



## Research Article

### HYPOGLYCEMIC, CELL MEMBRANE STABILITY, THROMBOLYTIC, TOTAL PHENOLIC CONTENT AND FREE RADICAL SCAVENGING ACTIVITIES OF METHANOLIC EXTRACT OF *ZIZIPHUS MAURITIANA* FRUITS (RHAMNACEAE)

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#### ABSTRACT

The purpose of the present study was to evaluate *in vitro* free radical scavenging activity and total phenolic content; hypoglycemic activity, cell membrane stabilizing activity and thrombolytic activity in mice model of methanolic crude extract of *Ziziphus mauritiana* fruits. The coarse powder of dried fruits was soaked with methanol for several days with occasional stirring. After that it was extracted at room temperature and dried extract was partitioned into pet ether, chloroform, carbontetrachloride and aqueous soluble fraction. Among all the fractions, aqueous soluble fraction revealed highest free radical scavenging activity having IC<sub>50</sub> value 26.77 µg/ml followed by chloroform soluble fraction of 43.46 µg/ml with respect to BHT having IC<sub>50</sub> value 24 µg/ml. The amount of total phenolic content of different extractives ranged from 29.30 mg to 52.18 mg of GAE / g of extractives of *Z. mauritiana* and pet ether soluble fraction showed the highest phenolic content 52.18 mg of GAE / g of extractives. Crude extract at a dose of 400 mg/kg body weight moderately lowered the blood glucose level after 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> hour having 37.81, 43.65 and 44.4 percent of fall of blood glucose level and exhibited highest thrombolytic activity 50.22 % clot lysis in mice model. In hypotonic solution induced condition carbontetrachloride and pet-ether soluble fraction inhibited 54.55 % and 52.8 % haemolysis of RBC with respect to 69.9 % revealed by acetyl salicylic acid (0.10 mg/mL); heat induced condition crude extract, pet ether and aqueous soluble fraction revealed moderate inhibition about 29.39, 30.90 % and 28.19 % compared with 40.20 % inhibition by acetyl salicylic acid in heat induced condition.

**Keywords:** *Ziziphus mauritiana*, Free radical scavenging activity, Total phenolic content, hypoglycemic activity, Cell membrane stability activity and Thrombolytic activity.

#### INTRODUCTION

Secondary metabolites those are obtained from medicinally important plants are harmless natural resources for ailment of human and give a new voyage for drug development. Interest towards the wide range of traditional natural resources is increasing day by day. Folk medicinal practices are very common in Bangladesh. *Ziziphus mauritiana*, also known as Kul or Boroi in Bangladesh, Chinese Apple, Jujube, Indian plum and Masau is a tropical fruit tree species belonging to the family Rhamnaceae. It is a common plant in Bangladesh. It bears citrus fruits which usually ripen during February to April in our country. The fruit is of variable shape and size. It can be oval, obovate and oblong or round and that can be 1-2.5 in (2.5-6.25 cm) long, depending on the variety. The flesh is white and crisp. When slightly under ripe, this fruit is a bit juicy and has a pleasant aroma. The fruit's skin is smooth, glossy, thin but tight. The fruits could be used to treat cuts and ulcers, fever and pulmonary ailments and mild laxative. Extensive investigation showed that this species revealed important biological activities such as antioxidant activity<sup>1</sup>, antimicrobial activity, anti-inflammatory activity<sup>2</sup>, anxiolytic property<sup>3</sup>, anti diabetic activity<sup>4</sup> and so on. Focused on different biological activities, present study has undertaken to evaluate free radical scavenging activity, total phenolic content, hypoglycemic, cell membrane stability and thrombolytic activity of methanolic crude extract of *Z. mauritiana* fruits in laboratory by following different standard protocols.

#### MATERIALS AND METHODS

##### Collection of the plant sample and experimental animal

Fruits of *Ziziphus mauritiana* were collected from Dhaka, Bangladesh in February, 2013. This plant was identified by botanists of the Botany Department of Dhaka University. The reference sample for the plant was DUSH, Accession Number 4257 and calls no 01. Swiss-albino mice of either sex, aged 4-5 weeks were the experimental animal and obtained from the Animal Resource Branch of the International Centre for Diarrheal Diseases and Research, Bangladesh (ICDDR, B). They were kept in standard environmental condition and fed ICDDR; B formulated rodent food and water. Experimental animals were collected, handled and kept by following standard protocol based on the ethical committee of our university

##### Preparation of the plant sample

After collection of fruits, it was sundried and grinded into coarse powder. Around one kg of powdered material was soaked into 2.5 liter of methanol for 15 days with occasional stirring. Then it was extracted at room temperature. Around 5 g of dried methanolic extract was partitioned into pet ether, carbontetrachloride, chloroform and aqueous soluble fraction by modified Kupkan partitioning methods<sup>5</sup>.

##### Evaluation of free radical scavenging activity

Methanolic crude extract of *Z. mauritiana* fruits and its different fractions were subjected for free radical scavenging activity on the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and estimated by the method of Brand-Williams<sup>6</sup>. The DPPH radical contains an odd electron, which is

responsible for the absorbance at 515-517 nm and also for a visible deep purple color. When it accepts electron donated by an antioxidant compound, it is decolorized, which can be quantitatively measured from the changes in absorbance. Two ml of methanol solution of the extract at different concentration were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml) Table 1. The antioxidant potential was assayed from the bleaching of purple colored methanol solution of DPPH radical by the extract as compared to that of tert-butyl-1-hydroxytoluene (BHT) at 517 nm against methanol as blank by UV spectrophotometer. Inhibition of free radical DPPH in percent (I %) was calculated as follows-

$$(I\%) = (1 - A_{\text{sample}}/A_{\text{blank}}) \times 100$$

Where  $A_{\text{blank}}$  is the absorbance of blank and  $A_{\text{sample}}$  is the sample

#### Assay for total phenolic content

Phenolic compounds become completely ionized in alkaline solution. When Folin-Ciocalteu reagent was used in this ionized phenolic solution the reagent readily oxidized the phenols and retained blue color from its original yellow color by oxidation process. The intensity of the color change was measured in a spectrophotometer at 760 nm and the absorbance value reflected the total phenolic content of the sample<sup>7</sup>. Extract solution of 0.5 ml (conc. 2 mg/ml), 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of  $\text{Na}_2\text{CO}_3$  (7.5 % w/v) solution were mixed and incubated for 20 minutes at room temperature. Then the absorbance was measured and using the standard curve prepared from gallic acid solution with different concentration, the total phenols content of the sample was measured and expressed as mg of GAE (gallic acid equivalent) / g of the extract.

#### Evaluation of hypoglycemic activity test by oral glucose tolerance test

In this method, methanolic crude extract and its different fractions of *Z. mauritiana* fruits were administered orally to Swiss albino mice at the fasting condition. Then the mice were given glucose load at a certain dose, 30 minutes after administration of crude extracts, their fractions, standard and control. The blood glucose levels of the experimental animals were measured by using a glucometer (Bio land G-423 S from Hong Kong) and Glucose oxidase - peroxidase reactive strips at 30 minutes, 90 minutes and 150 minutes interval. The hypoglycemic effect of the test samples were then compared with relative to that of control (vehicle containing 1 % Tween 80 and DMSO in saline) and standard (vehicle containing Glibenclamide) group<sup>8</sup>. Randomly twenty Swiss albino mice were selected and divided in to four groups (5 mice in each). Among that group-I, II received control and standard group-III, IV treated with crude extract at a dose of 200 and 400 mg/kg body wt respectively. Table 2

#### Evaluation of cell membrane stabilizing activity Hypotonic solution- induced hemolysis

The experiments were carried out with hypotonic solution of NaCl. The test sample was consisted of stock erythrocyte (RBC) suspension (0.50 mL) with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffer saline (pH 7.4) containing methanolic extract (1.0 mg/mL) or Acetyl Salicylic Acid (0.10 mg/mL). The Acetyl Salicylic Acid was used as a reference standard. The mixtures were incubated for 10 minutes at room temperature, centrifuged for 10 minutes at 3000 rpm and measured absorbance (O.D.) at 540 nm

using UV spectrophotometer. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation:

$$\text{Percent inhibition of haemolysis} = 100 \times \{(\text{OD}_1 - \text{OD}_2) / \text{OD}_1\}$$

Where,  $\text{OD}_1$  = Optical density of hypotonic-buffered saline solution alone (control) and  $\text{OD}_2$  = Optical density of test sample in hypotonic solution.

#### Heat- induced haemolysis

Aliquots (5 ml) of the isotonic buffer containing 1.0 mg/mL of methanolic crude extract of *Z. mauritiana* fruits (Table 3) were put into two duplicate sets of centrifuge tubes<sup>9</sup>. The vehicle, in the same amount was added to another tube as control. Erythrocyte suspension (30 µL) was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 54°C for 20 minutes in a water bath. The other pair was maintained at 0-5°C in an ice bath. The reaction mixture was centrifuged for 3 minutes at 1300 rpm and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition or acceleration of hemolysis in tests and was calculated according to the equation:

$$\text{Percent inhibition of haemolysis} = 100 \times [1 - (\text{OD}_2 - \text{OD}_1) / (\text{OD}_3 - \text{OD}_1)]$$

Where,  $\text{OD}_1$  = test sample unheated,  $\text{OD}_2$  = test sample heated and  $\text{OD}_3$  = control sample heated

#### Evaluation of thrombolytic activity

The thrombolytic activity of methanolic crude extract of *Z. mauritiana* fruits and its extractives were evaluated by a method using streptokinase (SK) as standard substance. The dry crude extract (100 mg) was suspended in 10 ml of distilled water and it was 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 micro centrifuge tube containing pre-weighed clot, 100 µl aqueous solutions 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 clot lysis. 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 according to the following equation

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

## RESULTS

#### Free radical scavenging activity

The methanolic crude extract and its different fractions of the *Z. mauritiana* fruits were subjected to determine free radical scavenging activity using DPPH assay method where BHT was used as standard. Among all the fractions aqueous soluble fraction revealed highest free radical scavenging activity having  $\text{IC}_{50}$  value 26.77 µg/ml flowed by chloroform soluble fraction of 43.46 µg/ml with respect to BHT having  $\text{IC}_{50}$  value 24 µg/ml. Table 4

**Table 1: Test samples of assay for total phenolic content and free radical scavenging activity test**

Plant part	Sample code	Test sample	Concentration (mg/ml)
Fruits of <i>Z. mauritiana</i>	MCE	Methanolic crude extract	2.0
	PESF	Pet ether soluble fraction	2.0
	CSF	Chloroform soluble fraction	2.0
	CTCSF	Carbon tetrachloride soluble fraction	2.0

**Table 2: Test samples used in the evaluation of hypoglycemic effect for *Z. mauritiana***

Sample code	Test samples	Group	Purpose	Dose (mg/kg)	Route of administration
1 % Tween 80 and DMSO in saline	-	I	Control Group	0.15 ml/10 g of body weight	Oral
Glibenclamide	-	II	Standard Group	10	Oral
MCE	Methanolic crude extract	III	Test sample	200	Oral
MCE	Methanolic crude extract	IV	Test sample	400	Oral

**Table 3: Test samples for evaluation of membrane stabilizing activity of *Z. mauritiana***

Sample code	Identification	Concentration
Hypotonic medium	-	50 mM
MCE	Methanolic crude extract	2 mg/ml
PESF	Pet-ether soluble fraction	2 mg/ml
CTSF	Carbon tetrachloride soluble fraction	2 mg/ml
CSF	Chloroform soluble fraction	2 mg/ml
AQSF	Aqueous soluble fraction	2 mg/ml
Acetyl salicylic acid	-	0.10 mg/ml

**Table 4: IC<sub>50</sub> values for free radical scavenging activity test of *Z. mauritiana***

Sample code	Identification	IC <sub>50</sub> value (µg/ml) ± SEM
BHT	<i>tert</i> -butyl-1-hydroxytoluene	24 ± 0.56
MCE	Methanolic crude extract	52.48 ± 0.049
PESF	Pet ether soluble fraction	67.46 ± 0.076
CTSF	Carbontetrachloride soluble fraction	95.45 ± 0.145
CSF	Chloroform soluble fraction	43.46 ± 0.067
AQSF	Aqueous soluble fraction	26.77 ± 0.032

Probability values (calculated as compared to control using one way-ANOVA followed by Dunnet's Test): P < 0.05.  
All values are means of individual data obtained where n = 3

**Table 5: Total phenolic content of the test samples of *Z. mauritiana***

Sample code	Test Sample	Total phenolic content (mg of GAE / g of extractives)
MCE	Methanolic crude extract	44.31
PESF	Petroleum ether soluble fraction	52.18
CTCSF	Carbon tetrachloride soluble fraction	39.31
CSF	Chloroform soluble fraction	29.30

**Table 6: Effects of methanolic crude extract of *Z. mauritiana* fruits on blood glucose level**

Group	% of fall of blood glucose level		
	After 1 <sup>st</sup> hour	After 2 <sup>nd</sup> hour	After 3 <sup>rd</sup> hour
Control	11.5	9.6	17.34
Standard	56.4	59.67	59.12
Methnolic crude extract at 200 mg/kg body wt	34.26	40.18	40.64
Methnolic crude extract at 400 mg/kg body wt	37.81	43.65	44.6

**Table 7: Effect of different extractives of *Z. mauritiana* on hypotonic solution-induced and heat induced haemolysis of erythrocyte membrane**

Samples	Concentration	% inhibition of haemolysis ± SEM	
		Heat induced	Hypotonic solution induced
Hypotonic medium	50 mM	--	--
Methanolic crude extract	1 mg/ml	29.39 ± 1.32	46.66 ± 1.95
Pet ether soluble fraction	1 mg/ml	30.90 ± 1.43	54.55 ± 1.59
Carbontetrachloride soluble fraction	1 mg/ml	18.46 ± 1.50	52.8 ± 1.63
Aqueous soluble fraction	1 mg/ml	28.19 ± 0.79	44.4 ± 0.90
Acetyl salicylic acid	0.10 mg/ml	40.20 ± 1.45	69.9 ± 0.95

Probability values (calculated as compared to control using one way-ANOVA followed by Dunnet's Test): P < 0.05.  
All values are means of individual data obtained where n = 3

**Table 8: Thrombolytic activity (in terms of % of clot lysis) of methanolic crude extract of *Z. mauritiana* fruits**

Fractions	W <sub>1</sub> (g)	W <sub>2</sub> (g)	W <sub>3</sub> (g)	% of lysis
MCE	4.754	5.176	4.964	50.22
PESF	4.755	5.118	4.94	49.03
CTSF	4.767	5.304	5.08	41.72
CSF	4.829	5.085	4.984	39.53
SK	4.880	5.780	5.16	68.88
Water	4.910	5.650	5.46	2.56

MCE = Methanolic crude extract, PESF = Pet-ether soluble fraction, CTSF = Carbon tetrachloride soluble fraction, CSF = Chloroform soluble fraction, SK = Streptokinase; W<sub>1</sub> = Weight of vial (3 ml/vial) alone, W<sub>2</sub> = Weight of clot containing tube; W<sub>3</sub> = Weight of clot containing tube after clot disruption

### Total phenolic content

The methanolic crude extract of *Z. mauritiana* fruits and its pet-ether, carbon tetrachloride and chloroform soluble fractions were tested for total phenolic content. Based on the absorbance values of the various extract solutions and the colorimetric analysis of the total phenolics of different extracts were determined and compared with the standard solutions of gallic acid equivalents. Total phenolic content of the samples are expressed as mg of GAE (gallic acid equivalent)/ g of extractives. The amount of total phenolic content of different extractives ranged from 29.30 mg to 52.18 mg of GAE / gm of extractives of *Z. mauritiana*. Among all extractives pet ether soluble fraction revealed the highest phenolic content 52.18 mg of GAE / g of extractives. Table 5

### Hypoglycemic activity

Methanolic crude extract of *Z. mauritiana* fruits was subjected for hypoglycemic activity test by oral glucose tolerance test. Crude extract at a dose of 400 mg/kg body weight moderately lowered the blood glucose level after 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> hour having 37.81, 43.65 and 44.4 percent of fall of blood glucose level. Table 6

### Cell membrane stabilizing activity

Methanolic crude extract of *Z. mauritiana* fruits and its different partitionates at concentration 2.0 mg/mL were tested to know 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). At 2.0 mg/mL in hypotonic solution induced condition the carbontetrachloride inhibited 52.8 % and pet-ether soluble fraction inhibited 54.55 % haemolysis of RBC with respect to 69.9 % revealed by acetyl salicylic acid (0.10 mg/mL). During heat induced condition crude extract, pet ether and aqueous soluble fraction revealed moderate inhibition about 29.39, 30.90 % and 28.19 % compared with 40.20 % inhibition by acetyl salicylic acid in heat induced condition. Table 7

### Thrombolytic activity

In case of thrombolytic activity, addition of 100 µl streptokinase (positive control) to the clots and subsequent incubation for 90 minutes at 37°C showed 61.50 % lysis of clot while distilled water exhibited 2.56 % of clot lysis. Among all the samples methanolic crude extract exhibited highest thrombolytic activity (50.22 %) and subsequently pet ether soluble fraction showed 49.03 % clot lysis. Table 8

### DISCUSSION

Methanolic crude extract and different fractions were subjected for evaluation of free radical scavenging activity, total phenolic content, hypoglycemic activity, cell membrane stabilizing activity and thrombolytic activity tests. Crude extract revealed significant free radical scavenging activity with high phenolic content. It has been determined that the antioxidant effect of plant products is mainly due to radical-scavenging activity of phenolic compounds such as flavonoids, polyphenols, tannins and phenolic terpenes<sup>10</sup>. The methanolic crude extract of *Z. mauritiana* fruits and its different partitionates at concentration 2.0 mg/mL were tested to know 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).

The present study shows that the fruits of *Z. mauritiana* have well membrane stabilizing activity and its anti-inflammatory activity may be due to its high contents flavonoids. The effect of synthetic and herbal anti-inflammatory agents on the stabilization of erythrocyte membrane exposed to hypotonic solution has been studied extensively. The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane. The results showed that the extracts were potent on human erythrocyte adequately protecting it against hypotonic induced lysis. The activity was comparable to that of standard anti-inflammatory drug (Acetyl Salicylic Acid). It has been reported that flavonoids exert profound stabilizing effects on lysosomes both *in vitro* and *in vivo* experimental animals<sup>11</sup>, while tannin and saponins have the ability to bind cations and other bio molecules, and are able to stabilize erythrocyte membrane Following thrombolytic activity test of methanolic crude extract, it could be concluded that the extract of *Z. mauritiana* fruits showed moderate to good clot lysis activity. Once found these herbal preparations may be incorporated as a thrombolytic agent for the improvement of the patients suffering from atherothrombotic diseases. Finally it could be concluded that fruit extract of *Z. mauritiana* contains important secondary metabolites which showed different biological activities. So it could be suggested further study on this plant to isolate and identify the secondary metabolites for ailment of human.

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