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ISOLATION OF ZIZYBERENALIC ACID AND BIOLOGICAL STUDIES OF *ZIZIPHUS MAURITIANA* LAM. GROWING IN BANGLADESH

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Abstract

Ziziphus mauritiana Lam. was assessed for phytochemical constituent, antioxidant and cytotoxic activities. Powder prepared from the air-dried leaf was extracted with methanol. The concentrated extract was partitioned with petroleum ether, carbon-tetrachloride, and chloroform. A triterpenoid acid was isolated from the petroleum ether soluble fraction of the plant extract by gel permeation chromatography using Sephadex LH-20 followed by preparative thin layer chromatography over silica gel. The structure of the isolated compound was elucidated as zizyberenalic acid (**1**) by extensive analysis of its NMR spectral data and confirmed by comparison with published value. The methanol extract (IC_{50} = 7.37 ± 0.38 µg/ml) and its chloroform soluble materials (IC_{50} = 24.14 ± 0.53 µg/ml) produced strong antioxidant effect where it was comparable to standard agent *tert*-butyl-1-hydroxy toluene (IC_{50} = 32.47 ± 0.72 µg/ml) in DPPH free radical assay. The petroleum ether soluble fraction also showed strong cytotoxicity (LC_{50} = 1.31 ± 0.01 µg/ml) in brine shrimp lethality assay which was comparable to the standard drug, vincristine sulfate (LC_{50} = 0.31 ± 0.01 µg/ml). The results of the present study suggest that the plant leaf could be considered as a significant natural source for the development of antioxidant as well as cytotoxic compounds.

Introduction

Plant-derived natural products have significantly contributed for thousand years to treat and/or prevent human diseases. The analysis of drugs developed from natural origin showed that 28% of drugs are derived from natural product- or directly used as natural product (Newman *et al*. 2000) and 25% are plant-derived drugs. It has been also found that, among the 252 essential and basic drugs approved by WHO, 11%, have been originated from flowering plants (Rates 2001). The indigenous knowledge regarding the medicinal uses of plants from local flora provides an inventory for an accurate source of traditional system of medicines. A recent report published that the ethnopharmacological uses of plant have contributed 80% to the drugs discovery from 122 plant-derived drugs (Fabricant and Farnsworth 2001). Thus, plant could be a better source to keep pace in the search of new drugs for human ailments.

Ziziphus mauritiana Lam. (Synonym: *Z. jujuba*, Family: Rhamnaceae) is a flowering tree which is widely grown for its edible fruit and also found in wild, road-side and bushes throughout Bangladesh (Ghani 2003). Leaves of the plant have been found to be useful for the treatment of diarrhea, typhoid, wounds, liver troubles, fever, asthma and as astringent in ethnomedicine (Goyal *et al*. 2012). Preliminary phytochemical studies reported that the plant leaves contain steroids, reducing sugar, tannins, alkaloids and glycosides (Karon *et al*. 2011, Parmar *et al*. 2012). Chromatographic separation of the plant extract led to the isolation of α-amyrin, β-sitosterol,

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β-amyrin, γ-fagarine, stigmasterol and lupeol (Hossain *et al*. 2015). GC-MS analysis of hexane, chloroform and methanol soluble extracts of the plant identified α-linolenic acid (26.45%), palmitic acid (38.55%) and methyl stearate (15.59%) as the major constituents (Ashraf *et al.* 2015). Pharmacological studies on crude extractives of the leaves revealed promising antimicrobial, analgesic, sedative (Karon *et al*. 2011), antidiarrheal (Hossain *et al.* 2015), antitumor, antidiabetic, cardiovascular, immunostimulant (Sharma and Gaur 2013), hepato-protective (Dahiru and Obidoa 2007), anti-inflammatory (Kumar *et al.* 2017) and phagocytic (Wadekar and Patil 2008) activities. Previous studies reported that chloroform soluble extract of the plant leaves possesses strong *in vitro* thrombolytic and anticancer effects (Hossain *et al*. 2015, Ashraf *et al*. 2015).

It has been reported that different parts of the plant including fruits (Vahedi *et al*. 2008, Esteki and Urooj 2012), seeds (Mishra *et al*. 2011, San *et al*. 2012), roots (Afzal *et al*. 2017) and barks (Rahman 2012) possess promising antioxidant and cytotoxic activities. Based on the findings of these previous reports, the current study was aimed to investigate the cytotoxic and antioxidant activities of crude methanol extract of leaves of *Z. mauritiana* growing in Bangladesh and its organic soluble extractives. Furthermore, the study was also conducted to isolate compound from its organic fraction which provided zizyberenalic acid (**1**). In the present study the isolation of zizyberenalic acid (**1**) and the results of the preliminary bioassays of crude methanol extract and its different organic soluble (petroleum ether, carbon-tetrachloride and chloroform) fractions are reported.

Materials and Methods

Ziziphus mauritiana leaves were collected from Dhaka, Bangladesh in September 2010 and identified by the scientists of Bangladesh National Herbarium, Mirpur, Dhaka. The experimental works were conducted at Phytochemical Research Laboratory, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University. About 1 kg coarse powder of the leaves was soaked in 3.0 litre methanol and kept for 7 days. The whole mixture was then filtered through cotton plug followed by Whatman No. 1 filter paper and the filtrate obtained was concentrated at 40°C by a rotary evaporator. The concentrated extract was then air dried to provide solid residue. The weight of the crude methanol extract was 25 g. An aliquot (10 g) of methanol extract was partitioned to obtain petroleum ether (2 g), chloroform (3 g) and carbon tetrachloride (3 g) soluble fraction by the modified Kupchan method (Van Wagenen *et al.* 1993).

An amount of 500 mg of petroleum ether soluble fraction was subjected to gel permeation chromatography over Sephadex LH-20 using *n*-hexane-dichloromethane-methanol (2:5:1) to afford 31 fractions. The column fractions were combined together to 3 groups (G1-G3) based on their TLC behavior. G1 was further purified over PTLC (silica gel 60 F_{254}, 20 × 20 cm) plate, developed in ethyl acetate-toluene (2 : 8) and the plates were examined under UV lamp (254 and 360 nm). A purple colored band was visualized after spraying with 1% vanillin-sulfuric acid reagent followed by heating in l10°C for 2 min. The selected band was scrapped off without spraying and eluted with 100% ethyl acetate to yield a pure compound (**1**, 3 mg).

Zizyberenalic acid (**1**): White gummy mass, ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 9.67 (1H, s, H-2), 6.53 (1H, s, H-3), 4.72 (1H, s, H-30), 4.59 (1H, s, H-30), 2.98 (1H, m, H-19), 1.95 (1H, s, H-28), 1.66 (3H, s, H-29), 1.13 (3H, s, H-27), 1.12 (3H, s, H-26), 0.98 (3H, s, H-25), 0.06 (6H, s, H-23, 24).

The antioxidant activity of Z. *mauritiana* extractives was determined by DPPH free radical assay as previously described by Brand-Williams *et al*. (1995). The extractives (2 mg) were dissolved in 200 µl of methanol. Then 100 µl of solution was taken in test tube each containing

2 ml of distilled methanol. Thus, the final concentration of the prepared solution in the first test tube was 500 µg/ml. A series of solutions of varying concentrations (500 - 0.78125 µg/ml) were prepared by serial dilution method. A concentration of 20 µg/ml (w/v) of DPPH solution was prepared in methanol. Then 2 ml of methanolic extract solution was added to 3 ml of DPPH solution and mixed well. The absorbance of the mixture was obtained at 517 nm followed by 30 min of incubation in a dark place. A similar experiment was carried out with *tert*-butyl-1-hydroxy toluene (BHT) which was used as a standard agent to compare the antioxidant activity of the plant extractives. A DPPH solution without any sample was taken as control. The DPPH free radical inhibition in per cent (%) was calculated as follows: % inhibition = $(1- A_{sample}/A_{blank}) \times 100$ where, A_{blank} and A_{sample} represent the absorbance of the DPPH solution with and without the test material, respectively. The concentration providing 50% inhibition (IC_{50}) was calculated from the graph by plotting percentage of inhibition against concentration of extract.

The plant extractives were also screened for cytotoxic activity following the brine shrimp lethality bioassay as described by Meyer *et al.* (1982). Each of extractives (4 mg) was dissolved in 200 µl of DMSO (dimethyl sulfoxide). Then 100 µl of the prepared solution was added in a test tube containing 5 ml of simulated seawater containing 10 shrimp nauplii. Thus, the final concentration of the prepared solution in the first test tube was 400 µg/ml. Then a series of solutions of varying concentrations (400 - 0.78125 µg/ml) were prepared by serial dilution. To serve as positive control, vincristine sulfate was used at the same concentration. A DMSO solution without sample and standard drug was used as negative control. The number of survived nauplii was counted after 24 hrs for each concentration and the LC_{50} (concentration of 50 % lethality) was calculated from per cent mortality.

The analysis of IC_{50} and LC_{50} values for antioxidant and cytotoxic effect was calculated using the program Graph Pad Prism (version 6.01), USA, respectively.

Results and Discussion

Repeated chromatographic separation and purification of the petroleum ether soluble fraction of the methanol extract of leaves of *Z. mauritiana* yielded a triterpenoid identified as zizyberenalic acid (**1**). No other compounds could be purified because of the limited amount of sample was available.

Compound **1** was obtained as white gummy mass. The compound provided purple colored spot on TLC plate upon spraying of 1% vanillin-sulfuric acid reagent followed by heating at l10 °C for 2 min which suggested its terpenoid nature. The ^{1}H NMR (400 MHz, $CDCl_3$) spectral data of the compound demonstrated four sharp singlets at δ 0.06 (6H, s) 0.98 (3H, s), 1.12 (3H, s), 1.13 (3H, s) which indicated the presence of five methyl groups in the compound. The singlets at δ 4.59 (1H, br s), 4.77 (1H, s) and multiplets from δ 2.26 to 2.99 (1H) could be attributed to the exo-methylene protons at C-30 and C-19 position, respectively. Therefore, a sharp singlet at δ 1.66 for three protons could be assigned to the methyl group at C-29. The spectrum further demonstrated a signal at δ 9.70 (1H, s) which could be attributed to an aldehyde proton at C-2 position of ring A. Thus, the proton signal at δ 6.53 (1H) could be assigned to the olefinic proton at C-3. Accordingly, the structure of the compound was deduced as zizyberenalic acid (**1**, Fig. 1). The spectral data of this compound was in agreement with that reported for zizyberanalic acid isolated from *Paliurus hemsleyanus* (Lee *et al*. 1997) and *Allophylus longipes* (Zhang *et al*. 2012). A very related compound, zizyberanalic acid (**2**), has previously been reported from *Z. jujuba* (Kundu *et al*. 1989). Although zizyberenalic acid (**1**) has been isolated from the fruits of *Z. mauritiana* (Lee *et al*. 2003) this is the first-time report of its isolation from the leaves of this plant.

The extractives of *Z. mauritiana* leaves demonstrated noticeable DPPH free radical inhibition. The methanol extract and its chloroform soluble material displayed strong inhibition of DPPH radicals with the IC_{50} value of 7.37 ± 0.38 µg/ml and 24.14 ± 0.53 µg/ml, respectively as compared to the standard agent, *tert*-butyl-1-hydroxy toluene (BHT) (32.47 ± 0.72 µg/ml) as shown in Table 1.

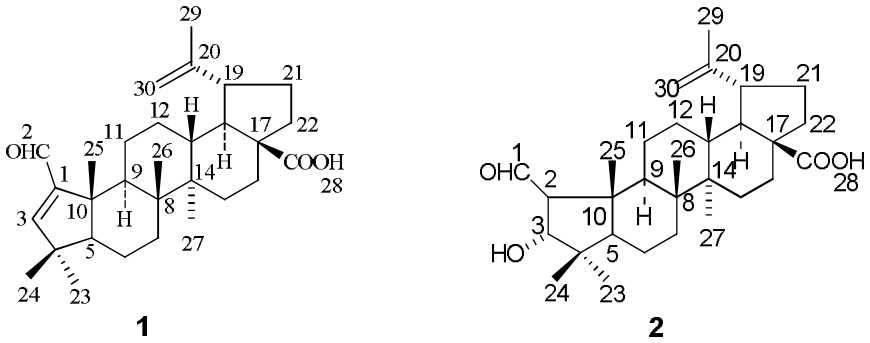

Fig. 1. Chemical structure of zizyberenalic acid (**1**) and zizyberanalic acid (**2**).

Table 1. Effect of *Z. mauritiana* extractives in DPPH free radical inhibition and brine shrimp lethality assay.

Sl. no.	Treatment	IC_{50} (µg/ml)	LC_{50} (µg/ml)
1	Standard	32.47 ± 0.72	0.31 ± 0.01
2	ME	7.37 ± 0.38	6.17 ± 0.12
3	PEF	225.80 ± 0.84	1.31 ± 0.01
4	CF	24.14 ± 0.53	1.38 ± 0.01
5	CTF	57.24 ± 0.69	9.43 ± 0.35

Data are presented as mean ± SEM (n = 3). BHT = *tert*-butyl-1-hydroxy toluene, ME = methanol extract, PEF = petroleum soluble fraction, CF = chloroform soluble fraction, CTF = carbon tetrachloride soluble fraction of *Z. mauritiana* leaves. Standard for DPPH free radical and brine shrimp lethality assay was *tert*-butyl-1-hydroxy toluene (BHT) and vincristine sulfate, respectively. IC_{50} = concentration of 50% DPPH free radical inhibition, LC_{50} = concentration of 50 % lethality.

The organic extractives of *Z. mauritiana* leaves also demonstrated remarkable cytotoxicity in brine shrimp lethality assay which is mentioned in Table 1. The petroleum ether soluble fraction (PEF) produced strong cytotoxic effect (LC_{50} = 1.31 ± 0.01 µg/ml) which was comparable to standard drug, vincristine sulfate (LC_{50} = 0.31 ± 0.01 µg/ml).

The present study reveals that the leaves of Z. *mauritiana* possess noticeable antioxidant and cytotoxic activities. Although the cytotoxic (Iqbal *et al*. 2016) and antioxidant (Al Ghasham *et al.* 2017) effects of the crude methanol extract of the leaves of Z. *mauritiana* have been previously reported, the present investigation reports the activities of methanol extract and its different organic fractions of the plant leaves collected in Bangladeshi sample. Further investigation is warranted with the isolated compounds for validation of the pharmacological effect of the plant. Consideration of the bioactive fractions revealed by the present investigations could be useful for

the isolation of lead compound(s) from the plant. The results of this study suggest that *Z. mauritiana* leaves could be a potential material for the screening of anticancer agents.

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