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ORIGINAL ARTICLE

Acute Toxicity and Cytogenotoxicity of Yangambin Isolated from *Ocotea duckei* Vattimo-Gil

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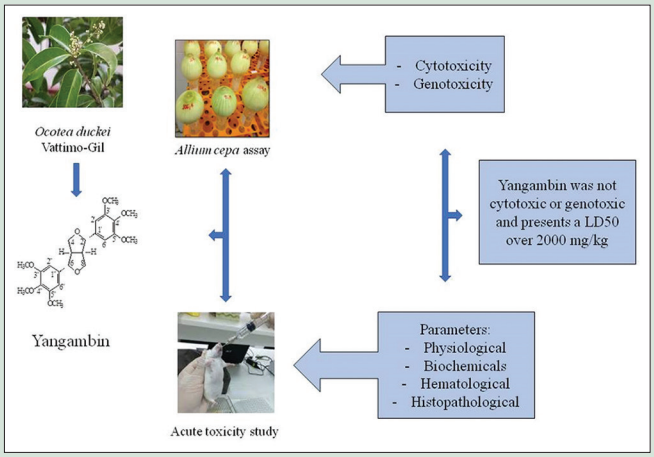
ABSTRACT

Background: Yangambin, a lignan isolated from *Ocotea duckei* leaves, has been reported to have antitumor, anxiolytic, hypotensive, and leishmanicidal activity, but there is little information on toxicity. **Objective:** The aim of this study was to investigate the acute toxicity of yangambin in mice, and cytotoxicity and genotoxicity through the *Allium cepa* assay. **Materials and Methods:** The yanganbin at a dose of 2000 mg/kg was orally administered once to mice to investigate acute toxicity, and meristem of the roots of *Allium cepa* was used to determine inhibition of root growth (root length) and mitotic index. **Results:** Yangambin did not cause hemolysis in sheep erythrocytes at concentrations of 12.5, 25, and 50 µg/mL, and did not show genotoxic changes in root meristem cells of *Allium cepa* at the same concentrations. In the acute toxicity study in Swiss mice at a dose of 2000 mg/kg, there were no apparent clinical signs or any behavioral changes and no deaths. **Conclusion:** Therefore, yangambin appears to a promising active compound for future pharmacological studies.

Key words: Acute toxicity, *Allium cepa*, Lauraceae

SUMMARY

- Yangambin isolated from *Ocotea duckei* leaves was evaluated according to toxicity, cytotoxicity and genotoxicity.-*Allium cepa* test system and acute toxicity test were performed on Swiss mice.- Cytotoxicity and genotoxicity were evaluated in the *Allium cepa* test system.- Physiological, biochemical, hematological and histopathological parameters were evaluated in the acute toxicity test.- Yangambin was not cytotoxic or genotoxic and presents a LD_{50} over 2000 mg/kg.



Abbreviations Used: NMR: Nuclear magnetic resonance; C: carbon; δC: carbon shift; H: hydrogen; δH: hydrogen shift; OECD: Economic Cooperation and Development; LD_{50}: Lethal dose 50; CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico; CAPES: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

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INTRODUCTION

The genus *Ocotea* belongs to the family Lauraceae, has a wide phytogeographic distribution in Brazil and includes a large number of species with medicinal properties.[1] Research has been carried out with different species of this genus to isolate and characterize their secondary metabolites and to identify the bioactive components as well.[2]

Ocotea duckei, popularly known as "Louro-de-cheiro" or "Louro-pimenta", is found in Northeast Brazil in the states of Pernambuco, Paraíba, Sergipe, and Ceará, and it contains a variety of secondary metabolites, including yangambin, a furofuran lignan which is the major constituent of the total fraction of lignoids.[3]

Studies have shown yangambin to have various pharmacological activities, such as antitumor against colorectal cells, apoptosis induction,[4] soporific,[5] hypotensive,[6] anti-PAF,[7,8] and leishmanicidal.[9,10]

For safety and pharmacological effectiveness, the World Health Organization (WHO) considers it important that medicinal plants and their active principles be tested for toxicity.[11] The guide for

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non-clinical toxicology and pharmacological safety studies necessary for drug development proposes that cytotoxicity and genotoxicity assays and single-and repeated-dose toxicity and carcinogenicity studies be conducted.[12]

The *in vitro* assay of hemolytic activity allows an evaluation of the cytotoxicity of herbal medicines by seeing if they cause the lysis of erythrocytes.[13] These cells are widely used for *in vitro* cytotoxicity studies, mainly due to their easy access and high availability, allowing the evaluation of the toxic or protective effect of active ingredients on the cell membrane.[14,15]

The *Allium cepa* test system is considered an excellent bioindicator of the genotoxicity of medicinal plants and phytotherapeutic compounds due to its reliability and agreement with other tests.[16] In addition, this method of evaluating chromosomal changes in *A. cepa* root has been validated by the International Chemical Safety Program (IPCS, WHO) and the United Nations Environment Program and deemed an effective test for *in situ* analysis and monitoring of the genotoxicity of test substances.[17]

Acute toxicity studies are those used to assess the toxicity caused by a test substance when administered at one or more doses for a period not exceeding 24 h, followed by observation of animals for 14 days after administration. The parameters to be evaluated in this test are mortality, clinical signs (including behavioral parameters), variations in body weight and feed and water intake, clinical pathology (hematology, biochemistry) and those from anatomical and histopathological investigations.[12]

Thus, to determine the pharmacological potential of yangambin by an essential evaluation of its preclinical safety, this study aimed to investigate the cytotoxicity, genotoxicity, and acute toxicity of yangambin isolated from *O. duckei* Vattimo-Gil.

MATERIALS AND METHODS

Extraction and isolation of yangambin

Yangambin was isolated from the leaves of *O. duckei* Vattimo-Gil (Lauraceae) according to the method described by Barbosa-Filho *et al*.[18] Botanical material was collected in the municipality of Santa Rita, Paraíba State, Brazil (Voucher Agra 4309). Fifteen kilograms of the plant material were used. After drying and ball-milling, the powder was extracted with ethanol and concentrated in a rotary evaporator, yielding 6.5% crude ethanol extract in relation to the dry plant powder. For the isolation of lignoids, 300 g of ethanol extract was suspended in 10% acetic acid and then filtered, obtaining an insoluble residue and the acidic aqueous solution, which was extracted in a separator funnel with two liters of dichloromethane. The dichloromethane phase was filtered dry with anhydrous sodium sulfate and concentrated under reduced pressure, yielding the total lignoid fraction, which was separated on a silica gel 60 column (Merck-0.063-0.200 mm) and eluted with hexane, chloroform, and methanol, pure or in binary mixtures, using an increasing polarity gradient. Fractions eluted with MeOH-$CHCl_3$ (5-95) yielded pure yangambin. The identification of yangambin was performed by ^{1}H and ^{13}C NMR spectral data analysis.

Allium cepa system test

Genotoxicity analysis was performed using the *A. cepa* test system as described by Bhattacharya, with modifications.[19] Yangambin was tested at 12.5, 25 and 50 μg/mL, diluted in distilled water. Healthy nongerminated bulbs weighing 80–100 g were purchased from a commercial establishment in Recife, PE, Brazil and allowed to germinate for 5 days at room temperature in 50-mL polypropylene tubes, with the lower part immersed in the yangambin test solutions and control solutions, constituting five treatments. Distilled water was used as negative control (NC) and 0.6 μg/mL copper sulfate as positive control (PC). After the germination period, the length of the three largest roots in each bulb of each treatment was measured. The results obtained from the test concentrations were compared with the negative and PCs. The rootlets were collected and fixed in of ethanol/acetic acid (3/1) for 24 h. Afterward, they were washed in running water and hydrolyzed with 45% HCl for 10 min. After HCl removal, the samples were again washed and stained with hematoxylin. The samples were then transferred to slides, where the meristem tic part was cut and crushed. Histological preparations were examined with light microscope (Leica, Wetzlar, Germany) with a Motic camera-attached ×100 objective and an image analysis system (Kinetic Imaging: Andor Technology, Nottingham, UK). A total of 5,000 cells from each *A. cepa* root treatment were analyzed; cells were observed in interphase, prophase, metaphase, anaphase and telophase and chromosomal abnormalities were determined. Subsequently, the mitotic index (MI) was calculated, that is the percentage of dividing cells relative the total number of cells analyzed: MI = NCM/NTC ×100, where NCM is the number of cells in mitosis and NTC the total number of cells counted.

Acute toxicity assay in female swiss mice

This study followed the guidelines of the Organization for Economic Cooperation and Development (OECD) for the acute toxicity class (Acute Toxic Class Method – OECD 423).[20] We complied with Brazilian Ministry of Health Ordinance 116/96 for the assessment of acute oral toxicity of chemicals and single-dose toxicity studies of new drugs.[12]

Twelve female Swiss albino mice (*Mus musculus*) weighing between 28 and 32 g, in the age group close to 60 days (young adults), were used; they were obtained from the bioterium of the Department of Antibiotics of the Federal University of Pernambuco (UFPE). The animals were kept under controlled temperature conditions of 21 ± 1°C under the natural day/night cycle (12 h light and 12 h dark), with water and food (Purina brand feed pellets) *ad libitum* during the experiment. All experimental procedures were analyzed and approved by the Ethics Committee on the Use of Animals of the Bioscience Center– UFPE, under process No. 23076.023060/2018-50. The animals were randomly divided into two groups of six animals: saline control group and treated group given 2000 mg/kg yangambin by oral gavage, using 10 mL/kg. The clinical signs of the animals were observed for 2 h after administration and daily for 14 days for mortality, body weight, and water and feed intake. Afterward, the animals were euthanized, and blood biochemical and hematological parameters were assessed. Histopathological analysis followed the procedures described by Katisart.[21]

Statistical analyses

Parametric data were submitted to one-way ANOVA with Tukey posttest at 5% probability. Nonparametric data were submitted to the Kruskal-Wallis test, with Dunn posttest at 5% probability. Correlation analysis was performed according to Spearman. Analyses were performed, and graphs were generated using GraphPad Prism 7 software (San Diego, California, USA).

RESULTS

Yangambin, isolated from *O. duckei* Vattimo-Gil, was identified by ^{1}H and ^{13}C NMR and comparisons were made with the literature data [Table 1].

According to the results obtained, there was no significant difference in the mean growth and MI of *A. cepa* roots exposed to the treatments at the test concentrations of yangambin when compared to the

Table 1: ^{13}C and ^{1}H nuclear magnetic resonance data (125 MHz) of yangambin and comparison with Nuclear magnetic resonance data from literature[18]

C	δC	Lit.[18]	H	δH	Lit.[18]
1	54.5	54.6	1	3.06 (m)	3.11 (m)
2	86.1	86.2	2	4.71	4.76 (d, *J*=4.2 Hz)
4	72.1	72.2	4ax	3.70-3.96 (m)	3.80-3.96 (m)
5	54.5	54.6	4eq	4.26-4.30 (m)	4.30-4.40 (m)
6	86.1	86.2	5	3.31 (m)	3.11 (m)
8	72.1	72.2	6	4.71	4.76 (d, *J*=4.2 Hz)
1',1"	136.8	136.9	8ax	3.78-3.96 (m)	3.80-3.96 (m)
2',2"	102.9	103.2	8eq	4.23-4.30 (m)	4.30-4.40 (m)
3',3"	153.5	153.7	2',2"	6.52 (s)	6.59 (s)
4',4"	137.5	137.8	6',6"	6.52 (s)	6.59 (s)
5',5"	153.5	153.7	OCH_3 (4',4")	3.80 (s)	3.86 (s)
6',6"	102.9	103.2	OCH_3 (3',3"5',5")	3.82 (s)	3.88 (s)
OCH_3 (4',4")	60.9	61.0			
OCH_3 (3',3",5',5")	56.2	56.0			

NMR: Nuclear magnetic resonance; C: Carbon; δC: Carbon shift; H: hydrogen; δH: Hydrogen shift; OECD: Economic cooperation and development; LD_{50}: Lethal dose 50; CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico; CAPES: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

Table 2: Cell numbers in the cell cycle and the mitotic index of *Allium cepa* roots exposed to different treatments

Treatment	Number of cells analyzed	Cells in interphase	Dividing cells	Mitotic index (%)	Growth of roots (cm)
Negative control	5000	4585	415	8.29[a]	2.96±0.241[a]
Positive control	5000	4756	248	4.82[b]	0.471±0.035[b]
12.5 (μg/mL)	5000	4639	361	7.22[a]	2.72±0.232[a]
25 (μg/mL)	5000	4631	369	7.38[a]	2.65±0.2881[a]
50 (μg/mL)	5000	4641	359	7.19[a]	2.52±0.376[a]

Same letters indicate no significant difference

NC (distilled water), but there was a significant difference ($P < 0.05$) when compared to the PC (0.6 μg/mL copper sulfate) [Table 2].

No chromosomal abnormalities were observed in the meristemtic cells of *A. cepa* treated with yangambin and showed similar cell division phases as in NC cells. On the other hand, the PC (0.6 μg/mL copper sulfate) showed abnormal mitotic cells, including delayed anaphase, anaphase bridges, chromosome breaks, disturbed telophase, micronuclei, lobulated nucleus, interphase with nuclear button, and binucleated cell [Figure 1].

In the acute toxicity test, 2000 mg/kg yangambin did not cause any animal deaths after administration or over the whole study period (14 days) and there was no statistically significant difference in water and feed intake, body weight or organ weight (liver and kidneys) in yangambin-treated versus control animals [Table 3].

According to the results described in Table 4, animals treated orally with 2000 mg/kg yangambin did not show statistically significant changes in hematological parameters compared to control groups ($P > 0.05$). On the other hand, in the biochemical parameters, there was a statistically significant effect ($P < 0.5$) but only in liver function monitored by the alanine aminotransferase (ALT) level; the other parameters analyzed (glucose, urea, creatinine, total cholesterol and aspartate aminotransferase showed no statistically significant difference ($P > 0.5$) between lignin-treated and saline-treated animals.

No pathological changes were detected in the liver and kidneys of animals treated with 2000 mg/kg yangambin when compared with the control group, and values obtained were in the normal range [Figure 2].

DISCUSSION

As previously described, yangambin is a lignan isolated from *O. duckei,* which has several possible pharmacological activities described in the literature. However, few studies have been conducted on the toxicological parameters of this substance.[3,22] Thus, studies to determine its toxicological profile are of great importance, and our study provided results on the *in vitro* and *in vivo* toxicity of yangambin.

We observed that yangambin did not cause hemolysis in sheep erythrocytes at the concentrations tested, indicating that there was no hemolytic activity [Table 1]. Similar results were found with a methanolic extract of cinnamon (*Cinnamomum tamala*), from a genus belonging to the family Lauraceae.[23]

The meristematic cells of *A. cepa* exposed to yangambin at the test concentrations used showed neither decrease nor increase in MI in relation to the NC [Table 2]. Therefore, there was no inhibition of onion root growth, since inhibition of root growth is generally related to changes in the cellular activity of the apical meristem.[24]

However, there was a difference when compared to the PC, since it caused a decrease in the MI and thus growth inhibition, which was expected since copper sulfate is proven to be a genotoxic and mutagenic substance. Thus, it appears that yangambin has no cytotoxic potential, as these results obtained "*in vivo*" corroborated those obtained "*in vitro*" by Monte Neto *et al*., where yangambin also showed no cytotoxicity against murine macrophages and sea urchin embryonic cells.[3]

Using the *A. cepa* test, it is also possible to observe the occurrence of chromosomal changes during the cell cycle, and therefore, this method has been used to determine risks of the consumption of different products of plant origin.[25] The efficiency of this bioassay is based on the observation that certain mutagenic mechanisms are similar in plants and animals.[26]

In the cell cycle of *A. cepa* meristematic cells treated with yangambin, normal mitotic cells were found, as in the NC but not the genotoxic PC, indicating that yangambin is not genotoxic [Figure 1]. In the literature, there is no scientific evidence of yangambin being genotoxic to plant cells. Moreover, Marques *et al*. did not find any genotoxic potential for yangambin and an ethanolic extract of *O. duckei* Vattimo-Gil did against *Salmonella typhimurium* strains in the Ames test.[22]

In the acute toxicity test at a dose of 2000 mg/kg, yangambin did not cause animal mortality. According to OECD Guideline 425, a substance

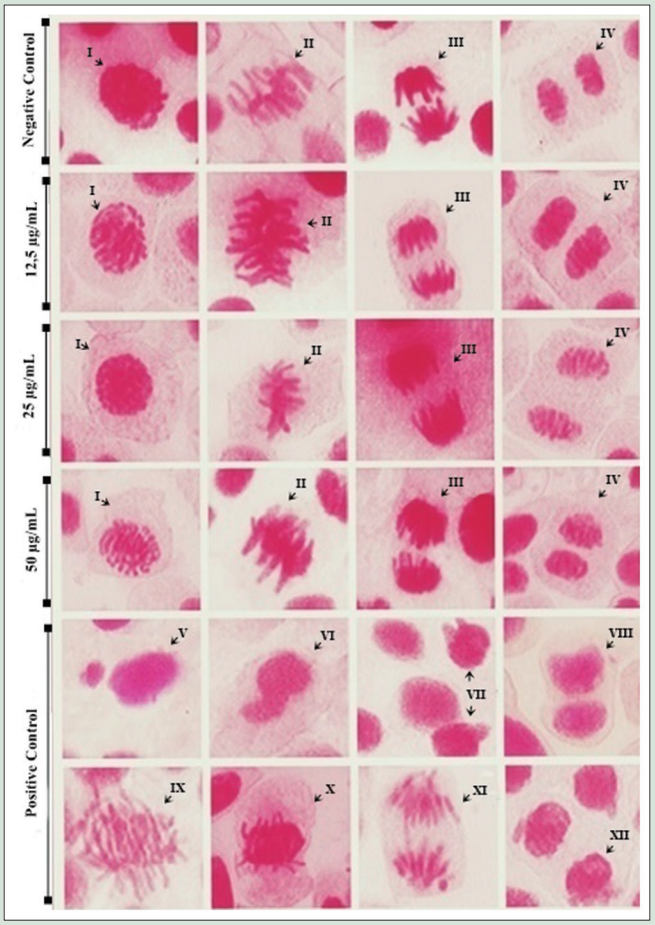

Figure 1: Photomicrographs of the cell division phases of meristematic cells (*Allium cepa*) exposed yangambin at 12.5, 25 and 50 µg/mL and negative and positive control cells. Cells were stained with hematoxylin and viewed with ×100 objective. Normal prophase (I), normal metaphase (II), normal anaphase (III), normal telophase (IV), micronucleus cell (V), lobulated nucleus (VI), interphase with nuclear button (VII), binucleated cell (VIII), multiple bridges in anaphase (IX), delayed anaphase (X), anaphase with bridge and chromosome break (XI) and binucleated cell (XII)

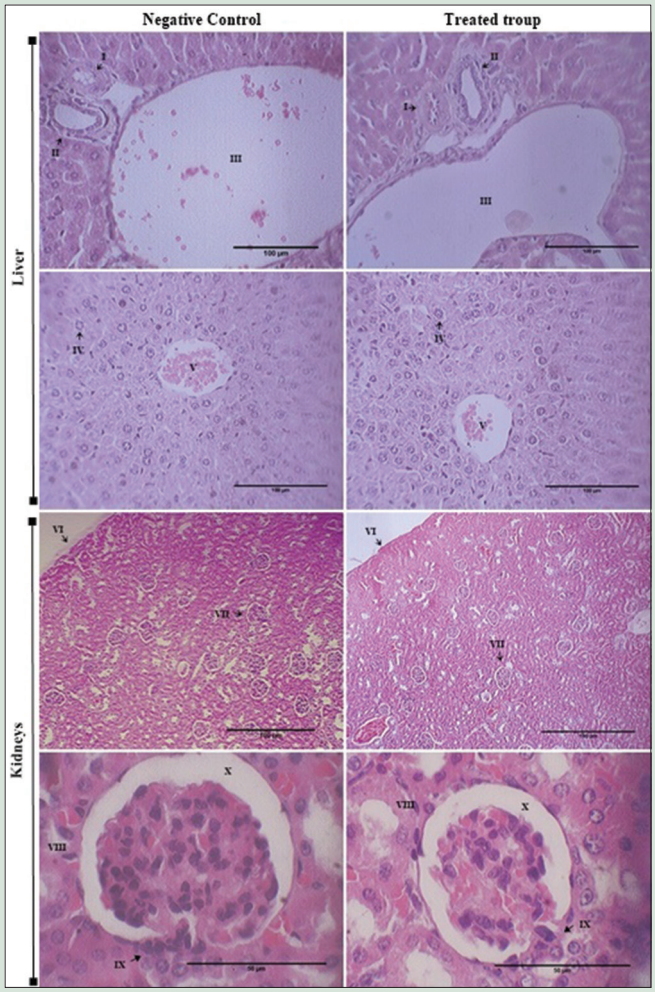

Figure 2: Photomicrographs of the organs of the group treated with 2000 mg/kg yangambin and control group (0.9% NaCl). Cells were stained with (H and E) and viewed with ×40 (scale bar = 100 µm) and ×100 objectives (scale bar = 50 µm) objectives. Indicated: artery (I), biliary duct (II), hepatic portal vein (III), hepatocyte (IV), centrolobular vein (V), connective tissue capsule (VI), glomerulus (VII), dense macula (IX) and Bowman's capsule (X)

Table 3: Mean water and feed intake, body weight and relative weight of organs of Swiss mice in the control group and group treated with 2000 mg/kg yangambin

Evaluations	Groups	
	Control	Yangambin, 2000 (mg/kg)
Intake		
Water	45±1.96 (NS)	44.3±2.76 (NS)
Feed	15.8±4.5 (NS)	16.8±3.9 (NS)
Body weight	36.11±0.315 (NS)	36.88±0.259 (NS)
Relative weight of organs		
Liver	2.29±0.30 (NS)	2.248±0.55 (NS)
Kidneys	0.552±0.015 (NS)	0.537±0.125 (NS)

Data presented as mean±SD of six animals, analyzed by Student's *t* test $P<0.05$. NS: Not significant; SD: Standard deviation

is considered practically non-toxic when it has an LD_{50} over 2000 mg/kg or between 2000 and 5000 mg/kg, falling into Class 5 and therefore, it is regarded as having low toxicity, indicating a certain margin of safety in its use.[21] In addition, as demonstrated by Dewi *et al*.[27] and indicated in OECD Guideline 423 (OECD 2001),[17] the study of toxicity in one gender (female) is generally considered sufficient. Therefore, our toxicity evaluation performed only with female Swiss mice was suitable.

According to Chebaibi *et al*. (2019),[28] the occurrence of changes in animal intake and weight in studie s evaluating acute toxicity is considered an important indicator of metabolic adverse effects for a given substance. In this study, female Swiss mice treated with yangambin did not show significant changes in feed intake, water intake, growth, and relative weight of liver and kidneys compared to the control group, indicating that there were no adverse effects at a dose of 2000 mg/kg [Table 3].

Yangambin at 2000 mg/kg also did not cause hematological changes in mice [Table 4]. These results indicate that yangambin was not toxic with regard to blood cell production and morphology in the experimental animals, since the hematopoietic system is extremely sensitive to the activities of toxic agents, especially those with mutagenic or cytotoxic potential, resulting in qualitative or quantitative, transient or permanent changes.[29] In the acute toxicity study in rodents, the ethanolic extract of *Litsea glutinosa* (Lauraceae) at a dose of 2000 mg/kg did not cause adverse effects regarding hematological parameters.[30]

Table 4: Effects of oral yangambin after single dose administration (2000 mg/kg) on hematological and biochemical parameters in female Swiss mice

Parameters	Dose (mg/kg)		Normal range
	Control	Yangambin 2000	
Hematological parameters			
Erythrocytes (10^6/mm^3)	9.745±0.4355	9.945±0.3088	5.2-10.4
Hemoglobin (g/dL)	14.24±0.2931	14.2±0.0678	11.1-14.8
Hematocrit (%)	43.9±1.24	44.58±1.536	32.1-46.5
MCV (µm^3)	46.85±0.5626	46.77±0.6048	44.2-58.5
MCH (pg)	15.32±0.2871	14.94±0.4479	14.0-18.7
MCHC (g/dL)	32.43±0.3323	32.2±0.5502	28.4-38.5
Platelets (10^3/mm^3)	754.5±27.23	757.2±35.05	315-758
Neutrophils (%)	19.11±4.286	21.02±2.998	10-23
Eosinophils (%)	0.202±0.081	0.192±0.1523	0-3
Basophils (%)	1±0.3215	0.8333±0.4372	0-1
Lymphocytes (%)	75.2±3.674	77.16±3.498	74-90
Monocytes (%)	2.364±0.85	1.012±0.2648	0-5
Biochemical parameters			
Glucose (mg/dL)	171.5±14.9	155.8±12.76	-
Urea (mg/dL)	35±3.033	32.67±2.261	-
Creatinine (mg/dL)	0.2±0.03162	0.22±0.03742	-
Total cholesterol (mg/dL)	58.67±3.383	63.5±2.941	-
AST (U/L)	151.5±3.797	184±17.48	-
ALT (U/L)	52±3.755	70.2±3.056**	-

Data presented as mean±SD of six animals, analyzed by Student's *t*-test $P<0.05$. NS: Not significant; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; SD: Standard deviation; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoblogin concentration

In the biochemical parameters, the indicators of renal function (urea and creatinine) and metabolic indicators (glucose and total cholesterol) did not show significant differences between the yangambin group and the control group, suggesting no effect on kidneys or glycolytic and lipid metabolism. Although the experimental animals that received 2000 mg/kg yangambin showed a significant increase in serum ALT level compared to control, this was not considered to be indicative of hepatotoxicity. Although changes in serum ALT concentrations characterize liver disease, they may not have any clinical significance, since the increase in cytoplasmic enzymes such as ALT is also due to other factors, such as increased permeability of the cell membrane to anesthetics.[31]

Histopathological analysis of the liver and kidneys of the animals that received 2000 mg/kg yangambin revealed no changes, where the histological architecture of the organs remained within normal limits, indicating that there was no hepatotoxicity or nephrotoxicity. Ethanolic extract of *Cinnamomum cassia* (Lauraceae) in the maximum acute dose tested in mice showed similar results; once no histopathological, physiological, biochemical, and hematological changes were noticed.[32]

CONCLUSION

Yangambin isolated from *O. dukei* leaves was not cytotoxic or genotoxic under the experimental conditions used. In addition, it had a LD_{50} over 2000 mg/kg, did not cause changes in body weight, feed and water intake, and the anatomorphological structure of the liver and kidneys of lignin-treated animals and was therefore considered to have little or no toxicity in Swiss mice, appearing to be a promising drug candidate in future studies.

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Conflicts of interest

There are no conflicts of interest.

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