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Antitumor and antileishmanial activities of limonene-thiosemicarbazones bearing heterocycles nucleous

Atividades antitumoral e antileishmania de limoneno-tiossemicarbazonas contendo núcleos heterocíclicos

Actividades antitumoral y antileishmanial de limoneno-tiosemicarbazonas con núcleo heterocíclico

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Abstract
In the present study, we provided the synthesis of a series of *R*-(+)- and *S*-(-)-limonene-based thiosemicarbazones containing different pentacyclic heterocyclic nucleus moiety focused in the search of novel antitumor and antileishmanial agents. In the antitumor assay, the derivative imidazole of *S*-(-)-limonene 8 was the most active compound, especially for U-251, UACC-62 and K562 human tumor cell lines with GI_{50} ranging from 1.0 to <0.25 $\mu g.mL^{-1}$. On the other hand, the imidazole-thiosemicarbazone of *R*-(+)-limonene 4 was the most promising derivative against the promastigote form of *L. amazonensis* (IC_{50}=5.9µM). Meanwhile, thiosemicarbazones without limonene moiety (9-12) showed the lowest activities in the biological assays performed. The results demonstrated the influence of the lipophilic molecular character and stereochemistry of chiral monoterpene on the evaluated activities.
Keywords: Limonene; Thiosemicarbazone; Imidazole; Antitumor activity.

Resumo
No presente estudo, apresentamos a síntese de uma série de tiossemicarbazonas derivadas do *R*-(+)- e *S*-(-)-limoneno contendo diferentes núcleos heterocíclicos pentacíclicos focando na busca de novos agentes antitumorais e antileishmania. No ensaio antitumoral, o derivado imidazol do *S*-(-)-limoneno 8 foi o composto mais ativo, especialmente para as linhagens de células tumorais humanas U-251, UACC-62 e K562 com GI_{50} variando de 1,0 a <0,25 $\mu g.mL^{-1}$. Por outro lado, o imidazol-tiossemicarbazona derivado do *R*-(+)-limoneno 4 foi o derivado mais promissor contra a forma promastigota de *L. amazonensis* (IC_{50}=5,9µM). Por outro lado, as tiossemicarbazonas sem a porção terpênica (9-12) apresentaram as menores atividades nos ensaios biológicos realizados. Os resultados demonstraram a influência do caráter lipofílico e da estereoquímica do monoterpeno nas atividades biológicas avaliadas.
Palavras-chave: Limoneno; Tiossemicarbazonas; Imidazol; Atividade antitumoral.

Resumen
En el presente estudio aportamos la síntesis de una serie de tiosemicarbazonas basadas en *R*-(+)- y *S*-(-)-limoneno que contienen diferentes núcleos heterocíclicos pentacíclicos enfocados a la búsqueda de nuevos agentes antitumorales y antileishmania. En el ensayo antitumoral, el derivado de imidazol de *S*-(-)-limoneno 8 fue el compuesto más activo, especialmente para las líneas de células tumorales humanas U-251, UACC-62 y K562 com GI_{50} entre 1,0 y <0,25 µg. mL^{-1}. Por otro lado, el imidazol-tiosemicarbazona derivada de *R*-(+)-limoneno 4 fue el derivado más promisorio contra la forma promastigote de *L. amazonensis* (IC_{50}=5.9µM). Por su parte, las tiosemicarbazonas sin la unidad limoneno mostraron las actividades más bajas en los ensayos biológicos realizados. Los resultados demostraron la influencia del carácter molecular lipofílico y de la estereoquímica del monoterpeno sobre las actividades evaluadas.
Palabras clave: Limoneno; Tiosemicarbazona; Imidazol; Actividad antitumoral.

1. Introduction

Thiosemicarbazone is considered an important scaffold for drug development and is embedded in diverse compounds with a broad spectrum of pharmacological activities such as antitumor (Oliveira et al., 2015; Vandresen et al., 2014; Lavanya et al., 2021), antiprotozoal (Batista et al., 2018; Pervez et al., 2013; Moreno-Rodríguez et al., 2014), antifungal (Neto et al., 2017) and antibacterial (Zhao et al., 2012).

The antitumor activity of thiosemicarbazones has been attributed to their ability to act as inhibitors of ribonucleotide reductase (RR), an enzyme containing a diferric center involved in the ratelimiting step of DNA synthesis (Kalinowski, Quach and Richardson, 2009). Some derived from either five- or six- membered heterocyclic carbonyl performed a potent antitumor activity. However, compounds with six-membered *N*-heterocyclic rings are more frequent in chemical structures of antitumor thiosemicarbazones, while molecules bearing five-membered *N*-heterocycles are not common as other. Triapine, the most antitumor recognized pyridinyl-thiosemicarbazone, has been a mechanism of action based in the inhibition of the enzyme ribonucleotide reductase. Furthermore, there is also evidence of topoisomerase II inhibition (Oliveira et al., 2015). Other heterocycle-thiosemicarbazones display potential as antitumor candidate drugs as di-2-pyridylketone thiosemicarbazones (DpT) with potent antiproliferative activity *in vitro* against SK-N-MC neuroepithelioma cells (Richardson et al., 2006). However, there have been few reports for pyrrole, imidazole and thiophene moieties embedded into a structures of thiosemicarbazones derivatives and its metal complexes focused on molecules with potential antitumor (Oliveira et al., 2015; Matesanz et al. 2021; Palamarciuc et al., 2019).

Also, thiosemicarbazones have remarkable antileishmanial activities (Batista et al., 2018; Melos et al., 2015; Schröder et al., 2013; Silva et al., 2020; Temraz et al., 2018). Among them, some heterocyclic-bearing thiosemicarbazones possess high activity and selectivity for *Leishmania* strains. For example, 4-methoxy-phenyl-2-furanyl-thiosemicarbazone (CP247129) showed an IC_{50} on CPB2.8ΔCTE for cysteine protease inhibitors in *L. mexicana* in the nanomolar range with complete selectivity over bovine Cat B (IC_{50} >30 µM) (Schröder et al., 2013).

On the other hand, hybrid molecules using monoterpene moieties as synthetic building blocks present a broad range of pharmacological properties (Kousar et al., 2017). Our research group has focused on the development of terpene-derivatives with antitumor, antiprotozoal, antifungal and antitubercular activities (Vandresen et al., 2014; Batista et al., 2018; Souza et al., 2019; Carvalho et al., 2021; Vandresen et al., 2017).

Based in the development of candidates molecules for antitumor and antileishmanial agents employing efficient and straightforward methods of synthesis to obtain different bioactive molecules, we prepared a series of *R*-(+)- and *S*-(−)-limonene-based heterocyclic-thiosemicarbazones with four different pentacyclic aromatic heterocycles (pyrrole, furan, thiophene and imidazole) with the specific aim of evaluating their potential antiproliferative and antileishmanial activities and *in silico* structural properties. Furthermore, in order to widen the purpose of this work, we also synthesised four heterocyclic-thiosemicarbazones derivatives without the terpenoid moiety to analyse the influence of stereochemistry and lipophilicity of monoterpene group on pharmacological activities evaluated.

2. Material and Methods

2.1 General

Reagents and solvents were used as obtained from commercial suppliers without further purification. The column chromatography used was silica gel 60, with 230-400 mesh (Merck). Melting points were determined on Micro-Química apparatus model MQAPF-301. The analysis of the optical rotations were determined at 20°C with $CHCl_3$ at a concentration of 5.0 mg/mL with a Perkin Elmer 343 polarimeter. Fourier transform infrared (FTIR) spectra were obtained using KBr pellets by a BOMEM-MICHELSON spectrophotometer. ^{1}H NMR, ^{13}C NMR, HSQC and HMBC experiments were run on VARIAN Mercury Plus apparatus operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. All deuterium solvents ($CDCl_3$ and CD_3OD-d_4 were bought from Sigma-Aldrich. Direct Infusion ESI-(+)-FTMS, performed in an LTQ-XL-Orbitrap Discovery (Thermo Scientific, Bremen, Germany), operating in 5.2 kV of capillary voltage, capillary temperature of 285ºC, sheath gas (nitrogen) flow rate at 10 arbitrary units and sample solution flow rate of 10 μL/min. Data acquisition was performed in the positive ion mode within the *m/z* range of 300-400. Raw data were treated on Xcalibur 2.1 (Thermo Scientific, San Jose, California, USA) and mass accuracy was the utilized parameter for compound characterization.

2.2 Synthesis of core products

The synthetic protocol to prepare terpenoids-thiosemicarbazones was established by our research group. Synthesis of isothiocyanomonoterpene and limonene-thiosemicarbazides were previously reported (Vandresen et al., 2014; Batista et al., 2018; Souza et al., 2021).

Synthesis of limonene-thiosemicarbazones (**1-8**): In a test tube, 20 mg of silica gel and 5% H_2SO_4 were taken. To this, 1.3 mmol of the corresponding heterocyclic aldehyde derivative (2-pyrrole-carboxaldehyde, 2-furancarboxaldehyde, 2-thiophenecarboxaldehyde and 2-imidazolecarboxaldehyde) was added. This mixture was manually stirred for approximately 5 min. Then, an equimolar amount of limonene-thiosemicarbazide was added. The reaction was processed by manual stirring for 20 minutes at room temperature and monitored by TLC (hexane:ethyl acetate 7:3). The products were extracted from silica gel using absolute ethanol. Limonene-thiosemicarbazones were obtained with yields ranging from 68 to 80%. The purity of compounds were confirmed by ESI-MS and NMR techniques.

Compound 1: (+)-(*R*)-*N*(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-*N*(1)- [(1H-pyrrol-2-yl)-methylene]-thiosemicarbazide (Vandresen et al., 2014)

Compound 2: (+)-(*R*)-*N*(1)-(furan-2-yl-methylene)]-*N*(4)- [2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-thiosemicarbazide (Vandresen et al., 2014)

Compound 3: (+)-(*R*)-*N*(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-*N*(1)- [(thiophen-2-yl)-methylene]-thiosemicarbazide (Vandresen et al., 2014)

Compound 4: (+)-(*R*)-*N*(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-*N*(1)- [(imidazo-2-yl)-methylene]-thiosemicarbazide**:** White crystals; yield 68%; mp. 138-139ºC; $[\alpha]_D$ +40 (c=5.2 mg/mL); IR (KBr/cm^{-1}): (NH) 3336 and 3315, (C=N) 1301, (C=S) 811, 1290; HRMS (ESI) calculated 306.4416 $[M+H]^+$ found 306.4405; ^{1}H NMR (300 MHz, CD_3OD): δ_H 1.48 (3H, s, H-8'), 1.50 (3H, s, H-9'), 1.64 (3H, s, H-10'), 1.79 (2H, m, H-6'), 1.98 (2H, m, H-2'), 2.05 (2H, m, H-5'), 2.56 (1H, m, H-1'), 5.36 (1H, m, H-3'), 7.29 (1H, d, J=1,2 Hz, H-3''), 7.95 (1H, s, H-5''), 8.79 (1H, s, HC=N, H-1''); ^{13}C NMR (75.5 MHz, CD_3OD: δ_C 23.5 (C-10'), 24.2 (C-9'), 24.4 (C-6'), 24.5 (C-8'), 26.7 (C-2'), 31.4 (C-5'), 41.2 (C-1'), 59.0 (C-7'), 118.6 (C-3''), 120.5 (C-3'), 121.8 (C-5''), 135.6 (C-2''), 133.6 (C-4'), 137.2 (C-1"), 177.3 (C=S, C-3).

Compound 5: (-)-(*S*)-*N*(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-*N*(1)- [(1H-pyrrol-2-yl)-methylene]-thiosemicarbazide: White crystals; yield 75%; mp. 115-116ºC; $[\alpha]^D$ -31 (c=5.1 mg/mL); IR (KBr/cm^{-1}): (NH) 3370 and 3288, (C=N) 1320, (C=S) 809, 1284; HRMS (ESI) calculated 305.1794 $[M+H]^+$ found 305.1789; ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 1.48 (3H, s, H-8'), 1.50 (3H, s, H-9'), 1.64 (3H, s, H-10'), 1.79 (2H, m, H-6'), 1.98 (2H, m, H-2'), 2.05 (2H, m, H-5'), 2.56 (1H, m, H-1'), 5.36 (1H, m, H-3'), 6.21 (1H, m, H-4''), 6.44 (1H, m, 5''), 6.87 (1H, m, 3''), 7.32 (1H, sl, NH, H-4), 7.55 (1H, s, HC=N, H-1''), 8.77 (1H, sl, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ_C 23.5 (C-10'), 24.2 (C-9'), 24.4 (C-6'), 24.5 (C-8'), 26.7 (C-2'), 31.4 (C-5'), 41.0 (C-1'), 59.1 (C-7'), 110.9 (C-4''), 115.2 (C-3''), 120.5 (C-3'),122.2 (C-5''), 126.6 (C-2''), 133.6 (C-4'), 134.0 (C-1") and 175.2 (C=S, C-3).

Compound 6: (-)-(*S*)-*N*(1)-(furan-2-yl-methylene)]-*N*(4)- [2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-thiosemicarbazide: White crystals; yield 72%; mp. 137-140ºC; $[\alpha]_D$ -42 (c=5.0 mg/mL); IR (KBr/cm^{-1}): (NH) 3339 and 3310, (C=N) 1309, (C=S) 800, 1300; HRMS (ESI) calculated 306.1635 $[M+H]^+$ found 306.1641; ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 1.48 (3H, s, H-8'), 1.50 (3H, s, H-9'), 1.64 (3H, s, H-10'), 1.79 (2H, m, H-6'), 1.98 (2H, m, H-2'), 2.02 (2H, m, H-5'), 2.56 (1H, m, H-1'), 5.36 (1H, m, H-3'), 6.51 (1H, m, H-4''), 6.69 (1H, m, H-3''), 7.53 (1H, s, NH, H-4), 7.53 (1H, m, H-5''), 7.64 (1H, s, HC=N, H-1''), 9.10 (1H, s, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ_C 23.5 (C-10'), 24.2 (C-9'), 24.4 (C-6'), 24.5 (C-8'), 26.7 (C-2'), 31.3 (C-5'), 41.0 (C-1'), 59.0 (C-7'), 112.3 (C-4''), 113.6 (C-3''), 120.5 (C-3'), 130.6 (C-1"), 133.8 (C-4'), 144.8 (C-5''), 149.2 (C-2'') and 175.4 (C=S, C-3).

Compound 7: (-)-(*S*)-*N*(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-*N*(1)- [(thiophen-2-yl)-methylene]-thiosemicarbazide: White crystals; yield 79%; mp. 186-188ºC; $[\alpha]_D$ -24 (c=5.3 mg/mL); IR (KBr/cm^{-1}): (NH) 3335 and 3315, (C=N) 1301, (C=S) 808, 1289; HRMS (ESI) calculated 322.1406 $[M+H]^+$ found 322.1418; ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 1.48 (3H, s, H-8'), 1.50 (3H, s, H-9'), 1.65 (3H, s, H-10'), 1.79 (2H, m, H-6'), 1.98 (2H, m, H-2'), 2.05 (2H, m, H-5'), 2.56 (1H, m, H-1'), 5.36 (1H, m, H-3'), 7.05 (1H, dd, J=5.1 and 3.6, H-3''), 7.24 (1H, dd, J=3.6 and 1.0 Hz, H-4''), 7.36 (1H, m, H-5''), 7.53 (1H, sl, NH, H-4''), 8.08 (1H, s, HC=N, H-1''), 10.06 (1H, sl, NH, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ_C 23.5 (C-10'), 24.2 (C-9'), 24.4 (C-6'), 24.5 (C-8'), 26.7 (C-2'), 31.3 (C-5'), 41.0 (C-1'), 59.0 (C-7'), 120.5 (C-3'), 127.9 (C-4''), 128.2 (C-5''), 130.5 (C-3''), 133.6 (C-4'), 136.1 (C-1"), 138.8 (C-2'') and 175.1 (C=S, C-3).

Compound 8: (-)-(*S*)-*N*(4)-[2-(4-methylcyclohex-3-en-1-yl)-propan-2-yl]-*N*(1)- [(imidazo-2-yl)-methylene]-thiosemicarbazide: White crystals; yield 69%; mp. 138-140ºC; $[\alpha]_D$ -24 (c=5.3 mg/mL); IR (KBr/cm^{-1}): (NH) 3340 and 3315, (C=N) 1301, (C=S) 819, 1285; HRMS (ESI) calculated 306.4416 $[M+H]^+$ found 306.4422; ^{1}H NMR (300 MHz, CD_3OD): δ_H 1.48 (3H, s, H-8'), 1.50 (3H, s, H-9'), 1.64 (3H, s, H-10'), 1.79 (2H, m, H-6'), 1.98 (2H, m, H-2'), 2.05 (2H, m, H-5'), 2.56 (1H, m, H-1'), 5.36 (1H, m, H-3'), 7.29 (1H, d, J=1,2 Hz, H-3''), 7.95 (1H, s, H-5''), 8.79 (1H, s, HC=N, H-1''); ^{13}C NMR (75.5 MHz, CD_3OD: δ_C 23.5 (C-10'), 24.2 (C-9'), 24.4 (C-6'), 24.5 (C-8'), 26.7 (C-2'), 31.4 (C-5'), 41.2 (C-1'), 59.0 (C-7'), 118.6 (C-3''), 120.6 (C-3'), 121.8 (C-5''), 135.6 (C-2''), 133.7 (C-4'), 137.2 (C-1"), 177.2 (C=S, C-3).

2.3 Biological assays

2.3.1 Antriproliferative activity

The heterocyclic-thiosemicarbazones were evaluated *in vitro* for their anti-proliferative activity in ten human tumor cells and in one strain of non- tumor human cells (VERO cell), according to the methodology recommended by the National Cancer Institute (NCI/NIH) (Frederick, WA, USA) (Monks et al., 1991; Shoemaker, 2006).The cell suspensions, cultivated in RPMI 1640 medium (Gibco BRL, Life Technologies) supplemented with 5% bovine fetal serum (BFS, Invitrogen) and 1% of a streptomycin/penicillin solution (VitroCell®) were plated in triplicate in 96-well plates (100 μL/well) and incubated at 37°C

for 24 h in a moist atmosphere with 5% CO_2. Experimental cultures were supplemented also with penicillin:streptomycin (10 μg/mL : 10 IU/mL). The cells in this plate being fixed with 50 g/100 mL trichloroacetic acid (TCA) (w/v) until addition of compounds **1-8**. After 24 hours of incubation, the thiosemicarbazones were added to the plates in four concentrations (0.25; 2.5; 25; and 250 μg/mL), previously diluted in RPMI 1640 medium, 5% BFS and 0.2% dimethylsulfoxide. The final DMSO concentration did not affect the cell viability. After 48 hours of incubation, all treated cells were fixed with 50% trichloroacetic acid (w/v) and the cell proliferation determined at 504 nm using colorimetric method with sulforhodamine B, according to NCI standard protocol. Doxorubicin (DOX) was used as standard drug. Thus three absorption measurements were taken: one at the start of incubation (T_0), the second after 48 h in the presence of compounds (T), and another in the absence (T_1) of target compounds. The cell growth was calculated from the mean absorbance values and considering the ratio between T and T_0. Thus when $T \geq T_0$, cell growth was determined according to the equation: $[(100 \times T\text{-}T_0)/(T1\text{-}T_0)]$, whereas when $T < T_0$, equation $100\times[(T\text{-}T_0)/(T_0)]$ was used. The results for each cell line were expressed as cell growth, expressed as a percentage, as a function of the concentration of thiosemicarbazones, using the software Origin Pro® 8.0 (OriginLab Corporation).

2.3.2 Antileishmanial activity

In order to evaluate the antileishmanial activity of thiosemicarbazones **1-12** against promastigote forms of *L. amazonensis*, the MHOM/BR/75/Josefa L. *amazonensis* strain (1×10^6 parasites per milliliter) were inoculated in Warren's medium supplemented with 10% of FBS. For the tests, the stock solutions of the derivatives were prepared with a concentration between 10 and 1000 mg/mL. Amphotericin B was used as positive control. Further, the experiments were carried out using DMSO to solubilise the stock solutions of all prepared compounds. To this end, the final concentration of DMSO did not exceed 1%. After 72 h maintained at 28 °C, leishmanial activity was determined by direct counting of free-living parasites in the Neubauer chamber (Dos Santos et al., 2010). In this case, all experiments were performed in duplicate. Hence, the experimental values of IC_{50} were obtained graphically, that is, by plotting the concentration vs. percentage growth inhibition of promastigote form of parasite.

2.3.3 ADME and *in silico* studies

In *silico* analysis of ADME parameters was carried out by Swiss ADME, OSIRIS Property Explorer and Molinspiration free software (Molinspiration Porperty Calculation Service). According to Lipinski et al., the following physicochemical parameters were evaluated: molecular weight, log P, H-bond donors (HBD) and H-bond acceptors (HBA) (Lipinski et al., 2001).

3. Results and Discussion

3.1 Synthesis and characterization

Limonene-based heterocycles-thiosemicarbazones (**1-8**) were prepared in three synthesis steps (Figure 1). Firstly, the isothiocyanomonoterpenes were obtained by chemoselective addition of thiocyanic acid (prepared with potassium thiocyanate and potassium bisulfate in chloroform) to the exocyclic double bond of limonene. Thiosemicarbazide was obtained via nucleophilic addition reaction between hydrazine ($NH_2NH_2.2HCl$) and isothiocyanates. Finally, the synthesized limonene-thiosemicarbazides reacted with 2-pyrrole-carboxaldehyde (to obtain compounds **1** and **5**), furaldehyde (to obtain compounds **2** and **6**), 2-thiophene-carboxaldehyde (to obtain compounds **3** and **7**) and 2-imidazolecarboxaldehyde (to prepare compounds **4** and **8**), in presence of a catalytic amount of sulfuric acid supported in silica gel (SiO_2:H_2SO_4 5%), in absence of solvent via condensation reaction, leading to the formation of compounds of interest in good yields (Vandresen et al., 2014; Batista et al.,

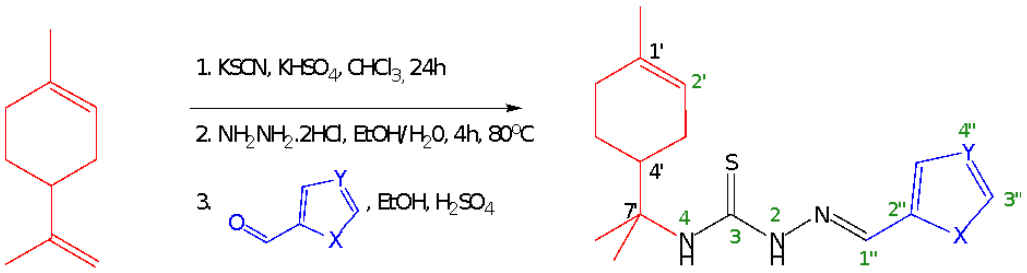
2018). Hence, all of the prepared compounds were characterised by spectrometric analysis (NMR, FTIR, and ESI-MS). In parallel, a known series of thiosemicarbazones without terpenoid moiety (**9-12**) were synthesized using thiosemicarbazide and the same four heterocycles aldehydes in good yields (78-92%). The spectra data were compared with literature (Dong et al., 2017).

The chemical structures of limonene heterocycle-thiosemicarbazones (**1-8**) were determined by ^{1}H and ^{13}C NMR. The terpene moiety showed typical signals at δ_H/δ_C 5.36 (1H, s, H-3′)/120.5 or 120.6(C-3′) and 133.6 or 133.7 (C-4’) attributed to olefinic hydrogen and carbon. The methyl groups showed signals at δ_H/δ_C 1.48 (3H, s, H-8′)/24.5 (C-8′), 1.50 (3H, s, H-9′)/24.2 (C-9′) and 1.64 (3H, s, H-10′)/23.5 (C-10’). The thiosemicarbazone moiety was characterised by signals at δ_H 8.77–10.06 and 7.32–7.53 (NH groups), the imine group at δ_H/δ_C 7.00–8.50 (CH=N)/140.0–143.0 (C=N) and δ_C175.1–177.3 (C=S) for thiocarbonyl group. The aromatic heterocycles showed signals ranging from δ_H 6.21 to 7.95 ppm.

Based on the fact that thiosemicarbazones and aromatic heterocyles as pyrrole, furan, thiophene and imidazole can play an important role as pharmacophoric groups, 8 (**1-8**) derivatives containing limonene moiety were prepared and their antileishmanial and anti-proliferative potential were evaluated (Figure 1). It was also verified whether the stereochemical profile of limonene can influence the biological activities evaluated. For this, other 4 derivatives (**9-12**) were synthesized without the terpenoid portion at N-4 position.

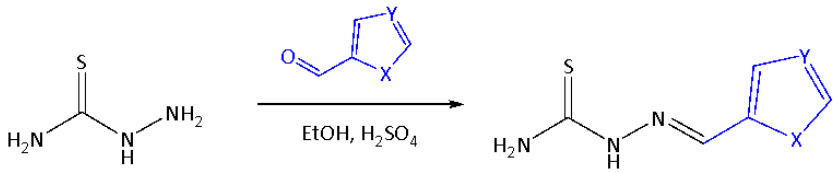
Figure 1: Synthesis of heterocycle-thiosemicarbazones.

Limonene-based heterocycle-thiosemicarbazones



Compound	Chiral-limonene	X	Y
1	*R*	NH	C
2	*R*	O	C
3	*R*	S	C
4	*R*	NH	N
5	*S*	NH	C
6	*S*	O	C
7	*S*	S	C
8	*S*	NH	N

Non-terpenic heterocycle-thiosemicarbazones



Compound	X	Y
9	NH	C
10	O	C
11	S	C
12	NH	N

Souce: Authors

3.2 Antiproliterative activity

The antitumor activity of heterocyclic-thiosemicarbazones was evaluated *in vitro* against ten different human tumor cell lines: glioma (U251), melanoma (UACC-62), breast (MCF-7), ovary with multidrug resistance phenotype (NCI-ADR/RES), kidney (786-0), lung, non-small cell type (NCI-H460), prostate (PC-3), ovary (OVCAR-3), colorectal (HT29) and leukemia (K562). The green monkey renal epithelial cell (VERO) was used as parameter to evaluate the toxicity of compounds. Doxorubicin was used as a positive control standard.

Antitumor activities of derivatives **1-3** were previously reported (Vandresen et al., 2014). According to the obtained data, all synthesized limonene-thiosemicarbazones (**1-8**) inhibited the growth of the cell lines (Table 1). The most active compound was the derivative **8** (*S*-limonene-imidazole derivative) with GI_{50} of 1.0 µg.mL^{-1} for U-251 and UACC-62 cells and

<0.25 μg.mL^{-1} for leukemia cells (K562). Also, compound **8** was the most effective derivative against human multi-resistant ovarian tumor cells (NCI/ADR-RES) (GI_{50}=2.0μg.mL^{-1}). Additionally, other compounds (**2**, **6** and **7**) showed growth inhibitory activity against NCI/ADR-RES better than standard drug doxorubicin (GI_{50}>25 μg.mL^{-1}). On the other hand, the other *S*-(-)-limonene derivatives (**5**, **6** and **7**) showed moderate activity against human tumor cells line tested.

Among the *R*-(+)-limonene derivatives **(1-4)**, the compound **1** (pyrrole-derivative) was active against glioma cells (GI_{50}=1.2 μg.mL^{-1}) and the compound **2** (furan derivative) was the most active of the series for non-small cell types of lung (GI_{50}=3.3 μg.mL^{-1}). Furthermore, differentelly of *S*-(-)-limonene-imidazole derivative, the compound **4** demonstrated a reasonable activity for all human tumor cells tested with GI_{50} ranged of 6.1 μg.mL^{-1} (U-251) to 79.5μg.mL^{-1} (HT-29), except for leukemia cell (K562).

In order to simulate the behavior of thiosemicarbazones against non-tumor cells, the VERO cell line was inserted into the evaluation panel. All terpenoids derivatives (**1**-**8**) presented a GI_{50} value for VERO cell line lower than 250 μg.mL^{-1}. These values can be suggest these compounds have toxicity to normal cells, highlighting compounds **2**, **3** and **6**, with GI_{50} 4.5, 4.3 and 4.3 μg.mL^{-1}, respectively. However, the most active derivatives (**1**, **5** and **8**) presented a GI_{50} value which might suggest these compounds have a low toxicity to normal cells. Considering the most active thiosemicarbazone (**8**), an approximately 33 and 16-fold better activity was found against U-261 and NCI/ADR-RES cells in comparison with their toxicity for VERO cells.

Table 1: *In vitro* antiproliferative activity (GI_{50} in μg.mL^{-1}) of heterocyclic-thiosemicarbazone derivatives **1**–**12**.

Cmpd.	U251	UACC-62	MCF-7	NCI-ADR/RES	786-0	NCI-H460	PC-3	OVCAR-3	HT-29	K562	VERO
1	1.2	15.2	3.5	163.8	53.3	17.5	>250	56.4	56.3	>250	31.4
2	25.0	4.3	16.4	4.3	3.0	3.3	8.3	4.2	8.2	>250	4.5
3	8.8	6.1	3.7	13.2	8.6	6.7	20.2	11.3	18.6	33.2	4.3
4	6.1	12.1	32.4	15.8	20.1	16.8	43.3	26.9	79.5	>250	7.9
5	22.0	45.1	>250	43.0	40.4	41.5	43.8	34.3	40.1	36.4	41.4
6	6.0	5.5	5.8	8.2	10.9	8.6	27.5	13.1	23.1	>250	4.3
7	7.3	7.1	7.8	8.6	26.9	11.3	24.9	8.1	25.7	>250	15.2
8	1.0	1.0	6.6	2.0	4.0	6.5	35.3	4.8	19.1	<0.25	32.9
9	123.8	140.1	200.0	>250	>250	>250	43.6	>250	>250	>250	42.0
10	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
11	130.0	7.6	>250	>250	>250	>250	>250	229.9	>250	>250	>250
12	122.0	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
DOX[a]	0.46	1.3	1.2	>25	1.0	1.1	>25	3.6	9.5	>25	4.1

[a] = doxorubicin. The lowest concentration tested was 0.25 μg.mL^{-1} . Data are means of representative experiment done in triplicate. Glioma (U251), melanoma (UACC-62), breast (MCF-7), ovary with multidrug resistance phenotype (NCI-ADR/RES), kidney (786-0), lung, non-small cell type(NCI-H460), prostate (PC-3), ovary (OVCAR-3), colorectal (HT29) and leukemia (K562). Source: Authors.

To investigate the effect of chiral-limonene in the antiproliferative activity, the compounds lacking the terpenic moiety (**9**-**12**) were evaluated (Table 1). In general, all compounds were inactive for inhibition of tumor cell growth. Compound **11** (thiophene derivative) was the unique analogous that showed moderate activity against UACC-62 cells (GI_{50}=7.6 μg.mL^{-1}). These results corroborate with the influence of limonene in antiproliferative activity on structures of heterocycle-thiosemicarbazones (**1-8**), because these monoterpenes are capable of inhibits the development of different types of tumors such as carcinomas breasts, lung cancer, tumors pancreatic, liver cancer, adenomas lung and stomach tumors (Vigushin et al., 1998).

3.3 Antileishmanial activity

The concentrations that correspond to 50% of growth inhibition (IC_{50}) of the promastigotes form of *Leishmania amazonensis* were determined for all heterocyclic-thiosemicarbazones. Compounds with IC_{50}>100 μM were considered inactive. Amphotericin B (IC_{50}=0.063 μM) was employed as the standard drug.

Analysis of IC_{50} data for the compounds **1-8** (Table 2) showed that all compounds *R*-(+)-limonene-based heterocycle-thiosemicarbazones (**1-4**) were more active than *S*-(-)-analogous (**5-8**). This activity is opposite to results previously reported to benzaldehyde-thiosemicarbazones derivatives, when the *S*-analogous were more active than *R*-derivatives (Batista et al., 2018). Compounds **1-4** showed IC_{50} values ranged of 5.9 to 7.5 μM, whereas *S*-(-)-derivatives presented IC_{50} ranged of 7.7 to 15.1 μM. On the other hand, derivatives lacking the terpenoid moiety (**9-12**) were inactive with IC_{50} greater than 100 μM, with exception for compound **11** (thiophene derivative) with IC_{50}=52.1 μM. These results demonstrate the importance of lipophilic limonene moiety in the antileishmanial activity.

When the antileishmanial activity of compounds **1-8** are compared with other limonene and thiosemicarbazones derivatives reported in literature (Melos et al., 2015; Silva et al., 2020), the results were extremely promising. The incorporation of distinct pharmacophoric groups (thiosemicarbazones, chiral terpenoids and pentacyclic heterocycles) in a unique bioactive molecule resulted in promising structures with antileishmanial activity.

As expected, the incorporation of terpenoid moiety alters significantly the lipophilic character of thiosemicarbazones (**1-8**). Limonene increases the lipophilicity of the target compounds which may result in higher hydrophobic membrane permeability, better bioavailability, and improved pharmacological profile. Furthermore, limonene is a potential anti-leishmanial agent. Studies confied the antiprotozoal action of this monoterpene against promastigote and amastigote forms from *Leishmania amazonensis, L. braziliensis, L. chagasi* and *L. major* (Arruda et al., 2009).

Table 2: Antileishmanial activity (in μM)[a] data for compounds **1-12.**

Compound	IC_{50}	Compound	IC_{50}	Compound	IC_{50}
1	6.9±0.5	**5**	10.1±4.2	**9**	172.0±49.3
2	7.5±0.0	**6**	15.1±3.3	**10**	327.6±27.0
3	6.2±2.2	**7**	10.4±9.0	**11**	52.1±9.1
4	5.9±0.0	**8**	7.7±2.5	**12**	>587.5

[a]IC_{50} values of promastigote form. Standard drug amphotericin IC_{50}=0.063 μM

3.4 *In silico* studies

Lipinski's rule of five is used frequently in drug design and development to predict oral bioavailability of potential bioactive molecules. According to Lipinski's ''rule of five'', to a biomolecule act as a candidate molecule to be orally active is

necessary molecular weight < 500, lipophilicity (Log P) < 5, number fewer than 5 atoms hydrogen bond donors (OH and NH groups) and less than ten hydrogen bond acceptors (Lipinski et al., 2001). The molecular properties of thiosemicarbazones were calculated by using Osiris(R) and SwissAdmet(R) cheminformatics softwares (MOLINSPIRATION Property Calculation Service, 2021; SwissADME, 2021; OSIRIS Predictor, 2021). According to Table 3, all the screened compounds fulfil all parameters of the Lipinski rule of five. Limonene derivatives (**1-8**) have a molecular weight in the range of 332.51–349.57 Daltons, indicating that they can be easily transported, diffused, and absorbed by organism. The parameter lipophilicity (Log P) measures hydrophobicity of a bioactive molecules with permeability across cell membrane. The log P values range of 2.72 to 4.37 for limonene derivatives (**1-8**) and -0.25 to 1.40 for molecules **9-12**. Among them, the more lipophilic thiosemicarbazones (**1-8**) were the most active compounds in the biological assays. This particular feature introduced in these compounds can be closely responsible for the better biological activities results. And also, all compounds have less than 5H-bond donors and less than 10H-bond acceptors.

Table 3: *In silico* parameters with data from Lipinski, Veber, logS (solubility).

Compound	LogP[a]	nHa[b]	nHb[c]	MW[d]	TPSA[e]	NRB[f]	LogS[g]
1	3.28	4	3	332.51	84.30	6	-4.97
2	3.70	4	2	333.50	81.65	6	-5.20
3	4.37	3	2	349.57	96.75	6	-6.16
4	2.72	5	3	333.50	97.19	6	-4.57
5	3.28	4	3	332.51	84.30	6	-4.97
6	3.70	4	2	333.50	81.65	6	-5.20
7	4.37	3	2	349.57	96.75	6	-6.16
8	2.72	5	3	333.50	97.19	6	-4.57
9	0.31	4	3	168.22	98.29	2	-1.95
10	0.72	4	2	169.21	95.64	2	-3.26
11	1.40	3	2	185.27	110.74	2	-2.07
12	-0.25	5	3	169.21	111.18	2	-1.54
Amphotericin B	0.32	18	12	924.04	319.61	3	-6.26
Doxorubicin	0.17	12	6	543.52	206.07	5	-5.26

Lipinski and Veber in silico parameters were done in Osiris® software and LogS in SwissADME® software. [a]Logarithm of partition coefficient between an organic solvent and water; [b]number of hydrogen bond acceptors; [c] number of hydrogen bond donors; [d] molecular weight;[e] topological polar surface area (TPSA); [f] number of rotatable bonds (NRB); [g] solubility of organic compounds in mol/L. Source: Authors.

Veber's rule are also applied to know the pharmacokinetic property of thiosemicarbazones. Topological polar surface area (TPSA) is correlated with the hydrogen bonding of a molecule and is a good parameter of the bioavailability of bioactive molecules. All compounds have TPSA <160 A, that would indicate a good oral biodisponibility (Hussain et al., 2016). The molecular flexibility can be indicated by the number of rotatable bonds (less than 10 rotatable bonds). The tested molecules were found to be flexible with the number of rotatable bonds ranging from 2 (compounds **9-12**) or 6 (compounds **1-8**). According to log S, values between -6 and -4 have moderate water solubility whereas log S values between -4 and -2 are compounds with good water solubility. All limonene derivatives (**1-8**) have moderate water solubility (log S ranged -6.16 to -

4.57). These results are similar to antitumor standard drug doxorubicin (log S ranged -5.26). The derivatives without terpenic moiety (**9-12**) have high solubility in water (log S ranged -3.26 to -1.54). Compounds with high solubility are easily metabolized and eliminated from the body, resulting in a lower probability of adverse effects.

The bioactivity scores of thiosemicarbazones were also predicted by Molinspiration and are presented in Table 4. In general, a molecule having bioactivity score more than 0.00 is most likely to exhibit considerable biological activities, while values -0.50 to 0.00 are expected to be moderately active and bioactivity score is less than -0.50 it is inactive. The results clearly reveal that the better physiological actions of thiosemicarbazones might involve multiple mechanisms and could be due to the interactions with GPCR ligands, ion-channel modulator and inhibit other enzymes.

Table 4: *In silico* predictions of bioactivity score in Molinspiration® software.

Compound	GPCR[a]	ICM[b]	KI[c]	NRL[d]	PI[e]	EI[f]
1	-0.12	-0.15	-0.66	-0.35	-0.49	0.21
2	-0.42	-0.45	-1.04	-0.64	-0.61	-0.11
3	-0.42	-0.47	-0.97	-0.58	-0.54	-0.10
4	0.02	-0.07	-0.55	-0.65	-0.32	0.34
5	-0.12	-0.15	-0.66	-0.35	-0.49	0.21
6	-0.42	-0.45	-1.04	-0.64	-0.61	-0.11
7	-0.42	-0.47	-0.97	-0.58	-0.54	-0.10
8	0.02	-0.07	-0.55	-0.65	-0.32	0.34
9	-2.10	-1.54	-1.84	-2.54	-1.78	-0.75
10	-2.64	-196	-2.54	-3.29	-2.08	-1.36
11	-2.69	-2.22	-2.52	-3.19	-1.90	-1.26
12	-1.80	-1.35	-1.62	-3.17	-1.41	-0.46
Amphotericin B	-3.06	-3.51	-3.54	-3.45	-2.45	-2.95
Doxorubicin	0.20	-0.20	-0.07	0.32	0.67	0.66

[a]G-protein coupled receptor (GPCR); [b]ion channel modulator; [c] kinase inhibitor; [d] nuclear receptor ligand;[e] protease inhibitor; [f] enzyme inhibitor. Source: Authors.

The most promising compounds were the both limonene-imidazole-thiosemicarbazones **4** and **8** with bioactivity associated to GPCR and enzyme inhibitors. G-protein coupled receptors have a crucial role in physiology acting in cell communication through recognition of diverse ligands types as peptides, lipides and nucleosides (Villamizar-Mogotocoro, Vargas-Méndez and Kouznetsov, 2020). Other derivatives presented moderate bioactivity score.

4. Conclusion

In this study, all limonene heterocyclic-thiosemicarbazones demonstrated promising antitumor and anti-leishmania activities, especially the imidazole derivatives **4** and **8**. Further *in vitro* and *in vivo* studies are necessary to increase our understanding of the mechanism of action of these compounds and determine whether they can be exploited as drug candidates for the treatment of cancer or leishmaniasis. The results corroborated for a positive effect of the chiral limonene moiety in the

thiosemicarbazones, and demonstrated the importance of the lipophilicity and the stereochemistry in the alteration of their pharmacological activities.

The promising results for antileishmanial and antitumor activities for heterocycles limonene-thiosemicarbazones encourage the continuity of the studies for a better understanding of pharmacological performance of most bioactive compounds and its mechanism of action.

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