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Phytochemical, Antioxidant, Antimicrobial, Thrombolytic as well as Cytotoxic Studies on the Stem Bark of *Manilkara zapota* (Sapotaceae)

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A total of seven compounds were isolated from the methanolic extract of the stem bark of *Manilkara zapota* (Family:Sapotaceae) and their structures were elucidated as chondrillasterol (**1**), stigmasterol (**2**), β-sitosterol (**3**), lupeol (**4**), lupenone (**5**), glut-5(6)-en-3β-acetate (**6**) and olean-12-en-3β-acetyl-11-one (**7**) by ^{1}H NMR spectra and confirmed by comparison with previously reported data. Among these chondrillasterol, glut-5(6)-en-3β-acetate and olean-12-en-3β-acetyl-11-one had been isolated for the first time from this plant. Different fractions of crude methanolic extract were also studied for antioxidant, antimicrobial, thrombolytic and cytotoxic activities. The methanolic extract of stem bark of *M. zapota* showed the significant free radical scavenging activity with IC_{50} value 1 µg/mL. Antimicrobial activity was carried out against six Gram-positive and eight Gram-negative bacteria using ciprofloxacin as standard. The maximum zone of inhibition produced by methanolic extract and its aqueous fraction was found to be 13 mm against *Sarcinalutea* and 17 mm against *Serratia* species respectively.

Keywords: *Manilkara zapota*, Chondrillasterol, Stigmasterol, β-Sitosterol, Lupeol, Lupenone, Glut-5(6)-en-3β-acetate.

INTRODUCTION

Manilkara zapota belongs to the family Sapotaceae, commonly known as sapodilla (sofeda/sobeda) is an evergreen, glabrous tree, one of the most interesting and desirable of all tropical fruit trees. The Sapodilla is believed native to southern Mexico, Central America and the Caribbean. It is grown in huge quantity in India, Thailand, Malaysia, Cambodia, Indonesia, Bangladesh and Mexico. Therapeutic value of *Manilkara zapota* has been recognized in different systems of traditional medication for the treatment of different types of disease and ailment of human being. However, scientific validation is still in high demand. This research aimed to phytochemically characterize the methanolic extract prepared and to determine if any significant biological properties were present. Successive chromatographic separation and purification of methanolic extract of stem bark of *Manilkara zapota* revealed a total of seven compounds namely chondrillasterol, stigmasterol, β-sitosterol, lupeol, lupenone, glut-5(6)-en-3β-acetate and olean-12-en-3β-acetyl-11-one. Previous phytochemical studies[1-3] revealed that the plant contains terpenoids, flavonoids, cynogenic glycosides, phenolics, reducing sugar, tannins, steroid, saponins and gum of compounds. Lupeol acetate, oleanolic acid, apigenin-7-O-α-L-rhamnoside, myricetin-3-O-α-L-rhamnoside and caffeic acid were identified from the leaves of *Manilkara zapota* cultivated in Egypt[4].

EXPERIMENTAL

The powdered stem bark of *Manilkara zapota* was extracted with methanol. The concentrated extract was then fractionated with vacuum liquid chromatography (VLC) using petroleum ether, ethyl acetate and methanol. Depending on the thin layer chromatography (TLC) behavior, various vacuum liquid chromatography fractions were subjected to gel permeation chromatography (GPC) using sephadex LH-20 for further fractionation. Finally the compounds were separated and purified from different column fractions of Gel permeation chromatography with preparative thin layer chromatography (PTLC). The ^{1}H NMR spectra of isolated compounds were obtained using a Varian Unity 500 spectrometer (500 MHz) instrument in $CDCl_3$.

The stem bark of *Manilkara zapota* was collected in the month of December, 2012, from Khulna, Bangladesh. A voucher specimen had been maintained in the National herbarium of

Bangladesh under the accession number 38580. The samples were cut into small pieces and sun dried for several days followed by oven drying for 24 h at 40 °C to facilitate grinding.

Extraction and isolation: 1250 g of the powdered stem bark was soaked in 4.5 L of methanol and kept for a period of 45 days accompanying occasional shaking and stirring. The whole mixture was then filtered through a fresh cotton plug and finally with a Whatman No.1 filter paper. The volume of the filtrate was then reduced using a rotary evaporator at low temperature and pressure. The weight of the crude extract was 67 g. 18 g of which was subjected to vacuum liquid chromatography for the initial rapid fractionation. The column was packed with fine vacuum liquid chromatography (VLC) grade silica (Kiesel gel 60H) under vacuum through which solvent systems were passed with progressively increasing polarity. Vacuum liquid chromatography column was first eluted with 100 % petroleum ether, followed by mixtures of petroleum ether and ethyl acetate and finally washed by methanol. Various vacuum liquid chromatography fractions were then subjected to gel permeation chromatography where the column was eluted with the mixtures of petroleum ether and chloroform; and finally with the mixtures of chloroform and methanol with increasing polarity.

An aliquot (5 g) of the crude methanolic extract was fractionated by the modified Kupchan partitioning method[5] for biological activity where ME = methanolic extract of stem bark of *Manilkara zapota*, PE = pet-ether soluble fraction of the methanolic extract of stem bark of *M. zapota*, CL4 = carbontetrachloride soluble fraction of the methanolic extract of stem bark of *M. zapota*, CL = chloroform soluble fraction of the methanolic extract of stem bark of *M. zapota*, AQ = aqueous soluble fraction of the methanolic extract of stem bark of *M. zapota*.

Compound 1: (2.1 mg), white crystal; isolated from vacuum liquid chromatography fraction that was eluted with 20 % EtOAc in petroleum ether. It was visualized as a dark quenching band under UV light (254 nm) on thin layer chromatography (TLC) plate and showed a purple spot after spraying with vanillin-sulfuric acid reagent followed by heating at 110 °C for 2 min. The ^{1}H NMR (500 MHz, $CDCl_3$): δ3.57 (1H, m, H-3), 5.12 (1H, bd, H-7),0.53 (3H, s, H_3-18), 0.77 (3H, s, H_3-19), 1.00 (3H, d, *J* = 6.5 Hz, H_3-21), 5.00 (1H, dd, *J* = 15.5, 9.0 Hz, H-22), 5.13 (1H, dd, *J* = 15.0, 9.0 Hz, H-23), 0.82 (3H, d, *J* = 6.0 Hz, H_3-26), 0.78 (3H, d, *J* = 7.5 Hz, H_3-27), 0.78 (3H, t, *J* = 7.5 Hz, H_3-29).

Compound 2: (6.1 mg), white crystal; isolated from vacuum liquid chromatography fraction that was eluted with 20 % EtOAc in petroleum ether. It was visualized as a dark quenching band under UV light (254 nm) on thin layer chromatography (TLC) plate and showed a purple spot after spraying with vanillin-sulfuric acid reagent followed by heating at 110 °C for 2 min. The ^{1}H NMR (500 MHz, $CDCl_3$): δ3.51 (1H, m, H-3), 5.35 (1H, br s, H-6), 0.66 (3H, s, H_3-18), 0.98 (3H, s, H_3-19), 1.00 (3H, d, H_3-21), 5.00 (1H, dd, H-22), 5.13 (1H, dd, H-23), 0.82 (3H, d, H_3-26), 0.78 (3H, d, H_3-27), 0.78 (3H, t, H_3-29).

Compound 3: (1 mg), white crystal; isolated from vacuum liquid chromatography fraction that was eluted with 50 % EtOAc in petroleum ether. It showed blue fluorescence under UV light (366 nm) on thin layer chromatography plate and no color was developed after spraying with vanillin-sulfuric acid reagent followed by heating at 110 °C for 2 min. The ^{1}H NMR (500 MHz, $CDCl_3$): δ3.51 (1H, m, H-3), 5.33 (1H, dd, *J* = 4.5, 0.5 Hz, H-6), 0.66 (3H, s, H_3-18), 0.99 (3H, s, H_3-19), 0.91 (3H, d, *J* = 7.0 Hz, H_3-21), 0.79 (3H, d, *J* = 7.0 Hz, H_3-26), 0.81 (3H, d, *J* = 7.0 Hz, H_3-27), 0.82 (3H, dd, *J* = 8.5, 7.5 Hz, H3-29).

Compound 4: (5.8 mg), white crystal; isolated from vacuum liquid chromatography fractions that were eluted with 12 and 15 % EtOAc in petroleum ether. On thin layer chromatography plates a dark quenching band was visualized under UV light (254 nm) which appeared as a purple spot after spraying with vanillin-sulfuric acid reagent followed by heating at 110 °C for 2 min. The ^{1}H NMR (500 MHz, $CDCl_3$): δ3.20 (1H, m, H-3), 0.97 (3H, s, H_3-23), 0.71 (3H, s, H_3-24), 0.85 (3H, s, H_3-25), 1.02 (3H, s, H_3-26), 0.88 (3H, s, H_3-27), 0.83 (3H, s, H_3-28), 4.54 (1H, br. s, H-29), 4.66 (1H, br. s, H-29), 1.65 (3H, s, H_3-30).

Compound 5: (7.4 mg), white crystal; isolated from vacuum liquid chromatography fraction that was eluted with 12 % EtOAc in petroleum ether. It showed blue fluorescence under UV light (366 nm) on thin layer chromatography plate and showed a violet spot after spraying with vanillin-sulfuric acid reagent followed by heating at 110 °C for 2 min. The ^{1}H NMR (500 MHz, $CDCl_3$): δ1.00 (3H, s, H_3-23), 0.77 (3H, s, H_3-24), 0.90 (3H, s, H_3-25), 1.04 (3H, s, H_3-26), 0.93 (3H, s, H_3-27), 0.85 (3H, s, H_3-28), 4.54 (1H, br. S, H-29), 4.66 (1H, br. s, H-29), 1.65 (3H, s, H_3-30).

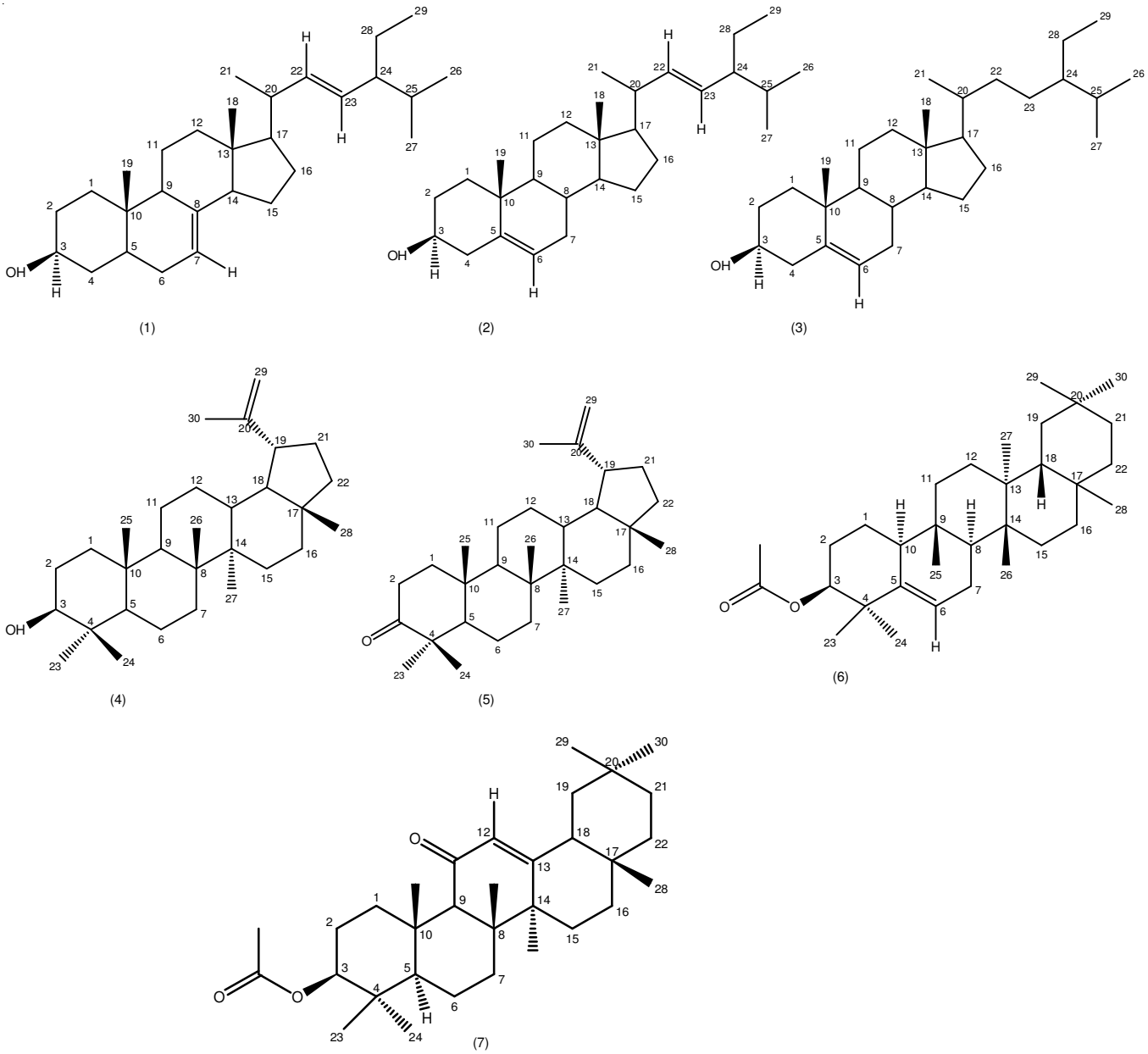
Compound 6: (6.8 mg), white crystal; isolated from vacuum liquid chromatography fraction that was eluted with 12 % EtOAc in petroleum ether. It was visualized as a dark quenching band under UV light (254 nm) on thin layer chromatography plate and showed a violet spot after spraying with vanillin-sulfuric acid reagent followed by heating at 110 °C for 2 min. The ^{1}H NMR (500 MHz, $CDCl_3$): δ4.48 (1H, m, H-3), 5.55 (1H, bs, H-6), 0.78 (3H, s), 0.85 (3H, s), 0.87 (3H, s), 0.91 (3H, s), 1.10 (3H, s), 1.12 (3H, s), 1.13 (3H, s), 1.16 (3H, s).

Compound 7: (6.8 mg), white crystal; isolated from vacuum liquid chromatography (VLC) fraction that was eluted with 12 % EtOAc in petroleum ether. It was visualized as a dark quenching band under UV light (254 nm) on thin layer chromatography plate and showed a violet spot after spraying with vanillin-sulfuric acid reagent followed by heating at 110 °C for 2 min. The ^{1}H NMR (500 MHz, $CDCl_3$): δ4.48 (1H, m, H-3), 5.51 (1H, bs, H-12), 0.78 (3H, s), 0.85 (3H, s), 0.87 (3H, s), 0.91 (3H, s), 1.10 (3H, s), 1.12 (3H, s), 1.13 (3H, s), 1.16 (3H, s).

RESULTS AND DISCUSSION

Repeated chromatographic separation and purification of the methanol extract of the stem bark of *Manilkara zapota* provided seven compounds (**1-7**). The structures of isolated compounds were solved by extensive NMR data analysis and comparison with published values.

Compound 1: ^{1}H NMR spectrum of compound **1** revealed a one proton multiplet at δ 3.57, the position and multiplicity of which was indicative of Hα-3 of the steroidal nucleus. The



signal for the olefinic H-7 of the steroidal skeleton was evident from a broad doublet at δ 5.12 integrating one proton. The spectrum further revealed two three proton singlets at δ 0.53 and δ 0.77 assignable to methyl groups attached to C-13 and C-10 (H_3-18 and H_3-19). The ^{1}H NMR spectrum also showed three doublets at δ 1 (J = 6.5 Hz), δ 0.82 (J = 6 Hz) and δ 0.78 (J = 7.5 Hz) that integrated for three protons, could be assigned to the primary methyl groups at C-20 and C-25 (H_3-21, H_3-26, H_3-27). Two double doublet centered at δ 5.00 (J = 15.5, 9 Hz) and δ 5.13 (J = 15, 9 Hz) was demonstrative of two protons at C-22 and C-23. The spectrum further revealed a three proton triplet at δ 0.78 (J = 7.5 Hz) assignable to methyl group at C-28 (H_3-29).

These spectral features were in close agreement to those observed for chondrillasterol[6]. On this basis, identity of compound **1** was confirmed as chondrillasterol. Although it is a known natural product, this is the first time isolation from this species.

Compound 2: ^{1}H NMR spectrum of compound **2** revealed a broad singlet at δ 5.35 integrating one proton which was indicative of the olefinic H-6 of the steroidal skeleton. One proton multiplet at δ 3.51 was indicative of Hα-3 and three proton singlets could be assigned to the methyl groups attached to the steroidal nucleus. The spectrum further revealed two double doublets at δ 5.00 and δ 5.13 (1H each) was demonstrative of two protons at C-22 and C-23, three doublets at δ 1.00, δ 0.82 and δ 0.78 (3H each) could be assigned to the primary methyl groups at C-20 and C-25 as well as a three proton triplet at δ 0.78 assignable to methyl group at C-28.

These spectral features when compared to those of stigmasterol[7] were found to be identical. On this basis the identity of compound 2 was assigned as stigmasterol.

Compound 3: ^{1}H NMR spectrum of compound 3 showed the presence of a proton corresponding to the proton connected to the C-3 hydroxyl group of the steroidal nucleus which appearedasa multiplet at δ 3.51. A double doublet at δ 5.33 (*J*

= 4.5, 0.5 Hz) integrating one proton was indicative of a proton corresponding to that of a trisubstituted olefinic bond (H-6) of the steroidal skeleton. The spectrum also revealed two methyl singlets at δ 0.66 and δ 0.99 assignable to methyl groups attached to C-13 and C-10 (H_3-18 and H_3-19) of the steroidal skeleton. The ^{1}H NMR spectrum further revealed three doublets at δ 0.91 (J = 7 Hz), δ 0.79 (J = 7 Hz) and δ 0.81 (J = 7 Hz) that integrated for three protons, could be assigned to the primary methyl groups at C-20 and C-25 (H_3-21, H_3-26, H_3-27). Another double doublet at δ 0.82 (J = 8.5, 7.5 Hz) integrating three protons assignable to a methyl group at C-28 (H_3-29).

The above spectral features were similar to those observed for β-sitosterol[7]. On this basis, together with the absence of protons corresponding to the double bond between C-22/C-23 in the ^{1}H NMR spectrum of compound **3**, its identity was confirmed as β-sitosterol.

Compound 4: ^{1}H NMR spectrum of compound **4** showed seven singlets at δ 0.97, 0.71, 1.02, 0.85, 0.88, 0.83 and 1.65 each integrated for three protons, assignable to the tertiary methyl group protons at C-4 (H_3-23, H_3-24), C-8 (H_3-26), C-10 (H_3-25), C-14 (H_3-27), C-17 (H_3-28) and C-20 (H_3-30), respectively. The spectrum also showed a multiplet at δ 3.20 integrating one proton which is typical for an α (alpha) proton at C-3 of a triterpene skeleton. The position and splitting pattern of this proton confirmed the β (beta) orientation of the C-3 secondary hydroxyl group. The ^{1}H NMR spectrum further showed the presence of two olefinic protons appeared as broad singlets (1H each) at δ 4.54 and δ 4.66, representing the exocyclic double bond protons at C-29.

The above spectral features were similar to those observed for lupeol[8]. On this basis the identity of compound **4** was confirmed as lupeol.

Compound 5: ^{1}H NMR spectrum of compound **5** showed seven singlets at δ 1.00, 0.77, 1.04, 0.90, 0.93, 0.85 and 1.65 each integrated for three protons, assignable to the tertiary methyl group protons at C-4 (H_3-23, H_3-24), C-8 (H_3-26), C-10 (H_3-25), C-14 (H_3-27), C-17 (H_3-28) and C-20 (H_3-30), respectively as observed for compound **4**. Like compound **4** the spectrum of compound **5** also revealed two broad singlets (1H each) at δ 4.54 and δ 4.66, representing two exocyclic olefinic protons at C-29.

The above spectral features were indicative of lupane skeleton. On this basis, together with the absence of a hydroxyl group unlike compound **4**, the identity of compound **5** was confirmed as the known compound lupenone[9].

Compound 6: ^{1}H NMR spectrum of compound **6** showed eight three proton singlets at δ 0.78, 0.85, 0.87, 0.91, 1.10, 1.12, 1.13 and 1.16. These were attributed to the methyl group protons at C-4 (H_3-23, H_3-24), C-9 (H_3-25), C-13 (H_3-27), C-14 (H_3-26), C-17 (H_3-28) and C-20 (H_3-29, H_3-30). The spectrum also revealed a one proton broad singlet at δ 5.55 assignable to the olefinic proton at C-6. A multiplet centered at δ 4.48 integrating one proton could be assigned to the proton at C-3 attached to the acetyl group.

^{1}H NMR signals suggested the presence of a typical pentacyclictriterpene skeleton. This was identified as glut-5(6)-en-3β-acetate by the comparison of these data with those of glut-5(6)-en-3β-ol[10].

Compound 7: ^{1}H NMR spectrum of compound **7** showed eight three proton singlets at δ 0.78, 0.85, 0.87, 0.91, 1.10, 1.12, 1.13 and 1.16 like compound **6**. These were attributed to the methyl group protons at C-4 (H_3-23, H_3-24), C-8 (H_3-26), C-10 (H_3-25), C-14 (H_3-27), C-17 (H_3-28) and C-20 (H_3-29, H_3-30). A multiplet centered at δ 4.48 integrating one proton could be assigned to the proton at C-3 attached to the acetyl group. The spectrum further revealed a broad singlet centered at δ 5.51 integrating one proton was a characteristic peak of a proton corresponding to the olefinic proton at the carbon atom (C-12) which directly attached to a ketone group.

^{1}H NMR signals suggested the presence of another pentacyclictriterpene skeleton which was identified as olean-12-en-3β-acetyl-11-one by comparing these data with those of β-amyrin acetate[11].

Antioxidant activity

DPPH assay[12]: In free radical scavenging activity (antioxidant capacity), the methanolic extract (ME), chloroform soluble fraction (CL) and aqueous soluble fraction (AQ) showed good free radical scavenging activity compared to the standard. The methanolic extract of stem barks of *Manilkara zapota* showed the significant free radical scavenging activity with IC_{50} value 1 µg/mL (Table-1).

TABLE-1
IC_{50} VALUES OF THE STANDARD AND PARTITIONATES OF *Manilkara zapota*

Sample code	IC_{50}value
BHT	27.5
ME	1
PE	160
CL	6
CL4	40
AQ	2

BHT = *tert*-Butyl-1-hydroxytoluene

Brine shrimp lethality bioassay[13,14]: In Brine shrimp lethality bioassay of CL and CL4 showed significant lethality whereas PE showed moderate activity while ME and AQ showed mild activity (Table-2).

TABLE-2
LC_{50} VALUES OF THE TEST SAMPLES OF *Manilkara zapota*

Test samples	LC_{50} (µg/mL)
VS	0.37
PE	18.91
CL4	8.68
CL	3.8
ME	236.18
AQ	88.41

VS = Vincristine sulphate

Antimicrobial screening: disc diffusion method[15]: The methanolic extract of the bark of *Manilkara zapota* (ME) and its aqueous soluble fraction (AQ) exhibited the highest inhibition against microbial growth (Table-3).

Thrombolytic activity[16]: From this experiment, it can be concluded that the extractives of stem bark of *Manilkara zapota* showed mild to moderate clot lysis activity compared to the standard substance streptokinase (SK) except the aqueous

TABLE-3
ANTIMICROBIAL ACTIVITY OF TEST SAMPLES OF *Manilkara zapota*

Test microorganisms	Diameter of zone of inhibition (mm)					
	ME	PE	CL4	CL	AQ	Ciprofloxacin
Gram-positive bacteria						
Bacillus subtilis	10	-	6	11	16	39
Bacillus megaterium	7	-	7	12	8	28
Bacillus cereus	8	-	7	13	9	42
Staphylococcus aureu	10	-	-	8	14	36
Sarcinalutea	13	-	-	-	13	33
Bacillus polymyxa	12	7	-	10	13	43
Gram-negative bacteria						
Shigella flexneri	12	-	-	-	-	39
Shigella boydii	-	-	-	7	-	26
Escherichia coli	-	-	-	-	-	50
Shigella dysenteriae	12	-	-	-	12	60
Salmonella B.	10	-	-	-	16	36
Klebsiella species	10	-	-	-	12	14
Shigella sonnei	12	-	-	7	13	37
Serratia species	-	-	-	-	17	51

soluble fraction (AQ), exhibiting very poor (10.46 %) thrombolytic activity compared to streptokinase (65.16 %) (Table-4).

TABLE-4
THROMBOLYTIC ACTIVITY (IN TERMS OF % OF CLOT LYSIS) OF THE EXTRACTIVES OF *M. zapota*

Fractions	% of thrombolysis
Blank	8.20
ME	57.58
PE	27.49
CL4	20.596
CL	57.53
AQ	10.46
Streptokinase	65.16

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