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BIOMEDICAL SCIENCES

In silico prediction of ADMET parameters and *in vitro* evaluation of antioxidant and cytotoxic activities promoted by indole-thiosemicarbazone compounds

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Abstract: Cancer is a complex and multifactorial disease characterized by uncontrolled cell growth and is one of the main causes of death in the world. This work aimed to evaluate a small series of 10 different indole-thiosemicarbazone compounds as potential antitumor agents. This is a pioneering study. For this, the antioxidant and cytotoxic capacity against normal and tumor cells was evaluated. The results showed that the compounds were able to promote moderate to low antioxidant activity for the ABTS radical scavenging assay. ADMET *in silico* assays showed that the compounds exhibited good oral bioavailability. As for toxicity, they were able to promote low cytotoxicity against normal cells, in addition to not being hemolytic. The compounds showed promising *in vitro* antitumor activity against the T47D, MCF-7, Jurkat and DU-145 strains, not being able to inhibit the growth of the Hepg2 strain. Through this *in vitro* study, it can be concluded that the compounds are potential candidates for antitumor agents.

Key words: thiosemicarbazones, antitumor, interaction, antioxidant

INTRODUCTION

The word cancer is a generic term for a group of diseases that can affect any part of the body (Bakherad et al. 2019, He et al. 2021, Jacob et al. 2023). It is defined by the abnormal and uncontrolled growth of cells that can spread to organs other than the original one, a process known as metastasis (Bakherad et al. 2019, He et al. 2021, Hong et al. 2023).

Cancer has been one of the leading causes of death worldwide, accounting for over 10 million deaths in 2020 (Ferlay et al. 2023). According to data from the World Health Organization (WHO), the most common types of cancer in terms of new cases in 2020 were: breast (2.26 million), lung (2.21 million), colon and rectum (1.93 million), prostate (1.41 million). million), skin (non-melanoma) (1.20 million) and stomach (1.09 million cases). On the other hand, those that caused the highest number of deaths were lung cancer (1.80 million), colon and rectum (916 thousand), liver (830 thousand), stomach (769 thousand) and breast (685 thousand) (WHO 2023). The predominance in the number of cases and deaths varies by country. It is known that one in five people in the world develop cancer during their lifetime. Thus, cancer prevention is one of the greatest public health challenges of the 21st century (Iarc 2023).

The main risk factors related to the development of cancer are: sedentary lifestyle,

smoking, poor diet, body weight, sexual habits, occupational factors, alcohol consumption, exposure to solar radiation and contamination with metals and synthetic substances (Blackadar 2016, Poudineh et al. 2023). Although advances have been achieved in treatment, side effects may occur during chemotherapy (Almeida et al. 2020). Therefore, it is necessary to search for new compounds that can be used as an alternative treatment, with a reduction in side effects.

Different synthetic and natural compounds have been promising antioxidant and antitumor agents (El-Naggar et al. 2022, Suleiman et al. 2022, Suleiman & Helal 2022, Abou El-Enain et al. 2023, Hendy et al. 2023, Jacob et al. 2023). Among the synthetic and natural compounds with potential for the treatment of different forms of cancer, we will highlight here the indole-thiosemicarbazone compounds. Thiosemicarbazones are compounds with several pharmacological applications, among which antimicrobial (Alam et al. 2023), antiparasitic (Rabelo et al. 2023) and antiviral (Arslan et al. 2021) action stand out. The biological activity of these compounds is linked to their chemical constitution, because as Schiff bases, they have N and S atoms that act as organic ligands (Manakkadan et al. 2023). One of the best-used strategies for obtaining bioactive compounds is the complexation of thiosemicarbazones with other pharmacophoric groups, creating heterocyclic compounds that increase biological activity (Balakrishnan et al. 2019, Yakan et al. 2023).

The indole nucleus is a group widely used in medicinal chemistry. As it is a constituent part of the amino acid tryptophan, it is able to bind to molecular targets and promote various biological activities (Balakrishnan et al. 2019, Yıldız et al. 2022, Goel et al. 2023). These compounds are described in the literature as having antitumor action against different cancer cell lines, acting through different mechanisms of action (Bakherad et al. 2019, He et al. 2021, Jacob et al. 2023).

In this context, this work aimed to evaluate the antioxidant, cytotoxic and antiproliferative activities in different tumor cell lines, predicting *in silico* the pharmacokinetic properties of indole-thiosemicarbazone compounds

MATERIALS AND METHODS

Reagents

The reagents used for the synthesis and analysis of thiosemicarbazones were: hydrazine solution (CAS:302-01-2), methylene chloride (CAS:75-09-2), 7-Bromo-5-methylindole-3-carboxaldehyde (CAS: 16077-60-4), 5-Bromo-7-methylindole-3-carboxaldehyde (CAS: 16076-86-1), phenyl isothiocyanate (CAS: 103-72-0), 4-Methoxyphenyl isothiocyanate (CAS: 2284) -20-0), 4-Methylphenyl isothiocyanate (CAS: 622-59-3), ethyl alcohol (CAS: 64-17-5), glacial acetic acid (CAS:1186-52-3), ascorbic acid (CAS: CAS: 50-81-7), dimethylsulfoxide (CAS:67-68-5), 2,2-diphenyl-1-picrylhydrazyl (DPPH; CAS:1898-66-4), ABTS (CAS:28752-68-3), ascorbic acid (Cewin), butylated hydroxytoluene (CAS:128-37-0), MTT (CAS:298-93-1) and RPMI 1640 Medium (CBasalab), Butylated Hydroxytoluene (CAS: 128-37-0), Asulacrine (PubChem CID 107924), Amsacrine hydrochloride (CAS: 54301-15-4), Doxirubicin (CAS: 25316-40-9). All reagents provided by Sigma/Merck. The solvents were ethyl alcohol, dichloromethane, dimethyl sulfoxide (DMSO), in addition to glacial acetic acid, provided by Dinâmica.

Indole-thiosemicarbazone compounds

The synthesis was carried out at the Chemistry and Therapeutic Innovation Laboratory of the Federal University of Pernambuco (UFPE), Recife, Pernambuco, Brazil and published by Silva et al. (2020). Obtaining the indole-thiosemicarbazone compounds (PR1 – PR10)

was carried out in two steps (Figure 1). Initially (a), the thiosemicarbazides were obtained from the linker hydrazinyl (hydrazine) with the unsubstituted and substituted isothiocyanates. Then (b), the thiosemicarbazones react with the substituted 3-indole-carboxaldehydes in the presence of acetic acid as a catalyst, originating the substituted Thiosecarbazones (PR1 -PR10).

In silico evaluation of absorption, distribution, metabolism, excretion and toxicity parameters (ADMET)

The *in silico* profile of absorption, distribution, metabolism, excretion and toxicity (ADMET) and bioavailability of the compounds evaluated in this study was obtained through the free pkCSM platforms (http://biosig.unimelb.edu.au/pkcsm/prediction) and SwissADME (http://www.swissadme.ch/) (Pires et al. 2015, Daina et al. 2017).

In vitro antioxidant activity promoted by indole-thiosemicarbazone compounds

Radical scavenging DPPH• (2,2 diphenyl-1-picrylhydrazyl)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) stable radical capture assay was performed according to the methodology proposed by Jacob et al. (2023) and Santos et al. (2023), with few modifications. Initially, the compounds were dissolved in 1% DMSO in concentrations ranging from 3.9 to 1000 µg/mL. To carry out the tests, 0.32 mL of the compounds and 2.0 mL of the DPPH solution in 1 mM methanol were used. Assays were incubated at 25 °C for 30 min in the absence of light. At the end of the reactions, the absorbances were determined in a spectrophotometer (Hewlett-Packard, model 8453) at a wavelength of 517 nm. The experimental control consisted of the DPPH

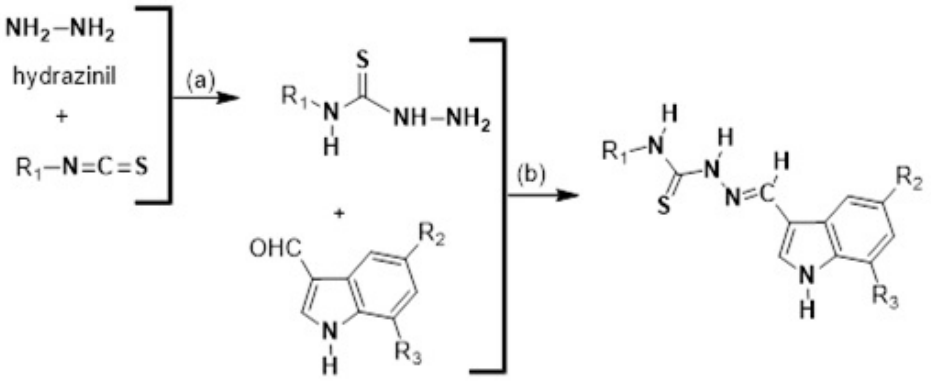

Figure 1. Reagents and Conditions: (a) hydrazine, substituted isothiocyanate, chloroform, temperature 30 ± 0.5ºC; (b) thiosemicarbazide, substituted 3-indole-carboxaldehyde, absolute ethanol, acetic acid as catalyst, temperature: 75 ± 0.5ºC.

Cpd.	R^1	R^2	R^3
PR1	phenyl	CH_3	Br
PR2	phenyl	Br	CH_3
PR3	*p*-methoxy-phenyl	CH_3	Br
PR4	*p*-methoxy-phenyl	Br	CH_3
PR5	*p*-methyl-phenyl	Br	CH_3
PR6	*p*-methyl-phenyl	CH_3	Br
PR7	Hydrogen	Br	CH_3
PR8	Hydrogen	CH_3	Br
PR9	*p*-ethyl-phenyl	Br	CH_3
PR10	*p*-ethyl-phenyl	CH_3	Br

solution without the addition of compounds, and the equipment blank was ethanol. In addition to the compounds, ascorbic acid and butylated hydroxytoluene (BHT) were used as experimental standards. The experiments were performed in triplicate. Antioxidant activity was calculated by Equation 1.

$$\text{Antioxidant activity (\%)} = \left(\frac{ABS_{control} - ABS_{sample}}{ABS_{control}}\right) * 100 \quad (1)$$

Where: ABS control = control absorbance; ABS sample = absorbance of the sample containing the compound after the assay.

The inhibition coefficient (EC_{50}), ie, the minimum compound concentration required to reduce the initial radical concentration by 50%, was determined by nonlinear regression fitting of antioxidant capacity versus compound concentrations. EC_{50} values were obtained in µg/mL and in µM (according to the molecular weight of the compound).

ABTS radical capture [2,2›-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)]

The ABTS+ (2,2›-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay was performed according to the methodology described by Jacob et al. (2023) and Santos et al. (2023) with few modifications. Compounds were dissolved under the same conditions as in the DPPH assay. The ABTS radical solution was prepared by reacting between ABTS (5 mL;7mM) and potassium persulfate (88 µL; 2.45 mM), followed by incubation at room temperature in the absence of light for 16 h. Subsequently, the solution was diluted in 80% ethanol, until reaching an absorbance of 0.70 ± 0.05 at 734 nm. ABTS solution (2.7 mL) was added to 0.3 mL of different concentrations of compounds. The system was incubated at 25 °C for 5 min, in the dark. At the end of the reaction, the absorbances were determined in a spectrophotometer (Hewlett-Packard, model 8453) at a wavelength of 734 nm. As a control of the experiment, the ABTS solution without the compounds was used, and the equipment blank was 80% ethanol. In addition to the compounds, ascorbic acid and butylated hydroxytoluene (BHT) were used as experimental standards. The experiments were performed in triplicate. Antioxidant activity was determined by Equation 1. EC_{50} was determined by non-linear regression fitting of antioxidant capacity versus compound concentrations. EC_{50} values were obtained in µg/mL and in µM (according to the mass of the compound).

Cytotoxicity assays on normal and mammalian tumor cells

The cytotoxicity of the compounds against normal and tumor cells of mammals was performed using the MTT assay (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) as described by Jacob et al. (2023) with few modifications. The normal cell lines used were: J774 macrophages, Vero cells and fibroblasts (V79). The tumor cell lines were: T47D and MCF-7 (breast cancer), Jurkat (leukemia/lymphoma), DU-145 (prostate) and Hepg2 (hepatoma). The cells were cultivated in RPMI 1640 medium supplemented with 10% FBS and penicillin: streptomycin solution (1000 IU/mL:1000 µg/mL), being kept in an oven at 37 °C and 5% CO_2 atmosphere.

The cells were cultivated in RPMI 1640 medium supplemented with 10% FBS and penicillin: streptomycin solution (1000 IU/mL:1000 µg/mL), being kept in an oven at 37 °C and 5% CO_2 atmosphere.

To carry out the tests, dilutions of the compounds were initially performed in 1% DMSO at concentrations ranging from 3.15 to 100 µM. Assays were performed in 96-well plates, containing $1x10^4$ cells/well. They were then incubated for 48 h in a humidified chamber with

5% CO_2. After treatment, 20 µL of MTT solution was added to each well, and the plates were incubated for 3 h. The formed formazan crystals were dissolved in DMSO, and the absorbance was determined at 570 nm in a varioskan plate reader. Cell viability was calculated according to Equation 2.

$$\text{Cell viability (\%)} = \left(\frac{VC}{TC}\right) * 100\% \quad (2)$$

Where: VC is the number of cells at different concentrations, TC is the concentration of cells in the control, which represents 100% viability.

The standards used were: m-AMSA (amsacrine), Asul (asulacrine) and Doxo (doxorubicin), used under the same conditions as the evaluated compounds. The values of CC_{50} (concentration that inhibits 50% of the growth of normal cells) and IC_{50} (concentration that inhibits 50% of the growth of tumor cells) were calculated by means of non-linear regression, using the GraphPad Prism 7 software. In addition, the selectivity index (SI) was determined for the ratio between the CC_{50} values for normal cells and IC_{50} antitumor cells for the compounds, amsacrine, asulacrine and doxorubicin these values were determined using Equation 3. All assays were performed in triplicate.

$$SI = \frac{CC50 \text{ Normal cells}}{IC50 \text{ Tumor cells}} \quad (3)$$

In vitro hemolytic activity

Hemolytic activity was performed according to Jacob et al. (2023). For the *in vitro* hemolytic activity assays, 5mL of blood from healthy mice were used. Erythrocytes were isolated by centrifugation (1500 rpm, 10 min at 4 °C) and washed three times with phosphate buffered saline (PBS; pH 7.4). In tubes were added a suspension (1.1 mL) of erythrocytes (1%) and 0.4 mL of the compounds and standards amsacrine, asulacrine and doxorubicin in concentrations that varied from 7.8 to 100 µM. Controls were erythrocytes only (negative) and Triton X100 (positive). After 60 min of incubation, the cells were centrifuged and the absorbance of the supernatant was determined in a plate reader at 540 nm. Hemolytic activity was calculated using Equation 4. This study was approved by the Animal Ethics Committee of the Instituto Aggeu Magalhães/Fundação Oswaldo Cruz, protocol number 164/2020.

$$\text{Hemolysis (\%)} = \left[\left(\frac{\text{ABS sample-ABS blank}}{\text{ABS Triton X -ABS blank}}\right)\right] * 100 \quad (4)$$

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

RESULTS AND DISCUSSION

In silico result of the parameters of absorption, distribution, metabolism, excretion and toxicity (ADMET)

The *in silico* prediction of pharmacokinetic and bioavailability parameters were obtained through the SwissADME and pkCSM platforms and the prediction results are presented in Table I. This prediction is fundamental for the selection and development of new drugs, as it reduces research time and effort, and eliminates costs. In addition to being one of the steps to determine whether a molecule is pharmacologically promising (Padole et al. 2022, Trivedi et al. 2022).

The parameters analyzed for absorption showed that the compounds are moderately soluble in water (-6 < LogS < -4). Water solubility is an essential parameter for drug absorption, and ideally it should maintain a balance between lipophilicity and hydrophilicity (Pires et al. 2015). As for the ability to cross cell membranes, represented by permeability in Caco2 cells (A

Table I. ADMET parameters.

	PR1	PR2	PR3	PR4	PR5	PR6	PR7	PR8	PR9	PR10	Unit
Absorption											
Water solubility	-4.58	-4.58	-4.87	-4.87	-4.81	-4.81	-4.00	-4.00	-4.91	-4.91	Numeric (log mol/L)
Caco2 permeability	1.00	1.00	0.99	0.99	0.99	0.99	1.00	1.00	0.97	0.97	Numeric (log Papp in 10^{-6} cm/s)
Intestinal absorption	85.9	85.9	86.9	86.9	85.9	85.9	88.8	88.8	85.9	85.9	Numeric (%Absorbed)
Skin Permeability	-2.84	-2.84	-2.85	-2.85	-2.85	-2.85	-2.87	-2.87	-2.84	-2.84	Numeric (log Kp)
P-glycoprotein substrate	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Categorical (Yes/No)
P-glycoprotein I inhibitor	No	No	Yes	Yes	No	No	No	No	Yes	Yes	Categorical (Yes/No)
P-glycoprotein II inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Categorical (Yes/No)
Distribution											
VDssa	0.23	0.23	0.31	0.31	0.30	0.30	-0.11	-0.11	0.45	0.45	Numeric (log L/kg)
Fraction unbound	0.08	0.08	0.08	0.08	0.08	0.08	0.34	0.34	0.05	0.05	Numeric (Fu)
BBB permeability	0.19	0.19	0.02	0.02	0.16	0.16	-0.15	-0.15	0.13	0.13	Numeric (log BB)
CNS permeability	-1.56	-1.56	-1.74	-1.74	-1.49	-1.49	-3.2	-3.2	-1.53	-1.53	Numeric (log PS)
Metabolism											
CYP2D6 substrate	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Categorical (Yes/No)
CYP3A4 substrate	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Categorical (Yes/No)
CYP1A2 inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Categorical (Yes/No)
CYP2C19 inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Categorical (Yes/No)
CYP2C9 inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Categorical (Yes/No)
CYP2D6 inhibitor	No	No	No	No	No	No	No	No	No	No	Categorical (Yes/No)
CYP3A4 inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Categorical (Yes/No)
Excretion											
Total clearance	-0.28	-0.20	-0.25	-0.17	-0.26	-0.34	-0.13	-0.21	-0.29	-0.37	Numeric (log mL/min/kg)
Renal OCT2 substrate	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes	Categorical (Yes/No)
Toxicity											
AMES toxicity	Yes	Yes	No	No	Yes	Yes	Yes	Yes	No	No	Categorical (Yes/No)
Maximum tolerated dose	0.21	0.21	0.16	0.16	0.21	0.21	0.61	0.61	0.3	0.3	Numeric (log mg/kg/day)
hERG I inhibitor	No	No	No	No	No	No	No	No	No	No	Categorical (Yes/No)
hERG II inhibitor	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Categorical (Yes/No)
Oral rat acutee Toxicity	2.56	2.56	2.57	2.57	2.60	2.60	3.04	3.04	2.59	2.59	Numeric (mol/kg)
Oral rat chronicf Toxicity	1.59	1.59	1.54	1.54	1.47	1.47	1.40	1.40	1.49	1.49	Numeric (log mg/kg_bw/day)
Hepatotoxicity	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Categorical (Yes/No)
Skin Sensitization	No	No	No	No	No	No	No	No	No	No	Categorical (Yes/No)
T. Pyriformis toxicity	1.31	1.31	.32	1.32	1.36	1.36	1.03	1.03	1.33	1.33	Numeric (log µg/L)
Minnow toxicity	-0.08	-0.08	0.17	0.17	0.14	0.14	1.53	1.53	0.01	0.01	Numeric (log mM)
Oral bioavailability											
Lipinski's rules	0	0	0	0	0	0	0	0	0	0	Violation (Numeric)
Veber's rules	0	0	0	0	0	0	0	0	0	0	Violation (Numeric)

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.

human colorectal adenocarcinoma cell line) and expressed in log Papp in 10^{-6} cm/s, the compounds ranged from low to moderate permeability. Compounds PR3, PR4, PR5, PR6, PR9 and PR10 were classified as having low permeability (log Papp in 10^{-6} cm·s^{-1} < 1.), while compounds PR1, PR2, PR7 and PR8 were considered as moderately permeable. permeable (1 < log Papp in 10^{-6} cm·s^{-1}< 10). Intestinal absorption was around 86% and the compounds showed good skin permeability (Log Kp >-2.5). Regarding p-glycoproteins, it was observed that all molecules can be substrates of these proteins. The PR1 and PR2 molecules were the only ones considered as non-inhibitors of the p-glycoprotein I action. All other compounds were classified as inhibitors of both p-glycoprotein I and p-glycoprotein II. P-glycoproteins are important efflux pumps that act to expel xenbiotic molecules from intestinal cells. The ability to inhibit the action of these enzymes is related to increased pharmacokinetic properties (Husain et al. 2022).

The distribution profiles were evaluated according to the volume of distribution (VDss), permeability to the blood-brain barrier (LogBB) and the ability to penetrate the Central Nervous System (CNS, represented by Log PS). The VDss determines whether the compound distributes more through the plasma (LogVDss < -0.15), or the tissues (LogVDss > 0.45). Compounds PR1, PR2, PR3, PR4, PR5, PR6, 7 and PR9 and PR10 are well distributed by tissues, while PR7 and PR8 have prevalence by plasmatic distribution. Regarding brain distribution, indicated by the ability to cross the blood-brain barrier, compounds PR7 and PR8 are not able to cross the barrier (Log BB <-1).

The other compounds have a moderate ability to cross the blood-brain barrier (-1<Log BB < 0.3), favoring the distribution of the compound throughout the brain region. Regarding CNS penetration, all compounds were evaluated as capable of penetrating the CNS (LogPS > -2), with the exception of PR7 and PR8, which were evaluated as non-penetrating (LogPS < -3) (Pires et al. 2015).

The metabolism of compounds was evaluated in relation to the performance of enzymes of the cytochrome P450 family (Pires et al. 2015, Husain et al. 2022). These enzymes are involved in drug oxidation reactions, which corresponds to phase I metabolism. The action of these enzymes increases water solubility, favoring excretion by the kidneys, and may cause drug inactivity. The analysis was carried out by determining the role of molecules as substrates or inhibitors of different proteins of the CYP family (Pires et al. 2015).

The compounds were classified as substrates of CYP2D6 and CYP3A4 enzymes, with the exception of compounds PR7 and PR8, which were not considered substrates for any of the enzymes. The ability of molecules to inhibit the action of P450 enzymes was also evaluated. No molecule was able to inhibit CYP2D2, while all can act as CYP1A2 inhibitors. For CYP2C19, CYP2C9 and CYP3A1 enzymes, only PR7 and PR8 were not classified as inhibitors. This ability may be related to the longer drug circulation time in the bloodstream, since metabolization by CYP enzymes is an important step for the inactivation and excretion of xenobiotics (Husain et al. 2022, Jacob et al. 2023).

The excretion parameters showed that the molecules have a low total clearance value, ranging from -0.377 to -0.134 (log mL/min/kg). However, PR1, PR2, PR5, PR6, PR9 and PR10 were classified as organic cation transporter2 (OCT2) substrates. OCT2 is an important cation transporter present in the basolateral membrane of the proximal tubules of the kidneys, and plays an important role in the clearance of drugs and endogenous compounds (Pires et al. 2015, Husain et al. 2022, Jacob et al. 2023).

Toxicity prediction showed that all molecules were considered hepatotoxic, but had a recommended maximum tolerated dose (MRTD) considered low > 0.477 log mg/kg/day. For the AMES test, only PR3, PR4, PR9 and PR10 compounds were not considered as potential mutagens. No compound was found to be hERG I inhibitors, while only PR7 and PR8 compounds were classified as hERG II inhibitors. Acute oral toxicity in rats ranges from 2.561 to 3.041 mol/kg, and chronic toxicity from 1.401 to 1.591 log mg/kg_bw/day. The toxicity shown against *T. Pyriformis* ranged from 1.038 to 1.583 log mM, being considered low (>-0.3 logs mM). No compound promotes skin irritability (Pires et al. 2015). In addition, the compounds showed good oral availability obeying the rules of Lipinsk and Veber. Finally, it is worth mentioning that the study presented here is in silico and the criterion for exclusion or inclusion of a compound will depend on different steps *in vitro* and *in vivo*.

In vitro antioxidant activity

Antioxidant activity assays are an important step for the evaluation of compounds with antitumor activity (Azeem et al. 2023). This is because antioxidants act to prevent the oxidation of macromolecules, promoting the removal or inactivation of free radicals formed during the initiation or propagation of the reaction, through the donation of hydrogen atoms to these molecules, interrupting the chain reaction (Bourais et al. 2022). In addition, they reduce cell growth and survival, an important factor in inducing cell death (Bourais et al. 2022, Azeem et al. 2023).

The literature describes different compounds with antioxidant activity. Here we will highlight the indole-thiosemicarbazonic compounds, which promote promising antioxidant activity (Bakherad et al. 2019, Jacob et al. 2023). The mechanism of antioxidant activity for these compounds is not yet fully established. However, it is known that the decrease or increase in activity will be related to its chemical structure, that is, difference and/or position of substituents (Bakherad et al. 2019). Figure 2 shows the curves of antioxidant activity versus concentration for each of the evaluated compounds.

The antioxidant activity curves (Figure 2) showed the same profile for the DPPH and ABTS assays, that is, they showed an increase in activity with increasing concentration. A similar profile was obtained by Bakherad et al. (2019) and Jacob et al. (2023) evaluating the in vitro antioxidant activity of different indole-thiosemicarbazonic compounds through DPPH and/or ABTS assays. From the curves, it was possible to determine the percentage values of antioxidant activity at the highest concentration of the tests (1000 µM) and the EC_{50} (minimum compound concentration necessary to reduce the initial concentration of the radical by 50%). Table II presents the results of *in vitro* antioxidant activity promoted by the indole-thiosemicarbazonic compounds against the DPPH and ABTS assays, respectively.

The results shown in Table II show that the compounds showed EC_{50} values for the DPPH assay ranging from 117.9 to 2000 µM. Regarding the ABTS assay, they showed higher EC_{50} results ranging from 23.36 to 240.1 µM. This difference may be associated with the method, the ABTS radical capture assay promotes good results for polar or non-polar compounds. The DPPH assay, on the other hand, presents better results for compounds of a polar nature (Jacob et al. 2023, Santos et al. 2023). Furthermore, the compounds were classified as sparingly soluble in water as shown in the *in silico* study, this profile is indicative of a higher affinity for ABTS radical scavenging assays.

Due to the better results for the ABTS assay, the compounds were classified using an arbitrary

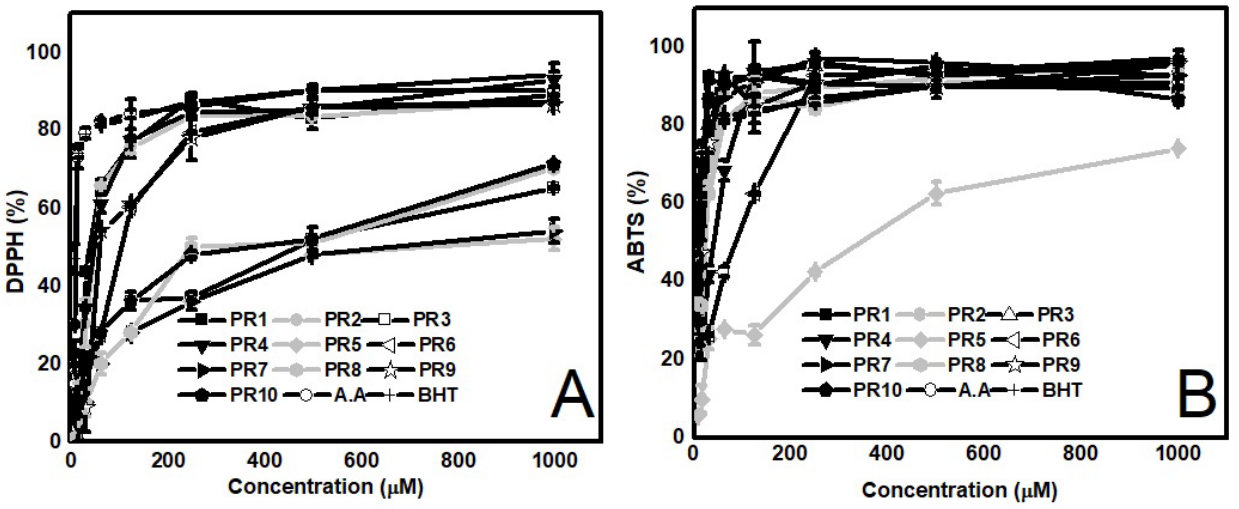

Figure 2. ***In vitro*** **antioxidant activity curves (DPPH and ABTS assays) versus concentration promoted by PR compounds and ascorbic acid (AA) and butylated hydroxytoluene (BHT) standards.**

scale as strong (EC_{50} < 100 μM), moderate (100 μM < EC_{50} < 200 μM) and weak (EC_{50} >200 μM) antioxidants. Compounds PR1, PR2, PR3, PR7, PR8, PR9 and PR10 were considered as strong antioxidants, compound PR4 was considered as moderate and compounds PR5 and PR6 were considered as weak.

The antioxidant activity results varied between the evaluated compounds; however, it was observed that the 7-bromo-5-methyl compounds (PR1, PR3, PR6, PR8 and PR10) were able to promote higher antioxidant activity results (lower EC_{50} values) when compared to those with 5-bromo-7-methyl (PR2, PR4, PR5, PR7 and PR9) for the ABTS assay. This difference in results may be associated with the mesomeric effect promoted by the bromine atom due to its proximity to the nitrogen atom. In addition, the effect promoted by the substituents must be considered. The 7-bromo-5-methyl compounds were classified in decreasing order of activity (from the lowest to the highest EC_{50} values) as follows: PR1> PR10 > PR8 > PR3 > PR6 respectively.

Compound PR1 (aromatic ring without substituents) showed higher antioxidant activity due to an electronic delocalization in the aromatic ring (Siddiqui et al. 2019). Compounds PR10 (aromatic ring with an ethyl group) and PR6 (aromatic ring with a methyl group) showed different activity results and this may be related to the increase in the alkyl group. This fact was also observed by Shah et al. (2022) evaluating thiazole derivatives of Schiff's base. The compound PR8 showed a promising result of antioxidant activity because it is a Schiff base. Schiff bases can act as a donor of electron pairs (reducing agent) and this characteristic is attributed to the presence of nitrogen atoms, mainly in the form of Schiff base, and sulfur as thiocarbonyl (Siddiqui et al. 2019). Finally, the compound PR3 that presents a methoxy group, described in the literature for being a good electron donor.

The literature presents other indole-thiosemicarbazonic compounds with promising antioxidant activity. Among the works we can mention those developed by Jacob et al. (2023) obtained activity results (EC_{50}) ranging from 89.67 to 1074.24 μM for the DPPH assay and 1.65 to 79.26 μM for the ABTS assay respectively. Bakherad et al. (2019) obtained values ranging from 0.016 to 0.0630 μM for the DPPH assay. These results show

Table II. ***In vitro*** **antioxidant activity (DPPH and ABTS assays) promoted by PR compounds and ascorbic acid (AA) and butylated hydroxytoluene (BHT) standards. Results in percentage at 1000 µg/mL concentration and EC_{50} values.**

	DPPH Test			ABTS Test		
PR compounds	**% (1000 µg/mL)**	**EC_{50} (µg/mL)**	**EC_{50} (µM)**	**% (1000 µg/mL)**	**EC_{50} (µg/mL)**	**EC_{50} (µM)**
PR1	88.8 ± 0.03	45.7 ± 0.36	117.9	89.5 ± 0.07	9.05 ± 0.82	23.36
PR2	87.1 ± 0.21	48.59 ± 1.37	125.4	95.7 ± 0.59	22.49 ± 2.9	58.06
PR3	65.3 ± 0.31	577.3 ± 4.5	1383	97.1 ± 0.08	14.94 ± 0.01	35.70
PR4	92.7 ± 0.19	50.76 ± 1.5	121.6	95.94 ± 2.47	41.9 ± 3.12	100.4
PR5	52.1 ± 1.3	802.7 ± 36.7	2000	74.0 ± 0.21	488.8 ± 0.0	1217
PR6	87.27 ± 0.15	104.8 ± 0.09	261.1	96.7 ± 2.3	96.36 ± 0.67	240.1
PR7	54.3 ± 0.9	829.3 ± 20.5	2664	92.7 ± 0.33	26.69 ± 1.07	85.76
PR8	70.1 ± 0.8	296.2 ± 64.8	951.7	90.9 ± 2.93	9.32 ± 1.1	29.94
PR9	86.45 ± 0.05	59.55 ± 1.5	143.3	95.6 ± 0.21	18.96 ± 0.82	45.65
PR10	71.4 ± 0.98	438.7 ± 64.8	1056	86.5 ± 0.99	11.18 ± 1.08	26.92
AA	90.0 ± 1.0	7.75 ± 0.01	44.01	91.1 ± 0.10	13.4 ± 0.01	76.09
BHT	94.5 ± 0.81	18.9 ± 0.03	85.77	93.7 ± 2.95	5.24 ± 0.02	23.78

Mean ± Standard deviation.

that indole-thiosemicarbazone (PR) compounds are promising *in vitro* antioxidant agents.

Cytotoxicity in normal and tumor cells of mammals

The evaluation of cytotoxicity in normal mammalian cells is a necessary step to obtain compounds with promising antitumor properties (Kalinowski et al. 2009, PapeVeronika et al. 2019). That is, the compounds should be less toxic for normal cells when compared to tumor cells (Kalinowski et al. 2009).

Table III presents the results of cytotoxicity promoted by normal mammalian cells. The results were expressed in CC_{50} (concentration that inhibits cell growth by 50%).

The results presented in Table III showed that the compounds presented CC_{50} values against J774 macrophage cells ranging from 53.23 to 357.97 µM. For vero cells CC_{50} ranging from 65.34 to 376.0 µM and CC_{50} ranging from 75.23 to 323.5 µM for fibroblasts. All evaluated compounds were less cytotoxic when compared to amsacrine, asulacrine and doxorubicin standards respectively. In addition, all compounds and standards were considered non-hemolytic at the evaluated concentrations, as they presented percentage values of hemolysis lower than 10%.

The literature presents different results of cytotoxic activity of indole-thiosemicarbazonic compounds against animal cells. Jacob et al. (2023) evaluating indole-thiosemicarbazonic compounds obtained CC_{50} for macrophages varying from macrophages showed that the compounds presented values ranging from 7.0 ± 0.6 to > 75 µM. Regarding HepG2 cells, the compounds showed CC_{50} ranging from 8.04 ± 0.09 to > 82.5 µM and hemolysis percentage values lower than 6%.

Silva et al. (2020) obtained CC_{50} values for macrophages ranging from 53.23 to 357.97 µM. Haribabu et al. (2021) evaluating water-soluble Ru-*p*-cymene binuclear complexes containing indole thiosemicarbazone ligand obtained CC_{50} values > 100 µM for the compounds. Balakrishnan et al. (2019) evaluating zinc (II) complexes of indole thiosemicarbazones obtained CC_{50} values ranging from 96.5 to 109.4 µM (MCF-10A),

Table III. Results of cytotoxicity promoted by PRs against J774, vero, fibroblasts and erythrocytes macrophages cells. Compared to the results of amsacrine, asulacrine and doxorubicin standards respectively.

PR compounds	J774 macrophages CC_{50} (μM)	Vero cells CC_{50} (μM)	Fibroblasts (V79) CC_{50} (μM)	Erythrocytes (%)
PR1	63.54 ± 0.1	89.72 ± 0.4	78.09 ± 0.9	< 5.0
PR2	100.65 ± 0.5	130.12 ± 1.0	81.23 ± 0.4	< 5.0
PR3	88.18 ± 1.0	93.21 ± 0.9	75.89 ± 0.2	< 5.0
PR4	120.08 ± 0.4	140.92 ± 0.2	91.23 ± 0.9	< 5.0
PR5	118.31 ± 0.9	157.21 ± 1.0	99.29 ± 0.1	< 5.0
PR6	135.25 ± 1.5	190.0 ± 2.0	116.13 ± 0.7	< 5.0
PR7	237.76 ± 2.1	300.9 ± 1.2	201.2 ± 3.0	< 5.0
PR8	357.97 ± 1.7	376.0 ± 0.1	323.5 ± 0.2	< 5.0
PR9	53.23 ± 0.2	65.34 ± 0.9	75.23 ± 0.9	< 5.0
PR10	93.17 ± 0.3	110.2 ± 0.6	88.91 ± 0.7	< 5.0
Amsacrine	3.0 ± 0.1	2.3 ± 0.1	3.5 ± 0.1	< 10.0
Asulacrine	3.1 ± 0.1	2.5 ± 0.01	2.7 ± 0.1	< 10.0
Doxorubicin	1.22 ± 0.2	3.0 ± 0.02	3.0 ± 0.1	< 10.0

Mean ± Standard deviation.

84.6 to 91.6 μM (HEK-293) and > 500 μM (L929) respectively.

Based on these findings, we can conclude that the indole-thiosemicarbazonic compounds evaluated in our study are considered to have low cytotoxicity against normal mammalian cells evaluated in our study.

Assuming that the compounds have low cytotoxicity, *in vitro* assays were performed to evaluate the antitumor potential. The cancer cell lines evaluated were: T47D and MCF-7 (breast cancer), Jurkat (leukemia/lymphoma), DU-145 (prostate) and HepG2 (hepatoma). IC_{50} and selectivity index (SI) results are shown in Table IV. Only the results for the HepG2 strain were not presented in Table IV. This is because the compounds were not capable of IC_{50} at the evaluated concentrations, indicating that they are not toxic against this strain.

The results presented in Table IV showed that the compounds presented IC_{50} values against the T47D strain ranging from 0.83 to 1.43 μM. For MCF-7 IC_{50} ranging from 0.68 to 1.50 μM. Regarding DU-145 IC_{50} ranging from 0.97 to 1.67 μM and finally, Jurkat with IC_{50} values ranging from 0.75 to > 100 μM. With the exception of compound PR3 (> 100 μM) for the Jurkat strain, all compounds showed IC_{50} values close to each other and close to amsacrine, asulacrine and doxorubicin standards, respectively. Furthermore, all compounds (except PR3 for the Jurkat strain) showed greater selectivity for tumor strains.

The literature presents other indole-thiosemicarbazone compounds that show promising in vitro antitumor activity. Balakrishnan et al. (2019) evaluating zinc (II) complexes of indole thiosemicarbazones obtained IC_{50} values ranging from 37.9 to 100.7 μM (A549) and 60.3 to > 200 μM (MCF-7). Haribabu et al. (2021) evaluating water-soluble Ru-p-cymene binuclear complexes containing indole thiosemicarbazone ligand obtained IC_{50} values for cells ranging from 7.7. a > 50 μM (A549), 5.18 a > 50 μM (MCF-7), 11.2 to 62.7 μM (HeLa), 11.5 a > 50 μM (HepG-2), 5.05 a > 50 μM (T24) and 18.5 a > 50 μM (EA. hy926). Finally, Jacob et al. (2023) obtained IC_{50} values close to those of our study against the T47D strain, ranging from 0.61 to 1.61 μM. For MCF-7 IC_{50} ranging from 0.82 to 1.43 μM.

Table IV. Results of cytotoxicity promoted by PRs against tumor cell lines T47D and MCF-7 (breast cancer), Jurkat (leukemia/lymphoma), DU-145 (prostate). Compared to the results of amsacrine, asulacrine and doxorubicin standards respectively.

PR compounds	T-47D IC_{50} (μM)	[a]SI	[b]SI	[c]SI	MCF-7 IC_{50} (μM)	[a]SI	[b]SI	[c]SI	DU-145 IC_{50} (μM)	[a]SI	[b]SI	[c]SI	Jurkat IC_{50} (μM)	[a]SI	[b]SI	[c]SI
PR1	0.83 ± 0.03	76.6	108.1	94.1	1.00 ± 0.17	63.5	89.7	78.1	1.07 ± 2.11	59.4	83.9	73.0	0.75 ± 0.03	84.7	119.6	104.1
PR2	1.21 ± 0.06	83.2	107.5	67.1	1.10 ± 0.33	91.5	118.3	73.8	1.67 ± 0.04	60.3	77.9	48.6	1.34 ± 0.07	75.1	97.1	60.6
PR3	1.35 ± 0.09	65.3	69.0	56.2	0.68 ± 0.56	129.7	137.1	111.6	1.07 ± 0.35	82.4	87.1	70.9	>100	> 0.9	> 0.9	> 0.8
PR4	0.96 ± 0.08	125.1	146.8	95.0	0.74 ± 0.18	162.3	190.4	123.3	1.29 ± 0.12	93.1	109.2	70.7	1.06 ± 0.89	113.3	132.9	86.1
PR5	0.93 ± 0.07	127.2	169.0	106.8	1.07 ± 0.17	110.6	146.9	92.8	1.04 ± 0.11	113.8	151.2	95.5	1.67 ± 0.04	70.8	94.1	59.5
PR6	1.04 ± 0.02	130.0	182.7	111.7	1.14 ± 0.62	118.6	166.7	101.9	1.03 ± 0.07	131.3	184.5	112.7	1.27 ± 0.13	106.5	149.6	91.4
PR7	1.42 ± 0.09	167.4	211.9	141.7	0.80 ± 0.44	297.2	376.1	251.5	0.97 ± 0.07	245.1	310.2	207.4	1.19 ± 0.16	199.8	252.9	169.1
PR8	1.27 ± 0.06	281.9	296.1	254.7	1.00 ± 0.22	358.0	376.0	323.5	1.36 ± 0.04	263.2	276.5	237.9	1.61 ± 0.05	222.3	233.5	200.9
PR9	1.43 ± 0.03	37.2	45.7	52.6	1.50 ± 0.59	35.5	43.6	50.2	1.41 ± 0.91	37.8	46.3	53.4	1.98 ± 0.00	26.9	33.0	38.0
PR10	1.12 ± 0.07	83.2	98.4	79.4	0.73 ± 0.17	127.6	151.0	121.8	1.14 ± 0.09	81.7	96.7	78.0	1.49 ± 0.08	62.5	74.0	59.7
Amsacrine	1.25 ± 0.38	2.4	1.8	2.8	1.14 ± 0.09	2.6	2.0	3.1	0.80 ± 0.03	3.8	2.9	4.4	1.41 ± 0.11	2.1	1.6	2.5
Asulacrine	1.26 ± 0.43	2.5	2.0	2.1	1.18 ± 0.07	2.6	2.1	2.3	0.66 ± 0.20	4.7	3.8	4.1	1.33 ± 0.08	2.3	1.9	2.0
Doxorubicin	1.03 ± 0.27	1.2	2.9	2.9	1.11 ± 0.86	1.1	2.7	2.7	1.12 ± 0.06	1.1	2.7	2.7	0.74 ± 0.03	1.6	4.1	4.1

Mean ± Standard deviation; IC_{50} (concentration that inhibits cell growth by 50%). [a]SI: macrophage CC_{50}/tumor cell IC_{50}; [b]SI: vero cell CC_{50}/tumor cell IC_{50}; cSI: CC_{50} fibroblasts/IC_{50} tumor cell

Regarding DU-145 IC_{50} ranging from 0.94 to 1.72 μM and finally, Jurkat with IC_{50} values ranging from 0.84 to > 50 μM. These results show that the compounds evaluated here are promising candidates for antitumor compounds.

CONCLUSIONS

Based on the results presented, we highlighted three compounds with potential candidates for anticancer drugs, where these compounds stood out in two tumor cell lines. The PR01 compounds; PR4 and PR7 showed antiproliferative activity on MCF7, T47D and DU-145 cell lines. Only the PR1 compound showed antiproliferative activity against the Jurkat strain. *In vivo* antitumor studies are needed.

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