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Original Article

MESUAFERRIN A-BIOACTIVE FLAVONOID ISOLATED FROM THE BARK OF *MESUA FERREA* L. AGAINST PHOSPHOLIPASE A_2, CYCLOOXYGENASE AND LIPOXYGENASE: AN *IN VITRO, IN VIVO* AND *IN SILICO* APPROACH

KRISHNA CHAITHANYA K.[a*], GOPALAKRISHNAN V. K.[a], ZENEBE HAGOS[a], GOVINDA RAO D.[b]

[a]Department of Chemistry, College of Natural and Computational Sciences, Aksum University, Axum, Ethiopia, [b]Department of Biochemistry and Molecular Biology, School of Biological Sciences, Central University of Kerala, India
Email: krishnachaitanyawc@gmail.com

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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 *Mesuaferrea* 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 (PLA_2, 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 onarachidonic acid metabolizing enzymes (PLA_2, 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 IC_{50} values of 45.22µg/ml and 35.74µg/ml respectively. Whereas Mesuaferrin-A showed less PLA_2 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 showeda 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 suggests that Mesuaferrin-A, may be an effective preventive and therapeutic approach for patients with inflammatory-related diseases.

Keywords: Mesuaferrin-A, Phospholipase-A_2(PLA_2), 5-Lipooxygenase (5-LOX), Cyclooxygenase-2 (COX-2), Generic Evolutionary Method for molecular Docking **(**GEM Dock).

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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 of pro-inflammatory mediators, such as nitric oxide (NO), prostaglandin E2 (PGE_2), 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 A_2 (PLA_2), cyclooxygenases (COXS) and lipoxygenase (LOX) and cytochrome P450 monooxygenases are act on phospholipids of membrane system and produce respective metabolites lysophospholipids, prostanoids, leukotrienes (LTs), hydroxyl eicosanoitetraic acids and epoxy eicosanoid tetraic 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 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

PLA_2, 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 A_2 assay

PLA_2 assay was performed using sPLA_2 enzyme inhibitory kit for assessing of anti-PLA_2 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 PLA_2, 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 thioester bond at the *sn*-2 position of diheptanoylthio-Phosphatidyl Choline (PC) (substrate) by PLA_2, the 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 percentage of inhibition of enzyme activity was calculated using the below formula.

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

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|-para tetra methyl-phenylenediamine (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 G_2 to prostaglandin H_2 by the activity of COX-2. The oxidation of TMPD represents the enzyme activity and measured at 603 nm using a spectrophotometer.

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

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 isomerisation of their double bonds and gives leukotriene A4 (LTA4). Hydrolysis of LTA4 leads to the formation of stable LTB4.

Linoleic acid is used as a substrate for the determination of 5-LOX enzyme inhibition assay 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 was compared with the standard positive control Zileuton.

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

$$\textit{Percentage of inhibition} = \left(\frac{\textit{OD of control} - \textit{OD of test}}{\textit{OD of control}}\right) \times 100$$

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 E_2 (PGE_2) 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 *adlibitum*. 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 (IGC), the second, and third groups (200 and 400 mg/kg body weight) received the Mesuaferrin-A isolated bioactive flavonoid for 10 d and the forth group received diclofenac sodium as a standard (10 mg/kg body weight). The paw volume was measured plethysommetrically (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.

$$\textit{Percentage inhibition} = \left(\frac{\textit{Control paw volume} - \textit{Test paw volume}}{\textit{Control paw volume}}\right) \times 100$$

Molecular docking studies of mesuaferrin-A on 5-LOX I GEM dock

The X-Ray crystal structure of protein 5-lipoxygenase (PDB ID: 308Y), COX-2 (PDB ID: 4COX) and PLA_2 (PDB: 1DB5) used in docking studies were retrieved from Protein Data Bank. Co-crystallized 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. Pymolis 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 different modes such as pain, swelling, raised temperature and erythema. 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 PLA_2 activity

Mesuaferrin-A was evaluated for PLA_2 inhibitory activity with different doses viz., 25, 50 and 100µg/ml as per the manufacturer's

directions. As shown in fig. 1the 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 IC_{50}-173.99µg/ml (**p≤0.01) as compared to that of Thio etheramide-PC whose IC_{50} was 6.65µg/ml. Our results also correlated with Apigenin-7-*O*-B-D-Glucuronide Methyl Ester Isolated from ethyl acetate of *Manilkarazapota* leaves have significant inhibitory effects on $spla_2$ activity [15].

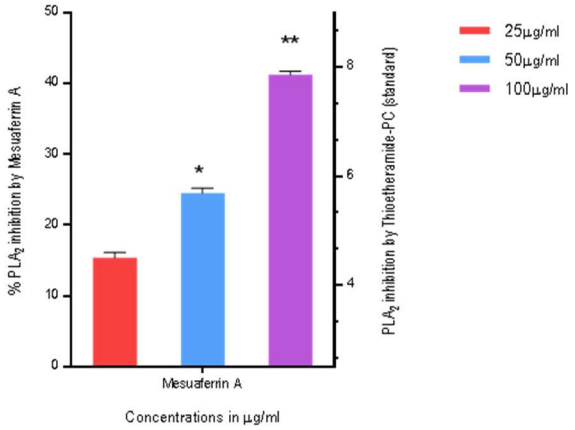


Fig. 1: Inhibitory effect of Mesuaferrin–A against PLA_2 activity compared with, Thioether amide-PC standard. Values are mean of three replicates±SEM. *p≤0.05, **p≤0.01, ***p≤0.001 represents a significant difference compared with the Thioetheramide-PC (5 µg/ml)

Effect of mesuaferrin-A on COX-2 activity

Mesuaferrin-A was tested on COX-2 activity by taking different doses viz., 25, 50 and 100µg/ml and corresponding percentage inhibition obtained 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 100µg/ml was found to be 78.67% with IC_{50} value of45.22µg/ml, (***p≤0.001)while that of Diclofenac was found to be 3.78µg/ml.

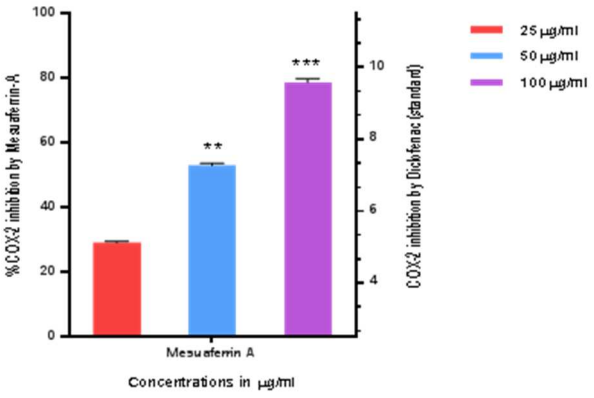


Fig. 2: Inhibitory Effect of Mesuaferrin–A against COX-2 activity compared with, Diclofenac sodium. Values are mean of three replicates±SEM. *p≤0.05, **p≤0.01, ***p≤0.001 represents a significant difference compared with the Diclofenac sodium (5 µg/ml)

Hong *et al.* [16] found that curcumin inhibited AA metabolism by blocking the phosphorylation of $cPLA_2$, down-regulating of COX-2 expression and inhibiting the 5-LOX catalytic activity. Cao*et al.*, [17] also reported that Senkyunolide O and cryptotanshinone were isolated from *Ligusticumchuangxiiong* and *Salvia miltiorrhiza* exhibited selective COX-2 inhibitors with IC_{50} values of 5 and 22 µM, respectively.

Effect of mesuaferrin-A on 5-LOX activity

The Mesuaferrin-A was tested for 5-LOX inhibitory activity by taking different doses viz., 25, 50 and 100µg/ml as shown in fig. 3,a significant dose-dependent percentage inhibition of 5-LOX activity was observed at 100µg/ml was found to be 81.03% with IC_{50} value of 35.74µg/ml, (***p≤0.001), while that of standard inhibitor zileuton whose IC_{50} value was 5.09µg/ml.

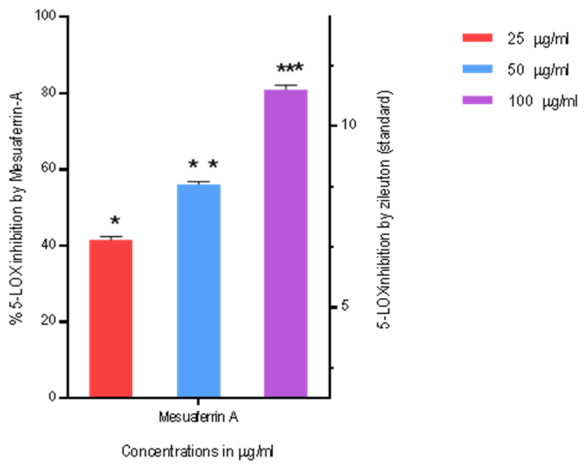


Fig. 3: Inhibitory Effect of Mesuaferrin–A against 5-LOX activity, values are mean of three replicates±SEM. *p≤0.05, **p≤0.01, ***p≤0.001 represents significant difference compared with the zileuton (5 µg/ml)

The anti-inflammatory activity of Mesuaferrin-A is possible due to inhibition of pro-inflammatory enzymes. Han *et al.* [18] reported that Garcinol, a polyisoprenylated benzophenone have 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. Yosie *et al.*, [19]

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 *Mesuaferra* 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≤0.001) and 57.36 % (**p≤0.01) and 68.34% (***p≤0.001) paw edema inhibition at 4^{th}h and 5^{th}h respectively, whereas Diclofenac (10 mg/kg body weight) showed 75.59 %(**p≤0.01), 82.70% (***p≤0.001) edema inhibition at 4^{th} h and 5^{th}h 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 *Buteaonosperma* 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 4^{th} to 5^{th} 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.

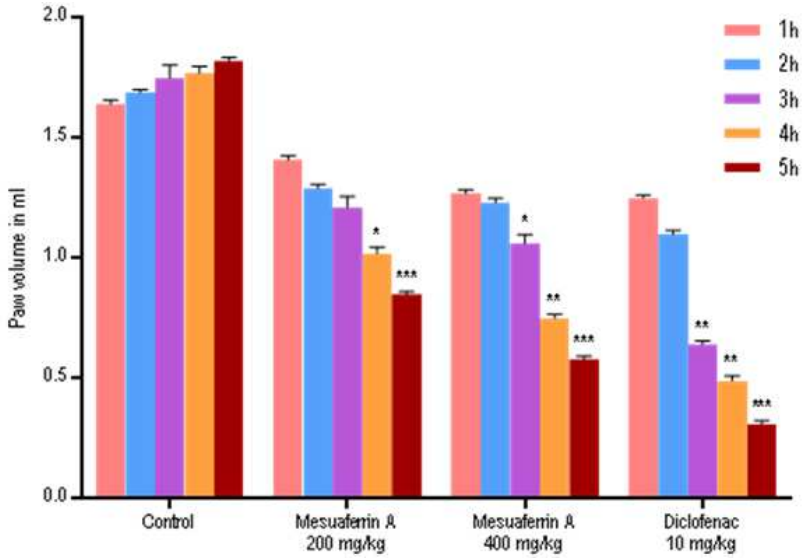
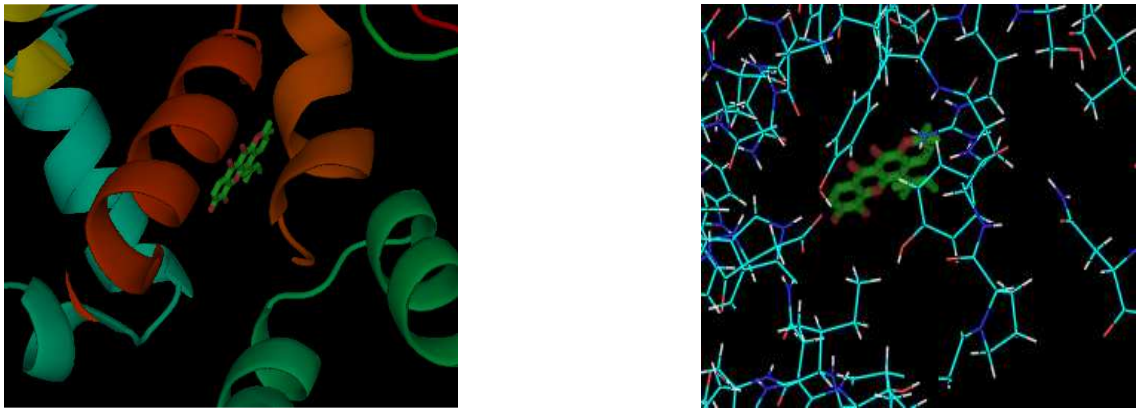


Fig. 4: Inhibitory effect of Mesuaferrin-A on carrageenan-induced paw volume (ml) in Wistar rats, values are mean of three replicates±SEM.*p≤0.05, **p≤0.01, ***p≤0.001 represents significant difference compared with the Diclofenac (10 mg/kg body weight)

Molecular docking studies (*in silico*) of isolated mesuaferrin-A on PLA_2, 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 PLA_2 and COX-2 crystal protein with binding energies-115.49 and-136.77 respectively. *In silico* studies validated that Mesuaferrin-Astrongly binds with 5-LOX and COX-2, the strong binding affinity of Mesuaferrin-Aon 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 ferra* L. stem bark possesses significant anti-inflammatory activity by inhibiting Cyclooxygenase-2 and 5-Lipoxygenase inflammatory enzymes.



A **B**

Fig. 5: Molecular docking studies of mesuaferrin-A on 5-LOX crystal protein, A. Binding orientation of mesuaferrin-A on 5-LOX crystal protein. B. Interactions of mesuaferrin-A with amino acid residues of 5-LOX

CONCLUSION

The isolated bioactive falvanoid Mesuaferrin-A from *Mesuaferrea* 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.

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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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