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Original Papers

Toxicogenetic studies of an antileishmania nanomedicine based on *Ocotea fasciculata*, a plant of the Brazilian flora

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Abstract

The lignoid fraction (LF) of *Ocotea fasciculata*, which is rich in yangambin and its epimer, epi-yangambin, showed promising activity against *Leishmania* sp. Subsequently, LF was incorporated into a solid lipid nanoparticle (SLN) in order to increase its pharmacological efficacy and decrease toxicity. In this regard, the present study was carried out to evaluate the cytotoxic and toxicogenetic potential of LF and LF-SLN in mammalian cells *in vitro* and *in vivo*. The cytotoxic activity was evaluated in a non-tumor human cell line (GM07492A) and the toxicogenetic potential was assessed *in vitro* in a Chinese hamster lung fibroblast cell line (V79) and in Swiss mice. LF-SLN showed no cytotoxic effect at the highest concentration tested (5,000 µg/mL), while LF exhibited an IC_{50} equivalent to 1,047 ± 4.50 µg/mL. The frequencies of micronuclei observed *in vitro* and *in vivo* in mammalian cells treated with different concentrations of LF and LF-SLN did not differ significantly from the negative control group. Therefore, LF and LF-SLN did not show genotoxic or cytotoxic effects under the experimental conditions used. These results contribute to the development of a drug for the treatment of leishmaniasis that is more effective and safer for human health.

Key words: Epi-yangambin, lignoid fraction, lipid nanoparticle, micronucleus, yangambin.

Resumo

A fração lignoide (LF) de *Ocotea fasciculata*, rica em yangambina e seu epímero, epi-yangambina, apresentou atividade promissora contra *Leishmania* sp. Posteriormente, a LF foi incorporada a uma nanopartícula lipídica sólida (SLN), visando aumentar a eficácia farmacológica e diminuir a toxicidade. Nesse sentido, o presente estudo foi realizado para avaliar o potencial citotóxico e toxicogenético de LF e LF-SLN em células de mamíferos *in vitro* e *in vivo*. A atividade citotóxica foi avaliada em linhagem celular humana não-tumoral (GM07492A) e o potencial toxicogenético *in vitro* foi avaliado em linhagem celular de fibroblastos pulmonares de hamster chinês (V79) e em camundongos Swiss. LF-SLN não apresentou efeito citotóxico na maior concentração testada, 5.000 µg/mL, enquanto LF apresentou IC_{50} equivalente a 1.047 ± 4,50 µg/mL. As frequências de micronúcleos observadas *in vitro* em células de mamíferos e *in vivo* tratadas com diferentes concentrações de LF e LF-SLN não diferiram significativamente do grupo controle negativo. Portanto, LF e LF-SLN não apresentaram efeitos genotóxicos e citotóxicos nas condições experimentais utilizadas. Esses resultados contribuem para o desenvolvimento de um medicamento para o tratamento da leishmaniose, mais eficaz e seguro para a saúde humana.

Palavras-chave: Epi-yangambina, fração lignoide, nanopartícula lipídica, micronúcleo, yangambina.

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Introduction

The species *Ocotea fasciculata* (Nees) Mez, popularly known as "louro-de-cheiro", belongs to the Lauraceae family and is native to the Atlantic Forest areas of northeastern Brazil (Castro *et al*. 2020). This plant species is rich in alkaloids, lignans, and essential oils. The most important component found in *O. fasciculata* is yangambin (YA), a lignan present mainly in the leaves and stem bark (Peixoto *et al*. 2021). Pharmacological activities of this compound, such as antileishmanial (Rossetti *et al*. 2014), hypotensive and vasorelaxant (Araújo *et al*. 2014), antiallergic, central nervous system depressant (Martins *et al*. 2020), analgesic, and antitumor properties (Park *et al*. 2021), have been demonstrated.

In the search for new antileishmanial products, Monte-Neto *et al*. (2007) observed that a purified fraction (lignan fraction-LF) from *O. fasciculata*, which is highly lipophilic and rich in YA and its epimer, epi-yangambin (EPI-YA), exhibited promising activity against *Leishmania amazonensis* and *L. chagasi*. Subsequently, a delivery system based on solid lipid nanoparticles (SLN) that contain LF was developed (LF-SLN) in order to its increase pharmacological efficacy. This system showed prominent antileishmanial activity (Marquele-Oliveira *et al*. 2016).

Since the registration of pharmaceuticals requires the assessment of their genotoxicity for safe use, the present study aimed to evaluate the cytotoxicity and genotoxicity of LF and LF-SLN in in vitro and in vivo test systems.

Material and Methods

Chemicals and general experimental procedures

The LF and SLN-LF were obtained and characterized according to Rossetti *et al.* (2014) and Marquele-Oliveira *et al*. (2016). Briefly, LF and the isolated pure YA (Fig. 1a) and EPI-YA (Fig. 1b) compounds were obtained from the dried leaves and stem bark of *O. fasciculata*. The LF extract contained 115.48 mg YA/g and 132.77 mg EPI-YA/g, resulting in 248.25 mg/g of total lignan. SLN were obtained by the microemulsion method, as follows: first, stearic acid (20%) was melted at 10 ºC above its melting point (65.0–70.0 ºC) and surfactant (10%) was then added and stirred until it was completely dissolved. Next, 2% LF was added to the resulting microemulsion. Unloaded SLN to which no lignan extract was added were also prepared.

In vitro test system

Cell line and culture conditions

Human lung fibroblast (GM07492A) and Chinese hamster lung fibroblast (V79) cells were kindly supplied by the Laboratory of Cytogenetics and Mutagenesis, University of São Paulo, Ribeirão Preto, São Paulo, Brazil. The cells were maintained as monolayers in plastic culture flasks (25 cm^3) in HAM-F10 (Sigma-Aldrich®) and DMEM (Sigma-Aldrich®) medium (1:1) supplemented with 10% fetal bovine serum (Nutricell®), antibiotics (0.01 mg/mL streptomycin, CAS:3810-74-0; 0.005 mg/mL penicillin, CAS:113-98-4; Sigma-Aldrich®), and 2.38 mg/mL Hepes (CAS:7365-45-9; Sigma-Aldrich®) at 36.5 ºC in a B.O.D. incubator (Fanem®,

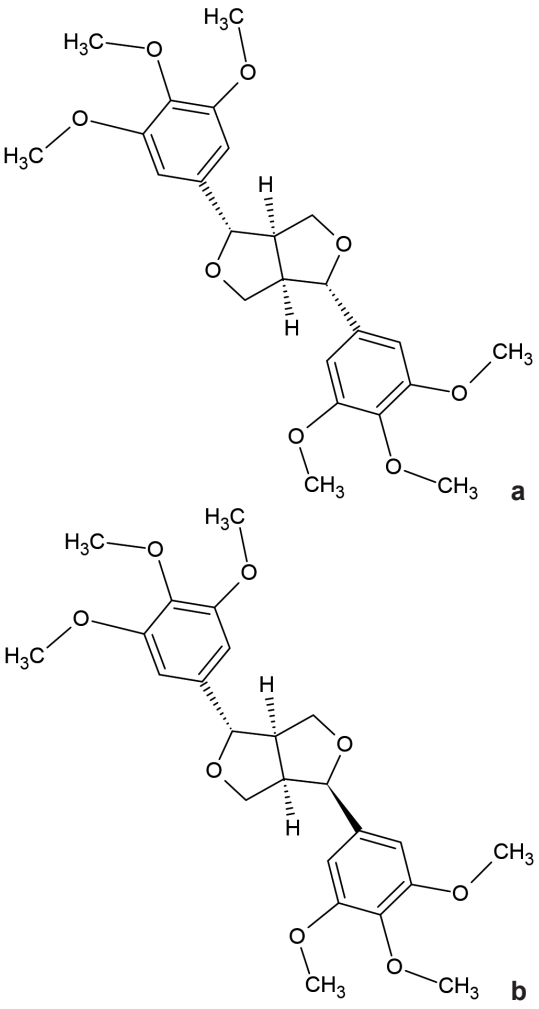

Figure 1 – a-b. Chemical structure of the compounds isolated from the lignan fraction – a. Yangambin; b. Epi-yangambin.

Brazil). All *in vitro* assays were performed on three different days to ensure reproducibility.

Cytotoxicity assay

The cytotoxicity assays were conducted using a non-tumor human cell line (GM07492A, human fibroblasts) since LF and LF-SLN were developed for use in humans. For this purpose, the colorimetric XTT Cell Proliferation Kit II (Roche®, Mannheim, Germany) was employed according to the manufacturer's instructions. The cells were treated with LF, YA or EPI-YA dissolved in dimethyl sulfoxide (DMSO, CAS: 67-68-5, Sigma-Aldrich®) and SLN and LF-SLN dissolved in cell medium (HAM-F10+DMEM) at concentrations ranging from 2.43 to 5000 µg/mL. Negative (no treatment), solvent (DMSO 1%), and positive (cisplatin, 0.39 to 50 µg/mL, CAS: 15663-27; Sigma-Aldrich®) controls were included. The treatment and analysis procedures were conducted as described by Carnizello *et al*. (2016). Non-linear regression analysis using the GraphPad Prism program was performed to calculate the sample concentration that inhibits 50% of cell viability (IC_{50}, half maximal inhibitory concentration). The experiments were carried out in triplicate.

Genotoxicity assay

The micronucleus assay was used to evaluate the genotoxic potential of LF and LF-SLN following OECD 487 recommendations (2016). V79 cell cultures were treated with different concentrations of LF (50, 100, and 200 µg/mL) and LF-SLN (1250, 2500, and 5000 µg/mL). The test concentrations of LF were chosen using cytotoxicity as a criterion, in which concentrations higher than 200 µg/mL were cytotoxic. Since limiting cytotoxicity was not observed for LF-SLN in the micronucleus assay, the highest test concentration evaluated was 5,000 µg/mL. Negative (no treatment), solvent (DMSO 1%), and positive (methyl methanesulfonate-MMS, 44 µg/mL, CAS:66-27-3; Sigma-Aldrich®) controls were included. The protocol was performed in triplicate on three different days to ensure reproducibility. The culture treatment, cell fixation and analysis procedures were performed as described by Reis *et al*. (2016). The frequency of micronuclei was obtained by the analysis of 3,000 binucleated cells per treatment. The cytokinesis-block proliferation index (CBPI) was also calculated to evaluate cytotoxicity of the treatments, in which 1,500 cells were analyzed per treatment.

In vivo test system

Animals

Male Swiss mice (*Mus musculus*), weighing approximately 30 g, were supplied by the Animal House of the School of Pharmaceutical Sciences of the University of São Paulo (Ribeirão Preto, São Paulo, Brazil). The animals were kept in plastic boxes in an experimental room under controlled conditions of temperature (23 ± 2 ºC) and humidity (50 ± 10%) on a 12-h light-dark cycle, with free access to regular laboratory chow and potable water. The study protocols were approved by the Animal Use Ethics Committee of the University of Franca (Approval No. 3120260417).

The micronucleus test was performed following OECD 474 recommendations (2016). Three different doses of LF and LF-SLN were evaluated: 500, 1,000 and 2,000 mg/kg b.w. The doses were dissolved in DMSO (5%) and administered to the mice (0.3 mL/animal) by gavage. Negative (water), solvent (DMSO 5%), and positive (MMS, 25 mg/kg b.w.) controls were included. Each treatment group consisted of five Swiss mice. The animals were euthanized with a single dose of thiopental sodium (840 mg/kg b.w., 1.0 g Thiopentax, Cristália®, Itapira, São Paulo, Brazil), administered intraperitoneally. For determination of the frequency of micronucleated polychromatic erythrocytes (MNPCE), 5,000 polychromatic erythrocytes (PCE) per animal were analyzed, totaling 25,000 PCE per treatment. The cytotoxicity of the treatments was also determined based on the PCE/PCE+NCE (normochromatic erythrocyte) ratio, in which 2,000 erythrocytes per animal were scored, totaling 10,000 erythrocytes per treatment. The slides used for this analysis were the same as those employed in the micronucleus test.

Statistical analysis

All data obtained were analyzed statistically by analysis of variance (ANOVA) using the GraphPad Prism 6® software. In cases in which $p < 0.05$, treatment means were compared by the Tukey test and the minimum significant difference was calculated for $\alpha = 0.05$.

Results and Discussion

The cytotoxicity results obtained by the XTT colorimetric assay are shown in Table 1. LF, which contains YA plus EPI-YA, had a

Table 1 – Cytotoxicity evaluation of LF and LF-SLN in GM07492A cell cultures after 24 h of treatment and respective controls.

Treatment	IC_{50} (µg/mL)
YA	2303 ± 214.65
EPI-YA	> 5000
SLN	3092 ± 333.50
LF	1047 ± 4.50
LF-SLN	> 5000
PC	32.1 ± 1.6

YA = yangambin; EPI-YA = epi-yangambin; SLN = solid lipid nanoparticles; LF = lignoid fraction; LF-SLN = solid lipid nanoparticle with lignoid fraction; PC = positive control (cisplatin); GM07492A = human lung fibroblasts. Values are the mean ± standard deviation.

lower IC_{50} (1,047 ± 4.50 µg/mL) than the values observed for each lignan alone (YA: 2,303 ± 214.65 µg/mL; EPI-YA: > 5,000 µg/mL), as occurred to LF that was more cytotoxic. Since YA and EPI-YA are optical isomers, they have similar physicochemical properties but differ in their optical activity and interaction with systems (Martins *et al*. 2020). Optical isomers can undergo the so-called chiral inversion - conversion from one optical configuration to another - under different conditions such as changes in temperature and in the presence of solvents, UV irradiation, enzymes, and other chiral compounds (Tverdislov *et al*. 2007). On the other hand, the chirality of biomolecules also produces strict requirements in the pharmaceutical industry since the optical isomers of drugs (enantiomers) often have different and sometimes opposite therapeutic activities (Lin *et al*. 2011; Ageeva *et al*. 2022).

Scientific investigation of natural botanicals is challenging because of their immense complexity and variability (Wagner & Ulrich-Merzenich *et al*. 2009; Efferth & Koch 2011). Within this context, the biological activity of mixtures containing LF can be due to the presence of compounds with synergistic, additive, or antagonistic activity. Additive and non-interactive combinations indicate that the combined effect of two compounds is a pure summation effect, whereas an antagonistic interaction results in a less than additive effect. Positive interactions, known as potentiation or synergy, occur when the combined effect of the constituents is greater than the expected additive effect. However, it is necessary to consider the multifactorial nature of botanical medicines (Pemovska *et al*. 2018; Caesar & Cech 2019). For example, LF was more cytotoxic (IC_{50} 1,047 ± 4.50 µg/mL) than YA or EPI-YA alone (YA: 2,303 ± 214.65 µg/mL; EPI-YA: > 5,000 µg/mL). This finding suggests an interaction between lignans in LF that enhances cytotoxicity.

When LF was encapsulated in SLN, no cytotoxicity was observed at the highest concentration tested, 5,000 µg/mL. Similarly, in a previous study using murine macrophages, which are the preferred cells for *Leishmania* infection, Marquele-Oliveira *et al*. (2016) observed lower toxicity of LF-SLN compared to LF. The reduction in LF cytotoxicity is likely related to encapsulation and the decreased amount of LF available in the medium.

Table 2 shows the data of the genotoxic evaluation of LF and LF-SLN. Cultures treated with different concentrations of LF and LF-SLN showed micronucleus frequencies that were not different from those of the negative control group. Regarding CBPI, no statistically significant differences were observed between the treatment groups and negative control, revealing the absence of cytotoxicity.

However, LF showed a cytotoxic effect at concentrations higher than 200 µg/mL in the micronucleus assay. In addition, SLN without LF showed cytotoxicity at concentrations higher than 3,000 µg/mL. On the other hand, when LF was encapsulated in SLN, no cytotoxic effect was observed at the highest concentration evaluated (5,000 µg/mL). It is important to emphasize that LF showed antileishmanial activity at concentrations (26.5 µg/mL) (Monte *et al*. 2007) lower than those at which it was cytotoxic to mammalian cells (GM07492A and V79 cells).

Table 2 – Frequencies of micronuclei in V79 cell cultures and in Swiss mice treated with LF and LF-SLN and respective controls.

Treatment	*In vitro*			*In vivo*		
	µg/mL	BNMN[a]	CBPI[b]	mg/kg	MNPCEs[c]	PCE/PCE+NCE[d]
Control		6.67 ± 2.52	1.57 ± 0.07		7.00 ± 2.45	0.03 ± 0.01
DMSO		7.67 ± 2.52	1.68 ± 0.18		8.20 ± 0.84	0.03 ± 0.01
MMS	44	31.67 ± 3.51*	1.51 ± 0.10	25	35.20 ± 7.33*	0.03 ± 0.01
SLN	750	7.33 ± 2.08	1.71 ± 0.10	500	10.20 ± 3.70	0.03 ± 0.00
	1500	9.00 ± 1.00	1.73 ± 0.04	1000	9.20 ± 3.30	0.03 ± 0.00
	3000	7.67 ± 1.15	1.62 ± 0.05	2000	15.40 ± 2.10	0.03 ± 0.00
LF	12.5	8.00 ± 1.73	1.76 ± 0.08	500	10.00 ± 3.54	0.02 ± 0.01
	50	13.00 ± 3.00	1.69 ± 0.05	1000	9.40 ± 0.90	0.02 ± 0.00
	200	10.30 ± 1.15	1.55 ± 0.07	2000	5.60 ± 0.90	0.03 ± 0.00
LF-SLN	1250	4.33 ± 1.53	1.58 ± 0.02	500	11.20 ± 3.11	0.03 ± 0.02
	2500	6.67 ± 2.02	1.70 ± 0.08	1000	11.40 ± 4.04	0.03 ± 0.01
	5000	13.00 ± 2.00	1.75 ± 0.08	2000	7.20 ± 2.95	0.03 ± 0.01

Control = no treatment; DMSO = dimethyl sulfoxide (solvent control 1% in vitro, 5% in vivo); MMS = methyl methanesulfonate (positive control); SLN = solid lipid nanoparticles; LF = lignoide fraction; LF-SLN = solid lipid nanoparticle with lignoide fraction. [a] = 1,000 binucleated cells with micronuclei (BNMN) were analyzed per culture, for a total of 3,000 cells/treatment. [b] = 500 cells were analyzed per culture, for a total of 1,500 cells/treatment for cytokinesis-block proliferation index (CBPI). [c] = 2,000 polychromatic erythrocytes (PCEs) were analyzed per animal, for a total of 12,000 cells/treatment. [d] = 400 erythrocytes were analyzed per animal, for a total of 2,400 cells/treatment (PCE/PCE +NCE [normocromatic erythrocytes]). * = Significantly different from control ($p < 0.05$). Values are the mean ± standard deviation.

The incorporation of LF into a lipid nanoparticle delivery system was important for a controlled release of the compound, with only 40–45% of LF being released into target cells, thus maintaining the desired therapeutic activity and the absence of cytotoxicity (Marquele-Oliveira *et al*. 2016). This fact is probably related to the lower cytotoxicity of SLN-LF when compared to LF, indicating that the lignan fraction of *O. fasciculata* associated with this delivery system is a promising leishmanicidal drug that should be further investigated clinically.

According to Zhao *et al*. (2015), nanoparticles are being widely studied because of their ability to reduce the cytotoxicity of the compound used. The entry of SLN into cells is facilitated, which may lead to a cytotoxic effect of the empty nanoparticle, albeit low. Therefore, encapsulation tests are crucial to ensure that the SLN has been loaded with LF, ensuring the effective and safe action of the lignan.

The animals treated with LF or LF-SLN showed micronucleus frequencies that did not differ from those of the negative control. Furthermore, no significant differences in the PCE/PCE+NCE ratio were observed between treatment groups (Table 2). In vivo tests are important to evaluate genotoxicity, metabolic, pharmacokinetic and pharmacodynamic factors and how they influence responses, thus explaining the difference in results compared to the in vitro tests. LF and LF-SLN did not show cytotoxic or genotoxic effects in the in vivo micronucleus test, considering that they were evaluated up to the highest recommended dose (2,000 mg/kg b.w., OECD 474, 2016). Negative results in appropriate in vivo assays are generally considered sufficient to demonstrate the absence of a significant genotoxic risk (ICH S2[R1], 2016). The data obtained in the present study indicate LF-SLN as safe for human health. Further studies using LF-SLN should be conducted for the development of a new drug for the treatment of leishmaniasis.

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Data availability statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

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