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Full Length Research Paper

Study of *Salmonella typhimurium* mutagenicity assay of (*E*)-piplartine by the Ames test

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Phytochemical studies carried out with Piperaceae species have shown great diversity of secondary metabolites among which are several displayed considerable biological activities. The species *Piper tuberculatum* has been intensively investigated and a series of amides have been described. For instance, (*E*)-piplartine showed significant cytotoxic activity against tumor cell lines, especially human leukemia cell lines; antifungal activity against *Cladosporium* species; trypanocidal activity and others. Considering the popular use of *P. tuberculatum* and the lack of pharmacological studies regarding this plant species, the mutagenic and antimutagenic effect of (*E*)-piplartine was evaluated by the Ames test, using the strains TA97a, TA98, TA100 and TA102 of *Salmonella typhimurium*. No mutagenic activity was observed for this compound.

Key words: Piperaceae, *Piper tuberculatum*, (*Z*)-piplartine, mutagenic activated, Ames test.

INTRODUCTION

Studies with plants from the Piperaceae have shown a great diversity of secondary metabolites such as pyrones, lignans, neolignans, terpenes, propenylphenols, chalcones, flavones, benzopyranes, chromenes, lactones and amides, with biological activities (Morandim et al., 2010; Morandim-Giannetti et al., 2010; Regasini et al., 2009; Bezerra et al., 2008; Batista-Junior et al., 2008; Lopes et al., 2008; Parmar et al., 1998; Felipe et al., 2007; Parka et al., 2007; Navickiene et al., 2006).

The *Piper* species are widely distributed in the tropical and subtropical regions of the world and includes more than 1000 species, is often used as food flavouring agents, psychotropic, antimicrobial, antioxidant and in traditional medicines for treating many diseases including gynaecological maladies, vaginitis, intestinal disorders, psychotropic, antimicrobial, antioxidant, cytotoxic effects, asthma, bronchitis, fever, hemorrhoidal afflictions, gastro-intestinal diseases and rheumatism (Parmar et al., 1998; Felipe et al., 2007; Parka et al., 2007; Navickiene et al., 2006).

Some preparations obtained from these plants have shown anti-inflammatory, insecticidal, anti-hypertensive, antidiabetics, immunomodulatory and antimutagenic effects, potential benzo(a)pyrene-induced DNA-damage, and neurotoxic properties. They also generate oxidative stress by changing thiol cellular status, and may be used as pest control agents (Parmar et al., 1998; Cotinguiba et

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Abbreviations: TLC, Thin layer chromatography; **5-FU,** 5-fluorouracil.

al., 2009; Lago et al., 2004; Duarte et al., 2004; Silva et al., 2002; Navickiene et al., 2000; Varanda, 2006).

The amide alkaloids, known as alkamides, are typical metabolites of this family, and are classified as isobutyl, pyrrolydine, pyridonil and piperidines with various biological effects. Indeed, the pharmacology of the these alkamides revealed interesting compounds with hormone-modulating, anticancer, anti-hypertensive, antioxidant, anti-lipidemic, enzyme-inhibiting, anxiolytic and antidepressant, as well as anti-inflammatory effects. For example, piperine and piperdardine, isolated of *Piper tuberculatum* Jacq. which showed an important hypotensive activity in rats (Cotinguiba et al., 2009).

(*E*)-Piplartine is an amide alkaloid component of *Piper* species. This secondary metabolite has shown significant cytotoxic activity against tumor cell lines, especially human leukemia cell lines, such as HL-60, K562, Jurkat and Molt-4, as well antifungal, antileishmanial, antitrypanosomal activities, anti-platelet aggregation, anxiolytic, antidepressant properties, genotoxic potential and the induction apoptosis in cultured mammalian cells, employing a permanent cell line derived from Chinese hamster lung fibroblasts (V79 cell line), as well as its mutagenic and recombinogenic potential in the simple eukaryote, *Saccharomyces cerevisiae* (Bezerra et al., 2008; Cotinguiba et al., 2009; Bernstein et al., 1982).

The objective of this study was to investigate the effect of piplartine and piperine in combination with the chemotherapeutic agent 5-fluorouracil (5-FU) using both *in vitro* and *in vivo* experimental models. Hematological, biochemical, histopathological and morphological analyses of treated animals were performed to assess the toxicological aspects related to different treatments (Bezerra et al., 2008; Cotinguiba et al., 2009; Varanda, 2006).

In spite of the proven pharmacological properties for several medicinal plants, several analyses should be made including the genotoxic activity, since their constituents can cause harmful changes in the DNA. The risks associated with genotoxic activity are significant when such alternative treatments are applied without criteria, without due attention to correct botanical identification, and not considering the correct part of the plant to be used or even the method of preparation and administration (Bezerra et al., 2008).

Therefore, the present study has been conducted in order to evaluate the mutagenic effect (genotoxic activity) of (*E*)-piplartine by the Ames test, using TA97a, TA98, TA100 and TA102 strains of *Salmonella typhimurium* microsome assay.

MATERIALS AND METHODS

Plant material

Specimen of *P. tuberculatum* Jacq. (Piperaceae) was obtained from greenhouse cultivation at the Institute of Chemistry, UNESP, Araraquara-SP, Brazil. Plant material was identified by Dr. Guillermo E. D. Paredes (Universidad Pedro Ruiz Gallo, Lambayeque, Peru). The voucher specimen (Kato-163) was deposited at the Herbarium of the Institute of Bioscience, USP, São Paulo-SP, Brazil.

Extraction and isolation of (*E*)-piplartitne

The shade-dried and powdered leaves (147.0 g) of *P. tuberculatum* were extracted with CH_2Cl_2:MeOH (2:1) (3 x 800 ml), for two weeks at room temperature. The crude extract (3.6 g) were fractionated by repeated column chromatographic on silica gel followed by preparative thin layer chromatography (TLC), resulting in the isolation of (*E*)-piplartine (45.0 mg), as previously described (Batista-Junior et al., 2008; Lopes et al., 2008; Cotinguiba et al., 2009).

Salmonella mutagenicity assay

Mutagenicity was tested by the *Salmonella*/microsome assay, based on the preincubation method using the *S. typhimurium* test strains TA97a, TA98, TA100 and TA102, kindly provided by Dr. B.N. Ames (University of California, Berkeley, CA), with and without metabolization. The test strains obtained from frozen cultures were grown overnight for 12 to 14 h in Oxoid Nutrient Broth No. 2 (London, UK).

(*E*)-Piplartine (100, 150, 200, 250 and 300 µg per plate for strains TA100, TA98, TA97a and TA102) dissolved in dimethyl sulfoxide were added to 100 ml of bacterial culture and 0.5 ml phosphate buffer (pH 7.4) or 0.5 ml of S9 mixture and incubated for 20 to 30 min at 37°C.

After the incubation, 2 ml of top agar was added, mixed and then poured onto a plate having a minimum amount of agar. The plates were incubated at 37°C for 48 h, and His^+ revertant colonies were enumerated with an automatic counter (ProtoCOL, Synbiosis, Cambridge, UK). All experiments were analyzed in triplicate. The concentrations were selected on the basis of a preliminary toxicity test. In all subsequent assays, the upper limit of the dose range tested was either the highest nontoxic dose or the lowest toxic dose determined in this preliminary assay. Toxicity was apparent either as a reduction in the number of His revert ants or as an alteration in the autotrophic background (background lawn).

Statistical analysis was performed with the Salanal computer program. The mutagenic index was also calculated for each dose, as the average number of revertants per plate divided by the average number of revertants per plate in the negative (solvent) control. A sample was considered positive when the mutagenic index was ≥2.

The standard mutagens used as positive controls in each experiment were 2-aminoanthracene (1.25 µg per plate) and sodium azide (1.25 µg per plate) for TA100, 4 nitro-ophenylenediamine (10 µg per plate) and 2-aminoanthracene (1.25 µg per plate) for TA98 and TA97a, and mitomycin C (0.25 µg per plate) and 2-aminofluorene (5 µg per plate) for TA102. Dimethyl sulfoxide served as the negative (solvent) control (Bernstein et al., 1982; Lima et al., 2008). The mutagenicity ratio (MR) was calculated for each concentration. The final data obtained from the test were analyzed using the statistical program Salanal (Salmonella Assay Analysis) version 1.0 of the Research Triangle Institute, RTP, NC, USA.

This program allows assessment of the dose-response effect by calculating the analysis of variance (ANOVA) between the measurements of the number of revertants tested at different concentrations and the negative control, followed by linear regression. This program also provides the slope of the linear part of the concentration-response curve, which is the number of revertants induced per unit of measurement of the sample.

Table 1. Mutagenic activity of (*E*)-piplartine from *P. tuberculatum* strains in the presence (+S9) and absent (-S9) of metabolic *activation*.

Treatment (mg per plate)	Number of revertants per plate in (*E*)-piplartine of *P. tuberculatum*							
	TA98		TA97a		TA100		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0.00[a]	22±7	29±7	185±18	200±33	139±1	127±8	371±12	291±18
100.00	22±1(1.0)	37±3(1.3)	163±1(0.9)	244±8(1.2)	124±1(0.9)	141±11(1.1)	279±22(0.8)	289±29(1.0)
150.00	19±4(0.9)	22±1 (0.7)	150±6(0.8)	256±0(1.3)	121±5(0.9)	109±4(0.9)	302±32(0.8)	249±25(0.9)
200.00	21±2(0.9)	27±4(0.9)	138±31(0.7)	252±33(1.3)	115±6 (0.8)	152±1(1.2)*	337±49(0.9)	250±16(0.9)
250.00	21±2(0.9)	27±1(0.9)	124±25(0.7)	244±13(1.2)	137±2(1.0)	134±18(1.1)	323±55(0.9)	272±36(0.9)
300.00	19±6(0.9)	32±2(1.1)	132±3(0.7)	278±4(1.4)*	107±9(0.8)	143±1(1.1)	290±0(0.8)	246±53(0.8)
C+[b]	2782±20	1014±309	1983±72	1206±14	1697±11	1180±48	2328±73	336±10

Data are mean ± SD values (mutagenic index). The negative control (0.0) was dimethyl sulfoxide (100.0 µL). In the presence of metabolic activation, the addition (+S9) was 2-aminoanthracene (1.25 µg per plate) for strains TA98, TA100 and TA97a and 2-aminofluorene (5.0 µg per plate) for strain TA102. In the absence of metabolic activation, the addition (-S9) was 4-nitro-o-phenylenediamine (10.0 µg per plate) for TA98 and TA97a, sodium azide (1.25 µg per plate) for TA100, and mitomycin C (0.25 µg per plate) for TA102. Statistically different values by ANOVA are indicated: *P < 0.05, **P < 0.01.

RESULTS

The Ames assay is commonly used to detect mutagenic and antimutagens activities and is a widely accepted method for identifying various chemicals and drugs that can cause gene mutations. It has a high predictive value for *in vivo* carcinogenicity and the most common test strains are TA97a, TA98, TA100 and TA102.

Hence, the mutagenicity of (*E*)-piplartine was investigated by Ames mutagenicity assay in the most sensitive Salmonella test strains currently available (TA100, TA98, TA97a and TA102). This test was performed in both the absence and the presence of a rat liver metabolizing system (S9 mix), providing a very sensitive study of potentially mutagenic pathways for the metabolism of these materials. Increasing doses of (*E*)-piplartine were tested and compared with the results of solvent control.

Analysis of mutagenic activities in (*Z*)-piplartine from *P. tuberculatum* showed no positive mutagenicity and the mutagenic index did not exceed 2 when compared with the standards and the solvent (Table 1).

DISCUSSION

There is a relationship between mutagenesis and carcinogenesis, in that, both show abrupt changes in a single cell, permanent and inherited by daughter cells. Because of this relationship, the mutagenicity evaluation for new drugs and also for medicinal plants should be highly recommended for detecting potential genotoxic compounds.

The amide (*E*)-piplartine from *P. tuberculatum* presented a decrease in the amount of revertants in strains TA100, TA98, TA97a and TA102. The highest mutagenicity ratios for TA98 at a concentration of 100 µg/plate were 1.3 in the presence, and 1.0 in the absence of metabolic activation, respectively. For TA97, the highest detected ratio was 1.4 at 300 µg/plate and 0.9 at 100 µg/plate, either in the presence or absence of metabolic activation.

Strains TA100 and TA102 showed maximum ratios in the presence of metabolic activation of 1.2 at a concentration of 200 mg/plate and 1.0 at a concentration of 100 mg/plate, respectively. In the absence of metabolic activation, maximum ratios at 250 mg/plate were 1.0 and 0.9, respectively.

An increase in the number of revertant colonies would yield positive results. In the present case, no increase was observed and mutagenic index values were below the limit (≥2). These results indicated efficiency for protecting bacterial genetic material againt different types of damage caused by several mutagenic agents. The present data in conjunction with the absence of mutagenicity of (*E*)-piplartine indicate promising use of this species against tumor, fungi and *Trypanossoma cruzi* and showed that (*E*)-piplartine is a potential natural product for further development of new drugs.

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