ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Anti-Inflammatory Activity of Isolated Bioactive Flavanoid Apigenin-7-O-B-D Glucuronide Methyl Ester from the Ethyl Acetate Leaf Extract of *Manilkara zapota*

Kamalakararao K[1]*, Krishna Chaithanya K[2], Gopalakrishnan VK[2], Zenebe Hagos[2], Debasish Pradhan[3], Padal SP[4], Tentu Kasi Naidu[5], Govinda Rao D[6].

[1]Department of Biochemistry, Aditya Degree and PG College, Kakinada, Andhra Pradesh, India
[2]Department of Chemistry, College of Natural and Computational Sciences Aksum University, Axum, Ethiopia
[3]Department of Pharmaceutical Sciences, Utkal University, Vani Vihar, Bhubaneshwar, India
[4]Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh, India.
[5]Department of Medical Laboratory Sciences, School of Health Sciences, Dilla University, Dilla, Ethiopia
[6]Dept of Biochemistry and Molecular Biology, School of Biological Science, Central University of Kerala,India

ABSTRACT

Manilkara zapota and its different parts have been traditionally used for alleviating inflammation related diseases such as arthritis, cancer and skin infections. The present study was undertaken to carry out the isolated bioactive compound apigenin-7-O-β-D-glucuronide methyl ester is studied for its anti-inflammatory activities by *in vivo, in vi*tro and *in silico* methods such as 5-LOX, COX-2, anti-inflammatory cytokines (IL-1β and TNF-α) and carrageenan induced paw edema. From the above study it can be concluded that the isolated bioactive compound can be used for treatment of inflammatory related diseases without having any side effects.

Keywords: *Manilkara zapota*; COX-2; LOX-2; Apigenin-7-O-β-D-glucuronide methyl ester; IL-1β; TNF-α

Corresponding author

ISSN: 0975-8585

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, is related with chronic inflammatory diseases such as inflammatory bowel diseases, arthritis, asthma, neurodegenerative diseases and autoimmune diseases [1].Inflammation is a vital response of vascular tissues to infectious and non-infectious agents.

TNF-α, IL-1β play a vital role in ROS and RNS induced inflammation [2]. Oxidative stress is an imbalance between reactive oxygen, nitrogen species (ROS/RNS) and antioxidant systems. Mitochondrial respiration is a major source of ROS such as hydrogen peroxide (H_2O_2), super oxide radicals (O_2^-) and hydroxyl radicals (OH^-) [3].Both steroidal and non-steroidal anti-inflammatory drugs are used for treatment of inflammatory diseases. 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. 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 and its different parts have been traditionally used and medicinal value has been reported. The acetone extract of *Manilkara zapota* leaves has shown significant antioxidant activity [4]. Petroleum ether and ethanolic leaf extracts of *Manilkara zapota* were reported to have analgesic activity [5].The ethanolic extract of *Manilkara zapota* possesses significant anti-arthritic activity [6].Ganguly et al.,[7] reported that ethyl acetate and methanolic extract of leaves of *Manilkara zapota* shown significant inhibition of paw edema. Kamalakararao et.al.,[8] reported that the in vitro and in vivo anti-inflammatory studies of ethyl acetate extract of *M. zapota* having significant anti-inflammatory activity. Apigenin-7-O-B-D-Glucuronide Methyl Ester isolated from *Manilkara zapota* leaves reported by Kamalakararaoet.al.,[9].The main objective of the study was to evaluate the isolated bioactive compound Apigenin-7-O-β-D-glucuronide methyl ester from *Manilkara zapota* by *in vitro* and *in vivo* and *in silico* approach.

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 Department Botany Herbarium, Andhra University, India.

Anti-inflammatory activity of isolated apigenin-7-O-β-D-glucuronide methyl ester from the ethyl acetate leaf extract of *M. zapota*

The isolated compound apigenin-7-O-β-D-glucuronide methyl ester is further studied for its anti - inflammatory activity by *in vitro, in vivo* and *in silico* methods. *In vitro* anti-inflammatory methods such as 5-Lipoxygenase assay, human COX-2 assay, and COX-2 gene expression in RAW 264.7 cell line, determination of pro inflammatory cytokine levels, such as IL-1β and TNF-α.

In vitro anti-inflammatory methods

Lipoxygenase assay

5-LOX inhibitory assay was performed by using UV kinetic method [39]. This method was performed by using an assay mixture consisting 3ml of 50mM phosphate buffer pH 6.3, along with 10µl of 80mM 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 Apigenin-7-O-β-D-glucuronide methyl ester was tested at different concentrations viz., 5,10,15,25 and 50 µg/ml. The activity of 5-Lipoxygenase was compared with the standard positive control Quercetin. The percent inhibition of 5-lipoxygenase inhibitory activity of Apigenin-7-O-β-D-glucuronide methyl esterwas calculated by using a formula.

ISSN: 0975-8585

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

COX-2 Assay

COX-2 inhibition was measured using a colorimetric human COX-2 inhibitor screening assay kit (Cayman Ann Arbor, MI, USA). The assay was performed according to instructions of the manufacturer. COX-2 enzyme activities were assessed according to the modified chromogenic method using N, N, N, N-tetramethyl p-phenylenediamine (TMPD). COX-2 enzyme (100 mg), hematin (15mM), EDTA (3mM), and 10, 50 and 100µg/ml of apigenin-7-O-β-D-glucuronide methyl ester were added in 100mM TrisHCl buffer (pH 8.0) and this assay mixture was pre incubated for 1 min at 25 ^{0}C. Further, sufficient amount of arachidonic acid and TMPD was added to the assay mixture to initiate the reaction. TMPD is oxidized during the reduction of the reduction of prostaglandin G2 to prostaglandin H2 of the activity of COX-2. The oxidation of TMPD represents the enzyme activity and measured at 603 NM using a spectrophotometer. Non enzymatic oxidation (if any) was deducted from the experimental values for calculating the percent of inhibition [10].

Cytokine analysis

RAW 264.7 macrophage cell culture

RAW 264.7, a mouse macrophage cell line was obtained from ATCC, Manassas, VA 20110, USA and cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100µg/ml streptomycin and 100ul/ml penicillin (Gibco BRL), maintained at 37^{0}C in a 5% CO_2 incubator.

Cell viability assay

To study the cytotoxicity of apigenin-7-O-β-D-glucuronide methyl ester RAW 264.7 cells were plated at a density of 1x10^5 cells for well in 24 well plates (BD Bioscience) using fresh DMEM media. Raw 264.7 cells were treated with 10, 25, 50µg/ml for one hour before addition of LPS. After 24 hours the medium containing test compounds were replaced with MTT solution and incubated in dark for another 4 hours at 37^{0}C. After removing the medium 100µL of DMSO was added to the cells. The absorbance was measured by using a microplate reader at 450nm. The control group consists of untreated cells was considered as 100% of viable cells. The results are expressed as percentage of viable cells when compared with control group.

$$\text{Percentage of viability} = \left(\frac{\text{O.D ofcontrol} - \text{O.D oftest}}{\text{O.D of control}}\right) \times 100$$

Measurement of TNF-α level in RAW 264.7 cells

The effect of different concentrations (25, 50µg/ml) of apigenin-7-O-β-D-glucuronide methyl ester on the production of TNF-α were measured by using Sandwich ELISA (e-biosciences, USA). 1x10^5 RAW 264.7 cells were seeded on 48 well plates for overnight, cells were pre-incubated with above mentioned concentrations for 2 hours, then stimulated with 1µg/ml LPS for another 24 hours to determine the TNF-α concentration, the capture antibody was used at a concentration of 0.5µg/ml in PBS. Serial dilutions of TNF-α standard from 0 to 1000pg/ml in dilute (0.05% tween 20) were used as internal standard. TNF-α was detected with biotinylated secondary antibody and an avidin peroxidase conjugate with TMB as detector agent. The color development was stopped using 0.5M sulphuric acid and absorbance was measured at 450nm.

Measurement of IL-1β levels in RAW 264.7 cells

The effect of apigenin-7-O-β-D-glucuronide methyl ester on the release of IL-1β from RAW 264.7 cell line was evaluated by using a commercial ELISA assay kit (e-biosciences, USA) according to the manufacturer's recommendation. 1x10^5 RAW 264.7 cells were seeded in 48 well plate for overnight, cells were pre-incubated with 10, 25, 50µg/ml concentrations for 2 hours, then stimulated with 1µg/ml LPS for another 24 hours to determine the IL-1β production.

***In vivo* methods**

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

Carrageenan-induced paw edema model is the most widely used for the evaluation of anti-inflammatory activity [11]. Male Wistar albino rats weighing160-180g were obtained from M/s Mahavir Enterprises (Hyderabad, Telangana, India). The animals were housed under standard conditions before and also during the experimentation. 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 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 inaugural group was given normal saline by intragastric catheter tube (IGC).The second and third groups (200 and 400 mg/kg body weight) received theapigenin-7-O-β-D-glucuronide methyl ester for 10 days and the fourth group received diclofenac sodium as a standard (10 mg/kg 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.

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

Molecular docking studies

Molecular docking is a method which predicts the preferred binding orientation of ligand to a target protein when bound to each other to form a stable complex [12]

Methods

iGEM Dock software

The proteins used in docking studies were obtained from Protein Data Bank. Phospholipase A_2 (PDB ID: 1DB5), cyclooxygenase 2 (PDB ID: 4COX) and 5-lipoxygenase (PDB ID: 308Y) were used in docking studies [13]. 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 until 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). PyMol is used for better visualization of interactions.

RESULTS AND DISCUSSION

Effect of Apigenin-7-O-β-D-glucuronide methyl ester on 5-LOX activity

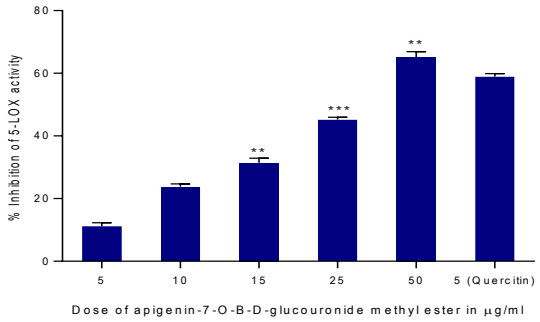
Lipoxygenase (LOXs) enzymes are reported to convert the archidonic, linoleic and other polyunsaturated fatty acid into biologically active metabolites that are involved in the inflammatory and immune response [14].

5-LOX inhibitory activity of Apigenin-7-O-β-D-glucuronide methyl ester has been evaluated by a UV kinetic method. The effect of this compound has been tested at different doses viz., 5, 10, 15, 25, 50 µg/ml on 5-LOX activity.

As shown in Fig.1 significant dose-dependent inhibition of 5-LOX activity by Apigenin-7-O-β-D-glucuronide methyl ester was observed implying the consistency of inhibitory activity of the compound. 5-LOX inhibitory activity was found significant with IC_{50} of 29.16µg/ml while that of standard inhibitor quercetin was 3.38µg/ml. Ravi Shankara et.al., [15] is also reported that the maximal LOX inhibitory activity (52.67%) at 800 µg/ml concentrations in the ethanolic extract of leaf galls of *Terminalia chebula*.

ISSN: 0975-8585

Figure 1: Dose-dependent inhibitory effect of Apigenin-7-O-β-D-glucuronide methyl ester on 5-LOX activity



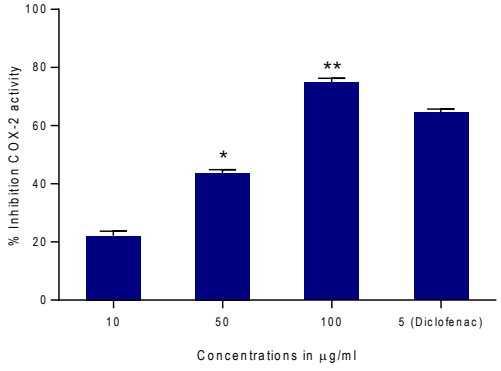
Values are expressed as mean±S.E.M.* P < 0.05, ** P < 0.01, *** P < 0.001 represents a significant difference compared with control group by student's t-test (n=3).

Effect of Apigenin-7-O-β-D-glucuronide methyl ester on COX-2 activity

The COX-2 enzyme inhibitory activity of Apigenin-7-O-β-D-glucuronide methyl ester has been evaluated by COX-2 assay kit, Cayman Chemical, USA, according to the manufacturer's instructions. The Apigenin-7-O-β-D-glucuronide methyl ester was tested for COX-2 inhibitory activity by taking different doses viz., 10, 50, 100µg/ml.

As shown in **Fig. 2,** a dose-dependent inhibition of COX-2 activity was observed with IC_{50} of 42.55µg/ml while that of diclofenac was found to be 3.38µg/ml. The inhibitory activity of the compound on COX-2 activity found significant at all the doses tested implying the consistency in COX-2 inhibitory activity. Romero-Estrada et.al., [16] is also reported that the inhibitory activity of COX-2 at 70µM of α-amrin acetate isolated from the Mexican copal resin of *Bursera copallifera.*

Figure 2: Dose-dependent inhibition of COX-2 activity by Apigenin-7-O-β-D-glucuronide methyl ester.



Values are expressed as mean±S.E.M.* P < 0.05, ** P < 0.01, *** P < 0.001 represents a significant difference compared with control group by student's t-test (n=3).

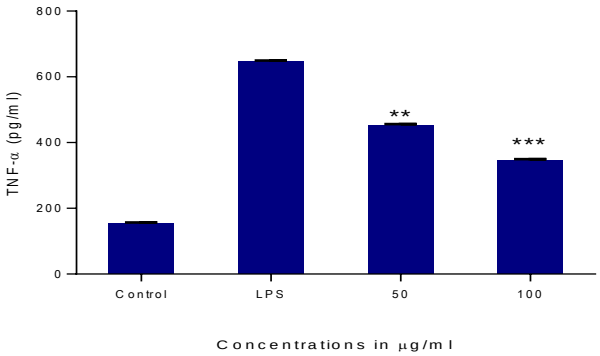
Inhibitory effect of Apigenin-7-O-β-D-glucuronide methyl ester on induction of TNF-α in LPS induced RAW 264.7 cells

As pro-inflammatory cytokines such as TNF-α and IL-1β are involved in inflammation, RAW 264.7 cell culture experiments were conducted in response to bacterial products like LPS. Pro-inflammatory cytokine production stimulated by LPS was measured in RAW 264.7 cell culture supernatants using a cytokine ELISA kit, eBiosciences USA according to manufacturer's instruction. Pro-inflammatory cytokine response is induced in RAW 264.7 cells against LPS, in the presence or absence of apigenin-7-O-β-D-glucouronide methyl ester at different doses tested viz., 50 and 100 µg/ml. As shown in **Fig. 3**, the apigenin derivative has inhibited the

production of TNF-α according to the doses employed. It is found that TNF-α levels were significantly inhibited by the compound with IC_{50} of 83.78µg/ml.

Our results are correlated with aqueous extract of *Orixa japonica* significantly reduce LPS induced expresion of inducible NO synthase (iNOS), cycloxygenase -2 (COX-2) and TNF-α at the transcriptional level[17].

Figure 3: Inhibitory effect of Apigenin-7-*O*-β-D-glucuronide methyl ester on TNF-α response in RAW 264.7 cell culture against LPS stimulation



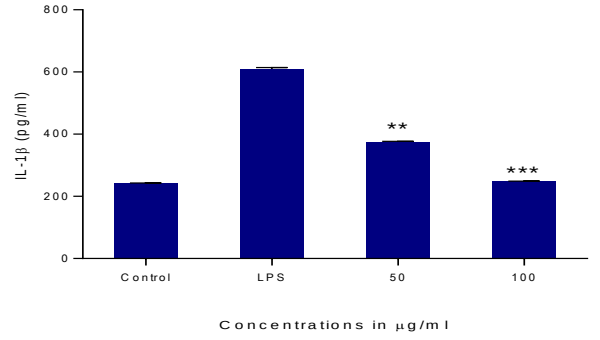
Values are expressed as mean±S. E. M.* P < 0.05, ** P < 0.01, *** P < 0.001 represents a significant difference compared with control group by student's t-test (n=3).

Inhibitory effect of Apigenin-7-O-β-D-glucuronide methyl ester on induction of IL-1β in LPS induced RAW 264.7 cells

Pro-inflammatory cytokine, IL-1β production stimulated by LPS was measured in RAW 264.7 cell culture supernatants using a cytokine ELISA kit, eBiosciences USA according to manufacturer's instruction. Pro-inflammatory cytokine response is induced in RAW 264.7 cells against LPS, in the presence or absence of Apigenin-7-O-β-D-glucuronide methyl ester at different doses tested viz., 50 and 100 µg/ml. As shown in **Fig.4**, the Apigenin derivative has inhibited the production of IL-1β according to the doses employed. It is found that IL-1β levels were significantly inhibited by the compound with IC_{50} of 73.08µg/ml

Li et.al.,[18] is also reported that that *Celastrus orbiculatus* extract inhibits IL-1β and TNF-α-induced RA-FLSs migration and invasion by suppressing NF-κB-mediated MMP-9 expression.

Figure 4: Inhibitory effect of Apigenin-7-*O*-β-D-glucuronide methyl ester on IL-1β response in RAW 264.7 cell culture against LPS stimulation



Values are expressed as mean±S.E.M.* P < 0.05, ** P < 0.01, *** P < 0.001 represents a significant difference compared with control group by student's t-test (n=3).

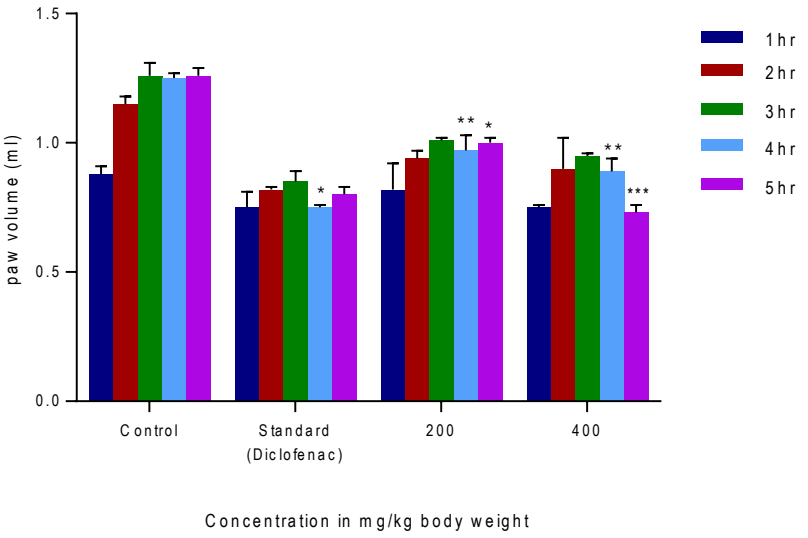
ISSN: 0975-8585

Anti-inflammatory effect of Apigenin-7-O-β-D-glucuronide methyl ester on carrageenan-induced inflammation in Wistar rats.

Different groups of Wistar male rats weighing about 150gms were treated with carrageenan and administered rats p.o. with different doses of Apigenin-7-O-β-D-glucuronide methyl ester i.e. 200 and 400mg/kg body weight. Similarly, two groups of rats served as negative and positive control received vehicle (1ml/kg BW) and standard drug diclofenac (10mg/kg BW). Apigenin-7-O-β-D-glucuronide methyl ester and standard diclofenac were administered 1h prior to the injection of carrageenan subcutaneously into hind paw. A mark was made at the ankle joint of the paw and pedal volume up to this point was measured using plethysmometer at 0h,1h, 2h, 3 h, 4h and 5h.

As shown in the **Fig.5,** a significant decrease in paw volume in the late phase, i.e. 4h and 5hr thus exhibiting its anti-inflammatory activity according to the dose of the Apigenin derivative. *In vivo* anti-inflammatory potential of Apigenin-7-O-β-D-glucuronide methyl ester is evaluated by carrageenan induced paw edema, which has showed potent anti-inflammatory activity at late phase with percent inhibition of 19.94 for 200mg/kg body weight and 41.49 for 400 mg/kg body weight. A similar results has been observed by Abdul Hafeezet.al.[19] who reported that Oral administration of *Ficus virens* extract (200 and 400 mg/kg) inhibited paw swelling dose-dependently at 1, 2, and 3, h after Carrageenan injection.

Figure 5: Paw volume in carrageenan induced paw edema by Apigenin-7-*O*-β-D-glucuronide methyl ester.



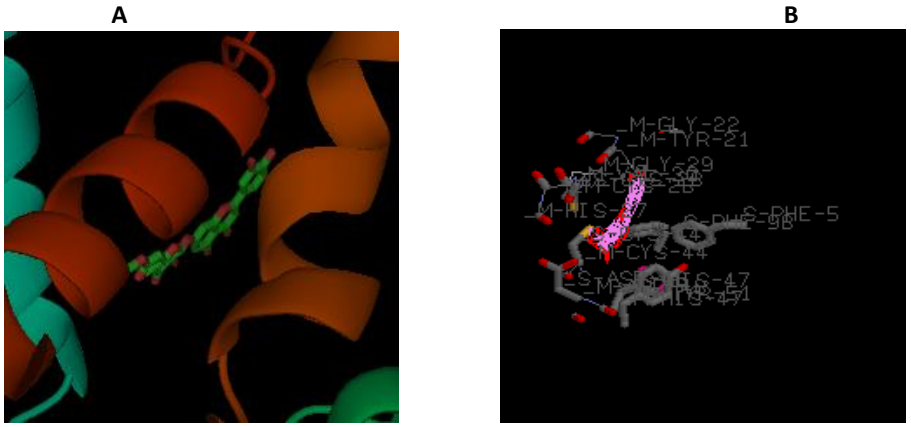
Values are expressed as mean±S.E.M.* P < 0.05, ** P < 0.01, *** P < 0.001 represents a significant difference compared with control group by student's t-test (n=3).

Molecular docking studies

Further, molecular docking studies of Apigenin-7-O-β-D-glucuronide methyl ester were performed on PLA_2, COX-2 and 5-LOX crystal proteins. Apigenin showed a good binding affinity on PLA_2 crystal protein with a binding energy of -145.89 Kcal/mol and binds in the vicinity of TYR21, GLY22, HIS27, CYS28, ASP48, TYR51 and PHE98 amino acid residues. Moreover, Apigenin-7-O-β-D-glucuronide methyl ester binds on COX-2 and 5-LOX crystal proteins with binding energies of -126.89 and -118.37 Kcal/mol respectively as shown in Table 1 and Fig 6, 7 and 8.

ISSN: 0975-8585

Figure 6: Molecular docking studies of Apigenin on PLA2 crystal structure.



A. Binding orientation of Apigenin on PLA_2.
B. Binding interactions of Apigenin with amino acids on PLA_2 crystal structure

Figure 7 : Molecular docking studies of Apigenin on 5-LOX crystal structure

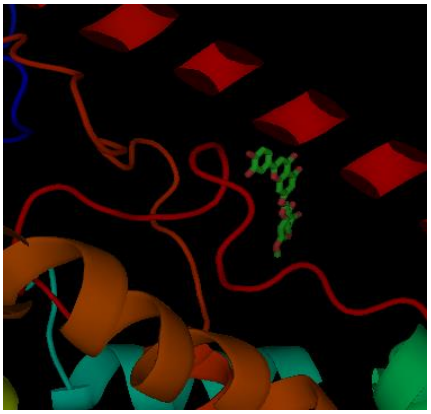


Figure 8 : Molecular docking studies of Apigenin on COX-2 crystal structure

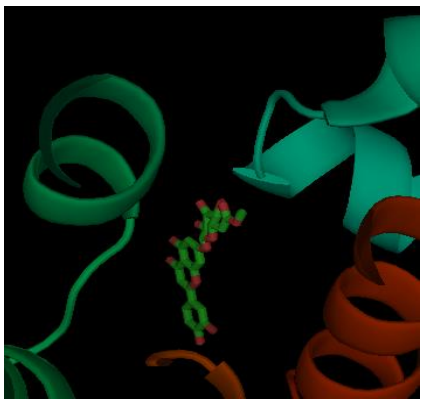


Table 1: Molecular docking studies of Apigenin-7-*O*-β-D-glucuronide methyl ester on PLA_2, COX-2 and 5-LOX using iGEM dock software

Enzymes	PDB ID	Binding energy (Kcal/mol)
PLA_2	1D85	-145.89
COX-2	4COX	-126.89
5-LOX	3O8Y	-118.37

CONCLUSION

In the present study we concluded that ethyl acetate extract of *Manilkara zapota* showed significant 5-LOX and PLA_2 anti-inflammatory activities, enabling to continue for further anti $sPLA_2$ guided fractionation by column chromatography.Apigenin-7-O-β-D-glucuronide methyl ester significantly inhibits the pro-inflammatory cytokines TNF-α and IL-1β in LPS induced RAW 264.7 cells and it also inhibits the COX-2 gene expressions in MCF-7 cancer cell lines therefore these results suggest that isolated compound Apigenin-7-O-β-D-glucuronide methyl ester from leaves of *Manilkara zapota* can be useful for curing of various inflammatory related diseases and can be used for development of novel anti-inflammatory drugs.

FINANCIAL SUPPORT

This work was partially supported by UGC-MRP.F. No.42-643/2013.

REFERENCES

[1] Libby P. Inflammation in atherosclerosis Nature, 2002;420:19-26
[2] Hussain SP, Harris CC. Inflammation and cancer: an ancient link with novel potentials. Int J Cancer. 2007; 121:2373–2380.
[3] Durackova Z. Some current insights into oxidative stress. Journal of Physiological Research, 2010;59: 459–469
[4] Kaneria M, Sumitra C. Evaluation of antioxidant and antimicrobial properties of *Manilkara zapota* L. (chiku) leaves by sequential soxhlet extraction method. Asian Pacific Journal of Tropical Biomedicine. 2012; S1526-S1533.
[5] Pankaj KJ, Prashant S, Neeraj U,Yogesh S. Evaluation of Analgesic Activity of *Manilkara zapota*(Leaves) European Journal of Experimental Biology. 2011; 1:14-17
[6] Singh M, PrashantS, NeerajU,Yogesh SIn-vitro anti-arthritic activity of *Manilkara zapota* Linn Asian J. Pharm. Tech. 2011; 1:123-124
[7] Ganguly A, Mahmud ZA, NasirUddin MM, AbdurRahmanSM *In vivo* anti-inflammatory and anti-pyretic activities of *Manilkara zapota* leaves in albino Wistar rats Asian Pac J Trop Dis. 2013; 3: 301-307
[8] Kamalakararao K, Krishna Chaithanya K, Gopalakrishnan VK, ZenebeHagos, HaftomKebedec, TentuKasi Naidu, Patricia Ponce Noyolae, John Dogulas P, GovindaRao D (2017a) Anti-Inflammatory activity of *Manilkara zapota* leaf extract. Int J Curr Pharm Res, 9(4): 130-134
[9] Kamalakararao K, Krishna Chaithanya K, Gopalakrishnan V K , ZenebeHagos, KalayuMesfinArefaye, Patricia Ponce Noyola, John Dogulas P, TentuKasi Naidu, GovindaRao D (2017b). Apigenin-7-O-B-D-Glucuronide Methyl Ester Isolated from *Manilkara zapota* Leaves. International Journal of Pharmaceutical Quality Assurance.8(2); 58-65
[10] Copeland RA, Williams JM, Giannaras J, Nurnberg S, Covington M, Pinto D, Pick S and Trzaskos JM. National Academy of Sciences. 1994; 91:11202-11206.
[11] Winter CA, Risley EA, Nuss GV. Carrageenin-induced edemas in hind paw of the rat as an assay for anti-inflammatory drugs. Proc Soc Exp Biol Med. 1962; 111:544-547.
[12] Lengauer T, RareyM. Computational methods for biomolecular docking.Curr.Opin.Struct.Biol.1996; 6: 402-406
[13] Yang JM, Chen CC (2004) GEMDOCK: A Generic Evolutionary Method for Molecular Docking. Proteins 55: 288-304.
[14] Catalano A and Procopio A (2005). New aspects on the role of lipoxygenases in cancer progression. Histol.Histopathol. 20:969-975.
[15] Ravi Shankara BE, Ramachandra YL, SundaraRajan S, SujanGanapathy PS, Austin RS, and Dhananjaya BL Anti-Lipoxygenase Activity of Leaf Gall extracts of *Terminalia chebula*(Gaertn.) Retz. (Combretaceae) Pharmacognosy Res. 2016;8:78–82
[16] Romero-Estrada A, Maldonado-Magaña A, González-Christen J, Bahena SM, Garduño-Ramírez ML, Rodríguez-López V, Alvarez L (2016). Anti-inflammatory and antioxidative effects of six pentacyclictriterpenes isolated from the Mexican copal resin of *Bursera copallifera* BMC Complement Altern Med. 16(1):422.
[17] Kang CH, Choi YH, Il-Whan Choi IW, Lee JD, Kim GY Inhibition of Lipopolysaccharide-Induced iNOS, COX-2, and TNF-_ Expression by Aqueous Extract of *Orixa Japonica* in RAW 264.7 Cells via Suppression of NF-κB Activity Tropical Journal of Pharmaceutical Research,2011; 10 : 161-168

[18] Guoqing Li, Dan Liu, ShiyuGuo, Masataka Sunagawa , Tadashi Hisamitsu and Yanqing Liu (2014) Anti-invasive effects of *Celastrus orbiculatus* extract on interleukin-1 beta and tumour necrosis factor-alpha combination-stimulated fibroblast-like synoviocytes. BMC Complementary and Alternative Medicine 2014, 14:62

[19] Abdul Hafeez, Upendra Jain, Pinky Sajwan, SirishSrivastava, Amit Thakur.Evaluation of Carrageenan induced anti-inflammatory activity of ethanolic extract of bark of *Ficus virens* Linn. In swiss albino mice. The Journal of Phytopharmacology,2013; 2(3): 39-43.