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Isolation and Characterization of Secondary Metabilites and Evaluation of Antimicrobial, Antioxidant and Thrombolytic Potentials of *Erythrina variegata* L. Bark

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Six compounds, sitosta-4-en-3-one (**1**), 3β,28-dihydroxyolean-12-en (**2**), scandenone (**3**), alpinum isoflavone (**4**), stigmasterol (**5**) and lupeol (**6**) were isolated from the methanol soluble extract of the stem bark of *Erythrina variegata*. The structure of the compounds was established by extensive NMR studies as well as co-TLC with authentic sample. The petroleum ether, carbon tetrachloride, chloroform and aqueous soluble fractions of the methanolic extract of *Erythrina variegata* were evaluated for antimicrobial, antioxidant and thrombolytic properties. In the antimicrobial study, most of the fractions of the extract exhibited mild to moderate antimicrobial activity where the zone of inhibition were ranging from 7.69 to 19.51 mm. The chloroform soluble fraction showed significant antioxidant activity with IC_{50} value 66.28 µg/mL as compared to standard BHT (IC_{50} value 23.09 µg/mL). The thrombolytic property of different extracts of *E. variegata* exhibited moderate activity ranging from 31.25 to 57.78 %.

Keywords: *Erythrina variegata*, Isoflavone, Triterpene, Sitosta-4-en-3-one, Antibacterial, Antioxidant, Thrombolytic.

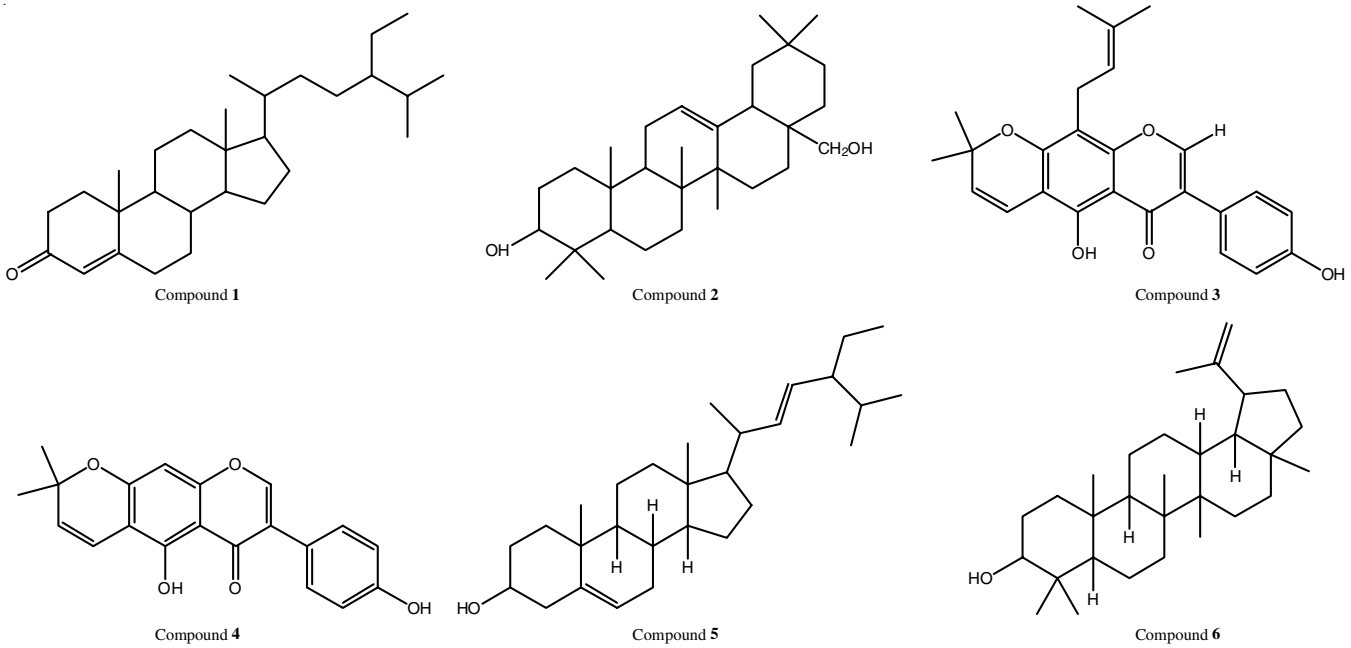
INTRODUCTION

The plant *Erythrina variegata* L. (Bangla/Vernacular Name: Palitamadar, Paniamadar, Raktamadar, Mandar, Madar) belonging to the Fabaceae family [1-3] is a thorny deciduous tree with thick clusters of scarlet or crimson or showy red flowers and black seeds [4,5].

The genus is comprised of more than 110 species and is native in the tropical and subtropical regions of the globe [6]. Traditionally various parts of the plant has been used as folk medicine for various ailments such as nervine sedative, febrifuge, antiasthmatic and antiepileptic [7] at various countries in the south Asian regions [8,9]. In Bangladesh the plant is found throughout the country and is often used as an astringent as well as in fever and leprosy; whereas the leave paste is externally used to relieve joint pain and to cure the inflammation [10]. Experimentally the plant has demonstrated potential remedial effects for the treatment of some diseases, namely, bacterial infection, convulsion, cuts and wounds, helminthiasis, cough and insomnia [11-14].

Earlier study on the plant revealed the presence of isoflavones, flavanones and a cinnamyl phenol [15-17]. The main isoflavones are erythrinins A, B and C, osajin and alpinum, along with styrene oxyresveratrol and dihydrostilbene dihydroxyresveratrol [18,19].

Various parts of *E. variegata* has found to be highly rich in multiple types of alkaloids namely spiroamine alkaloids, carboxylated indole-3-alkylamines [20], tetracyclic alkaloids [12], scoulerine, (+)coreximine, L-reticuline, erybidine [21], water-soluble bases, such as erysovine and stachydrine [22], spiroamine alkaloid [13], isoquinoline (erythritol) and isococcolinine alkaloids [23]. The present study has been undertaken to isolate and identify biologically active secondary metabolites. We, herein, report the isolation of two isoflavones and four triterpines [sitosta-4-en-3-one (**1**), 3β,28-dihydroxyolean-12-en (**2**), scandenone (**3**), alpinum isoflavone (**4**), stigmasterol (**5**) and lupeol (**6**)] (Fig. 1) from this plant and evaluated the antioxidant, thrombolytic and antimicrobial activities of different fractions of the extracts.



Compound **1** Compound **2** Compound **3**

Compound **4** Compound **5** Compound **6**

Fig. 1. Structure of the compounds **1-6** isolated from the stem of *E. variegata*

EXPERIMENTAL

Preparative TLC was conducted over glass plates coated with silica gel 60 PF254 (0.5 mm thickness, Merck) and compounds were detected with vanillin H_2SO_4 spray reagent. Gel permeation chromatography was performed using Sephadex LH-20. ^{1}H NMR spectra were recorded in $CDCl_3$ (δ values were reported in reference to $CHCl_3$ at 7.25 ppm) on a Bruker Avance100 and 400 MHz Ultrashield NMR spectrophotometer.

Stem bark of *Erythrina variegata* L. was collected from the campus of University of Dhaka in December 2015. Plant material was taxonomically identified by the taxonomist Shah Mohammad Ahsan Habib, Senior Herberium Technitian, Bangladesh National Herbarium, Dhaka, where a voucher specimen (accession number DACB No.46874) has been deposited for future reference.

Extraction and isolation: The stem bark of *E. variegata* was sun dried and then ground into course powder using a grinding machine and the powder plant material (900 g) of *E. variegata* was soaked in 2.5 L of methanol for 20 days and then filtered through a cotton plug followed by Whatman filter paper No. 1. The extract was concentrated at reduced pressure with a vacuum rotary evaporator at 40 °C. An aliquot of the crude methanolic extract (25.6 g) was fractionated by vacuum liquid chromatographic (VLC) technique using silica gel 60H and petroleum ether, petroleum ether-dichloromethane, dichloromethane-ethyl acetate, ethyl acetate and ethyl acetate-methanol in increasing order of polarity and thus 36 vacuum liquid chromatographic fractions were obtained.

The vacuum liquid chromatographic fraction of 2 % ethyl acetate in dichloromethane was subjected to gel permeation chromatography on Sephadex LH-20 using a mobile phase of petroleum ether, chloroform and methanol in order of increasing polarities to induce adsorption and partition quality like a normal phase column chromatography for better separation of components. Compound **1** (5 mg) was obtained as white crystal from the Sephadex column fraction eluted with 10-20 % petroleum ether in chloroform. The vacuum liquid chromatographic fraction of 8 % ethyl acetate in dichloromethane was further subjected to gel permeation chromatography following the previous procedure. The sephadex column fraction eluted with 10-20 % petroleum ether in chloroform was subjected to preparative TLC (toluene-EtOAc = 95:5) to obtain compound **2** (4 mg), compound **3** (4 mg) and compound **4** (3.5 mg). On the other hand, compound **5** (14 mg) was obatined as colourless crystal from the VLC fraction of 5 % ethyl acetate and the Sephadex column fraction eluted with 20 % petroleum ether in chloroform. Compound **6** (12 mg) was obtained from the VLC fraction of 10 % ethyl acetate in dichloromethane, which was purified by crystallization process.

Preparation of sample for biological investigation: The crude methanoic extract of *E. variegata* was subjected to solvent-solvent partitioning following the protocol designed by Kupchan [24] and modified by Van Wagenen *et al*. [25]. 5 g crude bark extract was dissolved in 10 % aqueous methanol to make the mother solution which was successively partitioned by petroleum ether, carbon tetrachloride, chloroform and water in order of increasing polarity by using separating funnel.

Antimicrobial activity: The antimicrobial activity of the extracts was determined against the test organisms by disc diffusion method described by Bauer *et al.* [26]. The sample solution of the extracts were prepared by dissolving a definite amount of material in appropriate solvent to give the desired concentration and then applied on sterile filter paper disc (6 mm diameter) followed by the drying off the solvent. Kanamycin (30 µg/disc) was used as standard and a blank disc impregnated with 10 µL of solvent followed by drying was used as a negative control. Discs containing the test materials, standard and negative control were placed onto nutrient agar medium uniformly seeded with the test microorganisms. The plates were kept at low temperature (4 °C) for 24 h to allow

maximum diffusion of the test materials and standard. The plates were then incubated at 37 °C for 24 h to allow maximum growth of the organisms. The test materials having antimicrobial activity showed a clear, distinct zone of inhibition surrounding the discs.

Antioxidant activity (DPPH free radical scavenging assay): Following the method developed by Brand-Williams *et al*. [27] the antioxidant activity of the different fractions of methanolic extract of *E. variegata* was assessed by evaluating the scavenging activities of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. 2 mL of the different concentrations (400 to 0.78125 µg/mL) of the test samples were mixed with 2 mL of DPPH solution (20 µg/mL) in methanol. After 0.5 h of reaction period at room temperature in dark, the absorbance was measured at 517 nm. Then the IC_{50} values (concentration of sample required to scavenge 50 % of free radicals) were calculated from the regression equation, developed by plotting concentration of the samples *versus* percentage inhibition of free radicals. Here, a synthetic antioxidant, butylated hydroxytoluene (BHT) was used as positive control.

Thrombolytic activity: The thrombolytic property was carried out according to the method developed by Prasad *et al.* [28] where standard streptokinase (100 µL) was used as positive control and water as negative control. Aliquots (5 mL) of venous blood were drawn from healthy volunteers who were distributed in 10 different pre-weighed sterile Eppendorf tubes (0.5 mL/tube) and incubated at 37 °C for 45 min. After clot formation, the serum was completely removed without disturbing the clot and each eppendorf tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). To each eppendorf tube containing pre-weighted clot, 100 µL aqueous solution of different extract along with the crude extract, positive and negative control were added separately. All the Eppendorf tubes were then incubated at 37 °C for 1.5 h and observed for clot lysis. Then the released fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption.

Clot lysis was expressed as percentage:

$$\text{Clot lysis (\%)} = \frac{\text{Weight of the lysis clot}}{\text{Weightof clot before lysis}} \times 100$$

Spectral data

Sitosta-4-en-3-one (1): Needle like crystal. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.73 (3H, *s*, CH_3 -18), 0.83 (3H, *d*, *J* = 7.0 Hz, CH_3 -26), 0.85 (3H, *d*, *J* = 6.6 Hz, CH_3 -27), 0.87 (3H, *t*, *J* = 7.0 Hz, CH_3 -29), 0.94 (3H, *d*, *J* = 6.6 Hz, CH_3 -21), 1.20 (3H, *s*, CH_3 -19), 5.74 (1H, *s*, H-4) [29].

3β,28-Dihydroxyolean-12-ene (2): Colourless solid, ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.18 (H-3), 5.18 (d, H-12 *J* = 10.8 Hz), 0.96 (s, H_3-23), 0.74 (3H, s, H-24), 0.87 (3Hs, H-25), 0.98 (3H, s, H-26), 1.15 (3H, s, H-27), 0.95 (3H, H-29), 0.88 (3H, H-29), 3.20 (d, H-28a, *J* = 10.8 Hz), 3.52 (d, H-28b, *J* = 10.8 Hz) [30].

Scandenone (3): Yellow crystals, ^{1}H NMR (400 MHz, $CDCl_3$): δ 13.17 (1H, s, OH-5), 7.88 (1H. s, H-2), 7.39 (2H, d, *J* = 8.0 Hz, H-2', H-6'), 6.90 (2H, d, *J* = 8.0 Hz, H-3', H-5'), 6.72 (1H, d, *J* = 10 Hz, H-4"), 5.60 (1H, d, *J* = 10 Hz, H-3"), 5.17 (1H, t, *J* = 7.2 Hz, H-2'"), 4.32 (1H, s, OH-4'), 3.39 (2H, d, *J* = 7.2 Hz, H_2-1'"), 1.80, 1.70 (6H, 2 x s, Me_2-3'"), 1.46 (6H, Me_2-2") [19].

Alpinum isoflavone (4): Yellow needles, ^{1}H NMR (400 MHz, $CDCl_3$-CD_3OD): δ 1.47 (6H, s, H-5', 6'), 5.63 (1H, d, *J* = 10 Hz, H-3"), 6.34 (1H, s, H-8), 6.73 (1H, d, *J* = 10 Hz, H-4"), 6.87 (2H, d, *J* = 8.7 Hz, H-3', 5'), 7.36 (2H, d, *J* = 8.7 Hz, H-2', 6'), 7.82 (1H, s, H-2), 13.10 (1H, s, 5-OH) [31].

Stigmasterol (5): White crystal, ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.70 (3H, *s*, CH_3 -13), 0.82 (3H, *t*, CH_3 -28), 0.84 (3H, *d*, *J* = 6.6 Hz, CH_3 -25), 0.86 (3H, *d*, *J* = 6.6 Hz, CH_3 -25), 0.94 (3H, *d*, *J* = 6.6 Hz, CH_3 -20), 1.02 (3H, *s*, CH_3 -10), 3.53 (1H, *m*, H-4), 5.04 (1H, *dd*, *J* = 15.0, 9.0 Hz, H-23), 5.16 (1H, *dd*, *J* = 15.0, 6.5 Hz, H-22), 5.36 (1H, *m*, H-6) [29].

Lupeol (6): Colourless crystalline mass was identified by co-TLC with the authentic sample in different solvent systems.

RESULTS AND DISCUSSION

Chemical investigation of *Erythrina variegate* L.: Extensive chromatographic separation and purification of the methanol soluble extract of the stem bark of *E. variegata* provided a total of six compounds (**1-6**), the structures of which were elucidated by ^{1}H NMR analysis. This is the first report of isolation of sitosta-4-en-3-one from this genous.

Biological investigations of *Erythrina variegate* L: The Kupchan fractions of the methanolic extract of *E. variegata* and the isoflavones demonstrated various degrees of bioactivities when subjected to antimicrobial, antioxidant and thrombolytic activities. In case of antimicrobial screening, the fractions and the compounds showed mild to moderate antimicrobial activity

TABLE-1
ANTIMICROBIAL ACTIVITY OF *E. variegata* EXTRACTS AND KANAMYCIN

Sample	Diameter of zone of inhibition (mm)					
	Gram-positive bacteria		Gram-negative bacteria			
	Bacillus subtilis	*Bacillus cereus*	*Pseudomonas aeruginosa*	*Salmonella paratyphi*	*Shigella dysenteriae*	*Vibrio cholerae*
Petroleum ether fraction (500 µg/disc)	10.76±0.63	18.53±89	NA	9.24±0.72	10.6±0.71	18.37±0.77
Carbon tetrachloride (500 µg/disc)	18.93±0.39	19.51±1.18	NA	13.12±0.33	11.87±0.68	15.78±0.88
Chloroform fraction (500 µg/disc)	12.74±0.78	17.88±0.69	12.78±0.44	9.01±0.75	10.84±0.70	13.64±0.55
Aqueous fraction (500 µg/disc)	13.55±0.94	NA	14.31±0.57	17.88±0.72	10.4±0.92	9.73±0.60
Scandenone (100 µg/disc)	NA	10.73±0.82	10.47±0.73	9.45±0.76	NA	NA
Alpinum isoflavone (100 µg/disc)	12.12±0.54	9.03±0.72	NA	11.0±1.06	NA	7.69±0.55
Kanamycin (30 µg/disc)	25.88±0.59	24.38±0.44	29.46±0.92	24.31±0.45	27.45±0.73	30.15±1.36

NA = Not active; Diameter of zone of inhibition was expressed as mean ± SD.

against most of the microorganisms. The petroleum ether, carbon tetrachloride and chloroform extracts exhibited good antimicrobial activity against *Bacillus cereus.* The isoflavones showed mild activity (some cases no activity) against the test microorganisms (Table-1).

In case of evaluation of antioxidant activity the IC_{50} value of butylated hydroxy toluene (BHT) (reference) was 23.09 µg/mL where, carbon tetrachloride, chloroform and aqueous fraction of the plant showed the moderate inhibitory concentration with IC_{50} value of 93.85, 66.28 and 75.02 µg/mL, respectively (Table-2).

TABLE-2
ANTIOXIDANT ACTIVITY OF
DIFFERENT FRACTIONS OF *E. variegata*

Sample	Free radical scavenging activity (IC_{50} in µg/mL)
Petroleum ether fraction	418.21 ± 6.40
Carbon tetrachloride	93.85 ± 1.04
Chloroform fraction	66.28 ± 3.64
Aqueous fraction	75.02 ± 2.62
Butylated hydroxy toluene	23.09 ± 1.37

The extractives of bark of *Erythrina variegata* showed moderate thrombolytic activity. Among all the fractions, the pet ether and aqueous fraction showed highest clot lysis activity (56.78 and 57.78 %, respectively), whereas standard streptokinase at 37 °C showed 76.45 % lysis of the clot as compared to distilled water showing a negligible lysis of clot (3.49 %) (Table-3).

TABLE-3
THROMBOLYTIC ACTIVITY OF
DIFFERENT FRACTIONS OF *E. variegata*

Sample	Clot lysis (%)
Negative control (water)	3.49 ± 0.28
Petroleum ether fraction	56.78 ± 0.55
Carbon tetrachloride	31.25 ± 0.45
Chloroform fraction	43.89 ± 0.76
Aqueous fraction	57.78 ± 0.24
Streptokinase	76.45 ± 0.90

It is evident from the bioassays, that various fraction of the crude methanol extract of *E. variegata* demonstrated significant antibacterial, antioxidant and thrombolytic activities. These bioactivities support the folk uses of *E. variegata* in various diseases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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