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Research Article

Apigenin-7-*O*-B-D-Glucuronide Methyl Ester Isolated from *Manilkara zapota* Leaves

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ABSTRACT

In nature there are numerous plants available with medicinal properties. Around 70 % of medicinal plants are found in tropical areas of India. The search for natural products and compounds derived from natural sources has played a vital role in drug discovery due to their pharmacological importance. Compounds isolated from botanical sources remain an important source of several clinically useful anti-inflammatory agents. *Manilkara zapota* is a large, evergreen forest tree belongs to family sapotaceae. It is commonly known as chiku (Hindi), sofeda (Bengali) sapodilla and sapoti (French), chickle tree, hase berry, tree potato (English). *Manilkara zapota* is a species of lowland rain forest. *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 aims to isolate, structurally characterize and analyze the bioactive compound from *Manilkara zapota* by using chromatographic and spectrophotometric techniques on the basis of inhibitory effects on $sPLA_2$ by activity guided fractionation of ethyl acetate extract of *Manilkara zapota* leaves. Among the six fractions (F1-F6) tested fraction-5 showed significant inhibitory effects on $sPLA_2$ activity hence fraction -5 was further subjected to structural analysis for identification of bioactive compounds by using analytical techniques such as TLC, HPLC, FT-IR, LC-MS and ^1H ,^{13}C NMR studies. The isolated compound identified as apigenin-7-*O*-β-D-glucuronide methyl ester.

Keywords: *Manilkara zapota*; TLC ; HPLC; FT-IR; LC-MS; NMR; Apigenin-7-*O*-β-D-glucuronide methyl ester.

INTRODUCTION

Nature has provided a complete store house of remedies to heal all ailments of mankind through medicinal plants[1]. Medicinal plants represent still a great unexploited resource of structurally novel compounds that might endow with a lead for the improvement of novel anti-inflammatory drugs[2,3]. Selection of the plants for their biological activity has been based on their chemotaxonomic investigations or ethno botanical knowledge of a particular disease. The importance of the plant lies in their biologically active principles. This shows a need for premeditated activity guided phytopharmacological evaluation of herbal drugs[4,5]. Plant derived natural products such as flavonoids, terpenes and alkaloids have received significant consideration due to their varied pharmacological properties including inflammatory, antipyretic and analgesic activities.

The high medical, scientific concern evoked by the involvement of phospholipase A_2 molecule in the different physiopathological process promoted an increased search for natural inhibitors aiming at PLA_2s neutralization[6]. Several plants with antiphospholipase A_2 activity were described throughout the world. Methanolic extracts of roots from *Hemidesus index* and *H. pluchea* inhibited the hemorrhagic and lethal effects of *Vipera russeli* venom[7-9]. Angulo and colleagues showed the antivenom activity of fucoidan a polysaccharide sulphate from the brown marine algae *Ficus vesiculus*[10]. De Silva *et.al* isolated Edunol a pterocarpan from *Harpalyce brasillana* with PLA_2s inhibitory properties[11]. Nunez *et al* isolated 4-neolidyl catechol from extracts of *Piper umbellatum* and *Piper peltatum* which are effective in PLA_2 neutralisation[12]. Borges,*et al* showed that the aqueous extracts of *Tabernaementan acatharinensis* neutralize PLA_2s[13].

Manilkara zapota and its different parts have been traditionally used and medicinal value has been reported. The acetone extract of *M. zapota* leaves has shown significant antioxidant activity[14]. Petroleum ether and ethanolic leaf extracts of *M. zapota* were reported to have analgesic activity[15]. The ethanolic extract of *M. zapota*

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Table 1: Solvent system and solvent ratio.

S.No	Solvent System	Solvent Ratio
1	*Manilkara zapota* – Chloroform Fraction (MCF-1)	100
2	*Manilkara zapota* – Chloroform – Ethyl acetate Fraction (MCEF-2)	6.4
3	*Manilkara zapota* – Chloroform – Ethyl acetate Fraction (MCEF-3)	5.5
4	*Manilkara zapota* – Ethyl acetate Fraction (MEF-4)	100
5	*Manilkara zapota* – Ethyl acetate- Methanol Fraction (MEMF-5)	9:1
6	*Manilkara zapota* – Ethyl acetate- Methanol Fraction (MEMF-6)	6.4

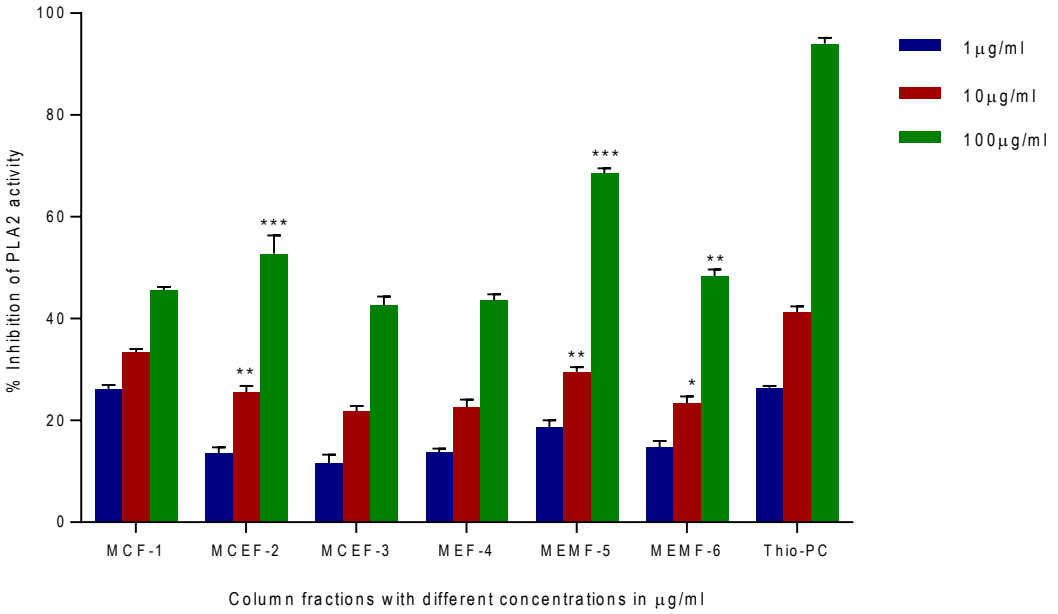

Figure 1: PLA_2 inhibitory activities of different fractions obtained from silica gel column. Values are expressed as mean±S.E.M.* P < 0.05, ** P < 0.01, *** P < 0.001 represents significant difference compared with control group by student's t-test (n=3).

possesses significant anti-arthritic activity[16]. The antitumor activity of *M. zapota* ethyl acetate extract was reported by Rashid and his colleagues against Ehrlich ascites carcinoma in mice. It has been reported that ethyl acetate and methanolic extract of leaves of *M. zapota* shows significant inhibition of paw edema[17]. Hence, the present study has been undertaken to carry out the activity-guided fractionation for the isolation of bioactive compound from the leaves of *M. zapota*.

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, India.

Isolation and identification of bioactive compound from leaves of M. zapota

Silica gel column chromatography is commonly used chromatographic technique to separate phyto compounds. In silica gel column chromatography column is packed with stationary phase (silica 100-200 mesh) and the mobile phase is pre-runned through the column. The Gradient elution method is observed to separate compounds from ethyl acetate leaf extract by using solvents from nonpolar to polar i.e. hexane to methanol in various proportions. Ethyl acetate leaf extract 35gms was taken, adsorbed to silica gel and subjected to silica gel column chromatography followed by gradient elution to separate the compounds. The flow rate was adjusted to 5ml/min, total 360 ml solvent was collected for each fraction. The obtained fractions were subjected to TLC to know the presence of compounds and their Rf values. Fractions depicting the presence of compounds as spots and experiencing the same Rf value are pooled, of all the fractions collected six fractions shown the presence of compounds. All the six fractions were individually pooled and concentrated by using rotavapour. The condensed fractions are evaluated for their PLA_2 inhibitory activity. The purity of bioactive compound is determined by HPLC.

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

Table 2 : Phytochemical analysis of six major fractions by thin layer chromatography.

Compounds	Column Fractions											
	F1		F2		F3		F4		F5		F6	
	Colour	Rf value	Colour	Rf value	Colour	Rf value	Colour	Rf value	Colour	Rf value	Colour	Rf value
Terpenoids	No Spot	-	Pink	0.68	No Spot	-	No Spot	-	No Spot	-	No Spot	-
Flavonoids	No Spot	-	No Spot	-	No Spot	-	No Spot	-	Yellow	0.84	No Spot	-

Rf- retardation factor or relative front

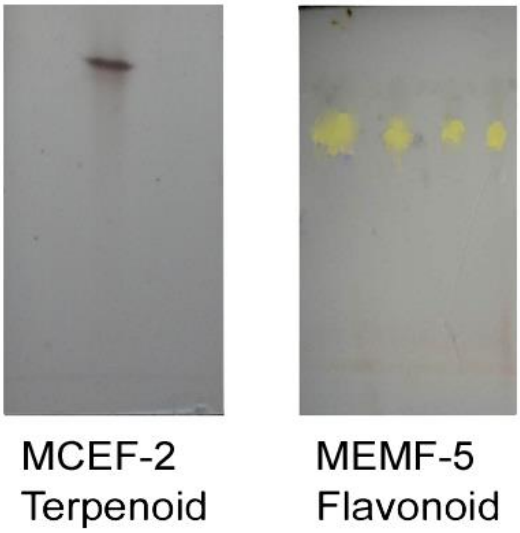

Figure 2 : TLC Profiles of phytoconstitutents

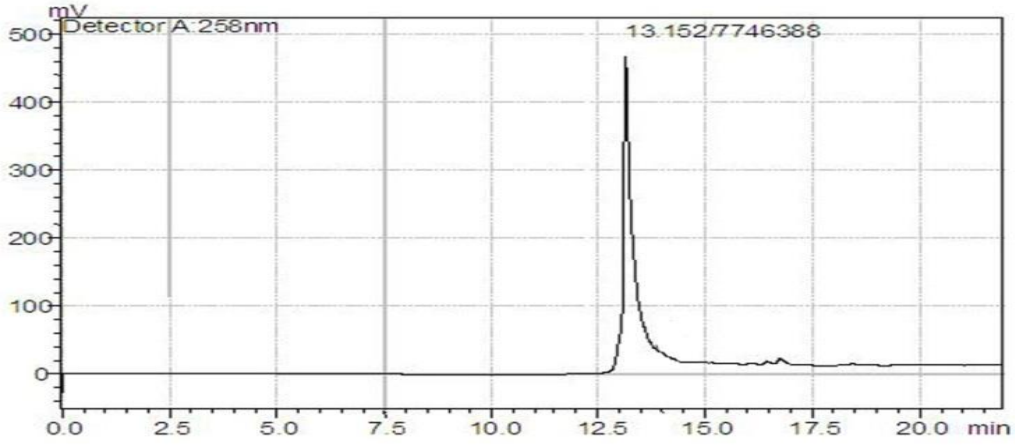

Figure 3: HPLC Chromatogram of MEMF-5.

mixture was contained 10µl of PLA_2, 1, 10 and 100 µg/ml of Apigenin-7-*O*-β-D-glucuronide methyl ester respectively in test wells, and 200µl substrate incubated for 15 minutes. 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 formula below:

Percentage inhibition

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

Thin layer chromatography

TLC is a simple, quick, and inexpensive procedure to know the number of components that are present in a mixture. TLC plays an important role in fractionation, isolation and detection of active compounds in the crude plant extracts and also widely employed in botanical extract analysis[18]. TLC also supports the identity of an unknown compound in a mixture by comparing the (Retardation factor) Rf of the compound with the Rf of a known compound. Further tests involve the use of phytochemical screening reagents, which on spraying

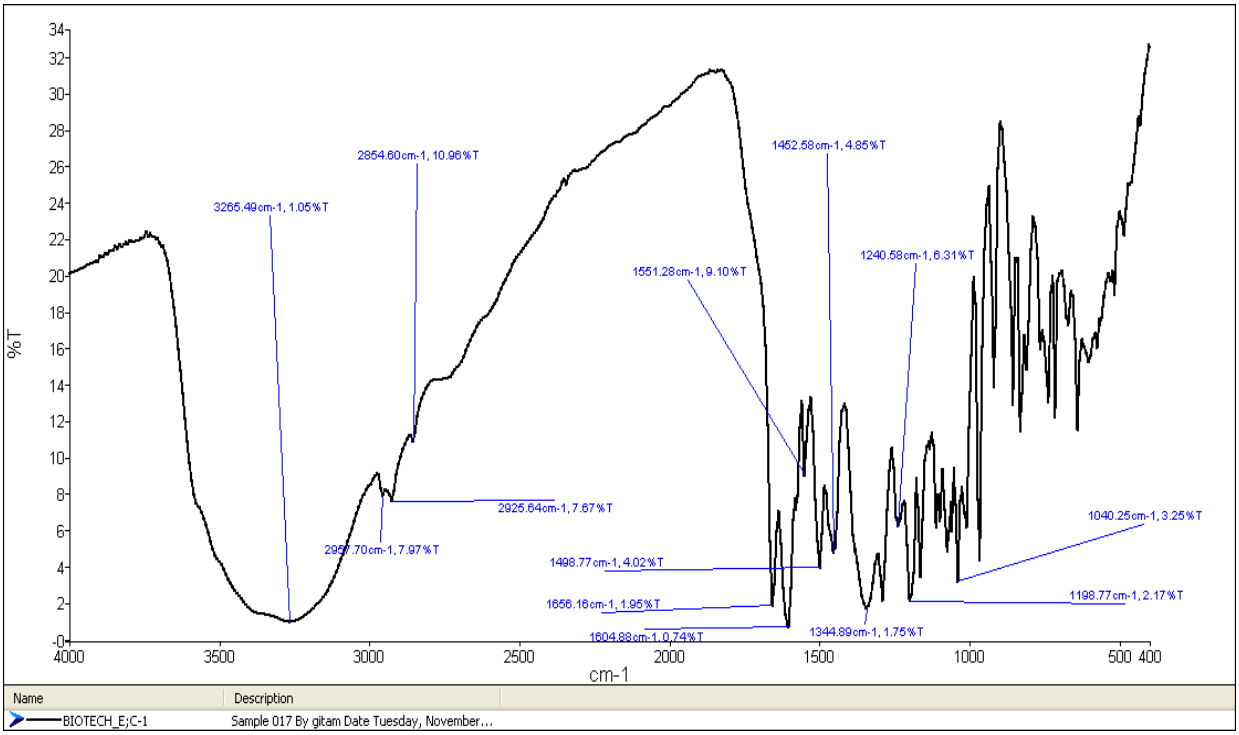

Figure 4: FTIR Spectrum of ME.

Table 3: Chromatogram report of HPLC fraction-I.

Index	Retention Time [Min]	Peak Area [%]
1	13.15	97.22

Table 4: IR spectra analysis of MFCE-F5.

Wavelength (cm^{-1})	Interpretation
3265	Broad OH^-
2957	
2925	CH_2 stretching
2854	
1740	Missing for Easter
1656	C=O
1604	C=C
1551	

cause color changes according to the constituents existing in the plant extract or by viewing the plate under the UV light.

Several chromogenic or fluorogenic reagents exist in the literature for the detection of specific types of compounds. Many of these spray reagents are specific to a type of compound, or to a broad range of compounds within a particular class. When applied to the plate, they specifically react with the separated compounds and produce distinct colors which indicate a particular class of compounds.

Thin layer chromatography (TLC) Identification of fractions constituents

The six column fractions (F1-F6) of ethyl acetate leaf extracts obtained from column fractionation were subjected to thin layer chromatography analysis to find the presence of chemical constituents (secondary metabolites) to support the phytochemical test. TLC separates the compounds based on the Rf values of phytoconstituents with respect to solvent system.

TLC analysis of flavonoids

Solvent mixture, chloroform and ethyl acetate in the proportion of 5:5 was used to separate the flavonoids from fractions. The color and Rf values of the separated flavonoids were recorded under the visible light after spraying with color developing solution, i.e. methanol: sulphuric acid (9:1).

TLC analysis for terpenoids

Solvent mixture, hexane and chloroform in the ration of 4:6 were used to separate the terpenoids from fractions. The colour and Rf values of the separated terpenoids were recorded under the visible light after spraying with colour developing solution i.e. methanol: sulphuric acid (9:1)

Bioactive fractions

Total six fractions are collected and screened for their anti-inflammatory activity by using phospholipase A_2 kit method. $sPLA_2$ assay was performed using Cayman Chemical kit USA, as per instructions of the manufacturer. The purified compound was structurally characterized and elucidated by Fourier transform infrared spectroscopy (FTIR), liquid chromatography-mass spectroscopy (LC-MS) and Nuclear magnetic resonance (NMR) ^1H and ^{13}C.

RESULTS AND DISCUSSION

PLA_2 inhibitory activity guided fractionation

All the six fractions are tested for their PLA_2 inhibitory activity taking thioetheramide PC as standard inhibitor. As shown in Figure 1 among the six fractions, MCEF-2 and MMEF-5 showed PLA_2 inhibitory activity at 52.8 and 68.6 µg/ml, respectively while that of standard inhibitor thioetheramide PC is 94.0µg/ml. It is found that MMEF-5

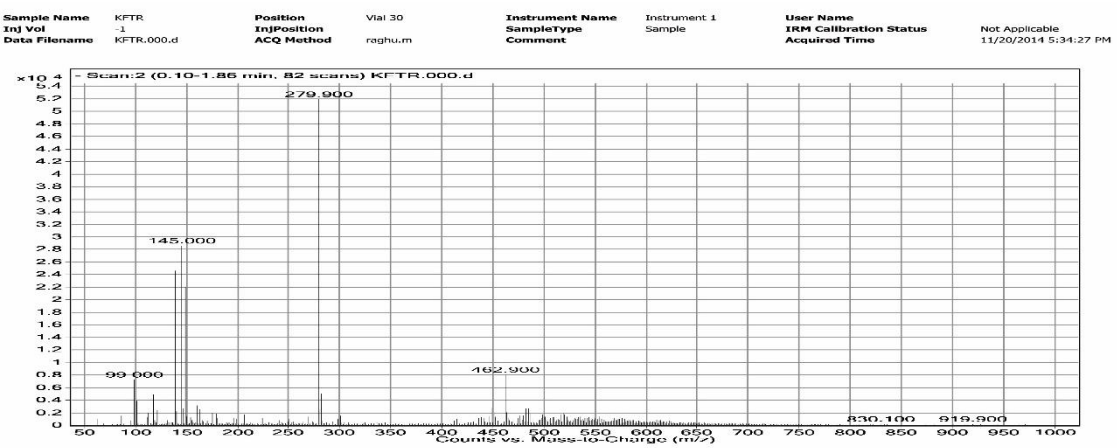

Figure 5: Mass Spectrum of MEMF-5.

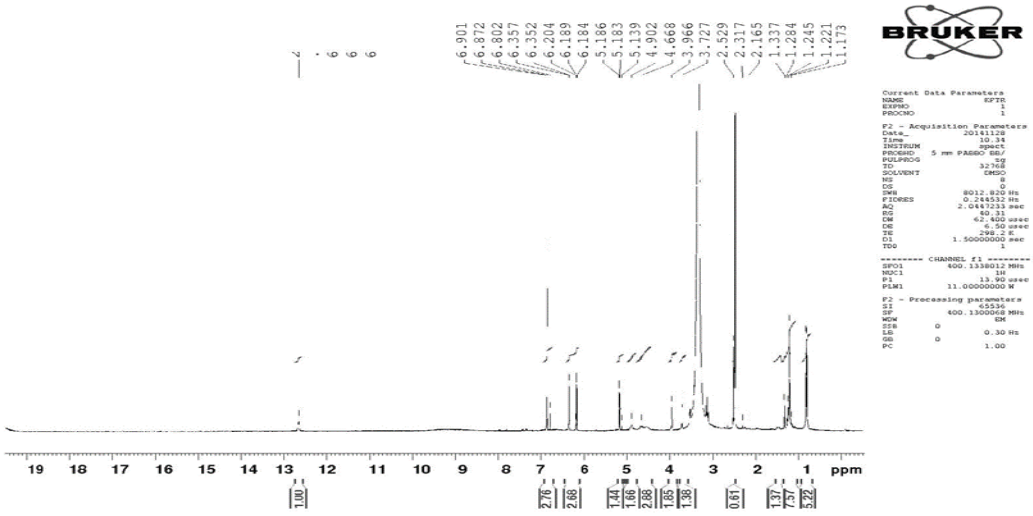

Figure 6: Proton NMR Spectrum of MEMF-5.

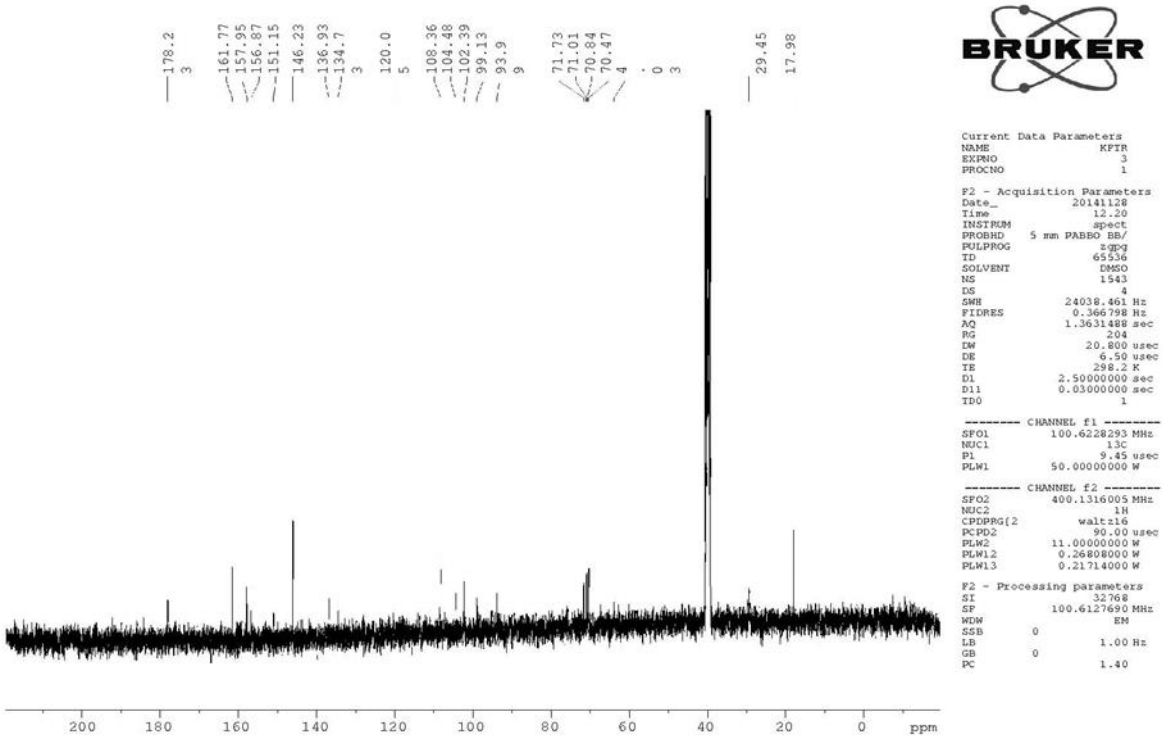

Figure 7 : ^{13}C NMR Spectrum of MEMF-5.

shown more PLA_2 inhibitory activity when compared with fraction MCEF-2.

Detection of compounds by TLC

The collected fractions were taken by capillary tube applied on pre-coated TLC plate with capillary tube. The dried TLC plates were runned in pure ethyl acetate solvent (mobile phase). Plates were taken out of the TLC chamber and the spots were visualized using UV light exposure, iodine vapors and using various spraying agents, including sulphuric acid: methanol (1:9) mixture and Rf values are calculated. The fractions showing the compounds with same Rf value were pooled into one fraction. The six fractions obtained after pooling are MCF-1 (chloroform), MCEF-2 (chloroform: ethyl acetate 6:4), MCEF-3 (chloroform: ethyl acetate 5:5), MEF-4 (ethyl acetate), MEMF-5 (ethyl acetate: methanol 9:1) and MEMF-6 (ethyl acetate: methanol 6:4). All the six fractions are subjected for phytochemical analysis to know the chemical constituents in these fractions shown in table 2. All the fractions except MCEF-2 and MEMF-5 contained a mixture of compounds, whereas fraction MCEF-2 showed positive for terpenoids and fraction MEMF-5 showed positive for flavonoids by exhibiting characteristic pink color spot for terpenoids and yellow color spot for flavonoids after spraying the TLC plate with color developing agent such as sulphuric acid:methanol (1:9) mixture shown in Figure 2. All the obtained six fractions are tested for PLA_2 inhibitory activity.

HPLC chromatogram of MEMF-5

Figure 3 shows that High Performance Liquid Chromatography (Waters C 18 column, USA) was performed for sample isolated by TLC. Sample was dissolved in HPLC grade methanol in concentration of about 1 to 10µg/ml and 20µl of the solution was injected in the column RP-C18 and analyzed by PDA detector. The wavelength range was 250 to 500nm. The mobile phase components acetonitrile: water was used in a gradient form, which varied with change in time. The sampling rate was kept 2 (points/sec).The total flow rate was kept 0.70 ml/min, filter time constant was 1.0000 sec and the software installed was Empower 2 software build 2154 SPs. Service pack H DB ID: 908711544.The retention time was shown in table 3.

Fourier Transform Infrared Spectroscopic Analysis (FT-IR)

The FTIR spectrum was used to identify the functional group of the active components based on the peak value in the region of infrared radiation. The purified fraction MEMF-5 of *Manilkara zapota* was passed into the FTIR and the functional groups of the components were separated based on its peak ratio. The results of the purified fraction MEMF-5 of *Manilkara zapota* FTIR analysis confirmed the presence of hydroxyl and keto groups present in the compound which shows major peaks at 3265 and 1656 cm^{-1}, respectively (Figure. 4 and Table 4).

LC-MS spectrum of MEMF-5

The Liquid chromatography/Mass spectrometry (LC/MS) chromatogram of the active pure compound of MEMF-5 was shown in Figure 5. The atmospheric pressure

Table 5 : ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR (100 MHz, $CDCl_3$) data of MFCE-F5.

Position	^{1}H NMR	δ (ppm)	^{13}C NMR	δ (ppm)
1	5`-C	6.90	5`-C	120
2	3`-C	6.80	3`-C	108
3	2` & 6`-C	6.35	2` & 6`-C	104
4	3	6.20	3	102
5	6	6.189	6	99
6	8	6.184	8	93
7	CH_3COO	1.24	CH_3COO	17
Sugar Moiety				
8	1``, 511	5.18	1``, 511	157, 156
9	2``, 4``	3.9	2``, 4``	151, 146
10	3``	3.72	3``	136
11	C=O	-	C=O	178.23
	C-O	-	C-O	161.77

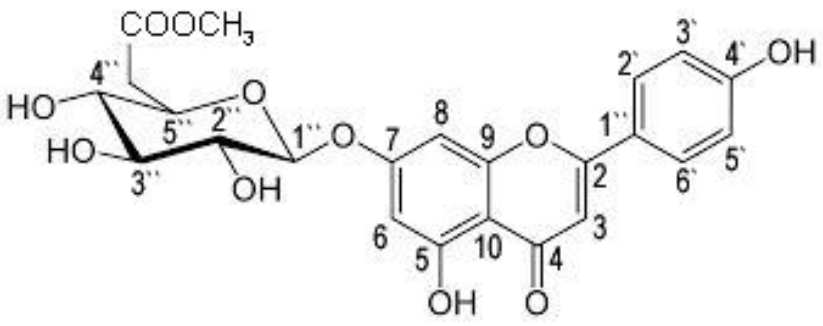

Figure 8: Chemical structure of Apigenin-7-O-β-D-glucuronide methyl ester.

ionization –electron spray (API-ES) mass spectrum of compound MEMF-5 was analyzed in positive ion mode. The MS fragmentation ions at m/z showed compound main peak $(M-H)^+$ at 462 Da. The mass spectral data of the compound MEMF-5 gave a molecular formula **$C_{22}H_{20}O_{11}$** which was supported by the Atmospheric pressure ionization-electron spray (API-ES).

Proton and ^{13}C NMR spectrum

The 400 MHz ^{1}Hand ^{13}C NMR Spectrum of the compound MEMF-5 in DMSO-De with TMS as internal standard .The various peaks assigned to ^{1}H and ^{13}C spectrum was shown in Figure 6 & 7 and data was given in the **Table 5**. In proton spectrum, δ shift (ppm) at 6.90, 6.80, 6.35, 6.20, 6.189 and 6.184, and ^{13}C peaks at 120.05, 108.36, 104.48, 102.39, 99.13, and 93.99 indicate aromatic protons in MEMF-5. δ shift (ppm) in ^{1}H NMR spectrum at 5.18, 3.72, 3.9 and^{13}C peaks at 157.95, 156.87, 151.15, 146.23, 136.93 and 134.73 indicates presence of the sugar moiety. δ shift (ppm) at 1.173 (^{1}H NMR) and 17.98 (^{13}C NMR) indicates presence of methyl groups in structure of MEMF-5.

Finally, bioactive compound shown in Figure 8 was isolated from column chromatography. The IR spectrum showed the presence of a hydroxyl group, C-H group, from the results of NMR data the compound is found to be Apigenin-7-*O*-β-D-glucuronide methyl ester ($C_{22}H_{20}O_{12}$) and the literature review revealed that compound has much biological activity. This is first report having anti-inflammatory activity of isolated bioactive compound from the leaves of *Manilkara zapota*.

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. The isolated bioactive is compound Apigenin-7-*O*-β-D-glucuronide methyl ester.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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