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BIOMEDICAL SCIENCES

Preliminary evaluation of the toxicological, antioxidant and antitumor activities promoted by the compounds 2,4-dihydroxy-benzylidene-thiosemicarbazones an *in silico, in vitro* and *in vivo* study

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Abstract: Thiosemicarbazones are promising classes of compounds with antitumor activity. For this study, six 2,4-dihydroxy-benzylidene-thiosemicarbazones compounds were synthesized. These compounds were submitted to different assays *in silico*, *in vitro* and *in vivo* to evaluate the toxicological, antioxidant and antitumor effects. The *in silico* results were evaluated by the SwissADME and pkCSM platforms and showed that all compounds had good oral bioavailability profiles. The *in vitro* and *in vivo* toxicity assays showed that the compounds showed low cytotoxicity against different normal cells and did not promote hemolytic effects. The single dose acute toxicity test (2000 mg/kg) showed that none of the compounds were toxic to mice. In *in vitro* antioxidant activity assays, the compounds showed moderate to low activity, with PB17 standing out for the ABTS radical capture assay. The *in vivo* antioxidant activity highlighted the compounds 1, 6 and 8 that promoted a significant increase in the concentration of liver antioxidant enzymes. Finally, all compounds showed promising antitumor activity against different cell lines, especially MCF-7 and DU145 lines, in addition, they inhibited the growth of sarcoma 180 at concentrations lower than 50 mg/kg. These results showed that the evaluated compounds can be considered as potential antitumor agents.

Key words: antioxidant, antitumor, cytotoxicity, sarcoma 180, thiosemicarbazones.

INTRODUCTION

Cancer is a multifactorial, life-threatening disease complex that can appear in different tissues and anywhere in the body (Shafiee et al. 2023). This disease is responsible for almost 10 million deaths in 2020 worldwide (Morgan et al. 2023). The cancers that most caused death in the respective year were: lung, colon and rectum, liver, stomach and breast respectively (Morgan et al. 2023, Shafiee et al. 2023). In Brazil, 704,000 new cases of cancer are expected for the three-year period 2023-2025 (Oliveira Santos et al. 2023).

In this alarming context, three cancer coping strategies are important: prevention, diagnosis and treatment. The removal of cancer-causing factors (such as alcohol and tobacco use) and a healthy diet are the main forms of prevention. In the initial stage, before metastasis, a quick and accurate diagnosis is extremely important to fight the tumor (Oliveira Santos et al. 2023, Morgan et al. 2023,

Shafiee et al. 2023). At this stage, the main forms of cancer treatment are surgery, radiotherapy and chemotherapy, which can be used together with the aim of obtaining a cure, prolonging and improving the quality of life (Dahan et al. 2023). Although many combined strategies have achieved good results, it is still far from having a good clinical outcome (Jin et al. 2023).

In order to help in the treatment of cancer, several chemotherapy drugs have been synthesized (Brianna & Lee 2023). These drugs act through different mechanisms to inhibit the growth of tumor cells, namely: DNA intrachain crosslinking; blocking DNA and/or RNA synthesis; inhibition of topoisomerase enzyme action, inhibition of kinases involved in growth factor receptor transduction, among others (Siqueira et al. 2023). Although there are several effective chemotherapeutics, many can cause different side effects (Brianna & Lee 2023, Siqueira et al. 2023). The level of exposure to the drug and the effect-to-damage ratio affects the degree of toxicity. It is necessary that there is a reduction in the side effects induced by these drugs, to improve the quality and survival of patients (Brianna & Lee 2023).

Given this scenario, medicinal chemistry presents itself as a strategy in the search for new alternatives for probable chemotherapy drugs, starting from a prospective sense where it suggests new chemical structures (Bai et al. 2022). The search for new therapeutic alternatives has been gaining prominence over the centuries all over the world, justified by the emergence of new diseases and, often, by the therapeutic failure of available drugs (Bai et al. 2022, Brianna & Lee 2023).

Faced with an infinity of compound classes, we will highlight the thiosemicarbazones here. This class of organic molecules presents heteroatoms of nitrogen and sulfur in its structure, of which the hydrazonic (HC=N-NH-) and thioamide (S=C-N-) moieties stand out. Thiosemicarbazones and their structural analogues are known in the literature for having different biological and pharmacological activities (Jacob et al. 2023). Regarding the antitumor activities promoted by different thiosemicarbazones, we will cite the works carried out by Bai et al. (2022) and Chaudhary et al. (2022), Jacob et al. (2023). The authors verified that thiosemicarbazones are potential drug candidates and that they showed promising results, that is, the compounds were less toxic for mammalian cells and more toxic for tumor cells, an important prerequisite for the screening of chemotherapy drugs.

The (2,4-dihydroxybenzylidene)-hydrazinyl) group is known for its strong antiproliferative activity, as seen in phenolic compounds found in natural products. The potency of the compound is influenced by the position and number of hydroxyl substituents, as the inhibitory activity increases with more hydroxyl groups. The position of hydroxyl substituents is more significant than the number of substituents in determining the potential inhibitory responses of the Schiff base (Leigh et al. 2011, Kang et al. 2016).

The objective of this study was to synthesize a small series of 6 compounds, specifically 2,4-dihydroxy-benzylidene-thiosemicarbazones, and to conduct a preliminary investigation of their toxicological, antioxidant, and antitumor properties *in silico*, *in vitro*, and *in vivo*. The results obtained here will contribute valuable information for potential applications of thiosemicarbazones as candidates for chemotherapy drugs.

This study was approved by the Animal Use Ethics Committee of Institute Aggeu Magalhães/ Oswaldo Cruz Foundation, protocol number 164/2020.

MATERIALS AND METHODS

Reagents and solvents

The reagents used for the synthesis and analysis of the hydroxylated thiosemicarbazone derivatives were 2-phenyl-ethyl isothiocyanate (Merck, CAS: 2257-09-2), 4-chloro-benzyl isothiocyanate (Merck, CAS: 2131-55-7), 4 -nitro-benzyl isothiocyanate (Merck, CAS: 2131-61-5), 4-methyl-benzyl isothiocyanate (Merck, CAS: 622-59-3), 4-ethyl-benzyl isothiocyanate (Merck, CAS: 18856-63- 8), 4-Bromo-benzyl isothiocyanate (Merck, CAS: 155863-32-4), 4-methoxy-benzyl isothiocyanate (Merck, CAS: 2284-20-0), Hydrazine Solution (Merck, CAS: 302-01 -2) and 2,4-dihydroxydo-benzaldehyde (Merck, CAS: 95-01-2). The solvents were ethyl alcohol, dichloromethane, dimethyl sulfoxide (DMSO), in addition to glacial acetic acid, provided by Dinâmica.

Synthesis of thiosemicarbazones

The compounds were synthesized at the Laboratory of Chemistry and Therapeutic Innovation (LQIT) at UFPE – Recife/PE/Brazil. Obtaining the compounds (PBs) was carried out from thiosemicarbazides from hydrazine hydrate (a) and substituted isothiocyanates (b) in diclomethane (Step 1). Then, the thiosemicarbazones were obtained according to Oliveira et al. (2015) and Jacob et al. (2021), from thiosemicarbazides (c) with 2,4-dihydroxy-benzylideyde (d) (Step 2) (Figure 1). All compounds have been previously described in the literature (Leigh et al. 2011, Kang et al. 2016)

For analysis of the synthesized compounds, thin layer chromatography (CCD) was performed using Polygram Sil G/UV 254 chromatographic plates (0.20 mm), developed under ultraviolet light at 254 nm. For melting point determination, a dry melting point apparatus (Model 431D - Fisatom, Brazil) was used. The characterization and structural verification were carried out through Infrared (IR) spectroscopy analysis, registered in an infrared spectrometer with Fourier transform, in UATR, Hydrogen Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR) and Carbon (^{13}C NMR) performed in Model Varian Plus Spectrophotometer (Varian, USA) at 400 MHz and 100 MHz, respectively, in DMSO-d_6 solvent (Characterization data are in the Supplementary Material Figures S1 to S17).

Evaluation of *in silico* parameters of absorption, distribution, metabolism, excretion and toxicity (ADMET)

ADMET and bioavailability profiles were predicted using the SwissADME (http://www.swissadme.ch/) and pkCSM (http://biosig.unimelb.edu.au/pkcsm/prediction) platforms (Daina et al. 2017, Pires et al. 2015). This study utilized the compounds synthesized in this research, along with the commercial antitumor drugs Doxorubicin and Amsacrine, respectively.

Ethics Committee

This study was approved by the Animal Ethics Committee of the Instituto Aggeu Magalhães/Fundação Oswaldo Cruz, protocol number 164/2020.

In vitro toxicity tests against normal and tumor cell lines

The *in vitro* toxicity tests promoted by thiosemicarbazones were carried out according to Jacob et al. (2023) with few modifications. The normal cell lines used were: J774 macrophages (strain

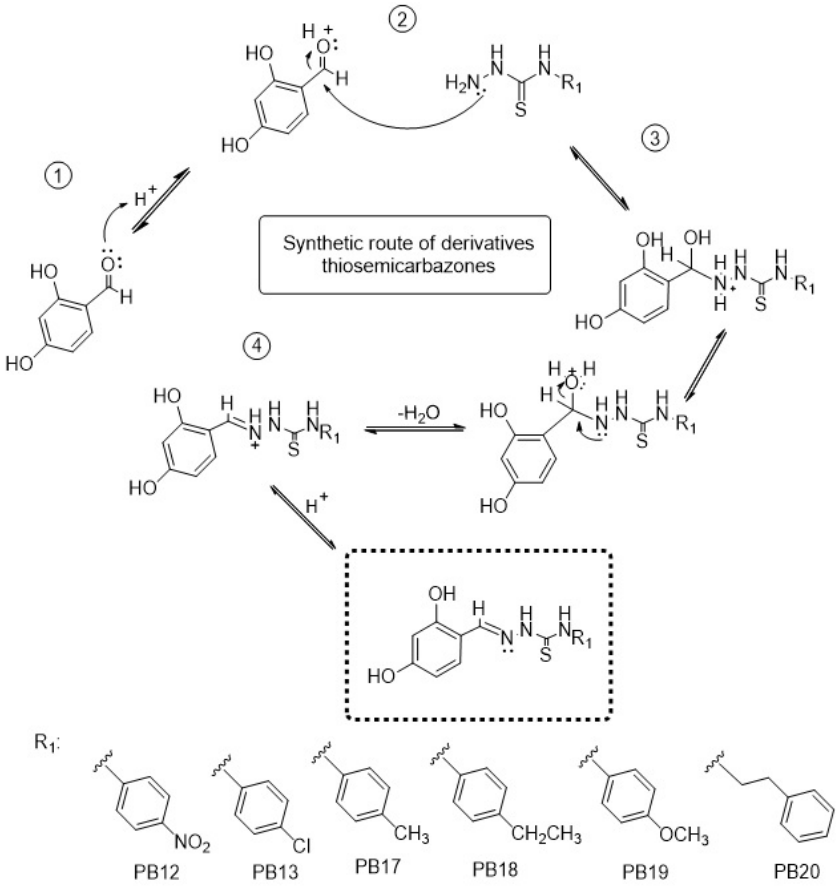

Figure 1. Chemical structures of compounds synthesized in LIQT/UFPE: Reagents and Conditions: (a) hydrazine, substituted isothiocyanate (b), chloroform, temperature 30 ± 0.5 ºC; (b) thiosemicarbazide (c), dihydroxy-benzylideyde (d), absolute ethanol, acetic acid as catalyst, temperature: 75 ± 0.5 ºC.

obtained from mice), Vero cells (renal epithelial cells extracted from an African green monkey) and V79 fibroblasts (cells obtained from the lung tissue of a Chinese hamster).

The tumor cell lines evaluated were: DU145 (prostate cancer), HCT-8 (human colon), HEp-2 (laryngeal squamous cell carcinoma), HepG2 (hepatoma), HL-60 (Acute promyelocytic leukemia), HT-29 (Adenocarcinoma colon cancer), Jurkat (T cell line derived from T cell leukemia/lymphoma), MCF-7 (Human breast adenocarcinoma), NCI-H292 (Human mucoepidermoid lung carcinoma), SF-295 (Human glioblastoma) and T-47D (human breast cancer).

Initially, the different strains, at a concentration of 2.0 x10^6 cells/well, were cultivated in 96-well plates containing RPMI culture medium and incubated in an atmosphere of 5% CO_2 at 37° C for 24 h. After the incubation period (24 h), the culture supernatant was removed and the cells were again incubated in an atmosphere of 5% CO_2 at 37 °C in the presence of compounds dissolved in DMSO1% at concentrations ranging from 1.5 to 500 µM per 72 h in RPMI medium.

After incubation, 20 µL of MTT solution (3-(4,5-Dimentyl-2-thiazole)-2,5-diphenyl-2-H-tetrazolium bromide, 3-(4,5- Dimethyl-thiazol-2-yl)-diphenyl-tetrazolium bromide) at a concentration of 5 mg/mL by diluting in PBS buffer (Phosphate Buffered Solution), and the cells were incubated for 3 h under the same culture conditions. After the incubation time, the supernatant was removed and the formed formazan crystals were diluted in 20 µL of DMSO.

The absorbance reading of formazan crystals was performed using a spectrophotometer with a microplate reader (Benchmark Plus (Bio-Rad)) at a wavelength of 590 nm. Afterwards, the percentages of cell viability were obtained. Tests were performed in triplicate. The negative control was formed only by means of culture and cells. The percentage of cell viability was determined from Equation 1.

$$\text{Cell viability (\%)}\left[\frac{\text{Treatment Absorbance}}{\text{Negative Control Absorbance}}\right]x100 \quad (1)$$

The values of CC_{50} (concentration (μM) that inhibits 50% the growth of normal cell cells) and IC_{50} (concentration (μM) that inhibits the growth of tumor cells by 50%) were determined by means of the dose-response curve using the statistical program GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA, USA). Furthermore, the selectivity index (SI) was obtained from the ratio between the CC_{50} and IC_{50} values. The experimental pattern used was the drug doxorubicin under the same conditions as the compounds.

In vitro hemolytic activity

The *in vitro* hemolytic activity assays were performed according to the methodology proposed by Cruz Filho et al. (2023) and Jacob et al. (2023) respectively. Erythrocytes were isolated by centrifugation under the following conditions: 2000 rpm, 15 min at 4 °C and the supernatant was removed. Then the erythrocytes were washed four times with phosphate buffered saline (PBS; pH 7.4). Assays were performed using 1.0 mL of erythrocyte suspension (1%) and 0.5 mL of various concentrations of compounds and doxorubicin at different concentrations (1.5 to 500 μM). Controls were solvent only (negative) and Triton X100 (positive). After 60 min of incubation the cells were centrifuged and the absorbance of the supernatant was recorded at 540 nm. The experiments were performed in triplicate. The percentage of hemolysis was determined using Equation 2.

$$\text{Hemolysis (\%)}=\left[\left(\frac{\text{ABS sample-ABS blank}}{\text{ABS Triton X -ABS blank}}\right)\right]*100 \quad (2)$$

Where: ABS sample = Sample absorbance; ABS blank = negative control absorbance; ABS Triton X = positive control absorbance.

In vivo toxicity tests

Animals: Swiss albino mice

Male Swiss albino mice (*Mus musculus*) aged 50 days weighing around 35 ± 0.6 g were obtained from the vivarium. The mice were fed daily with chow (Purina; Nestlé Ltda., Brazil) and water and kept at 22 °C under controlled light conditions in order to simulate a natural light/dark cycle of 12/12 h.

In vivo toxicity: hematological, biochemical and histological analyzes

Acute toxicity experiments were performed according to Oliveira et al. (2015) Albuquerque Nerys et al. (2022) with few modifications. In the acute oral toxicity test, a dose of 2000 mg/kg was administered orally (gavage) in female mice (n = 3 per cage). Compounds 1 - 6 were suspended in 10 mL of Tween 80 (Merck - CAS 9005-65-6) plus 1 mL saline q.s.p. The animals already separated into 7 groups (G): G1: without treatment, and the others treated with emulsions of different compounds G2: 1, G3:2, G4: 3, G5: 4, G6: 5, G7: 6. After administration, the animals were observed to identify possible behavioral

alterations, mortality and signs of toxicity, during the first 2 h, and subsequently for 14 days. During this period, when there was no death, the animals were fed and their boxes were changed.

After 14 days of treatment, the animals were euthanized by cervical dislocation according to the care, research and animal sacrifice protocols suggested by the principles and guidelines adopted by the Colégio Brasileiro de Experimentação Animal (COBEA). Next, blood was collected from the inferior vena cava and aliquots into three tubes, two tubes, one containing anticoagulant (ethylenediaminetetraacetic acid, EDTA) and the other without anticoagulant, which were submitted to hematological and biochemical analysis.

Hematological assays were performed in an automated analyzer (Animal Blood Counter: ABC Vet, Montpellier, France) and light microscopy was used to evaluate the following hematological parameters: number of erythrocytes, hemoglobin, hematocrit, mean corpuscular volume (MCV), corpuscular hemoglobin mean (HCM), mean corpuscular concentration of hemoglobin (CHCM), platelets (PLT), white blood cells (WBC), segmented (SEG), lymphocytes (LYM), monocytes (MON), basophils (BAS), eosinophils (EOS). We also analyzed the coagulogram by means of: activated partial thromboplastin (APTT), prothrombin time (PT) and thrombin time (TT).

Biochemical analysis was performed to evaluate albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin (BIL), gamma glutamyl transferase (GGT), total protein (TP), urea in blood (UR), creatimine (CRE), total cholesterol (TC), high density lipoprotein cholesterol (HDL), non-HDL (triglycerides (TG), low density lipoprotein cholesterol (LDL), very high density cholesterol low (VLDL), were evaluated using specific commercial kits (Diagnostica Stago, France; Labtest Diagnostic, Lagoa Santa, Brazil; COBAS Mira Plus analyzer (Roche Diagnostics Systems, Basel, Switzerland)) according to the manufacturer's instructions.

To evaluate the effects of the compounds at the histological level, the liver, kidney and spleen of animals in the control and treatment groups were analyzed by optical microscopy. Part of the organs were fixed in buffered formalin (10%, v/v) and subsequently dehydrated in a graded series of ethanol (70 – 100%), clarified in xylol and embedded in paraffin. Histological sections (5 µm) were stained with hematoxylin-eosin and mounted with Entellan resin coverslips (Merck). These were observed under a Motic BA200 microscope attached to a Moticam 1000 1.3 MP digital camera (Motic Incorporation Ltd, Causeway Bay, Hong Kong).

Evaluation of antioxidant activity

In vitro antioxidant activity assays

The evaluation of *in vitro* antioxidant activity was performed according to the methodology proposed by Jaishree et al. (2012), Ünver et al. (2014), Sens et al. (2018), Cruz Filho et al. (2023) and Jacob et al. (2023) respectively. In this study, the tests performed were: capture of DPPH, ABTS, hydroxyl radicals (OH), nitric oxide (NO) and reduction of iron ions.

The DPPH radical scavenging assays were performed using a 0.03 mM DPPH solution (3.0 mL/ethanolic solution) with the compounds diluted in DMSO at different concentrations (7.9 – 500 µg/mL) with a volume of 0.5 mL for 30 min. The reaction was carried out in the absence of light at a temperature of 25 °C. After this period, absorbance readings were performed in a spectrophotometer

(Hewlett-Packard, model 8453) at 517 nm. The control absorbance = 0.7 ± 0.05) used in this experiment was the DPPH solution.

The ABTS radical scavenging assays were performed by adding 30 µL (of the compounds diluted in 1% DMSO at different concentrations 7.9 – 500 µg/mL) to 1.0 mL of the ABTS radical solution (absorbance = 0.7 ± 0.05) this system was kept in the dark at 30 °C for 10 min. At the end of the reactions, the absorbances were determined in a spectrophotometer (Hewlett-Packard, model 8453) at 734 nm. The control used in this experiment was the ABTS solution.

The ability to capture the hydroxyl radical was evaluated in a 96-well plate, 25 µL of the compounds diluted in 1% DMSO at different concentrations (7.9 - 500µg/mL), 110 µL of 8 mM iron sulfate heptahydrate solution, 50 µL of 7.18 mM hydrogen peroxide solution, and subsequently 74.2 µL of 3 mM salicylic acid solution. The plate was shaken and incubated for 30 min at a temperature of 37 °C and then read in a Benchmark Plus plate reader (Bio-Rad), at a wavelength of 515 nm.

The ability to capture the nitric oxide radical was determined in a 96-well plate, 50 µL of sodium nitroprusside (20 mM SNP) and 50 µL of compounds diluted in 1% DMSO at different concentrations (7.9 – 500 µg/mL) were added. The mixture was incubated at room temperature, under the effect of light for 60 min. Afterwards, 50 µL of 2% phosphoric acid solution and 50 µL of Griess reagent were added. The microplate was incubated for 10 min in the dark at room temperature and then processed in a Benchmark Plus plate reader (Bio-Rad), at a wavelength of 562 nm.

The evaluation of the antioxidant capacity based on the reduction of iron was performed by two methods, the first using tripyridyltriazine (TPTZ) and the second using potassium ferricyanide. Initially, the working reagent preheated to 37 °C was prepared in a 10:1:1 ratio of 300 mM acetate buffer (pH 3.6), 10 mM tripyridyltriazine (TPTZ) solution in 40 mM hydrochloric acid and 20 mM ferric chloride mM, respectively. For the reaction, 20 µL of each compound (dissolved in DMSO1%) at different concentrations (7.9 – 500 µg/mL) were added to the wells of a 96-well microplate, adding 180 µL of the working reagent. After gently shaking it vertically on the bench and a reaction time of 30 min at room temperature away from light, the absorbance was read at 593 nm in a spectrophotometer with a microplate reader (Benchmark Plus (Bio-Rad)).

In the second method, samples containing 1.25 mL of phosphate buffer (0.2 M PBS; pH 6.6), 2.5 mL of potassium ferricyanide (1%) and 1 mL of different concentrations (7.9 - 500µg/mL) of the compounds were incubated in a water bath. Maria at 50 °C for 30 min. Subsequently, 1.25 mL of trichloroacetic acid (10%) was added and the samples centrifuged at 1500 rpm for 10 min. Then, 1.25 mL of supernatant was transferred to a new tube, with the addition of 1.25 mL of distilled water and 0.25 mL of ferric chloride (0.1%). After the addition of $FeCl_3$, the absorbances were read in the spectrophotometer at 700 nm.

All experiments described were performed in triplicate. The IC_{50} for the concentration of the compounds, capable of capturing or reducing the antioxidant activity by 50%, was calculated by non-linear regression. The standards used in this study were: ascorbic acid (AA) and butylated hydroxytoluene (BHT) under the same conditions as the compounds.

In vivo antioxidant activity assays

The *in vivo* antioxidant assay was performed under the same conditions as the *in vivo* toxicity assays. At the end of the test, the livers of the animals were removed. To obtain liver homogenate,

the procedure described by Celep et al. (2013) and Al-Mansury et al. (2022) with modifications. The method consists of homogenizing an aliquot of 1.0 g of liver in 3.0 mL of 0.1 M potassium phosphate buffer (pH 7.0), using a Dounce-type homogenizer. Then, the homogenate was centrifuged at 1010 xg for 30 min at 4 °C and the supernatant was centrifuged again at 11200 xg for 30 min at 4 °C. The supernatant from the ultracentrifugation consists of the cytosolic fraction of the liver and was stored at -70 °C until analysis for the determination of liver antioxidant enzymes.

Antioxidant liver enzymes (Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx) and Glutathione Reductase (GR) were determined according to the method proposed by Al-Mansury et al. (2022) with modifications.

The determination of catalase activity was carried out using an aliquot of 60 µL of 35% hydrogen peroxide transferred to a 100 mL volumetric flask and completing the volume with water. In an amber flask, 90 mL of this solution were mixed with 5 mL of 1M Tris buffer (pH 8.0 5mM EDTA) and 4 mL of water. In microtubes, 20 µL of the sample were then diluted in 380 µL of 0.1 M phosphate buffer pH 7.0 and 100 µL of this dilution were placed in a quartz cuvette. Then, 2.90 mL of the reaction medium were added to the same cuvette, reading at λ 230 nm, immediately and after 3 min. The final absorbance minus the initial absorbance generates the result expressed as ΔE. The final result was expressed in arbitrary units (ΔE/min/g of protein).

The glutathione reductase assay was prepared with a mixture of 0.0172 g of nicotinamide adenine dinucleotide (NADPH); 0.0654 g of oxidized glutathione (GSSG) and 20 mL of 0.005 M DTPA solution, in 50 mL of 0.1 MpH 7.0 phosphate buffer and 30 mL of distilled water. Then, 150 µL of sample and 2.85 mL of the reaction medium were added to a quartz cuvette, reading at λ 340 nm, immediately and after 1 min. The final absorbance minus the initial absorbance generates the result expressed as ΔE. The final result was expressed in arbitrary units (ΔE/min/g of protein).

The glutathione peroxidase (GPx) assay was performed on a microscale and in triplicate, initially preparing 50 mL of reaction medium consisting of 25 mL of 0.1 M phosphate buffer (pH 7.0), 8.6 mg of NADPH, 10 mL of DTPA 0.005 M, 24 mg of GSH, 3.8 mL of GR 5U and 15 mL of deionized water. In each microplate well, 2.5 µL of liver homogenate sample, 2.5 µL of t-BuOOH and 250 µL of reaction medium were added. For blank reading, the sample was replaced by deionized water. After quick and light agitation of the plate, the reading was performed at time zero and after one minute at 340 nm in a spectrophotometer with an automatic microplate reader. The result of GPx activity was expressed in arbitrary units, as ΔE/min/g of protein.

SOD activity was performed in a 96-well plate in triplicate, with the addition of 30 µL of liver homogenate sample, 99 µL of 50 mM phosphate buffer (pH 7.0), 6 µL of MTT (methylformazan) 1.25 mM and 15 µL of 100 µM pyrogallol. For the standard, 129 µL of buffer, 6 µL of MTT and 15 µL of pyrogallol were added to each well, and for the blank, 144 µL of buffer and 6 µL of MTT were added. The plate was incubated in an oven for 5 min at 37°C and then 150 µL of DMSO were added to all wells, including the blank, to stop the reaction. The reading was performed at 570 nm in a spectrophotometer with an automatic microplate reader. The result was expressed as a unit of SOD per milligram of protein (U of SOD/mg of protein), with 1U being related to the autoxidation of pyrogallol given by the standard.

In vivo antitumor activity

Transplantation of the solid tumor of Sarcoma 180 was performed following the methodology of Stock et al. (1954) modified. The solid tumor, Sarcoma 180, was found seven days after implantation in the right axillary region of male Swiss albino mice (Mus musculus). These were sacrificed 24 h after the end of chemotherapy through cervical dislocation, the tumor being surgically removed and placed in a Petri dish containing 0.9% saline solution and 80mg of garamycin.

Tumors extracted from donor animals were sectioned into fragments of approximately 2 to 3 mm in diameter and placed in a Petri dish containing 0.9% saline solution and 80mg of garamycin. Recipient animals were anesthetized with 10% ketamine hydrochloride and after asepsis at the implant site, they received the tumor fragment through a trocar.

Treatment began 48 hours after tumor transplantation. The animals (n = 5 per cage) were divided into groups (n=6), treated with emulsions of different compounds G2: 1, G3:2, G4:3, G5:4, G6: 5, G7: 6. Compounds 1, 2, 3, 4, 5, 6 were suspended in 10 mL Tween 80 (Merck - CAS 9005-65-6) plus 1 mL saline q.s.p. The animals were treated with concentrations 0, 10, 20 and 40 mg/kg. All substances were administered orally for seven days. On the eighth day, the animals were sacrificed and the tumors removed, dissected, weighed and then the difference between the treated and control groups was calculated to obtain the tumor inhibition index according to Equation 3.

$$I(\%) = \left(\frac{C-T}{C}\right)*100\% \quad (3)$$

I% = % tumor inhibition; C = Mean weight of tumors in animals in the control group; T = Mean tumor weights of animals in the treated group.

IC_{50} values (concentration (μM) that inhibits tumor growth by 50%) were determined by linear regression using the statistical program GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA, USA).

Statistical analysis

All statistical analyzes were performed using Prisma software version 6.0. Cell tests were compared using one-way ANOVA test and post Tukey test. Values were expressed using mean and standard deviation. Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Evaluation of *in silico* parameters of absorption, distribution, metabolism, excretion and toxicity (ADMET)

The evaluation of ADMET parameters is an important step in the search for new drug candidates (Daina et al. 2017). Obtaining these parameters is an *in silico* methodology that is easy to perform, fast and low-cost, which allows the investigation of different pharmacokinetic parameters and, in addition to predicting oral bioavailability, an important parameter in the development of new drugs (Pires et al. 2015, Daina et al. 2017). Table I presents the ADMET profiles for the 2,4-dihydroxy-benzylidene-thiosemicarbazone compounds evaluated in this study compared to commercial antitumor drugs.

The results presented in Table I show the profile of ADMET promoted by the compounds, with the absorption profile being the first to be evaluated.

Table I. ADMET parameters for 2,4-dihydroxy-benzylidene-thiosemicarbazones compounds.

Parameters	PB12	PB13	PB17	PB18	PB19	PB20	Dox	mAMSA	Unit
Absorption									
Water solubility	-3.55	-3.71	-3.51	-3.71	-3.34	-3.35	-2.91	-4.88	Numeric (log mol/L)
Caco2 permeability	-0.16	1.05	1.08	1.01	1.09	0.98	0.45	0.57	Numeric (log Papp in 10^{-6} cm/s)
Intestinal absorption	70.54	84.90	86.36	86.33	87.35	87.70	62.37	94.93	Numeric (%Absorbed)
Skin Permeability	-2.78	-3.05	-3.09	-3.05	-3.18	-3.05	-2.73	-2.73	Numeric (log Kp)
P-glycoprotein substrate	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Categorical (Yes/No)
P-glycoprotein I inhibitor	No	No	No	No	No	No	No	Yes	Categorical (Yes/No)
P-glycoprotein II inhibitor	No	No	No	No	No	No	No	Yes	Categorical (Yes/No)
Distribution									
VDssa	-0.36	-0.09	-0.03	0.07	-0.04	0.09	1.64	-0.98	Numeric (log L/kg)
Fraction unbound	0.14	0.25	0.26	0.23	0.25	0.26	0.21	0.12	Numeric (Fu)
BBB permeability	-0.95	-0.92	-0.77	-0.76	-0.95	-0.68	-1.37	-0.09	Numeric (log BB)
CNS permeability	-2.51	-2.19	-2.23	-2.26	-2.48	-2.55	-4.30	-2.2	Numeric (log PS)
Metabolism									
CYP2D6 substrate	No	No	No	No	No	No	No	No	Categorical (Yes/No)
CYP3A4 substrate	Yes	No	No	No	No	No	No	Yes	Categorical (Yes/No)
CYP1A2 inhibitor	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Categorical (Yes/No)
CYP2C19 inhibitor	No	Yes	Yes	Yes	Yes	No	No	Yes	Categorical (Yes/No)
CYP2C9 inhibitor	No	No	No	Yes	No	No	No	Yes	Categorical (Yes/No)
CYP2D6 inhibitor	No	No	No	No	No	No	No	No	Categorical (Yes/No)
CYP3A4 inhibitor	Yes	No	No	No	No	No	No	Yes	Categorical (Yes/No)
Excretion									
Total clearance	-0.11	-0.26	-0.18	-0.22	-0.09	-0.09	0.98	0.24	Numeric (log mL/min/kg)
Renal OCT2 substrate	No	No	No	No	No	No	No	No	Categorical (Yes/No)
Toxicity									
AMES toxicity	Yes	No	No	No	No	No	No	Yes	Categorical (Yes/No)
Maximum tolerated dose	0.294	0.102	0.06	0.117	0.047	0.075	0.08	0.26	Numeric (log mg/kg/day)
hERG I inhibitor	No	No	No	No	No	No	No	No	Categorical (Yes/No)
hERG II inhibitor	No	Yes	Yes	Yes	No	Yes	Yes	Yes	Categorical (Yes/No)
Oral rat acutee Toxicity	3.607	2.273	2.273	2.32	2.161	2.291	2.40	1.96	Numeric (mol/kg)
Oral rat chronicf Toxicity	2.289	1.622	1.575	1.589	1.615	2.651	3.33	1.4	Numeric (log mg/kg_bw/day)
Hepatotoxicity	No	No	No	No	No	No	Yes	Yes	Categorical (Yes/No)
Skin Sensitization	No	No	No	No	No	No	No	No	Categorical (Yes/No)
T. Pyriformis toxicity	0.638	1.014	0.995	1.044	0.942	1.018	0.28	0.28	Numeric (log µg/L)
Minnow toxicity	2.334	1.605	1.822	1.75	2.042	1.207	4.41	-0.07	Numeric (log mM)

Papp: Apparent permeability; Pgp: P-glycoprotein; BBB: Blood-Brain Barrier; CYP: Cytochrome P450; OCT2: Organic cation transporter 2; hERG: Human Ether-a-go-go Related Gene. Dox: Doxorubicin; mAMSA: Amsacrine.

Absorption is related to the passage of active substances present in drugs into the bloodstream (Daina et al. 2017, Chandrasekaran et al. 2018). Therefore, the first predicted parameter was water solubility (LogS). The degree of solubility of a drug in water is an important parameter for its absorption. In the case of oral administration, the drug has to be soluble or partially soluble in the gastric liquid so that it can be absorbed and transported, via the systemic circulation, to its site of action (Daina et al. 2017). Thus, the compounds presented LogS ranging from -3.71 to -3.34 log mol/L and can be classified as soluble (values in the range -4 < soluble < -2 are considered soluble) (Cruz Filho et al. 2023, Jacob et al. 2023). The drug doxorubicin was classified as soluble and amsacrine moderately soluble.

Regarding permeability in caco-2 cells (extracted from human colon adenocarcinoma). These cells mimic the enterocytes of the human intestine responsible for the absorption of substances due to the presence of microvilli (Pires et al. 2015, Daina et al. 2017). The compounds PB12, PB20, doxorubicin, and amsacrine were classified as having poor absorption. (< 1.10^{-6} cm·s^{-1}), while the other compounds were moderately absorbed (1 and 10.10^{-6} cm·s^{-1}). Regarding absorption in the human small intestine, the compounds and amsacrine showed a moderate to high probability of being absorbed, with values > 70%. Doxorubicin exhibited moderate to low absorption. In addition to the evaluation of intestinal absorption, it was verified that the compounds presented high permeability in the skin (logKp < –2.5).

The last parameter evaluated was the interaction of the compounds with different p-glycoproteins. These are transmembrane glycoproteins of the active efflux system, participating in the multidrug resistance phenomenon (Pires et al. 2015, Yalcin 2020). Acting in the protection of tissues against toxic xenobiotics and endogenous metabolites, through the excretion of these compounds into the intestinal lumen, bile, urine and also promoting their expulsion from the central nervous system (Pires et al. 2015). The compounds were considered as glycoprotein substrates in addition to being non-inhibitors of P-glycoprotein I (it is a drug transporter) and P-glycoprotein II (related to the biliary efflux of phosphatidylcholine). This prediction indicates that these glycoproteins can eject these compounds out of the cell. Different from amsacrine, which can act as an inhibitor and substrate for glycoproteins.

The second profile to be evaluated was the distribution of compounds. The distribution of a drug is primarily through the systemic circulation, and secondarily through the lymphatic circulation. It is from this moment on that the metabolites are able to reach the target organs (Cruz Filho et al. 2023, Jacob et al. 2023). Initially, the volume of distribution (VDss) was evaluated; this parameter indicates whether a drug has a greater tendency to be distributed more easily in plasma (Log VDss) < -0.15 or in tissues (Log VDss) > 0.45 (Pires et al. 2015).

The compounds and the standards showed intermediate values when compared to the classification parameters for plasma and tissue. However, the results showed that the compounds had a greater tendency to be distributed in the blood plasma. The results also showed that the compounds have a low fraction bound to serum proteins, which indicates that only the free fraction is responsible for the pharmacological effects. This low free fraction can help reduce toxicity (Cruz Filho et al. 2023).

The blood-brain barrier (BB) is a structure whose function is to regulate the transport of substances between the blood and the central nervous system, blocking the entry of toxic substances and excess

plasma hormones (Pires et al. 2015, Daina et al. 2017, Chandrasekaran et al. 2018). The compounds were evaluated with those that have the ability to cross (logBB > 0.3) or not (logBB < –1) the blood-brain barrier (Pires et al. 2015). The compounds presented intermediate values when compared to the classification parameters for easily and hardly distributed. However, the results showed that the compounds had a greater tendency to be poorly distributed across the blood-brain barrier.

Regarding the central nervous system, the compounds presented intermediate values LogPS > -2 are considered penetrating while those with LogPS < -3 are considered incapable of penetrating the CNS (Pires et al. 2015). Therefore, the compounds are classified as moderate penetrants.

Metabolism was assessed by predicting the interaction of compounds with cytochrome P450 a broad and diverse superfamily of proteins responsible for oxidizing a large number of a large number of substances to make them more polar and water soluble (Daina et al. 2017, Chandrasekaran et al. 2018). For this study, the effect of the compounds as substrates and inhibitors of different cytochrome P450 isoforms was evaluated.

All compounds were considered as non-substrates of the CYP2D6 isoform. Only 1 was considered as a CYP3A4 substrate. In addition to the evaluation as a substrate, it was verified whether these compounds could act as inhibitors of the different isoforms. For the CYP1A2 isoform, only the compound 5 is considered as non-inhibitor. Regarding the CYP2C19 isoform, compounds 1 and 6 were classified as non-inhibitors. As for the CYP2C9 and CYP2D6 isoforms, all compounds were classified as non-inhibitors and, finally, only the compound 1 was considered as an inhibitor of the CYP3A4 isoform. Doxorubicin is not classified as an inhibitor or substrate of cytochrome isoforms. however, amsacrine can be classified as an inhibitor and as a substrate of some isoforms.

Excretion was initially evaluated by clearance; this parameter is related to the body's ability to eliminate a drug. This measure is given by the sum of the biotransformation capacity of all metabolized organs (Cruz Filho et al. 2023, Jacob et al. 2023). All compounds showed low clearance. Furthermore, they are not substrates of Renal OCT2 (responsible for the renal excretion of xenobiotics).

The last profile studied was toxicity, through different parameters possible damage to different organisms can be predicted. The first parameter to be evaluated was the AMES test, a method for detecting mutagenic chemical agents. Only compound 1 and amsacrine have mutagenic potential. Compounds were evaluated for the maximum tolerated dose (MRTD), that is, the highest dose of a drug or treatment that does not cause unacceptable side effects (Pires et al. 2015). All compounds showed low MRTD values (less than or equal to 0.477 Log(mg/kg/day) (Pires et al. 2015). All compounds were non-hERG I inhibitors and only compound 1 was considered non-hERG II inhibitors. The compounds exhibit moderate to low acute and chronic toxicity. They are non-hepatoxic and do not irritate the skin. In addition, the prediction of toxicity in relation to *T. Pyriformis*, a parasite used as a model in cytotoxicity assays, was evaluated (Pires et al. 2015, Cruz Filho et al. 2023, Jacob et al. 2023).

The compounds were considered of low toxicity against the parasite, presenting values > -0.5 Log µg/L. The toxicity towards Minnow (a fish) was also evaluated and it was verified that all compounds were not toxic towards the fish.

Finally, the compounds obeyed Lipinski and Veber's rules indicating good oral availability. Even though it is a prediction, the compounds with the exception of 1 showed similar ADMET profiles. In addition to being of low toxicity, however, *in vitro* and *in vivo* assays are essential to confirm these results. Only doxorubicin violates the rules.

In vitro and *in vivo* toxicity tests

The evaluation of *in vitro* cytotoxicity is one of the main steps to determine the pharmacological potential of new compounds (Queiroz et al. 2020). In addition to the desired biological activity, these molecules must be considered safe (Sousa et al. 2022). Table SI presents the CC_{50} results, concentration capable of inhibiting 50% of cell growth, of the benzylidene-thiosemicarbazone derivatives against different mammalian cell lines.

The CC_{50} of the compounds varied between 49.27 ± 0.9 and 74.1 ± 5.1 μM for J774 macrophages, between 45.21 ± 2.0 μM for Vero cells and between 62.65 ± 0.1 and 81, 45 ± 1.3 μM for V79 fibroblasts All compounds showed hemolytic activity lower than 10%, being considered non-hemolytic. Macrophages are cells that are part of the innate immune system, the body's first line of defense. The vero cell lineage was obtained from the renal epithelium of the green monkey *Chlorocebus aethiop*s, one of the main organs involved in the excretion of xenobiotics. Fibroblasts are responsible for collagen production and are linked to the functional integrity of the lungs.

The literature presents different results of cytotoxicity promoted by thiosemicarbazones. Queiroz et al. (2020) studying thiosemicarbazone and thiazole derivatives, obtained CC_{50} values for macrophages ranging from 31.80 to 196.38 μM. Sousa et al. (2022) evaluated the cytotoxicity of acridine-thiosemicarbazone derivatives and found CC_{50} values ranging from 9.61 to >70 μM for MCR-5 fibroblasts. Manakkadan et al. (2023) found CC_{50} values for Vero cells all greater than 50 μM. Jacob et al. (2023) evaluating the cytotoxicity of indo-thiosemicarbazone compounds obtained CC_{50} ranging from 7.0 ± 0.6 to >75 μM for macrophages. In addition, it was observed that the compounds were not hemolytic, presenting a percentage of hemolysis lower than 6%.

The CC_{50} values found for the compounds in this study showed that the 2,4-dihydroxy-benzylidene-thiosemicarbazones compounds are potentially safe, due to the low toxicity in cells that interact directly with xenobiotic drugs administered in human beings. In addition, the evaluated compounds were less toxic when compared to doxorubicin (antitumor drug).

The acute oral toxicity experiments were carried out in 7 groups (n = 3/group) in which the first group (G1) was administered water and the others, the emulsion of the compounds at a concentration of 2000 mg/kg (mass ratio of the compound/animal weight) by gavage. Then, the animals were observed for a period of 14 days. After treatment, it was verified that there was no death or behavioral changes in the animals. At the end of the experiments, blood was collected and the animals were euthanized. Then, the organs were removed, weighed, analyzed macroscopically, in addition, no signs of hemorrhage or necrosis were observed.

Table SII presents the results obtained during the experiments, that is, average values of water consumption, food and evolution of the average weight and weight of organs, kidneys, spleen and liver during the 14 days of treatment.

The results presented in Table SII showed that the compounds during treatment were not able to promote significant differences ($p > 0.05$) for the following parameters: water consumption, feed and weight of animals and organs when compared to the control (animals treated with water).

The literature presents different thiosemicarbazones that were also not able to promote toxic effects *in vivo* in mice. Oliveira et al. (2015) performing acute oral toxicity tests on a single dose of 600 mg/kg of thiophene-2-thiosemicarbazone found that the compound did not cause death in mice

over a period of 14 days. Furthermore, it was observed that the animals did not show any signs of toxicity or change in general behavior or other physiological activities.

However, some thiosemicarbazone compounds linked to metals may have effects at the CNS level and at the level of the liver and kidney. El-Sisi et al. (2021) evaluating thiosemicarbazones complexed with Cd, found that after oral administration there was a significant increase in the levels of aspartate aminotransferase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP), creatinine, urea and uric acid. Cd intoxication significantly increased in liver and kidney malondialdehyde (MDA) and decreased levels of reduced glutathione (GSH) and catalase (CAT) in liver and kidney tissues

Lira et al. (2022) evaluating the acute toxicity promoted by metal complexes of 2-acetylpyridine-N-(4)-orthochlorophenyl thiosemicarbazone found that these compounds promoted behavioral alterations of stimulation of the central nervous system (CNS) at a dose of 300 mg/kg. One hour after administration, a dose of 2000 mg/kg caused depressive signs. All changes disappeared after 24 h, with no deaths, suggesting an estimated LD_{50} of 5000 mg/kg. Therefore, the tested thiosemicarbazone compounds do not show toxicity when administered orally at the tested dose (2000 mg/kg).

Histological analyzes of the organs were carried out with the aim of evaluating the morphological changes in different organs caused by the administration of drug candidate compounds. For this, only the liver, spleen and kidney were analyzed.

The liver is an organ that is very sensitive to injuries due to its important role in xenobiotic metabolism, since there are many synthetic drugs and natural substances that can promote damage to this organ. The kidneys are also sensitive to the toxicity of different compounds, damage to their structure can compromise the body's homeostasis. The last organ to be evaluated was the spleen responsible for the production of immune cells. Damage to this organ compromises the body's functioning by decreasing defenses against different pathogens (Kanso et al. 2021).

The PB compounds evaluated in our study were not able to promote changes in macroscopic and histological analyzes (Figure S18). Furthermore, it was observed that the compounds were not able to promote significant hematological and biochemical changes, confirming the low toxicity (Table SIII).

Antioxidant activity *in vitro* and *in vivo*

Thiosemicarbazones are able to promote antioxidant activities *in vitro* and *in vivo*. This activity is directly related to its chemical structure. These compounds have the ability to donate pairs of electrons (reducing agent) (Bingul et al. 2019). This characteristic is due to the presence of nitrogen atoms, mainly in the form of Schiff's base, and sulfur as carbathioamide (Siddiqui et al. 2019, Yang et al. 2020).

Table SIV shows the results of *in vitro* antioxidant activity promoted by the compounds 2,4-dihydroxy-benzylidene-thiosemicarbazones in different assays of radical capture DPPH, ABTS, hydroxyl (OH), nitric oxide (NO) and reduction of iron ions by the TPTZ and potassium ferricyanide methods respectively.

The results presented in Table SIV showed that antioxidant activity is directly correlated with the chemical structure. The compounds showed EC_{50} ranging from 598.90 μM to values greater than 1500 μM for DPPH. EC_{50} ranging from 84.25 to > 1500 μM (ABTS). Regarding the hydroxyl radical (OH) and nitric oxide (NO) capture assays, values ranging from 1245 to > 1500 μM and 1479 to > 1500 μM were

obtained. Finally, for the iron ion reduction assays, EC_{50} values greater than > 1500 μM were obtained for the TPTZ) and potassium ferrocyanide assays, respectively.

Through these results it was possible to verify that the compounds presented better results for the DPPH and ABTS tests when compared to the other tests. The DPPH radical inhibition method is based on the transfer of electrons from an antioxidant compound to an oxidant. In this reaction, the color changes from purple to yellow, because when the DPPH radical reacts with an antioxidant compound that can donate hydrogen, it is reduced and discoloration occurs. The ABTS radical is generated through a chemical, electrochemical or enzymatic reaction. With this method, the antioxidant activity of both hydrophilic and lipophilic compounds can be determined (Yousef & El-Reash 2020). The reaction is accompanied by a color change from green to colorless.

Therefore, we highlight compounds PB17 that presented EC_{50} for DPPH radical capture, hydroxyl (OH) and nitric oxide (NO) tests respectively. In addition to promoting greater activity for the ABTS test. The presence of the methyl group promoted an increase in antioxidant activity when compared to the other substituents, this fact may be related to the solubility of the compound. Bingul et al. (2019) evaluating the in vitro antioxidant activity of different carbazole-based bis-thiosemicarbazone compounds found that compound 4c (bi-substituted with a methyl group) showed greater activity when compared to the other compounds in the DPPH and ABTS assays, respectively. In view of the results, it can be concluded that the compounds can promote moderate to low antioxidant activity *in vitro*

In addition to the *in vitro* assays, *in vivo* assays were performed to evaluate the antioxidant activity. The results of antioxidant liver enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR)), which are responsible for reducing the damage caused by oxidative stress, are shown in Table SV.

The results presented in Table SV showed that the compounds 1, 3 and 5 were able to promote a significant increase in the concentration of liver antioxidant enzymes ($p < 0.05$). This increase in the concentration of enzymes suggests a decrease in reactive oxygen species, promoting a decrease in damage to these organs.

Nguyen et al. (2013) evaluating substituted benzaldehyde thiosemicarbazones and N-(tetra-O-acetyl-β-D-galactopyranosyl) thiosemicarbazide found that the compounds were able to promote an increase in the enzymes superoxide dismutase, glutathione peroxidase and catalase respectively. Thanh & Hoai (2012) evaluating a series of substituted benzaldehydes (2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl) thiosemicarbazones found that the compounds were capable of promoting an increase in the concentration of liver enzymes.

These results showed that the compounds are promising antioxidant agents and that they can contribute to the reduction of cell damage caused by excess free radicals.

In vitro and *in vivo* antitumor activity

Thiosemicarbazones are known as potential antitumor agents, being able to inhibit the growth of different types of tumor cell lines (Bai et al. 2022, Jacob et al. 2023). Table SVI presents the results of in vitro antitumor activity promoted by compounds compared to doxorubicin against different tumor cell lines (IC_{50} values and SI selectivity index) respectively.

The IC_{50} results presented in Table SVI for the compounds against different tumor cell lines were classified according to the arbitrary scale: compounds that presented IC_{50}< 50μM were considered

active, moderately active 50 μM < IC_{50} < 100 μM and inactive the compounds that presented IC_{50} > 100 μM. all compounds were active for all strains evaluated except for the HepG2 strain, which showed moderate activity. This hepatoma lineage in many cases is used to mimic hepatocytes. In addition, it was observed that all compounds had higher IC_{50} when compared to doxorubicin, being less toxic.

Among the strains, we highlight a greater effectiveness of all compounds for the MCF-7 strain, showing IC_{50} values ranging from 1.12 to 1.92 μM and the DU145 strain, showing values ranging from 1.83 to 2.33 μM. These values were close to those obtained for the doxorubicin standard. The literature presents other thiosemicarbazone compounds with promising in vitro antitumor activity for MCF-7 and DU145 strains. Jacob et al. (2023) evaluating indole-thiosemicarbazone compounds obtained IC_{50} values ranging from 0.74 to 1.43 μM for the MCF-7 strain and for DU145 values ranging from 0.94 to 1.72 μM. Chaudhary et al. (2022) evaluating the potential of N (4)-ring incorporated-5-methoxyisatin thiosemicarbazones obtained IC_{50} values ranging from 2.93 to 7.41 μM against the MCF-7 strain.

Due to the promising results obtained for *in vitro* antitumor activity, all compounds were submitted to *in vivo* antitumor activity assays against the inhibition of sarcoma 180 (a solid tumor). The results of *in vivo* antitumor activity promoted by the compounds are presented in Table SVII.

The results shown in Table SVII showed that the compounds were able to promote inhibition of sarcoma size 180 at different non-toxic concentrations for the animals. In order of effectiveness, that is, from the lowest IC_{50} values (most effective) to the highest (least effective): 2 > 3 > 1 > 4 > > 6. Therefore, these results showed that the compounds are promising antitumor agents.

CONCLUSIONS

This work proposed a preliminary study for the evaluation of 2,4-dihydroxy-benzylidene-thiosemicarbazones compounds as antitumor agents. The *in silico* study showed that these compounds can have good oral availability, an important property when administering different drugs. The *in vitro* and *in vivo* toxicity tests showed that these molecules have low toxicity and can be used safely. Antioxidant activity assays showed that the compounds are able to capture free radicals, preventing cellular damage in different structures. Finally, in the *in vitro* and *in vivo* antitumor activity, it was verified that the compounds were able to inhibit different tumor cell lines, especially MCF-7 (human breast cancer) and DU145 (human prostate cancer) lines. Furthermore, it was observed that the compounds were able to reduce sarcoma 180 using concentrations lower than 50 mg/kg. The results obtained for this study showed that the compounds 2,4-dihydroxy-benzylidene-thiosemicarbazones are promising candidates for antitumor drugs and future studies of increased solubility with the evaluated series need to be carried out in order to potentiate the activity of these compounds.

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SUPPLEMENTARY MATERIAL

Figure S1-S18.
Table SI-SVII.

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