Antiproliferative Effects of Two Amides, Piperine and Piplartine, from *Piper* Species

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The present work evaluated the cytotoxicity of piplartine {5,6-dihydro-1-[1-oxo-3-(3,4,5-trimethoxyphenyl)-*trans*-2-propenyl]-2(1*H*)pyridinone} and piperine {1-[5-(1,3)-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidine}, components obtained from *Piper* species. The substances were tested for their cytotoxicity on the brine shrimp lethality assay, sea urchin eggs development, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay using tumor cell lines and lytic activity on mouse erythrocytes. Piperine showed higher toxicity in brine shrimp (DL_{50} = 2.8 ± 0.3 μg/ml) than piplartine (DL_{50} = 32.3 ± 3.4 μg/ml). Both piplartine and piperine inhibited the sea urchin eggs development during all phases examined, first and third cleavage and blastulae, but in this assay piplartine was more potent than piperine. In the MTT assay, piplartine was the most active with IC_{50} values in the range of 0.7 to 1.7 μg/ml. None of the tested substances induced hemolysis of mouse erythrocytes, suggesting that the cytotoxicity of piplartine and piperine was not related to membrane damage.

Key words: Piplartine, Piperine, Cytotoxicity

Introduction

The genus *Piper* (Piperaceae), widely distributed in the tropical and subtropical region of the world, includes many species and is often used as food flavouring, traditional medicines and pest control agents. The chloroform extract of the stems of *P. arborescens* was found to display significant activity against a KB cell culture system and a P-388 lymphocytic leukemia system in cell culture. An extract of the black pepper shows carcinogenesis in mice. The evidence of malignant tumors and of multiple tumors was greater in the pepper treated mice than in vehicle treated mice. Many amides from *Piper* species have insect antifeedant activity. Phytochemical investigations of *Piper* species have led to the isolation of several classes of physiologically active compounds such as alkaloids, amides, pyrones, terpenes, steroids, kawapyrones, piperolides, flavonoids, phenylpropanoids, lignans and neolignans (revised by Parmar *et al.*, 1997). Constituents of *Piper* species have inhibitory activity on prostaglandin and leukotriene biosynthesis *in vitro* (Stohr *et al.*, 2001).

Piperine was the first amide isolated from *Piper* species and was reported to display central nervous system depression, antifeedant, analgesic, antipyretic, anti-inflammatory, and antioxidant activities (Parmar *et al.*, 1997; Mittal and Gupta, 2000). It also prevents liver and stomach carcinogenesis induced by chemical carcinogens (Khajuria *et al.*, 1998) and protects aflatoxin B_1 (AFB_1)-induced cytotoxicity and genotoxicity in H4IIEC3 rat hepatoma cells (Singh *et al.*, 1994). Piperine was a non-genotoxic chemical when tested in Ames test using *Salmonella typhimurium*, micronucleus test, sperm shape abnormality test and dominant lethal test using Swiss albino mice (Karekar *et al.*, 1996), but inhibits drug metabolizing enzymes (Atal *et al.*, 1985) and thus, may enhance drug bioavailability. In addition, piperine has recently been reported to inhibit lung metastasis induced B16F-10 melanoma cells in mice (Pradeep and Kuttan, 2002), and also to be cytotoxic in the brine shrimp assay (Padmaja *et al.*, 2002), to stimulate melanocyte proliferation and melanocyte differentiation (Venkatasamy *et al.*, 2004), to protect Swiss albino

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mice from benzo[α]pyrene-induced lung carcinogenesis (Selvendiran e*t al.*, 2004), and immunomodulatory and antitumor activities (Sunila and Kuttan, 2004).

Piplartine, also an amide isolated from *Piper* species, was found to display significant activity against KB (nasopharyngeal carcinoma), P-388 (lymphocytic leukaemia), A-549 (lung carcinoma) and HT-29 (colon carcinoma) cell lines, all in cell culture system (Duh *et al.*, 1990; Duh and Wu, 1990).

In this paper, the cytotoxicity of piplartine and piperine was evaluated as their ability to inhibit the sea urchin eggs' development, tumor cell lines proliferation, brine shrimp lethality and lysis of mouse erythrocytes.

Material and Methods

Plant material

The roots of *Piper tuberculatum* were harvested on September 2004 from a wild population on the Pici Campus of Federal University of Ceará, Fortaleza-Ceará, Brazil. A voucher specimen (# 34736) was deposited at the Prisco Bezerra Herbarium (EAC), Department of Biology, Federal University of Ceará.

Piplartine isolation

420.0 g of ground roots of *P. tuberculatum* were macerated with a mixture of petroleum ether/ethyl acetate 1:1 (1.5 l) for 24 h (3 ×). The solvent mixture was rotaevaporated under reduced pressure to yield a yellowish solid (13.2 g), which gave a first crop of piplartine (4.3 g) after crystallization from hot MeOH. Piplartine was characterized particularly by uni and bidimensional NMR analysis and m.p. 122.2–122.6 °C (Lit. 128–130 °C and 124 °C, Braz-Filho *et al.*, 1981).

Piperine, from black pepper seeds, was purchased from Acros Organics (Morris Plains, New Jersey, USA).

Brine shrimp toxicity

Brine shrimp (*Artemia salina* Leach) eggs were hatched in a beaker filled with sea-water under constant aeration. After 48 h the photographic nauplii were collected by pipette. The nauplii were counted macroscopically in the stem of a pipette against a lighted background. Ten shrimp were transferred to each well of 24-multiwell plates containing the samples. The concentration of piplartine and piperine ranged from 0.1 to 100 μg/ml. The plates were maintained under illumination. Survivors were counted after 24 h of incubation and the percentage of deaths at each dose and control (sea water plus vehicle) was determined (Meyer *et al.*, 1982).

Cytotoxicity against tumor cell lines

The cytotoxicity of piplartine and piperine was tested against CEM and HL-60 (human leukemias), B16 (murine melanoma) and HCT-8 (human colon) cell lines obtained from the National Cancer Institute, Bethesda, MD, USA. Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin, and incubated at 37 °C with a 5% CO_2 atmosphere. For all experiments, cells were plated in 96-well plates (10^5 cells/well for adherent cells or 0.5×10^5 cells/well for suspended cells in 100 μl of medium). After 24 h, piplartine and piperine (0.39 to 25.0 μg/ml) dissolved in 1% DMSO were added to each well and incubated for 72 h. Control groups received the same amount of DMSO. Doxorubicin (Doxolem®, Zodiac Produtos Farmacêuticos S/A, São Paulo, Brazil) was used as positive control. The tumor cell growth was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to a purple formazan product (Mosmann, 1983). At the end of the incubation, the plates were centrifuged and then the medium was replaced by fresh medium (200 μl) containing 0.5 mg/ml MTT. 3 h later, the MTT formazan product was dissolved in 150 μl DMSO, and the absorbance was measured using a multiplate reader (Spectra Count, Packard, Ontario, Canada). The drug effect was quantified as the percentage of control absorbance of reduced dye at 550 nm.

Antimitotic activity on sea urchin eggs

The assay was performed following the method described by Jimenez *et al.* (2003). Adult sea urchins (*Lytechinus variegatus*) were collected at Lagoinha beach, on the northeastern coast of Brazil. Gamete elimination was induced by injecting 3.0 ml of 0.5 M KCl into the urchin's coelomic cavity. For fertilization, 1 ml of a sperm suspension (0.05 ml of concentrated sperm in 2.45 ml of filtered sea-water) was added to every 50 ml of egg

solution. The assay was carried out in 24-multiwell plates. Piplartine and piperine were added immediately after fecundation (within 2 min) to get concentrations ranging from 0.1 to 100 μg/ml in a final volume of 2 ml. Doxorubicin was used as positive control. At appropriate intervals, 200 μl aliquots were fixed in the same volume of 10% formaldehyde to obtain first and third cleavages and blastulae. 100 eggs or embryos were counted for each concentration of test substance to obtain the percentage of normal cells.

Hemolytic assay

The test was performed in 96-well plates using a 2% mouse erythrocyte suspension in 0.85% NaCl containing 10 mM $CaCl_2$, following the method described by Jimenez *et al.* (2003). Piplartine and piperine were tested at concentrations ranging from 0.8 to 200 μg/ml. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed and the liberated hemoglobin was measured spectrophotometrically as the absorbance at 540 nm.

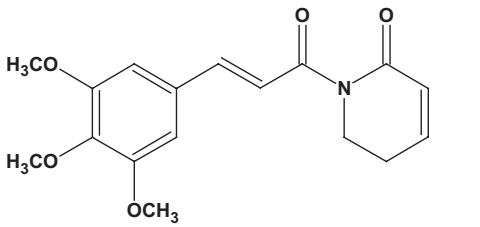
Statistical analysis

Data are presented as mean ± S.E.M. The IC_{50} values and their 95% confidence intervals (CI 95%) were obtained by nonlinear regression using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA). DL_{50} values with brine shrimp were obtained from 24 h counts using the probit analysis method described by Litchfield and Wilcoxon (1949). The differences between experimental groups were compared by Student's t test.

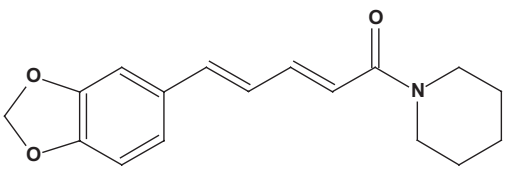
Results and Discussion

The cytotoxic activity of piplartine and piperine was evaluated in different bioassays. The antimitotic activity was determined as the ablility to inhibit sea urchin eggs development and four tumor cell lines' growth. Their toxicity in the brine shrimp lethality assay and the lytic activity on mouse erythrocytes was also determined.

Piperine showed higher toxicity to brine shrimp nauplii (DL_{50} = 2.8 ± 0.3 μg/ml) than piplartine (DL_{50} = 32.3 ± 3.4 μg/ml). The toxicity of piperine in the brine shrimp assay was already described in previous studies, where it (DL_{50} = 2.4 μg/ml) was tested in a screening program with Indian medicinal plants (Padmaja *et al.*, 2002). Piperine exhibited the most potent antifeedant activity when compared with other amides and the authors concluded that the presence of a methylenedioxyphenyl and an alicyclic amide group in the compound might be crucial for high antifeedant activity (Parmar *et al.*, 1997). These groups appear to be also important to its brine shrimp activity, since piperine was the most active in this assay.



Piplartine



Piperine

Fig. 1. Chemical structure of piplartine {5,6-dihydro-1-[1-oxo-3-(3,4,5-trimethoxyphenyl)-*trans*-2-propenyl]-2(1*H*)-pyridinone} and piperine {1-[5-(1,3)-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidine}.

Both piplartine and piperine (Fig. 1) induced a dose-dependent inhibition on egg development during all phases examined, first and third cleavage and blastulae. The IC_{50} values are presented in Table I. Piplartine was 2, 2.3 and 3.5 times more active than piperine at the first and third cleavage and blastulae phases, respectively. Piperine seems

Table I. Antimitotic activity of piplartine and piperine on sea urchin (*Lytechinus variegatus*) eggs development. Doxorubicin was used as positive control. Data are presented as IC_{50} values and 95% confidence intervals for first and third cleavages and blastulae obtained by nonlinear regression.

Substance	1st cleavage [μg/ml (μM)]	3rd cleavage [μg/ml (μM)]	Blastulae [μg/ml (μM)]
Doxorubicin	6.3 (10.8)	0.3 (0.7)	0.5 (0.9)
	4.3–9.1	0.2–0.7	0.3–1.1
Piplartine	3.4 (10.6)[a]	2.5 (7.8)[a]	1.0 (3.3)[a]
	3.0–3.8	2.0–3.0	1.0–1.1
Piperine	6.8 (23.9)	5.7 (20.1)	3.7 (13.1)
	5.4–8.2	4.8–6.7	2.9–4.6

[a] $p < 0.05$ as compared by Student's t test with piperine.

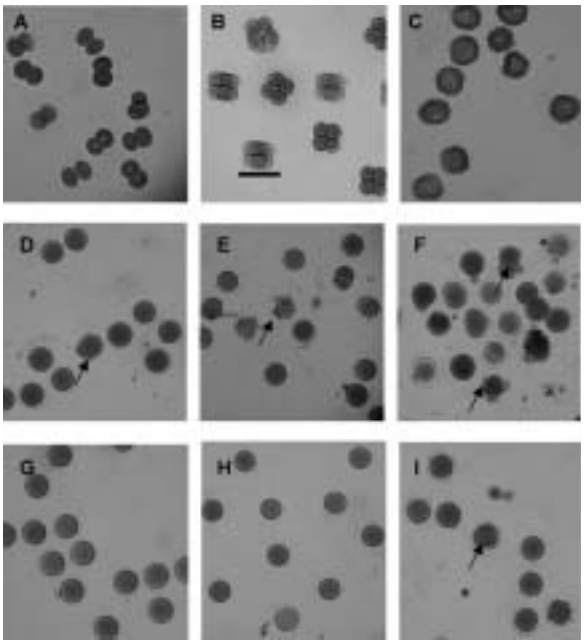


Fig. 2. Photomicrographs showing the effect of piperine and piplartine on the sea urchin eggs development. A, B and C, control; D, E and F, treated with 30 μg/ml piperine; G, H and I, treated with 30 μg/ml piplartine at first and third cleavages and blastulae stages, respectively. Horizontal bar = 100 μm.

to induce membrane disruption associated with mitosis inhibition beginning at the first cleavage, while for piplartine cell destruction was only observed after few hours of contact (Fig. 2). According to Jacobs *et al.* (1981), if a substance promotes 100% inhibition in this assay at a concentration of 16 μg/ml or less, it may be considered to be very active. Thus, both amides could be considered very active, completely inhibiting sea urchin mitosis at concentrations lower than 7 μg/ml.

Several tumor cell lines were treated with increasing concentrations of piplartine and piperine for 72 h and analyzed by the MTT assay. Table II shows the obtained IC_{50} values. As observed in the sea urchin assay, piplartine was stronger active than piperine. The cytotoxic activity of piplartine and piperine on tumor cell lines was already described in previous studies (Duh *et al.*, 1990; Duh and Wu, 1990; Sunila and Kuttan, 2004). As previously mentioned, piplartine demonstrated a strong cytotoxicity towards KB (nasopharyngeal carcinoma), P-388 (lymphocytic leukaemia), A-549 (lung carcinoma) and HT-29 (colon carcinoma) cell lines and the authors suggested that the presence of two α,β-unsaturated carbonyl moieties would be responsible for its cytotoxic activity (Duh *et al.*, 1990; Duh and Wu, 1990). Our data corroborate this hypothesis, since piperine does not possess this group and was just weakly active. Piperine was cytotoxic towards Dalton's lymphoma ascites (DLA), Ehrlich ascites carcinoma (EAC), L929 and B16 cells at concentrations equal or higher than 25 μg/ml (Sunila and Kuttan, 2004; Pradeep and Kuttan, 2004). Piperine also was able to inhibit the growth of solid tumors induced by DLA cells and ascites tumors induced by EAC cells and the authors suggested that this may be due to the combined action of humoral and cell-mediated immune responses (Sunila and Kuttan, 2004). Thus, piperine acts as a non-toxic immunomodulator, which also possesses an antitumor property (Sunila and Kuttan, 2004).

Table II. Cytotoxic activity of piplartine and piperine on tumor cell lines. Doxorubicin was used as positive control. Data are presented as IC_{50} values and 95% confidence intervals for leukemia (HL-60 and CEM), colon (HCT-8) and skin (B-16) cancer cells. Experiments were performed in triplicate.

Cell line	Doxorubicin IC_{50} [μg/ml (μM)]	Piplartine IC_{50} [μg/ml (μM)]	Piperine IC_{50} [μg/ml (μM)]
CEM	0.02 (0.04)	1.4 (4.5)[a]	> 25.0 (87.6)
	0.02–0.03	1.3–1.5	
HL-60	0.02 (0.03)	1.7 (5.4)[a]	> 25.0 (87.6)
	0.01–0.02	1.5–1.9	
HCT-8	0.01 (0.02)	0.7 (2.2)[a]	18.8 (66.0)
	0.01–0.02	0.6–0.8	12.5–28.2
B-16	0.03 (0.06)	1.7 (5.3)[a]	19.9 (69.9)
	0.02–0.04	1.4–2.0	17.0–23.3

[a] $p < 0.05$ as compared by Student's t test with piperine.

Since the inhibition of urchin eggs mitosis seems to be related to membrane disruption, the compounds were tested for their ablility to induce lysis of mouse erythrocytes. The erythrocyte membrane is a dynamic structure that can dictate significant changes in its interaction with drugs (Aki and Yamamoto, 1991). However, none of the amides were hemolytic even at the highest tested concentration (200 μg/ml). This result suggested that the mechanism of cytotoxicity is probably related to a more specific pathway.

The present result showed that piplartine and piperine possess *in vitro* antimitotic activity. Therefore, further investigations to elucidate the

mechanisms of cytotoxicity exhibited and on their *in vivo* activities are required.

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