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Antishigellosis and Cytotoxic Potency of Crude Extracts and Isolated Constituents from *Duranta repens*

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The crude ethanol extracts (stem and fruits), their fractions and two triterpenes, β-Amyrin and 12-Oleanene 3β, 21β-diol, isolated as a mixture from the chloroform soluble fraction of an ethanolic extract of *Duranta repens* stem, were evaluated for antibacterial, antifungal activities by the disc diffusion method and cytotoxicity by brine shrimp lethality bioassay. The structures of the two compounds were confirmed by IR, ^{1}H-NMR, ^{13}C-NMR and LC-MS spectral data. The chloroform soluble fraction of stem and ethanol extract of fruits possess potent antishigellosis activity and also exhibited moderate activity against some pathogenic bacteria and fungi but the isolated compound 1 (mixture of β-Amyrin and 12-Oleanene 3β, 21β-diol) showed mild to moderate inhibitory activity to microbial growth. The minimum inhibitory concentrations (MICs) of the extracts (stem and fruits), their fractions and compound 1 were found to be in the range of 32~128 μg/ml. The chloroform soluble fractions of stem and ethanol extract of fruit showed significant cytotoxicity with LC_{50} value of 0.94 μg/ml and 0.49 μg/ml, respectively against brine shrimp larvae.

KEYWORDS : β-Amyrin, Antimicrobial activity, Cytotoxicity, *Duranta repens*, 12-Oleanene-3-β, 21-β-diol

Shigellosis or bacillary dysentery is endemic throughout the world and accounts for about 15% of diarrhea-associated deaths among the children worldwide (Victora *et al.*, 1993). In Bangladesh, shigella infection is most frequent and the fatality rate between the children is 3.5% (Stoll *et al.*, 1982). The pathogens responsible for this fatal disease are growing resistance day by day in Bangladesh (Bennish *et al.*, 1992). To fight against the resistance of microbes, scientists all over the world have been searching new and potent bioactive principles from plants as plants deserve various bioactive principles and accounts for about 25% of current drugs have derived from plants (Wanyoike *et al.*, 2004). Ethnopharmacological survey of antimicrobial and cytotoxic principles from medicinal plants are extensively going on throughout the world (Somchit *et al.*, 2003; Asha *et al.*, 2003). In continuation of this search we studied the antishigellosis activities of stem and fruit of *Duranta repens* against some bacteria and also examined the cytotoxic properties using brine shrimp nauplii (*Artemia salina*) in artificial sea water.

Duranta repens Linn. (Syn. *Duranta plumieri* Jacq., *D. erecta* Linn. and Eng: Golden dewdrop) is commonly known as pigeon berry and locally called 'Kata mehedi' belongs to the family Verbenaceae. It is shrubs, herbs or small tree usually 1 to 3 m. in height and also grown as a hedge plant in various parts of our country (David, 1981). The plant is not browsed by cattle and is believed to be poisonous (Nelson, 1996). Ethyl acetate and aqueous extracts of leaves showed significant antimalarial activity when administered to mice (Castro *et al.*, 1996). The fruits are used in the treatment of malaria and intestinal worms (Whistler, 2000). The leaves are used in the treatment of abscess (Xiao, 1992).

Previously, wide range of chemical compounds had been isolated from different parts of *Duranta repens*. Especially from stem durantosides I, II, III, duranterectoside A and lamiidoside were isolated (Takida *et al.*, 1995) but there was no report on biological activities of these compounds. Thus, the aim of this investigation was to evaluate the *in vitro* antimicrobial activity and cytotoxiciy of the crude extracts (stem and fruits), solvent partionates of the crude ethanolic extracts and isolation of new bioactive compounds from the chloroform soluble fraction of an ethanolic extract of *Duranta repens* stem.

Materials and Methods

Plant material. The plant parts (stems and fruits) of *Duranta repens* Linn. were collected from the adjoining areas of Rajshahi University Campus, Bangladesh during September to November and were identified by Professor A.T.M. Nadiruzzaman, Department of Botany, University of Rajshahi, Bangladesh where a voucher specimen number (Alam 78, collected on September 19, 1997) has been deposited.

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Extraction and fractionation. *Duranta repens* stem were sun dried and pulverized into a coarse powder. The ground plant materials (1 kg) were then extracted in cold with ethanol (5.0 lit.). After concentration, the ethanol extract was fractionated with chloroform and diethyl ether. The solvents were concentrated by rotary evaporator at 40°C under reduced pressure to afforded a semisolid mass of ethanol extract, diethyl ether soluble fraction and chloroform soluble fraction (90.0, 15.6 and 20.8 gm), respectively. Similar extraction process was followed for the 950 gm of fruit to obtain a semisolid mass of ethanol extract, chloroform soluble fraction and petroleum ether soluble fraction (30.0, 8.0 and 6.0 gm), respectively.

TLC screening. All extracts were run on pre-coated silica gel plate using hexane and ethyl acetate (2 : 1 and 1 : 1) as the mobile phase and vanillin-H_2SO_4 reagent was used as spray reagent. Stem ethanol extract and diethyl ether soluble fraction gave positive tests for steroids and chloroform soluble fraction showed the presence of flavonoids and terpens. On the other hand, fruit ethanol extract gave positive test for steroids and glycosides and the chloroform and petroleum ether soluble fractions mainly showed the presence of flavonoids (Harbone, 1984).

Isolation. The chloroform soluble fraction (5 gm) of stem was subjected to a column chromatography over silica gel eluting with n-hexane and ethyl acetate with increasing polarity which gave a total of 33 fractions. Among these, fractions 4-15 eluted with n-hexane and ethyl acetate (2 : 1) showed similar spots on TLC and were combined together. The combined fractions were then subjected to PTLC using the solvent system n-hexane-ethyl acetate (5 : 1). The pink colored band was observed in an edge of the chromatogram by spraying with vanillin-H_2SO_4 reagent and was scrapped off and eluted with ethyl acetate and evaporated off under reduced pressure to afford a compound **1** (480 mg) as amorphous powder. From spectral analysis, compound **1** was found to be a mixture of two compounds, but their separation was not possible due to similar R_f value and used as such for antimicrobial and cytotoxic activity.

Antimicrobial activity. Antimicrobial assay was performed by disc diffusion method (Vander and Vlietnck, 1991; Rois *et al.*, 1988). Crude extracts (stem and fruits), their fractions and isolated compound were tested for antishigellosis activity against five Gram negative shigella bacteria and also tested for antimicrobial activity against six pathogenic Gram-positive and Gram-negative bacteria and six pathogenic fungi. The sample solution of the materials (extracts, fractions and compound **1**) were prepared by dissolving definite amounts of materials in appropriate solvent to attain the desired concentration and then applied on to sterile disc (6 mm diameter, filter paper) followed by drying off the solvent in an aseptic hood. To compare the activity with standard antibiotics, *Kanamycin* (30 μg/disc) and *Nystatin* (50 μg/disc) were used for antibacterial and antifungal test, respectively.

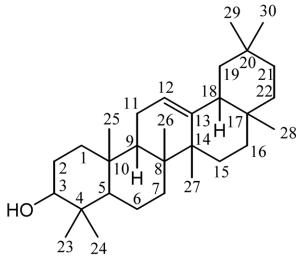
Minimum inhibitory concentration. The minimum inhibitory concentration (MICs) of extracts (stem and fruits), their fractions and compound **1** were determined by serial dilution technique (Reiner, 1982).

Cytotoxicity. For cytotoxicity screening, DMSO solutions of the plant extracts were applied against *Artemia salina* in a 1-day *in vivo* assay (Meyer *et al.*, 1982) according to published protocol. For the experiment 3mg of extracts (stem and fruits), their fractions and compound **1** were dissolved in 0.6 *ml* (600 μl) of DMSO to get a concentration of 5 mg/*ml* and by serial dilution technique, solutions of varying concentrations such as 0.25, 0.50, 1.0 and 2.0 μg/*ml* were obtained. After 24 h. of incubation, the vials were observed using a magnifying glass and the number of survivors in each vial were counted and noted. From this data, the percentage of mortality of the nauplii was calculated for each concentration and the LC_{50} values were determined using Probit analysis as described by Finney (1971).

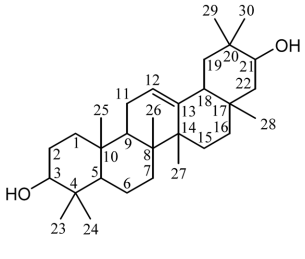
Results and Discussion

Compound **1** isolated from the chloroform fraction of ethanol extract of the stem of *Duranta repens* Linn. as white amorphous powder, decomposed between 121~125°C. IR spectrum of compound **1** showed O-H stretching band between 3445~3888 cm^{-1} and C-O stretching vibration at 1099 cm^{-1}. The C-H and >C=C-H stretching vibrations observed between 2877~2924 cm^{-1} and >C=C< stretching showed a strong band at 1689 cm^{-1}. Although the TLC examination of compound **1** showed a single spot, but LC-MS and the NMR data (both ^1H and ^{13}C) suggested that, compound **1** was not a single one. The ^1H-NMR spectrum (500 MHz, $CDCl_3$) of compound **1** showed two triplets (J=3.6 Hz) at δ_H 5.26 and 5.30 which suggested the presence of two oleanene type triterpenes having double bond at C_{12}-C_{13}. By comparison of ^1H and ^{13}C-NMR data to those published in literature (Hossain and Ismail, 2006; Rahman, 2002) and from LC-MS, it was possible to identify these two triterpenes existed in compound **1** as a mixture of β-Amyrin (major) and 12-Oleanene 3β, 21β-diol (minor) at the ratio of 3 : 1. So far, to our best knowledge, previously α-amyrin was isolated from this species (Mokboul *et al.*, 1981). But both the isolated compound β-Amyrin (Fig. 1) and 12-Oleanene 3β, 21β-diol (Fig. 1) are reported for the first time from this plant.

The results of *in vitro* antishigellosis activity of the



β-Amyrin



12-Oleanene 3β, 21β-diol

Fig. 1. Structures of β-amyrin and 12-Oleanene 3β, 21β-diol.

chloroform soluble fraction of the stem of *Duranta repens* exhibited better activity than ethanol extract, diethyl ether soluble fraction and compound **1** against five shigella bacteria at 30 μg/disc and 100 μg/disc. In contrast, ethanol extract of fruit showed stronger antishigellosis activity than petroleum ether and chloroform soluble fractions against all shigella bacteria (Table 1). Among the two types of samples, fruit ethanol extract showed comparatively better activity than chloroform soluble fraction of stem and both samples showed highest activity against *Shigella dysenteriae* and produced inhibition zone ranging from 10 to 22 mm (Table 1). Again all the samples were tested against six pathogenic (three Gram-positive and three Gram-negative) bacteria at 30 μg/disc and 100 μg/disc (Table 2). All the tested samples (except chloroform fraction of stem and fruit ethanol extract) showed no zone of inhibition at 30 μg/disc and mild to moderate activities at 100 μg/disc. Similarly, the ethanol extract of fruit was more bioactive than the chloroform soluble fraction of stem and both of them exhibited inhibitory activity against *Klebsiella* sp. at 30 μg/disc (09~10 mm) and 100 μg/disc (15~21 mm) (Table 2). *Kanamycin* (30 μg/disc) was used as standard disc for comparison the data in two cases. MIC values were evaluated against five Gram-negative bacteria and the lowest MIC values were observed for chloroform soluble frac-

Table 1. *In vitro* antishigellosis activity of *Duranta repens* (stem and fruits)

Test samples	*Shigella boydii*		*Shigella shiga*		*Shigella dysenteriae*		*Shigell flexneri*		*Shigella sonnie*	
	A	B	A	B	A	B	A	B	A	B
	Zone of inhibition (mm)									
Stem										
Ethanol extract	00	10	00	09	08	12	00	13	00	14
Diethyl ether fraction	00	11	00	09	09	11	00	11	00	14
Chloroform fraction	09	18	08	16	10	19	09	14	00	15
Compound **1**	00	10	00	09	09	13	00	10	00	13
Fruit										
Ethanol extract	09	20	10	19	10	22	08	18	00	20
Petroleum ether fraction	00	11	00	10	08	12	00	10	00	13
Chloroform fraction	00	13	00	13	09	14	00	13	00	15
Standard antibiotic										
Kanamycin (30 μg/disc)	26	-	25	-	26	-	24	-	25	-

A = 30 μg/disc; B = 100 μg/disc

Table 2. *In vitro* antibacterial activity of *Duranta repens* (stem and fruits)

Test samples	*Bacillus subtilis*		*Staphylococcus aureus*		*Streptococcus-β-haemoliticus*		*Escherichia coli*		*Pseudomonas aeruginosa*		*Klebsiella* sp.	
	A	B	A	B	A	B	A	B	A	B	A	B
	Zone of inhibition (mm)											
Stem												
Ethanol extract	00	10	00	11	00	11	00	11	00	09	00	12
Diethyl ether fraction	00	11	00	10	00	10	00	11	00	09	00	14
Chloroform fraction	00	10	00	12	00	14	00	13	00	11	09	15
Compound **1**	00	10	00	10	00	13	00	12	00	10	00	13
Fruit												
Ethanol extract	09	18	09	16	09	17	00	20	09	19	10	21
Petroleum ether fraction	00	10	00	09	00	11	00	12	00	13	00	11
Chloroform fraction	00	13	00	11	00	12	00	14	00	15	00	14
Standard antibiotic												
Kanamycin (30 μg/disc)	21	-	23	-	23	-	22	-	24	-	25	-

A = 30 μg/disc; B = 100 μg/disc

Table 3. Minimum inhibitory Concentrations of *Duranta repens* (stem and fruits)

Test samples	Minimum inhibitory concentrations (μg/ml)				
	Shigella dysenteriae	*Shigella sonnie*	*Shigella flexneri*	*Escherichia coli*	*Klebsiella* sp.
Stem					
Ethanol extract	64	128	128	64	64
Diethyl ether fraction	128	64	128	64	128
Chloroform fraction	32	32	64	64	32
Compound **1**	64	64	128	64	64
Fruit					
Ethanol extract	32	64	32	32	32
Petroleum ether fraction	64	128	128	128	64
Chloroform fraction	64	64	128	64	64

Table 4. *In vitro* antifungal activity of *Duranta repens* (stem and fruits)

Test samples	*Aspergillus niger*		*Aspergillus flavus*		*Aspergillus fumigatus*		*Candida albicans*		*Trichoderma* sp.		*Fusarium* sp	
	A	B	A	B	A	B	A	B	A	B	A	B
	Zone of inhibition (mm)											
Stem												
Ethanol extract	08	12	08	11	08	13	00	08	00	08	00	08
Diethyl ether fraction	08	12	08	11	09	13	00	09	00	09	00	08
Chloroform fraction	10	15	09	13	09	14	00	13	00	12	00	11
Compound **1**	08	13	08	11	08	12	00	09	00	09	00	09
Fruit												
Ethanol extract	10	18	09	16	10	17	00	14	00	12	00	11
Petroleum ether fraction	08	14	08	12	09	13	00	09	00	10	00	09
Chloroform fraction	09	13	08	12	09	11	00	10	00	10	00	10
Standard antibiotic												
Nystatin (50 μg/disc)	22	-	19	-	22	-	20	-	25	-	25	-

A = 50 μg/disc; B = 100 μg/disc

tion of stem (32 μg/ml) against *Shigella dysenteriae*, *Shigella sonnie*, *Klebsiella* sp. and for ethanol extract of fruit (32 μg/ml) against *Shigella dysenteriae, Shigella flexneri*, *E. coli* and *Klebsiella* sp. (Table 3). Six pathogenic fungi were used for antifungal activities at 50 μg/disc and 100 μg/disc and *Nystatin* (50 μg/disc) was used as standard antibiotic disc (Table 4). Chloroform soluble fraction of stem showed better antifungal activity than ethanol extract, diethyl ether soluble fraction and compound **1** and produced zone of inhibition between 09 to 15 mm against *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus fumigatus*. Again the ethanol extract of fruits exhibited good antifungal activity against the same three fungi and produced the inhibitory zone between 09 to 18 mm. In all cases the isolated compound **1** showed mild to moderate antimicrobial activities compared to its crude extract and fractions (Table 1, 2, 3 and 4). The present result is similar to Dhembare and Sarla (2003) who reported that the alcoholic extract of *Duranta repens* showed antibacterial activity against human pathogenic bacterial strains, *Proteus*, *Pseudomonas*, *Klebsiella*, *E. coli* and *Staphylococcus aureus.* Patil *et al*. (2002) also reported that the fruit extracts of *D. repens* showed potent antibacterial and antifungal activity against *Xanthomonas citrii* and *Aspergillus* and *Penicillum* sp., respectively at 50, 100 and 500 ppm concentrations and they showed spectacular activities at higher concentrations.

The toxicity of stem and fruits of *Duranta repen* Linn. were observed against brine shrimp nauplii and all the samples showed strong toxicity. Among the samples the chloroform soluble fraction of stem and the ethanol extract of fruit showed the highest toxicity and LC_{50} value was 0.94 μg/*ml* and 0.49 μg/*ml*, respectively and compound **1** exhibited moderate activity (LC_{50} 1.23 μg/*ml*) (Table 5).

The ethanol extract of fruits has potent antimicrobial and cytotoxic activities than other extract, fraction and isolated compound **1**. In conclusion, our results reveal that the stem and fruits of *Duranta repen* Linn were effective against both Gram-positive and Gram-negative bacteria, especially shigella bacteria and also effective against pathogenic fungi. Cytotoxicity of the plant on brine shrimp nauplii was also evaluated. However, more research should

Table 5. Cytotoxicity of *Duranta repens* (stem and fruits) against brine shrimp nauplii

Test samples	LC_{50} ($\mu g/ml$)
Stem	
Ethanol extract	1.36
Diethyl ether fraction	1.06
Chloroform fraction	0.94
Compound **1**	1.23
Fruit	
Ethanol extract	0.49
Petroleum ether fraction	1.21
Chloroform fraction	0.81

be directed towards the isolation of bioactive compounds from fruits and further toxicological studies (acute, subacute and chronic toxicity) are needed, in order to establish its safety and evaluate possible clinical application in therapy of infections diseases.

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