

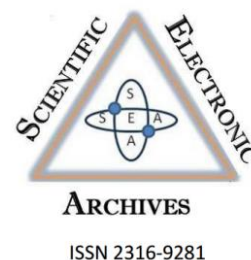
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Preliminary evaluation of the interaction with Albumin/DNA and *in vitro* evaluation of the antioxidant properties promoted by thiosemicarbazones and thiazole compounds

Pedro Henrique do Bomfim Nascimento

Federal University of Pernambuco

Keriolaine Lima dos Santos.

Federal University of Pernambuco

Arthur Van Lauter Albuquerque Pereira

Federal University of Pernambuco

Júlia Conceição Bezerra dos Santos

Federal University of Pernambuco

Corresponding author

Diego Santa Clara Marques

Federal University of Pernambuco

diego.scmarques@ufpe.br

Iranildo José da Cruz Filho

Federal University of Pernambuco

Maria do Carmo Alves de Lima

Federal University of Pernambuco

Abstract. Thiosemicarbazones and thiazoles are known for their diverse biological activities. This study introduced two series of molecules, 4-(3-(4-nitrophenyl)-4-phenylthiazol-2(3H)-ylidene)-hydrazine)-methyl)-phenol and 4-(3-(4-chlorophenyl)-4-phenylthiazol-2(3H)-ylidene)-hydrazine)-methyl)-phenol, which exhibit biological potential as antioxidant agents. The study also assessed the interaction of these compounds with various HSA/DNA macromolecules. The results of the antioxidant activity showed that thiazoles in the DPPH assay exhibited IC_{50} values ranging from 439.4 to 691.67 μ M. In the ABTS assay, thiosemicarbazones exhibited significant activity, ranging from 39.19 to 50.03 μ M. Interaction assays were carried out with human serum albumin (HSA) and DNA. All compounds were able to interact with both DNA (low to moderate interaction) and HSA (moderate to high interaction).

Keywords: antioxidant, macromolecules, biological potential

Introduction

The development of new therapeutic agents is based on the search for compounds with relevant biological activities, such as antioxidant potential and the ability to interact with biological macromolecules such as DNA and HSA (Human Serum Albumin) (Jacob et al. 2023). Thiosemicarbazones and thiazoles are important scaffolds used in the synthesis of new compounds

with pharmaceutical potential, due to their versatile biological activities, which include antioxidant, antimicrobial, and antitumor properties (Salar et al. 2019; Geronikaki et al. 2013).

Oxidative stress, caused by the imbalance between the generation of free radicals and the body's antioxidant capacity, leads to a state of oxidative stress. This state of stress is related to the pathogenesis of several diseases, such as cancer,

cardiovascular diseases, and neurodegenerative diseases (Baschieri&Amorati, 2021). Therefore, the identification of new antioxidants is crucial for the development of effective therapeutic strategies.

For a molecule to be considered a promising drug candidate, it is crucial that it is effectively distributed throughout the body. Understanding the mechanism of action enables the selection of the most appropriate therapeutic approach (Jacob et al. 2023; Shafiei et al. 2020). The interaction of the drug with HSA is a fundamental aspect in the modulation of biological processes in drug development, contributing to solubility, stability, and low toxicity (Ribeiro et al. 2019). DNA is an important molecular target involved in the mechanism of action of several drugs (Souza et al. 2022). Compounds can interact with DNA in a variety of ways, such as forming covalent or noncovalent bonds, intercalating nitrogenous bases, or binding to the grooves of the DNA molecule (Jacob et al. 2023; Hanifa et al. 2022). Interaction with DNA can influence gene expression and cell division, constituting a possible mechanism of action for several drug candidate molecules (Almeida et al. 2021; Hoogenboezem& Duvall, 2018).

The objective of the study was to evaluate two series of compounds, thiosemicarbazones and thiazoles, for their *in vitro* antioxidant activity and their ability to interact with DNA and HSA. Both classes of compounds have chemical structures that suggest the potential to act as free radical scavengers, in addition to having functional groups that can facilitate interaction with biological macromolecules (Yildiz et al. 2022; Petrou et al. 2021). This work aims to contribute to the understanding of the mechanisms of action of these compounds and evaluate their potential as candidates for new drugs, providing valuable information for the development of new therapeutic strategies.

Material and Methods

Reagents

Reagents and solvents obtained commercially from Sigma-Aldrich, Fluka, Vetec and

Merck were used as listed below: ascorbic acid (CAS 50-81-7), dimethylsulfoxide (DMSO; CAS 67-68-5), 2,2-diphenyl -1-Picrylhydrazyl (DPPH; CAS 1898-66-4), absolute ethanol (CAS 64-17), ABTS (CAS 28752-68-3), ascorbic acid (Cewin), butylated hydroxytoluene (CAS 128-37-0), potassium persulfate (CAS 7727-21-1), MTT (CAS 298-93-1), acridine orange (CAS 494-38-2) 4', 6-Diamidine-2'-phenylindole dihydrochloride (DAPI) (CAS 28718-90-3).

General procedure for the synthesis of thiosemicarbazones and thiazoles compounds

The proposals were provided and a synthesis carried out at the Laboratory of Chemistry and Therapeutic Innovation of the Federal University of Pernambuco, Recife, Pernambuco, Brazil. The synthesis of the proposals was carried out in three stages, methodology by Oliveira et al. (2015) with modifications. Initially, thiosemicarbazides were obtained through the nucleophilic addition of hydrazine with substituted isothiocyanates. For this, equimolar amounts of hydrazine hydrate (1 mmol) were slowly added in solution with equimolar amounts of isothiocyanate (1 mmol) and 20 mL of dichloromethane. The reaction was refluxed for 2 hours at room temperature (30 ± 5 °C). The product was filtered, washed with dichloromethane and dried in a vacuum desiccator.

Thiosemicarbazones in the presence of 4-hydroxy-benzaldehyde condense to form thiosemicarbazones. The reaction was carried out in 1 mmol equimolar amounts of thiosemicarbazides by reacting with 1 mmol of 4-hydroxy-benzaldehyde in absolute ethanol (10 mL) using a catalytic amount of acetic acid (0.75 mL). The reaction was run for 3 hours at room temperature (30 ± 5 °C), followed by thin layer chromatography. The thiosemicarbazones were cyclized in the presence of acetophenones with different substitutions, to obtain the thiazoles (Oliveira-Filho et al. 2017).

Figure 1 shows the thiosemicarbazones 3 and 4. Figure 2 of shows the structures of the different thiazoles starting from intermediates 3 and 4 respectively.

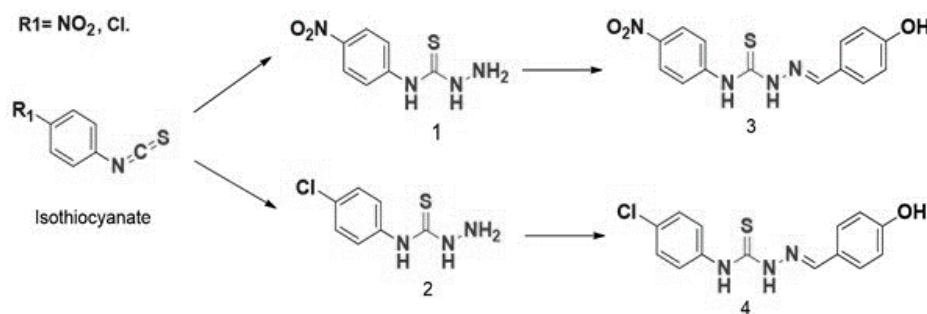


Figure 1. Synthesis of hydroxylated thiosemicarbazones.

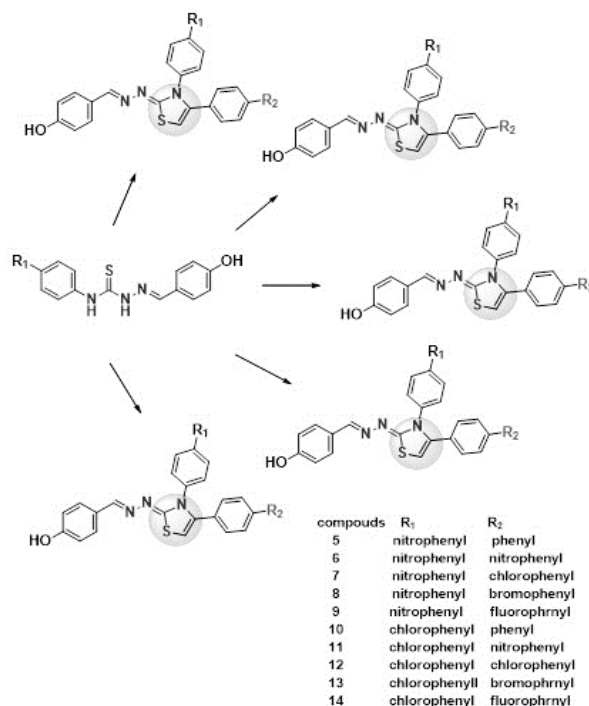


Figure 2. Structures of thiazoles and their substituents.

In vitro antioxidant activity

Method of capturing DPPH radicals

The determination of the antioxidant activity of the 1,1-diphenyl-2-picrylhydrazine (DPPH) method was performed according to the methodology described by Mic et al. (2021) and Marc et al. (2021) with modifications. The assay consists of the reaction of the free radical DPPH 0.03mM (3.0 mL/ethanolic solution) with the compounds diluted in DMSO at different concentrations (7.9 - 1000µg/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 used in this experiment was the DPPH solution. The standards used were ascorbic acid and butylated hydroxytoluene (BHT). The spectrophotometer blank was ethanol. All experiments were performed in triplicate. The antioxidant activity values were obtained by Equation 1.

$$\text{DPPH \%} = \left(\frac{A_c - A_s}{A_c} \right) * 100\% \quad 1)$$

Onde A_c = Absorbância do controle; A_s = Absorbância da amostra

With the results obtained from Equation 1, it was possible to obtain a curve of the antioxidant activity (in percentage) as a function of the concentration of the compounds. Then, the concentration necessary to obtain the IC₅₀, a

concentration that captures 50% of the DPPH radicals, was calculated by non-linear regression.

ABTS radical capture method

The methodology used to evaluate the antioxidant activity by the ABTS radical scavenging method (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) was performed according to Mic et al. (2021) and Marc et al. (2021) with modifications. The ABTS radical was produced by reacting 5 mL of 7 mM ABTS with 88 µL of 140 mM potassium persulfate solution. This mixture was stored in the absence of light and under refrigeration for at least 16 hours before the analysis. The ABTS radical solution was diluted in ethanol until an absorbance of 0.7 ± 0.05 at 734 nm was obtained.

Activity assays were performed as follows: compounds were dissolved in DMSO and diluted at different concentrations (7.9 – 1000 µg/mL) and volumes of 30 µL were added to 1.0 mL of the ABTS radical solution. This system was kept in the dark. at 30 °C for 10 min. After this time interval, the absorbance of the assays was determined in a spectrophotometer (Hewlett-Packard, model 8453) at 734 nm. The control used in this experiment was the ABTS solution. The standards used were ascorbic acid and butylated hydroxytoluene (BHT). The spectrophotometer blank was ethanol. All experiments were performed in triplicate.

The antioxidant activity values for this assay were also obtained by Equation 1. Through the results it was possible to obtain different antioxidant activity curves as a function of the concentration of the compounds. Finally, the IC₅₀ required

concentration of 50% radical capture was calculated by nonlinear regression.

Interaction assays of compounds with human albumin (HSA)

Albumin is a highly circulating protein and one of its main functions is the transport of endogenous and exogenous compounds, being responsible for the distribution of drugs in the body (Hoogenboezem& Duvall 2018). Therefore, it is necessary to search for drugs that bind reversibly to albumin and that do not promote changes in its conformation (Ribeiro et al. 2021). The human serum albumin used in this interaction experiment was kindly provided by the Molecular Biology Laboratory of the University of Pernambuco, located in Garanhuns, Pernambuco, Brazil.

The experiments of interaction with albumin were carried out according to the methodology proposed by Alves et al. (2021) with few modifications. Compounds were dissolved in with dimethylsulfoxide (DMSO) to obtain a stock concentration of 1 mM. Then, they were diluted in the following concentrations: 5, 10, 15, 20, 25 and 30 μ M the dilutions were carried out in Tris-HCl buffer (0.1 M, pH 7.6) to obtain a final concentration of 1% DMSO. Subsequently, 10 μ L of each solution was added to a 1.0 mL of HSA at a concentration fixed at 10 μ M. The system was homogenized and left at room temperature for 10 min to stabilize in Tris-HCl buffer (0.1 M, pH 7.6) and then subjected to fluorescence analysis. The generated emission spectra were recorded in the region of 310 - 400 nm using an excitation wavelength of 280 nm. The experiments were carried out at a temperature of 25 $^{\circ}$ C. The determinations were carried out at room temperature, in a rectangular quartz cuvette with an optical length of 1 cm. The fluorescent emission suppression behavior was analyzed through Equation 2 proposed by de Stern-Volmer (Ksv) (Lakowicz 2006).

$$\frac{F_0}{F} = 1 + K_{sv}[Q] \quad (2)$$

Where: F_0 and F are the fluorescence intensities in the absence and presence of the compounds. K_{sv} is the linear suppression constant. $[Q]$ represents the concentration of the compound.

The graph of relative emission intensity (F_0/F) versus $[Q]$ was used to obtain the constant based on the linear regression of the graph. Binding data was obtained from SigmaPlot 10.0 software. The changes in the maximum emissive peak (λ_{max}) of the spectra generated from free and bound HSA to the compounds were also analyzed. All experiments were performed in triplicate.

Interaction assays of compounds with DNA

Preparation of the ssDNA solution

The tests for the preparation of the probes were carried out according to the methodology proposed by Alves et al. (2021). The ssDNA was dissolved in Tris-HCl buffer (0.1 M, pH 7.6) and left for 24 hours at 8 $^{\circ}$ C for stabilization. After this period, the solution was homogenized for 30 minutes and its concentration determined using the molar extinction coefficient $6600 \text{ M}^{-1} \text{ cm}^{-1}$ at 260 nm. The ratio of absorbance values at 260 and 280 nm was used to verify the degree of purity of the ssDNA solution. These parameters were determined by Equations 3 and 4 respectively.

$$ABS_{260} = \epsilon_{260} \cdot b \cdot c \quad (3)$$

$$\text{Degree of purity} = \frac{ABS_{260}}{ABS_{280}} \quad (4)$$

Where: ABS: Absorbance at different wavelengths ϵ : extinction coefficient, b: optical path (1 cm); c: concentration.

All absorbances in this study were determined in aspectrophotometer (Hewlett-Packard, model 8453) using a rectangular quartz cuvette with an optical length of 1 cm at a temperature of 25 $^{\circ}$ C. The tests were performed in triplicate.

Fluorescent emission spectroscopy with probes and DNA

The DNA interaction assays with the synthesized compounds were performed according to Alves et al. (2021) with few modifications. Initially, compounds were dissolved in with dimethylsulfoxide (DMSO) to obtain a stock concentration of 1 mM. Then, they were diluted in the following concentrations: 5, 10, 15, 20, 25 and 30 μ M, the dilutions were carried out in Tris-HCl buffer (0.1 M, pH 7.6) to obtain a final concentration of 1% DMSO. For the DNA interaction assays, the fluorescent markers 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) and acridine orange (AO) were used.

The fluorescence spectra of the DAPI-ssDNA complex were obtained by excitation at 364 nm and emission scanning from 400 to 600 nm. Then, solutions containing DAPI-ssDNA were analyzed in the presence of various concentrations of the compounds. The fluorescence spectra of the acridine orange -ssDNA complex was obtained by excitation at 480 nm and emission scanning from 500 to 600 nm. The system consisted of 1mL of DNA solution with the respective probes and 10 μ L of each concentration of compounds, after which they were incubated at 25 $^{\circ}$ C for 10 min. Determinations were performed using a rectangular quartz cuvette with an optical length of 1 cm and a JASCO FP-6300 spectrofluorometer (Tokyo, Japan). Assays were performed in triplicate. The solutions were homogenized and stabilized for 10 minutes and subsequently submitted to fluorescence analysis. The emission suppression behavior was analyzed using Equation 5 proposed by Stern-Volmer (K_{sv}).

$$\frac{F_0}{F} = 1 + K_{sv}[Q] \quad (5)$$

Where: F₀ and F represent the steady-state fluorescence intensities of compounds in the absence and presence of ctDNA and compounds, K_{sv} is the Stern-Volmer constant and [Q] is the inhibitor concentration. Binding data were obtained using SigmaPlot 10.0 software.

Results and discussion

In vitro antioxidant activity promoted by thiosemicarbazones and thiazoles

Thiosemicarbazones and thiazoles are capable of promoting antioxidant activities *in vitro* and *in vivo* (Geronikaki et al. 2013; Bingul et al. 2019; Salar et al. 2019; Yousef et al. 2020). This activity is directly related to its chemical structure. Thiosemicarbazones have the ability to donate electron pairs (reducing agent) (Geronikaki et al. 2013; Bingul et al. 2019). This characteristic is due to the presence of nitrogen atoms, mainly in the form of Schiff base, and of sulfur as thiocarbonyl (Siddiqui et al. 2019; Yang et al. 2020). On the other hand, thiazole compounds are described in the literature for being able to scavenge radicals, which makes them potential antioxidant agents (Geronikaki et al. 2013; Bingul et al. 2019; Salar et al. 2019; Yousef et al. 2020; Petrou et al. 2021).

In this context, different methods can be used to evaluate the antioxidant activity promoted by thiosemicarbazones and thiazoles (Geronikaki et al. 2013; Bingul et al. 2019; Salar et al. 2019; Yousef et al. 2020). Among the most commonly used methods to assess antioxidant activity are DPPH (2,2-diphenyl-1-picrylhydrazine) and ABTS (2,2'-

azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging methods. Both are characterized by their excellent reproducibility under established conditions, but can show significant differences in their antioxidant responses (Bingul et al. 2019; Salar et al. 2019; Yousef et al. 2020).

The DPPH radical inhibition method is based on the transfer of electrons from an antioxidant compound to an oxidant (Baschieri&Amorati2021). 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, causing discoloration (Geronikaki et al. 2013). The ABTS radical is generated through a chemical, electrochemical or enzymatic reaction (Moharram & Youssef 2014). With this method, one can determine the antioxidant activity of compounds of both hydrophilic and lipophilic nature (Moharram & Youssef 2014 2014; Bingul et al. 2019; Salar et al. 2019; Yousef et al. 2020). The reaction is accompanied by a color change from green to colorless.

Figure 3 shows the antioxidant activity curves promoted by the compounds at different concentrations for the DPPH and ABTS assays, respectively. The results showed an increase in antioxidant activity with increasing concentration. Similar profiles were obtained by Bingul et al. (2019), Salar et al. (2019) and Yousef et al. (2020) evaluating different thiosemicarbazones and thiazoles for the DPPH and ABTS assays.

Table 1 presents the results of antioxidant activity represented by IC₅₀ (minimum concentration capable of reducing free radicals by 50%).

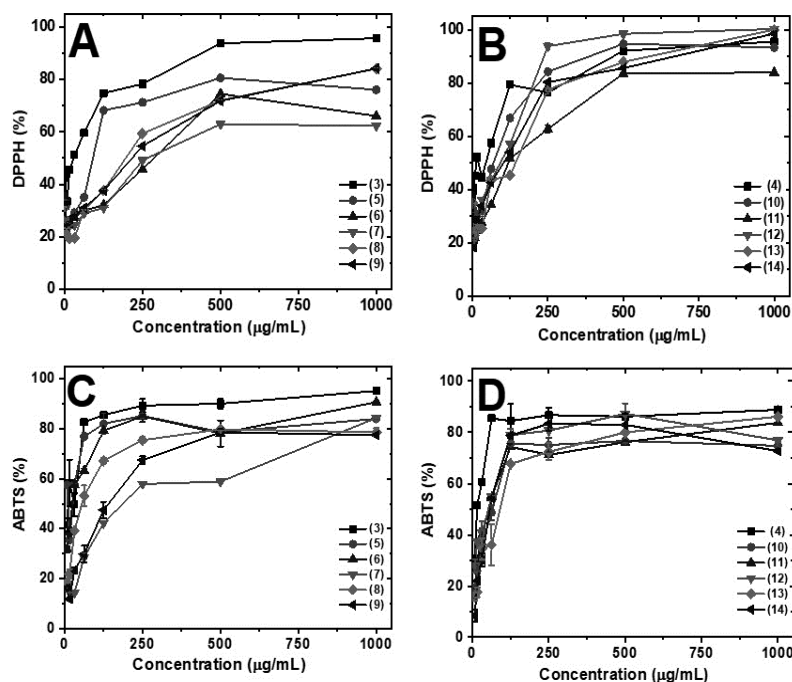


Figure 3. *In vitro* antioxidant activity for DPPH (A and B) and ABTS (C and D) assays, promoted by compounds synthesized at different concentrations

Table 1. Results of *in vitro* antioxidant activity promoted by the compounds synthesized in this study for the DPPH and ABTS radical scavenging assays.

Compounds	IC ₅₀ (DPPH [•])		IC ₅₀ (ABTS [•])	
	µg/mL	µM	µg/mL	µM
3	139.0 ± 0.0	439.4	12.4 ± 3.0	39.19
4	211.5 ± 1.0	691.67	15.3 ± 0.1	50.03
5	85.5 ± 0.2	205.30	25.0 ± 0.1	60.03
6	51.8 ± 0.2	112.4	65.5 ± 0.8	141.94
7	271.5 ± 1.6	602.12	28.5 ± 3.0	63.20
8	321.5 ± 2.6	649.0	193.5 ± 0.3	390.63
9	194.1 ± 1.0	446.78	61.81 ± 0.3	142.27
10	252.8 ± 1.4	622.8	156.4 ± 1.3	385.31
11	82.6 ± 3.2	183.1	67.4 ± 3.9	149.47
12	176.3 ± 1.8	400.36	52.5 ± 1.3	119.22
13	97.0 ± 1.5	200.08	53.4 ± 4.8	110.14
14	125.9 ± 1.1	297.01	82.2 ± 4.8	193.91
Ascorbic acid	7.78 ± 0.1	44.17	14.67 ± 0.8	83.29
BHT	18.85 ± 0.7	85.54	5.64 ± 0.2	25.29

Mean ± Standard Deviation

The IC₅₀ results presented in Table 1 showed that thiazoles present better results when compared to thiosemicarbazones for the DPPH radical scavenging assays. The thiazoles presented IC₅₀ ranging from 112.4 to 649.0 µM and the thiosemicarbazones presented values ranging from 439.4 to 691.67 µM. In general, the compounds evaluated showed lower results of antioxidant activity when compared to the standards used (ascorbic acid and BHT). These results show that for the DPPH assay the synthesized compounds present moderate to low antioxidant activity.

In relation to the ABTS radical scavenging assay, thiosemicarbazones presented higher results when compared to thiazoles and the ascorbic acid standard, however, they presented lower results when compared to the BHT standard. The thiosemicarbazones presented IC₅₀ values ranging from 39.19 to 50.03 µM (high activity). The thiazoles presented values between 60.03 to 390.63 µM (moderate to low activity). These better results when compared to the DPPH radical capture assays are associated, in addition to the chemical structure, to the versatility of the ABTS assay, that is, it presents good results for compounds of polar and non-polar nature.

The literature presents promising results for the antioxidant activity of thiosemicarbazones and thiazoles for the DPPH and ABTS assays. Among these studies, we can mention those carried out by Yildiz et al. (2022) who evaluating a range of novel monomeric and dimeric indole-based thiosemicarbazone derivatives 17 – 28 obtained IC₅₀ results ranging from 26.88 to 99.50 µM for the DPPH assay and 17.38 to 96.10 µM for the ABTS assay. Salar et al. (2017) evaluating new chromones substituted by hybrid hydrazinyl thiazole obtained good results for DPPH (IC₅₀ = 0.09 - 2.233 µM) and ABTS (IC₅₀ = 0.584 – 3.738 µM). These results show that the antioxidant activity promoted by the compounds synthesized in this study is directly related to the chemical structure, mainly to the

effects promoted by the different substituents (Geronikaki et al. 2013; Bingul et al. 2019; Salar et al. 2019; Yousef et al. 2020; Petrou et al. 2021).

Interaction assays of compounds with human albumin (HSA)

Human serum albumin (HSA) is the most abundant plasma protein in human blood, present at a concentration around 40 to 50 mg/mL (Ribeiro et al. 2021). As it is a widely circulated protein, one of its main functions is the transport of endogenous and exogenous ligands, including being responsible for the distribution of drugs in the body (Hoogenboezem & Duvall 2018). Thus, the pharmacological properties of a drug can be highly influenced by its ability to bind albumin protein (Hoogenboezem & Duvall, 2018; Ribeiro et al. 2021). An important feature of the drug-albumin interaction is that the binding is reversible in nature, leading to an equilibrium between the bound molecule and its free portion in the circulation (Ribeiro et al. 2021).

Through the use of spectroscopic techniques, it is possible to predict whether a molecule is able to bind albumin, and thus influence the intended therapeutic effect (Alves et al. 2021; Ribeiro et al. 2021). If binding affinity is proven, the result can directly influence patterns such as drug solubility, absorption, distribution, metabolism and excretion (Alves et al. 2021).

Figure 4 presents the fluorescence emission spectra of HSA (λ_{exc} = 280 nm), at different concentrations of the synthesized compounds (0 to 30 µM) at 25 °C in a fluorescence emission range between 300 and 400 nm. In addition, it presents the Stern-Volmer linearized plots for the fluorescence quenching of HSA for one of the compounds. The spectroscopic results of the fluorescent emission of HSA, as a function of varying concentrations of the compounds are shown in Table 2.

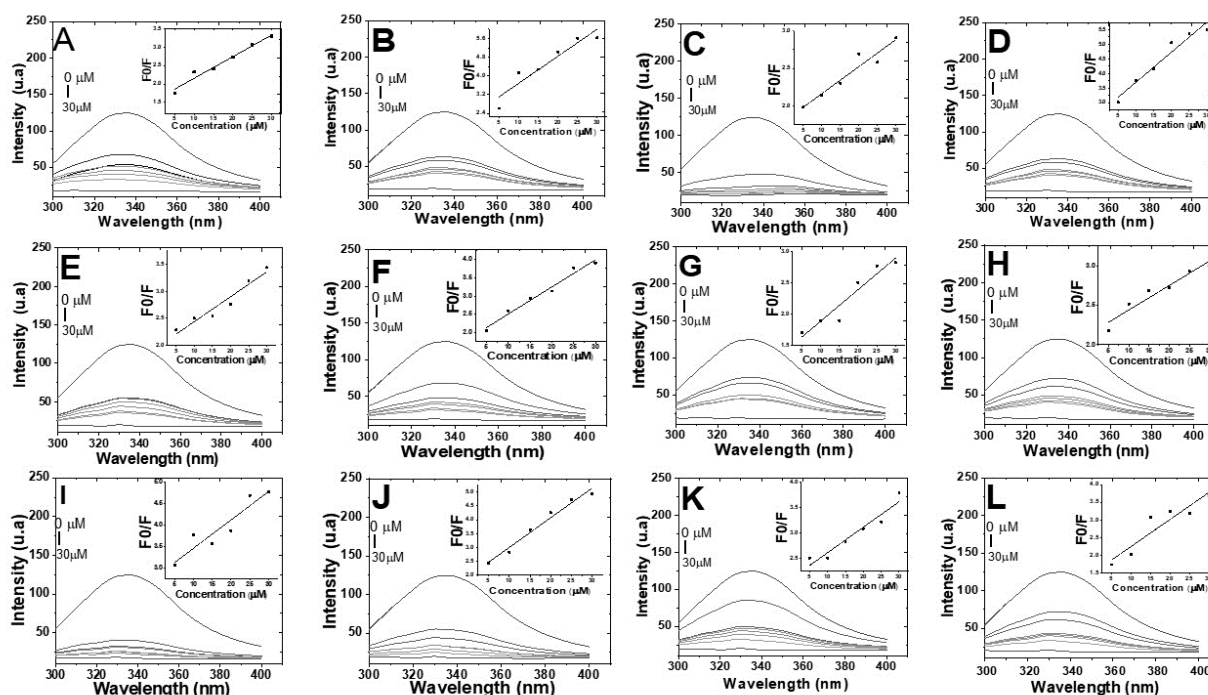


Figure 4. Fluorescence spectrum of HSA (10 μM) exposed to different concentrations of synthesized 8 compounds. In addition to the linearized graphics of the model proposed by de Stern-Volmer. The compounds are represented in the following order: compound 3 (A), 4 (B), 5(C), 6 (D), 7 (E), 8 (F), 9 (G), 10 10 (H), 11 (I), 12 (J), 13 (K) and 14 (L) respectively

The curves obtained in Figure 4 show that the fluorescence intensity of the protein (HSA) showed a gradual decrease in the intensity of maximum fluorescence emission of HSA at different concentrations of the compounds. The increase in the concentration of these compounds in solution causes the suppression of fluorescence emission (hypochromic effect) of the tryptophan amino acid residue of the protein, this is a clear indication of an interaction between HSA and the compounds (Alves et al. 2021; Wang et al. 2021). In addition, a slight shift in the albumin fluorescence emission band to

the blue region (hypsochromic effect) was also observed, indicating the occurrence of interactions of the compound in the hydrophobic cavity of albumin, causing small structural changes in this region (Alves et al. 2021; Wang et al. 2021; Szymaszek et al. 2022). The results presented in Table 2 show that the compounds were able to promote 100% fluorescence inhibition (hypochromism). Furthermore, through the curves obtained in Figure 4 and Equation 2, it was possible to determine the values of the suppression constant (K_{sv}).

Table 3. Spectroscopic results of the interaction of human albumin and the synthesized compounds.

Compounds	$\lambda_{\text{máxfree}}$ (nm)	$\lambda_{\text{máxon}}$ (nm)	hypochromism (%)	K_{sv} (L/mol)
3	335	329	100	5.85×10^4
4	335	358	100	1.16×10^5
5	335	331	100	3.63×10^4
6	335	359	100	1.03×10^5
7	335	331	100	4.64×10^4
8	335	330	100	7.42×10^4
9	335	330	100	5.09×10^4
10	335	330	100	3.09×10^4
11	335	329	100	6.60×10^4
12	335	329	100	1.06×10^5
13	335	329	100	5.01×10^4
14	335	330	100	7.63×10^4

^aHypochromism resulting from the interaction of albumin fixed at 10 μM with varying concentrations of test compounds (5-30 μM) compared to free albumin. ^bStern–Volmer suppression constant (K_{sv}) obtained based on fluorescence data with HSA.

The thiosemicarbazones presented values that varied from 5.85×10^4 to 1.16×10^5 L/mol. The thiazoles presented values that varied from 3.09×10^4 to 1.06×10^5 L/mol. The higher the value of the constants, the stronger the binding of the

compounds with the protein (Wang et al. 2021; Szymaszek et al. 2022). Thus, the K_{sv} values suggest that the compounds that bound more strongly to the protein follow the following order of strength: $4 > 12 > 6 > 14 > 8 > 11 > 3 > 9 > 13 > 7 >$

5 > 10. In studies of interaction with human serum albumin (HSA), the K_{sv} values found were of the order of 10⁴ and 10⁵, indicating that the interactions of the compounds with albumin are moderate to high (Alves et al. 2021; Wang et al. 2021; Szymaszek et al. 2022).

As all compounds proved to be good albumin binders, the compounds that showed the highest K_{sv} values were compounds 4 (K_{sv}= 1.16x10⁵ L/mol) and 12 (K_{sv}= 1.06 x10⁵ L/mol). Both present the presence of the ligand chlorine (-Cl), in the position R1, and only for the 4 the presence of chlorine in R1, but absence of substitution in R2. Results similar to those found by Silva Filho et al. (2019), evaluating the interaction of acridine-thiosemicarbazone derivatives (3a-3h) with bovine serum albumin, obtained K_{sv} values ranging from 1.62 x10⁴ to 8.71x10⁵ L/mol. They observed that compound 3e (with the highest K_{sv} value) had a chloro substituent in its structure. Pricopie et al. (2020) evaluating the interaction of new 2,4-disubstituted-1,3-thiazole derivatives (4a – d and 7a – d) with bovine albumin, they obtained K_{sv} ranging from 1.41 x10⁴ to 1.332x10⁵ L/mol. Among these compounds, compound 7b (with the highest K_{sv} value) also had a chloro substituent in its structure. These results indicate that the chemical structure is the main factor of increase or decrease of the interaction between the different compounds and albumin

Fluorescent studies with DAPI and acridine orange probes: interaction of compounds with DNA

DNA has a fundamental role in cellular functions, in this linear polymer are encoded the instructions for the formation of all other cellular components (Madsen&GOTHELF 2019). Furthermore, it provides a model for the production of identical DNA molecules to be distributed to progeny when a cell divides (Almeida et al. 2021). Thus, drugs that interact with DNA in such a way as to damage it compromises the functionality of the organism (Ceze et al. 2019; Almeida et al. 2021). However, it is worth noting that for the drug to interact with DNA it needs to cross cell and nuclear membranes (Ceze et al. 2019; Almeida et al. 2021). Therefore, the interaction assays are of a preliminary nature and carried out in order to evaluate the effect of different compounds on DNA, being necessary to carry out *in vitro* cytotoxicity assays (Sharma et al. 2020; Almeida et al. 2021).

Compounds can interact with DNA via non-covalent or covalent interactions (Sharma et al. 2020). This type of interaction can cause irreversible binding, forming adducts between the ligands of the complex or even a possible alkylation of nitrogenous DNA bases (Almeida et al. 2021). The non-covalent interaction is a reversible interaction in which a molecule interacts with biomolecules via intercalation, electrostatic forces or groove interaction, triggering changes in DNA conformation, breaks in its structure or even inhibiting the protein-DNA interaction (Sharma et al. 2020). Intercalative

and sulcus bonding can occur simultaneously and in a variety of ways, close to or within the double helix of this biomolecule, while electrostatic bonding occurs in its outer part (Sharma et al. 2020). Therefore, to evaluate the type of interaction mechanism, different fluorescent probes are used (Sharma et al. 2020; Almeida et al. 2021; Alves et al. 2021).

Fluorescent probes are increasingly used in biological investigations and can be applied in several areas (Sharma et al. 2020; Alves et al. 2021). In studies involving DNA, probes can help in the identification of target compounds, since they can interact with DNA in different ways (Alves et al. 2021). One such probe that has been widely used in DNA studies is 4',6-diamidino-2-phenylindole (DAPI). DAPI has high affinity and specificity for the minor groove of DNA, which is a region rich in AT (adenine-thymine) sequences. One of the most striking features of this binding is the increased fluorescence intensity of the DNA-DAPI complex (Parameswaran et al. 2021).

Thus, this probe was used in a competition experiment to investigate the ability of compounds to interact with the minor groove of DNA (Alves et al. 2021). Figure 5 the fluorescence spectra of interactions of compounds with DNA using the DAPI probe. The spectroscopic results of the fluorescent emission of DNA, as a function of different concentrations of the compounds are presented in Table 4.

Through Figure 5, we can see that after adding the compounds, there was a deviation from the maximum emissive peak (difference between the wavelength of the bound and free form) varying on average from 1 to 10 nm (Table 4).

All synthesized compounds were able to promote the hypsochromic effect (left shift) with different percentages. This effect suggests the formation of a complex between DAPI-DNA-compounds (Alves et al. 2021). In addition to this effect, the hypochromic effect was also observed, which is related to the decrease in the intensity of the emissive peak. The thiosemicarbazones presented values that varied from 1.07 x10⁴ to 1.26 x10⁴ L/mol. The thiazole compounds showed a range ranging from 1.10 x10⁴ to 9.42x10⁴L/mol. Fluorescent emission suppression (K_{sv}) can be observed in the following order of strength: 11 > 6 > 8 > 12 > 14 > 5 > 10 > 4 > 7 > 13 > 9 > 3. These values suggest that the interaction between the compounds with DNA is moderate (10⁴ L/mol). Among the compounds evaluated, we highlight compound 11, which in addition to causing a 100% hypochromic effect, also presented the highest K_{sv} value (9.42x10⁴ L/mol). The observed hypochromism suggests that all compounds in this study may be competing with the DAPI-DNA binding site, or supposedly, may form a ternary complex in the minor groove of DNA (Rahman et al. 2017; Alves et al. 2021). These results show that the synthesized compounds can interact with DNA grooves in a moderate way (Alves et al. 2021).

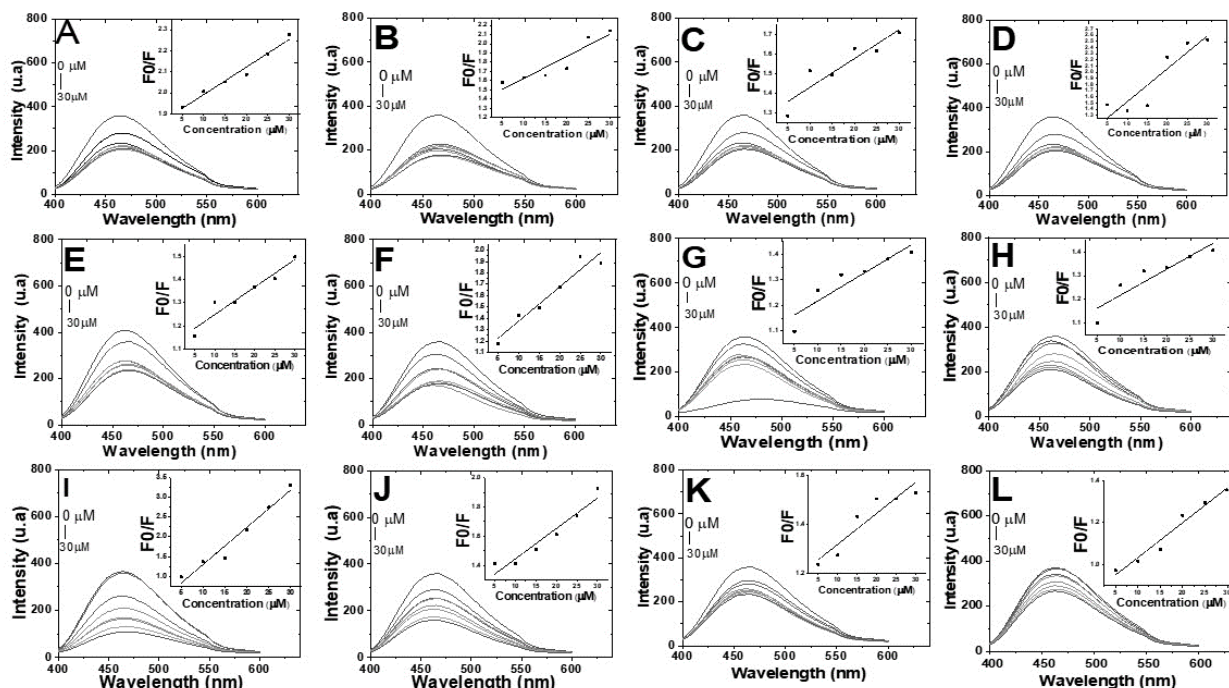


Figure 5. Fluorescence spectrum of compounds + DAPI (3 μM) + DNA (30 μM). In addition to the 12 linearized graphics of the model proposed by de Stern-Volmer. The compounds are represented in the following order: compound 3 (A), 4 (B), 5(C), 6 (D), 7 (E), 8 (F), 9 (G), 10 (H), 11 (I), 12 (J), 13 (K) and 14 (L) respectively.

Table 4. Results of fluorescent emission of the DAPI-ctDNA complex in the presence of compounds.

Compounds	$\lambda_{\text{m\acute{a}x}}$ free (nm)	$\lambda_{\text{m\acute{a}x}}$ on (nm)	hypochromism (%)	Ksv (L/mol)
3	468	472	100	1.07×10^4
4	468	467	74.26	1.26×10^4
5	468	466	71.24	1.47×10^4
6	468	473	100	5.25×10^4
7	468	465	49.77	1.19×10^4
8	468	468	88.84	3.02×10^4
9	468	465	40.79	1.10×10^4
10	468	464	27.78	1.41×10^4
11	468	466	100	9.42×10^4
12	468	463	92.37	2.08×10^4
13	468	464	52.99	1.16×10^4
14	468	458	35.38	1.65×10^4

Another probe used in DNA studies is the acridine orange dye (Meng et al. 2012; Alves et al. 2021). This dye has the ability to interact with deoxyribonucleic acid through the interaction between its base pairs, causing an increase in fluorescence, due to the formation of the complex, a characteristic that has already been well elucidated (Sayed et al. 2021; Meng et al. 2012). In addition, this dye can bind efficiently to both the DNA of living cells and the DNA of dead cells, so it is widely used in studies in various areas such as biology, medicine, engineering (Amado et al. 2017; Guzaev et al. 2017).

Thus, in order to investigate how the compounds interact with DNA, a competition assay

by fluorescence spectroscopy was performed in order to determine whether the derivatives evaluated here had the ability to intercalate with DNA, as well as acridine orange. presents as one of its binding mechanisms (Amado et al. 2017).

Figure 6 shows the fluorescence spectra of the compounds' interactions with DNA using the acridine orange probe. The spectroscopic results of the fluorescent emission of DNA, as a function of different concentrations of the compounds are presented in In Table 5.

All synthesized compounds were able to promote the hypsochromic effect (left shift). It was not possible to observe a hyperchromic or hypochromic effect, which caused a lack of linearity

of the data for the Stern-Volmer equation to obtain Ksv. Even with this lack of linearity from the equation to the data, we calculated by extrapolation the Ksv values in order to compare them to the DAPI probe.

The thiosemicarbazones presented values that varied from 3.8×10^3 to 5.9×10^3 L/mol. The thiazole compounds showed a range ranging from 3.0×10^2 to 3.55×10^4 L/mol. Thus, the compounds were classified in decreasing order of fluorescence

suppression: $8 > 12 > 11 > 7 > 6 > 3 > 14 > 4 > 5 > 10$ and $13 > 9$. In general, the competition assays with the probes and DNA, indicate that all compounds evaluated here can compete for DNA binding sites in a moderate to low manner. However, these have higher affinity for sulcus bonds when compared to base pairs (Alves et al. 2021).

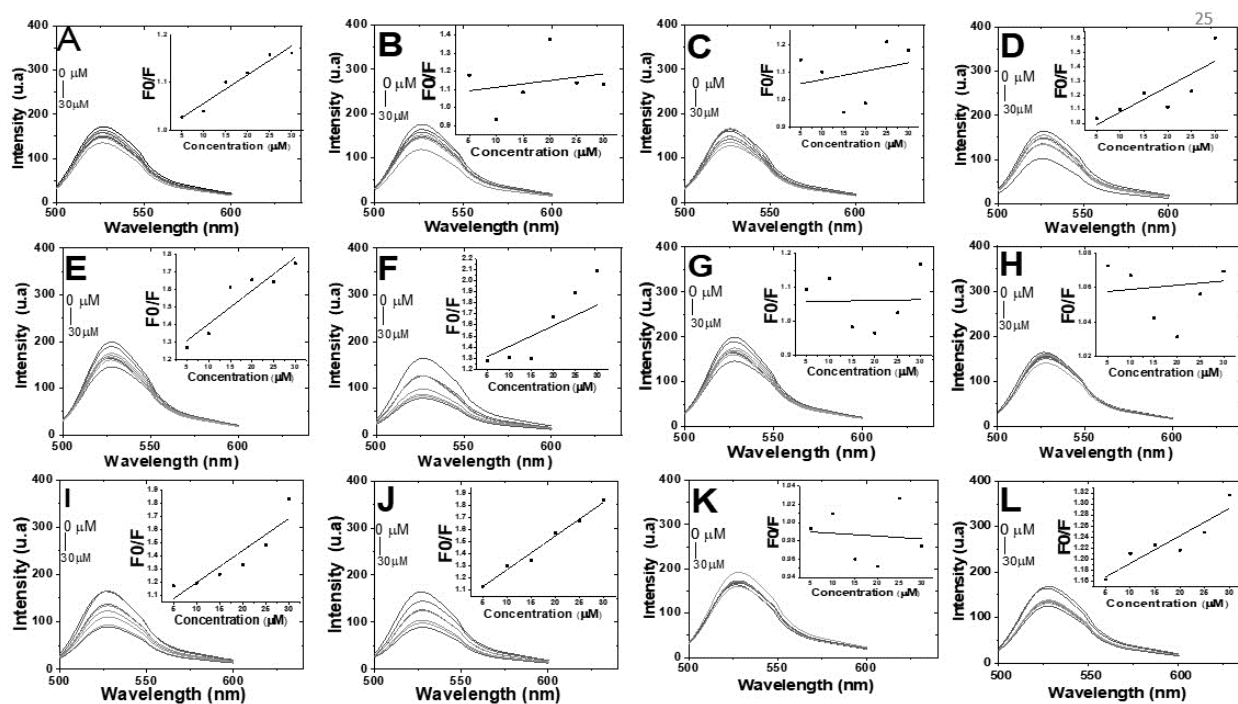


Figure 6. Fluorescence spectrum of compounds + acridine orange (3 μ M) + DNA (30 μ M). In addition to 16 the linearized graphics of the model proposed by de Stern-Volmer. The compounds are represented in the following order: compound 3 (A), 4 (B), 5 (C), 6 (D), 7 (E), 8 (F), 9 (G), 10 (H), 11 (I), 12 (J), 13 (K) and 18 14 (L) respectively.

Table 5. Results of fluorescent emission of acridine orange-ctDNA complex in the presence of compounds.

Compounds	$\lambda_{\text{máxfree}}$ (nm)	$\lambda_{\text{máxon}}$ (nm)	hypochromism (%)	Ksv (L/mol)
3	526	526	3.9	5.9×10^3
4	526	526	12.96	3.8×10^3
5	526	528	17.91	3.0×10^3
6	526	526	60.29	1.80×10^4
7	526	527	74.52	1.88×10^4
8	526	526	100	3.55×10^4
9	526	527	16.81	2.0×10^2
10	526	528	6.95	3.0×10^2
11	526	528	83.71	2.43×10^4
12	526	528	84.52	2.8×10^4
13	526	528	3.5	3.0×10^2
14	526	528	31.59	5.0×10^3

Conclusion

This study investigated the antioxidant activity and interaction with macromolecules of two series of compounds: thiosemicarbazones and thiazoles. These compounds are known for their versatility in biological activities. The results highlighted the significant activity of these compounds both as antioxidant agents and in their ability to interact with DNA and HSA. These findings suggest a promising potential for these molecules as bioactive drug candidates. Furthermore, the ability of these compounds to interact with essential biomolecules such as DNA and HSA suggests potential mechanisms of action that warrant further investigation. This research contributes not only to the understanding of the structure and activity of these compounds but also to the development of potential new therapies for a variety of medical conditions. However, it is important to highlight the need for additional studies, including *in vivo* assays and toxicity analyses, to fully validate the therapeutic potential of these compounds. Ultimately, this study represents an important step towards developing new pharmaceutical agents with significant benefits for human health.

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