**Entada rheedii** phaseoloidin, protocatechuic acid and entadamide A against protozoal diseases: trypanosomiasis and leishmaniasis

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**ABSTRACT**

**Background:** African plant extracts and their isolated constituents remains a hot area for discovering novel drugs. *Entada rheedii* Spreng. and its previously isolated major phytochemicals were evaluated for their antiprotozoal potency. **Method:** In vitro antiprotozoal activity against *Trypanosoma brucei brucei*, *T. b. rhodesiense*, *T. cruzi* and *Leishmania infantum* was determined, including cytotoxicity evaluation for the determination of selectivity. **Results:** The crude extract was inactive. Phaseoloidin exhibited pronounced activity against *T. b. brucei*, *T. cruzi*, *T. b. rhodesiense*, and *L. infantum* (IC$_{50}$ of 9.70, 8.00, 7.83 and 6.96 μg/mL, respectively). Entadamide A showed pronounced activity against *T. cruzi* and *L. infantum* (IC$_{50}$ of 8.98 and 10.77 μg/mL, respectively). Protocatechuic acid showed pronounced activity against *T. b. brucei* (IC$_{50}$ of 8.12 μg/mL) and moderate activity against *T. cruzi* and *T. b. rhodesiense* (IC$_{50}$ of 14.42, 12.23 μg/mL, respectively). All the active compounds exhibited low cytotoxicity score 2 (CC$_{50}$$>$13 μg/mL). **Conclusion:** The major phytochemicals of the African *E. rheedii* seeds were potent against sleeping sickness, Chagas disease, and leishmaniasis. They acted in their pure form rather than acting collectively in the crude extract. **Keywords:** Sulfuramide, phaseoloidin, antitrypanosomal, MRC-5stress.

**INTRODUCTION**

Protozoal infections are one of the major worldwide health problems especially, African sleeping sickness, Chagas disease and leishmaniasis are among the neglected tropical diseases that do not receive attention like many others. Neglected tropical diseases tend to thrive in developing countries where health care, water purity, and sanitation are poor. The WHO estimates that not less than one-sixth of people suffer from at least one neglected tropical disease and it is predicted that 7-8 million people have the Chagas disease [1]. Conventional medicines for neglected tropical diseases are unaffordable, especially for poor African people, and of course they can cause many side effects. This encouraged the authors to search for more effective and less harmful medicinal agents from medicinal plants that are thought to be an excellent source of new antiprotozoal drugs [2]. Africa is highly diverse ethnobotanically, the documentation of the African plant-based chemical components by in silico procedures to explore their mechanisms of action is nowadays hot research topic [3-7]. Many isolated compounds from African medicinal plants were evaluated in vitro and/or in vivo against parasitic protozoal infections [8-11]. In this
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study, the emphasis is laid on isolates from *Entada rheedii* Spreng. (Fabaceae) seed; an African medicinal plant for drug discovery and further development of new agents for parasitic diseases treatment especially sleeping sickness, leishmaniasis, and Chagas disease. No studies have been traced reporting *E. rheedii* phytochemicals antiprotozoal activity.

2. Materials and methods

2.1 Plant material, extraction, fractionation, and isolation of the major constituents

*E. rheedii* Spreng. seeds were purchased from Pharmacognosy Department, Faculty of Pharmacy, Cairo University experimental station. Then, the seeds were powdered and defatted with *n*-hexane. The marc was then extracted with ethanol (70%) and isolation of the major constituents was done as reported by Okba, et. al. [12].

2.2 Chemicals

All reference drugs were obtained from WHO-TDR and Sigma-Aldrich (Bornem, Belgium).

2.3 In vitro biological assays

Standard protocols used by the Lab of Microbiology, Parasitology and Hygiene (LMPH), Faculty of Pharmaceutical Sciences, Biomedical and Veterinary Sciences of the University of Antwerp, Belgium were applied [13,14]. Test plate production, antitrypanosomal and antileishmanial activity in addition to evaluation of cytotoxicity on MRC-5 cells (human embryonic lung fibroblasts) were all carried according to Abdel-Sattar et.al. [15].

2.4 HPLC characterization of the crude extract

HPLC apparatus Agilent Series 1100 equipped with Quaternary pump; and UV detector series 1100 was used for HPLC analyses. HPLC analysis was done on a Hypersil-ODS (4.6x250 mm, 5µm) column.

Isocratic elution was adopted with acetonitrile and 15% acetic acid (40:60 v/v) as mobile phase. The flow rate of the mobile phase was 1 ml/min, and the injection volume was 5 µl for both standards and sample extracts. Detection was carried out by a UV detector set at 270 nm for phenolic acids. Components of the samples were identified by comparing their retention times with that of the standards (prepared as 50-600 µg/ml solutions in methanol). Quantification was based on measuring the peak areas of both standards and samples related to external standards.

3. Results

In a continuation of our interest in exploring plants with antiprotozoal potential [15-21], the ethanol (70%) extract of *E. rheedii* Spreng. seed and its major isolated phytochemicals [12] were tested against *T. cruzi*, *T. b. rhodesiense*, *T. b. brucei* and *L. infantum*, along with MRC-5 cell line for cytotoxicity together with an assessment of their selectivity. Analysis of the collected data is based on the scoring system [8] and IC50-values calculation (µg/mL) adopted by LMPH (Table 1). IC50 of tested compounds expressed as µM/mL were recorded in Table 2. Structures of the previously [12] isolated compounds were illustrated in Figure 1.

Results in (Table 1) revealed that the total ethanol extract of *E. rheedii* Spreng. seeds was inactive (score 1) against all tested protozoa. On the other hand, phaseoloidin exhibited pronounced activity (score 3) against all tested protozoa; *T. b. brucei*, *T. cruzi*, *T. b. rhodesiense* and *L. infantum* (IC50 of 9.70, 8.00, 7.83 and 6.96 µg/mL, respectively). Entadamide A had pronounced activity (score 3) against *T. cruzi* and *L. infantum* only with an IC50 of 8.98 and 10.77 µg/mL, respectively. Protocatechuic acid also showed pronounced activity (score 3) against *T. b. brucei*, (IC50 of 8.12 µg/mL), while it’s methyl ester was inactive. A moderate (score 2) antiprotozoal activity was given by protocatechuic acid against *T. b. rhodesiense* and *T. cruzi* (IC50 of 12.23 and 14.42 µg/mL respectively). All the active tested compounds have low toxicity against MRC-5 cell line (cytotoxicity score 2). The best selectivity index (SI) [2] was presented by phaseoloidin (4.51 *L. infantum*, 4.01 *T. b. brucei* and 3.93 *T. b. rhodesiense*).
Entadamide A showed the best SI (3.82) towards T. cruzi.

Results of the HPLC analysis enabled the quantification of the isolated compounds, being 3315.48 mg/100g protocatechuic acid and 11487.7 mg/100g for phaseoloidin as markers for the total crude extract of E. rheedi seeds Figure (2).

4. Discussion
Parasitic diseases still represent a global threat, especially among poor countries. This is due to the absence of vaccines and the developed resistance against the available drugs. Nothing was reported in the literature concerning the antiprotozoal potency of E. rheedi seeds constituents. One study has reported the interesting activity of monomethyl ester-15- kolaric acid terpenoid isolated from E. abyssinica against T. brucei [22]. It has been reported that Fabaceae phenolics [23], terpenoids [22, 24], flavonoids [25-27] and crude extracts [24, 28] are good candidates for discovering novel antiprotozoal drugs, but it is the first time to conduct an antiprotozoal screening on Fabaceae plants sulphur compound; entadamide A. In addition to the antiviral activity of phasoloidin, the current study represented the first report on its antiprotozoal activity. The current study enthuses further in vivo study on phasoloidin.

The pronounced antiprotozoal activity of protocatechuic acid against T. b. brucei and its moderate activity against T. b. rhodesiense and T. cruzi while the lack of activity of its methyl ester activity against all studied protozoa is in accordance with the reported data that protocatechuic acid is more potent than its methyl ester as an antibacterial agent against gram positive and gram negative bacteria, and Mycobacterium [12]. It is worthy to mention that this is the first study to test the activity of protocatechuic acid and its methyl ester on T. b. brucei, T. b. rhodesiense and L. infantum. Protocatechuic acid and its ethyl ester activity against T. cruzi was studied once before [29].

It is also worthy to note that phaseoloidin and entadamide A were, respectively, the major isolated phenolic compound and thioamide compound from E. rheedi seed and they both exhibited potent antifulcerogenic and antibacterial activities.

To conclude, the isolated compounds of E. rheedi were more active than the crude extract. This was in accordance with our finding during evaluation of the antiprotozoal activity of other Fabaceae plants [15].

Our study revealed that all the tested compounds were less active than the used reference standard drugs except for phaseoloidin which exhibited pronounced antileishmanial potency with an IC50 of 6.96 µg/mL which is less than that of miltefosine IC50 (10.7 µg/mL).

5. Conclusion
Three different compounds were previously isolated from E. rheedi seeds crude extract. Those isolated compounds were more potent antiprotozoal candidates than the crude extract. This indicate that E. rheedi may contain more active compounds yet to be discovered. However, all isolated compounds demonstrated non-specific activity. Translation of some in vitro results into in vivo follow-up studies is recommended in a future study.

6. Funding
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

7. Ethical conduct of research
The use of laboratory rodents was performed in accordance to (European Union directive 2010/63/EU on the protection of animals used for scientific purposes and the Declaration of Helsinki) mandatory guidelines and was approved by the ethical committee (UA-ECD 2015-90) of the University of Antwerp.

8. Conflict of interest
We wish to confirm that there are no known conflicts
of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

List of Abbreviations

CC\textsubscript{50}: 50% cytotoxic concentration
IC\textsubscript{50}: The half maximal inhibitory concentration
LMPH: Lab of Microbiology, Parasitology and Hygiene
SI: Selectivity Index (SI = CC\textsubscript{50}/IC\textsubscript{50})
WHO: World Health Organization
WHO-TDR: Special Program for Research and Training in Tropical Diseases

Table 1. Antiprotozoal activity of E. rheedii Spreng. seed crude extract and isolates and their cytotoxicity against MRC-5 cells.

<table>
<thead>
<tr>
<th>Tested sample</th>
<th>unit</th>
<th>MRC-5</th>
<th>T. b. brucei</th>
<th>T. b. rhodesiense</th>
<th>T. cruzi</th>
<th>L. infantum</th>
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<tr>
<td></td>
<td></td>
<td>CC\textsubscript{50}</td>
<td>SC</td>
<td>IC\textsubscript{50}</td>
<td>SI</td>
<td>SC</td>
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<tr>
<td>Crude ethanol (70%) extract</td>
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<td>&gt; 64.00</td>
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<td>&gt; 64.00</td>
<td>1</td>
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<tr>
<td>Protocatechuic acid</td>
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<td>32.22</td>
<td>2</td>
<td>8.12</td>
<td>3.97</td>
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<tr>
<td>nM</td>
<td>0.209</td>
<td>0.053</td>
<td>0.079</td>
<td>0.094</td>
<td>0.211</td>
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<tr>
<td>Protocatechuic acid methyl ester</td>
<td>µg/ml</td>
<td>&gt; 64.00</td>
<td>1</td>
<td>41.21</td>
<td>&gt; 1.55</td>
<td>=3</td>
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<tr>
<td>nM</td>
<td>0.380</td>
<td>0.245</td>
<td>0.209</td>
<td>0.207</td>
<td>0.381</td>
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<tr>
<td>Phaseoloidin</td>
<td>µg/ml</td>
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<td>7.83</td>
<td>4.01</td>
<td>3</td>
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<tr>
<td>nM</td>
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<td>0.029</td>
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<tr>
<td>Entadamide A</td>
<td>µg/ml</td>
<td>34.27</td>
<td>2</td>
<td>37.65</td>
<td>0.91</td>
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<tr>
<td>nM</td>
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<td>0.234</td>
<td>0.219</td>
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Standards:

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<th>MRC-5</th>
<th>T. b. brucei</th>
<th>T. b. rhodesiense</th>
<th>T. cruzi</th>
<th>L. infantum</th>
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<tr>
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<tr>
<td>Miltefosine</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>10.7</td>
<td></td>
</tr>
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</table>

Scores adopted by LMPH for assessment of antiprotozoal and cytotoxic activities T. cruzi, score 1: >30, 2: >11, 3: >4; T. brucei brucei, score 1: >24, 2: >9, 3: >3; T. brucei rhod, score 1: >24, 2: >9, 3: >3; L. infantum, score 1: >30, 2: >11, 3: >4; Cytotoxicity scores: non-cytotoxic score 1: >37, low cytotoxicity score 2: >13, moderate cytotoxicity score 3: >5, high cytotoxicity: score 4: >1.8; MRC-5: diploid human embryonic lung fibroblasts; CC\textsubscript{50}: concentration causing 50% cytotoxicity; IC\textsubscript{50}: concentration causing 50% inhibition; SI: selectivity index (SI = CC\textsubscript{50}/IC\textsubscript{50}); Sc: score; Activity score 1: inactive, 2: moderate, 3: pronounced activity.
Figure (1): Structures of the *Entada rheedii* major phytochemicals

- Protocatechuic acid
- Protocatechuic acid methyl ester
- Protocatechuic acid methyl ester
- Phaseoloidin
- Entadamide A

Figure (2): HPLC chromatogram of phenolics in *E. rheedii* Spreng. crude extract.
REFERENCES


Entadamide A و protocatechuic acid و phaseoloidin
نشاط مركبات الانتادا ريدياي
المضاد الكائنات الأولية: داء المثقبيات وداء الليشمانيات
من على عقبة، ان ماثيسون، عماد عبد السارية، نعم يوسف، قردية الديب، 2018.
كلية الصيدلة، جامعة القاهرة، مصر
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ملخص

الخلفية العلمية: تبقى المستخلصات النباتية الأفريقية والمركبات المغفولة منها مجالاً مهماً للكشف أدوية جديدة.
الهدف: تقييم نشاط ديت (الانتادا ريدياي) والمواد الكيميائية المغفولة منها ضد الكائنات الأولية. 
الطريقة: تقييم نشاط ديت (Entadamide A) و Trypanosoma, T. b. rhodesiense, T. cruzi brucei brucei والمضاد للأنتادا الأولية ضد T. b. rhodesiense، T. cruzi، T. b. brucei، T. b. brucei، T. cruzi
نشاط واضح ضد Phaseoloidin نشاط واضح مضاد ضد L. infantum، T. brucei، T. b. brucei، T. b. brucei، T. cruzi، T. b. brucei، T. b. brucei، T. cruzi
وشكل معدل 9.70، 6.96، 7.83، 8.00، 7.83، 8.00، 7.83، 8.00، 7.83، 8.00، 7.83، 8.00
التركيز المطلق للنص L. infantum و T. cruzi Entadamide A على النان (على النان). أظهر حمض البروتوكاتيشوك Protocatechuic والمضادات المغفولة (على النان) T. b. brucei T. brucei، T. b. brucei، T. cruzi، T. b. brucei، T. cruzi
أظهرت جميع المركبات النشطة درجة مبطنة خليفة منخفضة (CC50>10 μg/mL). 
الخصائص: المواد الكيميائية النباتية الرئيسية المغفولة من ذاكرة الانتادا ريدياي مغفولة فعلاً ضد مرض النوم، ومرض شامس، وداء الليشمانيات. تأثير المركبات القوي ضد الكائنات الأولية يظهر في كل مركبات النتيجة المغفولة وليس له تأثير بشكل جماعي في
المستخلص الخام.

الكلمات الدالة: السلفوراميد، فاصوليدين، مضاد المثقبيات 5-MRC.