

(+)-Spectaline, a Piperidine Alkaloid from *Senna spectabilis* DC. Effective in Reducing the *In Vitro* Infection of *Leishmania major*

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Keywords: *Senna spectabilis*, Leishmaniasis, *in vitro* intracellular amastigote assay model, *Leishmania major*, bioassay-guided fractionation, (+)-spectaline.

Abstract. *Senna spectabilis* is known to have antimicrobial, laxative, antiulcerogenic, analgesic, and anti-inflammatory properties in folk medicine. Piperidine alkaloids extracted from various parts of this plant have been shown to have anticonvulsant (iso-6-spectaline), antinociceptive [(-)-spectaline] and lipid peroxidation [(+)-3-*O*-feruloylcassine, (-)-spectaline and (-)-3-*O*-acetylspectaline] activities. In the present study, the ethyl acetate extract from *S. spectabilis* exhibited antileishmanial activity via intracellular promastigote assay or leishmanicidal assay and was further fractionated by using bioassay-guided isolation approach. The antiprotozoal principle was isolated from the ethyl acetate portion through solvent fractionation and few series of chromatographic processes. The isolated active compound **1** was identified as (+)-spectaline on the basis of its spectral analysis (MS, 1D & 2D NMR) with EC₅₀ value of 0.063 ± 0.005 μM for antileishmanial activity and selectivity index of 3.76.

Introduction

Leishmaniasis is considered as one of the most neglected tropical diseases (NTDs) in the world [1, 2]. It is endemic in 98 countries worldwide with an estimated 1.3 million new cases and an annual mortality rate of 20,000 to 40,000 [3]. In addition, Global Burden of Disease Study 2010 showed that it results in 3.32 million DALY (disability-adjusted life years) [1] which is second to malaria [4]. Current treatments are complicated because of the intramacrophagic location of the infectious form [5]. Although more efforts to come on vaccine development for leishmaniasis, there is no registered human leishmaniasis vaccine to date and pentavalent antimony or antimonials still act as the primary therapy [6, 7]. Other drugs available in the market face great challenges such as acquired resistance by certain common species, depleted immune capability in HIV co-infection patients and organs toxicities [2]. The unfavorable side effects and resistance together with the increasing number of patients intensified the demand for alternative remedies to curtail this disease [8-10].

Leishmania spp infections typically originate via the bite of infected *Phlebotomus spp* or *Lutzomya spp* female sandflies. On being bitten, promastigotes are released into the skin and bloodstream which are then phagocytosed by the host macrophage [2]. The promastigotes are then transformed to amastigotes and then multiply, causing host cells to be burdened with parasites and thus becoming prone to lysis. The lysis will release amastigotes which then may infect other macrophages, thus ensuring their survival in the host [8, 10]. The disease is characterized by a spectrum of clinical manifestations which are visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL), and also rarer ones such as mucosal leishmaniasis (MCL) [6]. The varied

spectrum of clinical manifestations normally range from appearance of lesions, papule or nodules, or even mucosal tissue destruction of CL [10].

Plant natural products have been used in the treatment of infectious diseases, especially in developing countries [11], with some displaying antileishmanial activities [12]. These activities have been attributed to the flavonoids, phenylpropanoids, steroids, quinones, chalcones and a few more, but the most listed in reviews are alkaloids and terpenes [10, 12]. To date, a list of compounds from plants which have entered investigational stage had been summarized by Sundar and Chakravarty [13] which includes amarogentin from *Swertia chirata*, maesabalide III triterpenoid saponins from *Maesa balansae*, prenyloxy-naphthoquinone from *Plubago zeylanica*, plumbagin from *Pera benensis* and a list of 2-substituted quinaline alkaloids derived from *Galipea longiflora*.

Senna spectabilis (*S. spectabilis*) which is native in tropical countries, is from the genus *Senna* used in the folk medicines of the Brazilians, Indians (Ayurvedic), Thais, Indonesians and Malaysians [14]. The majority of them use it for anti-inflammatory, analgesic, antibacterial, antifungal, antiulcerogenic treatment, for flu and colds, fevers and headaches, as a laxative and purgative, and for ringworm as well as skin diseases [15, 16]. In the present study, we have examined the antileishmanial potential of this plant to reduce the parasitic burden of macrophages infected with *Leishmania sp. in vitro*.

Materials and methods

General experiment procedure. Chemicals and standard drug used in this study were Amphotericin B (Amp-B) (A241 Sigma), Schneider's Drosophila medium (S0146 Sigma), Fetal Bovine Serum (FBS 10270 Gibco, Invitrogen), RPMI 1640 medium (R5886 Sigma), L-Glutamine reagent (G7513 Sigma), and MEM medium (Gibco). The initial working solutions of all fractions, compound and Amp-B were prepared in 1% dimethylsulfoxide (DMSO) as the vehicle.

Thin layer chromatography (TLC) was performed using 0.20 m precoated silica gel aluminium sheets (Merck Kiesel Gel 60F₂₅₄). Spots were visualized under UV-light (254nm) and sprayed with vanillin reagent. Melting point (MP) was measured by differential scanning calorimetry (DSC) instrument. Infrared spectrum was obtained using FTIR spectrometer and LCMS-QTOF. ¹H NMR (400 MHz) and ¹³C NMR (100 MHz) spectra were recorded on a Bruker Avance 400 spectrometer, with deuterated chloroform (CDCl₃) as the solvent and tetramethylsilane (TMS) as internal standard. COSY, DEPT, HMQC and HMBC NMR experiments were performed on the same spectrometer. Specific optical rotation was recorded with a Jasco DIP-370 Digital Polarimeter.

Plant Material. Leaves of the *S. spectabilis* plant were collected from Universiti Sains Malaysia (USM) campus Penang, Malaysia. The plant was pre-tagged by the Herbarium of the School of Biological Sciences, USM. The taxonomic identification of the plant was identified by a botanist, Mr. Kamarudin Saleh from Forest Research Institute Malaysia (FRIM), Selangor, Malaysia. A voucher specimen (PID 070412-01) has been deposited at the herbarium of IPharm Natural Compound Library.

Preparation of extracts and fractions. Fresh leaves of *S. spectabilis* were dried in an oven for 3 days at 50°C. The dried leaves were then ground and soaked with 1.5 L of methanol (MeOH) for 72 hours. The extract obtained was concentrated using a rotary evaporator at 40-50°C under reduced pressure. These brought to yield a product of 54 g (27%) of MeOH extract which designated as CS-MeOH. Next, it was partitioned (3 x 400 mL) using n-hexane (Hex), ethyl acetate (EtOAc) and butanol (BuOH) to give Hex, EtOAc and BuOH extracts designated as CS-Hex [3.11 g (5.87%)], CS-EA [7.0 g (13.21%)] and CS-BuOH [3.52 g (6.64%)] extracts respectively.

CS-EA exhibited the highest antileishmanial activity (Table 1) and was fractionated by vacuum liquid chromatography (270 g silica gel kiesel gel 60F, 0.040 – 0.063 mm, 230-400 mesh ASTM) and eluted with mixture of increasing polarity Hex-MeOH (100:0, 95:5, 90:10, 80:20, 70:30, 55:45, 45:55, 40: 60, 35:65, 30:70, 20:80, 10:90, 5:95 *n*-Hex : EtOAc, 95:5, 90:10, 85:15,

75:25, 65:35, 50:50, 0:100 v/v). The eluent size used was 400 mL of each eluent system to obtain 26 fractions. Based on the TLC profiles of these fractions, they were pooled into 8 final fractions which designated as CS-EA-CC1-F1 ~ F8.

CS-EA-CC1-F8 exhibited highest antileishmanial activity and was further fractionated via silica gel column chromatography (silica gel 60F, 70-230 mesh, 0.063-0.100 mm, Merck) and eluted with mixture of increasing polarity using dichloromethane (DCM)-MeOH (100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 60:40, 40:60, 30:70, 60:40, 30:70, 20:80, 0:100 v/v). Again, the eluent size used was 400 mL of each eluent system to obtain 42 fractions. Based on the TLC profile of these fractions, they were pooled into 4 final fractions (CS-EA-CC1-F8-CC2-F1 ~ F4) and were subjected to antileishmanial test. CS-EA-CC1-F8-CC2-F4 exhibited the highest antileishmanial activity and were further fractionated.

Isolation of Compound 1. The active fraction; CS-EA-CC1-F8-CC2-F4 was further purified by silica gel flash column chromatography (flash CC) and eluted with an isocratic gradient of DCM:MeOH (99:1 v/v). Flash CC conditions: column, Hi-Flash M (20 x 65 mm); flow rate, 30 mL/min; time, 4 minutes; wavelength, 254 nm; fraction volume, 5 mL/piece. 100 fractions were collected and made up into 7 fractions (CS-EA-CC1-F8-CC2-F4-CC3-F1 ~ F7) based on the TLC analysis. All fractions were tested for their antileishmanial activities. CS-EA-CC1-F8-CC2-F4-CC3-F1 was obtained as a single compound, designated as compound **1** (5.18 mg) and was found to be active against *L. major* infection.

Leishmania parasite and cell line. *L. major* promastigotes (MHOM/SA/85/JISH118) were obtained from The London School of Hygiene and Tropical Medicine (LSHTM), United Kingdom. The parasites were grown and maintained as reported previously [17]. THP-1 and Vero cells (ATCC) were cultured as reported previously by Gebre-Hiwot and colleagues [18] also Carbonell and colleagues [19], respectively.

Leishmanicidal assay against promastigotes. This assay was performed as described in Poorrajab and colleagues [20] with minimal modification. Briefly, 2×10^6 cells/mL of log phase cultured promastigotes were incubated with a serial range of drug concentrations on a 96-well plate for 48 hours at 26°C. The activity was measured via reduction of resazurin to resorufin using Alamar Blue[®]. After 2 hours of incubation, the assay plates were read using an ELISA microplate reader at a wavelength of excitation of 530 nm and emission of 590 nm. The IC₅₀ value was determined via analysis using Excel[®] and GraphPad Prism[®] 5.

In vitro intracellular antileishmanial assay. The assay was carried out according to Seifert and Croft [21]. Briefly, log-phase THP-1 cells were harvested at 1.0×10^6 cells/mL. Cells were diluted to 2.0×10^5 cells/mL with the complete medium, seeded in 16-well Nunc[™] Lab-Tek[™] tissue culture chamber slide (Fisher Scientific) at a seeding density of 5.0×10^4 macrophages/well (100 μ L) and allowed to adhere by the addition of PMA (Phorbol -12 myristate Acetate, P8139 Sigma) for 3 days at 37°C in a 5% CO₂ – 95% air mixture. Macrophages were then infected with long-slender (stationary stage) *L. major* promastigotes at a macrophage-promastigote ratio of 1:5. Infected macrophages were maintained at 34°C in a 5% CO₂ – 95% air mixture. After 48 hours, extracellular parasites were removed by substituting the overlay with new fresh RPMI-1640 medium supplemented with 1% L-glutamine. Extracts, fractions and standard reference drug with various concentrations were added and their activities or effective concentration to decrease the parasite burden (EC₅₀) were determined from the percentage of infected cells in treated cultures in relation to non-treated cultures after MeOH fixation and Giemsa staining.

Cytotoxicity assay. The cytotoxicity assay was performed as described by Konowalchuk and colleagues [22] as well as Carbonell and colleagues [19] with some modifications. Briefly, confluent monolayers of Vero cells were removed with trypsin EDTA, re-suspended to

approximately 1×10^5 cells/mL in MEM. Next, 0.1 mL of the cells was pipetted into each well of a 96-well microtiter plate. After incubation at 37°C in 5% CO₂/95% air for overnight, the cells were treated with compound **1**. Amp-B was used as positive control and MEM with 1% DMSO was used as negative control. All samples were tested in triplicates. Cytotoxic activity of Vero cells was measured via fluorescence reduction of resazurin to resorufin [23] by microplate reader and IC₅₀ value was determined using Excel[®] and GraphPad Prism[®] 5.

Results and Discussion.

The leaf extracts from *S. spectabilis* can be used for oedema inhibition, treatment of constipation, poisoning and protozoic infections' of the gut [24]. In addition, extracts from the leaves also showed good activities against various bacteria, fungi and yeast [15, 25]. Antioxidant and anticonvulsant activities have also been observed from alkaloids found in the leaf [26].

In antileishmanial area of study, a few alkaloids have been proven to be effective against extracellular promastigotes which have been extensively listed by Chan-Bacab and Pena Rodriguez [27]. In a more recent study [28], mixed compounds of major piperidine alkaloids from *S. spectabilis* flowers extract which consist of (-)-spectaline and (-)-casein have been claimed to be effective against promastigotes of *L. major*. Through intracellular amastigotes assay model, several pure alkaloids such as berberine [29], liriodenin [30], harmaline [27] and gabunine [31] have been proven for their antileishmanial activity.

Table 1 shows the results on the antileishmanial activity of extracts and fractions of *S. spectabilis*. In the promastigote assay, IC₅₀ refers to the 50% inhibitory concentration to inhibit the growth of promastigotes [20], while EC₅₀ in the intracellular amastigote assay model means the effective concentration of the substance to decrease as much as 50% parasite burden of infected macrophage [21].

Based on the results presented in Table 1, the ethyl acetate extract (CS-EA) from the leaves of *S. spectabilis* showed antileishmanial activity by inhibiting the promastigotes' growth (IC₅₀ = 70.29 ± 0.38 µg/mL). The CS-EA extract was then further purified using silica gel column chromatography by subjecting it to series of bioassay-guided fractionations. Further fractionation results in only fraction 8 (CS-EA-CC1-F8) exhibited antileishmanial activity as much as 20% in decreasing intracellular amastigotes at 33.33 ± 0.05 µg/mL. Subsequently, CS-EA-CC1-F8 was further fractionated via silica gel column chromatography. Results showed that fraction 4 (CS-EA-CC1-F8-CC2-F4) is the most active in decreasing the intracellular amastigotes (EC₅₀ = 31.75 ± 10.04 µg/mL) and the fraction was further purified using flash column chromatography. As a result, pure compound was isolated from fraction 1 (CS-EA-CC1-F8-CC2-F4-CC3-F1) and was designated as compound **1** which possessed the highest activity in decreasing intracellular amastigotes (EC₅₀ = 20.58 ± 1.65 µg/mL). Compound **1** was identified as (+)-spectaline, a known piperidine alkaloid (Figure 1) that agrees in every respect (IR, NMR and MS) with those in literature [32] which the comparison of NMR data are summarised in Table 2 and 3.

With an EC₅₀ value of 20.58 ± 1.65 µg/mL, compound **1** decreased the parasite burden or, it has the activity in macrophage clearance. This value seems to be more potent than activity of Amp-B against *L. donovani* infection on THP-1 which is around 231.02 µg/mL (0.25 µM) [33]. However, this value is less potent compared to berberine (a quaternary alkaloid found in several plant families) with an EC₅₀ value of 9.08 µg/mL (0.027 µM) [29]. Berberine has been used clinically and applied parenterally for the treatment of CL. However, it is topically inactive [34]. With IC₅₀ for cytotoxicity = 0.237 ± 0.004 µM and EC₅₀ in antileishmanial assay = 0.063 ± 0.005 µM, (+)-spectaline (Compound **1**) has selectivity index of 3.76.

(+)-spectaline has been successfully isolated from the leaves of *S. spectabilis* [32]. However, no antileishmanial studies being done on this compound thus far. On the other hand, a mixed compounds of (-)-spectaline and (-)-cassein from the flower of *S. spectabilis* has been reported to have antileishmanial properties (IC₅₀ = 24.9 ± 1.4 µg/mL) via extracellular promastigote assay by De Albuquerque Melo and colleagues [28]. To the best of our knowledge, this is the first report on the antileishmanial activity of (+)-spectaline via intracellular amastigotes assay model.

Acknowledgment.

This project was financially supported by the Ministry of Science, Technology and Innovation (MOSTI) Malaysia under Research Grant [09-05-IFN-BPH-003]. The authors would like to express their gratitude to: The team of the Drug Neglected Disease Initiative (DNDi), Professor Simon L. Croft and Dr. Vanessa Yardley from The LSHTM, UK for guidance in the bioassay transfer and for providing the *L. major* strain MHOM/SA/85/JISH118.

Table 1: Antileishmanial activity of *Senna spectabilis* extracts and fractions against *Leishmania major*.

a) Partition Extracts	
Samples	Promastigote assay activity (IC₅₀ µg/mL)
Ethyl acetate (CS-EA)	70.29 ± 0.38
<i>n</i> -Hexane (CS-Hex)	> 200
<i>n</i> -Butanol (CS-BuOH)	> 200
b) Amphotericin B^a	0.037 ± 0.075 µM (34.19 µg/mL)
c) Fractions (from CS-EA)	
Samples	Intracellular amastigote assay activity (EC₅₀ in µg/ml)
CS-EA-CC1-F1	> 100
CS-EA-CC1-F2	> 100
CS-EA-CC1-F3	> 100
CS-EA-CC1-F4	> 100
CS-EA-CC1-F5	> 100
CS-EA-CC1-F6	> 100
CS-EA-CC1-F7	> 100
CS-EA-CC1-F8	20 % inhibition at 33.33 ± 0.05
d) Fractions (from fraction CS-EA-CC1-F8)	
CS-EA-CC1-F8-CC2-F1	> 100
CS-EA-CC1-F8-CC2-F2	85.51 ± 10.65
CS-EA-CC1-F8-CC2-F3	> 100
CS-EA-CC1-F8-CC2-F4	31.75 ± 10.04
e) Fractions (from fraction CS-EA-CC1-F8-CC2-F4)	
CS-EA-CC1-F8-CC2-F4-CC3-F1	20.58 ± 1.65
CS-EA-CC1-F8-CC2-F4-CC3-F2	25.05 ± 1.19
CS-EA-CC1-F8-CC2-F4-CC3-F3	24.33 ± 1.31
CS-EA-CC1-F8-CC2-F4-CC3-F4	27.38 ± 1.55
CS-EA-CC1-F8-CC2-F4-CC3-F5	27.76 ± 1.37
CS-EA-CC1-F8-CC2-F4-CC3-F6	24.49 ± 2.15
CS-EA-CC1-F8-CC2-F4-CC3-F7	71.65 ± 3.53
f) Amphotericin B^b	0.50 ± 0.03 µM (462.04 µg/mL)

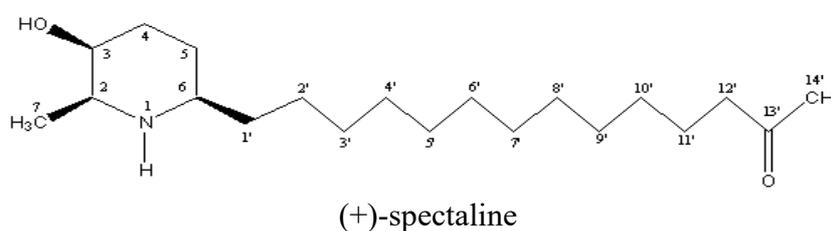
*Amphotericin B values are almost similar with previously reported by Escobar and colleagues [35] (Amphotericin B^a) and Siqueira-Neto and colleagues [33] (Amphotericin B^b) respectively. The mean ± SEM of triplicate experiments are displayed for all IC₅₀ & EC₅₀ values.

Table 2: ^1H NMR data of compound **1** compared with Christophidis and colleagues [32].

Position	Compound 1 (400 MHz NMR)	(+)-spectaline [32] (100 MHz PMR)
H-7	1.25 d 3H J=8Hz	1.10 d 3H J=6.5 Hz
H- (2'-10')	1.17 s 22H	1.28 s 22H
H-14'	2.06 s 3H	2.13 s 3H
H-12'	2.35 t 2H J=8Hz	2.42 t 2H J=6.5 Hz
H-2	2.86 s 1H bd	2.70 m 1H bd
H-6	3.09 s 1H bd	2.90 m 1H bd
H-3	3.78 s 1H	3.55 s 1H bd

Table 3: ^{13}C NMR data of compound **1** compared with Christophidis and colleagues [32].

Position	Compound 1 (400 MHz NMR)	(+)-spectaline [32] (100 MHz CMR)
2	57.1 (d)	57.0 (d)
3	66.1 (d)	67.6 (d)
4	30.8 (s)	32.3 (t)
5	26.1 (d)	26.5 (t)
6	51.4 (d)	55.7 (d)
7-CH ₃	15.9 (s)	19.0 (q)
1'	33.7 (s)	37.1 (t)
2'	} 29.4-29.9 (s)	} 30.0 (s)
3'		
4'		
5'		
6'		
7'		
8'		
9'		
10'		
11'		
12'	44.1 (d)	43.7 (t)
13' COCH ₃	209.9(s)	206.4 (s)
14' - CH ₃	30.2 (s)	26.0 (q)

Figure 1: The chemical structures of (+)-spectaline (compound **1**).

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