12]. The shortcomings of drugs available today have propelled the discovery of new pharmaco-therapeutic agents from medicinal plants. To determine the potential and promote the use of herbal medicine, it is essential to intensify the study of those medicinal plants that has prominent place in folklore [2].

Inflammation is the pathophysiological response of mammalian tissues to a variety of hostile agents, and the complex events and mediators involved in inflammation can induce, maintain and aggravate many disorders. Non-steroidalanti-inflammatory drugs (NSAIDs) are the main drugs used to reduce the untoward consequences of inflammation [1]. The frequent usage of NSAIDs has many side effects; to minimize these there is a need to explore natural sources of herbal drugs that are effective against inflammatory disorders and have fewer side effects.

B. biternata (Asteraceae) is a medicinal herb found throughout India, especially in Western Ghats of Kerala. Many medicinal uses like antidiabetic, antiallergic, antipyretic, antihepatic activities of this plant have been reported by Pradeesh *et al*. [15-18]. Though the plant and its extracts have been extensively used in the tribal medicine, information from organized search of published literature does not provide evidence for its anti-inflammatory activities. So the present study aimed to investigate the *in vitro* anti-inflammatory effects of methanolic extract and isolated bioactive compound from leaves of *B. biternata*, using COX and LOX for anti-inflammatory activity assays.

MATERIALS AND METHODS

Collection and preparation of sample

Leaves of *B. biternata* were collected fresh from Western Ghats of Kerala. These were shade dried, ground well using mechanical blender in to fine powder and transferred to airtight containers for future studies. Experiments were done in triplicate, graph pad Instat DTCG was employed followed by IC_{50} values for the calculation and comparison.

Chemical extraction

The fine powder was used for extraction by using solvent like methanol. Fifty gram of sample powder was covered with cotton cloth and kept into the soxhlet apparatus for distillation. Three hundred ml of methanol was taken into the round bottom flask and heated in a mantle for 8 hours at 70°C. After completing the process, extract was collected in beaker and kept in oven at 37°C-40°C for evaporation. The crude concentrated extract was weighed and used for further studies.

The crude methanolic leaf extract was used for the isolation of bioactive compound from *B. biternata*. The anti-inflammatory activity was analyzed *in vitro* initially with crude methanolic extract of *B. biternata* leaves and later with isolated compound. Column chromatography and Thin Layer Chromatography (TLC) were used for separation of compounds followed by UV, IR, mass and NMR spectroscopic analysis, for the characterization of phytochemical from methanolic leaf extract of *B.biternata*. Anti-inflammatory activity of crude methanol extract and isolated compound of *B. biternata* leaves was tested using lymphocyte culture

Human peripheral blood lymphocytes were cultured in RPMI 1640 (HIMEDIA) media, supplemented with 20% heat inactivated foetal bovine serum (FBS), antibiotics (Penicillin). The culture was filtered using 0.2 µm pore sized cellulose acetate filter (Sartorios) in completely aseptic conditions. Fresh plasma was aseptically added to the culture at a concentration of $1õ10^6$ cells/ml. The culture was then incubated for 72 hours. After 72 hours culture was activated by adding 1µl LPS (Lipopolysaccharides). A standard drug (Penicillin) was added in the concentration of 100 µg/ml from a stock of 100 mg/ml and the sample was added in the concentration of 100 µg/ml, 500 µg/ml and 1000 µg/ml from a stock of 100 mg/ml incubated for 24 hours. The isolation was done by spinning at 6,000 rpm for 10 minutes. Supernatant was discarded and 200 µl of cell lysis

buffer (1M Tris-HCl, 0.25 M EDTA, 2M NaCl, 0.5% Triton) was added. The incubation was done for 30 minutes at 4°C and anti-inflammatory assay was done in pellet suspended in a small amount of supernatant [13].

Cycloxygenase (COX-2) inhibition assay

Enzymatic activity of COX-2 was measured according to the method of Copeland *et al*. [5] with slight modifications. It is based on the oxidation of N, N, N, N-tetra methyl-P-phenylene diamine (TMPD) during the reduction of PGG2 (Prostaglandin G_2) to PGH_2. The assay mixture contained Tris-HCl buffer (100 mM, p^H 8.0), haematin (15 µM), EDTA (3 µM), enzyme (100 µg COX-2) and the test drugs (crude methanolic extract). The mixture was pre-incubated at 25°C for 15 minutes and then the reaction was initiated by the addition of arachidonic acid and TMPD in total volume of 1 ml. The enzyme activity was measured by estimating the initial velocity of TMPD oxidation for the first 25 seconds of the reaction by following the increase in absorbance at 603 nm. A low rate of non-enzymatic oxidation observed in the absence of COX-2 was subtracted from the experimental value while calculating the percentage inhibition.

5-Lipoxygenase (5-LOX) inhibition assay

5-LOX enzyme inhibitory activity of *B. biternata* was measured by using the method of Reddanna *et al*. [20] modified by Ulusu *et al*. [25]. The assay mixture contained 80 mM linoleic acid and 10 µl of enzyme 5-LOX in 50 mM phosphate buffer (p^H 6.3). The reaction was initiated by the addition of enzyme buffer mix to linoleic acid and the enzyme activity was monitored as the increase in absorbance at 234 nm. The reaction was monitored for 120 seconds and the inhibitory potential of the test substance was measured by incubating various concentrations of test substances (crude methanolic extract) for 2 minutes before the addition of linoleic acid. Percentage of inhibition was calculated by using the formula:

$$\% \text{ inhibition} = (C\text{-}T/C) \times 100$$

where

C = Optical density of control , T = Optical density of test

RESULTS AND DISCUSSION

Antiinflammatory activity of methanol extract of *Bidens biternata* leaves and isolated compound, quercetin

Cycloxygenase and 5-Lipoxygenase inhibition assay

Cyclooxygenase (COX) is an enzyme that is responsible for the formation of prostanoids. The three main groups of prostanoids - prostaglandins, prostacyclins, and thromboxanes are involved in the inflammatoryresponse. Lipoxygenases are non-heme iron-containing enzymes that catalyze the stereo specific incorporation of molecular oxygen into polyunsaturated fatty acids with a 1, 4-*cis, cis*-pentadiene motif leading to production of leukotrienes which finally cause inflammation [11]. In mammalian cells, eicosanoid biosynthesis is usually initiated by the activation of phospholipase-A_2 and the release of arachidonic acid from membrane phospholipids in response to the interaction of a stimulus with a receptor on the cell surface. Arachidonic acid is subsequently transformed by the enzyme cyclooxygenase (COX) to prostaglandins (PGs) and thromboxane (TX). The COX pathway is of particular clinical relevance because it is the major target for non-steroidal anti-inflammatory drugs, which are commonly used for relieving inflammation, pain and fever. COX exists in two distinct isozymes (COX-1 and COX-2), one of which, COX-2, is primarily responsible for inflammation but apparently not for gastrointestinal integrity or platelet aggregation [4].

The biosynthesis of PGs is initialized by COX isoenzymes, namely COX-1 a constitutively expressed enzyme in numerous cell types, thought to provide PGs mainly for physiological functions and COX-2, an inducible isoform cause inflammation, fever and pain [8]. After conversion of arachidonic acid to PGH_2 (Prostaglandin H_2) by COX enzymes, it is subsequently isomerized to three different PGE_2 (Prostaglandin E_2). PGE_2 plays a major role in the pathophysiology of inflammation, pain and pyresis but it also regulates physiological

functions in the gastrointestinal tract, kidney and in the immune and nervous system [23]. The non-steroidal anti-inflammatory drugs (NSAIDs) reduce PGE_2 biosynthesis by inhibiting both COX isoenzymes and they are potent suppressors of inflammation, fever and pain [6].

Chronic use of these drugs is associated with severe side effects, mainly gastrointestinal injury and renal irritations, apparently due to suppression of COX-1 derived PGE_2 [19]. COX-2 selective inhibitors were designed to minimize gastrointestinal complications of traditional NSAIDs but recent clinical studies indicated small but significantly increased risks for cardiovascular events [10]. Suppression of leucotriens (LT) and prostaglandin (PG) synthesis by interfering with the 5-LOX and COX pathways represent an efficient pharmacological approach for the treatment of inflammatory diseases [6].

The enzyme 5-lipoxygenase (5-LOX) initiates the synthesis of leukotrienes (LTs) from arachidonic acid (AA). The expression of 5-LOX is restricted to certain leukocytes, including neutrophils, eosinophils, mast cells, basophils, monocytes, macrophages and B-lymphocytes. These cells will, upon stimulation, synthesize and secrete LTs, which move short distances to target cells, bind specific receptors, and trigger cell-specific responses [3].

In cyclooxygenase (COX-2) inhibitory assay of crude methanolic leaf extract, the percentage of inhibition was found to be 36.71%, 63.92% and 87.35% (IC_{50} = 24.21 ìg/ml) as given in Fig 1 for sample concentration 100 μgml^{-1}, 500 μgml^{-1} and 1000 μgml^{-1} respectively. In the lipoxygenase assay (5-LOX) percentage of inhibition were found to be 35.64%, 61.76% and 85.95% (IC_{50} = 23.32 ìg/ml) as shown in Fig 2. The methanol leaf extract of *B. biternata* showed inhibitory effect on both COX and 5-LOX enzymes but it has higher inhibition on COX-2 than LOX.

Anti-inflammatory activity of isolated compound quercetin could be due to its inhibitory effects on inflammation producing enzymes (Cyclooxygenase, lipoxygenase) and the

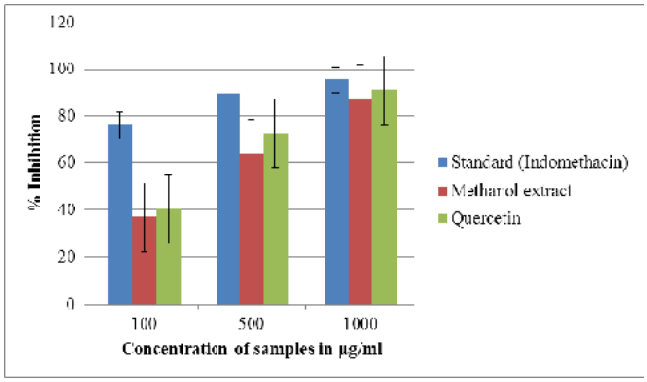

Fig 1 Cyclooxygenase inhibitory effect of methanolic extract and quercetin from *Bidens biternata*

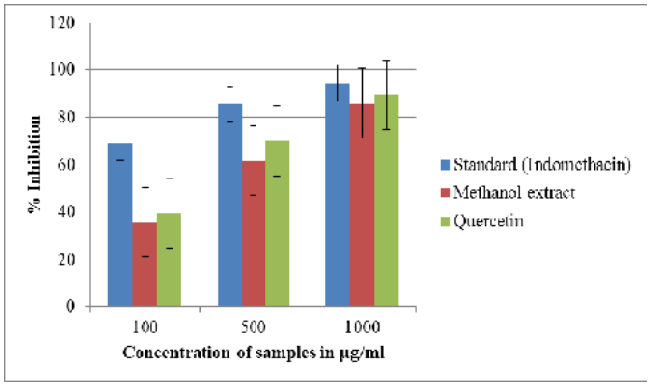

Fig 2 Lipoxygenase inhibitory effect of methanolic extract and quercetin from *Bidens biternata*

subsequent inhibition of inflammatory mediators including, leukotrienes and prostaglandins. Inhibition of histamine release by mast cells and basophils also contributes to quercetin's anti-inflammatory activity [9]. The compound isolated quercetin from methanolic leaf extract of *B. biternata* showed higher inhibitory effects in both COX (40.36%, 72.61% and 90.73%) and LOX (39.25%, 70.14% and 89.68%) than crude extract as shown in Fig 1 and 2. *In vitro* anti-inflammatory activity of isolated compound was comparable with standard anti-inflammatory drug indomethacin (COX-95.34% and LOX-94.31% for concentration 1000 μgml^{-1}). In isolated compound (quercetin), the IC_{50} values for COX (22.25 ìg/ml) and LOX (20.24 ìg/ml) were compared with the standard (20.13 ìg/ml).

Quercetin has the molecular formula of $C_{15}H_{10}O_7$, 316°C melting point, 303.0 m/z molecular ion peak and lambda maximum of 360 nm. It was characterized as 3, 3', 4', 5, 7-pentahydroxy

flavones/quercetin [7]. Quercetin, one of the flavonoids in fruits and vegetables reported to have powerful antioxidant activity [24]. Quercetin is included in flavonoids present in plants that are used as foods. Quercetin act as antioxidants, scavenges oxygen radicals, inhibits xanthine oxidase and lipid peroxidation. It also inhibits oxidation of low density lipoprotein (LDL) cholesterol, probably by inhibiting LDL oxidation itself, by protecting vitamin-E in LDL from being oxidised or by regenerating oxidized Vitamin-E. It possessess useful pharmacological effects due to its strong antioxidant effects such as prevention of circulatory disease, antiinflammatory effects and anticancer effects [21]. Quercetin belongs to the group of flavonoids that are largely effective as antioxidant, anti-inflammatory, hepatoprotective and anticancer agents [**22**].

ACKNOWLEDGMENTS

The authors are grateful to the Western Ghats Cells and Kerala State Council for Science Technology and Environment (KSCSTE), Pattom, Thiruvananthapuram for funding this research work.

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