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 E2 (PGE2) 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 synergistically induce arginid 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 methanol 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 sPLA2 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 Manilkara zapota 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-inflammatory activity using in vitro and in vivo methods.
In vitro phospholipase A<sub>2</sub> assay

PLA<sub>2</sub> assay was performed using sPLA<sub>2</sub> enzyme inhibitory screening kit as per instructions of manufacturer (Cayman Chemical, Ann Arbor, Michigan, USA). The reaction mixture was contained 10 µl of PLA<sub>2</sub> 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-bis-(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<sub>2</sub>, the released free thiols were detected using DTNB, which has an absorbance at 415 NM. The control wells contain only PLA<sub>2</sub> substrate and DTNB. Thioetheramide-PC was used as positive control. The percent inhibition of enzyme activity was calculated using below formula:

\[
\text{Percentage Inhibition} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \right) \times 100
\]

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 25 µg/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:

\[
\text{Percentage Inhibition} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \right) \times 100
\]

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/IACUC/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/kg body weight) received the ethyl acetate and methanol 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 plethysmometrically (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.

\[
\text{Percentage Inhibition} = \left( \frac{\text{Control paw volume} - \text{Treated paw volume}}{\text{Control paw volume}} \right) \times 100
\]

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<sub>2</sub> 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<sub>2</sub> inhibitory activity. A significant inhibition of PLA<sub>2</sub> was found with ethyl acetate extract when compared to methanol extract. As shown in [fig. 1] a dose dependent inhibition of PLA<sub>2</sub> activity was observed with the doses tested, indicating the uniformity of anti PLA<sub>2</sub> activity. Further, the IC<sub>50</sub> 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<sub>50</sub> was 7.66µg/ml respectively.

[Fig. 1: Inhibitory effect of *Manilkara zapota* ethyl acetate and methanol leaf extracts on PLA<sub>2</sub> activity in vitro]
**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.
DISCUSSION

Current active research is focused on herbal medicine in treating inflammation. Herbal medicines obtained from wide arrays 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 Z. zapota by in vivo and in vitro anti-inflammatory methodologies. 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 anti-inflammatory agents. 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 Z. zapota extract was apparent in each concentration of extract 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 Solanum nigrum [22], Phyllanthus amarus [29], Syringa patula [30], Plumeria acuminata [31] and Pistia stratiotes [32]. There are few references with respect to the anti-inflammatory activity of Z. zapota. As seen in this study ethyl acetate extract of Z. zapota especially at the high dose has an inhibitory effect on edema formation in both early and late phases of carrageenan induced rat paw edema model. The significant inhibitory activity shown by the extract of Z. zapota over a period of five hours in carrageenan 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 A2 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 M. zapota has been found to have significant anti-inflammatory activity with methanolic leaf extract evaluated by PLA2 assay, 5-LOX assay and in vivo carrageenan induced paw edema model. The ethyl acetate extract has shown significant PLA2 inhibitory activity with IC50 of 122.1±µg/ml while that of standard inhibitor Thioetheramide-PC, 7.66µg/ml Similarly ethyl acetate leaf extract exhibited significant 5-LOX inhibitory activity with IC50 value of 15.85µg/ml while that of standard quercetin 4.85µg/ml. Further, it is concluded that between PLA2 and 5-LOX inhibitory activities of ethyl acetate extract of M. zapota, 5-LOX activity is more significant than PLA2 inhibitory activity as indicated by the IC50 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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