Evaluation of the *in vitro* antileishmanial activities of bioactive guided fractionations of two medicinal plants

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Abstract. The organisms of the genus *Leishmania* are flagellated protozoan parasites and are the causative agents of leishmaniasis. This disease is a major health problem, especially in tropical countries. Currently, cutaneous leishmaniasis is treated by chemotherapy using pentavalent antimonials, but these drugs have serious organo-toxicity, drug resistance on several occasions, and low efficiency in controlling the infection. The present work is carried out to evaluate the *in vitro* antileishmanial activity of methanolic extracts and phytochemical fractions of two plants ethnobotanically used against leishmaniasis and skin infection, *Calotropis procera* and *Rhazya stricta* leaves against *Leishmania major* promastigote and amastigote stages and cytotoxicity against the Vero cell line. The leaves of *C. procera* and *R. stricta* were extracted with methanol and fractionated by petroleum ether, chloroform, ethyl acetate, n-butanol, and water. The methanolic extracts of the leaves of *C. procera* and *R. stricta* exhibited antileishmanial activity against *L. major* promastigotes with IC_{50} values of 66.8 and 42.4 µg mL^{-1}, respectively. While their CC_{50} 2.3 and 298 µg mL^{-1} and their SI 0.03 and 7.03 respectively. However, the fractionations of the methanolic extract of *C. procera* leaves revealed antiparasitic activity against both *L. major* promastigote and amastigote stages *in vitro*, which significantly increased with polarity with the exception of n-butanol. Hence the best activity was revealed by the water fraction (IC_{50} of 26.3 and 29.0 µg mL^{-1}) for the two stages. In conclusion, further phytochemical investigation should be performed for the *C. procera* water extract in terms of antileishmanial active ingredient isolation that may enhance the possibility of avoiding toxic substances and overcome the low SI (1.1 and 1.01).

INTRODUCTION

Leishmaniasis is a serious parasitic disease that occurs due to infection with flagellated protozoans from the genus *Leishmania*, mainly in the subtropical and tropical parts of the world, resulting in significant morbidity and mortality. It is divided into two clinical forms, namely, visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL). CL is the major form and has reached endemic proportions in approximately 88 countries throughout the world, with 1-1.5 million cases being reported annually (WHO, 2002; Blum *et al.*, 2004). The majority of CL cases were reported in Saudi Arabia, Pakistan, Afghanistan, Algeria, Iran, Peru and Syria

(Desjeux, 2004). The situation has become increasingly serious due to the absence of vaccines at present (Herwaldt *et al.*, 1999; Carrión *et al.*, 2008).

Leishmaniasis is endemic in various provinces of Saudi Arabia, mainly in the Al-Hassa oasis, Al-Madinah Al-Munawarah, and Al Qassim provinces, due the prevalence of desert rodents (*Psammomys obesus* and *Meriones libycus*), which serve as reservoir hosts, and *Phlebotomus papatasi* sand flies, which serve as vectors (Peters *et al.*, 1987; Al-Tawfiqa *et al.*, 2004; El-Beshbishy *et al.*, 2013). *L. major* (Zymodeme LON-4) is known as the main causative organism of Zymodeme cutaneous leishmaniasis (ZCL) in most endemic areas in Saudi Arabia, while

L. tropica has been identified as the causative organism of anthroponotic cutaneous leishmaniasis (ACL) in the southwestern region at the high plateau of Asir (Shalaby *et al.*, 2011; Yehia *et al.*, 2012; El-Beshbishy *et al.*, 2013).

Pentavalent antimonials, developed six decades ago and administered via intravenous or intramuscular injection, often result in serious side effects, such as electrocardiographic abnormalities, elevations in serum aminotransferases and chemical pancreatitis (Hepburn, 2003). Moreover, the current treatment options with antimonials are not sufficient due to the emergence of antimony resistance (Sundar *et al.*, 2000; Adler-Moore *et al.*, 2002; Croft *et al.*, 2003). Therefore, there is an urgent need to develop and explore new drugs for the safe and effective treatment of leishmaniasis.

Natural product research has shown the potential to serve as a niche for novel active ingredients and plants that are regarded as highly valuable sources, particularly for the screening of bioactive compounds against parasitic diseases (Poloni *et al.*, 2008; Awadh *et al.*, 2017). It is noteworthy that the only medicines active against *Leishmania* are the major forms of accessible treatment against malaria in developing countries where modern medicines are unavailable. Although these folkloric remedies have been playing an important role in the treatment of many clinical disorders, only a limited number of them have been subjected to scientific scrutiny despite the validation of traditional medicines, which has led to innovative strategies for controlling many protozoal diseases such as malaria and leishmaniasis (Al-Musayeib *et al.*, 2012; Mothana *et al.*, 2014; Al-Sokari *et al.*, 2015). Phytochemicals could potentially represent a constant source of molecules with novel mechanisms of action against various diseases, including bacterial and protozoal infections.

R. stricta Decne of the Apocynaceae family is famous for its exclusive array of terpenoid indole alkaloids. This plant is an evergreen dwarf shrub widely distributed in the Indian subcontinent and the Middle East (Hooper, 1906). These plants possess a range of biological activities, including antitumor, antimicrobial and antihypertensive properties (Verpoorte, 1998). *R. stricta* has long been used in Arabian communities to cure diabetes, helminthiasis, and inflammatory conditions (El-Ghonemy, 1993). In Saudi Arabia, the leaves of *R. stricta* are commonly used as a purgative, vermifuge and acaricide against mange (Al-Yahia *et al.*, 1990). In addition, *R. stricta* is used for the treatment of helminthiasis in camels (Abbas *et al.*, 2002). This plant used locally by traditional healer against allergy and skin wound infection.

C. procera Linn belongs to the Asclepiadaceae family and has been shown by several researchers to contain pentacyclic triterpenes, alkaloid cardenolides, phytosterols and triterpenoid saponins (Chundattu *et al.*, 2016). The aqueous extracts of its latex have demonstrated beneficial effects in hepatocellular carcinoma of mice by inhibiting cellular infiltration and the development of neoplastic changes (Choedon *et al.*, 2006). The chloroform extract of the root has been proved to be hepatoprotective against induced liver damage (Basu *et al.*, 1992), while the flower ethanol extract revealed good anti-microbial and anti-inflammatory properties (Mascolo *et al.*, 1998) as well as antimalarial activities (Sharma *et al.*, 1999). This plant used locally by traditional healer against leishmaniasis and other skin diseases.

The exploration of these molecules is limited but could prove to be highly beneficial to improve the health situation of individuals, as occurred for quinine, i.e., a major antimalarial compound that was isolated from *Cinchona succiruba*. This discovery is an achievement in the historical backdrop of antimalarial drugs from plants pursued by the collection of artemisinin from *Artemisia annua* and hydroxynaphthoquinones and encourages researchers to explore traditionally used plants to discover better molecules against protozoal diseases (Hooper, 1906; El-Ghonemy, 1993; Verpoorte, 1998; Al-Musayeib *et al.*, 2006; Poloni *et al.*, 2008).

With this background, the present study aims to evaluate the *in vitro* antileishmanial

activities and cytotoxicity of the methanolic extracts and fractions of *C. procera* and *R. stricta* leaves against both promastigote and amastigote stages of *L. major*.

MATERIALS AND METHODS

Plant material and preparation of extracts
The leaves of *C. procera* and *R. stricta* were collected from fields in the Al Qassim region during their growing seasons. Subsequently, the collected plants were identified and authenticated by a taxonomist and voucher specimens were prepared and deposited in the herbarium of the botany department of Qassim University. The collected plant materials were shade dried, reduced to a fine powder using a laboratory blender, passed through a 60-mesh sieve (BS), packed in airtight containers and stored at 4°C until further use.

Each sample of the plant material (500 g) was soaked in 5 L of analytical grade methanol at room temperature for 24 h and extracted in a mechanical shaker for 16 h. Then, the extract was filtered through Whatman No. 1 filter paper, and the residue was transferred to a container, extracted again with 2.5 L of fresh methanol for 16 h and filtered. The two filtrates were pooled and evaporated to dryness under vacuum using a rotary vacuum evaporator at 40°C. The dried extracts were weighed to determine the yield and stored in airtight containers at 4°C until further use (Al Nasr *et al.*, 2019).

Phytochemical fractionation of plant crude extracts
The crude methanolic extract of the leaves of *C. procera* was dissolved in distilled water (not completely soluble) and then partitioned in different solvents with increasing polarity (through a liquid-liquid extraction system). A 5 L separating funnel was used to obtain petroleum ether (4.9 g) extract F1, chloroform (11.3 g) extract F2, ethyl acetate (3.1 g) extract F3, n-butanol (5.1 g) extract F4 and the remaining water extract (8.2 g) F5. The same technique was used for *R. stricta*

leaves to obtain (1.5 g) F6, (5.2 g) F7, (3.9 g) F8, (6.2 g) F9 and (4.5 g) F10 (Cuca-Suarez *et al.*, 2011).

Parasite maintenance and bioassays
The promastigotes of *L. major* were isolated from an indoor patient at Al Rass General Hospital in February 2016. Schneider's *Drosophila* medium (Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, USA) and antibiotics were used, maintained at 26°C, in a tissue culture flask with weekly transfers. The promastigotes were cryopreserved in liquid nitrogen at a concentration of 3×10^6 parasite/mL. Passing of the parasite via a group of female BALB/c mice was used for maintaining the virulence of *L. major*. The hind footpads were injected with 1×10^6 stationary-phase promastigotes. After 8 weeks, *L. major* amastigotes were isolated from the mice as described by (Osorio *et al.*, 2011). Culturing at 26°C in Schneider's medium supplemented with 10% FBS and antibiotics was used to transform the isolated amastigotes to promastigote forms (Al Nasr *et al.*, 2019).

Activity of crude extracts and fractions against *L. major* promastigotes
The promastigotes from the logarithmic phase cultured in phenol red-free RPMI 1640 medium (Invitrogen, USA) with 10% FBS were suspended on 96-well plates to yield 10^6 cells mL^{-1} (200 µL/well) after hemocytometer counting. Crude extracts/fractions were added to obtain the final concentrations (100, 50, 25, etc. µg mL^{-1}). Negative control wells containing cultures with DMSO (1%) and without extracts/fractions and positive control wells containing cultures with decreasing concentration of Amphotericin B (AmB) (reference compound, 100, 50, 25, etc. µg mL^{-1}) were used. After that, the plates were incubated at 26°C and 72 h to evaluate the anti-proliferative effects. The colorimetric method was used to assess the number of viable promastigotes by using tetrazolium dye (MTT). The samples were analyzed using an ELISA reader at 570 nm. The assays were performed in three independent experiments (Al Nasr *et al.*, 2019).

Activity of crude extracts and fractions against *L. major* intramacrophage amastigotes

After peritoneal collections of macrophages from female BALB/c (6-8 weeks of age), 5×10^4 cells/well were seeded in 96-well plates in phenol red-free RPMI 1640 medium with 10% FBS for 4 h at 37°C in a 5% CO_2 atmosphere to promote cell adhesion. The medium was discarded, and the cells were washed with Phosphate Buffered Saline (PBS). Then, 200 µl of *L. major* promastigote-containing solution (at a ratio of 5 promastigotes to 1 macrophage in phenol red-free RPMI 1640 medium with 10% FBS) was added per well. To allow infection and amastigote differentiation, the plates were incubated for 24 h at 37°C in a humidified 5% CO_2 atmosphere. Then, the infected macrophages were washed three times with PBS to remove the free promastigotes and overlaid with fresh phenol red-free RPMI 1640 medium containing crude extract/ fractions at final concentrations (100, 50, 25, etc. µg mL^{-1}), and the cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 72 h. Negative control cultures containing DMSO (1%) and without crude extract/ fractions and positive control wells containing cultures with decreasing concentrations of AmB (reference compound, 100, 50, 25, etc. µg mL^{-1}) were used. The percentage of infected macrophages was evaluated microscopically after removing the medium, washing, fixing and Giemsa staining. The assays were performed in three independent experiments (Al Nasr *et al.*, 2019).

Toxicological evaluation of crude extracts and fractions *in vitro* by using MTT assay

To confirm the safety or toxicity of the plant extracts against host cells and to determine the plant extract/fractions concentration that can be safely used without negatively affecting the cell viability, an MTT assay was performed. Briefly, 96-well plates were used for Vero cell culture (5×10^3 cells/well/200 µL) for 24 h in RPMI 1640 medium with 10% FBS and 5% CO_2 at 37°C. The cells were washed with PBS and treated with AmB for 72 h (positive control) or plant extract/ fractions at varying concentrations (100, 50, 25, etc. µg mL^{-1}) in a medium containing 10% FBS. For the negative control, the cells were treated in a medium with only 10% FBS. The cells were incubated with the plant extract/ fractions for 72 h. Thereafter, the supernatant was removed and 50 µL RPMI 1640 medium containing 14 µL MTT (5 mg mL^{-1}) was added and incubated for 4 h. After that, the supernatant was removed and 150 µL DMSO was added in order to dissolve the formazan. Microplate absorbance spectrophotometer was applied for colorimetric analysis ($\lambda = 540$ nm). Cytotoxic effects were expressed by IC$_{50}$ values (concentration that caused a 50% reduction in viable cells) (Al Nasr *et al.*, 2019).

Statistical analysis

The data were expressed as the mean ± SD of triplicate determinations. IC$_{50}$ values, were calculated by Microsoft excel program (ver. 2003) from linear regression equation by plotting concentration for X axis and inhibition % at Y axis. From the equation $Y = a * X + b$, IC$_{50}$ = $(0.5 - b)/a$. The same technique was used for CC$_{50}$ calculation but Y axis was used for mortality %.

RESULTS

Both *C. procera* and *R. strica* crude methanol extracts showed dose-dependent antileishmanial activity against *L. major* promastigotes *in vitro* ranging from 52.6 to 35.5% parasitic inhibition and 74.4 to 61.8% parasitic inhibition for the tested concentrations of 100 to 12.5 µg mL^{-1} with IC$_{50}$ values of 66.8 and 42.4 µg mL^{-1}, respectively (Table 1).

The percentage inhibitory activity against promastigotes of 5 fractions from *C. procera* (F1 to F5) eluted by the solvents petroleum ether, chloroform, ethyl acetate, n-butanol, and water ranged from 0 to 94%. All the fractions showed a positive dose-dependent trend along with the serial dilutions of 100, 50, 25 and 12.5 µg mL^{-1}. The

Table 1. CC_{50}, IC_{50} ± S.D and SI values of selected samples in µg mL^{-1}

S. No	Fractions	CC_{50}	IC_{50} *Leishmania* Promastigote	IC_{50} *Leishmania* Amastigote	SI *Leishmania* Promastigote	SI *Leishmania* Amastigote
1	*C. procera* extract	2.31 ± 0.5	66.8 ± 9.2	X	0.03	X
2	*R. stricta* extract	298 ± 26	42.4 ± 6.8	X	7.03	X
3	F1	9.5 ± 3.1	>150	275 ± 56.2	< 0.06	0.03
4	F2	9.4 ± 2.7	44.2 ± 8.1	37.5 ± 6.6	0.21	0.25
5	F3	11.7 ± 4.2	33.5 ± 6.6	43.7 ± 12.7	0.35	0.27
6	F4	10.8 ± 3.6	64.1 ± 10.5	104.1 ± 38.9	0.17	0.10
7	F5	29.21 ± 6.8	26.3 ± 5.5	29 ± 6.4	1.11	1.01
8	F6	112.3 ± 35	85 ± 20.4	67.5 ± 26.8	1.32	1.66
9	F7	14.12 ± 3.6	99.5 ± 22.3	75 ± 27.1	0.14	0.19
10	F8	73.6 ± 31.3	>200	180 ± 60.3	< 0.368	0.41
11	F9	70.6 ± 16.5	>200	293.9 ± 38.4	< 0.353	0.24
12	F10	92.6 ± 21.7	>200	246.9 ± 53.9	< 0.463	0.38
13	AmB	7.5 ± 1.1	0.81 ± 0.2	0.45 ± 0.07	9.3	16.7

maximum activity was demonstrated by the water fraction, with an IC_{50} value of 26.3 µg ml^{-1}, while the petroleum ether fraction was the least effective, with an IC_{50} value of > 150 µg mL^{-1} (Table 1).

The fractions (F6 to F10) of *R. stricta* eluted with petroleum ether, chloroform, ethyl acetate, n-butanol, and water showed antipromastigote activity that ranged from 0 to 48% for serially diluted doses of 100, 50, 25 and 12.5 µg mL^{-1}. The maximum antipromastigote potential was shown by fractions F6 and F7, with IC_{50} values of 85 and 99.5 µg mL^{-1}, while the rest of the fractions showed marginal activities, with values more than 200 µg mL^{-1} (Table 1).

Among the fractions of *C. procera*, the maximum activity against amastigotes was recorded for F5 with an IC_{50} value of 29 µg mL^{-1}, while F1 was the least effective, with an IC_{50} value of 275 µg mL^{-1} (Table 1).

The more apolar fractions (F6: petroleum ether and F7: chloroform) of *R. stricta* demonstrated similar maximum activities, with IC_{50} values of 67.5 and 75 µg mL^{-1}, respectively, while the fractions with polar solvents were the least effective, with IC_{50} values of 293.9 and 246.9 µg mL^{-1} for F9 and F10, respectively (Table 1).

The cytotoxicity and Selectivity Index (SI) derived by CC_{50} rather than IC_{50} values of all the tested extracts and fractions

revealed that the crude extract of *R. stricta* was the least toxic, with an SI value of 7.03 against, while of its fractions, F6 was the least toxic, with an SI value of 1.66 against amastigotes. *C. procera* was the most toxic, with an SI value as low as 0.03, while its F5 fraction was the least toxic, with an SI value of 1.01 against amastigotes, quite comparable to the least toxic fraction F6 of *R. stricta*. While AmB, the reference drug, was found very active against both promasitoge and amastigote with IC_{50} 0.81 and 0.45 µg mL^{-1} with SI 9.3 and 16.7 respectively (Table 1).

DISCUSSION

Leishmaniasis is still a major health concern, particularly in tropical and subtropical countries. However, there is not an efficient vaccine that is active against this disease. Moreover, the commonly used clinical medication possesses toxicity and multiple side effects. For all these reasons, there is an urgent need for the discovery of new therapeutic agents with affordable prices for common people (Bouyahya et al., 2016). To date, screening studies of more than 100 medicinal plants have reported anti-leishmanial activities (Rochaa et al., 2005; Bouyahya et al., 2018).

In the first part of the present study, two medicinal plants (methanolic extract *of C. procera* and *R. strica* leaves) that are commonly used locally against infectious diseases (Al-Yahia *et al.*, 1990; Basu *et al.*, 1992; El-Ghonemy, 1993; Abbas *et al.*, 2002; Mascolo *et al.*, 1998; Sharma *et al.*, 1999; Choedon *et al.*, 2006; Chundattu *et al.*, 2016) were investigated for their activity against *L. major* promastigotes for the first time. *R. stricta* proved to be more active than *C. procera*, while *C. procera* was found to be more toxic than *R. stricta*. Both plants have previously shown antiparasitic activities, e.g., *R. stricta* exhibited anthelmintic activity (Abbas *et al.*, 2002), while *C. procera* revealed antiprotozoal activity against malaria parasite (Sharma *et al.*, 1999). In the second part of this study, methanolic extracts of the leaves of the abovementioned plants were phytochemically fractionated by different solvents according to polarity by using petroleum ether, chloroform, ethyl acetate, n-butanol, and water. Then, these fractions were tested against two different stages of *L. major* (promastigotes and amastigotes) *in vitro*. Although the crude methanolic extract of *R. stricta* leaves was found to be the most active extract in the first part of this study, with an IC_{50} value of 42.4 µg mL^{-1}, all the fractions revealed less activity, suggesting the possibility that the synergistic active ingredient is distributed in solvents of different polarities. This type of relationship between fractions and compounds was previously observed several times (Sharma *et al.*, 2003). On the other hand, the fractions obtained from the methanolic extracts of *C. procera* leaves showed antileishmanial activities against *L. major* promastigotes that were proportional to the polarity, with the exception of n-butanol. Their IC_{50} values decreased from 44.2 to 33.5 and 26.3 µg mL^{-1} for the chloroform, ethyl acetate, and water fractions, respectively. These results strongly agree with the previous finding of (Chalise *et al.*, 2010), who proved that more polar extracts have greater efficacy, owing to the larger quantities of phenolic derivatives (Chalise *et al.*, 2010). Our findings, in agreement with the aforementioned outcomes, also reveal that water fractions (the most polar one) obtained from the methanolic extracts of *C. procera* leaves appeared to be the most active against *L. major* reproductive stages of promastigotes and amastigotes, with IC_{50} values of 26.3 and 29 µg mL^{-1}, respectively.

Since *Leishmania* parasites can be found in two different forms, i.e., the extracellular flagellates (promastigotes) in sand fly midguts and the intracellular (amastigotes) form engulfed in host macrophages, there exist obvious differences in the chemical nature and temperature and subsequently, a different response for different types of antileishmanial medication, as documented previously (Berman *et al.*, 1982). However, some compounds had been identified to be active against both promastigotes and amastigotes, such as Amphotericin B. As per our current finding, the water fraction of *C. procera* was potent against both amastigote and promastigote stages, which is consistent with the findings of Callahan (Callahan *et al.*, 1997), but the possibility of the presence of different compounds in the same extract remains.

Although the fractions of the methanolic extract of *C. procera* leaves resulted in better biological activities than *R. stricta* fractions against both *L. major* promastigotes and amastigotes, their toxicity was very high, with CC_{50} values ranging from 9.4 to 29.2 µg mL^{-1}. This toxicity results in a very low value of the selectivity index (SI), i.e., less than 1, except for the water fraction, which has a value of 1 for both amastigote and promastigote stages. Therefore, in con-clusion, we advise further phytochemical isolation of the active ingredient, particularly for the *C. procera* water fraction, as the most active extract is not the most toxic extract, thus suggesting the possibility of nontoxic active compounds. For the *R. stricta* fractions, it may not be productive to continue the studies of the isolation of the active ingredients because the activity in the crude extract was better in comparison to the fractions, hence limiting the scope of further investigation.

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