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Phytochemical and Biological Investigations of Methanol Extract of Leaves of *Ziziphus mauritiana* Lam.

[Investigaciones fitoquímicas y biológicas de extractos metanólicos de hojas de *Ziziphus mauritiana* Lam.]

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Abstract: Seven compounds were isolated from the methanol extract of leaves of *Ziziphus mauritiana*. The structures of the isolated compounds were elucidated as γ-fagarine (1), β-sitosterol (2), stigmasterol (3), α-amyrin (4), lupeol (5), β-amyrin (6) and betulin (7) by extensive spectroscopic studies and by co-TLC with authentic samples. This is the first report of isolation of compounds 1-7 from this plant species. The extractives from leaves were investigated for thrombolytic, membrane stabilizing, antimicrobial, antidiarrheal and analgesic potentials along with sleep inducing property on animal model. The thrombolytic and membrane stabilizing activities were assessed by using human erythrocyte comparing with standard streptokinase and acetylsalicylic acid, respectively. The methanolic extract reduced frequency of diarrheal faeces when compared to the standard loperamide. In castor oil induced diarrhea in mice a dose dependent decrease of gastrointestinal transits were observed. In radiant heat tail-flick method the methanol extract showed moderate antinociceptive activity, compared to standard morphine.

Keywords: *Ziziphus mauritiana*, triterpene, thrombolytic, membrane stabilizing, antimicrobial, antidiarrheal.

Resumen: Siete compuestos fueron aislados del extracto metanólico de hojas de *Ziziphus mauritiana*. Las estructuras de los compuestos aislados fueron identificadas como γ-fagarina (1), β-sitosterol (2), estigmasterol (3), α-amirina (4), lupeol (5), β-amirina (6) y betulina (7) por medio de extensivos estudios espectroscópicos y por co-TLC con muestras auténticas. Este es el primer informe de aislamiento de los compuestos 1-7 a partir de esta especie vegetal. Los extractos de las hojas fueron investigados por efectos trombolíticos, estabilizadores de membrana, antimicrobianos, antidiarreicos y potenciales analgésicos junto a la propiedad de inducir sueño en el modelo animal. Las actividades trombolíticos y la estabilización de la membrana se evaluaron mediante el uso de eritrocitos humanos comparando con estreptoquinasa estándar y el ácido acetilsalicílico, respectivamente. El extracto metanólico redujo la frecuencia de las heces diarreicas en comparación con el estándar de loperamida. En la inducción por aceite de ricino de diarrea en ratones, se observó una disminución dosis dependiente de los tránsitos gastrointestinales. En el método del coletazo producido por calor radiante, el extracto metabólico mostró una actividad antinociceptiva moderada, en comparación con la morfina estándar.

Palabras clave: *Ziziphus mauritiana*, triterpenos, trombolíticos, estabilizadora de membrana, antimicrobianos, antidiarreicos.

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INTRODUCTION

Ziziphus mauritiana Lam. (Bangla: Boroi or Kul) is a fruit tree belonging to the family Rhamnaceae. *Z. mauritiana* has folkloric implications against many ailments and diseases. Traditionally, the fruit has been used as anodyne, sedative, tonic anticancer, potent wound healer and has also been used against asthma (Morton & Miami, 1987; Verheij & Coronel, 1991). Different parts of the plant are used in cuts and ulcers, pulmonary ailments, fevers, constipation, nausea, rheumatism, wounds and abscesses, swelling, gonorrhea (Michel, 2002). The extracts from fruits (Ndhala *et al.*, 2006), leaves (Dahiru & Obidoa, 2007; Dahiru *et al.*, 2005), and seeds (Bhatia & Mishra, 2009) of *Z. mauritiana* have been reported to exhibit antioxidant activity, whereas bark (Pisha *et al.*, 1995; Ramadoss *et al.*, 2000) and pulp (Vahedi *et al.*, 2008) are reported to possess cytotoxicity against different cancer cell lines. Extensive phytochemical investigations of Ziziphus species have proven it to be a rich source of many chemically unique and biologically interesting compounds. Cyclopeptide alkaloids mauritines A and B (Tschesche et al., 1972a), C-F (Tschesche et al., 1974), G and H (Tschesche et al., 1977), J (Jossang et al., 1996), K (Singh et al., 2007), L-M (Panseeta et al., 2011), frangufoline (Tschesche et al., 1972a), amphibines D (Tschesche et al., 1972b), E (Tschesche et al., 1974; 1972b), B and F (Tschesche et al., 1974), sativanine K (Singh et al., 2007), nummularines H and B (Panseeta et al., 2011), hemsine A (Panseeta et al., 2011), sedative flavonoids such as swertish and spinosin, puerarin (6'''-feruloylspinosin), apigenin-6-C-□-D-glucopyranoside, 6'''- feruloylisospinosin, isospinosin and isovitexin-2''-O-b-D-glucopyranoside (Cheng et al., 2000) have previously been reported from Z. mauritiana. Hysodricanin-A (Tschesche et al., 1977), lupane and ceanothane triterpenes (Suksamrarn et al., 2006) were also reported from the genus Ziziphus. Due to low natural abundance and unique structural features mauritines A-C and F (Cristau et al., 2005), amphibine E and their epimers (Kim et al., 2003; Joullie and Richard, 2004) and frangufoline (Xiao et al., 1998) have been synthesized. Some of these cyclopeptide macrocycles exhibited sedative (Han et al., 1989), analgesic (Trevisan et al., 2009), antibacterial (Morel et al., 2002), antifungal (Pandy and Devi, 1990), immunostimulant (Lin et al., 2000), antiplasmodial and antimycobacterial (Suksamrarn et al., 2005; Panseeta et al., 2011) activities.

Keeping above points as well as part of our continuing studies on medicinal plants of Bangladesh in view, the present study was undertaken and we herein, report γ-fagarine (**1**), β-sitosterol (**2**), stigmasterol (**3**), α-amyrin (**4**), lupeol (**5**), β-amyrin (**6**) and betulin (**7**) for the first time. The results of thrombolytic, membrane stabilizing, antimicrobial, antidiarrheal and analgesic potentials along with sleep inducing property of leaves of *Z. mauritiana* have also been reported.

MATERIALS AND METHODS

General Experimental Procedures

^{1}H NMR spectra were recorded by using a Bruker AMX-500 (500 MHz) instrument in $CDCl_3$ and the δ values for ^{1}H data were referenced to the residual nondeuterated solvent signal.

Plant materials

The leaves of *Z. mauritiana* were collected from Dhaka in October 2011. It was identified by Prof. M.A. Hassan at the Department of Botany, University of Dhaka, where a voucher specimen has been maintained in the herbarium of (Accession number- 6452) for future reference.

Extraction and Isolation

The leaves were sun dried for several days and then oven dried for 24 hours at considerably low temperature (below 40 ºC) to facilitate grinding. The powdered material of *Z. mauritiana* (500 g) was macerated in 2.0 L of methanol for 15 days and then filtered through a cotton plug followed by Whatman filter paper number 1. The extract was concentrated with a rotary evaporator at low temperature (40 - 45 ºC) and reduced pressure. The concentrated methanol extract (ME) was partitioned by the modified Kupchan method (Van Wagenen *et al.*, 1993) and the resultant partitionates i.e., petroleum ether (PESF, 0.85 g), carbon tetrachloride (CTCSF, 0.95 g), chloroform (CSF, 0.80 g), and aqueous (AQSF, 1.60 g) soluble fractions were used for the experiment.

The petroleum ether, carbon tetrachloride and chloroform soluble partitionates were separately chromatographed over silica gel (Kiesel gel 60H, mesh 70 - 230) and the columns were eluted with petroleum ether followed by mixtures of petroleum ether and ethyl acetate in order of increasing polarities. Successive chromatographic separation and purification of the carbon tetrachloride soluble partitionate yielded two compound **1**, while the

chloroform soluble material afforded β-sitisterol (**2**). Similar treatment of the petroleum ether soluble partitionate provided compounds **4**, **5**, **6** and **7**.

Spectroscopic properties of compounds

γ-Fagarine (**1**); 6 mg, 0.0063% yield; amorphous powder; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.61 (1H, d, *J* = 2.0 Hz, H-2), 7.04 (1H, d, *J*= 2.0 Hz, H-3), 4.41 (3H, s, OMe-4), 7.82 (1H, br. d, *J*= 8.5 Hz, H-5), 7.33 (1H, t, *J*= 8.5 Hz, H-6), 7.03 (1H, br. d, *J*= 8.5 Hz, H-7), 4.05 (3H, s, OMe-8).

β-sitosterol (**2**); 10 mg, 0.012% yield; colorless crystalline mass; ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.35 (1H, d, *J* = 5.2 Hz, H-6), 3.51 (1H, m, H-3), 1.00 (3H, s, H_3-19), 0.92 (3H, d, *J* = 6.4 Hz, H_3-21), 0.85 (3H, m, H_3-29), 0.83 (3H, d, *J* = 7.0 Hz, H_3-26), 0.81 (3H, d, *J* = 7.0 Hz, H_3-27), 0.67 (3H, s, H_3-18).

Stigmasterol (**3**); 8 mg, 0.0094% yield; White power; ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.34 (1H, m, H-6), 5.12 (1H, dd, *J* = 15.0, 6.5 Hz, H-22), 5.01 (1H, dd, *J* = 15.0, 9.0 Hz, H-23), 3.52 (1H, m, H-3), 1.00 (3H, s, H_3-19), 0.92 (3H, d, *J* = 6.0 Hz, H_3-21), 0.83 (3H, d, *J* = 6.0 Hz, H_3-26), 0.85 (3H, t, *J* = 6.5 Hz, H_3-29), 0.80 (3H, d, *J* = 6.0 Hz, H_3-27), 0.67 (3H, s, H_3-18).

α-Amyrin (**4**); 4 mg, 0.0047% yield; white gum; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.23 (1H, dd, J = 11.0, 5.0, H-3), 5.29 (1H, m, H-12), 0.82 (3H, s, H_3-23), 0.79 (3H, s, H_3-24), 0.96 (3H, s, H_3-25), 1.01 (3H, s, H_3-26), 1.07 (3H, s, H_3-27), 0.96 (3H, s, H_3-28), 0.88 (3H, s, H_3-29), 0.91 (3H, s, H_3-30).

Lupeol (**5**); 5 mg, 0.0058% yield; gummy mass; ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.68 (1H, br. s, H_a -29), 4.56 (1H, s, H_b-29), 3.19 (1H, dd, J = 11.00, 4.76 Hz, H-3), 1.04 (1H, m, H-12), 0.96 (3H, s, H_3-23), 0.74 (3H, s, H_3-24), 0.82 (3H, s, H_3-25), 1.02 (3H, s, H_3-26), 0.94 (3H, s, H_3-27), 0.78 (3H, s, H_3-28), 1.67 (3H, s, H_3-30).

β-Amyrin (**6**); 6 mg, 0.0069% yield; amorphous powder; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.18 (1H, dd, = 10.5, 4.7 Hz, H-3), 5.17 (1H, m, H-12), 0.93 (3H, s, H_3-23), 0.92 (3H, s, H_3-24), 0.98 (3H, s, H_3-25), 0.98 (3H, s, H_3-26), 1.07 (3H, s, H_3-27), 0.86 (3H, s, H_3-28), 0.89 (3H, s, H_3-29), 0.89 (3H, s, H_3-30).

Betulin (**7**); 7 mg, 0.0081% yield; white crystal; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.12 (1H, dd, J = 10.8, 4.4 Hz, H-3), 2.90 (1H, m, H-19), 0.78 (3H, s, H_3-23), 0.82 (3H, s, H_3-24), 0.94 (3H, s, H_3-25), 0.96 (3H, s, H_3-26), 1.02 (3H, s, H_3-27), 3.25 (1H, d, J = 10.8 Hz, H_a-28), 3.28 (1H, d, J = 10.8 Hz, H_b-28), 4.68 (1H, br. s, H_a -29), 4.56 (1H, s, H_b-29), 1.67 (3H, s, H_3-30).

Animals

Healthy Long Evan's rats 4-5 months of age, weighing between 170-220 g and Swiss-albino mice of either sex, aged 4-5 weeks, were used for the experiment. The animals were bought from the Animal Resources Branch of the International Centre for Diarrhoeal Diseases and Research, Bangladesh (ICDDR, B). They were kept in standard environmental condition and fed with ICDDR, B formulated rodent food and water. They were housed individually in cages and were kept at constant room temperature (25.0 ± 3.0 ºC), humidity 35-60% and 12 hours light and 12 hours dark cycle to get them adapted with the new environment of the laboratory, before being employed in any experiment (Hawk *et al.*, 1954). All the studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals and the protocol was approved by the Institutional Ethics Committee. All the functions were performed according to guidelines and all efforts were made to minimize sufferings. Following the guidelines of Bangladesh Medical Research Council (BMRC) an Institutional Ethics Review Committee has provisionally been formulated at the State University of Bangladesh (SUB), where all the biological studies were conducted as per their guidelines.

Streptokinase (SK)

Commercially available lyophilized Altepase (Streptokinase) vial (Beacon pharmaceutical Ltd.) of 15,00,000 I.U., was collected and 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100 µl (30,000 I.U) was used for *in vitro* thrombolysis.

Blood sample

Blood (n = 5) was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy and 1ml of blood was transferred to the previously weighed microcentrifuge tubes and was allowed to form clots.

Thrombolytic activity

The thrombolytic activity of all extracts was evaluated by the method developed by Daginawala *et al.* (2006) and slightly modified by Kawsar *et al.* (2011) using streptokinase (SK) as the standard. .

Different extractives (100 mg each) were suspended in 10 ml of distilled water and were kept overnight. Then the soluble supernatant was decanted and filtered. Aliquots (5 ml) of venous blood were drawn from healthy volunteers which were distributed in five different pre-weighed sterile micro centrifuge tube (1 ml/tube) and incubated at 37 °C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone).

To each microcentrifuge tube containing pre-weighed clot, 100 µl aqueous solution of different partitionates along with the crude extract was added separately. As a positive control, 100 µl of streptokinase (SK) and as a negative non thrombolytic control, 100 µl of distilled water, were separately added to the control tubes. All the tubes were then incubated at 37 °C for 90 minutes and observed for lysis of clot, if any. After incubation, the released of fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis as shown below:

$$\% \text{ of clot lysis} = (\text{wt of released clot} / \text{clot wt}) \times 100$$

Membrane stabilizing activity

The membrane stabilizing activity of the extractives was assessed by using hypotonic solution and heat-induced hemolysis of human erythrocyte by the method developed by Shinde *et al.* (1999) and modified by Sikder *et al.* (2011). To prepare the erythrocyte suspension, whole blood was obtained using syringes (containing anticoagulant EDTA) from mice through cardiac puncture. The blood was centrifuged and blood cells were washed three times with solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4) through centrifugation for 10 min at 3000 g. The test sample consisted of stock erythrocyte (RBC) suspension (0.50 mL) mixed with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extract (2.0 mg/mL) or acetyl salicylic acid (0.1 mg/mL).

Antimicrobial activity

The antimicrobial screening, which is the first stage of antimicrobial drug discovery, was performed by the disc diffusion method (Bauer *et al.,* 1966) against twelve bacteria (Table 4) collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh. Standard disc of Ciprofloxacin (30 µg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control, respectively. The antimicrobial activity of the test agents (400 µg/disc) was determined by measuring the diameter of zone of inhibition expressed in mm.

Antidiarrheal activity by castor oil challenge

The antidiarrheal activity of the methanolic crude extract of *Z. mauritiana* was evaluated using the method of castor oil induced diarrhea in mice (Shoba & Thomas, 2001). According to this method each mice was fed with 1ml of highly pure analytical grade of castor oil to induce diarrhea. The numbers of fecal stools were recorded for each individual mouse. The observations of the experimental groups were compared against that of the control to evaluate the antidiarrheal activity of the samples after oral administration.

Analgesic activity by tail flicking method

In this method (Pizziketti *et al.,* 1985) the basal reaction time of animals to radiant heat was recorded by placing the tip (last 1-2 cm) of the tail on the radiant heat source. The tail withdrawal from the heat (flicking response) is taken as the end point. A cut off period of 15 second was set to avoid damage to the tail. The measurements of withdrawal time using the tail flick apparatus was conducted at 0 hr, 1 hr, 2 h and 3 h after administration of drugs. Here a total of 12 rats divided into 4 groups were used. Group-I served as normal control and received 1% Tween-80

in saline mixture (0.1 ml/10 mg body weight), group-II served as positive control and received morphine (10 mg/kg b.w., intraperitonially), group-III and group-IV served as treatment groups and received 200 mg/kg and 400 mg/kg body weight extractive orally, respectively. The pain inhibition percentage (PIP) was calculated according to the following formula:

$$\text{Pain inhibition percentage (PIP)} = ((T_1-T_0)/T_0) \times 100$$

where, T_1 is post-drug latency and T_0 is pre-drug latency.

Phenobarbitone induced sleeping time

Phenobarbitone induced sleeping time test was carried out according to the method of Williamson *et al.*, (1996). The test animals (rats) were divided in three groups consisting of three rats per group. Group I was the control group whereas group II and III belonged to experimental groups. The experimental groups were administered orally with test samples prepared with normal saline water and Tween-80 at doses of 200 and 400 mg/kg body weight, respectively while the control group was administered with normal saline water containing 1% Tween 80 solution. Thirty minutes later phenobarbitone sodium (25 mg/kg body weight) was administered intraperitonially to all the groups to induce sleep. The onset of sleep and total sleeping time were recorded for both control and treated groups.

Statistical analysis

For all bioassays, three replicates of each sample were used for statistical analysis and the values are reported as mean ± SD.

RESULTS

Successive chromatographic separation and purification from the petroleum ether, carbon tetrachloride and chloroform soluble fractions of a methanol extract of leaf of *Z. mauritiana* yield a total of seven compounds (**1** – **7**). The structures of the isolated compounds were solved by NMR data analyses and comparison with published values as well as by co-TLC with authentic samples.

As a part of discovery of cardio protective drugs from natural resources the extractives of *Z. mauritiana* were assessed for thrombolytic activity and the results are presented in Table 1. Addition of 100µl SK, a positive control (30,000 I.U.), to the clots and subsequent incubation for 90 minutes at 37 °C, showed 69.66% lysis of clot. On the other hand, distilled water which was treated as negative control which exhibited a negligible percentage of lysis of clot (2.56%).

Table 1
Thrombolytic activity (in terms of % of clot lysis) of *Z. mauritiana*.

Test sample	W_1	W_2	W_3	% clot lysis
ME	4.65 ± 0.041	5.17 ± 0.062	4.96 ± 0.049	40.61 ± 1.24
PESF	4.25 ± 0.018	5.11 ± 0.029	4.94 ± 0.051	20.62 ± 1.54
CTCSF	4.76 ± 0.049	5.30 ± 0.041	5.08 ± 0.034	41.72 ± 1.12
CSF	4.82 ± 0.034	5.08 ± 0.055	4.98 ± 0.049	39.53 ± 1.32
SK (standard)	4.89 ± 0.055	5.78 ± 0.062	5.16 ± 0.041	69.66 ± 0.66
Water (blank)	4.91 ± 0.004	5.65 ± 0.006	5.46 ± 0.006	2.56 ± 0.35

ME = Methanol extract; PESF = Petroleum ether soluble fraction; CTCSF = Carbon tetrachloride soluble; CSF = Chloroform soluble fraction; W_1 = Weight of vial alone (g); W_2 = Weight of clot containing vial (g); W_3 = Weight of clot containing vial after clot disruption and removal of fluid; SK =Streptokinase.

The methanol extract of *Z. mauritiana* and its different partitionates, at concentration 2.0 mg/mL, were tested to see the activity against lysis of human erythrocyte membrane induced by hypotonic solution as well as heat, as compared to the standard acetyl salicylic acid (0.10 mg/mL) (Table 2). At 2.0 mg/mL in hypotonic solution induced condition the carbon tetrachloride soluble fraction (CTCSF) inhibited 52.80% and petroleum ether soluble fraction (PESF) inhibited 54.55% haemolysis of RBC as compared to 69.90% revealed by acetyl salicylic acid (0.10 mg/mL).

Table 2
Effect of different extractives of leaves of *Z. mauritiana* on hypotonic solution and heat-induced haemolysis of erythrocyte membrane.

Sample code	Concentration	% Inhibition of haemolysis	
		Heat induced	Hypotonic solution induced
Hypotonic medium	50 mM	--	--
ME	2 mg/mL	29.39 ± 1.32	46.66 ± 1.95
PESF	2 mg/mL	30.90 ± 1.43	54.55 ± 1.59
CTCSF	2 mg/mL	18.46 ± 1.50	52.80 ± 1.63
AQSF	2 mg/mL	28.19 ± 0.79	44.40 ± 0.90
Acetyl salicylic acid	0.10 mg/mL	40.20 ± 1.45	69.90 ± 0.95

Table 3
Antimicrobial activity of *Z. mauritiana* extractives.

Test microorganisms	Test sample					
	Diameter of zone of inhibition (mm)					
	ME	PESF	CTCSF	CSF	AQSF	Ciprofloxacin
Bacillus cereus	15.2 ± 0.37	11.2 ± 0.45	13.5 ± 0.17	9.67 ± 0.57	-	41.67 ± 1.5
B. megaterium	16.4 ± 0.57	10.4 ± 0.63	12.9 ± 0.21	-	-	41.33 ± 1.5
B. subtilis	14.5 ± 0.51	-	12.4 ± 0.51	11.0 ± 1.0	-	53.33 ± 0.57
Staphylococcus aureus	18.8 ± 0.72	-	14.8 ± 0.51	14.0 ± 1.2	-	51.67 ± 0.57
Sarcina lutea	14.7 ± 0.37	7.5 ± 0.21	12.8 ± 0.78	10.0 ± 1.0	-	47.67 ± 0.57
Escherichia coli	-	-	-	9.33 ± 1.0	-	51.0 ± 1.0
Pseudomonas aeroginosa	16.9 ± 0.57	8.2 ± 0.16	14.7 ± 0.52	14.33 ± 1.5	-	49.0 ± 1.0
Salmonella paratyphi	16.4 ± 0.57	7.5 ± 0.08	15.1 ± 0.91	-	-	54.67 ± 1.5
S. typhi	17.4 ± 0.51	8.2 ± 0.57	13.9 ± 0.89	-	-	44.33 ± 1.5
Shigella boydii	15.8 ± 1.12	-	14.2 ± 0.51	12.33 ± 0.57	-	44.33 ± 1.5
S. dysenteriae	14.9 ± 0.32	7.5 ± 0.11	13.5 ± 0.42	10.67 ± 1.2	-	51.0 ± 1.0
Vibrio mimicus	17.9 ± 1.2	-	14.6 ± 0.21	11.33 ± 1.5	-	49.67 ± 0.57

ME= Methanol extract; PESF= petroleum ether soluble fraction; CTCSF= Carbon tetrachloride soluble fraction; CSF = Chloroform soluble fraction; AQSF =Aqueous soluble fraction of methanol extract of *Z. mauritiana*.

The extracts were also subjected to screening of *in vitro* antibacterial activity against five gram positive and seven gram negative bacteria at 400 µg/disc by the standardized disc diffusion method. The results of antibacterial activity revealed moderate inhibitory activity against most of the tested pathogens (Table 3).

The methanol extract showed antidiarrheal property in castor oil induced diarrhea in mice (Table 4). The methanolic crude extract (400 mg/kg) reduced diarrheal feces by 52.02% whereas the same extract at 200 mg/kg dose showed 47.98% reduction of diarrhoeal feces when compared to the standard loperamide (67.24%).

The methanol extract of the leaves of *Z. mauritiana* was subjected to screening for centrally acting antinociceptive property by radiant heat tail-flick method. The test was performed by taking samples at doses of 200 and 400mg/kg body weight. The extract moderately elongated the reaction time in a dose dependent manner (Table 5 and 6).

Table 4
Effect of methanol extract on castor oil (1ml/mice) induced diarrhea in mice

Treatment	Dose	Number of diarrheal faeces	% Reduction of diarrhoea
Control (saline)	**10 ml/kg**	**17.3 ± 1.50**	**---**
Standard (loperamide)	**50 mg/kg**	**5.67 ± 0.57**	**67.24 ± 1.78**
Methanol extract	**200 mg/kg**	**9.00 ± 1.20**	**47.98 ± 2.01**
Methanol extract	**400 mg/kg**	**8.30 ± 1.12**	**52.02 ± 1.89**

Values are expressed as Mean ± SD from the experiments.

Table 5
Effect of morphine and *Z. mauritiana* extracts on tail flicking time of mice

Group	Latency period (sec)			
	0 minutes	30 minutes	60 minutes	90 minutes
Control	**6.03 ± 0.47**	**6.26 ± 0.57**	**6.16 ± 0.89**	**6.23 ± 0.47**
Morphine (standard)	**10.8 ± 0.91**	**16.5 ± 1.03**	**15.96 ± 1.17**	**16.16 ± 1.07**
Methanol extract (200 mg/kg)	**7.77 ± 0.69**	**9.7 ± 0.41**	**9.30 ± 0.78**	**9.23 ± 1.16**
Methanol extract (400 mg/kg)	**10.33 ± 0.34**	**13.37 ± 0.67**	**13.30 ± 0.89**	**12.73 ± 0.47**

Table 6
Antinociceptive activity of methanol extract of leaves of *Z. mauritiana*

Group	Dose (mg/Kg)	% Pain inhibition		
		30 min	60 min	90 min
Control	**-**	**3.81 ± 0.69**	**2.16 ± 0.98**	**3.37 ± 0.78**
Morphine (standard)	**2**	**52.77 ± 2.38**	**47.78 ± 2.03**	**49.6 ± 1.89**
Methanol extract	**200**	**24.83 ± 1.07**	**19.69 ± 1.83**	**18.83 ± 2.57**
Methanol extract	**400**	**29.42 ± 1.37**	**28.75 ± 1.93**	**23.30 ± 1.27**

Each value represents the mean ± SD (n = 3)

The methanol extract of *Z. mauritiana* leaf potentiated the phenobarbitone sodium–induced sleeping time in a dose dependent manner (Table 7). The time of onset of sleep was 15.8 min in control group whereas in experimental group it was 27.6 min and 29.6 min at doses of 200 and 400 mg/kg body weight, respectively. The total sleeping time was about 79.0 min and 89.8 min at 200 and 400 mg/kg, respectively while it was 118.6 min in the control group.

Table 7
Effect of methanol extract of *Z. mauritiana* on phenobarbitone sodium–induced sleep.

Group	Dose	Time of onset of sleep (min)	Total sleeping time (min)
Control (Only Phenobarbitone treated)	0.1 ml/10 g of b.w (1%Tween 80 solution)	15.8 ± 1.19	118.6 ± 2.81
Methanolic extract	200 mg/kg	27.6 ± 1.76	79.0 ± 3.210
Methanolic extract	400 mg/kg	29.6 ± 2.20	89.8 ± 2.85

Each value represents the mean ± SD (n= 3)

Discussion

The ^{1}H NMR spectrum of compound **1** revealed the presence of a two dishielded three proton singlet at δ 4.05 and 4.41 attributable to the two aromatic methoxy groups. Two doublets (J = 2.0 Hz) centered at δ 7.04 and 7.61 were assigned to C-3 and C-2 protons, respectively of a furan ring. The ^{1}H NMR spectrum of compound **1** also displayed two broad doublets (J = 8.5 Hz) at δ 7.82 and 7.03, respectively which could be assigned to H-5 and H-7 in a furoquinoline alkaloid. The characteristic low field resonance of H-5 resulted from the deshielding influence of the oxygen in *peri* position (Robertson, 1963). The remaining one proton triplet at δ 7.33 was attributable to H-6 of furoquinoline ring. These spectral features are in close agreement to those observed for γ-fagarine (Haque *et al.*, 2013). On this basis, the identity of compound **1** was established as γ -fagarine (**1**).

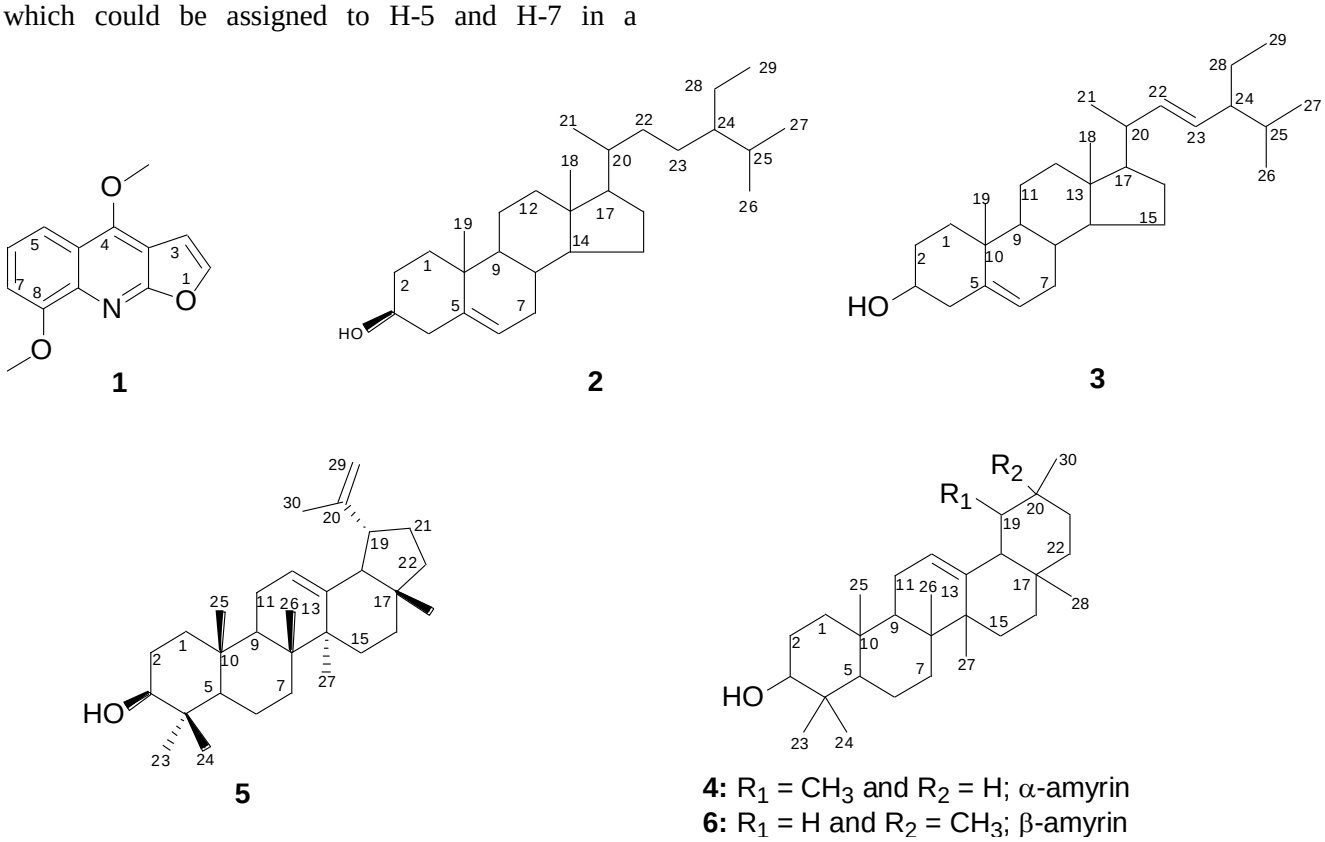

Figure 1
Structures of compounds (1-6) isolated from *Z. mauritiana*

Compounds **2** and **3** were readily characterized as β-sitosterol and stigmasterol by direct comparison of their ^{1}H NMR spectra previously acquired in our laboratory as well as by co-TLC with authentic samples.

The ^{1}H NMR spectrum of compound **4** displayed eight three proton singlets at δ 0.84, 0.79, 0.96, 1.01, 1.07, 0.88 and 0.91 which suggested the presence of eight methyl groups in compound **4**. It also showed a multiplet centered at δ 5.29 which could be assigned to an olefinic proton at C-12. The double doublet (J = 11.0, 5.0 Hz) centered at δ 3.23 could be demonstrative of the α-orientation of C-3 proton. The above spectral features are in close agreement to those observed for α-amyrin (Rao, 2012). On this basis the compound **4** was as characterized as α-amyrin (**4**), the identity of which was further substantiated by co-TLC with the authentic sample.

The ^{1}H NMR spectrum of the compound **5** revealed presence of a pentacyclic lupene type triterpene with vinylic protons at δ 4.56 and 4.68 (H_2-29) and seven singlets assignable to the tertiary methyl groups at 0.74, 0.78, 0.82, 0.94, 0.96, 1.02, 1.67 (H-24, H-28, H-25, H-27, H-23, H-26 and H-30, respectively). The identity of compound **5** as lupeol was confirmed by comparison of these data with published values (Aratanechemuge *et al.*, 2004). Thus, the major constituent in compound **5** was characterized as lupeol.

The ^{1}H-NMR spectrum of the compound **6** showed a broad singlet centered at δ 5.17 which could be assigned for an olefinic proton for H-12. The double doublet (J = 10.5, 4.7 Hz) at δ 3.18 can be demonstrated for a β-oriented proton at C-3. It also revealed eight three proton singlets at δ 0.86 (H_3-28), 0.89 (H_3-29, H_3-30), 0.92 (H_3-24), 0.93 (H_3-23), 0.98 (H_3-25, H_3-26) and 1.07 (H_3-27) which suggested the presence of eight methyl groups. The above spectral features are in close agreement to those observed for β-Amyrin (Rao, 2012). On this basis the identity of compound **6** was confirmed as β-amyrin.

The ^{1}H NMR spectrum displayed a one proton double doublet (J = 4.8, 10.8) at δ 3.12, the position and multiplicity of which were indicative of H-3 of the terpenoidal nucleus. The spectrum demonstrated two singlets at δ 4.68 and δ 4.56 (1H each) assignable to the vinylic protons at C-29. The ^{1}H NMR spectrum also showed six singlets of three proton intensity each at δ 0.78 , 0.82 , 0.94, 0.96 , 1.02 and 1.67 assignable to protons of methyl groups at C-4 (H_3-23, H_3-24), C-10 (H_3-25), C-8 (H_3-26), C-14 (H_3-27) and C-30 (H_3-30), respectively. The multiplet at δ 2.90 could be ascribed to H-19. Two mutually coupled doublet (J = 10.8 Hz) at δ 3.25 and δ 3.28 suggested the presence of hydroxyl methyl group at C-28. Thus, it was identified as betulin (**7**). Its identity was further substantiated by comparison with published data (Sharma *et al*., 2010).

In this study, the carbon tetrachloride soluble fraction exhibited highest thrombolytic activity (41.72%). However, good thrombolytic activity was demonstrated by the crude methanol extract (40.61%) of *Z. mauritiana* and its chloroform soluble fraction (39.53%). The methanol extract (ME) and aqueous soluble fraction (AQSF) showed moderate inhibition of haemolysis of RBCs. During heat induced condition different partitionates of methanol extract of *Z. mauritiana*. i.e., CTCSF, PESF, and AQSF inhibited 18.46%, 30.90%, and 28.19% haemolysis of RBCs, respectively whereas the percent inhibition of ASA was 40.20%. The methanol extract (at 200 mg/kg and 400 mg/kg) of *Z. mauritiana* demonstrated moderate antinociceptive activity having 18.83% and 29.42% elongation of reaction time, respectively while the standard morphine exhibited 52.77%, of elongation of reaction time. The methanol extract at 400 mg/kg body weight showed better results in potentiating of phenobarbitone sodium–induced sleeping time than 200 mg/kg body weight. The methanol extract also showed significant antidiarrheal property in castor oil induced diarrhea in mice.

CONCLUSION

The methanol extract of *Z. mauritiana* and different partitionates derived from it were screened for the antimicrobial, antinociceptive, analgesic, antidiarrheal, and thrombolytic and membrane stabilizing activities. Among all extractives the carbon tetrachloride soluble fraction exhibited highest thrombolytic activity, whereas the methanolic crude extract showed moderate inhibition of haemolysis of RBCs and antinociceptive activity. These results are consistent with some of the folkloric uses of the plant. Further bioassay guided isolation is warranted to isolate and characterize the active thrombolytic agents, as a part of cardioprotective drug discovery program.

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REFERENCES

Aratanechemuge Y, Hibasami H, Sanpin K, Katsuzaki H, Imail K, Komiya T. 2004. Induction of apoptosis by lupeol isolated from mokumen (*Gossampinus malabarica* L. Merr) in human promyelotic leukemia HL-60 cells. **J Oncol Rep** 11: 289 - 292.

Bauer AW, Kirby WMM, Sherris JC, Turck M. 1966. Antibiotic susceptibility testing by a standardized single disc method. **Am J Clin Pathol** 45: 493 - 496.

Bhatia A, Mishra T. 2009. Free radical scavenging and antioxidant potential of *Ziziphus mauritiana* (Lamk.) seed extract. **J Compl Integr Med** 8: 42 - 46.

Cheng G, Bai Y, Zhao Y, Tao J, Liu Y, Tu G, Ma L, Liao N, Xu X. 2000. Flavonoids from *Ziziphus jujuba* Mill var. *spinasa*. **Tetrahedron** 56: 8915 - 8920.

Cristau P, Temal-Laib T, Bois-Choussy M, Martin M-Th, Vors J-P, Zhu J. 2005. Total synthesis of mauritines A, B, C, and F: cyclopeptide alkaloids with a 14-membered paracyclophane unit. **Chem Eur J** 11: 2668 - 2679.

Daginawala HF, Prasad S, Kashyap RS, Deopujari JY, Purohit HJ, Taori GM. 2006. Development of an *in vitro* model to study clot lysis activity of thrombolytic drugs. **Thrombosis J** 4: 14.

Dahiru D, Obidoa O. 2007. Pretreatment of albino rats with aqueous leaf extract of *Zizyphus mauritiana* protects against alcohol induced liver damage. **Trop J Pharm Res** 6: 705 - 710.

Dahiru D, William ET, Nadro MS. 2005. Protective effect of *Ziziphus mauritiana* leaf extract on carbon tetrachloride-induced liver injury. **Afr J Biotechnol** 4: 1177 - 1179.

Han BH, Park MH, Park JH. 1989. Chemical and pharmacological studies on sedative cyclopeptide alkaloids in some Rhamnaceae plants. **Pure Appl Chem** 61: 443 - 448.

Haque MM, Begum S, Sohrab MH, Ahsan M, Hasan CM, Ahmed N, Haque R. 2013. Secondary metabolites from the stem of *Ravenia spectabilis* Lindl. **Pharmacogn Magazine** 9: 76 - 80.

Hawk PB, Oser L, Summerson WH. 1954. **Practical Physiological Chemistry**. 13th ed. McGraw Hill Book Company, USA.

Jossang A, Zahir A, Diakite D. 1996. Mauritine J, a cyclopeptide alkaloid from *Zizyphus mauritiana*. **Phytochemistry** 42: 565 - 567.

Joullie M, Richard DJ. 2004. Cyclopeptide alkaloids: chemistry and biology. **Chem Commun** 2011 - 2015.

Kawsar MH, Sikder MA, Rana MS, Nimmi I, Rashid MA. 2011. Studies of thrombolytic and cytotoxic properties of two asteraceous plants of Bangladesh. **Bang Pharm J** 14: 103 - 106.

Kim YA, Shin HN, Park MS, Cho SH, Han SY. 2003. Studies toward a synthesis of C3-epimauritine D: construction of the macrocycle. **Tetrahedron Lett** 44: 2557 - 2560.

Lin HY, Chen CH, You BJ, Liu KCSC, Lee SS. 2000. Cyclopeptide alkaloids from *Paliurus ramossisimus*. **J Nat Prod** 63: 1338 - 1343.

Michel A. 2002. **Tree, Shrub and Liana of West African Zones.** Margraf Publishers GmbH, Paris, France.

Morel AF, Maldaner G, Ilha V, Missau F, Silva UF, Dalcol II. 2005. Cyclopeptide alkaloids from *Scutia buxifolia* Reiss and their antibacterial activity. **Phytochemistry** 66: 2571 - 2576.

Morton JF, Miami FL. 1987. **Indian jujube. Fruits of Warm Climates.** Florida Flair Books, Miami, USA.

Ndhala AR, Mupure CH, Chitindingue K, Benhura MAN, Muchuweti M. 2006. Antioxidant potentials and degree of polymerization of six wild fruits. **Scientific Research and Essay** 1: 87 - 92.

Pandy VB, Devi S. 1990. Biologically active cyclopeptide alkaloids from Rhamnaceae plants. **Planta Med** 56: 649 - 650.

Panseeta P, Lomchoey K, Prabpai S, Kongsaeree P, Suksamrarn A, Ruchirawat S, Suksamrarn S. 2011. Antiplasmodial and antimycobacterial cyclopeptide alkaloids from the root of *Ziziphus mauritiana*. **Phytochemistry** 72: 909 - 915.

Pisha E, Chai H, Lee IS. 1995. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. **Nature Med** 1: 1046 - 1051.

Pizziketti RJ, Pressman NS, Geller EB, Adler MW. 1985. Rat cold water tail-flick: A novel analgesic test that distinguishes opioid agonist from mixed agonist-antagonist. **Eur J Pharm** 119: 23 - 29.

Ramadoss S, Jaggi M, Siddiqui MJA. 2000. Use of betulinic acid and its derivatives for inhibiting cancer growth and a method of monitoring this. Assignee: Dabur Research Foundation. **US patent** No. 6048847.

Rao V. 2012. **Phytochemicals - A Global Perspective of their role in Nutrition and Health**. InTech Europe, Croatia.

Robertson AV. 1963. The Proton Magnetic Resonance of Furoquinoline Alkaloids and Related Compounds, **Aust J Chem** 16: 451‑458.

Sharma PP, Roy B, Anurag RK, Gupta AD. 2010. Pentacyclic Triterpinoids from *Betula utilis* and *Hyptis suaveolens*. **Int J PharmTech Res** 2: 1558 - 1532.

Shinde UA, Phadke AS, Nair AM, Mungantiwar AA, Dikshit VJ, Saraf MN. 1999. Membrane stabilizing activity - a possible mechanism of action for the anti-inflammatory activity of *Cedrus deodara* wood oil. **Fitoterapia** 70: 251 - 257.

Shoba FG, Thomas M. 2001. Study of antidiarrheal activity of four medicinal plants in castor oil induced diarrhea. **J Ethnopharmacol** 76: 73 - 76.

Sikder MA, Rahman MA, Kaisar MA, Rahman MS, Hasan CM, Rashid MA. 2011. *In vitro* antioxidant, reducing power, free radical scavenging and membrane stabilizing activities of seeds of *Syzygium cumini* L. **Lat Am J Pharm** 30: 781 - 785.

Singh AK, Pandey MB, Singh VP, Pandey VB. 2007. Mauritine K, a new antifungal cyclopeptide alkaloid from *Zizyphus mauritiana*. **J Indian Chem Soc** 84: 781 - 784.

Suksamrarn S, Suwannapoch N, Aunchai N, Kuno M, Ratananukul P, Haritakun R, Jansakul C, Ruchirawat S. 2005. Ziziphine N, O, P and Q, new antiplasmodial cyclopeptide alkaloids from *Ziziphus oenoplia* var. Brunoniana. **Tetrahedron** 61: 1175 - 1180.

Suksamrarn S, Panseeta P, Kunchanawatta S, Distaporn T, Ruktasing S, Suksamrarn A. 2006. Ceanothane- and lupane-type triterpenes with antiplasmodial and antimycobacterial activities from *Ziziphus cambodiana*. **Chem Pharm Bull** 54: 535 - 537.

Trevisan G, Maldaner G, Velloso NA, da Silva SAG, Ilha V, de Campos VGC, Rubin MA, Morel AF, Ferreira J. 2009. Antinociceptive effects of 14-membered cyclopeptide alkaloids. **J Nat Prod** 72: 608 - 612.

Tschesche R, Wilhelm H, Fehlhaber HW. 1972a. Alkaloids from Rhamnaceae. XIV. Mauritine-A and mauritine-B, two new peptide alkaloids from *Zizyphus mauritiana*. **Tetrahedron Lett** 26: 2609 - 2612.

Tschesche R, Kaussmann EU, Fehlhaber HW. 1972b. Alkaloids from Rhamnaceae, XIII. Amphibine-B, -C, -D, and -E, four Peptide Alkaloids from *Ziziphus amphibia* A. Cheval. **Chem Ber** 105: 3094 - 3105.

Tschesche R, Wilhelm H, Kaussmann EU, Eckhardt G. 1974. Alkaloids from Rhamnaceae. XVII. Mauritine-C, -D, -E and -F, new peptide alkaloids from *Ziziphus mauritiana*. **Justus Lieb Ann Chem** 10: 1694 - 1701.

Tschesche R, Hillebrand D, Wilhelm H, Ammermann E, Eckhardt G. 1977. Hysodricanin-A, mauritin-H, scutianin-F und aralionin-C, vier weitere cyclopeptidalkaloide aus Zizyphus, Scutia und Araliothamnus. **Phytochemistry** 16: 1025 - 1028.

Vahedi F, Najafi MF, Bozari K. 2008. Evaluation of inhibitory effect and apoptosis induction of Zyzyphus Jujube on tumor cell lines, an in vitro preliminary study. **Cytotechnology** 56: 105 - 111.

Van Wagenen, BC, Larsen R, Cardellina JH, Ran dazzo D, Lidert ZC, Swithenbank C. 1993. Ulosantoin, a potent insecticide from the sponge *Ulosa ruetzleri*. **J Org Chem** 58: 335 - 337.

Verheij EWM, Coronel RE. 1991. **Plant Resources of South-East Asia - Edible fruits and nuts.** version: 1.0. Margraf Publishers GmbH, Weikersheim, Germany.

Williamson EM, Okpako DT, Evans FJ. 1996. **Pharmacological Methods in Phytotherapy Research: Selection, preparation and pharmacological evaluation of plant material.** 1st edition, John Willey & Sons, England.

Xiao D, East SP, Joullie MM. 1998. Total synthesis of sanjoinine A (frangufoline). **Tetrahedron Lett** 39: 9631 - 9632.