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Entada rheedii seeds thioamides, phenolics, and saponins and its antiulcerogenic and antimicrobial activities

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ABSTRACT

Entada rheedii (Fabaceae) seeds are used against diarrhea and stomach aches. This study aims at scientifically validate its ethnomedicinal uses. Seeds ethanol (70%) extract (EE) was prepared by percolation. Antibacterial and antiviral activities of EE and isolated compounds were determined using agar well diffusion and MTT assays, respectively. Anti-ulcerogenic activity was evaluated using ethanol-induced ulcer model. Four phenolics: protocatechuic acid **C1**, protocatechuic acid methyl ester **C2**, 1,3,4-trihydroxybenzene glucoside **C3**, phaseoloidin **C4**, three thioamides: entadamide A **C5**, entadamide A-*β*-d-glucopyranoside **C6**, entadamide C **C7**, and two saponins: rheedeioside A **C8** and rheedeioside B **C9** were isolated from EE. EE, **C4**, **C5**, and **C8** evidenced significant ($p < 0.05$) antiulcerogenic activity. Strong antibacterial activity was reported for EE, **C1**, and **C7**. **C4** exhibited moderate (35% inhibition) antiviral activity. This study provides scientific validation of the seed ethnomedicinal use in treating gastric ailments and as antimicrobial.

INTRODUCTION

Entada rheedii Spreng. (Fabaceae) forests are found in tropical Asia; East Africa, Australia, Indian Ocean islands (Langran *et al.*, 2009). It is widely distributed in tropical and subtropical countries bordering the Indian ocean. Its synonyms are *E. pursaetha* Candolle; *E. pursaetha* subsp. *sinohimalensis.* Long; *E. pursaetha* var. *sinohimalensis* (Grierson & D. G), *E. scandens* (L) Benth. (Joshi, 2000). Most of its traditional uses were previously scientifically proved e.g. anti-inflammatory (Bhogireddy *et al.*, 2015), wound healing (Vidya *et al.*, 2012) and antidiarrheal activities (Dey *et al.*, 2013). The plant folklore use in stomach problems (Bako *et al.*, 2005; Uddin, 2006; Dey *et al.*, 2013) and its use as antimicrobial (Dash and Padhy, 2006) (Vidya *et al.*, 2012; Nzowa *et al.*, 2013) is not yet fully investigated. This study investigates *E. rheedii* seeds potential in the treatment of stomach ulcers especially that high levels of glutamic acid, a powerful antiulcerogenic amino acid, was reported before in the plant (Okba *et al.*, 2013). Special emphasis was made on the evaluation of its antibacterial and antiviral potential. In addition, the scarcity of reports concerning the bioactive phytochemicals of the seeds of the cited plant encouraged the authors towards their isolation, identification, and assessment of their bioactivities.

MATERIAL AND METHODS

Plant material

E. rheedii Spreng. Seeds were purchased from the Experimental Station of Medicinal Plants, Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Giza. Dr. Mohammed El-Gibali senior botanist confirmed its identity. The plant name has been checked with the International Plant Names Index (IPNI, 2004). Voucher samples (herbarium no. 14.4.2013.1) were deposited at the Museum of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

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Seeds extraction and fractionation

Powdered seeds (3 kg) were defatted with *n*-hexane. Defatted mark was extracted using ethanol (70%) by percolation at room temperature for 48 h yielding the ethanol extract (EE). EE (300 gm) was suspended in distilled water and successively fractionated using methylene chloride, and ethyl acetate. The EE fractions; methylene chloride (EE.MC), ethyl acetate (EE.EA), and remaining aqueous (EE.AQ), were used for isolation of the major compounds and biological evaluation.

Isolation of the major constituents

General

Stationary phases: Thin layer chromatography (TLC) was carried out on precoated silica gel F254 plates, (20 × 20 cm, Sigma-Aldrich Chemicals-Germany). Meanwhile, various stationary phases were used for column chromatography (Cioffi *et al.*, 2006) viz., non-ion exchange resin (Diaion HP20 Sigma-Aldrich, St. Louis, MO, USA), Sephadex LH 20 (Pharmacia Fine Chemicals AB Uppsala, Sweden), silica gel 60 H (70-230 mesh, Fluka, Sigma-Aldrich chemicals-Germany) and Silica gel RP-18 (70-230 mesh, Fluka, Sigma-Aldrich chemicals-Germany) for medium pressure liquid chromatography (MPLC). Silica gel H was used for vacuum liquid chromatography (VLC). Solvents (of analytical grades) and authentic reference samples used were all purchased from E-Merck (Darmstadt, Germany). Solvent systems and spray reagents: S1: methylene chloride: methanol (9.1:0.9 v/v), S2: methylene chloride: methanol: formic acid (8.5:1.5:4 drops v/v/v), S3: methylene chloride: methanol: formic acid (6:4:20 drops v/v/v), S4: n-Butanol: acetic acid: water: methanol (4:1:1:0.5 v/v/v/v), S5: methylene chloride: methanol (7:3 v/v) were used. Spray reagents used were p-anisaldehyde-sulfuric acid, ferric chloride and iodine vapors (Smith, 1960; Stahl, 1969). Equipment and Apparatus: Chromatographic jars and glass columns of different dimensions were used for TLC and CC, respectively. UV lamp (λ_{max} = 254 and 330 nm, Shimadzu), a product of Hanovia lamps, was used for spot visualization on the chromatograms and a UV/Visible spectrophotometer, Shimadzu UV-1650 PC for recording UV spectra. Varian Mercury NMR-spectrometers (Japan) were used: 600 MHz for recording ^{1}H-NMR and 125 MHz for ^{13}C-NMR; spectra were recorded in DMSO or CD3OD using TMS as internal standard and chemical shift values expressed in δ ppm. The mass of the compounds was detected using ESI-MS (electrospray ionization mass spectrometry) in negative mode.

Phenolics isolation

Part of the EE.EA (870 mg; TLC: 2 major spots, R_f 0.62 S_1 & 0.44 S_1, both gave dull brown color under UV 254 nm & bluish black color with ferric chloride) was chromatographed on Sephadex LH 20 and silica gel 60 H columns resulted in isolation of **C1** [R_f = 0.62 S_1] and **C2** [R_f = 0.44 S_1].

Thioamides isolation

From EE.MC (2.5 g; TLC: 3 major spots, R_f 0.58 S_1, 0.35 S_1 and 0.16 S_5, the first two gave a dull brown color under short U.V. 254 nm while the third gave green color with *p*-anisaldehyde/sulphuric) was enriched with fraction A from EE.EA (stated in 2.3.3.2 section) was fractionated on several silica gel 60 H column resulted in isolation of **C5** [R_f = 0.58 S_1 & 0.82 S_2], **C7** [R_f = 0.35 S_1] and **C3** [R_f = 0.16 S_5].

From EE.EA (4.5 g; TLC: 2 major spots, R_f 0.82 S_2 (=0.58 S_1) & 0.25 S_2, both gave a dull brown color under short U.V. 254 nm, brown with iodine vapors and faint yellow and faint green colors after spraying with *p*-anisaldehyde/sulphuric reagent, respectively) was fractionated on Sephadex LH 20 column afforded two collective fractions A and B. Fraction A was added to methanol fractions of EE.MC and fractionated as mentioned before (section 2.3.3.1). Fraction B was purified over silica gel 60 H yielding **C6** [R_f = 0.25 S_2].

Saponins isolation

EE.AQ (150 g; TLC: 3 spots, R_f 0.66 S_4, 0.28 S_3 and 0.21 S_3, the first gave green, greyish brown & greyish brown color with *p*-anisaldehyde/sulphuric acid reagent respectively) were chromatographed over non-ion exchange resin (diaion). Two collective fractions I & II were obtained. Fraction I was refractionated over a VLC, sephadex LH-20 followed by repeated CC using silica gel 60 H columns to yield **C4** [R_f = 0.66 S_4 & 0.17 S_2]. Fraction II was rechromatographed on a silica gel 60 H. The eluted fractions showed the presence of **C4** and another spot [R_f = 0.28 S_3 and 0.21 S_3]. They were rechromatographed on a sephadex LH 20 column affording two main sub-fractions, the first revealed on TLC a minor spot [R_f = 0.28 S_3] of **C9**; while the second upon purification on MPLC afforded **C8** [R_f = 0.21 S_3].

Biological evaluation

Done in accordance with ethical procedures and policies approved by the Animal Care and Committee of National Research Center, Dokki, Cairo.

LD_{50}

It was estimated using the acute toxic class method as per OECD guideline (Organization for Economic Co-operation and Development, Guideline-423, adopted on 17th December 2001). After that, a dose-response curve was conducted for median effective dose determination.

Antiulcerogenic activity

Ethanol-induced ulcer model was adopted. Lesions were examined under an illuminated magnifier (Amaral *et al.*, 2013).

Antibacterial activity

The agar well diffusion assay method as described by (Holder and Boyce, 1994) was adopted. Ten pathogenic bacteria were used *Mycobacterium africanum, M. bovis, M. caprae, M. microti, M. orygis, Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhimurium* (RCMB 0023645, 0023642, 00236940, 00236948, 00236941, 000106, 010010, 010043, 010052, 010072, respectively). The tested bacteria were subcultured on nutrient agar medium (Oxoid laboratories, UK). Gentamicin (Misr Company for Pharmaceutical Industry, Mataria, Cairo, Egypt) was used as a positive control. The diameter of inhibition zone was measured and the experiment was done in triplicate (Agwa *et al.,* 2000). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration were estimated according to

(Doughari, 2006).

Antiviral activity

VERO cells (kidney epithelial cells of African green monkey) incubated into culture bottle were checked using the inverted microscope for its proper physical conditions. Healthy cells propagation, determination of extract cytotoxicity and MTT assay protocol were done according to (Gong, 2013).

RESULTS AND DISCUSSION

Chromatographic fractionation of the EE.ME, EE.EA and EE.AQ allowed the isolation of nine compounds. They were identified through their physicochemical characters, co-TLC comparison to authentic reference samples, spectroscopic analysis and through previously published data.

Compound C1: 29 mg, colorless needles. EI/MS at *m/z* 153.12 $(M-H)^-$. Its ^{1}H-NMR spectral data showed signals at 7.5 (1H, s, H-2), 6.8 (1H, d, J = 8.1 Hz, H-5) and 7.45 (1H, J = 8.1 Hz, H-6). Its ^{13}C-NMR spectral data showed the following peaks: 124.1 (C1), 117.6 (C2), 151.09 (C3), 145.44 (C4), 115.7 (C5), 122.7 (C6) and 170.9 (C7).

Compound C2: 104 mg, pale buff needles. EI/MS at *m/z* 167.14 $(M-H)^-$. Its ^{1}H-NMR spectral data showed signals at: 7.48 (1H, s, H-2), 6.78 (1H, d, J = 8 Hz, H-5), 7.39 (1H, d, J = 2.1 Hz, H-6) and 3.821 (3H, s, H-8). Its ^{13}C-NMR spectral data showed theses peaks: 122.5 (C1), 115.8 (C2), 146.12 (C3), 151.64 (C4), 117.41 (C5), 123.6 (C6), 168.83 (C7) and 52.22 (C8).

Compound C3: 18 mg, white needles. EI/MS at *m/z* 287.13 $(M-H)^-$. Its ^{1}H-NMR spectral data showed signals at 7.05 (1H, d, J = 6 Hz, H-6), 6.668 (1H, d, J = 1.6 Hz, H-3) and 6.607 (1H, dd, H-5). Its ^{13}C-NMR spectral data showed the following peaks 145.3 (C1), 153.84 (C2), 119.11 (C3), 150.6 (C4), 118.6 (C5), 115.23 (C6), 104.7 (C1'), 75.07 (C2'), 77.9 (C3'), 71.49 (C4'), 78.13 (C5') and 62.65 (C6').

Compound C4: 197 mg, white needles. EI/MS at *m/z* 329.14 $(M-H)^-$. Its ^{1}H-NMR spectral data showed: 6.67 (1H, d, J = 2.7 Hz, H-3), 6.63 (1H, dd, J = 8.7 Hz, 3Hz, H-5) and 7.045 (1H, d, J = 8.7 Hz, H-6). Its ^{13}C-NMR spectral data showed the following peaks 149.15 (C1), 126.3 (C2), 117.08 (C3), 152.5 (C4), 117.8 (C5), 114.25 (C6), 35.5 (C7), 175.2 (C8), 103.2 (C1'), 73.7 (C2'), 76.5 (C3'), 70.04 (C4'), 76.7 (C5') and 61.26 (C6').

Compound C5: 78 mg, light brown syrup. EI/MS at *m/z* 160.21 $(M-H)^-$. Its ^{1}H-NMR spectral data showed signals at: 5.81 (1H, d, J = 14.7 Hz, H-2), 7.534 (1H, d, J = 14.7 Hz, H-3), 6.2 (1H, s, NH), 3.377 (2H, J = 4.2 Hz, CH2 attached to NH), 3.646 (2H, t, J = 5.4 Hz, CH2 beside OH) and 2.311 (3H, s, CH3 attached to S). Its ^{13}C-NMR spectral data showed the following peaks 164.12 (C1), 116.38 (C2), 143.29 (C3), 42.7 (NHCH2), 61.45 (CH2OH) and 14.4 (SCH3).

Compound C6: 23 mg, light brown syrup. EI/MS at *m/z* 322.36 $(M-H)^-$. Its ^{1}H-NMR spectral data showed: 5.86 (1H, d, J = 14.7 Hz, H-2), 7.58 (1H, d, J = 14.7 Hz, H-3), 2.32 (3H, s, CH_3 attached to S) and 4.29 (1H, d, J = 7.5 Hz, anomeric proton). Its ^{13}C-NMR spectral data showed the following peaks 167.49 (C1), 116.63 (C2), 143.82 (C3), 40.7 (NHCH2), 69.84 (CH2OH), 14.4 (SCH3), 104.56 (C1'), 75.14 (C2'), 78.006 (C3'&C5'), 71.605 (C4') and 62.708 (C6').

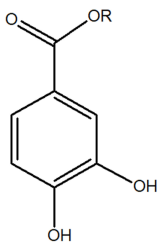
Compound C7: 14 mg, white amorphous powder. EI/MS at *m/z* 176.21 $(M-H)^-$. Its ^{1}H-NMR spectral data showed: 6.707 (1H, s, J = 15 Hz, H-2), 7.616 (1H, d, J = 15 Hz, H-3), 3.644 (2H, t, J = 5.4 Hz, CH2 attached to NH), 3.4 (2H, t, J = 5.4 Hz, CH2 beside OH) and 2.743 (3H, s, CH3 attached to S). Its ^{13}C-NMR spectral data showed the following peaks 165.268 (C1), 129.3 (C2), 147.48 (C3), 43.3 (NHCH2), 61.339 (CH2OH) and 39.894 (SCH3).

Compounds C8: 83 mg, transparent amorphous powder. Its ^{13}C-NMR spectral data showed the following peaks 38.35 (C1), 25.6 (C2), 88.27 (C3), 38.37 (C4), 54.77 (C5), 18.01 (C6), 36.30 (C7), 40.03 (C8), 46.68 (C9), 36.40 (C10), 23.08 (C11), 123.81 (C12), 143.22 (C13), 47.32 (C14), 67.00 (C15), 77.6 (C16), 47.44 (C17), 40.37 (C18), 46.41 (C19), 30.13 (C20), 32.97 (C21), 30.19 (C22), 27.50 (C23), 16.3 (C24), 15.30 (C25), 17.10 (C26), 19.70 (C27), 174.90 (C28), 32.886 (C29), 24.05 (C30), 103.6 (C1'), 55.13 (C2'), 79.56 (C3'), 73.21 (C4'), 75.74 (C5'), 67.806 (C6'), 102.7 (C1''), 73.44 (C2''), 76.44 (C3''), 69.29 (C4''), 64.05 (C5''), 101.8 (C1'''), 71.91 (C2'''), 81.61 (C3''') 66.92 (C4'''), 76.33 (C5'''), 63.31 (C6'''), 106.2 (C1''''), 74.79 (C2''''), 76.74 (C3''''), 69.55 (C4''''), 65.89 (C5''''), 92.34 (C1'''''), 79.14 (C2'''''), 81.95 (C3'''''), 69.46 (C4'''''), 77.33 (C5'''''), 60.58 (C6'''''), 108.77 (C1''''''), 77.58 (C2''''''), 79.19 (C3''''''), 73.39 (C4''''''), 69.39 (C5''''''), 108.99 (C1'''''''), 76.115 (C2'''''''), 78.661 (C3'''''''), 72.16 (C4'''''''), 63.49 (C5'''''''), 20.65 ($NHCO\mathbf{C}H_3$), 168.50 ($NH\mathbf{C}OCH_3$), 19.97 ($OCO\mathbf{C}H_3$), 170.20 ($O\mathbf{C}OCH_3$).

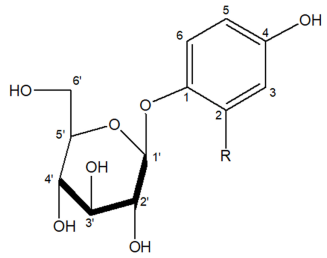
Compounds C9: 23 mg transparent amorphous powder. Its ^{13}C-NMR spectral data showed the following peaks 38.43 (C1), 25.91 (C2), 88.39 (C3), 38.45 (C4), 54.65 (C5), 18.08 (C6), 36.26 (C7), 40.15 (C8), 46.55 (C9), 36.39 (C10), 23.16 (C11), 124.10 (C12), 143.20 (C13), 47.45 (C14), 67.19 (C15), 77.93 (C16), 47.63 (C17), 40.47 (C18), 46.39 (C19), 30.14 (C20), 33.04 (C21), 30.22 (C22), 27.69 (C23), 16.45 (C24), 15.32 (C25), 17.41 (C26), 19.73 (C27), 174.98 (C28), 32.89 (C29), 24.12 (C30), 105.00 (C1'), 57.25 (C2'), 82.4 (C3'), 75.01 (C4'), 76.91 (C5'), 70. 03 (C6'), 105.8 (C1''), 76.25 (C2''), 78.5 (C3''), 72.68 (C4''), 66.09 (C5''), 103.8 (C1'''), 74.76 (C2'''), 84.70 (C3'''), 69.78 (C4'''), 79.98 (C5'''), 64.82 (C6'''), 111.5 (C1''''), 79.8 (C2''''), 81.23 (C3''''), 74.36 (C4''''), 70.9 (C5''''), 93.9 (C1'''''), 80.56 (C2'''''), 83.39 (C3'''''), 71.24 (C4'''''), 78.68 (C5'''''), 62.34 (C6'''''), 107.00 (C1''''''), 76.09 (C2'''''''), 77.60 (C3'''''), 72.10 (C4'''''), 68.03 (C5'''''), 111.8 (C1'''''''), 79.24 (C2'''''''), 81.5 (C3'''''''), 75.36 (C4'''''''), 66.47 (C5'''''''), 22.34 ($NHCO\mathbf{C}H_3$), 169.97 ($NH\mathbf{C}OCH_3$).

Compounds C1 and C2 ^{1}H and ^{13}C-NMR in agreement with the reported data of protocatechuic acid and protocatechuic acid methyl ester respectively (Kwak *et al.,* 2009). Compounds C3, C4, C5, C6, and C7 ^{1}H and ^{13}C-NMR in agreement with the reported data of 1,3,4 trihydroxybenzene glucoside (Pan and Lundgren, 1995), phaseoloidin (Barua *et al.*, 1988), entadamide A (Fumio *et al.*, 1985), entadamide A-*β*-D-glucopyranoside (Dai *et al.*, 1991), entadamide C (Ikegami *et al.*, 1989) respectively. Compounds C8 and C9 ^{1}H, ^{13}C-NMR, COSY, HSQC (Table 1) and HMBC in agreement with the reported data of rheedeioside A and rheedeioside B respectively (Sugimoto *et al.*, 2011).

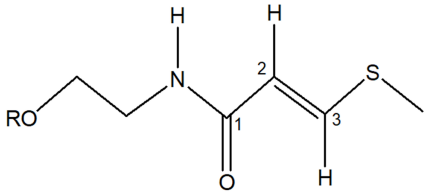
Structures of C1-9 are presented in Figure 1. Phaseoloidin, entadamide A-*β*-D-glucopyranoside, rheedeioside A and rheedeioside B were previously reported in *E. rheedii* seeds (Tapondjou *et al.*, 2005; Sugimoto *et al.*, 2011). However, this is the first report for the presence of protocatechuic acid, protocatechuic acid methyl ester, 1,3,4 trihydroxybenzene glucoside, entadamide A and entadamide C in this seeds.



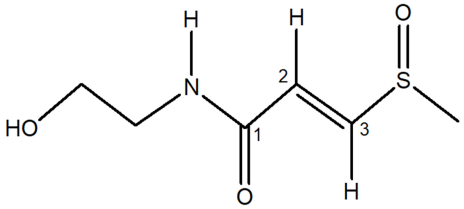
C1: R=H
C2: R=CH_3



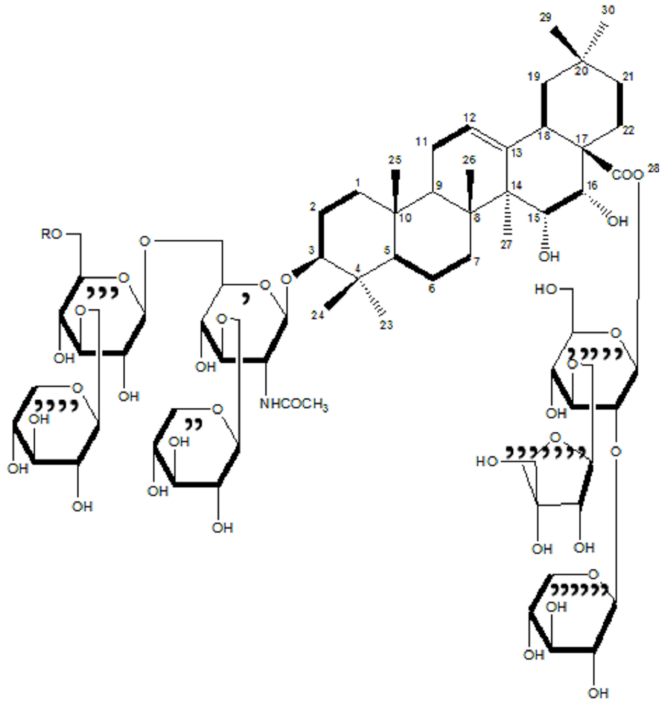
C3: R=OH
C4: R=CH_2COOH



C5: R=H
C6: R=Glucose



C7



C8: R=$COCH_3$
C9: R=H

Fig. 1: Structures of the isolated compounds **C1-9.**

Table 1: HSQC Spectroscopic correlation between anomeric carbons and protons of Compounds **C8** and **C9**.

	C8		C9	
	Anomeric c	**Anomeric H**	**Anomeric c**	**Anomeric H**
C3 sugars				
’	103.606	4.51	105	4.62
”	102.792	4.44	105.8	4.41
”’	101.824	4.36	103.8	4.59
””	106.228	4.23	111.5	4.95
C28 sugars				
”””	92.34	5.38	93.9	5.45
””””	108.77	5.27	107	4.43
”””””	108.99	4.77	111.8	5.3

Previous investigations have shown that several *Entada* species contain saponins in considerable amounts. Several workers isolated saponins from different *Entada* species (Tapondjou *et al.*, 2005; Cioffi *et al.*, 2006; Sugimoto *et al.*, 2011). Many *Entada* pharmacological activities were related to its saponins (Liu *et al.*, 1972; Yasuraoka *et al.*, 1977; Dai *et al.*, 1991; Cioffi *et al.*, 2006). The lack of information concerning the relationship between *E. rheedii* biophytochemicals and pharmacological activities encouraged the authors to study the relation between each studied activity and the isolated thioamides, phenolics, and saponins.

No mortality was observed after one-day administration of EE limit dose up to 4 g/kg b. wt. The experiment was repeated using 3 additional animals at the same dose and no mortality was observed again. In accordance with the Acute Toxic Class Method reported in OECD guideline# 423. The EE is considered to be GHS unclassified with $\mathbf{LD_{50}}$ cut-off > 5000 mg/kg. **Antiulcerogenic activity** (Table 2). Its activity exceeded that of omeprazole (20 mg/kg) and approached that of sucralfate (400 mg/kg) and ranitidine (50 mg/kg). EE reduced ulcers number and severity by 95.24 and 97.98% respectively. Thus *E. rheedii* seeds antiulcerogenic activity is more than *E. phaseoloides* seeds antiulceroginic activity reported before by (Ramakrishna *et al.*, 2008). All tested EE fractions and isolated compounds (C4, C5 & C8 major isolated phenolic, thioamide, and saponin respectively) have significant ($p < 0.05$) antiulcerogenic activity. EE.EA was the most powerful in the reduction of both ulcer number and severity followed by EE.MC. It reduced ulcer number and severity by 92.86 and 97.25% respectively. It deserves to mention that EE.EA is the fraction from which thioamides and phenolics were isolates. Rheedeioside A **C8** was more potent antiulcerogenic than phasoledin **C4** and entadamide A **C5**. It reduced ulcer number and severity by 92.86 and 96.33% respectively. Thus, the antiulcerogenic activity was not attributed to specific biophytochemical class. The powerful antiulcerogenic activity of *E. rheedii* seeds might be also attributed to high levels of glutamic acid (Okba *et al.*, 2013). Glutamic acid is known in the treatment of ulcers (Blitz *et al.*, 1963; Repetto and Llesuy, 2002).

EE (1 mg/ml) showed moderate to strong **antibacterial activity** against 8 strains of the 10 tested pathogenic bacteria except for *M. orygis* and *P. aeruginosa* which were resistant (Table 3). EE has high antibacterial activity relative to standard reference gentamicin (SRG). It exhibited its antibacterial activity through bactericidal action against all sensitive strains. The activity of EE against *S. typhi* reached 96% of SRG. The seeds can be used in urinary tract infections due to the potent bactericidal activity of EE against *E. coli* and *S. aureus* as same as SRG potency. This potent antimicrobial activity is possibly due to the seed content of various classes of phytochemicals. Saponins (Pavithra *et al.*, 2010), phenolics (Okoro *et al.*, 2010), and sulfur compounds (Kim *et al.*, 2006) are among classes of well documented antibacterial activity.

Table 2: Effect of *E. rheedii* seed on gastric ulcer number and severity.

Treatment	Dose (mg/kg)	Ulcer number		Ulcer severity	
		Mean ± SE	**% Reduction**	**Mean ± SE**	**% Reduction**
Control		0		0	
99.5% Ethanol		8.4 ± 0.005*		21.8 ± 0.124*	
EE	200	0.40 ± 0.244*@	95.24	0.44 ± 0.040*@	97.98
EE	100	0.66 ± 0.333*@	92.14	0.97 ± 0.214*@	95.52
EE.MC		0.8 ± 0.065*@	90.48	2.6 ± 0.135*@	88.1
EE.EA		0.6 ± 0.002*@	92.86	0.6 ± 0.001*@	97.25
C5		0.8 ± 0.001*@	90.48	1.4 ± 0.015*@	93.58
C4		0.8 ± 0.003*@	90.48	5.6 ± 0.08*@	74.32
C8		0.6 ± 0.011*@	92.86	0.8 ± 0.04*@	96.33
Omeprazole	20	1 ± 0.003*@	88.1	2 ± 0.021*@	90.83
Sucraflate	400	0.4 ± 0.004*@	95.24	0.4 ± 0.002*@	98.2
Ranitidine	50	0.33 ± 0.004*@	96.1	0.33 ± 0.001*@	98.5

Each value represents the mean of 6 rats ± SE of the mean. Statistical analysis was carried out using Kruskal-Wallis non parametric one way ANOVA. EE: ethanol 70% extract. Fractions of ethanol 70% extract: methylene chloride (EE.MC), and ethyl acetate (EE.EA). *Statistically significant from the normal $p < 0.05$, @Statistically significant from the control ulcer $p < 0.05$. phaseoloidin C4, entadamide A C5 and rheedeioside A C8.

Fractionation of EE showed that both EE.EA and EE.MC had potent antibacterial activity. EE.AQ had no activity against all sensitive strains. It is worth to note that the potent fractions (EE.EA and EE.MC) are the two fractions from which thioamides and

phenolics were isolated. It is obvious that fractionation of the EE to EE.EA increases the antibacterial activity against five strains by 4-16% of SRG and decreased the activity against *S. aureus* and *M. microti* by 4-12% of SRG. EE.EA exerted significantly higher activity than EE.MC against all sensitive strains except *M. africanum*. The absence of activity in EE.AQ, the fraction from which saponins C8 & C9 were isolated, indicates that the antibacterial activity is not due to the seed saponins content. On the other hand, the potent activity of EE.EA and EE.MC indicated that the antimicrobial activity is related to thioamides and phenolics. This was also confirmed when testing the antibacterial potency of the isolated saponins C8 and C9. C8 exhibits moderate to weak activity against 3 strains only while C9 was completely inactive.

Table 3: Antibacterial activity of *E. rheedii* seeds.

			Extract and fractions				Phenolics			Thioamides			Saponins		RSG
			EE	EE.MC	EE.EA	EE.AQ	C1	C2	C4	C5	C6	C7	C8	C9	
Mycobacteria	M.a.	D (mm)*	22	24	22	-	23	20	18	18	14	20	-	-	24
		D (%)**	92	100	92		96	83	75	75	58	83			100
		MIC	0.24	0.12	0.49	-	0.24	3.9	15.63	7.81	31.25	0.98	-	-	0.06
		MBC	0.24	0.49	0.49	-	0.24	3.9	15.63	31.25	62.5	0.98	-	-	0.06
	M.b.	D (mm)*	20	21	23	-	24	21	19	21	16	22	-	-	19
		D (%)**	105	111	121		126	111	100	111	84	116			100
		MIC	3.9	1.95	0.24	-	0.12	1.95	7.81	1.95	31.25	0.49	-	-	1.95
		MBC	3.9	7.81	0.24	-	0.12	1.95	7.81	3.9	62.5	0.49	-	-	1.95
	M.c.	D (mm)*	23	23	24	-	25	22	21	21	18	22	-	-	24
		D (%)**	96	96	100		104	92	88	88	75	92			100
		MIC	0.24	0.24	0.24	-	0.12	0.49	0.98	0.98	15.63	0.24	-	-	0.06
		MBC	0.24	0.98	0.24	-	0.12	0.49	0.98	3.9	31.25	0.24	-	-	0.06
	M.m.	D (mm)*	24	20	21	-	22	19	22	19	16	22	-	-	24
		D (%)**	100	83	88		92	79	92	79	67	92			100
		MIC	0.12	1.95	1.95	-	0.98	3.9	0.49	7.81	31.25	0.49	-	-	0.06
		MBC	0.12	3.9	1.95	-	0.98	3.9	0.49	15.63	62.5	0.49	-	-	0.06
Gram +	S.a.	D (mm)*	23	20	22	-	22	20	nd	16	16	19	13	-	25
		D (%)**	92	80	88		88	80		64	64	76	52		100
		MIC	0.49	3.9	0.49	-	0.24	1.95	Nd	31.25	31.25	3.9	500	-	0.03
		MBC	0.49	3.9	0.49	-	0.24	1.95	Nd	62.5	62.5	3.9	1000	-	0.03
	S.p.	D (mm)*	18	21	22	-	24	17	nd	19	13	21	15	-	20
		D (%)**	90	105	110		120	85		95	65	105	75		100
		MIC	7.81	0.98	0.98	-	0.12	31.25	Nd	3.9	125	0.98	125	-	1.95
		MBC	7.81	7.8	0.98	-	0.12	62.5	Nd	31.25	500	0.98	500	-	1.95
Gram –	E.c.	D (mm)*	24	-	23	-	24	15	nd	18	15	15	-	-	24
		D (%)**	100		96		100	63		75	63	63			100
		MIC	0.12	-	0.24	-	0.06	31.25	Nd	15.63	62.5	62.5	-	-	0.06
		MBC	0.12	-	0.24	-	0.06	62.5	Nd	62.5	125	62.5	-	-	0.06
	S.t.	D (mm)*	25	-	24	-	22	20	nd	19	15	16	12	-	26
		D (%)**	96		92		85	77		73	58	62	46		100
		MIC	0.12	-	0.12	-	0.24	1.95	Nd	3.9	62.5	62.5	500	-	0.03
		MBC	0.12	-	0.12	-	0.24	7.81	Nd	3.9	62.5	62.5	500	-	0.03

*Mean zone of inhibition in mm beyond good diameter (6 mm) produced on a range clinically pathogenic bacteria using (1 mg/ml) concentration of tested samples. **% inhibition relative to gentamicin. MICs and MBCs (µg/ml). EE: 70% extract. Fractions of ethanol 70% extract: methylene chloride (EE.MC), ethyl acetate (EE. EA), n-butanol (EE.BU) and aqueous (EE.AQ) M. a., *Mycobacterium africanum*, M. b., *Mycobacterium bovis*, M.c., *Mycobacterium caprae*, M.m., *Mycobacterium microti*, S.a., *Staphylococcus aureus*, S.p., *Streptococcus pneumoniae*, E.c., *Escherichia coli*, S.t., *Salmonella typhimurium*. nd., not done. protocatechuic acid C1, protocatechuic acid methyl ester C2, phaseoloidin C4, entadamide A C5, entadamide A-*β*-D-glucopyranoside C6, entadamide C C7, rheedeioside A C8 and rheedeioside B C9. nd; not done. RSG; reference standard genatmycin.

Concerning phenolics: protocatechuic acid **C1** is more active than its methyl ester **C2**. Its activity exceeds SRG against certain strains e.g. *M. bovis* (126%), *S. pneumonia* (120%) and *M. caprae* (104%) of SRG. Phaseolidin **C4** activity ranges from 75% to 100% of SRG on the sensitive strains. *Concerning thioamides*: entadamide C **C7** was much more active than entadamide A **C5** and its glucoside **C6** against all sensitive gram-positive and mycobacterium bacteria, while entadamide A **C5** was more active

than other thioamides against sensitive gram-negative bacteria. Entadamide A **C5** had a potent antibacterial activity than its corresponding glucoside **C6** against all sensitive strains except *S. aureus* which is affected equally by both. Both saponins (**C8** and **C9**) are inactive against all *mycobacterium* strains. Only rheedeioside A **C8** showed a moderate activity (46%, 52%, and 75% of SRG) against *S. typhimurium, S. aureus,* and *S. pneumonia* respectively. Thus, it is concluded that the antibacterial activity is related to phenolics and thioamides not to the saponin content.

Studying MIC and MBC revealed that, EE.EA, Protocatechuic acid **C1**, phaseoledine **C4** and entadamide C **C7** exhibited bactericidal action against all sensitive strains. EE.MC and rest of tested compounds showed bacteriostatic activity against some strains and bactericidal activity against others as shown in Table 3.

Table 4: Antiviral effect of *E. rheedii* seed against *CoxB4* virus.

Test sample	MNTC (mg/ml) [MNTC]	O.D	Viabilityy %	Cytotoxicity %	Anti-viral effect
VERO cell line		0.113	100%	0%	0%
Virus control		0.035	31	69	
EE	0.1	0.057	50	50	19%*
C4	0.1	0.075	66	34	35%*
C5	0.1	0.042	37	63	6%
C6	0.1	0.057	40	50	19%*
C7	0.1	0.045	40	60	9%
C8	0.01	0.032	28	72	0%
C9	0.1	0.044	39	61	8%

EE: ethanol 70% extract. MNTC, Maximum non-toxic concentration; O.D., Optical density; *average of six determinations. phaseoloidin C4, entadamide A C5, entadamide A-*β*-D-glucopyranoside C6, entadamide C C7, rheedeioside A C8 and rheedeioside B C9.

M. bovis was the most sensitive bacteria to *E. rheedii* seed tested fractions and isolates Table 3, its sensitivity ranged from 79% to 126% of SRG. *M. bovis* was inhibited by some samples with very low MICs ranging from 0.12, 0.24, 0.49 µg/ml by protocatechuic acid **C1**, EE.EA and entadamide C **C7**. These MICs are lower than that of SRG (1.95 µg/ml). *M. bovis* is the causative agent of tuberculosis in cattle, known as bovine TB, and can jump the species barrier and cause tuberculosis in human (Grange *et al.*, 1996). The pronounced activities against *M. bovis* and *M. caprae,* which can cause tuberculosis also (Rodríguez *et al.*, 2009), validate the seeds ethnomedicinal use in tuberculosis treatment (Vidya *et al.*, 2012). All isolated compounds exert lower antityphoid activity (46-85% of SRG) when compared to EE (96% of SRG) which indicated the necessity of synergism between different classes of biophytochemicals to exert such potent antityphoid activity. *S. aureus* and *E. coli* are among bacteria that cause gastroenteritis and diarrhea (Jarraud *et al.*, 2001; Becker *et al.*, 2003; Al-Gallas *et al.*, 2007; Rodríguez *et al.*, 2009). EE and isolated compounds exhibited potent activity against this species, this validates the seeds ethnomedicinal use in gastroenteritis and diarrhea.

EE has weak **antiviral activity** against *Coxsackie B4* virus Table 4. It reduced the virus activity by 19%. This virus can trigger an autoimmune reaction resulting in the destruction of pancreatic insulin-producing beta cells causing diabetes mellitus (Ylipaasto *et al.*, 2004). Phasoloidin **C4** and entadamide A-*β*-D-glucopyranoside **C6** were the most active antiviral constituents. They reduced the activity of *Coxsackie B4* virus by 35 and 19% respectively. Phasoloidin **C4** the major isolated phenolic is much more potent than EE while entadamide A-*β*-D-glucopyranoside **C6** has antiviral activity equal to that of EE. Subsequently, the antiviral activity is related mainly to the seed phenolics especially that the other tested thioamides and saponins have almost neglected activities ranged from 0-9%.

This is the first report on antibacterial, antiviral and anti-ulcerogenic activities of *E. rheedii* seeds biophytochemicals.

CONCLUSION

E. rheedii seeds possess potent antiulcerogenic and antimicrobial activities. This explains its traditional use in the treatment of stomach aches and as an antimicrobial.

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