MESUAFERRIN A-BIOACTIVE FLAVONOID ISOLATED FROM THE BARK OF MESUA FERREA L. AGAINST PHOSPHOLIPASE A₂, CYCLOOXYGENASE AND LIPOXYGENASE: AN IN VITRO, IN VIVO AND IN SILICO APPROACH

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Received: 13 Nov 2017 Revised and Accepted: 21 Dec 2017

ABSTRACT

Objective: The main objective of the present study was to evaluate the anti-inflammatory activity of isolated bioactive flavonoid Mesuaferrin-A from the bark of Mesua ferrea L. by in vitro, in vivo and in silico approach.

Methods: To evaluate the effect of isolated bioactive flavonoid Mesuaferrin-A on arachidonic acid metabolizing enzymes (PLA₂, COX-2 and 5-LOX) using in vitro methods, followed by carrageenan-induced paw edema model by in vivo and to determine the binding orientation and interactions of Mesuaferrin-A on arachidonic acid metabolizing enzymes (PLA₂, COX-2 and 5-LOX) crystal proteins using molecular docking (in silico) studies.

Results: Mesuaferrin-A exhibited a dose-dependent significant 5-LOX inhibitory and considerable COX-2 inhibitory activity by in vitro. The inhibitory activities of 5-LOX and COX-2 at 100µg/ml were found to be 78.67%, 81.03% with IC₅₀ values of 45.22µg/ml and 35.74µg/ml respectively. Whereas Mesuaferrin-A showed less PLA₂ inhibitory activity. Mesuaferrin-A showed 68.34% inhibitory activity at 400 mg/kg body weight at the late phase of carrageenan-induced paw edema, and in silico studies demonstrated that Mesuaferrin-A strongly binds with 5-LOX and COX-2, these strong binding affinity of Mesuaferrin-A on active site amino acids of 5-LOX and COX-2 may be responsible for inhibition of enzyme activity. Mesuaferrin-A showed comparable 5-LOX and COX-2 inhibition activity with (positive control).

Conclusion: It was concluded that Mesuaferrin-A act as 5-LOX and COX dual inhibitor, from the results it was suggested that Mesuaferrin-A may be an effective preventive and therapeutic approach for patients with inflammatory-related diseases.

Keywords: Mesuaferrin-A, Phospholipase-A₂ (PLA₂), 5-Lipoxygenase (5-LOX), Cyclooxygenase-2 (COX-2), Generic Evolutionary Method for Molecular Docking (GEM Dock).

INTRODUCTION

Inflammation is an innate immune response activated by a variety of factors such as physical and chemical factors, immunological reactions, microbial infections, and tissue damage [1]. Its main functions are to protect the body against a wide variety of harmful agents and to promote the renewal of normal tissue. During inflammation up-regulation of inflammatory macrophages releases pro-inflammatory mediators, such as nitric oxide (NO), prostaglandin E₂ (PGE₂), and various cytokines, in response to activation signals, include chemical mediators, cytokines, and bacterial lipopolysaccharide (LPS) [2]. The inflammatory response in the host is important for interruption and resolution of the infectious diseases, but it is also often responsible for the signs and symptoms of the disease [3].

Arachidonic acid (AA) pathway is an important pathway in which phospholipase A₂ (PLA₂), cyclooxygenases (COXs) and lipoxygenase (LOX) and cytochrome P450 monooxygenases are act on phospholipids of membrane system and produce respective metabolites hydroxyphospholipids, prostanoids, leukotrienes (LTs), hydroxy eicosanoid tetrac acids and epoxy eicosanoid tetrac acids are involved in normal and various pathophysiological functions [4]. Understanding the role of AA pathway in several inflammatory-related diseases, considerable efforts are being made to the discovery and development of inhibitors of AA pathway as inflammatory preventive and therapeutic agents. Non-steroid anti-inflammatory drugs (NSAIDs) have been explored as chemo preventive agents for several cancers. Though, several side effects associated with usage of NSAIDs hindered their clinical applications. Therefore, naturally occurring anti-inflammatory agents with a high therapeutic index and less side-effects are required as substitutes for synthetic anti-inflammatory drugs. Mesua ferrea L. had been used as traditional medicine for treatment of inflammatory related diseases such as arthritis, leprosy and cancer. There are only few studies reported on anti-inflammatory activities of Mesua ferrea L. From the literature, phytochemical analysis of Mesua ferrea L. bark extract, showed the presence of secondary metabolites such as flavonoids, terpenoids, glycosides, steroids, quinones and coumarins [5]. Narendra Prasad et al., [6] reported that that ethanolic leaf extract of Mesua ferrea L. has shown potent antioxidant activities (DPPH, ABTS and NBT). Pinkesh et al., [7] reported that in vivo carrageenan-induced rat paw edema is significantly inhibited by ethanolic extracts of Mesua ferrea L. flowers. No further work has been carried out on the isolated bioactive flavonoid Mesuaferrin-A. Hence the present study was to evaluate the anti-inflammatory activity of isolated bioactive flavonoid Mesuaferrin-A by in vitro, in vivo and in silico approach for the development of novel therapeutic plant derived anti-inflammatory agents.

MATERIALS AND METHODS

Chemicals and materials

PLA₂ and COX-2 kits from Cayman chemical company, Ann Arbor, Michigan, USA. 5-LOX from Invitrogen, USA. All other reagents used in this study were of analytical grade.

Phospholipase A₂ assay

PLA₂ assay was performed using sPLA₂ enzyme inhibitory kit for assessing of anti-PLA₂ activity of Mesuaferrin-A. This assay was performed as per the instructions of the manufacturer. The reaction mixture is a combination of 10 µl of PLA₂, 25, 50 and 100µg/ml of Mesuaferrin-A and 200 µl substrate. The whole reaction mixture was
incubated for 15 min, after incubation, 10 μl of 5, 5’-dithio-bis-2- nitrobenzoic acid (DTNB) was added to develop color and color is read at a wavelength of 415 nm. After hydrolysis of the diolester bond at the sn-2 position of diheptanoylthio-Phosphatidyl Choline (PC) substrate by PLAs, the free thiols were detected using DTNB, which has an absorbance at 415 nm. The control wells contain only PLas substrate and DTNB. Thioetheramide-PC was used as positive control. The percentage of inhibition of enzyme activity was calculated using the below formula.

\[
\% \text{ Inhibition} = \left( \frac{O.D. \text{ of control} - O.D. \text{ of test}}{O.D. \text{ of control}} \right) \times 100
\]

Cyclooxygenase (COX-2) assay
The COX-2 inhibitory assay was performed using a colorimetric COX-2 inhibitory assay kit for screening of COX-2 inhibitory activity of Mesuaferrin-A. This assay was performed according to the modified chromogenic method, using N, N, N, N-penta tetra methyl phenylethylenediamine (TMPD). Assay mixtures containing a COX-2 enzyme (100μg), hematin (15 mmol), EDTA (3 mmol) and 25, 50 and 100 μg/ml of Mesuaferrin-A and 100 mmol Tris HCl buffer (pH 8.0). The assay mixture was pre-incubated for 1 min at 25°C. The Reaction is activated by adding a sufficient amount of substrate arachidonic acid and TMPD. TMPD is oxidized during the reduction of prostaglandin G2 to prostaglandin H2 by the activity of COX-2. The oxidation of TMPD represents the enzyme activity and measured at 605 nm using a spectrophotometer.

\[
\% \text{ Inhibition} = \left( \frac{O.D. \text{ of control} - O.D. \text{ of test}}{O.D. \text{ of control}} \right) \times 100
\]

5-Lipoxygenase (5-LOX) inhibition assay
The anti-inflammatory activity of Mesuaferrin-A was determined using in vitro 5-LOX inhibition assay. This assay analyses the inhibitory activity against the 5-LOX enzyme, which is involved in the synthesis of inflammatory mediators known as leukotrienes. This assay was first developed by [8]. Later it is modified by [9]. Lipoxygenases are a group of dioxygenases which are involved in the insertion of molecular oxygen into proinflammatory ω-6 fatty acids such as arachidonic acid and linoleic acid. Leukotrienes are formed from the initial attack on arachidonic acid by 5-lipoxygenase which adds molecular oxygen to carbon 5, leading to the formation of hydroperoxyeicosatetraenoic acid (5-HPETE). Dehydration of 5-HPETE gives the epoxide, these epoxides then undergoes isomerization and provides double bond hydroperoxide which gives leukotriene A4 (LTA4). Hydrolysis of LTA4 leads to the formation of stable LTβ4. Linoleic acid is used as a substrate for the determination of 5-LOX enzyme inhibitory activity because it shares a structural resemblance with arachidonic acid [10]. The increase in absorbance at 234 nm is due to the formation of 1, 3-diene from 1, 4-diene in linoleic acid hydroperoxide which is used in the determination of 5-lipoxygenase inhibitory assay.

Mesuaferrin-A is tested at different concentrations viz., 25, 50 and 100μg/ml. The activity of 5-lipoxygenase is compared with the standard positive control Zileuton.

The percentage inhibition of 5-lipoxygenase inhibitory activity of Mesuaferrin-A was calculated by using a formula.

\[
\% \text{ Inhibition} = \left( \frac{O.D. \text{ of control} - O.D. \text{ of test}}{O.D. \text{ of control}} \right) \times 100
\]

Assessment of anti-inflammatory activity
For screening and assessment of anti-inflammatory compounds, carrageenan-induced paw edema is widely used animal model for acute inflammation and was introduced by [11]. Carrageenan is a mucopolysaccharide, derived from Iris sea moss, produce non-immunological edema. There are two phases of carrageenan-induced inflammatory reactions, i.e. Initial phase and late phase. The initial phase (1-3h) involves the release of histamine, serotonin and kinins from mast cells responsible for swelling and pain [12]. During the late phase (4-5 h) two important inflammatory mediators such as prostaglandin E2 (PGE2) and leukotriene B4 are synthesized from arachidonic acid-dependent pathways by cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) enzymatic system involved with the commencement of inflammatory reactions.

Anti-inflammatory effect of Mesuaferrin-A on carrageenan-induced inflammation in Wistar rats
Carrageenan-induced paw edema used for the evaluation of the anti-inflammatory activity. Male Wistar albino rats weighing 160-180 g were obtained from M/s Mahavir Enterprises (Hyderabad, Andhra Pradesh, India). The animals were fed with standard laboratory diet, which was purchased from M/s Rayan’s Biotechnology Pvt. Ltd. (Hyderabad, Andhra Pradesh, India) during the experiment the rats were fed with 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 each group containing six animals (n=6). The first group was given normal saline by intragastric catheter tube (IGT), the second, and third groups (200 and 400 mg/kg body weight) received the Mesuaferrin-A isolated from the dried leaves of Dioscorea communis root (10 mg/kg body weight). The paw volume was measured plea thysometrically (ug obasile, 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.

Molecular docking studies of mesuaferrin-A on 5-LOX I GEM dock
The X-Ray crystal structure of protein 5-lipoxygenase (PDB ID: 30BY, COX-2: PDB ID: 4COX) and PLA2A (PDB: 1DB5) used in docking studies were retrieved from Protein Data Bank. Co-crystalized ligands and water molecules were removed from target protein using Argus lab. Ligands are prepared using chemoffice (Cambridge). Energy minimization was done using molecular mechanics. The minimization was executed by the root mean square value reached below 0.001 Kcal/mol. Such energy minimized ligands and receptors were used for docking studies using GEMDOCK (Generic Evolutionary Method for molecular docking). A population size of 300 with 70 generations and 3 solutions were used in docking accuracy setting. Pymolk used for better visualization of the interactions.

Statistical analysis
The results were expressed as the mean±Standard error of the mean (SEM). The statistical difference between the test and control groups were evaluated by one-way analysis of variance (ANOVA) by Graph pad prism 6.0 software and followed by Dunnett’s t-test.*p≤0.05, **p≤0.01, ***p≤0.001 represents a significant difference between the control with the test group.

RESULTS AND DISCUSSION
In general the processes of wound heal occurring in the body with the lapse of time is the greatest gift of nature mother to mankind. During this process, the body responds/reflects this process through the lapse of time is the greatest gift of nature mother to mankind. In order to reduce the incidence of this process, allopathic doctors prescribe anti-inflammatory drugs called NSAIDs (Non-Steroidal Anti-Inflammatory Drugs). Although these drugs provide temporary relief, research data suggests that it could give undesirable side effects such as gastric ulceration, liver damage and even stimulates the likelihood of getting myocardial infarction and stroke [13]. In this case, natural anti-inflammatory compounds are of immense interest and have been used to mediate the anti-inflammatory process often with lesser side effects [14].

Effect of mesuaferrin-A on PLA2 activity
Mesuaferrin-A was evaluated for PLA2 inhibitory activity with different doses viz., 25, 50 and 100μg/ml as per the manufacturer’s...
directions. As shown in fig. 1, the inhibitory effect of Mesuaferrin-A was found to be increased concentration-dependent manner. The percent inhibition was observed to be 15.38, 24.49 and 41.23% respectively. However, the inhibitory effect of Mesuaferrin-A was not so significant IC₅₀ value of 17.95 μg/ml (**p≤0.01) as compared to that of Thio etheramide-PC whose IC₅₀ was 5.65 μg/ml. Our results also correlated with Apigenin-7-O-B-D-Glucuronide Methyl Ester isolated from ethyl acetate of Manilkara zapota leaves having significant inhibitory effect on sPLA₂ activity [15].

Effect of mesuaferrin-A on COX-2 activity

Mesuaferrin-A was tested on COX-2 activity by taking different doses viz., 25, 50 and 100 μg/ml as shown in fig. 2. A dose-dependent percentage inhibition of COX-2 activity was observed at 100 μg/ml. The percent inhibition was found to be 29.12, 52.98 and 78.67% respectively. As shown in fig. 2, a dose-dependent percentage inhibition of COX-2 activity was observed at 100 μg/ml was found to be 78.67% with IC₅₀ value of 3.78 μg/ml. Our results also correlated with the bacteria associated with H. amboinensis having anti-inflammatory to inhibit the COX-1, COX-2, and sPLA₂ enzymes activity. Yosie et al. [19] reported that Garcinol, a polyisoprenylated benzophenone has to possess 5-LOX inhibitory activity and suppressive effect on LTB₄ production in cancer cells. Our results also correlated with that the bacteria associated with H. amboinensis having anti-inflammatory to inhibit the COX-1, COX-2, and sPLA₂ enzymes activity.

Anti-inflammatory effect of mesuaferrin-A on carrageenan-induced acute inflammation in wistar rats

It is well known that carrageenan-induced paw edema is characterized by biphasic event with the involvement of different inflammatory mediators. The results of the present investigations revealed that the flavonoid fraction isolated from the stem bark of Mesuaferrin L. Possess significant anti-inflammatory activity against acute inflammatory models carrageenan-induced paw edema. As shown in the fig. 4, the isolated bioactive flavonoid Mesuaferrin-A (200 and 400 mg/kg body weight) showed 42.31% (**p≤0.05) and 53.52% (**p≤0.01) paw edema inhibition at 4th and 5th respectively, whereas Diclofenac (10 mg/kg body weight) showed 75.59% (**p≤0.01), 82.70% (**p≤0.001) paw edema inhibition at 4th and 5th respectively. Hence it can be concluded that Mesuaferrin-A has shown potent anti-inflammatory activity comparable to standard drug Diclofenac in both early and late phases. Muralidhar et al. [20] reported that the flavonoid fraction isolated from the stem bark of Butea monosperma significantly reduced the inflammation in the carrageenan-induced rat paw edema and cotton pellet induced granuloma in rats. Ishwar Bhat and Abhishek kumar [21] also reported that synthetic novel 1,5 benzodiazepine derivatives having anti-inflammatory activities against carrageenan-induced paw edema in rats. It was known that levels of COX-2 and 5-LOX are more in 4th to 5th h, of the second phase of acute inflammation. This demonstrated that in vivo anti-inflammatory activity of Mesuaferrin-A probably due to COX-2 and 5-LOX inhibition.
Molecular docking studies (in silico) of isolated mesuaferrin-A on PLA₂, 5-LOX and COX-2 crystal proteins

Molecular docking studies demonstrated that Mesuaferrin-A showed good binding affinity on 5-LOX crystal protein with binding energy of -158.67/mol and binds in the vicinity of amino acid residues (fig. 5). Mesuaferrin-A binds on PLA₂ and COX-2 crystal protein with binding energies -115.49 and -136.77 respectively. In silico studies validated that Mesuaferrin-A strongly binds with 5-LOX and COX-2, the strong binding affinity of Mesuaferrin-A on active site amino acids of 5-LOX and COX may be responsible for inhibition of enzyme activity.

From the above studies, it is quite apparent that the flavonoid fraction of Mesuaferrin-A isolated from Mesua ferrea L. stem bark possesses significant anti-inflammatory activity by inhibiting Cyclooxygenase-2 and 5-Lipoxygenase inflammatory enzymes.

CONCLUSION

The isolated bioactive flavonoid Mesuaferrin-A from Mesua ferrea L. bark ethyl acetate extract acts as a dual inhibitor by inhibiting 5-LOX, COX-2 enzymes and inhibiting carrageenan-induced paw edema in the late phase. Mesuaferrin-A exhibited comparable anti-inflammatory activity with standard inflammatory drugs. Hence, it can be concluded that to development of novel plant-derived anti-inflammatory drugs without having side effects.

ACKNOWLEDGMENT

I am very happy to convey my sincere thanks to esteemed Professor U. S. N Murthy, Chief Scientist, Head, Department of Biology, Indian Institute of Chemical Technology, and Hyderabad, India has permitted me to carry out pharmacological studies in his laboratory.

FINANCIAL SUPPORT

This work was partially supported by UGC-MRP. F. No. 4N 2-643/2013, New Delhi, India sanctioned to Prof. Duddakuri Govinda Rao, GITAM University, Visakhapatnam.

AUTHORS CONTRIBUTIONS

Corresponding author (Dr. K. Krishna Chaithanya) contributed in performing the experiment and writing of the manuscript, Dr. V. K. Gopalakrishnan has contributed to compilations of the manuscript, Mr. Zenebe Hagos has contributed for statistical analysis and Dr. D. Govinda Rao has contributed in experiment design and valuable guidance to Krishna Chaithanya for his Ph. D research work.

CONFLICT OF INTERESTS

The author(s) declare(s) that there is no conflict of interest.
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