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Antiprotozoal activities of *Vincetoxicum stocksii* and *Carum copticum*

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Abstract

The antileishmanial and antimalarial activities of different fractions of *Vincetoxicum stocksii, Carum copticum, Gentiana olivierii* and *Zygophyllum fabago* were determined. Amongst these, *V. stocksii* and *C. copticum* showed moderate to good antimalarial activity. The ethyl acetate fractions of *C. copticum* and hexane fraction of *V. stocksii* showed 94 and 52% inhibition of *Plasmodium falciparum* (D6 Clone) respectively. The hexane ethyl acetate fraction of *C. copticum V. stocksi* exhibited antileishmanial activity against, *Leishmania donovani promastigotes* with IC_{50} of 30 µg/mL and 51 µg/mL, respectively.

Introduction

The parasitic diseases such as malaria and leishmaniasis have a high death rate in developing countries. Every year more than 500 million people, mostly those living in the world's poorest countries are at risk of malaria (Mansoor, 2003). Approximately 350 million people living in tropical and sub-tropical countries, suffer from this disease (Bharate, 2007). Therefore, there is a need to develop effective antimalarial and antileishmanial drugs for the treatment of these diseases.

The natives of Balochistan from centuries have been using more than 400 species of medicinal plants. The medicinally important plants *Carum copticum* Linn. Syn (Umbelliferae), *Vincetoxicum stocksii* (Asclepiadaceae) Ali & Khatoon, *Gentiana olivieri* Griseb (Gentianaceae) and *Zygophyllum fabago* (Zygophyllaceae) are widely distributed in Pakistan. *C. copticum* is used for curing diarrhea, amebiasis, dyspepsia and killing worms in stomach and other stomach disorders. It is also used as an antihelmintic, antispasmosadic and antiseptic agent (Sahaf, 2007). It is traditionally used, as antileishmanial herb *Vincetoxicum stocksii is* used by the natives as poultice for the treatment of wounds, injuries and external cancers (Staerk et al., 2000). *G. olivieri* is used in traditional use folk medicine for the treatment of skin diseases, abscesses, ulcer, blood pressure (Mansoor et al., 1998) and fever caused by malaria. *Z. fabago* is used to treat skin diseases, septic, injuries and external wounds (Zaidi, 2006) and Leishmania. In this paper the antimalarial and antileishmanial effects of these four medicinal plants were determined.

Materials and Methods

Plant material

The whole plant of *C. copticum, V. stocksii, G. olivieri and Z. fabago* (2 kg each) were collected in June 2006 from the mountains of Quetta. These were identified and a voucher specimen of all the plants were deposited in the herbarium of Botanical Garden, University of Balochistan Quetta. Plants were cleaned, dried in shade, ground, packed and then sent to USA for analysis. These were soaked in ethanol and extracted repeatedly 3-4 times at room temperature. The extract was filtered

and concentrated under reduced pressure in a rotary evaporator at 40°C, to obtain thick crude extract, which yielded *C. copticum* (23.4 g), *V. stocksii* (20.7 g), *G. olivieri* (27.5 g) *and Z. fabago* (22.8 g). The crude ethanolic extract of these plants was evaluated for antimalarial and antileishmanial activity.

Preparation of fractions

Only biologically active plant extracts were subjected to further fractionation. The crude ethanol extract of *C. copticum* was fractionated by vacuum liquid chromatography. Elution was initiated by hexane (Fraction ACCH-1 4.6 g), followed by hexane/ethyl acetate (Fraction ACCHE-2, 2.8 g), ethyl acetate (Fraction ACCE-3, 1.9 g), ethyl acetate/acetone (Fraction ACCEA-4, 2.5 g), acetone (Fraction ACCA-5, 2.8 g), acetone/methanol (Fraction ACCAM-6, 2.7 g), methanol (Fraction AccM-7, 3.5 g) and finally with water (ACCW-8, 2.1 g).

Ethanol extract of *V. stocksii* was dissolved in water and partitioned first with hexane (Fraction AVH, 3.9 g) and then with ethyl acetate (Fraction AVE, 5.3 g) and finally with butanol (Fraction AVB, 9.8 g). The activity of each fraction was determined for antimalarial and antileishmanial assay.

Antileishmanial activity

Antileishmanial activity of the extracts of the plants was tested *in vitro* against a culture of *Leishmania donovani promastigotes* grown at 26°C, in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco Chem. Co.). A 3-day-old culture was diluted to 5 x 10^5 *promastigotes*/mL. Extract dilutions were prepared in cell suspension in 96-well plates, which were incubated at 26°C for 48 hours. Almar blue assay was used to determine the growth of *L. donovani promastigotes* (Mikus and Steverding, 2000). Fluostar Galaxy plate reader (BMG Lab Technologies) was used to measure standard fluorescence at excitation wavelength of 544 nm and emission wavelength of 590 nm. Amphotericin B and pentamidine were used as the standard antileishmanial agents. IC_{50} and IC_{90} values were computed from dose-response curves generated by plotting percent growth versus drug concentration.

Assay for in vitro antimalarial activity

The plasmodial LDH activity is the bases for the determination of antimalarial activity. A red blood cells suspension was infected with D6 or W2 strains of *P. falciparum* D6 Clone (200 µL, with 2% hematocrit and 2% parasitemia, in RPMI 1640 medium, with 60 µg/mL amikacin and 10% human serum) was added to the wells of a 96 well plate containing 10 µg/mL of test samples diluted in medium at various concentrations. The plate was kept in a modular incubation chamber (Billups-Rothenberg, CA) flushed with 90% N_2, 5% O_2, and 5% CO_2 gas mixture, and incubated at 37°C, for 72 hours. Malstat™ reagent was used to determine parasitic LDH activity (Flow Inc., Portland, OR) as described by elsewhere (Makler and Hinrichs, 1993). Incubation mixture (20 µL) was mixed with 100 µL of the Malstat™ reagent at room temperature and incubated for 30 min. Then twenty microliters of of NBT/PES (Sigma, USA) 1:1 mixture was added. The plate was further incubated for 1 hour in the dark. Hundred microliter of a 5% acetic acid solution was then added to stop the reaction. EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont) was used to read the plate at 650 nm. IC_{50} values were computed from the dose–response curves. Chloroquine and artemisinin were used as the drug controls in each assay. DMSO (0.25%) was used as vehicle for control.

Results and Discussion

Ethanol extracts of all the four plants were evaluated for *in vitro* antileishmanial and antimalarial, activities. Only the extracts of *C. copticum* and *V. stocksii* showed moderate to mild antileishmanial activities with IC_{90} values of 60 and 70 µg/mL respectively. IC_{50} of 50 µg/mL was detected for *V. stocksii* (Table I). No such activity was shown by *G. olivieri* and *Z. fabago. C. copticum* showed good antimalarial activity, whereas, moderate activity was shown by *V. stocksii.* Poor activity was shown by *G. olivieri and* no such activity was observed by *Z. fabago.* Therefore, further fractionation of the extracts of the two active medicinal plants

Table I

Antiprotozoal activity of the ethanol extracts of Gentiana olivieri, Carum copticum, Zygophylum fabago and Vincetoxicum stocksii

Sample Name	P. falciparum	*L. donovani*	*L. donovani*
	% Inhibition	IC_{50} (µg/mL)	IC_{90} (µg/mL)
Gentiana olivieri	28	Inactive	Inactive
Carum copticum	60	22	60
Zygophyllum fabago	Inactive	Inactive	Inactive
Vincetoxicum stocksii	40	50	70
Pentamidine	Not tested	1.2	6
Amphotericin B	Not tested	0.3	0.7
Chloroquine	99.2	Not tested	Not tested
Artemisinin	98.8	Not tested	Not tested

IC_{50} and IC_{90} are the sample concentrations that kill 50 and 90% *Leishmanial* cells compared to the solvent controls; Test = 3 concentrations: 100, 20, 4 µg/mL

Table II

In vitro* antimalarial and antileishmanial activities of fractions of *Carum copticum

Samples Name	*P. falcipa-rum*	*L. donovani*	
	% Inhibition	IC_{50} (µg/mL)	IC_{90} (µg/mL)
Carum copti-cum (ACCH -1)	0	90	Inactive
Carum copti-cum (ACCHE-2)	90	30	90
Carum copti-cum (ACCE -3)	94	50	90
Carum copti-cum (ACCEA-4)	82	50	98
Pentamidine	Not tested	2.1	6.2
Amphoteri-cin B	Not tested	0.09	0.2
Chloroquine	99	Not tested	Not tested
Artemisinin	99.4	Not tested	Not tested

IC_{50} and IC_{90}, are the concentrations that affords 50 and 90% inhibition of leishmanial growth compared to the solvent controls; Test = 3 concentrations; *Carum copticum* concentration: 100, 20, 4 µg/mL

Table III

In vitro* antimalarial and antileishmanial activity of the fractions of *vincetoxicum stocksii

Samples Name	*P. falcipa-rum*	*L. donovani*	
	% Inhibi-tion	IC_{50} (µg/mL)	IC_{90} (µg/mL)
Vincetoxi-cum stocksii AVH	52	51	90
Vincetoxi-cum stocksii AVE	21	Inactive*	Inactive*
Vincetoxi-cum stocksii AVB	15	Inactive*	Inactive*
Pentamidine	Not tested	1.2	5.2
Amphoteri-cin B	Not tested	0.5	0.9
Chloroquine	99.6	Not tested	Not tested
Artemisinin	99	Not tested	Not tested

IC_{50} and IC_{90}, are the concentrations that affords 50 and 90% inhibition of leishmanial growth compared to the solvent controls; *at 40 µg/mL

i.e., C. copticum and *V. stocksii* were carried out.

The most potent antilimalarial activity (94%) was exhibited by fraction ACCE-3 and ACCHE-2 (90%) of *C. copticum* (Table II). The significant antimalarial activity of ACCE-2 and ACCHE-3 indicates that the active compounds must be present in the less-polar fractions of hexane/ethyl acetate (Fraction ACCHE-2) and ethyl acetate (ACCE-3). The chemical composition of the essential oil from dry seeds of *C. copticum* was found to have thymol (41.3%), α-terpinolene (17.5%) and ρ-cymene (11.8%) as the major constituents of the oil and have shown to exhibit strong insecticidal activity. The mortalities of the insect species reached 100% at concentrations higher than 185.2 µL/L and 12 hours exposure time (Sahaf, 2007). The findings indicate that *C. copticum* oil had potential antiprotozoal activities.

Since good antiprotozoal activity was shown by *Vincetoxicum stocksii,* so its ethanol extract was dissolved in water and partitioned first with hexane (Fraction AVH) and then with ethyl acetate (Fraction AVE) and finally with butanol (Fraction AVB) (Table III). Moderate activity was shown against *P. falciparum* and *L. donovani* by the hexane fraction of *V. stocksii* whereas poor or no activity was shown by the other two fractions.

Vincetoxicum is known for its poisonous as well as its medicinal properties. It has also been found to be allelopathic and active against a number of microorganisms (Zaidi and Crow, 2005). Pronounced cytotoxicity has also been reported against *Candida albicans* by Staerk et al. (2000). This effect is probably due to the presence of phenanthroindolizidine alkaloids reported from other species of *Vincetoxicum.*

Conclusion

This study demonstrates good antimalarial and moderate anitleishmanial activity of the extracts of *C. copticum* and *V. stocksi*, which parallels the traditional use of these herbs as an antiprotozoal medicine.

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