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In-vitro anti-oxidant and anti-platelet activity of *Manilkara zapota* (L.) P Royen latex

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Abstract
In the present study latex of *Manilkara* zapota (L.) P.Royen is evaluated for anti-oxidant and anti-platelet activities. In DPPH method the latex sample (300 µg) exhibited 90% of radical scavenging activity with IC_{50} value of 96 µg. In TAA and FRAP assay methods showed 60 ± 2.2 and 83 ± 2.6 GAE values at 300 µg, respectively. The latex sample showed a maximum platelet aggregation inhibition of 86% at the concentration of 12 µg. Further, phytochemical analysis of latex sample by GC-MS study revealed 42 phytocompounds.

Keywords: *manilkara zapota*, anti-oxidant activity, anti-platelet activity, free radicals

1. Introduction
The living cells constantly undergo biochemical oxidation and reduction reactions leading to generation of free radicals such as reactive oxygen species (hydrogen peroxide, superoxide anion, hydroxyl radical) and nitrogen species (nitric oxide and nitrogen dioxide) [1]. The oxidative stress induced by free radicals damage the cellular bio-molecules (proteins, lipids, nucleic acids) [2], which in turn alter many biological processes including vascular endothelial injury. Damage caused by free radicals to endothelium stimulates the platelets to adhere to the damaged vascular endothelium and increase the chance of exposure to agonists such as Thrombin, Adenosine diphosphate (ADP), epinephrin, collagen and serotonin. Frequent exposure to agonists results in activation and aggregation of platelet. Hyperactivity and hyper-aggragability of platelets leads to thrombotic disorders such as thromboembolism and atherosclerosis [3]. The physiological system possesses anti-oxidant enzymes (catalase, glutathione peroxide, superoxide dismutase) to check free radicals generation [4]. In addition prostacyclin and endothelial AD Pase acts as antagonist in hyper activation/ hyper aggrag ability of platelets [5, 6]. However, these are not enough to reduce the cellular/vascular damage, hyperactivation of platelets and cardiovascular diseases in oxidative stress related conditions. External supplements needs to be provided to augment free radical generation and its associated effects. Hence, there is a need to find an alternative anti-oxidant and anti-platelet molecule with less/no side effects. Medicinal plants are known to be rich source of bioactive molecules with anti-oxidant activity. Recent studies have reported several medicinal plant extract/s exhibiting antioxidant activity and inhibiting associated side effects with respect to hyperactivity of platelets [7, 8]. The *Manilkara zapota* is an evergreen tree and member of the family Sapotaceae. The plant is native of tropical America and Mexico and spread in all tropical regions [9]. Folklore medicinal practitioners use parts of *M. zapota* plant for medication purposes; different parts of the plant reported to possess anti-inflammatory, anti-microbial, anti-pyretic, anti-oxidant, hepato-protective activities [10]. Fruits, barks and leaves were used to treat pulmonary diseases and diarrhea. Fruits were used to reduce inflammation and pain in gastritis [11]. The seed paste is used to reduce pain and inflammation caused by insect bites and stings [12]. The plant extract is used to treat bleeding of piles and injuries [13]. Chickle prepared from latex of *M. zapota* is used to prepare chewing gums. Resins present in latex sap are used in manufacturing paints, adhesives, and water resistant varnishes [14, 15].
Earlier, we have reported the presence of serine protease and its hemostatic activity from the *M. zapota* latex [16]. In present study, we are evaluating anti-oxidant and anti-platelet activities of *M. zapota* latex.

2. Materials and Methods
2.1. Materials
ADP, 2, 4, 6-Tripyridyl-S-triazine (TPTZ), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), Ascorbic acid were procured from Sigma Aldrich (St. Louis, MO, USA). All other reagents used in this study were of analytical grade.

2.2. Latex collection
The *M. zapota* latex was collected in the month of March near Tumkur district, Karnataka, India. The plant was identified by Dr. P. Sharanappa, Professor, Department of Studies and Research in Bioscience, Hemagangotri, University of Mysore, Hassan district, Karnataka, India. The voucher specimen for *M. zapota* plant (TU16DOSRBC004) is deposited in the herbarium of the Department of Studies and Research in Botany, Tumkur University, Tumkur, Karnataka, India

2.3. Processing of latex
The latex of *M. zapota* plant was collected into clean container by plucking the fruit and freeze to –20 °C for 24 h. After thawing, the wax materials were removed by centrifuging the latex at 12,000 rpm for 20 min. The supernatant was lyophilized to powder and stored in –20 °C until further use.

2.4 Phytochemical analysis by GC-MS (gas chromatography – mass spectrometry)
The *M. zapota* latex was subjected to GC-MS analysis in a silica capillary column (VF-5ms) of diameter 0.25 mm,

length 30 m, and film thickness of 0.25 µm. The ionization of the sample was performed in electron impact mode (70 eV). The column oven temperature was set from 80 °C to 310 °C for 2 °C min−1 and the injector and detector temperature was fixed to 270 °C and 230 °C, respectively. The carrier gas (Helium) flow rate was set to 1.21 mL/min. The 50 µg of *M. zapota* latex (extracted in methanol for GC-MS study) in 2 µl of extract was manually injected using Hamilton syringe in split injection technique. The running time for GC-MS was 50 min. The phytochemical molecules of *M. zapota* latex were identified by comparing their retention indices and patterns of mass spectra using ChemStation software.

2.5. Anti-oxidant activities

2.5.1. Total anti-oxidant activity

The total anti-oxidant activity was performed according to the method of Prieto *et al*. [17] with slight modifications. Different concentration of *M. zapota* latex ranging from 50 – 300 µg was treated with 1 mL of reagent (28 mM sodium phosphate, 0.6 M H_2SO_4 and 4 mM ammonium molybdate) at 95 ºC for 60 min. after cooling to room temperature absorbance was read at 695 nm. Total anti-oxidant capacity was expressed as Gallic acid equivalents (GAE). Each test was performed in triplicates.

2.5.2. FRAP (Ferric reducing anti-oxidant power)

The anti-oxidant activity by FRAP was performed according to the method of Benzie *et al*. [18]. Different concentrations of *M. zapota* latex ranging from 50 – 300 µg was incubated with 1 mL of FRAP reagent (10:1:1 ratio of 300 mM acetate buffer, 10 mL TPTZ dissolved in 40 mM HCl, 20 mM $FeCl_3$) at 37 ºC for 30 min. Absorbance was read at 593 nm. The values were expressed as GAE. Each test was performed in triplicates.

2.5.3. DPPH

The anti-oxidant activity by DPPH was performed according to the method Braca *et al*. [19]. Different concentration of *M. zapota* latex ranging from 0 – 300 µg in 50 µL aliquot was treated with 140 µL of 0.1 mM DPPH and incubated at room temperature for 30 min. Absorbance was read at 517 nm. The percent inhibition of DPPH radical was calculated using the formula;

$$\% \text{ inhibition of DPPH radical} = \frac{(Ac - At)}{Ac} \times 100$$

Where, Ac is absorbance of control and At is absorbance of test sample at 517 nm. Ascorbic acid was used as positive control. Each test was performed in triplicates.

2.6. Platelet aggregation

The fresh blood was mixed with 3.2% of sodium citrate in 9:1 ratio. The platelet rich plasma (PRP) was obtained by centrifuging citrated fresh blood at 900 rpm for 15 min. The platelet poor plasma (PPP) was obtained by centrifuging same blood at 3,000 rpm for 20 min.

The platelet aggregation was monitored using Chrono-Log Model 700–2D aggregometer. Different concentrations of *M. zapota* latex (4, 8, 12 µg) was pre-treated with 0.25 µL of PRP at 37 ºC, aggregation was initiated by adding 10 µM of ADP as agonist. The extent of aggregation was constantly monitored for 6 min by using an optical method for identifying change in turbidity, with PRP and PPP representing 0% and 100% transmittance, respectively.

Statistical analysis

The Statistical Package for Social Sciences (SPSS) with the version 15.0 (SPSS Inc., Chicago, IL, USA) was used for all analysis. All the statistical results were represented as mean ± standard error. One-way ANOVA was used for statistical significance of intergroup differences and the Tukey's test was used for comparison of means. The data were considered significant at $p < 0.05$.

3. Results and Discussion

3.1. GC-MS analysis

The GC-MS analysis of *M. zapota* latex revealed the presence of 42 different phytocomponents based on retention time and molecular mass. The GC-MS chromatographic profile of the latex is shown in Fig. 1. In Table 1, the peak area (in %), molecular weight and molecular formula of each compound is tabulated. The major phytocomponents present in *M. zapota* latex are found to be; Phosgene (11.74%), 1-(Benzyloxy)-2-methoxy-3-[(E)-2-nitroethenyl]benzene (30.57%), Benzo (c) phenanthren-3-amine (5.11%), Inositol, 1-deoxy (2.435%), 2-Methylhexadecan-1-ol (5.96%), 17-Pentatriacontene (2.802%), 1-Hexacosene (2.17%), 2,6,10-Tri methyl tetradecane (5.41%), 1-Monolinoleoylglycerol tri methyl silyl ether (9.12%), 2,6,10,10-Tetramethyl-1-oxaspiro [4.5]decan-6-ol (5.03%).

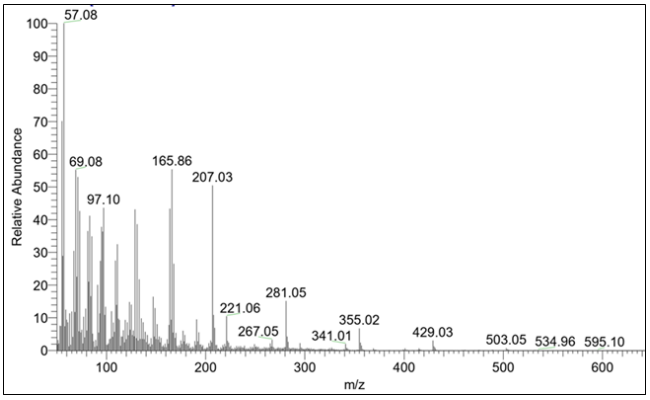


Fig 1: Chromatogram of GC-MS analysis

3.2. Anti-oxidant activities

3.2.1 TAA

TAA method works on the phosphomolybdenum method. Where, the sample reduces Mo (VI) to Mo (V) at acidic pH (4.0) forming green coloured phosphate/ Mo (V) complex. The TAA values are represented as mg GAE calculated using standard Gallic acid calibration graph. The *M. zapota* latex sample has increased the TAA in dose dependent manner (50 – 300 µg). The latex sample exhibited maximum TAA of 60 ± 2.2 at a concentration of 300 µg. The results are tabulated in table 2.

3.2.2. FRAP

FRAP method works by reducing Fe3þ- tetra (2-pyridyl) pyrazine complex to Fe2þ-tripyridyltriazine. This is the effective method to find reducing potential of an anti-oxidant in association with presence of compounds responsible for breaking the free radical chain through donation of hydrogen atom. The FRAP values are

represented as mg GAE, calculated using standard Gallic acid calibration graph. The *M. zapota* latex showed effective anti-oxidant potential in dose dependent manner (50 – 300 µg). The *M. zapota* latex showed maximum FRAP value of 83 ± 2.6 at concentration of 300 µg. The results are tabulated in table 2.

3.2.3 DPPH

DPPH is a stable free-radical; hence anti-oxidant activity is commonly performed using this method. The molecule with anti-oxidant capacity scavenges or donates hydrogen to reduce DPPH. The *M. zapota* latex showed significant antioxidant activity with DPPH. The inhibition percentage is increased with increase in *M. zapota* latex concentration (Fig. 2). At the concentration 300 µg the latex showed 90% of inhibition (IC_{50} value: 96 µg). However, the standard ascorbic acid showed 100% inhibition at 250 µg concentration.

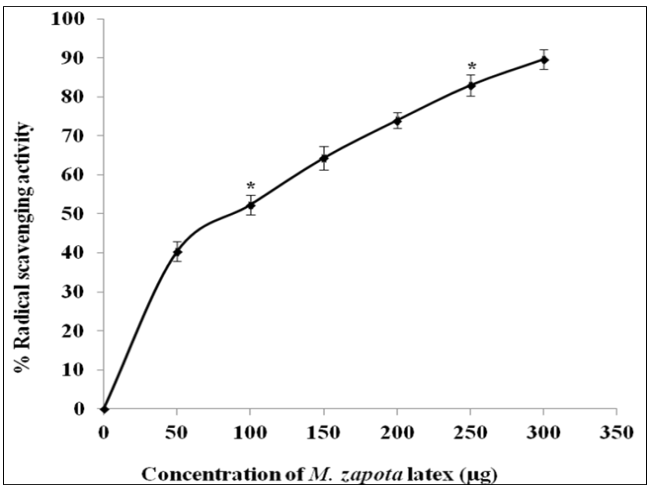
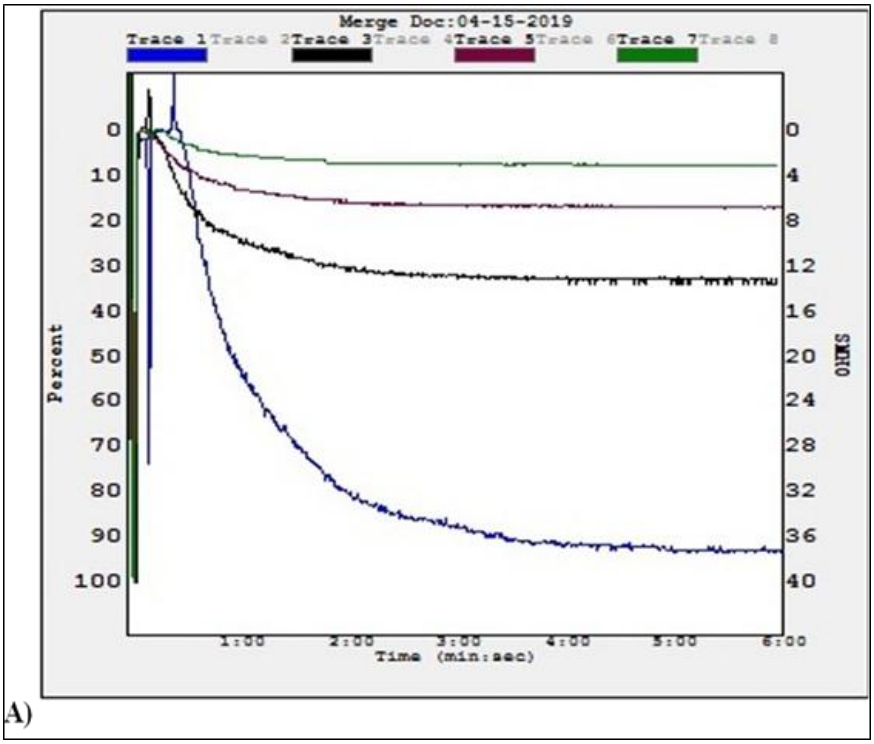


Fig 2: Anti-oxidant activity by DPPH method. Each value presented as mean ± SE of the mean (*n*=3). Statistically significant results are indicated by asterisks, * – $p < 0.05$. DPPH, 1, 1-diphenyl-2-picrylhydrazyl.

3.3. Platelet aggregation

Platelet aggregation is a crucial step in blood coagulation cascade. After blood vessel wall damage, platelets adhere and aggregate at site of injury, forming the platelet plug [20, 21]. The plasma membrane of platelets is made of phospholipid bilayer. Where, different lipid rafts and surface receptors are involved in intracellular signaling and trafficking [22]. Many platelet agonists (ex., Thrombin, ADP, epinephrin, collagen and serotonin) plays crucial role in activation of platelet aggregation through various receptors. ADP is one such activator through the receptor P2Y12 which is coupled to inhibitory G-proteins and conciliate ADP induced release of Ca^{2+}. This process inhibits adenylate cyclase and activates the GPIIb/IIIa receptor which leads to platelet aggregation [23].

The effect of *M. zapota* latex on platelet aggregation was studied using PRP. The latex inhibited the aggregation of platelets induced by ADP. The traces of platelet aggregation are shown in Fig. 3A. The inhibition of aggregation of platelets was found to be dose dependent. Agonist ADP alone showed platelet aggregation of 93% while with *M. zapota* latex sample of 4, 8 and 12 µg showed 32%, 16% and 9% of aggregation respectively (Fig. 3B). If the aggregation induced by ADP was considered as 100%, the latex concentrations 4, 8, and 12 µg inhibited the platelet aggregation by 63%, 77% and 86% respectively (Fig. 3C). The free radicals are well known to contribute various diseases including thrombosis, atherosclerosis and cardio vascular diseases. Hyperactivity of platelets is one of the consequences of these disorders [3, 24 - 26]. The platelet aggregation inhibition and anti-oxidant property of *M. zapota* latex could be a promising hope in managing hyperactivity of platelets.



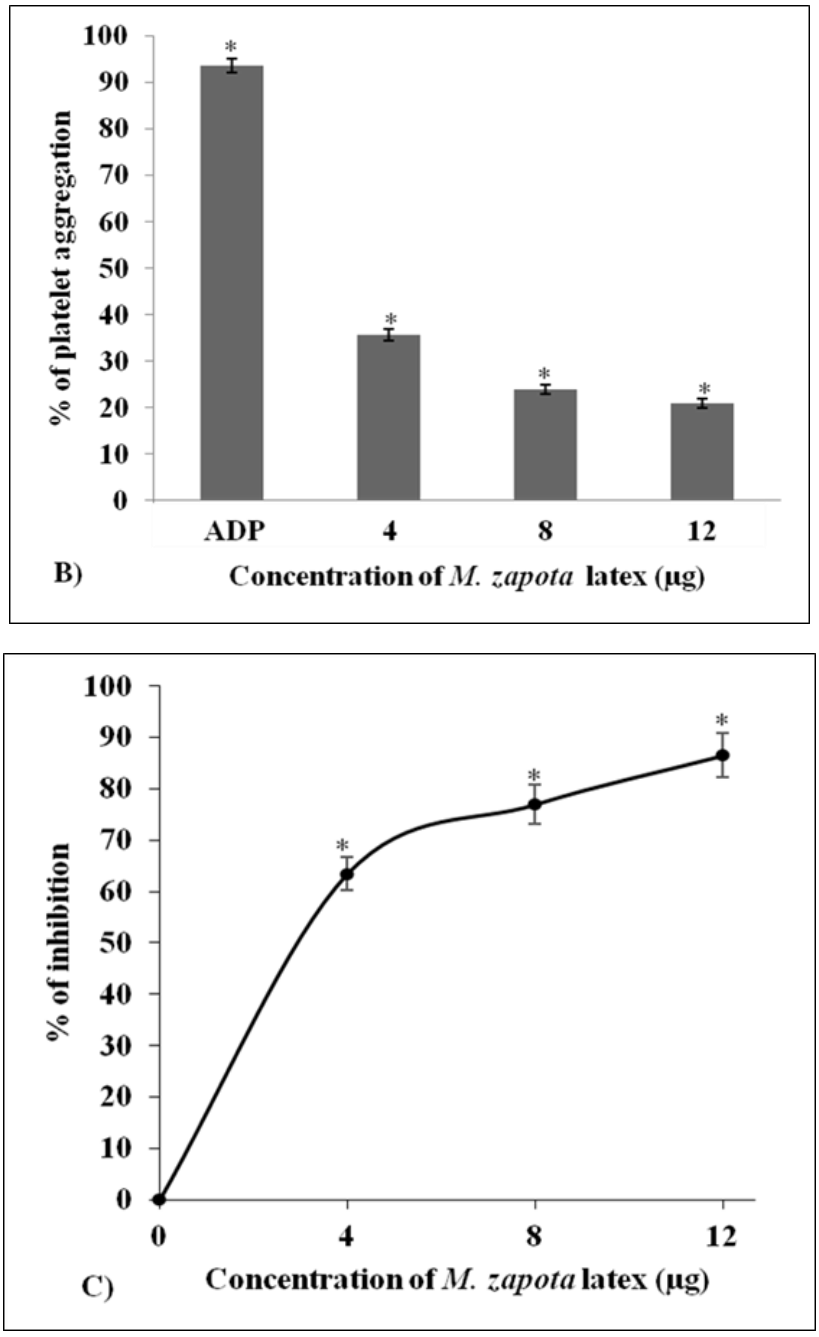


Fig 3: Platelet aggregation A) Traces of platelet aggregation: Trace 1 (ADP 10 µM); trace 2 (ADP 10 µM + 4 µg of *M. zapota* latex); trace 3 (ADP 10 µM + 8 µg of *M. zapota* latex); trace 4 (ADP 10 µM + 12 µg of *M. zapota* latex). B) Concentration dependent platelet aggregation in percent. C) Concentration dependant inhibition of platelet aggregation. Each value presented as mean ± SE of the mean (n=3). Statistically significant results are indicated by asterisks, * – $p < 0.05$. ADP, adenosine diphosphate.

Table 1: Phytocompound analysis by GC-MS.

Retention time	Compound name	Molecular formula	Molecular weight	Peak area (%)
2.27	Phosgene	CCl_2O	98	11.749
3.1	1-(Benzyloxy)-2-methoxy-3-[(E) -2-nitroethenyl]benzene	$C_{16}H_{15}NO_4$	285	30.572
3.5	2,2-Dimethoxybutane	$C_6H_{14}O_2$	118	0.364
4.16	Tetrachloroethylene	C_2Cl_4	164	0.367
5.5	Acetic acid, phenyl(trimethylsiloxy)-, trimethylsilyl ester	$C_{14}H_{24}O_3Si_2$	296	0.9
5.85	Guanine, 1-methyl	$C_6H_7N_5O$	165	0.232
6.7	Nickel, nitrosyl[(1,2,3,4,5-ü)- 1,2,3,4,5-pentamethyl-2, 4-cyclopentadien-1-yl]-	$C_{10}H_{15}NNiO$	223	0.287
7.09	1,3-Cyclopentadiene,5-diazo	$C_5H_4N_2$	92	0.068
7.48	2,7-Diphenyl-1,6-dioxopyridazino[4,5:2',3']pyrrolo[4',5'-d]pyridazine	$C_{20}H_{13}N_5O_2$	355	0.067
7.9	Anodendroside E 2, monoacetate	$C_{32}H_{40}O_{12}$	616	0.090
8.68	Astaxanthin	$C_{40}H_{52}O_4$	596	0.417
10.17	1,2-Benzisothiazol-3-amine tbdms	$C_{13}H_{20}N_2SSi$	264	0.065
10.9	Tetrabutyl orthotitanate	$C_{16}H_{36}O_4Ti$	340	0.027

11.55	2',3'-O-(1-Methylethyliden)-guanosin	$C_{13}H_{17}N_5O_5$	323	0.046
12.18	Octadecanoic acid, 1-[(tetradecyloxy)carbonyl]pentadecyl ester	$C_{48}H_{94}O_4$	734	0.013
13.85	1-Propyl-3,6-diazahomoadamantan-9-ol	$C_{12}H_{22}N_2O$	210	0.015
14.81	1,3,5-Trimethyl-4-n-octadecylcyclohexane	$C_{27}H_{54}$	378	0.015
15.12	Rhodopin	$C_{40}H_{58}O$	554	0.034
15.81	Oleic acid, 3-(octadecyloxy)propyl ester	$C_{39}H_{76}O_3$	592	0.028
16.16	1,1-Di(4-methylcyclohexyl)dodecane	$C_{26}H_{50}$	362	0.30
16.64	Octadecanal, 2-bromo	$C_{18}H_{35}BrO$	346	0.026
16.91	17-Pentatriacontene	$C_{35}H_{70}$	490	0.032
18.72	2-Methylhexadecan-1-ol	$C_{17}H_{36}O$	256	0.073
19.42	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	$C_{19}H_{34}O_2$	294	0.158
20.13	1-Heptatriacotanol	$C_{37}H_{76}O$	536	0.044
21.11	1,1-Bis(dodecyloxy)hexadecane	$C_{40}H_{82}O_2$	594	0.518
24.77	2-Methyl-3-methoxy-6-phenyl-5-oxo-2,5-dihydro-1,2,4-triazine	$C_{11}H_{11}N_3O_2$	217	0.371
26.81	9H-Fluorene-9-methanol, à-phenyl-, acetate	$C_{22}H_{18}O_2$	314	0.364
27.48	4-Benzyloxybenzyl chloride	$C_{14}H_{13}ClO$	232	1.598
27.89	Benzo(c)phenanthren-3-amine	$C_{18}H_{13}N$	243	5.110
29.01	Inositol, 1-deoxy	$C_6H_{12}O_5$	164	2.435
30.32	2-Methylhexadecan-1-ol	$C_{17}H_{36}O$	256	5.966
31.31	i-Propyl 5,9,19-octacosatrienoate	$C_{31}H_{56}O_2$	460	1.159
31.9	7-Isopropyl-4a-methyloctahydro-2(1H)- naphthalenone	$C_{14}H_{24}O$	208	1.408
32.23	17-Pentatriacontene	$C_{35}H_{70}$	490	2.802
32.7	Ingol 12-acetate	$C_{22}H_{32}O_7$	408	0.162
33.96	1-Hexacosene	$C_{26}H_{52}$	364	2.178
35.04	2,6,10-Trimethyltetradecane	$C_{17}H_{36}$	240	5.416
35.47	1-Monolinoleoylglyceroltrimethylsilyl ether	$C_{27}H_{54}O_4Si_2$	498	9.125
37.19	2-Bromooctadecana	$C_{18}H_{35}BrO$	346	1.378
46.78	2,6,10,10-Tetramethyl-1oxaspiro[4.5]decan-6-ol	$C_{13}H_{24}O_2$	212	5.036
48.86	9,12,15-Octadecatrienoic acid, 2-phenyl-1,3-dioxan-5-yl ester	$C_{28}H_{40}O_4$	440	1.606

Table 2: Anti-oxidant activity by TAA and FRAP methods. Each value presented as mean±SE of the mean (n=3). Statistically significant results are indicated by asterisks, * – $p < 0.05$. TAA, total antioxidant activity; FRAP, Ferric reducing anti-oxidant power.

Concentration of *M. zapota* (µg/ml)	TAA (mg GAE)	FRAP (mg GAE)
50	6 ± 0.7*	25 ± 0.6*
100	12 ± 1.6*	38 ± 2*
150	24 ± 1.4*	55 ± 1.3*
200	35 ± 1.3*	62 ± 2.1*
250	44 ± 2.3*	70 ± 2.5*
300	60 ± 2.2*	83 ± 2.6*

4. Conclusion

Herein, we have evaluated chemical profile of *M. zapota* latex. The pharmacological properties like anti-oxidant and anti-platelet activities seem to be interesting. Further, isolating pure compound responsible for these activities could help in managing hyperactivity of platelets in thrombotic disorders.

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