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Original Article

ANTI-INFLAMMATORY ACTIVITY OF *MANILKARA ZAPOTA* LEAF EXTRACT

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ABSTRACT

Objective: *Manilkara zapota* is a medicinal plant which is native to Mexico and Central America, and widely distributed in India. Various parts of this plant are traditionally used for treatment of several diseases, including inflammation-associated ailments. The main aim of the present study is to evaluate the anti-inflammatory potential of ethyl acetate and methanolic extracts of *M. zapota* leaf.

Methods: *In vitro* secretary phospholipase A_2 (PLA_2) and 5-Lipoxygenase (5-LOX) assays and *In vivo* studies using carrageenan induced rat paw edema model were performed to assess the anti-inflammatory activity of *M. zapota* leaf extracts.

Results: *In vitro* studies suggest that *M. zapota* leaf extracts exhibited significant $_sPLA_2$ and 5-LOX inhibitory activities. In *in vivo* studies *M. zapota* leaf extracts showed dose dependent inhibition of carrageenan induced paw edema in rats. The anti-inflammatory activity of ethyl acetate leaf extract was superior to methanolic extract.

Conclusion: This study concluded that ethyl acetate leaf extract of *M. zapota* exhibited significant anti-inflammatory activity and warranted further investigation to isolate and identify the components.

Keywords: *Manilkara zapota*, COX-2, Phospholipase A_2, TNF-α, IL-1β,IL-6, IFN-γ

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INTRODUCTION

The immune system has developed gradually as a unique complex network that defends the host body from both infectious and non-infectious foreign substances. Malfunctioning of the immune network either innate or adaptive branches, leads to chronic inflammatory diseases such as inflammatory bowel disorders, arthritis, asthma, neurodegenerative ailments and autoimmune diseases. Inflammation is a vital response of vascular tissues to infectious and non-infectious agents, both exogenous and endogenous inflammatory inducers such as lipopolysaccharides (LPS), proinflammatory cytokines such as Tumor Necrosis Factor (TNF-α), Interleukin-1β (IL-1β),Interleukin-6 (IL-6) and Interferon-γ (IFN-γ) stimulates inflammatory macrophages M1, which elevate inflammatory mediators such as prostaglandin E_2 (PGE_2) and leukotrienes (LT-4) and nitrous oxide (NO) by cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX) and inducible nitric oxide synthase (iNOS) [1]. Nuclear factor kappa B (NF-κB) plays a central role in the up-regulation of inflammatory pathways. Proinflammatory cytokines such as TNF-α and IL-1β act syngertically induce archidonic acid dependent and independent inflammatory pathways [2]. Inflammatory response leads to a cascade activation of NF-κB and signal transducer activator of transcription 3 (STAT3) controls stress response. TNF-α, IL-1β play a vital role in ROS and RNS induced inflammation [3]. Both steroidal and non-steroidal anti-inflammatory drugs are used for treatment of inflammatory diseases [4] though these drugs have potent anti-inflammatory activity, long term administration is required for treatment of chronic diseases. Furthermore, these anti-inflammatory drugs have several serious side-effects on organ functions [5]. Therefore, naturally occurring anti-inflammatory agents with a high therapeutic index and less side-effects are required as substitutes for synthetic anti-inflammatory drugs.

Manilkara zapota (Sapotaceae) and its different parts have been traditionally used for medicinal purpose. The acetone extract of *M. zapota* leaves has shown significant antioxidant activity [6]. Petroleum ether and ethanolic leaf extracts of *M. zapota* were reported to have analgesic activity. The ethanolic extract of *M. zapota* possesses significant anti-arthritic activity [7]. It is likewise reported that ethyl acetate and methanolic extract of leaves of *M. zapota* shown significant inhibition of paw edema in rats. The present work deals with evaluation of anti-inflammatory activity of both ethyl acetate and methanol leaf extracts by *in vitro* assays such as $sPLA_2$ inhibitory assay, 5-lipoxygenase inhibitory assay and *in vivo* anti-inflammatory carrageenan induced paw edema model.

MATERIALS AND METHODS

Plant material collection

Fresh leaf material of *M. zapota* plant was collected from Vizag steel plant area, Visakhapatnam district, Andhra Pradesh during month of May 2011. Plant leaf material was authenticated by Dr. S. B. Padal, Associate Professor, Department of Botany, Andhra University. A voucher specimen (Accession Number AU (BDH) 21913) of this plant was deposited in Botany department Herbarium, Andhra University.

Preparation of methanol/ethyl acetate leaf extract

Fresh leaves of *Manilkarazapota* were collected and shade dried. Further, dried leaves were pulverized into powder and used in extract preparation.

Approximately 500 grams of dried powder was extracted with solvent methanol/ethyl acetate by a hot percolation method using a Soxhlet apparatus. The obtained extracts were Rota-vaporized to obtain a crude methanol leaf extract weighing about 25 and 35 grams. The methanol and ethyl acetate extracts were used to assess their anti-inflamnmatory activity using *in vitro* and *in vivo methods.*

In vitro phospholipase A_2 assay

PLA_2 assay was performed using $sPLA_2$ enzyme inhibitory screening kit as per instructions of manufacturer (Cayman Chemical, Ann Arbor, Michigan, USA). The reaction mixture was contained 10 µl of PLA_2, 25, 50 and 100 µg/ml of ethyl acetate and methanol plant extracts, respectively in test wells, 200 µl substrates and incubated for 15 min. Further, 10 µl of 5, 5′-dithio-bi's-(2-nitrobenzoic acid) (DTNB) was added to develop color and read at a wavelength of 415 nm. After hydrolysis of the thioester bond at the *sn*-2 position of diheptanoyl Thio-PC (substrate) by PLA_2, the released free thiols were detected using DTNB, which has an absorbance at 415 NM. The control wells contain only PLA_2, substrate and DTNB. Thioetheramide-PC was used as positive control. The percent inhibition of enzyme activity was calculated using below formula:

$$Percentageinhibition = \left(\frac{Absorbance\ of\ control - Absorbance\ of\ test}{Absorbance\ of\ control}\right) \times 100$$

Lipoxygenase assay

5-LOX inhibitory assay was performed by using UV kinetic method [8, 9]. This method was performed by using an assay mixture consisting 3 ml of 50 mM* phosphate buffer pH 6.3, along with 10 µl of 80 mM* of linoleic acid and potato 5-LOX enzymes. This assay solution was kept in ice and measured the enzyme activity throughout the experiment for every two minutes at 234 NM in UV visible spectrophotometer. The 5-LOX inhibitory activity of *M. zapota* methanol and ethyl acetate leaf extracts was tested at different concentrations viz., 5, 10, 15 and 25µg/ml. The activity of 5-Lipoxygenase was compared with the standard positive control Quercetin. (Vendor, city, country)

The percent inhibition of 5-lipoxygenase inhibitory activity of plant extracts was calculated by using a formula.

$$Percentageinhibition = \left(\frac{Absorbance\ of\ control \quad Absorbance\ of\ test}{Absorbance\ of\ control}\right) \times 100$$

In vivo methods

Carrageenan-induced hind paw edema in rats (acute inflammation model)

Carrageenan-induced paw edema model, developed by [10] the most widely used for the evaluation of anti-inflammatory activity. Male Wistar albino rats weighing 150-200 g were obtained from M/s Mahavir Enterprises (Hyderabad, Telangana, India). The animals were housed under standard conditions (Temperature of 22±100C with an alternating 12h light-dark cycle and relative humidity of 60±5%. The animals were fed with standard laboratory diet, which was purchased from M/s Rayans Biotechnology Pvt. Ltd. (Hyderabad, Telangana, India). During the experiment, the rats were allowed to have access to water and food ad *libitum*. Animal experiments were conducted according to CPCSEA guidelines. The animal experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of GITAM University (IAEC no. 517/IAEC/2012). The animals were divided into four groups (n=6). The first group was given normal saline by gastric incubation. The second and third groups (200 and 400 mg/kilogram body weight) received the ethyl acetate and methanol

M. zapota leaf extracts for 10 d and the fourth group received diclofenac sodium as a standard (10 milligram/kilogram body weight). The paw volume was measured plethysommetrically (UgoBasile, Italy) at 0h, 1h, 2h, 3h, 4h, and 5h after the injection of carrageenan. The percentage of inhibition of paw volume of treated groups was calculated by comparing with a mean paw volume of the control group.

$$Percentageinhibition = \left(\frac{Controlpawvolume - Testpawvolume}{Controlpawvolume}\right) \times 100$$

Statistical analysis

Experimental results are expressed as mean±SEM A P-Value less than 0.05 represents significant difference compared with control group by Students *t*-test (n=3-6)

RESULTS

M. zapota ethyl acetate and methanol leaf extracts on PLA_2 activity *in vitro*

Various doses (25, 50 and 100 µg/ml) of Ethyl acetate and methanol leaf extracts of *M. zapota* were evaluated for PLA_2 inhibitory activity. A significant inhibition of PLA_2 was found with ethyl acetate extract when compared to methanol extract. As shown in [fig. 1] a dose dependent inhibition of PLA_2 activity was observed with the doses tested, indicating the uniformity of anti PLA_2 activity. Further, the IC_{50} of both ethyl acetate and methanol extracts was determined and found to be 122µg/ml and 172.1µg/ml respectively. Etheramide-PC was employed as positive control whose IC_{50} was 7.66µg/ml respectively.

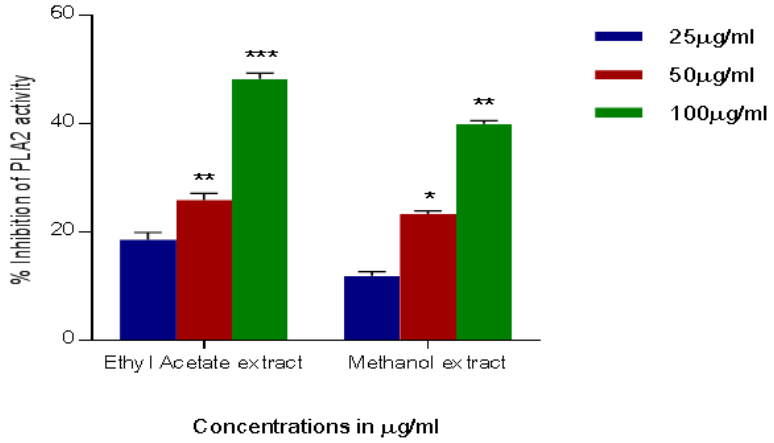

Fig. 1: Inhibitory effect of *Manilkara zapota* ethyl acetate and methanol leaf extracts on

M. zapota ethyl acetate and methanol leaf extracts exert inhibition of 5-LOX activity: *In vitro*

5-LOX inhibitory activity of *M. zapota* has been evaluated by a UV kinetic method. The 5-LOX inhibitory activity of *M. zapota* ethyl acetate and methanol leaf extracts was performed with various doses viz., 5, 10, 15, 25µg/ml. As shown in fig. 2 and 3 a dose dependent inhibition of 5-LOX activity of ethyl acetate and methanol leaf extract was observed. *M. zapota* leaf ethyl acetate and methanol extracts shown significant 5-LOX inhibitory activity with IC_{50} of 15.85µg/ml for ethyl acetate extract and 33.24µg/ml for methanol extract respectively. Ethyl acetate extract showed significant 5-LOX inhibitory activity than methanol extract. Quercetin was employed as positive control whose IC_{50} was 4.85µg/ml.

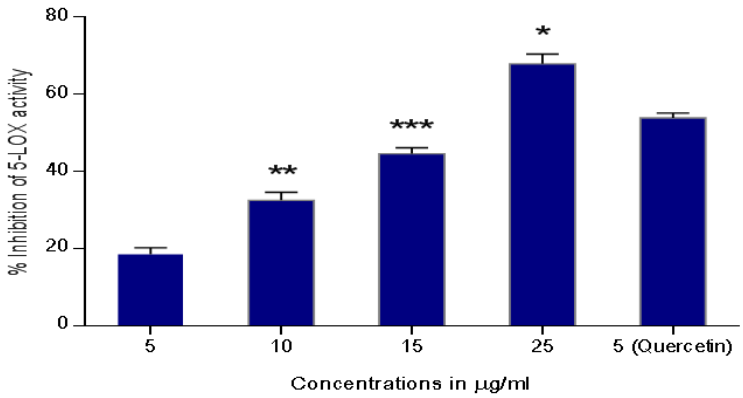

Fig. 2: Inhibitory effect of ethyl acetate leaf extract of *Manilkara zapota* on 5-LOX activity, Values are expressed as mean±SEM* P<0.05, ** P<0.01, *** P<0.001 represents significant difference compared with control group by student's t-test (n=3)

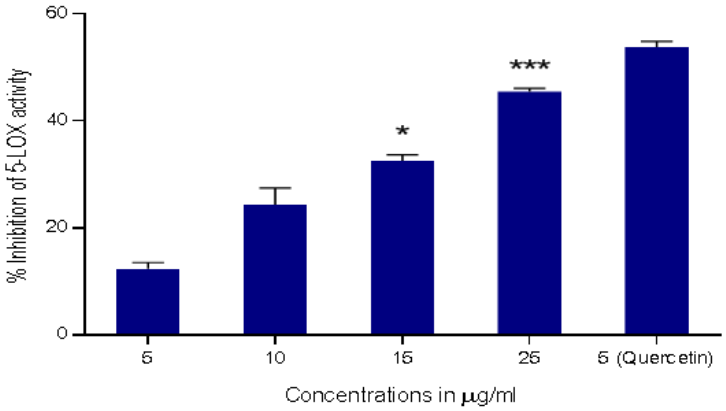

Fig. 3: Inhibitory effect of methanol leaf extract of *Manilkara zapota* on 5-LOX activity, values are expressed as mean±SEM* P<0.05, ** P<0.01, *** P<0.001 represents significant difference compared with control group by student's t-test (n=3)

M. zapota* ethyl acetate and methanol leaf extracts display Anti-inflammatory effect: *in vivo

Anti-inflammatory activity of *M. zapota* has been evaluated by using carrageenan induced hind paw edema in rats (acute inflammatory model) developed by [10]. As shown in the fig. 4 ethyl acetate and methanol leaf extracts of *M. zapota* exhibited dose dependent decrease in paw edema up to 5 h. However, ethyl acetate leaf extract showed significant decrease in paw volume in late phase 4hr and 5hr when compared to methanol leaf extract as a significant decrease in paw edema was observed in late phase 4h and 5h when compared to methanol leaf extract as a significant inhibition of paw edema was observed in late phase 4h and 5h. It is substantiated that inhibition of inflammation is probably due to the inhibition of inflammatory enzymes such as PLA2, COX-2 and 5-LOX.

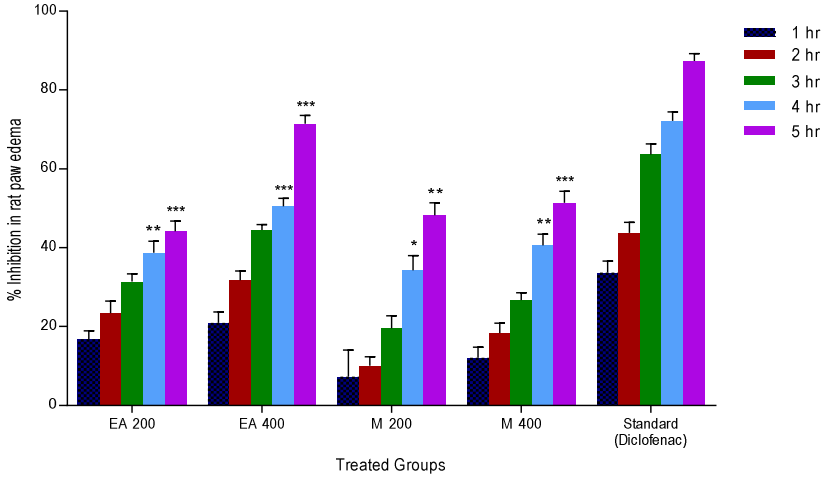

Fig. 4: Inhibition of carrageenan induced paw edema by ethyl acetate and methanol extracts, values are expressed as mean±SEM* P<0.05, ** P<0.01, *** P<0.001 represents significant difference compared with control group by student's t-test (n=3)

DISCUSSION

Current active research is focused on herbal medicine in treating inflammation. Herbal medicines obtained from wide array of plant extracts are in high usage to cure a wide variety of inflammatory diseases [11-13]. Side effects associated with Non Steroidal Anti-inflammatory Drugs (NSAIDS) made researchers to think about alternative medicine to NSAIDS which is to be natural and free from side effects [14-16]. The significance of natural anti-inflammatory compounds raised interest in pharmacological assessment of variety of plants used in traditional medicine, this interest resulted in the scientific study of herbal drugs having lesser side effects, thus providing relief to inflammation [17-19]. Thus in the present study an effort was made to evaluate the anti-inflammatory potential of methanol and ethyl acetate leaf extracts of *M. Zapota* by *in vivo* and *in vitro* anti-inflammatory methods. Carrageenan induced paw edema in rats is generally used as *in vivo* experimental model for assessing anti edematous effect of natural compounds, and assess the role of inflammatory mediators in acute inflammation [20-23]. The carrageenan induced paw edema is broadly used to evaluate potential anti-inflammatory agents particularly non-steroidal type. The time line of edema development in carrageenan induced paw edema model in rats is commonly represented by a biphasic curve. The first phase occurs within an hour of injection and is partly due to the trauma of injection and partly due to the release of 5-HT, histamine and kinins [24-28]. The anti-inflammatory effect of *M. zapota* extract was apparent in each concentration of extracts as early as the first hour of carrageenan injection and highest inhibition was during the fifth hour. It maintained the suppression of the inhibition throughout the duration of the study. This shows that the plant extract may hamper any of all process of inflammation and acts as an anti-inflammatory agent. The anti-inflammatory potential of medicinal plants has been reported in plants *Solanumnigrum* [22], *Phyllanthus amarus* [29], *Syringa patula* [30], *Plumeria acuminate* [31] and *Pistia stratiotes* [32]. There are few references with respect to the anti-inflammatory activity of *M. zapota*. As seen in this study ethyl acetate extract of *M. zapota* especially at the high dose has an inhibitory effect on edema formation in both early and late phases of carrageen an induced rat paw edema model. The significant inhibitory activity shown by the extract of *M. zapota* over a period of five hours in carrageen an induced inflammation was quiet similar to that exhibited by the group treated with diclofenac sodium. Animal data is valuable for developing cost effective and successful anti-inflammatory agents. This further supports the association of reverse pharmaceutics with ayurvedic drug actions. The result of this study indicates that referring to folk literature is a helpful approach to identify plants with bioactive potentials. The tested extracts showed prospective anti-inflammatory bioactivities in *in vivo* and *in vitro* models of inflammation. The nature of these bioactive compounds and their mechanism of action were not determined and will be subject to further investigation.

In vitro anti inflammatory activity was evaluated by 5-lipoxygenase and phospholipase A_2 assays, [33-37] for 5-lipoxygenase inhibitory activity both ethyl acetate and methanol extracts were tested, among the two extracts ethyl acetate extract shown significant 5-LOX inhibitory activity when compared to methanol leaf extract. The inflammatory enzyme phospholipase A2 is well known for its capability of formation of mediators of inflammation such as prostaglandins and leukotrienes. Phospholipase A2 catalyses the conversion of phospholipid to arachidonic acid which is effectively converted to prostaglandins by cyclooxygenase, the formed prostaglandins cause inflammation [38-40]. PLA2 inhibitory activity of ethyl acetate and methanol leaf extracts were evaluated, among the two extracts ethyl acetate leaf extract shown promising PLA2 inhibitory activity when compared to methanol leaf extract.

CONCLUSION

From the results of *in vitro* and *in vivo* assays, it is concluded that the ethyl acetate extract of *Manilkara zapota* has been found to have shown good anti-inflammatory activity over methanolic extract, as evaluated by PLA_2 assay, 5-LOX assay and *in vivo* carrageenan induced paw edema model. The ethyl acetate extract has shown significant PLA_2 inhibitory activity with IC_{50} of 122.1µg/ml while that of standard inhibitor Thioetheramide–PC, 7.66µg/ml Similarly ethyl acetate leaf extract exhibited significant 5-LOX inhibitory activity with IC_{50} value of 15.85µg/ml while that of standard quercetin 4.851µg/ml. Further, it is concluded that between PLA_2 and 5-LOX inhibitory activities of ethyl acetate extract of *Manilkara zapota*, 5-LOX activity is more significant than PLA_2 inhibitory activity as indicated by the IC_{50} values.

In vivo studies in carrageen an induced acute inflammation model reveal that significant anti-inflammatory activity in terms of edema inhibition was observed with ethyl acetate leaf extract in doses tested in comparison to the standard diclofenac drug

The potential anti-inflammatory and antioxidant activities of *M. zapota* were found to be referable to the presence of compounds such as flavonoids, terpenoids, steroids (glycosides, cardiac glycosides).

From these *in vitro* and *in vivo* anti-inflammatory studies ethyl acetate extract of *M. zapota* showed significant anti-inflammatory activity. The anti-inflammatory activities of *M. zapota* extracts were found to be due to its 5-LOX and PLA2 inhibitory activity. Further studies are in progress to isolate and identify novel anti-inflammatory molecule from the ethyl acetate extract of *M. zapota* leaves.

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CONFLICT OF INTERESTS

Declared none

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