

# Antitumour Efficacy of *Piper tuberculatum* and Piplartine Based on the Hollow Fiber Assay

## Authors

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## Key words

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## Abstract

*Piper tuberculatum*, popularly known in Brazil as “jaborandi falso” and “pimenta darta”, is widely used in folk medicine for the treatment of several diseases. In this study, the *in vivo* hollow fiber assay was used to investigate the antitumour efficacy of the crude extract and piplartine obtained from *P. tuberculatum* roots. Human glioblastoma (SF-295) and colon carcinoma (HCT-8) cell lines were used. *In vitro* cytotoxicity was assayed by the MTT assay. In the hollow fiber assay, nude

mice implanted with tumour cells in hollow fibers were treated for four consecutive days via the intraperitoneal route, and tumour cell populations were assessed by the MTT assay. Both the crude extract and piplartine displayed cytotoxicity. In the hollow fiber assay, tumour growth inhibition rates were 24.6–54.8% for the crude extract and 33.7–62.2% for piplartine. No signal of toxicity was noticed. In conclusion, the crude extract and piplartine obtained from *P. tuberculatum* roots displayed *in vitro* and *in vivo* anticancer efficacy.

## Introduction

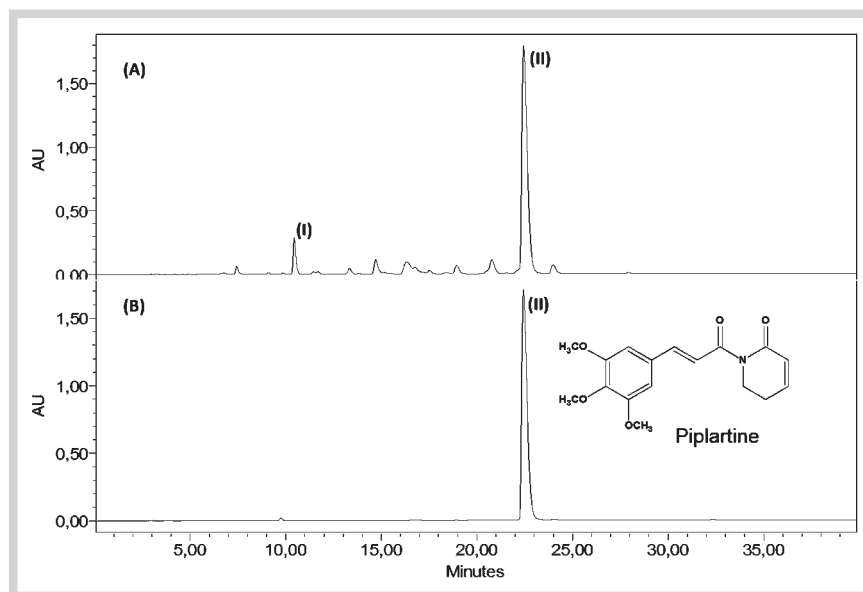
*Piper tuberculatum* Jacq. is a species of pepper belonging to the Piperaceae family. It is popularly known in Brazil as “jaborandi falso” and “pimenta darta”, and is widely used in folk medicine as an analgesic, sedative, antidote for snakebites, and treatment for digestive disorders [1,2]. Some pharmacological studies have reported larvicidal [3], molluscicidal [4], and gastroprotective [5] activities of extracts and fractions of *P. tuberculatum*. Phytochemical analyses of *P. tuberculatum* extracts have identified the presence of a variety of amides with different biological activities [1,2,5–7]. Among these compounds, piplartine, also known as piperlongumine, is the most commonly studied.

The reported pharmacological activities of piplartine include cytotoxic [6,8–10], antitumour [10–12], genotoxic [13,14], antiangiogenic [10], antimetastatic [10], anxiolytic [2], antidepressant [2], leishmanicidal [15], and trypanocidal [16] activities. Among the multiple pharmacological effects of piplartine, its anticancer property is the most promising [17]. In this study, the *in vivo* hollow fiber assay (HFA) was used to investigate the antitumour efficacy of the crude extract and piplartine obtained from *P. tuberculatum* roots.

## Results and Discussion

The present work investigated the *in vivo* antitumour efficacy of the crude extract and piplartine obtained from *P. tuberculatum* roots through the *in vivo* HFA. The HFA is a standard *in vivo* model that allows simultaneous evaluation of multiple cell lines. It was developed by Hollingshead et al. [18] as a model with a shorter evaluation time and a reduced compound requirement compared to traditional xenograft models. The *in vitro* cytotoxic effect was also assayed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. As cited above, the *in vitro* cytotoxic effect of piplartine has been previously examined [6,8–10]; however, *in vitro* cytotoxicity of the crude extract obtained from *P. tuberculatum* roots has never been investigated. In addition, the *in vivo* antitumour efficacy of the crude extract and piplartine obtained from *P. tuberculatum* roots was, in this study, evaluated through the *in vivo* HFA for the first time.

The extraction of *P. tuberculatum* roots resulted in a crude extract and piplartine. ● Fig. 1 shows representative chromatograms from HPLC analysis of the crude extract and piplartine obtained from *P. tuberculatum*. Piplartine is the main compound found in the crude extract.



**Fig. 1** Representative chromatograms from HPLC analysis of the crude extract and piplartine obtained from *P. tuberculatum*. **A** Crude hexane/ethyl acetate 1 : 1 extract from the roots of *P. tuberculatum*. **B** Piplartine precipitate from the crude hexane/ethyl acetate 1 : 1 extract in methanol. **I** Fatty material. **II** Piplartine.

Cell lines	Histotype	IC <sub>50</sub> (µg/mL)		
		Crude extract	Piplartine	5-FU
SF-295	Glioblastoma	> 10	0.8	0.3
			0.6–1.2	0.2–0.4
HCT-8	Colon carcinoma	4.3	0.7	0.2
		2.8–6.7	0.5–0.9	0.2–0.3

**Table 1** *In vitro* cytotoxic activity of the crude extract and piplartine obtained from *P. tuberculatum* roots against tumour cell lines.

Data are presented as IC<sub>50</sub> values and their 95% confidence interval obtained by nonlinear regression from two independent experiments performed in duplicate; 5-Fluorouracil (5-FU) was used as a positive control

The *in vitro* cytotoxicity was evaluated for human tumour cell lines SF-295 (glioblastoma) and HCT-8 (colon carcinoma) using the MTT assay. 5-Fluorouracil (5-FU) was used as a positive control. **Table 1** summarises the half maximal inhibitory concentration (IC<sub>50</sub>) data for cytotoxic activity. The IC<sub>50</sub> values were > 10 and 4.3 µg/mL for the crude extract obtained in SF-295 and HCT-8 cell lines, respectively, and 0.8 and 0.7 µg/mL for piplartine obtained in the same cell lines, respectively. 5-FU, used as a positive control, showed IC<sub>50</sub> values of 0.3 and 0.2 µg/mL obtained in SF-295 and HCT-8 cell lines, respectively.

According to the preclinical anticancer drug-screening program used in this study, a crude extract that presents IC<sub>50</sub> values below 30 µg/mL and a lead compound that presents IC<sub>50</sub> values below 4 µg/mL in tumour cell line-based assays are considered promising for anticancer drug development [19]. Therefore, the cytotoxic activity observed for the crude extract and piplartine obtained from *P. tuberculatum* is considered promising.

In a second set of experiments, the *in vivo* HFA was used to assess the antitumour efficacy of the drugs. With this aim, tumour cells (i.e., SF-295 and HCT-8) were cultivated within biocompatible, semipermeable polyvinylidene fluoride (PVDF) hollow fibers subcutaneously implanted in the dorsal part of BALB/c nude mice. The crude extract and piplartine obtained from *P. tuberculatum* and 5-FU, used as a positive control, were administered intraperitoneally during four consecutive days. On day five, the fibers were removed to quantify cell proliferation. As shown in **Table 2**, the crude extract reduced cell proliferation of tumour cells SF-295 (40.3 and 54.8%) and HCT-8 (24.6 and 42.4%) at doses of 100 and 200 mg/kg/day, respectively, and piplartine re-

duced cell proliferation of tumour cells SF-295 (62.2 and 61.5%) and HCT-8 (33.7 and 50.8%) at doses of 50 and 100 mg/kg/day, respectively. In addition, 5-FU reduced cell proliferation of tumour cells SF-295 (53.7%) and HCT-8 (70.0%) at doses of 20 mg/kg/day. In the *Piper* genus, the extract of *Piper capense* displayed cytotoxicity against leukaemia CCRF-CEM, CEM/ADR5000, and MDA-MB-231 cell lines. In addition, it induced apoptosis in CCRF-CEM cells via the alteration loss of the mitochondrial membrane potential and enhanced the production of reactive oxygen species [20]. In a screening of Venezuelan medicinal plant extracts for anticancer activity, the extracts of *Piper san-vicentense* and *Piper arboretum* showed the most cytotoxic and cytostatic activity, respectively [21]. Moreover, the extracts of *Piper imperiale* exhibited concentration-dependent cytotoxic effects against MCF-7 cancer cells [22]. The extract of the plant *Piper longum* showed potent immunomodulatory and antitumour activity, as demonstrated by different *in vitro* and *in vivo* animal models [23]. Herein, the crude extract and piplartine obtained from *P. tuberculatum* showed interesting *in vitro* and *in vivo* anticancer activity. Moreover, piplartine is a known cytotoxic agent. Its molecular pathway includes induction of oxidative stress selectively in cancer cells by the inhibition of GSTp1 and CRB1. Piplartine can be used to induce genotoxicity as an alternative strategy to killing tumour cells. In addition, piplartine has excellent oral bioavailability and inhibits tumour growth in animal models. This compound also demonstrated weak systemic toxicity that makes it suitable as a novel antineoplastic agent [10–12, 17].

The hollow fibers were quite well tolerated by the animals, and no signals of rejection were detected. The treatments with the

**Table 2** Antitumour efficacy of the crude extract and piplartine obtained from *P. tuberculatum* roots assessed by the *in vivo* hollow fiber assay.

Treatment	Dose (mg/kg/day)	Survival	Proliferation (OD 595 nm)		Inhibition (%)	
			SF-295	HCT-8	SF-295	HCT-8
5% DMSO	–	6/6	0.86 ± 0.13	1.12 ± 0.11	–	–
5-FU	20	6/6	0.40 ± 0.05*	0.33 ± 0.09*	53.7	70.0
Crude extract	100	6/6	0.51 ± 0.10*	0.84 ± 0.14	40.3	24.6
	200	6/6	0.39 ± 0.03*	0.64 ± 0.06*	54.8	42.4
Piplartine	50	5/5	0.32 ± 0.04*	0.74 ± 0.13	62.2	33.7
	100	5/5	0.33 ± 0.06*	0.55 ± 0.05*	61.5	50.8

Data are presented as mean ± SEM for 5–6 animals; Starting one day after hollow fiber implantation, the animals were treated for four consecutive days via the intraperitoneal route; The negative control was treated with the vehicle used for diluting the test substance (5% DMSO). 5-Fluorouracil (5-FU) was used as a positive control; \*  $p < 0.05$  compared with the 5% DMSO group by ANOVA followed by Student-Newman-Keuls

crude extract and piplartine obtained from *P. tuberculatum* and 5-FU did not affect the conditions of the mice beyond acceptable limits, and no deaths were noticed. No significant changes in body weight were seen in the crude extract- or piplartine-treated groups. Moreover, in order to evaluate the toxicological aspects related to drug treatment, histological analyses of the liver, kidneys, heart, lungs, and spleen of the treated animals were performed; however, the treatments with the crude extract and piplartine obtained from *P. tuberculatum* did not affect the morphology of the analysed organs.

In conclusion, the crude extract and piplartine obtained from *P. tuberculatum* displayed *in vitro* and *in vivo* anticancer efficacy. The data presented here reinforce the anticancer potential of the genus *Piper*.

## Materials and Methods



### Cells

Cytotoxicity and antitumour assays were determined in human tumour cells, namely, SF-295 (glioblastoma) and HCT-8 (colon carcinoma), donated by Children's Mercy Hospital, Kansas City, MO, USA. Cells were maintained in RPMI-1640 medium (Gibco-BRL) supplemented with 10% FBS (Cultilab) and 2 mM L-glutamine (Vetec Química Fina). Cells were harvested by treatment with 0.25% trypsin EDTA solution (Gibco-BRL). All cell lines were cultured in cell culture flasks at 37 °C in a 5% CO<sub>2</sub> atmosphere and subcultured every 3–4 days to maintain exponential growth. Cytotoxicity and antitumour experiments were conducted with cells in the exponential growth phase.

### Animals

A total of 34 female BALB/c nude (nu/nu) mice, aged 6–8 weeks, were obtained from the animal facilities of the State University of São Paulo, Faculty of Medicine, São Paulo, Brazil. They were kept in well-ventilated, sterile cages (Alesco) under standard conditions of light (12 h with alternate day and night cycles) and temperature (22 ± 1 °C), and were housed with access to a commercial sterile rodent stock diet (Nutrilabor) and water *ad libitum*. The animals were treated according to the ethical principles for animal experimentation of the SBCAL (Brazilian Association of Laboratory Animal Science), Brazil, and the study was approved by the local Ethical Committee on Animal Research (# 102/2007).

### Plant material

Roots of *P. tuberculatum* were collected at Campus do Pici of the Universidade Federal do Ceará, Fortaleza, Ceará State, Northeastern Brazil, in January 2014. The identification was performed by E.R. Silveira, after comparison with the voucher specimen EAC 34736 deposited at the Prisco Bezerra Herbarium, Departamento de Biologia, Universidade Federal do Ceará.

### Preparation of the crude extract and piplartine isolation

The dried powdered roots (331.0 g) of *P. tuberculatum* were macerated with a mixture of hexane/ethyl acetate 1 : 1 (1 × 2.0 L and 2 × 1.3 L). After filtration, the solutions were pooled together and evaporated under reduced pressure to yield a yellowish solid crude extract (3.47 g). The crude extract (2.58 g) was suspended in MeOH to yield 895.2 mg of a precipitate and 1.44 g of the mother liquors. The precipitate was recrystallised from methanol to yield piplartine (purity > 99%).

The chromatographic analysis was carried out using a Waters 1525 (Milford) chromatograph, equipped with a binary pump and a photodiode-array detector (Waters-2996 PDA). A gradient elution mode, using hexane-ethyl acetate, varying from 20% to 100% ethyl acetate, over a 40-min total running time, was accomplished through a Phenomenex silica column (250 × 4.6 mm) using an injection volume of 50 µL at a flow rate of 1.0 mL/min, while observing at 275 nm.

### *In vitro* cytotoxic activity assay

Tumour cell growth was determined by the ability of living cells to reduce the yellow dye MTT to a purple formazan product, as described by Mosmann [24]. For all experiments, cells were seeded in 96-well plates (0.7 × 10<sup>5</sup> cells/mL in 100 µL of medium). After 24 h, the drugs (0.039 to 10 µg/mL) were dissolved in pure DMSO and added to each well using high-throughput screening (HTS; Biomek 3000, Beckman Coulter, Inc.). Then, the cells were incubated for 72 h. 5-FU (purity > 99%; Sigma Chemical Co.) was used as a positive control. At the end of incubation, the plates were centrifuged and the medium was replaced by fresh medium (150 µL) containing 0.5 mg/mL MTT. Three hours later, the formazan product was dissolved in 150 µL DMSO and absorbance was measured using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter, Inc.). The drug effects are expressed as the percentage of control absorbance of reduced dye at 595 nm.

### *In vivo* hollow fiber assay

The *in vivo* HFA was performed as previously described by Hollingshead et al. [18] with minor modifications. PVDF hollow fibers with a 1-mm internal diameter and a molecular weight cut-off point of 500 kDa were used (Spectrum Laboratories). The fibers were cut into 12–15 cm long pieces, washed 2× with sterile distilled water, and were then kept in sterile conditions.

Before use, under sterile conditions, the fibers were incubated in complete RPMI with 20% FBS overnight (packaging time). Cell viability was assessed by the trypan blue exclusion assay. A cell suspension of  $1 \times 10^7$  cell/mL (equivalent to 200 000 cells/20  $\mu$ L/2 cm PVDF fiber) at 4°C was injected into the fiber, with the ends thereof immediately heat-sealed. The fibers were cut into 2 cm each, transferred to petri plates, and incubated in complete RPMI medium for 24 h prior to implantation in the mice. Each cell was injected into one fiber of a different color (HCT-8, yellow fibers; SF-295, blue fibers).

Before implantation, the mice were anaesthetised with ketamine (90 mg/kg)-xylazine (4.5 mg/kg). A small incision in the neck was made to permit subcutaneous (s.c.) implantation of the fibers in the dorsal part of the animal. Each animal received two fibers at the s.c. site. All incisions were sealed with a surgical stapler. The treatments were started one day after hollow fiber implantation. At the beginning of the experiment, the mice were divided into six groups, as follows: group 1 – negative control (5% DMSO, n = 6); group 2 – positive control (5-FU, 20 mg/kg/day, n = 6); group 3 – crude extract (200 mg/kg/day, n = 6); group 4 – crude extract (100 mg/kg/day, n = 6); group 5 – piplartine (100 mg/kg/day, n = 5); group 6 – piplartine (50 mg/kg/day, n = 5). The dosages were determined based on previous articles [11, 12]. The test compounds were administered intraperitoneally during four consecutive days. On day five, the fibers were removed to quantify the antiproliferative capacity.

Tumour cell proliferation was quantified through the MTT assay. For this purpose, the fibers removed from the animals were incubated with 1 mg/mL MTT in 6-well plates during 4 h at 37°C and 5% CO<sub>2</sub>. The MTT solution was aspirated and the fibers were washed with saline solution containing protamine sulphate 2.5% and incubated in protamine solution overnight at 4°C. The fibers were transferred to 24-well plates, cut into 2 or 3 pieces, and put to dry. The formazan was dissolved in 500 mL DMSO, the aliquots (150  $\mu$ L) were transferred to 96-well plates, and absorbance was measured at 595 nm (DTX-880, Beckman Coulter). The drug effect was quantified as the percentage of control absorbance.

After the treatment described above, the liver, kidneys, heart, lungs, and spleen were excised and fixed in 10% formaldehyde. After fixation with formaldehyde, the organs were submitted to a gross examination for size or colour changes and haemorrhage. Portions were then cut into small pieces, followed by staining of the histological sections with haematoxylin and eosin. Histological analysis was performed by light microscopy. The presence and extent of liver, kidneys, heart, lungs, and spleen lesions attributed to the drugs were considered.

### Statistical analysis

Data are presented as mean  $\pm$  SEM or IC<sub>50</sub> values and their 95% confidence intervals (CI 95%) were obtained by nonlinear regression. The differences between experimental groups were compared by ANOVA (analysis of variance) followed by the Student-Newman-Keuls test ( $p < 0.05$ ). All statistical analyses were performed using the GraphPad program (Intuitive Software for Science).

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### Conflict of Interest

The authors have declared that there is no conflict of interest.

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