In Vitro Antiparasitic Activity and Chemical Composition of the Essential Oil Obtained from the Fruits of *Piper cubeba*

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Abstract

Protozoans of the trypanosomatid family cause the neglected tropical diseases leishmaniasis and trypanosomiasis, for which few drugs are available. In this context our group has recently reported that the essential oil obtained by steam distillation of the fruits of *Piper cubeba* is active against *Schistosoma mansoni*. Therefore, we have investigated the *in vitro* effects of the essential oil against the trypomastigote and amastigote forms of *Trypanosoma cruzi* isolated from an $LLCMK_2$ cell line culture and the promastigote forms of *Leishmania amazonensis*. The *in vitro* activity of the essential oil against trypomastigotes of *T. cruzi* increased upon rising concentrations, giving IC_{50} values of 45.5 and 87.9 $\mu g \cdot mL^{-1}$ against trypomastigotes and amastigotes, respectively. The essential oil was not active against *L. amazonensis*, since it displayed lyses of only 24% at 400 $\mu g \cdot mL^{-1}$, and an IC_{50} of 326.5 $\mu g \cdot mL^{-1}$. Therefore, the essential oil should be further investigated to determine the compounds responsible for the observed activities, as well as its mechanism of action.

Key words

Piper cubeba · Piperaceae · essential oil · trypanocidal activity · leishmanicidal activity

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Protozoans of the trypanosomatid family cause two of the seventeen neglected tropical diseases – leishmaniasis and trypanosomiasis [1], both affecting millions of people worldwide. The flagellate protozoan *Trypanosoma cruzi* is the etiological agent of Chagas' disease [2]. In 2009, approximately 10 million people were infected with this parasite, with an estimated number of 10000 deaths per year [3]. Today it is reckoned that eight million people are infected with *T. cruzi* in Latin America alone [4], 30–40% of which have or will develop cardiomyopathy or mega digestive syndrome [5,6]. Leishmaniases, a group of diseases caused by various species of the protozoan parasite genus Leishmania, are endemic in 88 countries, have an overall prevalence of 12 million cases worldwide and culminate in approximately 50000 deaths annually. Currently, 350 million people are at risk of infection with the protozoans of the trypanosomatid family [7].

The medications currently available to treat trypanosomiasis and leishmaniases have undesirable side effects and low efficiency, not to mention that parasites have developed resistance to them. In recent years, many pharmacologically active compounds derived from plants have been discovered [8,9], and species belonging to the genus *Piper* (Piperaceae) are a good source for prospecting antiparasitical compounds [10,11].

Considering the lack of vaccines, the toxicity of the chemotherapies used to treat trypanosomiasis and leishmaniasis, and the resistance of the parasites to the current drugs, as well as the previous work reported by our group on the activity of *Piper cubeba* L. essential oil (PC-EO) against *Schistosoma mansoni* [12], we have undertaken the *in vitro* assay of PE-EO against *Trypanosoma cruzi* and *Leishmania amazonensis.*

GC-MS analysis of PC-EO revealed that sabinene (19.99%), eucalyptol (11.87%), 4-terpineol (6.36%), *β*-pinene (5.81%), and camphor (5.61%) are the major components (**Table 1S**; Supporting Information). This chemical composition was found to be similar to that reported by other authors for other *Piper* species [12].

Currently, the treatment of Chagas' disease in Brazil primarily depends on benznidazole (Bz) [13], and the first-line chemotherapy to treat leishmaniosis relies on pentavalent organoantimonials (Pentostam® and Glucantime®), which display variable efficacy among strains and species, and high toxicity [14].

Previous works have shown that PC-EO is effective against *Schistosoma mansoni* [12]. Additionally, PC-EO has been reported to exhibit antibacterial activity against *Bacillus subtilis, Streptococcus faecalis*, and *Staphylococcus aureus*, as well as antifungal action against *Aspergillus fumigatus, Aspergillus flavus*, and *Fusarium solani*, among others. It also displays anthelmintic activity against earthworms and tapeworms *in vitro* [15].

The activity of PC-EO against a culture of *T. cruzi* trypomastigotes increased with rising concentrations of the oil; the highest concentration of PC-EO tested (400 $\mu g \cdot mL^{-1}$) yielded 65.7% lysis and an IC_{50} equal to 45.5 $\mu g \cdot mL^{-1}$ (**Table 1**). In the case of *T. cruzi* amastigotes, this oil afforded over 50% lysis at dosages between 100 and 200 $\mu g \cdot mL^{-1}$, and an IC_{50} value of 87.9 $\mu g \cdot mL^{-1}$ (**Table 1**). Benznidazole, used as the positive control, gave 66.3 and 78.3% of amastigote lysis at 100 and 200 $\mu g \cdot mL^{-1}$, respectively, and an IC_{50} equal to 53.2 $\mu g \cdot mL^{-1}$. Therefore, PC-EO exhibited activity similar to the currently employed benznidazole.

PC-EO was not active against the promastigote forms of *Leishmania amazonensis* in the evaluated concentrations (**Table 2**). Amphotericin B displayed an IC_{50} value of 73.4 at 12.5 $\mu g \cdot mL^{-1}$.

It is suggested that the activity might be a consequence of the additive effect of several compounds, majorly monoterpenes, which correspond to about 90% of the total extract. The major compounds in the oil are sabinene, eucalyptol, 4-terpineol, *β*-pinene, camphor, and *α*-copaene, but sometimes they are not the ones responsible for the activity. However, some of these compounds have been reported to display antiparasitic activity. Tóro et al. [16] reported that the pinenes from *Pinus ellioti* are active against the fish parasite *Lerneae cyprinacea*. Yadav et al. [17] reported that sabinene is active against *Hymenolepis diminuta*, and Chen et al. [18] reported that camphor is active *in vivo* against *Eimeria tenella*.

It is important to point out that PC-EO did not display cytotoxicity against fibroblast cells at the analyzed concentration range [12]. There are indications that, owing to their lipid solubility, essential

Table 1 Trypanocidal activity of the essential oil of *P. cubeba* L. against trypomastigote and amastigote forms of *Trypanosoma cruzi*.

Samples	% Lysis ± SEM/concentrations (μg·mL^{-1})							
	6.25	12.5	25	50	100	200	400	IC_{50} (μg·mL^{-1})
1	10.8 ± 1.2	12.9 ± 2.2	13.1 ± 2.2	29.8 ± 0.4	55.5 ± 1.2	62.7 ± 1.0	65.7 ± 0.7	45.5
2	13.9 ± 8.6	14.4 ± 7.9	25.7 ± 11.3	39.8 ± 10.0	53.9 ± 8.7	58.5 ± 9.7	59.8 ± 8.4	87.9
3	7.9 ± 5.6	16.4 ± 9.9	23.8 ± 7.7	47.7 ± 8.6	66.3 ± 10.0	78.3 ± 10.8	79.7 ± 7.8	53.2

1: *Piper cubeba* essential oil (PC-EO) against trypomastigotes; 2: Benznidazole (Bz) against amastigotes; 3: *Piper cubeba* essential oil (PC-EO) against amastigotes

Table 2 Leishmanicidal activity of the essential oil of *P. cubeba* L. against promastigote forms of *Leishmania amazonensis*.

Samples	% Lysis ± SEM/concentrations (μg·mL^{-1})							
	6.25	12.5	25	50	100	200	400	IC_{50} (μg·mL^{-1})
1	0 ± 0	0.6 ± 0.5	8.6 ± 13.6	13.9 ± 7.2	18.1 ± 13.3	20.6 ± 8.6	24.3 ± 2.2	326.5
2	67.4 ± 4.5	69.6 ± 5.3	79.2 ± 8.3	81.1 ± 7.5	84.4 ± 5.2	90.5 ± 4.3	97.3 ± 1.3	73.41

1: *Piper cubeba* essential oils (PC-EO) against promastigotes; 2: Amphotericin B

oils present properties such as low density and rapid diffusion across cell membranes. Therefore, they could damage the parasite cell membrane structure and lead to cellular lysis [19], not to mention that synergistic and/or additive effects among the constituents of PC-EO could take place [20].

In conclusion, it was found that *Piper cubeba* displays trypanocidal activity and should be further investigated.

Materials and Methods

The PC-EO obtained by steam distillation (batch number S-4113526) was purchased from Suraj Bala Exports. It was obtained and analyzed according to the method previously described [12].

To obtain the trypomastigotes of *T. cruzi* for the trypanocidal assay, $LLCMK_2$ cells (epithelial cells of monkey kidney) were cultured in RPMI. Approximately 1×10^6 trypomastigotes were added to each well of a 96-well microtiter plate, and PC-EO was tested at concentrations ranging from 6.25 to 400 μg·mL^{-1}. The biological activity was analyzed by colorimetric MTT tetrazolium salt [21].

Amastigotes were obtained from $LLCMK_2$ cell cultures as previously described [22]. The microplates were incubated for further 48 h, followed by using the colorimetric Giemsa method. The biological activity was verified quantitatively by counting the infected cells and determining the percentage of parasitic reduction.

Promastigotes of *Leishmania amazonensis* for the Leishmaniasis assay were grown at the Parasitology Laboratory of the University of Franca, Brazil. Biological assays were performed according to the method previously described [22].

Results are expressed as the mean ± SEM. The statistical tests were performed using Graphpad Prism software (version 5.0). Data were statistically analyzed by one-way analysis of variance (ANOVA), followed by Dunnet's comparison test.

Supporting information

A representative chromatogram of PC-EO and its chemical constituents identified by GC-MS are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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