Yangambin Cytotoxicity: A Pharmacologically Active Lignan Obtained from *Ocotea duckei* Vattimo (Lauraceae)

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The *in vitro* cytotoxic potential of yangambin was evaluated. Yangambin is a pharmacologically active furofuran lignan obtained from the leaves of *Ocotea duckei*. It is the major compound from the lignoids fraction. Yangambin presented low cytotoxicity in all *in vitro* models analyzed. Its cytotoxicity to murine macrophages was measured by the Trypan blue dye exclusion test and MTT reduction assay, resulting in high CC_{50} values of 187.0 μg/mL (383.3 μM) and 246.7 μg/mL (504.3 μM), respectively. The difference obtained in the inhibitory concentrations aforementioned can be explained, at least in part, by the different principles of the methods. While the MTT reduction assay evaluates the ability of yangambin to inhibit the activity of the mitochondrial enzyme succinate dehydrogenase, the Trypan blue dye exclusion test evaluates possible damages to the integrity of the cytoplasmic membrane which result in cell death. The capacity of yangambin to inhibit the sea urchin embryonic development showed that it has low antimitotic and teratogenic potential, once continued exposure of embryos to concentrations up to 500 μg/mL (1.025 μM) did not result in an inhibitory effect on the first egg cleavages. Such low *in vitro* cytotoxicity is correlated with the low acute toxicity previously studied. All these data, together with the various therapeutic properties of yangambin, make this lignan a promising one for a new drug.

Key words: Yangambin, Cytotoxicity, *Ocotea duckei*

Introduction

Ocotea duckei Vattimo is a member of the Lauraceae family found in the northeast of Brazil. From its leaves yangambin was isolated, a furofuran lignan found as major constituent in the total lignoids fraction of this material (Morais *et al.*, 1996; Barbosa Filho *et al.*, 1999).

Some pharmacological properties of the lignan yangambin [7*S*,7′*S*-di-(3,4,5-trimethoxyphenyl) furofuran] (Fig. 1) have already been reported: (a) it is an antagonist which selectively blocks PAF receptors on platelets in several *in vitro* and *in vivo* experimental models (Castro Faria Neto *et al.*, 1995a, b; Herbert *et al.*, 1997); (b) it is an effective pharmacological agent against cardiovascular collapse and mortality due to endotoxin shock (Ribeiro *et al.*, 1996; Tibiriça *et al.*, 1996; Araújo *et al.*, 2001); (c) it may also be a useful drug in the treatment of some allergic inflammatory responses (Serra *et al.*, 1997); (d) it increases the pentobarbital-induced sleeping time and may act as a central nervous system depressant, anticonvulsive and sedative-hypnotic (Pachú *et al.*, 1993; Almeida *et al.*, 1995); (e) it presents antileishmanial activity against *Leishmania* parasites (Monte Neto *et al.*,

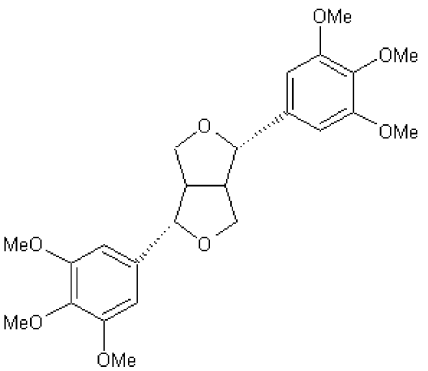

Fig. 1. Chemical structure of yangambin.

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2007); (f) it presents antitumoural activity in colorectal cells by inducing apoptosis (Haussot *et al.*, 2003). All these pharmacological properties, allied to a good isolation yield (Barbosa Filho *et al.*, 1999), makes this lignan a promising prospect for a new drug.

Little is known about the toxicity of yangambin. It is reported that this lignan presents low acute toxicity in murine models (Pachú *et al.*, 1993), and it does not present mutagenic activity in bacteria (Marques *et al.*, 2003). The aim of this paper was to enlarge the studies on the cytotoxicity of yangambin using different *in vitro* models.

Material and Methods

Obtainment of yangambin

The lignan yangambin was obtained as previously described (Barbosa Filho *et al.*, 1999; Morais *et al.*, 1999). A specimen of *Ocotea duckei* Vattimo (Lauraceae) was collected in March 2004, near the city of Santa Rita, State of Paraíba (PB), Brazil, and the material was identified by Dr. Maria de Fátima Agra, Setor de Botânica of Laboratório de Tecnologia Farmacêutica (LTF), João Pessoa, PB, Brazil. A voucher specimen is deposited at the Herbarium Prof. Lauro Pires Xavier (under registry JPB-4309) of Departamento de Sistemática e Ecologia, Universidade Federal da Paraíba, João Pessoa, PB, Brazil.

The ethanolic extract of dried and powdered leaves of *Ocotea duckei* (5 kg) was obtained by extraction with 95% EtOH at room temperature for 3 d. After being concentrated under reduced pressure, the ethanolic extract was dissolved and stirred in a solution of 10% acetic acid and filtered over celite; the precipitate was discarded and the filtrate was extracted with $CHCl_3$. After concentration under reduced pressure, the $CHCl_3$ extract was called "Acid Chloroform Fraction". The deffated acetic acid solution was made alkaline with NH_4OH to pH 9 and extracted with $CHCl_3$. The residue of this $CHCl_3$ extract was called "Basic Chloroform Fraction", which was dissolved in MeOH and left in a freezer for 24 h. The precipitate yielded 3 g (0.06%) of yangambin after recrystallization in MeOH. The supernatant was concentrated and the residue was subjected to column chromatography over silica gel yielding more yangambin (2 g, 0.04%), resulting in a total yield of 0.1%. Yangambin occurred in the form of white crystals with a melting point of 119–120 °C (from MeOH). The molecular formula of yangambin is $C_{24}H_{30}O_8$ and its molecular weight is 446 g/mol. The ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were obtained in $CDCl_3$ with the solvent as reference, employing a Bruker AMX-400 spectrometer. Standard Bruker pulse sequences were used to perform the 2D experiments. Low resolution mass spectra were obtained on a Hewlett Packard 5988 GC/MS instrument. Column chromatography was carried out with silica gel 60 (Merck). Thin layer chromatography was performed on silica gel 60 PF_{254} (Merck).

The purified compound was diluted in dimethyl sulfoxide (DMSO; Vetec, Brazil) for the cytotoxicity analysis. A final content of DMSO until 0.5% in the test solution proved to have no effect on the cellular models evaluated.

Cytotoxicity assay using murine macrophages

Thioglycolate-elicited peritoneal macrophages from Swiss mice (Laboratório de Tecnologia Farmacêutica, UFPB, João Pessoa, Brazil) were collected in phosphate buffered saline (PBS) supplemented with 3% fetal bovine serum (FBS) and cultivated in RPMI-1640 medium (Cultilab, Campinas, Brazil), supplemented with 10% FBS, 100 μg/mL streptomycin and 100 U/mL penicillin. The cultures were maintained at 37 °C in a 5% CO_2 atmosphere. All experimentation protocols involving animals were approved by the Committee for Ethics in Animal Research of Laboratório de Tecnologia Farmacêutica, UFPB, Brazil under no. 0711/06.

Trypan blue exclusion assay

In order to evaluate the effect of yangambin on the cellular viability of murine macrophages, the Trypan blue dye exclusion method was used (Freshney, 1994). Thioglycolate-elicited peritoneal macrophages were harvested and cultivated as described above. Aliquots of 1 mL (containing 10^6 cells/mL) of the cell suspension were transferred to polypropylene tubes and incubated with different concentrations of yangambin for 24 h. Then, both living and dead cells were quantified in a Neubauer chamber in the presence of Trypan blue (0.4%).

MTT reduction colorimetric assay

The cellular viability was determined by the ability of living cells to reduce the yellow MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], a tetrazole, to purple formazan (Mosmann, 1983). Murine macrophage cultures

were incubated in 96-well cellular culture plates (TPP, Switzerland) with $4 \cdot 10^5$ cells/well in 200 μL of RPMI medium for 4 h for cell adhesion. Non-adherent cells were removed, and the adherent macrophages were washed with RPMI medium previously warmed. Different concentrations of yangambin were added to the wells, and the cells were incubated in RPMI medium at 37 °C in a 5% CO_2 atmosphere for 24 h. Control groups were established in the absence of yangambin, only in RPMI medium (white) and in the presence of DMSO (negative control). After 24 h of incubation, 100 μL of medium were removed and 10 μL of 5 mg/mL MTT solution were added to it. After 4 h of incubation at 37 °C in a 5% CO_2 atmosphere, the formed product formazan was dissolved in SDS to 10% for 16 h and the absorbance was measured by spectrophotometry at the wavelength of 570 nm. The percentage viability was calculated from the ratio of OD readings in wells with yangambin versus wells without yangambin.

Antimitotic assay using sea urchin embryonic cells

Antimitotic and teratogenic potentials were investigated by means of the sea urchin embryonic development model (Costa Lotufo *et al.*, 2002). Sea urchins (*Echinometra lucunter* Linnaeu, 1758) were collected at the northeast coast of the State of Paraíba (7°01′55.55′′S 34°49′14.96′′W), approx. 1 km from Intermares beach, Cabedelo, PB, Brazil and kept in the laboratory until the end of the experiments.

For fertilization, gametes were collected after stimulation of males and females with 3 mL of 0.5 M KCl administrated into their coelomic cavity. The eggs were collected in filtered sea water, washed two times and suspended in 50 mL of filtered sea water. The sperms were similarly collected, but they were recovered with a Pasteur pipette, and suspended in filtered sea water (mixing of 0.05 mL of concentrated sperm with 2.45 mL sea water). Fertilization was performed by adding 1 mL of sperm solution to 50 mL of egg suspension. Each well (24-multiwell plate; TPP, Trasadingen, Switzerland) received 1 mL of fertilize eggs ($2 \cdot 10^4$ cells/mL). After fertilization, several concentrations of yangambin (ranging from 50 to 500 μg/mL) were added to a final volume of 2 mL, and the plates were incubated at 25 °C. DMSO (0.4%) was utilized as negative control. At appropriate intervals (1.5 h after fecundation – first cleavage; 6 h – blastulae, and 28 h – larvae), aliquots of 200 μL were fixed in an equal volume of Isoton (0.05 M citric acid, 0.12 M NaCl, 0.5% formaldehyde, pH 7.2). 100 eggs, embryos or larvae were counted for each concentration of the test substance to obtain the percentage of normal cells.

All data are expressed as means ± standard error of mean (SEM). The values of CC_{50} (cytotoxic concentration for 50% of cells) were obtained using the probit model of linear regression (Finney, 1971). The differences among the experimental groups were compared using the variance analysis (ANOVA) followed by the test of multiple comparisons of Dunnett's, admitting $p < 0.05$ as significance level.

Results

Cytotoxicity of yangambin to murine macrophages

Table I shows the effect of yangambin on murine macrophages using the Trypan blue dye exclusion as a parameter of cell viability. Yangambin was not toxic until a concentration of 50 μg/mL (102.5 μM), presenting a CC_{50} value of 187 μg/mL (383.8 μM).

Table I. Effect of different concentrations of yangambin on the viability of peritoneal murine macrophages incubated for 24 h in RPMI-1640 medium at 37 °C in a 5% CO_2 atmosphere. The viability was assessed using the Trypan blue dye exclusion method and employing the tetrazolium bromide reduction method (MTT).

Yangambin [μg/mL]	Murine macrophages	
	Cell viability[a] (%) Trypan blue assay Mean ± SEM	Cell viability[b] (%) MTT assay Mean ± SEM
0	72.43 ± 4.93	100.00 ± 0.00
50	57.14 ± 0.59	105.97 ± 0.68
100	37.63* ± 6.09	93.13 ± 0.19
200	41.05* ± 6.64	34.31* ± 4.12
400	ND	22.84* ± 0.1

[a] Values represent the average of three independent experiments performed in triplicate.
[b] Values represent the average of two independent experiments performed in quadruplicate.
* $p < 0.01$; ND, not determined.

The cytotoxicity results of yangambin to murine macrophages using the MTT reduction assay are shown in Table I. Until the concentration of 100 μg/mL (205 μM), yangambin presented no cytotoxic effect on the evaluated model, as demonstrated by percentage viability values obtained by spectropho-

tometry at 570 nm. The calculated CC_{50} value for yangambin was 246.7 μg/mL (504.3 μM).

Antimitotic assay against sea urchin embryonic cells

From the first egg cleavage to the blastulae stage, all treatments (concentrations of yangambin ranging from 50 to 500 μg/mL) presented 100% of the cells at the same developmental stage of control, evidencing that yangambin is not toxic to early stages of the sea urchin (*Echinometra lucunter*) embryonic development. After 28 hours of exposure, (73.66 ± 2.33) and 100% of the embryos treated with yangambin at 50 and 500 μg/mL were in the gastrulae stage respectively, differently from control groups, in which all analyzed cells were in the larval development.

Discussion

In this study, the cytotoxicity of yangambin to a culture of murine macrophages and sea urchin embryonic cells was investigated. The *in vitro* cytotoxic potential was established as the ability of yangambin to reduce the macrophages culture viability or inhibit the sea urchin embryonic development. Yangambin showed low cytotoxicity to murine macrophages in both tests. The assay that evaluated the cytoplasmic membrane integrity by the capacity of the cells to exclude the Trypan blue dye resulted in a CC_{50} value of 187.0 μg/mL (383.3 μM), and the test which evaluated the ability of the mitochondrial enzyme succinate dehydrogenase to reduce MTT to formazan crystals resulted in a CC_{50} value of 246.7 μg/mL (504.3 μM). Pessoa *et al.* (2000), using the MTT reduction assay to search for antineoplasic drugs, showed that new purified compounds can be considered significantly toxic when they present IC_{50} (inhibitory concentration for 50% of the cellular growth) values less than 1 μg/mL or 1 μM.

The difference obtained in the inhibitory concentrations of yangambin in *in vitro* models using murine macrophages can be partly explained by the different principles of the methods used. Additionally, one of the factors, that might have resulted in the greater cytotoxicity of yangambin in the Trypan blue exclusion test, may be due to the fact that the cells were in suspension and, therefore, presented a greater surface area of contact for interaction with yangambin, in comparison with cells adhered on plates in the MTT reduction assay.

In the present paper, the ability of yangambin to inhibit the sea urchin embryonic development was also investigated. This model has been applied to investigate the antimitotic and teratogenic activity of new compounds (Morale *et al.*, 1998; Costa Lotufo *et al.*, 2002, 2004). The results obtained in this study demonstrate that yangambin has low antimitotic and teratogenic potential, since continuous exposure of embryos to concentrations up to 500 μg/mL (1.025 mM) resulted in no inhibitory effect in the first egg cleavages, and only in the blastulae stage it was possible to identify some effect of this lignan. Using this model to analyze the ability of different compounds to inhibit cell division, Jacobs *et al.* (1981) concluded that a substance is considered significantly active when it inhibits 100% of the first division of the sea urchin eggs, at a concentration ≤ 16 μg/mL. After 28 h of continued exposure to yangambin, the embryos of the control group were in the larvae stage, while all the embryos treated with concentrations ranging from 50 to 500 μg/mL of this lignan were in the gastrulation phase, implying that besides being late, the effect is not concentration-dependent.

Studies using certain related chemical groups, such as anticancer compounds and metal salts, have been demonstrating a significant correlation between *in vitro* cytotoxic potential and acute toxicity in animals and humans (Garle *et al.*, 1994; Evans *et al.*, 2001; Clemedson *et al.*, 2002). The results obtained with yangambin corroborate this correlation. The low cytotoxicity of this lignan observed in the present study is correlated with the data of low acute toxicity reported on the murine model (Pachú *et al.*, 1993), because no clinical effect was identified up to 48 h after the treatment of mice with doses of 1g/kg body weight administered intraperitoneally. Thus, *in vitro* tests using the Trypan blue dye to evaluate the integrity of the cytoplasmic membrane, analysis of the activity of mitochondrial dehydrogenases by MTT reduction, as well as the study of sea urchin embryonic development proved to be good indicative models of the acute toxicity of yangambin. Considering some advantages of these *in vitro* tests, such as high reproducibility, simplicity, speed and use of small quantities of drugs, these tests may initially be used as a reference of toxicity for this class of compounds.

This paper presents the first experimental evidence that yangambin displays little cytotoxicity to the models evaluated. Furthermore, the low *in vitro* cytotoxicity demonstrated a correlation with toxi-

city rates *in vivo*. All these data, in association with various therapeutical properties of yangambin, make this lignan a promising prospect for a new drug.

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