Scientific Electronic Archives
Issue ID: Sci. Elec. Arch. Vol. 17 (4)
Jul/Ago 2024
DOI: http://dx.doi.org/10.36560/17420241946
Article link: https://sea.ufr.edu.br/SEA/article/view/1946

ISSN 2316-9281

In silico studies (ADME) and *in vitro* evaluation of the cytotoxic and antimicrobial properties of thiosemicarbazones and thiazole compounds

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Abstract. The thiosemicarbazones and thiazoles are known for their versatility of biological activities, among which we can mention: antioxidant, antimicrobial, anticancer activity and the ability to interact with biological macromolecules, HSA and DNA. This study presented 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 with biological potential against different microorganisms. The *in silico* ADME profile showed that thiosemicarbazones and thiazoles have good oral bioavailability. The cytotoxicity results in J774 macrophages cells showed that the compounds showed toxicity ranging from 49.15 to 61.28 µM for thiosemicarbazones and from 10.75 to 39.76 µM for thiazoles. Finally, all thiosemicarbazones and thiazoles synthesized were also able to inhibit microbial growth. In yeasts of the genus *Candidas* sp. we obtained close results ranging from 12.5 to 50 µg/mL. This study demonstrates that the compounds assessed have the potential to be antimicrobial agents *in vitro*.
Keywords: thiosemicarbazone; thiazole, antimicrobial

Introduction

In the last decades, the difficulty in the treatment of microbial infections has increased, due to the emergence of resistance to several classes of antimicrobial compounds (Vila-Costa.2021; Wang et al. 2020). Microbial resistance becomes a problem because it threatens public health, this characteristic in microorganisms has been driven by the inappropriate use of antimicrobial drugs over the years, which are used in human and animal health (Wang. 2020). According to Vila-Costa et al. (2021) microbial resistance may be linked to several

mechanisms developed by these organisms, such as: gene transfers from one cell to another; degradation of the chemical structure aided by specific enzymes produced by the cell; and activation of membrane efflux pumps.

The World Health Organization (WHO) advocates the importance of surveillance as an essential part of developing alternatives that can combat resistant strains, since these microorganisms are a unique problem, leading to thousands of deaths a year (Murray et al. 2022). The WHO has identified certain microorganisms as a priority for research and development (R&D) of new therapies alternatives, among which are *Acinetobacter baumannii* and *Pseudomonasaeruginosa*. These bacteria represent a high incidence of microbial resistance, which has been worrying many researchers, since few drugs are able to combat them and new effective drugs are not emerging (Coates et al. 2020). Based on these data, there is a need for R&D of new drugs with antimicrobial activities.

The discovery of new drugs goes through several stages, among which the planning and synthesis are at the beginning of the process and pre-clinical in vitro studies corroborate its effectiveness (Leigh et al.2011). The thiosemicarbazones are a group of molecules that have different biological activities and that are used as prototypes for new drugs in medicinal chemistry. The groups found in thiosemicarbazones structures (OH, NH, and CN) contribute to stabilizing polar interactions formed between inhibitors and amino acid residues in the access channel of biological enzymes (Leigh et al.2011).

Sibuh et al. (2021) highlight that, due to their privileged structure, thiosemicarbazones present good availability of binding modes, excellent complexation and variety of coordination modes with deoxyribonucleic acid (DNA), proving to be a target of interest for new thiosemicarbazones prototypes. This class of molecules has been widely reported to perform several biological activities, such as: Antimicrobial, antioxidant, antiparasitic, anticancer (Sibuh et al. 2021; Eğlence-Bakır et al. 2021; Silva et al. 2021; Ahmed et al. 2021).

Although thiosemicarbazones have great potential for biological activities, they are also used as reactive intermediates for the formation of heterocyclic rings such as: thiazolidines, thiazolidionones and thiazoles (Mishra et al. 2015). Heterocyclic compounds play a crucial role in biological systems, in addition to being present in a wide variety of drug candidates such as antibiotic, antitumor, anti-inflammatory, antiviral, antimicrobial, antifungal and antidiabetic characteristics (Kaur Manjal et al 2017).

Babu et al. (2019) understand that the heterocyclic azole compounds are important chemical constructs for the development and improvement of bioactive compounds, highlighting the thiazole nucleus as an important component of drugs used in the clinic that perform a wide versatility of biological assignments. Thiazole is a molecular nucleus metabolized by routine biochemical reactions, without carcinogenic properties, and is capable of performing various biological activities as evidenced by its presence in many biologically active compounds (El-Achkar et al. 2015).

This nucleus has several biological activities described in the literature such as: antimicrobial, anti-inflammatory, antioxidant, anticancer (Sultan et al. 2017; Nastasă et al. 2015; Pund et al. 2022; Oliveira et al. 2021). Thiazole compounds derived from thiosemicarbazones contain Schiff bases (–C=N) in their structure, which are found in numerous bioactive compounds due to their physiological and pharmacological properties, in these compounds the group corroborates with performance in biological activities such as antibacterial and antifungal (Ayati et al. 2015).

Based on these observations, the present study aimed to demonstrate the potential of the new derivatives 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 as drug candidates. To achieve this, the pharmacokinetic properties *in silico* (ADME) were investigated. Furthermore, the compounds underwent various *in vitro* studies, including cytotoxicity testing against a macrophage lineage and assessment of antimicrobial activity.

Material and Methods

Reagents

Reagents and solvents obtained commercially from Sigma-Aldrich, Fluxa, Vetec and Merck were used as listed below: ascorbic acid (CAS 50-81-7), dimethylsulfoxide (DMSO; CAS 67-68-5), MTT (CAS 298-93-1), Griess reagent, resazurin (CAS 62758-13-8). In addition to these, the following antimicrobials were used: gentamicin (Medley), Oxacillin (Medley) Ampicillin+Sulbactam (Novafarma). Mueller Hinton Culture Media (Kasvi) and Medium Rpmi 1640 Medium (CBasalab).

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 proposals were synthesized in three stages, following the methodology of 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 h 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 h 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 thiosemicarbazones 3 and 4. Figure 2 shows the structures of the different thiazoles starting from intermediates 3 and 4 respectively.

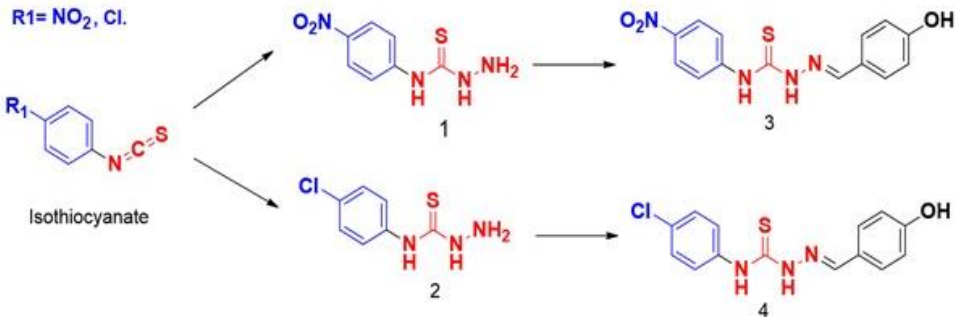


Figure 1. Synthesis of hydroxylated thiosemicarbazones.

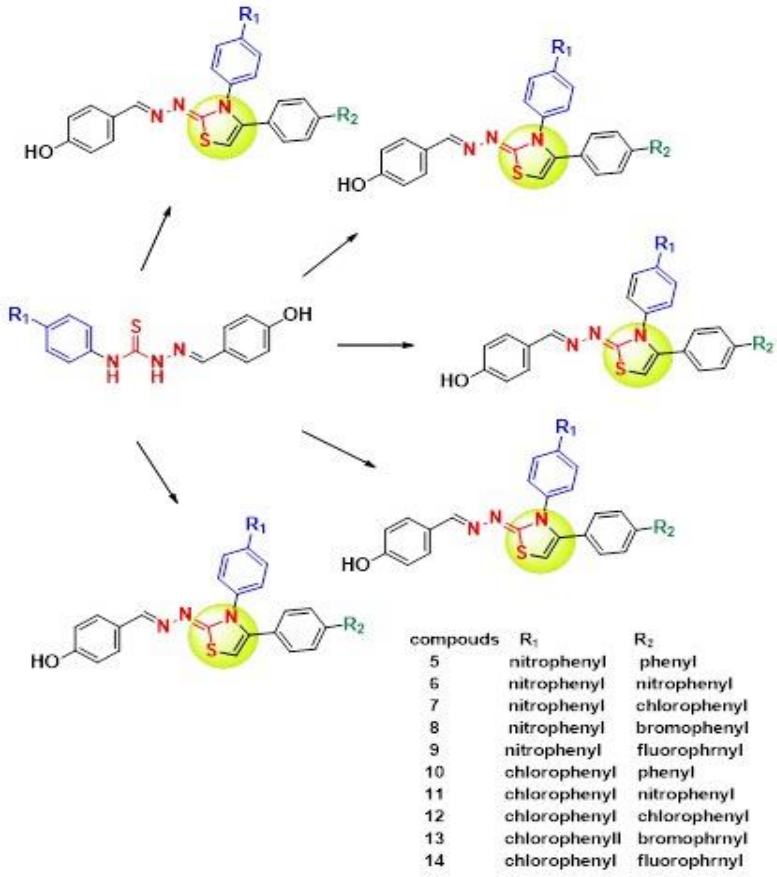


Figure 2. Structures of thiazoles and their substituents

In silico evaluation of theoretical pharmacokinetic and toxicological parameters: absorption, distribution, metabolism and excretion (ADME)

ADME profiles were predicted in order to assess whether the synthesized compounds have good oral availability (Veber et al. 2002; Lipinski et al. 1997). For the analysis of these profiles, SwissADME (http://www.swissadme.ch/) and pkCSM (http://biosig.unimelb.edu.au/pkcsm/prediction) (Daina et al. 2017; Pires et al. 2015) software were used for free. The evaluation parameters were: (1) Total polar surface area (TPSA); (2) LogP; (3) Molecular weight; (4) Number of hydrogen bond acceptors; (5) Number of hydrogen bond donors; (6) permeability in Caco2; (7) Rotatable links; (8) Volume of distribution (VDss); (9) Lipophilicity expressed as log P (C logP) calculations; (10) intestinal absorption; (11) Solubility in aqueous media (LogS); (12) Total clearance or clearance (a measure of the organism's ability to eliminate a drug) and (13) unconsolidated fraction.

Cytotoxicity assays on J774 macrophage cells. and production of nitric oxide

The toxicity tests were carried out according to the methodology proposed by Leite et al. (2019) with modifications. Initially, the compounds were dissolved in with dimethylsulfoxide (DMSO) to obtain a concentration of 1000 µg/mL. Then, they were diluted at the following concentrations: 6.25, 12.5, 25, 50 and 100 µg/mL. Dilutions were performed in RPMI culture medium in order to obtain a DMSO concentration of less than 1%. Finally, all solutions were filtered through 0.22 µm membranes.

The cytotoxicity assay in mammalian cells was evaluated using the MTT 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazoline bromide assay, which is based on the reduction of yellow tetrazolium salts by mitochondrial reductases. of metabolically active cells. Cells grown in RPMI culture medium were seeded in a 96-well plate ($1x10^5$ cells/well) containing different concentrations (6.25, 12.5, 25, 50 and 100 µg/mL) of the compounds. These were incubated at 37 °C in 5% CO_2 for 48 h.

To read the absorbances, the formazan crystals (formed during the tests) were solubilized in DMSO and then the plates were analyzed with a plate reader, Benchmark Plus (Bio-Rad, California, USA) at a wavelength of 490 nm. The concentration capable of causing 50% loss of cell viability (IC_{50}) was determined by non-linear regression analysis of data obtained by SPSS 8.0 software for Windows and converted to micromolar concentration (µM) according to the molecular weight of each compound. Each experiment was performed in technical triplicate and biological duplicate.

During cultivation, the production of nitric oxide was evaluated. The supernatant of the control cultures (untreated) and treated with the compounds in the test concentrations (6.25 to 100 µg/mL) were analyzed using the Griess reagent method. 100 µL of J774 macrophage culture supernatant was used. A1 and incubated with 100 µL of Griess reagent at room temperature for 10 min. Absorbance was determined using a length of 540 nm in a Benchmark Plus reader (Bio-Rad, California, USA).

In vitro antimicrobial activity promoted by thiosemicarbazones and thiazoles

Tested compounds and antimicrobials

For the evaluation of the antimicrobial activity, the 12 synthesized compounds were tested and these were divided into two series. Series I consist of compound number 3 (thiosemicarbazone) and its derivatives 5 to 9 (thiazoles). Series II was formed by compound number 4 (thiosemicarbazone) and its derivatives 10 to 14 (thiazoles). The tests were carried out according to a methodology adapted from Althagafi et al. (2019) and Prajapati et al. (2019) with modifications. Initially, solutions were prepared with the compounds in a concentration of 1000 µg/mL using dimethylsulfoxide (DMSO) at 5% (v/v) as solvent. The solutions were diluted in different concentrations that varied from 150 to 0.58 µg/mL for bacterial strains and from 100 to 0.39 µg/mL for yeasts.

The experimental standards used (positive control) were: the antibiotics Gentamicin, Oxacillin (diluted in sterile water) and Ampicillin+Sulbactam (diluted in phosphate buffer (0.1M, pH, 7.4) defined according to the species. the antifungal fluconazole (diluted in sterilized water) these were filtered through 0.22 µm filters. The results for these antimicrobials were expressed in µg/mL and in µM. The results obtained were compared with the defined breakpoints for defined resistance, intermediate resistance and sensitivity by the Clinical Laboratories Standard Institute (CLSI), document M100 (2017) for bacteria and M60 (2018) for yeast.

Microorganisms and standardization of the inoculum

The microorganisms used in this study were obtained from the Collection and Culture of Microorganisms, from the Antibiotics Department of the Federal University of Pernambuco (UFPEDA). The bacterial strains used were: Staphylococcus aureus UFPEDA-02, *Enterococcus faecalis* UFPEDA-138, *Pseudomonas aeruginosa* UFPEDA-416 and *Candida albicans* yeast strain UFPEDA-1007. In addition to these strains, clinical isolates of bacteria identified as Enterococcus faecalis UFPEDA-69, *Pseudomonas aeruginosa* UFPEDA-261, *Staphylococcus aureus* UFPEDA-709, *Acinetobacter baumannii* UFPEDA-1024 and yeast strains also isolated from clinical samples were included: *Candida guilliermondii* UFPEDA-6390 and *Candida glabrata* UFPEDA-6393.

Standardization of the inoculum for both bacteria and yeast were performed following the CLSI recommendations (CLSI 2018: CLSI 2017). Isolated colonies were inoculated into Mueller Hinton broth to achieve turbidity equivalent to Mc Farland's 0.5 scale (1.5×10^8 colony forming units/mL - CFU/mL). Then, the bacterial suspensions were diluted in saline solution to obtain the final inoculum (1.5×10^7

CFU/mL). As for the inoculum for yeasts, isolated colonies were inoculated in sterile saline solution until turbidity equivalent to 0.5 of the Mc Farland scale (1.0 x 10^6 to 5.0 x 10^6 CFU/mL).

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC/CFM)

The MIC of the synthesized compounds, as well as of Gentamicin, Oxacillin and Ampicillin+Sulbactam, against the bacteria, was determined using the microdilution technique in Mueller Hinton broth, using 96-well plates, following the CLSI recommendations, according to the document M100 (CLSI2017). After the microdilution of the compounds in the plate, aliquots of previously prepared and standardized inoculum of the bacteria were added, in order to obtain a final concentration of 1.5 x 10^5 CFU/mL in each well. The plates were incubated at 37 °C for 24 h.

The MIC of the synthesized compounds and fluconazole, against yeast strains, was also determined using the microdilution technique in RPMI 1640 broth, in a 96-well plate, following the recommendations of the CLSI document M60 (CLSI 2018). After microdilution of the compounds on the plate, aliquots of the previously prepared yeast inoculum were added to the wells, as recommended, in order to obtain a concentration of 2.5 x 10^3 CFU/mL per well. The antifungal fluconazole was included as a positive control. The plates were incubated at 30 °C for 48 h. After the incubation time, the microplates were developed using 20 µL of 0.01% resazurin (blue indicator dye) in each well. The microplates were incubated for 4 h at 37 °C. Maintenance of blue color is interpreted as inhibition of microbial growth, while pink wells indicate growth. The MIC is considered as the concentration of the last well of the dilution that showed the blue color. That is, the lowest concentration of each compound capable of inhibiting microbial growth on a plate.

In order to determine MBC or CMF, after 24 h (bacteria) or 48 h (yeasts) of incubation of the plates, 10 µL were collected from each well that showed visible inhibition of microbial growth, which were inoculated on Mueller-Hinton agar (for bacteria) and on Sabouraud agar (for yeast). The plates were incubated at 37 °C for 24 or 48 h and the colonies were counted. The MBC or CMF was defined as the lowest concentration of each compound that resulted in a 99.9% reduction in microbial growth when compared to an untreated growth control. Compounds were classified as bacteriostatic/fungistatic when the MBC/MIC ratio was ≥ 8 and bactericidal/fungicide when the MBC/MIC ratio was ≤ 4 (Jones 2006).

Statistical analysis

For significance analysis, *p*-values < 0.05 were considered significant, evaluated using the ANOVA test and Tukey's post-test using the GraphPad Prism 5.0 program (Graphpad, California, USA) for Windows (test version).

Results and discussion

For the pharmaceutical industry, the evaluation and prediction of pharmacokinetic parameters related to absorption, distribution, metabolism and excretion (ADME) *in silico* is of great importance (Kumar et al. 2021). This methodology accelerates the process of discovery and development of new drug candidates (Kar et al. 2021). The two methodologies commonly used to assess the oral bioavailability potential of a drug are based on the rules of Lipinski and Veber (Veber et al. 2002; Lipinski et al. 1997).However, other studies of prediction of pharmacokinetic parameters can be performed, as is the case of the parameters proposed by Pires et al. (2015). The ADME results obtained for each of the thiosemicarbazonic compounds (3 and 4) and thiazoles (5 to 14) in silico were presented in Table 1 of the supplementary material.

According to the results obtained from the *in silico* approach, it was possible to observe that all compounds evaluated present desirable molecular properties. These obeyed Lipinski's rule of 5, that is, molecular weight ≤ 500g/mol; number of hydrogen acceptors (ALH) ≤ 10, number of hydrogen donors (DLH) ≤ 5 and lipophilicity coefficient (cLogP) ≤ 5, in addition to having obeyed Veber's rule, rotatable bonds ≤ 10 and polar surface area (TPSA) ≤ 140 $Å^2$. TPSA is a parameter related to passive molecular transport across membranes and allows the prediction of human intestinal absorption and blood-brain barrier passage (Pires et al. 2015). Therefore, TPSA values ≤ 140 $Å^2$ are considered desirable to indicate good gastrointestinal absorption and blood-brain barrier passage (Ertl et al. 2020).

These results suggest that these compounds theoretically have good oral bioavailability and differences in their bioactivity cannot be attributed to this property (Pires et al. 2015). Other studies presented a profile similar to ours, that is, they followed the rules of Lipinski and Veber. Among these works we can mention those carried out by Matsa et al. (2019) and Ferreira et al. (2021) evaluating different thiosemicarbazones and thiazoles.

The low solubility of different drugs in aqueous media is a problem not only for the development of formulations, but also for absorption (Pires et al. 2015). For a drug to be absorbed, it must be part of an aqueous solution (Sharma et al. 2010). Therefore, the solubility profile in aqueous medium (LogS) was evaluated. LogS values can vary in the following order: insoluble <-10 < slightly soluble < - 6 < moderately < - 4 < soluble < -2 < very soluble < 0 < highly soluble. The *in silico* results showed that compounds 3 and 4 have moderate solubility, while the other compounds are classified as poorly soluble.

The process of evaluating the oral absorption of compounds is quite complex and requires several *in vitro* methods, such as the monolayer system of Caco-2 cells derived from colon adenocarcinoma (Pires et al. 2015). Because it is based on human

cells, it is widely used in intestinal permeability studies. According to the literature, compounds can be classified according to Caco-2 permeability. Thus, compounds with values < 1.10^{-6} cm·s^{-1} are poorly absorbed, between 1 and 10.10^{-6} cm·s^{-1} are moderately absorbed and > 10.10^{-6} cm·s^{-1} are well absorbed (Pires et al. 2015). Thus, compounds 4 and 14 are classified as moderately absorbed, while the other compounds are classified as poorly absorbed. Another parameter evaluated was gastrointestinal absorption. This parameter can be classified as follows: poorly absorbed (0 - 20%), moderately absorbed (20 - 70%) and well absorbed (70 - 100%) drugs. The compounds in this study have absorption values greater than 70%, indicating that they can be well absorbed (Pires et al. 2015).

The volume of distribution (VDss) was also evaluated, this parameter describes the extent of drug distribution. VDss values (log VDss) > 0.45 indicate that the drug will be distributed into the tissue. Log VDss values <−0.15 indicate that the drug will be distributed into plasma (Lambertucci et al 2018). The compounds showed varying values of VDss compounds 3, 4,5,6,7,8,9 and 11 show distribution in plasma. On the other hand, compounds 10, 12, 13 and 14, even with a low VDss value, that is, less than 0.45, tend to be distributed in the tissues. In addition, it was observed that all compounds presented had a low fraction not bound to proteins (Pires et al. 2015). The high lipophilicity of these compounds (as seen by the LogS values) leads to a high degree of binding to plasma proteins and thus the presence of low levels of unbound drug at the site of infection (Pires et al. 2015).

Finally, there is the total clearance. A drug with a high total release value will present a fast excretion process and consequently a shorter action time (Moraes et al 2018). This profile was not observed in the compounds under study, since the release values ranged from -0.447 – 0.037 log mL/min/kg. In general, these predictive results show that these compounds may be potential drug candidates.

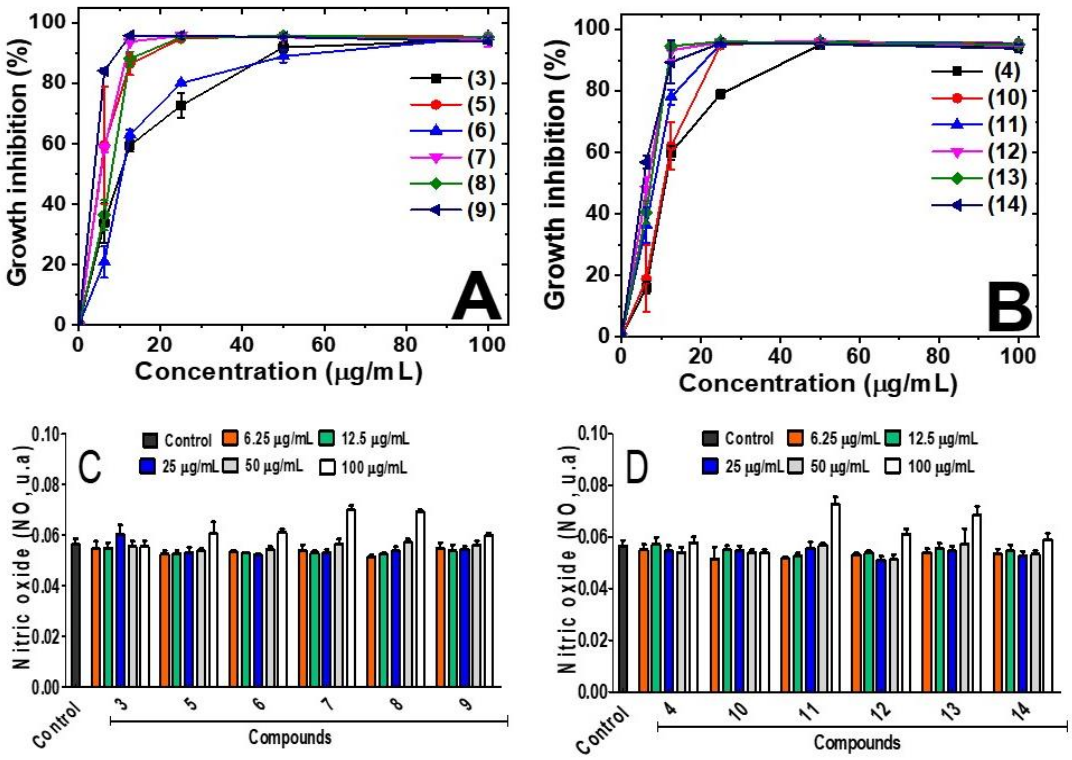


Figure 3: Cytotoxicity curves against J774 macrophage cells. A1 promoted by the synthesized compounds. Curves obtained for compounds 3, 5, 6, 7, 8 and 9 (A) and curves obtained for compounds 4, 10, 11, 12, 13 and 14 (B) respectively. Levels of nitric oxide produced by macrophages in contact with compounds 3, 5, 22 6, 7, 8 and 9 (C) and by compounds 4, 10, 11, 12, 13 and 14 respectively

The results presented by Figures 3A and 3B show that the compounds evaluated promote an increase in cytotoxicity with increasing concentration. Furthermore, Figures 3C and 3D show that a non-significant increase in oxide occurred at the highest concentrations. This increase is associated with the cytotoxicity promoted by the compounds. With the results from the cytotoxicity curves (Figure 3A and 3B) it was possible to determine the IC_{50} (concentration capable of inhibiting cell growth by 50%) as shown in Table S6 of the supplemental material.

The thiosemicarbazones presented IC_{50} ranging from 49.15 to 61.28 µM less toxic when compared to the thiazole compounds which presented IC_{50} ranging from 10.75 to 39.76 µM. These results show that the cyclization of thiosemicarbazone compounds into thiazoles promoted an increase in

cytotoxicity against macrophage cells. The literature presents different values of cytotoxicity against J774 macrophage cells, thiosemicarbazones and thiazoles. Among the works we can mention those listed by: Aliança et al.(2017) evaluating the cytotoxicity of phthalimido-thiazole derivatives obtained IC_{50} ranging from 112.9 to 1582.9 µM and Jacob et al. (2021) evaluating indole-based thiosemicarbazone compounds obtained IC_{50} ranging from 7.0 to values greater than 75 µM. the results presented show that the IC_{50} values vary according to the chemical structure.

Tables 1 and 2 present the results of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) promoted by the compounds, as well as the MIC of the positive controls.

The compound thiosemicarbazone 3 (Table 1) showed antibacterial activity against strains of *S. aureus* UFPEDA-02, *A. baumannii* UFPEDA-1024, *P. aeruginosa* UFPEDA-26 and *P. aeruginosa* UFPEDA-416, with MIC ranging from 75 to 150 µg/mL and MBC ranging from 75 to > 150 µg/mL, but did not show activity against the other bacterial strains. Similar behavior to that presented by compound 3, was also observed in its compounds derived from 5 to 9, but only compound 9 was able to inhibit the growth of *E. faecalis* UFPEDA-13802. All thiazole compounds derived from compound 3 showed activity against *A. baumannii* UFPEDA-1024, *P. aeruginosa* UFPEDA-261 and *P. aeruginosa* UFPEDA-416 strains, with emphasis on compound 5, which showed a MIC of 37.5 µg/mL against of *P. aeruginosa* UFPEDA-261 and *P. aeruginosa* UFPEDA-416 strains.

The compound thiosemicarbazone 4 (Table 2) showed activity against all strains tested, both for gram-positive and gram-negative bacteria, with MIC and MBC ranging from 37.5 to 150 µg/mL, with emphasis on activity against of the *S. aureus* strain UFPEDA-709, since this strain was characterized as methicillin resistant *S. aureus* (MRSA) (MIC oxacillin - 512 µg/mL). The thiazole compounds derived from compound 4 showed activity against most strains tested with MIC ranging from 37.5 to > 150 µg/mL, especially compound 12, which showed antibacterial activity against all strains, with the exception of *E. faecalis* UFPEDA-69, in addition this compound demonstrated a MIC of 37.5 µg/mL for the *P. aeruginosa* UFPEDA-261 strain.

A study developed by Khan & Asiri (Khan et al. 2018) reported antibacterial activity of thiosemicarbazone steroid derivatives against Gram positive and Gram-negative bacteria, with MIC ranging from 32 to 128 µg/mL, corroborating the data found in this study, since the compounds tested showed a similar spectrum of action. Another study demonstrated the antibacterial activity of 17 compounds derived from chlorophenylthiosemicarbazone against Gram positive bacteria (*S. aureus, S. epidermidis, Bacillus subtilis, B. cereus* and *Micrococcus luteus*), with MICs ranging from 3.91 to 500 µg/mL, however no activity was identified in this study against Gram negative bacteria (Trotsko et al. 2018).

The thiazole compounds, derived from compounds 3 and 4, showed promising results, since all were able to inhibit the *in vitro* growth of *P. aeruginosa* UFPEDA-261, *P. aeruginosa* UFPEDA-416 and *A. baumannii* UFPEDA-1024 strains, where compounds 5 and 12 stand out with which they presented the lowest MICs against *P. aeruginosa* UFPEDA-261.

According to the World Health Organization (WHO) list, the species *P. aeruginosa* and *A. baumanii* are considered priorities for the development of new antibiotics with activity against this species, since they have important resistance profiles (WHO 2017). For the *S. aureus* UFPEDA-709 strain, compounds 12, 13 and 14 are highlighted, which showed activity with MIC ranging from 75 to 150 µg/mL, demonstrating important activity, since this strain is resistant to methicillin (SARM), which makes it resistant to multiple drugs, especially all β-lactam antibiotics and cephalosporins, with few therapeutic options available for treatment (Nag et al. 2020).

Most of the compounds tested showed bactericidal activity verified by the MBC/MIC ratio, especially compound 4 showed bactericidal characteristics for all strains tested. All thiazole compounds of the proposed series showed bactericidal activity against *P. aeruginosa* UFPEDA-261 and *P. aeruginosa* UFPEDA-416, with MBC/MIC ratios ranging from 1 to 2.

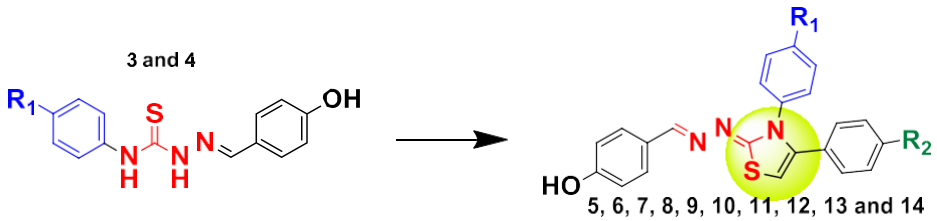
The compound thiosemicarbazone 3 (Table 1) showed antibacterial activity against strains of *S. aureus* UFPEDA-02, *A. baumannii* UFPEDA-1024, *P. aeruginosa* UFPEDA-26 and *P. aeruginosa* UFPEDA-416, with MIC ranging from 75 to 150 µg/mL and MBC ranging from 75 to > 150 µg/mL, but did not show activity against the other bacterial strains. Similar behavior to that presented by compound 3, was also observed in its compounds derived from 5 to 9, but only compound 9 was able to inhibit the growth of *E. faecalis* UFPEDA-13802. All thiazole compounds derived from compound 3 showed activity against *A. baumannii* UFPEDA-1024, *P. aeruginosa* UFPEDA-261 and *P. aeruginosa* UFPEDA-416 strains, with emphasis on compound 5, which showed a MIC of 37.5 µg/mL against of *P. aeruginosa* UFPEDA-261 and *P. aeruginosa* UFPEDA-416 strains.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (expressed in µg/mL and µM) for the compound thiosemicarbazone 3 and its corresponding thiazoles 5, 6, 7, 9 and 9 respectively.

Compounds		Gram positive bacteria								Gram negative bacteria					
		E. faecalis UFPEDA-69		*E. faecalis* UFPEDA-138		*S. aureus* UFPEDA-02		*S. aureus* UFPEDA-709		*A. baumannii* UFPEDA-1024		*P. aeruginosa* UFPEDA-261		*P. aeruginosa* UFPEDA-416	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
3	µg/mL	>150	>150	>150	>150	150	>150	>150	>150	75	> 150	75	75	150	150
	µM	> 475	> 475	> 475	> 475	475	> 475	> 475	> 475	237	> 475	237	237	475	475
5	µg/mL	>150	>150	>150	>150	> 150	>150	>150	>150	75	150	37.5	75	37.5	75
	µM	>360	>360	>360	>360	>360	>360	>360	>360	180	360	90	180	90	180
6	µg/mL	>150	>150	>150	>150	> 150	>150	>150	>150	150	> 150	75	75	75	75
	µM	> 325	> 325	> 325	> 325	> 325	> 325	> 325	> 325	360	> 325	163	163	163	163
7	µg/mL	>150	>150	>150	>150	> 150	>150	>150	>150	150	150	75	75	75	75
	µM	> 333	> 333	> 333	> 333	> 333	> 333	> 333	> 333	325	333	167	167	167	167
8	µg/mL	>150	>150	>150	>150	> 150	>150	>150	>150	150	150	75	75	75	75
	µM	> 303	> 303	> 303	> 303	> 303	> 303	> 303	> 303	333	303	152	152	152	152
9	µg/mL	>150	>150	150	150	> 150	>150	>150	>150	150	150	75	75	75	75
	µM	> 346	> 346	346	346	> 346	> 346	> 346	> 346	303	346	173	173	173	173
Amp+Sulbac	µg/mL	-		-		-		-		16 (I)		-		-	
	µM	-		-		-		-		26		-		-	
Gentamicin	µg/mL	8 (S)		8 (S)		0.25 (S)		-		-		0.5 (S)		0.5 (S)	
	µM	17		17		0.5		-				1.0		1.0	
Oxacillin	µg/mL	-		-		-		512 (R) - MRSA		-		-		-	
	µM	-		-		-		1275		-		-		-	

Legend: Amp+Sulbac – Ampicillin combined with Sulbactam; R - Resistant; S – Sensitive; I - Intermediate resistance

Table S1. *In silico* estimation of the pharmacokinetic parameters for the thiosemicarbazones and thiaozoles synthesized in this study determined by the SwissADME and pkCSM platforms.



Compounds	Substituents		MW (g/mol)[a]	HBA[b]	HBD[c]	LogP[d]	TPSA (Å)[f]	Rotatable bonds[g]	LogS[e]	Caco-2 perm.[h]	Int.abs. (%)[i]	VDss (logL/kg)[j]	Fract. Unb.[k]	Totalclearence (logmL/min/kg)[l]
	R1	R2												
3	-NO2	-	316.34	4	3	2.43	135.56	6	-5.11	0.914	78.79	-0.159	0.022	-0.297
4	-Cl	-	305.78	2	3	2.80	88.74	5	-4.97	1.367	85.25	-0.188	0.067	-0.447
5	-NO2	-H	416.45	5	5	2.83	123.94	5	-7.36	0.569	100	-0.3	0.22	0.037
6	-NO2	-NO2	461.45	7	1	1.98	169.76	6	-8.15	0.431	97.156	-0.544	0.224	-0.057
7	-NO2	-Cl	450.90	5	1	3.31	123.94	5	-8.02	0.486	100	-0.234	0.222	-0.093
8	-NO2	-Br	495.35	5	1	3.42	123.94	5	-8.08	0.488	100	-0.222	0.222	-0.115
9	-NO2	-F	434.44	6	1	3.20	123.94	5	-7.47	0.558	100	-0.405	0.247	-0.223
10	-Cl	-H	405.90	3	1	4.27	78.12	4	-7.23	0.967	89.81	0.027	0.249	-0.114
11	-Cl	-NO2	450.90	5	1	3.31	123.94	5	-8.02	0.503	100	-0.243	0.226	-0.209
12	-Cl	-Cl	440.35	3	1	4.74	78.12	4	-8.71	0.958	89.00	0.116	0.254	-0.245
13	-Cl	-Br	484.80	3	1	4.85	78.12	4	-7.95	0.956	88.94	0.131	0.253	-0.266
14	-Cl	-F	423.89	4	1	4.64	78.12	4	-7.33	1.005	89.99	0.037	0.285	-0.374

a. SwissADME Molecular Weight; b. SwissADME Number H-bonds acceptors; c. SwissADME Number H-bonds donors; d. SwissADME origuchi logo foctanol – water partition coefficient; e. SwissADME Ali log of aqueous solubility; f. SwissADME calculation of Topological Polar Surface Area (TPSA); g. SwissADME Number rotatable bonds; h. pkCSM prediction of Caco-2 cell permeability as an estimation of absorption at the human intestinal mucosa (logPapp in 10-6cm/s); i. pkCSM prediction of the proportion of compound absorption through the human small intestine; j. pkCSM prediction of the log of steady state volume of distribution (VDss); k. pkCSM prediction of compound fraction unbound in plasma (not bound to serum proteins); l. pkCSM prediction of the log of total drug clearance.

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (expressed in µg/mL and µM respectively) of the compound thiosemicarbazone 4 and its corresponding thiazoles 10, 11, 12, 13 and 14 respectively.

Compounds		Gram positive bacteria								Gram negative bacteria					
		E. faecalis UFPEDA-69		*E. faecalis* UFPEDA-138		*S. aureus* UFPEDA-02		*S. aureus* UFPEDA-709		*A. baumannii* UFPEDA-1024		*P. aeruginosa* UFPEDA-261		*P. aeruginosa* UFPEDA-416	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
4	µg/mL	150	150	37.5	150	37.5	150	37.5	75	37.5	37.5	37.5	75	37.5	75
	µM	495	495	124	495	124	495	124	247	124	124	124	247	124	247
10	µg/mL	> 150	> 150	150	150	> 150	> 150	> 150	> 150	150	150	75	75	75	75
	µM	> 370	> 370	370	370	> 370	> 370	> 370	> 370	370	370	185	185	185	185
11	µg/mL	> 150	> 150	150	150	> 150	> 150	> 150	> 150	150	150	75	75	75	75
	µM	> 333	> 333	333	333	> 333	> 333	> 333	> 333	333	333	167	167	167	167
12	µg/mL	> 150	> 150	150	150	150	> 150	75	150	150	150	37.5	75	75	75
	µM	> 342	> 342	342	342	342	> 342	171	342	342	342	85	171	171	171
13	µg/mL	> 150	> 150	> 150	> 150	150	150	75	150	75	> 150	75	75	37.5	75
	µM	> 311	> 311	> 311	> 311	311	311	155	311	155	> 311	155	155	78	155
14	µg/mL	> 150	> 150	> 150	> 150	150	> 150	75	150	150	> 150	75	75	75	75
	µM	> 355	> 355	> 355	> 355	355	> 355	177	355	355	> 355	177	177	177	177
Amp+Sulbac	µg/mL	-		-		-		-		16 (I)		-		-	
	µM	-		-		-		-		26		-		-	
Gentamicin	µg/mL	8 (S)		8 (S)		0.25 (S)		-		-		0.5 (S)		0.5 (S)	
	µM	17		17		0,5		-				1,0		1.0	
Oxacillin	µg/mL	-		-		-		512 (R) - MRSA		-		-		-	
	µM	-		-		-		1275		-		-		-	

Legend: MIC – Minimum inhibitory concentration; MBC – Minimum bactericidal concentration; Amp+Sulbac – Ampicillin combined with Sulbactam; R - Resistant; S – Sensitive; I - Intermediate resistance

The compound thiosemicarbazone 4 (Table 2) showed activity against all strains tested, both for gram-positive and gram-negative bacteria, with MIC and MBC ranging from 37.5 to 150 µg/mL, with emphasis on activity against of the *S. aureus* strain UFPEDA-709, since this strain was characterized as methicillin resistant *S. aureus* (MRSA) (MIC oxacillin - 512 µg/mL). The thiazole compounds derived from compound 4 showed activity against most strains tested with MIC ranging from 37.5 to > 150 µg/mL, especially compound 12, which showed antibacterial activity against all strains, with the exception of *E. faecalis* UFPEDA-69, in addition this compound demonstrated a MIC of 37.5 µg/mL for the *P. aeruginosa* UFPEDA-261 strain.

Khan & Asiri (2018) reported antibacterial activity of thiosemicarbazone steroid derivatives against Gram positive and Gram-negative bacteria, with MIC ranging from 32 to 128 µg/mL, corroborating the data found in this study, since the compounds tested showed a similar spectrum of action. Another study demonstrated the antibacterial activity of 17 compounds derived from chlorophenylthiosemicarbazone against Gram positive bacteria (*S. aureus, S. epidermidis, Bacillus subtilis, B. cereus* and *Micrococcus luteus*), with MICs ranging from 3.91 to 500 µg/mL, however no activity was identified in this study against Gram negative bacteria (Trotsko et al. 2018).

The thiazole compounds, derived from compounds 3 and 4, showed promising results, since all were able to inhibit the *in vitro* growth of *P. aeruginosa* UFPEDA-261, *P. aeruginosa* UFPEDA-416 and *A. baumannii* UFPEDA-1024 strains, where compounds 5 and 12 stand out with which they presented the lowest MICs against *P. aeruginosa* UFPEDA-261.

According to the World Health Organization (WHO) list, the species *P. aeruginosa* and *A. baumanii* are considered priorities for the development of new antibiotics with activity against this species, since they have important resistance profiles (WHO 2017). For the *S. aureus* UFPEDA-709 strain, compounds 12, 13 and 14 are highlighted, which showed activity with MIC ranging from 75 to 150 µg/mL, demonstrating important activity, since this strain is resistant to methicillin (SARM), which makes it resistant to multiple drugs, especially all β-lactam antibiotics and cephalosporins, with few therapeutic options available for treatment (Nag et al. 2020).

Most of the compounds tested showed bactericidal activity verified by the MBC/MIC ratio, especially compound 4 showed bactericidal characteristics for all strains tested. All thiazole compounds of the proposed series showed bactericidal activity against *P. aeruginosa* UFPEDA-261 and *P. aeruginosa* UFPEDA-416, with MBC/MIC ratios ranging from 1 to 2.

Determination of the minimum inhibitory concentration (MIC) and minimum fungicide (CFM)

Table 3 presents results of *in vitro* antifungal activity promoted by the compounds obtained in this study.The results presented in Table 3 shows that thiosemicarbazone (compound 4) showed antifungal activity, with MIC ranging from 12.5 to 50 µg/mL, with emphasis on the activity against the *C. albicans* UFPEDA-1007 strain, where the compound showed a MIC of 12.5 µg/mL, this result when compared to the MIC of fluconazole (16 µg/mL) for this strain, shows that the activity of compound 4 is promising, since this strain has shown to have reduced sensitivity to fluconazole.

It is also noteworthy that compound 4 was the only one that showed activity against the *C. glabrata* UFPEDA-6393 strain with a MIC of 50 µg/mL, with the other compounds being inactive against this strain.

The results presented in Table 3 shows that thiosemicarbazone (compound 4) showed antifungal activity, with MIC ranging from 12.5 to 50 µg/mL, with emphasis on the activity against the *C. albicans* UFPEDA-1007 strain, where the compound showed a MIC of 12.5 µg/mL, this result when compared to the MIC of fluconazole (16 µg/mL) for this strain, shows that the activity of compound 4 is promising, since this strain has shown to have reduced sensitivity to fluconazole (Table S9). It is also noteworthy that compound 4 was the only one that showed activity against the *C. glabrata* UFPEDA-6393 strain with a MIC of 50 µg/mL, with the other compounds being inactive against this strain.

Results of antifungal activity of derivatives structurally similar to the thiosemicarbazones and thiazoles under study also stood out against *C. albicans* [45]. Where they demonstrate activity against this species presenting MICs of the compounds ranging from 6.25 to 25 µg/mL. In this study, we obtained similar results ranging from 12.5 to 50 µg/mL.

The antifungal activity of thiazoles derived from compounds 3 and 4 was very promising in inhibiting the growth of *C. albicans* UFPEDA-1007 and *C. guilliermondii* UFPEDA-6390, especially compound 9, which presented the lowest MICs against these two strains, when compared with the MICs of the other thiazole compounds. In addition, compounds 3 and 4 showed antifungal activity against *C. guilliermondii* UFPEDA-6390 with MICs ranging from 12.5 to 100 µg/mL.

Table 3. Antifungal activity promoted by the compounds synthesized in this work against different yeast species. the results of minimum inhibitory concentration and minimum fungicidal concentration were expressed in µg/mL and µM, respectively.

Compounds	*C. albicans* UFPEDA-1007				*C. glabrata UFPEDA-6393*				*C. guilliermondii UFPEDA*-6390			
	MIC		CFM		MIC		CFM		MIC		CFM	
	µg/mL	µM	µg/mL	µM	µg/mL	µM	µg/mL	µM	µg/mL	µM	µg/mL	µM
3	> 100	> 316	> 100	> 316	> 100	> 316	> 100	> 316	> 100	> 316	> 100	> 316
4	12.5	41	100	330	50	165	50	165	12.5	41	> 100	> 330
5	50	120	> 100	> 240	> 100	> 240	> 100	> 240	50	120	100	240
6	> 100	> 217	> 100	> 217	> 100	> 217	> 100	> 217	25	54	50	108
7	> 100	> 222	> 100	> 222	> 100	> 222	> 100	> 222	100	222	> 100	> 222
8	25	51	> 100	> 202	> 100	> 202	> 100	> 202	50	101	> 100	> 202
9	25	58	> 100	> 230	> 100	> 230	> 100	> 230	12.5	29	50	115
10	> 100	> 247	> 100	> 247	> 100	> 247	> 100	> 247	100	247	> 100	> 247
11	> 100	> 222	> 100	> 222	> 100	> 222	> 100	> 222	25	56	> 100	> 222
12	50	114	> 100	> 228	> 100	> 228	> 100	> 228	25	57	50	114
13	50	104	> 100	> 207	> 100	> 207	> 100	> 207	50	104	100	207
14	50	118	> 100	> 236	> 100	> 236	> 100	> 236	25	59	> 100	> 236
Fluc.	16 (SSD)	52			8 (S)	26			4 (S)	13		

Legend: MIC – Minimum inhibitory concentration; CFM – Minimum fungicidal concentration; S- Sensitive; SSD- Dose-dependent sensitivity; Fluc. – Fluconazole

Relationship chemical structure and biological activity (SAR) of thiosemicarbazones and thiazoles derivatives against microorganisms under study

We observed that the thiosemicarbazones and thiazoles derivatives showed a better response in the antibacterial activity for Gram negative, *A. baumannii* UFPEDA-1024, *P. aeruginosa* UFPEDA-261 and *P. aeruginosa* UFPEDA-416. As for the antifungal activity, we highlight *C. albicans* UFPEDA-1007 and especially *C. guilliermondii* UFPEDA-6390. In this way, the relationship between the chemical structure and the biological activity (SAR) of the derivatives reveals that the non-classical bioisosterism strategy between thiosemicarbazones and thiazoles were responsible for the electronic and volume effects of the substituents, which culminated in an antimicrobial profile and promising antifungal.

The 4-NO_2-phenyl group of thiosemicarbazone 3 has an -I and -M effect being a strongly electron-withdrawing group. The 4-Cl-phenyl group of thiosemicarbazone 4, on the other hand, presents the -I and +M effects, being considered a weak electron withdrawer. These differences in effects were not relevant to the increase in the lipophilicity of the compounds, with LogP values of 2.43 and 2.80, respectively. However, they led to distinct bactericidal effects, whereas compound 3 was active only for strains of *A. baumannii* UFPEDA-1024, *P. aeruginosa* UFPEDA-26 and *P. aeruginosa* UFPEDA-416, with MIC ranging from 75 µg/mL and MBC ranging from 75 to > 150 µg/mL. Compound 04 showed activity against *E. faecalis* UFPEDA-138, *S. aureus* UFPEDA-02, *S. aureus* UFPEDA-709, *A. baumannii* UFPEDA-1024, *P. aeruginosa* UFPEDA-261, *P. aeruginosa* UFPEDA-416 with MIC and MBC ranging from 37.5 to 150 µg/mL.

The thiazole derivatives 5 to 9, derived from thiosemicarbazone 3, in addition to the 4-NO_2 and 4-OH substituents already existing in the molecule, when cyclized with substituted acetophenones, add in their structure at C4 of the thiazole plus an unsubstituted phenyl ring or with different substituents in position 4 (NO_2, Br, Cl and F). The absence of a phenyl substituent on C4 - thiazole, compound 5, and the presence of a strongly electron-withdrawing group o (-NO_2) in compound 6, led to subtle changes in the lipid solubility of the molecules. Compound 5 was less lipid soluble than 6, with the compounds having LogP values of 2.83 and 1.98, respectively. Despite this small variation in LogP, compound 5 was more effective with MIC of 37.5 µg/mL against *P. aeruginosa* UFPEDA-261 and *P. aeruginosa* UFPEDA-416 strains, while compound 6 for these same microorganisms showed MIC ranging from 75 to 150 µg/mL.

In contrast, in thiazoles from 7 to 9, and the insertion of halogens in position 4 of the phenyl C4 of the thiazole. did not affect the pharmacokinetic properties of compounds that were preserved within the desired ADME parameters. It was only possible to observe a decrease in lipid solubility in relation to compounds 5 and 6. The -I and +M effects of the halogens in this portion of the molecule did not contribute to an improvement in the bactericidal activity of this series of compounds, showing MICs ranging from 75 to 150 µg/mL.

On the other hand, thiazole derivatives 10 to 14, derived from thiosemicarbazone (compound 4), showed activity against most strains tested with MIC ranging from 37.5 to > 150 µg/mL. It is possible to highlight the compound 12 substituted with 4-Cl-phenyl of the C4-thiazole having MIC of 37.5 µg/mL for the *P. aeruginosa* UFPEDA-261 strain and for the compound 13 substituted with 4-Br-phenyl of the C4-thiazole having MIC of 37.5 µg/mL for the *P. aeruginosa* UFPEDA-416 strain. The insertion of these halogens in the molecules described does not effectively determine their pharmacological activity, since the intrinsic characteristics of the molecules can be influenced and it is a result of the environment in which the molecule is inserted and interacts (Victorio&Andricopulo 2008).

The response of antifungal activity to these compounds reinforces the potential of thiosemicarbazone 4 as a promising antimicrobial. The presence of halogens with -I and +M effects on phenyl seems to lead to an improvement in antifungal activity. The 4-Cl-phenyl present in 04 provided an MIC ranging from 12.5 to 50 µg/mL against different *Candida* species. Highlighting the inhibition against *C. albicans* UFPEDA-1007, where the compound had an MIC of 12.5 µg/mL, being better than fluconazole, the reference drug, which had a MIC of 16 µg/mL.

The antifungal activity of thiazoles derived from thiosemicarbazones (compounds 3 and 4) were promising in inhibiting the growth of *C. albicans* UFPEDA-1007 and especially *C. guilliermondii* UFPEDA-6390, which had MIC ranging from 12.5 to 100 µg/mL. With emphasis on compound 09 that presented MIC of 25 µg/mL and 12.5 µg/mL against these two strains respectively. Compound 9 when compared with thiosemicarbazone (compound 4), in molar concentration it is noticed that a smaller molar amount of compound was needed to reach the same stage of inhibition. While for compound 4 41 µM of product was needed to reach MIC of 12.5 µg/mL in *C. guilliermondii*UFPEDA-6390, 9 used only 29 µM of the product and obtained the same MIC in the same fungus.

Conclusion

The results of the *in silico* study (ADME) showed that the compounds have good oral availability, obeying the rules of Lipinski and Veber. In relation to the toxicity tests against animal cells, the thiosemicarbazones promoted less toxicity. The results of antimicrobial activity showed that thiosemicarbazone (compound 4) stood out in the proposed *in vitro* activities, presenting a broad spectrum of antimicrobial activity (showing greater activity against yeast fungi), at a non-toxic concentration for J774 macrophage cells. However, compounds 12, 13 and 14 were the final compounds that showed the best *in vitro* activities compared to

oxacillin, this potential is more evident in the fight against resistant strains of *S. aureus*.

Acknowledgment

This study was supported by Fundação de Amparo à Pesquisa do Estado de Pernambuco - FACEPE (Process APQ-0498-4.03/19), Research Fellowship - FACEPE (Process BFP-0038-04.03/21) and National Council Scholarship of Scientific and Technological Development - CNPq (Process 306865 /612020-3).

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