Journal of Molecular Structure 1232 (2021) 130014

Contents lists available at ScienceDirect

Journal of Molecular Structure

journal homepage: www.elsevier.com/locate/molstr

Synthesis, characterization and antichagasic evaluation of thiosemicarbazones prepared from chalcones and dibenzalacetones

Aline Alves da Silva[a], Pedro Ivo da Silva Maia[b], Carla Duque Lopes[c,d], Sergio de Albuquerque[c], Marcelo Siqueira Valle[a,*]

[a] Departamento de Ciências Naturais, Universidade Federal de São João del-Rei, 36301-160, São João del-Rei, MG, Brazil
[b] Departamento de Química, Universidade Federal do Triângulo Mineiro, 38025-440, Uberaba, MG, Brazil
[c] Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto e FCFRP-USP, Universidade de São Paulo, Avenida do Café s/n, 14040-903, Ribeirão Preto, SP, Brazil
[d] Centro Universitário Estácio de Ribeirão Preto, Abraão Issa Halach Street, 980, 14096-160, Ribeirão Preto, SP, Brazil

ARTICLE INFO

Article history:
Received 13 August 2020
Revised 10 November 2020
Accepted 24 January 2021
Available online 28 January 2021

Keywords:
Chagas disease
Thiosemicarbazones
Trypanosoma cruzi

ABSTRACT

Chagas disease is a neglected disease, being one of the leading causes of death from infectious diseases. In view of the severity of this pathology, this work describes the synthesis of new thiosemicarbazones derived from chalcones and dibenzalacetones as potential drugs for the treatment of this disease. The structures of all compounds were elucidated by infrared (IR) and nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectroscopies. The chalcone derived thiosemicarbazones **10**-**14** were tested against the intracellular amastigote form of the protozoan *Trypanosoma cruzi* and had their cytotoxicity assessed using LLC-MK2 cells. The compound **10** (IC_{50} = 12.25 μM) presented the best activity when compared with the standard drug benznidazole (IC_{50} = 5.64 μM).

1. Introduction

Chagas disease (CD) is a neglected disease, caused by hemoflagellate protozoan *Trypanosoma cruzi* (TC) [1,2]. This disease is endemic in countries of Central and South America, but, in the last decades the number of cases of CD increase in other countries in the South of United States of America, Canada, West Mediterranean and Western Pacific [2,3]. It is estimated that approximately 6 to 7 million people are potentially infected with TC which causes approximately 20,000 deaths per year and is the leading cause of infectious myocarditis [1,2]. Acute infections are usually asymptomatic but one third of chronically infections progress to death, showing diverse manifestations affecting the heart, intestines, and nervous systems with determines the debilitating character of this disease. Although Chagas disease was identified more than 100 years ago, current therapeutic options are limited only two nitroheterocyclic drugs: Benznidazole (BZN) and Nifurtimox (NFX). In some countries, NFX was discontinued due to serious side effects, such as neuropathy and anorexia, among others. BZN is only effective in the acute stage, with low cure infection but its efficacy, in the chronic phase of disease, remain questionable [4–6]. Moreover, BZN present limitations due to side effects, long treatment regiments [5,7–9]. In terms to improve the treatment options for the CD, many therapeutic options have been tested. Recently, the inhibitor of ergosterol synthesis, Posoconazol, was promising in reduce the infection. Unfortunately it failed in the clinical trial [5,10]. Therefore, one effective therapeutic option for patients with Chagas' disease are clearly needed.

With this concern in mind, the search for new drug candidates for the treatment of Chagas disease patients is urgent. Many molecules having thiosemicarbazones moiety has shown a broad pharmacological applications that include antineoplastic, antibacterial, antiviral, antiprotozoal and antifungal activity [11–15]. Regarding the antichagasic activity, a series of thiosemicarbazones, semicarbazones and aminoguanidine derivatives (**1**-**3**, Fig. 1) have shown trypanocidal activity which is supposedly related to their capacity in inhibiting essential enzymes for parasite survival inside the host cells [16,17]. Also interesting, the coordination of thiosemicarbazones to metal ions have shown to be a good strategy to potentialize their activity. For example, organometallic gold(III) complexes, such as **4**, were evaluated both by *in vitro* and *in vivo* tests, being more effective than benznidazole in eliminating both the extracellular trypomastigote and intracellular amastigote forms of the parasite without cytotoxic effects on mammalian cells [18].

* Corresponding author.
E-mail address: marcelovalle@ufsj.edu.br (M.S. Valle).

https://doi.org/10.1016/j.molstruc.2021.130014
0022-2860/

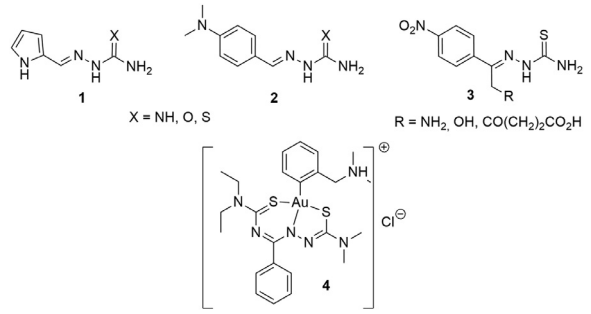


Fig. 1. Selected semicarbazones, thiosemicarbazones and aminoguanidine derivatives with properties against Chagas disease (**1**-**4**).

In view of the promising antichagasic properties of thiosemicarbazones, we propose the synthesis of new derivatives of this class of substances by conjugation with chalcones and its structural analogues dibenzalacetones (dbas). Chalcones are known for their varied medicinal activities [19,20]. Since chalcones and dbas are α,β-unsaturated ketones, they are liable to react with thiosemicarbazides to provide the respective thiosemicarbazones.

2. Experimental section

2.1. Materials and instruments

All reagents were purchased from commercial sources and used without further purification. The melting point measurements were performed on the MICRO-CHEMICAL equipment, model MQAPF-302. The FT-IR spectra were obtained using the BRUKER ALPHAFT-IR MB 102 spectrophotometer, in the 4000-400 cm^{-1} region on KBr pellets. The ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were performed on the BRUKER ADVANCE spectrometer, using deuterated chloroform ($CDCl_3$) or dimethylsulfoxide ($(CD_3)_2SO$).

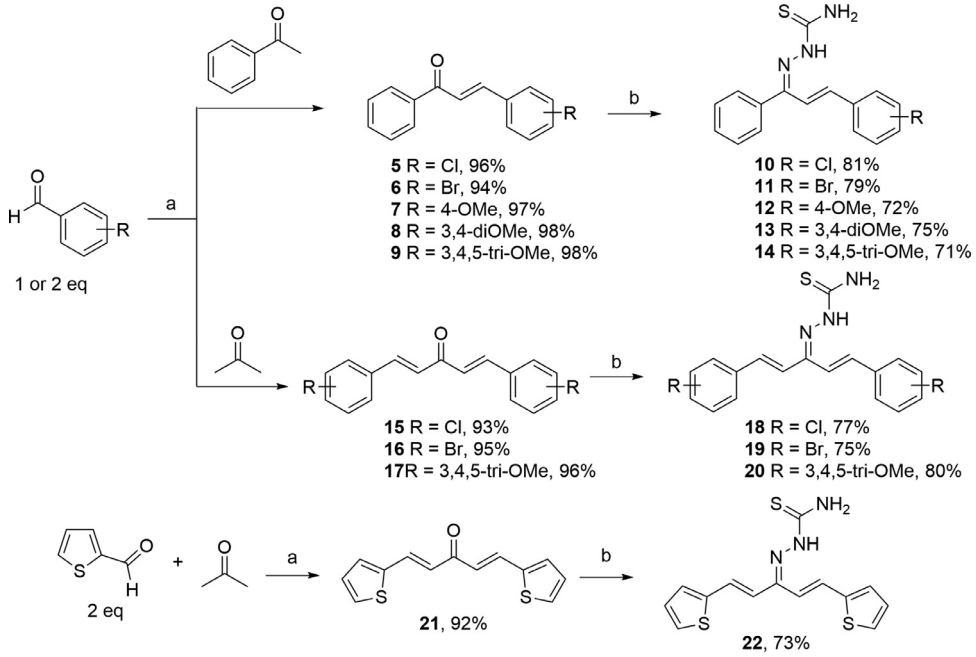
2.2. Synthesis

The α,β-unsaturated ketones (**5**-**9, 15**-**17, 21**) were obtained using Claisen-Schmidt aldolic condensation (Scheme 1) and the experimental procedure used for the synthesis of these compounds was based on the work of Aljamali and collaborators [21]. Briefly, 5.0 mmol of acetophenone and 20 mL of ethanol were added to a round-bottom flask in the presence of sodium hydroxide (5.0 mmol). The reaction system was kept under magnetic stirring until the mixture was completely solubilized. Immediately thereafter, the substituted aromatic aldehyde (5.0 mmol) was added, and the system remained under magnetic stirring until the precipitation of crystals. The reaction time varied from 1 to 4 hours at room temperature. The end of the reaction was verified by TLC (thin layer chromatography) (eluent: 7:3 hexane/ethyl acetate; developer: ultraviolet light and iodine vapor). The generated solids were vacuum filtered and left in a desiccator till dryness. The same procedure was made to the synthesis of dibenzalacetones but using acetone instead of acetophenone and 2 equivalents of aldehyde were required.

The corresponding thiosemicarbazones (**10**-**14, 18**-**20, 22**) were synthesized from the chalcones and dibenzalacetones previously obtained (Scheme 1). The procedure was performed as described by Góes et al. [22] 5.0 mmol of the chalcones (**5**-**9**) or dibenzalacetones (**15**-**17, 21**) were subjected to a condensation reaction with thiosemicarbazide (5.0 mmol) in ethanol in the presence of catalytic amounts of sulfuric acid (H_2SO_4). The reaction time varied from 1 to 4 hours at room temperature. The end of the reaction was verified by TLC (eluent: 7:3 hexane/ethyl acetate; developer: ultraviolet light and iodine vapor). The generated solids were filtered off and dried under vacuum.

2.3. Spectroscopic data

(2E)-3-(4-chlorophenyl)-1-phenylprop-2-en-1-one (**5**). Pale yellow solid; Yield: 96%; mp: 108–110°C (lit. 108.0 – 110.0°C) [23]; FT-IR (KBr) $1/\lambda$ (cm^{-1}): 1660 (C=O), 1591 (C=C $_{olefin}$), 1465 and 1402 (C=C $_{aromatic}$), 1090 (C-Cl), 3241 (=C-H sp^2), 813 (*p*-substituted), 2356 (*p*-substituted harmonic); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36 – 7.59 (m, 8H, H_α and aromatic hydrogens), 7.75 (d, 1H, *J* 15.7 Hz, H_β), 7.98 (d, 2H, *J* 7.5 Hz, H_3, H_5); ^{13}C NMR (125 MHz, $CDCl_3$) δ 122.5 (C_α), 128.5 ($C_{2'}$, $C_{6'}$), 128.7 (C_3, C_5), 129.2 ($C_{3'}$, $C_{5'}$), 129.6 (C_2, C_6), 132.9 (C_1), 133.4 ($C_{1'}$), 136.4 (C_4), 138.0 ($C_{4'}$), 143.3 (C_β), 190.2 (C=O).



Scheme 1. Synthetic route for the synthesis of thiosemicarbazones **10**-**14, 18**-**20** and **22**. *Reactional conditions*: a) NaOH, EtOH/H_2O, rt; b) $NH_2NHCSNH_2$, $H_2SO_{4(cat)}$, EtOH, rt.

(2E)-3-(4-bromophenyl)-1-phenylprop-2-en-1-one (**6**). Pale yellow solid; Yield: 94%; mp: 121–122°C (lit. 121.0 – 123.0°C) [24]; FT-IR (KBr) 1/λ (cm^{-1}): 3419 (=C-H sp^2), 1662 (C=O), 1598 (C=C $_{olefin}$), 1482 and 1444 (C=C $_{aromatic}$), 1069 (C-Br), 813 (*p*-substituted); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.47–7.59 (m, 8H, H_α and aromatic hydrogens), 7.73 (d, 1H, *J* 15.7 Hz, H_β), 7.98 (d, 2H, *J* 7.3 Hz, H_3, H_5); ^{13}C NMR (125 MHz, $CDCl_3$) δ 122.5 (C_α), 124.8 (C_4), 128.5 ($C_{2'}$, $C_{6'}$), 128.7 (C_2, C_6), 129.8 ($C_{3'}$, $C_{5'}$), 132.2 (C_3, C_5), 132.9 (C_1), 133.8 ($C_{4'}$), 138.0 ($C_{1'}$), 143.3 (C_β), 190.2 (C=O).

(2E)-3-(4-methoxyphenyl)-1-phenylprop-2-en-1-one (**7**). Pale yellow solid; Yield: 97%; mp: 64–66°C (lit. 65.0 – 67.0°C) [25]; FT-IR (KBr) 1/λ (cm^{-1}): 3261 (=C-H sp^2), 2834 (C-H sp^3), 2048 (*p*-substituted harmonic), 1602 (C=O), 1511 (C=C $_{olefin}$), 1471 and 1422 (C=C $_{aromatic}$), 1252 (C-O), 822 (*p*-substituted); ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.83 (s, 3H, CH_3), 6.92 (d, 2H, *J* 8.5 Hz, H_2, H_6), 7.37–7.59 (m, 5H, aromatic hydrogens), 7.41 (d, 1H, *J* 15.7 Hz, H_α), 7.78 (d, 1H, *J* 15.6 Hz, H_β), 7.98 (d, 2H, *J* 7.6 Hz, H_3, H_5); ^{13}C NMR (125 MHz, $CDCl_3$) δ 55.4 (C_7), 114.4 (C_3, C_5), 119.8 (C_α), 127.6 (C_1), 128.4 ($C_{2'}$, $C_{6'}$), 128.6 ($C_{3'}$, $C_{5'}$), 130.2 (C_2, C_6), 132.6 ($C_{1'}$), 138.5 ($C_{4'}$), 144.7 (C_β), 161.7 (C_4), 190.6 (C=O).

(2E)-3-(3,4-dimethoxyphenyl)-1-phenylprop-2-en-1-one (**8**). Pale yellow solid; Yield: 98%; mp: 81–83°C (lit. 83.0 – 84.0°C) [26]; FT-IR (KBr) 1/λ (cm^{-1}): 3344 (=C-H sp^2), 2968 (C-H sp^3), 1604 (C=O), 1519 (C=C $_{olefin}$), 1468 and 1420 (C=C $_{aromatic}$), 1261 (C-O); ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.00 (d, 6H, CH_3), 5.95 (d, 1H, *J* 8.4 Hz, H_5), 6.21 (s, 1H, H_2), 6.26 (t, 1H, $H_{4'}$), 6.29 (t, 1H, H_6), 6.45 (d, 1H, *J* 15.6 Hz, H_α), 6.55 (t, 2H, $H_{3'}$, $H_{5'}$), 6.82 (d, 1H, *J* 15.6 Hz, H_β), 7.06 (d, 2H, *J* 7.3 Hz, $H_{2'}$, $H_{6'}$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 56.0 (C_7), 56.1 (C_8), 110.1 (C_2), 111.1 (C_5), 120.1 (C_α), 123.2 (C_6), 127.9 (C_1), 128.4 ($C_{2'}$, $C_{6'}$), 128.6 ($C_{3'}$, $C_{5'}$), 132.6 ($C_{4'}$), 138.5 ($C_{1'}$), 145.0 (C_β), 149.2 (C_4), 151.4 (C_3), 190.6 (C=O).

(2E)-3-(3,4,5-trimethoxyphenyl)-1-phenylprop-2-en-1-one (**9**). Pale yellow solid; Yield: 98%; mp: 135–137°C (lit. 135.0 – 136.0°C) [27]; FT-IR (KBr) 1/λ (cm^{-1}): 3344 (=C-H sp^2), 2827 (C-H sp^3), 1581 (C=O), 1504 (C=C $_{olefin}$), 1473 and 1419 (C=C $_{aromatic}$), 1114 (C-O); ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.89 (s, 9H, CH_3), 6.84 (s, 2H, H_2, H_6), 7.24 – 7.99 (m, 7H, H_β, H_α and aromatic hydrogens); ^{13}C NMR (125 MHz, $CDCl_3$) δ 56.2 (C_7, C_9), 61.0 (C_8), 105.7 (C_2, C_6), 121.5 (C_α), 128.5 (C_1), 128.6 ($C_{2'}$, $C_{6'}$), 130.4 ($C_{3'}$, $C_{5'}$), 132.7 ($C_{4'}$), 138.3 ($C_{1'}$), 140.5 (C_4), 145.0 (C_β), 153.5 (C_3, C_5), 190.6 (C=O).

(Z)-2-((E)-3-(4-chlorophenyl)-1-phenylallylidene) hydrazinecarbothioamide (**10**). Pale yellow solid; Yield: 81%; mp: 218–220°C; FT-IR (KBr) 1/λ (cm^{-1}): 3416 (=C-H sp^2), 3230 (N-H $_{stretch}$), 1639 (C=N), 1619 (C=C $_{olefin}$), 1479 and 1400 (C=C $_{aromatic}$), 1253 (N-H $_{folding}$), 1085 (C-Cl), 815 (*p*-substituted); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.38 (d, 1H, *J* 16.2 Hz, H_α), 6.47 (s, 1H, NH), 7.03 (d, 1H, *J* 16.2 Hz, H_β), 7.39 – 7.79 (m, 7H, aromatic hydrogens), 8.03 – 8.04 (d, 2H, H_3, H_5), 8.54 (s, 2H, NH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 122.5 (C_α), 128.2 ($C_{3'}$, $C_{5'}$), 128.3 (C_3, C_5), 129.0 (C_2, C_6), 129.2 ($C_{2'}$, $C_{6'}$), 130.0 ($C_{4'}$), 130.4 (C_1), 134.7 ($C_{1'}$), 137.4 (C_4), 143.3 (C_β), 152.0 (C=N), 178.5 (C=S).

(Z)-2-((E)-3-(4-bromophenyl)-1-phenylallylidene) hydrazinecarbothioamide (**11**). Pale yellow solid; Yield: 79 %; mp: 228–230°C; FT-IR (KBr) 1/λ (cm^{-1}): 3407 (=C-H sp^2), 3234 (N-H $_{stretch}$), 1661 (C=N), 1639 (C=C $_{olefin}$), 1484 and 1392 (C=C $_{aromatic}$), 1221 (N-H), 1071 (C-Br), 812 (*p*-substituted); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.30 (d, 1H, *J* 16.3 Hz, H_α), 6.40 (s, 1H, NH), 7.00 (d, 1H, *J* 16.3 Hz, H_β), 7.40–7.70 (d, 2H, H_3, H_5), 7.40–8.00 (m, 7H, aromatic hydrogens), 8.50 (s, 2H, NH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 122.5 (C_α), 124.8 (C_4), 128.2 (C_2, C_6), 128.7 ($C_{3'}$, $C_{5'}$), 129.8 ($C_{2'}$, $C_{6'}$), 130.4 ($C_{4'}$), 132.0 (C_3, C_5), 132.2 (C_1), 134.7 ($C_{1'}$), 143.4 (C_β), 152.0 (C=N), 178.5 (C=S).

(Z)-2-((E)-3-(4-methoxyphenyl)-1-phenylallylidene) hydrazinecarbothioamide (**12**). Orange solid; Yield: 72 %; mp: 226–228°C; FT-IR (KBr) 1/λ (cm^{-1}): 3435 (=C-H sp^2), 3236 (N-H $_{stretch}$), 2364 (C-H sp^3), 1637 (C=N), 1618 (C=C $_{olefin}$), 1512 (N-H $_{folding}$), 1402 and 1383 (C=C $_{aromatic}$), 1258 (C-O), 804 (*p*-substituted); ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.92 (s, 3H, CH_3), 6.31 (d, 1H, *J* 16.3 Hz, H_α), 6.42 (d, 1H, *J* 16.3 Hz, H_β), 6.92 (s, 1H, NH), 7.00 – 7.01 (d, 2H, H_3, H_5), 7.24 – 8.08 (m, 7H, aromatic hydrogens), 8.55 (s, 2H, NH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 55.4 (C_7), 114.4 (C_3, C_5), 119.8 (C_α), 125.6 (C_1), 127.6 ($C_{3'}$, $C_{5'}$), 128.4 ($C_{2'}$, $C_{6'}$), 128.6 (C_2, C_6), 130.2 ($C_{4'}$), 132.5 (C_β), 138.5 ($C_{1'}$), 144.7 (C=N), 161.7 (C_4), 178.3 (C=S).

(Z)-2-((E)-3-(3,4-dimethoxyphenyl)-1-phenylallylidene) hydrazinecarbothioamide (**13**). Orange solid; Yield: 75 %; mp: 245–249°C; FT-IR (KBr) 1/λ (cm^{-1}): 3470 (=C-H sp^2), 2826 (C-H sp^3), 1645 (C=N), 1500 (C=C $_{olefin}$), 1467 (N-H $_{folding}$), 1410 and 1329 (C=C $_{aromatic}$), 1265 (C-O); ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.97 (s, 6H, CH_3), 6.32 (d, 1H, *J* 16.3 Hz, H_α), 6.35 (d, 1H, *J* 16.3 Hz, H_β), 6.83 (s, 1H, NH), 6.93–8.04 (m, 8H, aromatic hydrogens), 8.52 (s, 2H, NH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 56.0 (C_7, C_8), 110.1 (C_2), 111.1 (C_5), 120.1 (C_α), 123.2 (C_6), 127.9 (C_1), 128.4 ($C_{3'}$, $C_{5'}$), 128.6 ($C_{2'}$, $C_{6'}$), 129.9 ($C_{4'}$), 132.6 ($C_{1'}$), 138.5 (C_4), 145,0 (C_β), 149.3 (C_3), 151.5 (C=N), 178.3 (C=S).

(Z)-2-((E)-1-phenyl-3-(3,4,5-trimethoxyphenyl)allylidene) hydrazinecarbothioamide (**14**). Orange solid; Yield: 71 %; mp: 247 – 251°C; FT-IR (KBr) 1/λ (cm^{-1}): 3425 (=C-H sp^2), 3154 (N-H $_{stretch}$), 2826 (C-H sp^3), 1645 (C=N), 1601 (N-H), 1504 (C=C $_{olefin}$), 1473 and 1419 (C=C $_{aromatic}$), 1123 (C-O); ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.87 (s, 9H, CH_3), 6.38 (d, 1H, *J* 16.2 Hz, H_α), 6.55 (s, 1H, NH), 7.01 (d, 1H, *J* 16.2 Hz, H_β), 7.27–7.29 (d, 2H, H_2, H_6), 7.58–7.61 (m, 3H, $H_{3'}$, $H_{4'}$, $H_{5'}$), 8.02–8.04 (d, 2H, $H_{2'}$, $H_{6'}$), 8.52 (s, 2H, NH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 56.2 (C_7, C_9), 61.0 (C_8), 104.2 (C_2, C_6), 105.7 (C_1), 121.5 (C_α), 128.5 ($C_{3'}$, $C_{5'}$), 128.6 ($C_{2'}$, $C_{6'}$), 130.0 ($C_{4'}$), 130.4 ($C_{1'}$), 132.7 (C_β), 138.3 (C_4), 140.5 (C_3, C_5), 145.0 (C=N), 177.8 (C=S).

(1E,4E)-1,5-bis(4-chlorophenyl)penta-1,4-dien-3-one (**15**). Pale yellow solid; Yield: 93 %; mp: 190–193°C (lit. 193°C) [28]; FT-IR (KBr) 1/λ (cm^{-1}): 3043 (=C-H sp^2), 1650 (C=O), 1588 (C=C $_{olefin}$), 1492 and 1405 (C=C $_{aromatic}$), 1087 (C-Cl), 821 (*p*-substituted); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.08 (d, 2H, *J* 15.6 Hz, H_α, $H_{\alpha'}$), 7.43 (d, 4H, H_3, H_5, $H_{3'}$, $H_{5'}$), 7.58 (d, 4H, H_2, H_6, $H_{2'}$, $H_{6'}$), 7.73 (d, 2H, *J* 15.6 Hz, H_β, $H_{\beta'}$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 125.7 (C_α, $C_{\alpha'}$), 129.3 (C_3, C_5, $C_{3'}$, $C_{5'}$), 129.5 (C_2, C_6, $C_{2'}$, $C_{6'}$), 133.2 (C_1, $C_{1'}$), 136.5 (C_4, $C_{4'}$), 142.0 (C_β, $C_{\beta'}$), 188.3 (C=O).

(1E,4E)-1,5-bis(4-bromophenyl)penta-1,4-dien-3-one (**16**). Pale yellow solid; Yield: 95 %; mp: 209–212°C (lit. 211.0 – 213.0°C) [29]; FT-IR (KBr) 1/λ (cm^{-1}): 2930 (=C-H sp^2), 1653 (C=O), 1592 (C=C $_{olefin}$), 1490 and 1402 (C=C $_{aromatic}$), 1076 (C-Br), 822 (*p*-substituted); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.07 (d, 2H, *J* 15.6 Hz, H_α, $H_{\alpha'}$), 7.50 (d, 4H, H_3, H_5, $H_{3'}$, $H_{5'}$), 7.58 (d, 4H, H_2, H_6, $H_{2'}$, $H_{6'}$), 7.70 (d, 2H, *J* 15.6 Hz, H_β, $H_{\beta'}$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 124.9 (C_4, $C_{4'}$), 125.8 (C_α, $C_{\alpha'}$), 129.7 (C_2, C_6, $C_{2'}$, $C_{6'}$), 132.2 (C_3, C_5, $C_{3'}$, $C_{5'}$), 133.6 (C_1, $C_{1'}$), 142.1 (C_β, $C_{\beta'}$), 188.3 (C=O).

(1E,4E)-1,5-bis(3,4,5-trimethoxyphenyl)penta-1,4-dien-3-one (**17**). Orange solid; Yield: 96 %; mp: 124–127°C (lit. 123.0 – 124.0°C) [29]; FT-IR (KBr) 1/λ (cm^{-1}): 3000 (=C-H sp^2), 2830 (C-H sp^3), 1591 (C=O), 1510 (C=C $_{olefin}$), 1464 and 1415 (C=C $_{aromatic}$), 1128 (C-O); ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.93 (s, 18H, H_7, H_8, H_9, $H_{7'}$, $H_{8'}$, $H_{9'}$), 6.87 (s, 4H, H_2, H_6, $H_{2'}$, $H_{6'}$), 6.98 (d, 2H, *J* 15.0 Hz, H_α, $H_{\alpha'}$), 7.66 (d, 2H, *J* 15.0 Hz, H_β, $H_{\beta'}$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 56.2 (C_7, C_9, $C_{7'}$, $C_{9'}$), 61.0 (C_8, $C_{8'}$), 105.6 (C_2, C_6, $C_{2'}$, $C_{6'}$), 124.8 (C_α, $C_{\alpha'}$), 130.2 (C_1, $C_{1'}$), 140.4 (C_4, $C_{4'}$), 143.3 (C_β, $C_{\beta'}$), 153.5 (C_3, C_5, $C_{3'}$, $C_{5'}$), 188.5 (C=O).

2-((1E,4E)-1,5-bis(4-chlorophenyl)penta-1,4-dien-3-ylidene) hydrazinecarbothioamide (**18**). Orange solid; Yield: 77 %; mp: 226–228°C; FT-IR (KBr) 1/λ (cm^{-1}): 3435 (=C-H sp^2), 3236 (N-H $_{stretch}$), 1627 (C=N), 1601 (C=C $_{olefin}$), 1482 and 1402 (C=C $_{aromatic}$), 1318 (N-H $_{folding}$), 1084 (C-Cl), 814 (*p*-substituted); ^{1}H NMR

(500 MHz, $CDCl_3$) δ 7.04 (d, 2H, *J* 16.2 Hz, H_α, $H_{\alpha'}$), 7.24 (s, 1H, NH), 7.29 – 7.58 (m, 8H, aromatic hydrogens), 7.72 (d, 2H, *J* 16.2 Hz, H_β, $H_{\beta'}$), 9.08 (s, 2H, NH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 125.7 (C_α, $C_{\alpha'}$), 126.4 (C_4, $C_{4'}$), 128.3 (C_1, $C_{1'}$), 128.5 (C_2, C_6, $C_{2'}$, $C_{6'}$), 129.3 (C_3, C_5, $C_{3'}$, $C_{5'}$), 129.5 (C_β, $C_{\beta'}$), 142.1 (C=N), 178.7 (C=S).

2-((1E,4E)-1,5-bis(4-bromophenyl)penta-1,4-dien-3-ylidene) hydrazinecarbothioamide (**19**). Orange solid; Yield: 75 %; mp: 230–232°C; FT-IR (KBr) 1/λ (cm^{-1}): 1655 (C=N), 1596 ($C=C_{olefin}$), 1487 and 1398 ($C=C_{aromatic}$), 1202 (N-H), 1076 (C-Br), 822 (*p*-substituted); ^1H NMR (500 MHz, $CDCl_3$) δ 7.03 (d, 2H, *J* 15.3 Hz, H_α, $H_{\alpha'}$), 7.31 (s, 1H, NH), 7.45 – 7.53 (m, 8H, aromatic hydrogens), 7.66 (d, 2H, *J* 15.3 Hz, H_β, $H_{\beta'}$), 8.95 (s, 2H, NH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 124.9 (C_α, $C_{\alpha'}$), 125.8 (C_4, $C_{4'}$), 128.7 (C_1, $C_{1'}$), 129.7 (C_2, C_6, $C_{2'}$, $C_{6'}$), 132.2 (C_3, C_5, $C_{3'}$, $C_{5'}$), 133.6 (C_β, $C_{\beta'}$), 142.1 (C=N), 188.3 (C=S).

2-((1E,4E)-1,5-bis(3,4,5-trimethoxyphenyl)penta-1,4-dien-3-ylidene)hydrazinecarbothioamide (**20**). Pale red solid; Yield: 80 %; mp: 250–252°C; FT-IR (KBr) 1/λ (cm^{-1}): 2919 (C-H sp^3), 1582 (C=N), 1500 (N-H), 1456 ($C=C_{olefin}$), 1339 and 1237 ($C=C_{aromatic}$), 1128 (C-O); ^1H NMR (500 MHz, $CDCl_3$) δ 3.87 (s, 18H, H_7, H_8, H_9, $H_{7'}$, $H_{8'}$, $H_{9'}$), 6.37 (d, 2H, *J* 15.3 Hz, H_α, $H_{\alpha'}$), 6.74 (d, 4H, H_2, H_6, $H_{2'}$, $H_{6'}$), 6.95 (s, 1H, NH), 7.35 (d, 2H, *J* 15.3 Hz, H_β, $H_{\beta'}$), 9.01 (s, 2H, NH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 56.2 (C_7, C_9, $C_{7'}$, $C_{9'}$), 61.0 (C_8, $C_{8'}$), 104.3 (C_2, C_6, $C_{2'}$, $C_{6'}$), 104.6 (C_1, $C_{1'}$), 114.9 (C_4, $C_{4'}$), 125.5 (C_α, $C_{\alpha'}$), 131.5 (C_β, $C_{\beta'}$), 137.4 (C_3, C_5, $C_{3'}$, $C_{5'}$), 153.6 (C=N), 187,0 (C=S).

(1E,4E)-1,5-di(thiophen-2-yl)penta-1,4-dien-3-one (**21**). Brown solid; Yield: 92 %; mp: 110–113°C (lit. 113.0 – 114.0°C) [30]; FT-IR (KBr) 1/λ (cm^{-1}): 3073 (=C-H sp^2), 1609 (C=O), 1555 ($C=C_{olefin}$), 1420 and 1370 ($C=C_{aromatic}$); ^1H NMR (500 MHz, $CDCl_3$) δ 6.80 (d, 2H, *J* 15.7 Hz, H_α, $H_{\alpha'}$), 7.05 (t, 2H, H_3, $H_{3'}$), 7.31 (d, 2H, H_2, $H_{2'}$), 7.38 (d, 2H, H_4, $H_{4'}$), 7.82 (d, 2H, *J* 15.7 Hz, H_β, $H_{\beta'}$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 124.4 (C_α, $C_{\alpha'}$), 128.3 (C_3, $C_{3'}$), 128.8 (C_2, $C_{2'}$), 131.8 (C_β, $C_{\beta'}$), 135.6 (C_4, $C_{4'}$), 140.3 (C_1, $C_{1'}$), 187.7 (C=O).

2-((1E,4E)-1,5-di(thiophen-2-yl)penta-1,4-dien-3-ylidene) hydrazinecarbothioamide (**22**). Dark brown solid; Yield: 73 %; mp: 217–220°C; FT-IR (KBr) 1/λ (cm^{-1}): 3422 (=C-H sp^2), 3264 (N-H), 1610 (C=N), 1484 ($C=C_{olefin}$), 1420 and 1360 ($C=C_{aromatic}$), 1094 ($N-H_{folding}$); ^1H NMR (500 MHz, $CDCl_3$) δ 6.80 (d, 2H, *J* 15.3 Hz, H_α, $H_{\alpha'}$), 7.05–7.42 (m, 5H, H_3, $H_{3'}$, H_2, $H_{2'}$, NH), 7.82 (d, 2H, *J* 15.3 Hz, H_β, $H_{\beta'}$), 8.99 (s, 2H, NH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 114.2 (C_β, $C_{\beta'}$), 127.8 (C_3, $C_{3'}$), 128.0 (C_α, $C_{\alpha'}$), 128.5 (C_2, $C_{2'}$), 128.8 (C_4, $C_{4'}$), 135.7 (C_1, $C_{1'}$), 145.7 (C=N), 178.6 (C=S).

2.4. Biological assays

2.4.1. In vitro trypanocidal activity assay

Trypanocidal activity of the thiossemicarbazones complexes and benznidazole (BZN) was evaluated against amastigotes forms of the Tulahuen strain expressed beta-galactosidase. Briefly, LLCMK-2 cells (2.5×10^4 cells. mL^{-1}) were ressuspended in RPMI-1640 medium without phenol red (Sigma-Aldrich), supplemented with 5% bovine foetal serum (GIBCO, Grand Island, NY, USA), 100 IU mL^{-1} penicillin G, and 100 mg mL^{-1} streptomycin (Gibco-BRL, Grand Island, NY, USA) and seeded in 96-well microplates. After 3 h, the cells were infected with 5.0×10^5 trypomastigotes forms of *T. cruzi* Tulahuen strain stably expressing the β-galactosidase gene from *Escherichia coli* and incubated for 48 h. After the infection period, the plates were washed to remove the free trypomastigotes forms of *T. cruzi*. The infected cells with the intracellular amastigotes forms were incubated with the compounds or benznidazole (BZN), in serial concentrations between 500 to 3.9 mM. After 72 h, 50 µL of PBS (phosphate buffered saline) containing 0.3% of Triton X-100 and 400 mM chlorophenol Red-β-D-galactopyranoside (CPRG) were added. Plates were incubated at 37°C for 4 h and the absorbance was read at 570 nm. The BZN was used as positive control and the culture media as negative control. The concentration of the compound corresponding to 50% trypanocidal activity in amastigote forms was expressed as the IC_{50} amastigote.

2.5. In vitro *cytotoxicity assay*

The LLC-MK2 cell viability was assessed using the classical [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) colorimetric assay to determine selectivity against *T. cruzi*. For this propose, the same protocol established to trypanocidal activity was maintained. Briefly, 2.5×10^4 cells were incubated for 48 h in 96-well microplate for cell culture. After this incubation period, the compounds or BZN was added (concentrations 500 to 3.9 mM in serial dilution / compounds solubilized in DMSO 0.5%) in a final volume of 200 mL. Cells were incubated for 72 hours at 37 °C. After incubation with the ligands and their complexes, the medium was removed and added 50 mL of MTT (5.0 mg mL^{-1}) diluted in phosphate buffered saline (PBS). The precipitated blue MTT formazan was then dissolved in 50 mL of DMSO, and the absorbance was measured at 570 nm in a VARIAN CARY-50 plate reader MPR multiwell. Cell viability was expressed as the percentage of absorption values in treated cells compared with untreated (control) cells. CC_{50} (cytotoxic concentration of 50% of the cells) was also calculated. Thus, SI is defined by the ratio of CC_{50} to IC_{50}.

3. Results and discussion

3.1. Synthesis

For the synthesis of thiossemicarbazones, we started preparing the α,β-insaturated ketones chalcones and dibenzalacetones by Claisen-Schmidt aldolic condensation of acetophenone or acetone with different aromatic aldehydes in basic medium (Scheme 1). The reaction time varied from 1 to 4 hours and the solids generated were filtered under vacuum and washed with cold water and left in a desiccator till dryness. The products were obtained in excellent yields (94-98% for the chalcones **5-9** and 92-96 % for the dibenzalacetones **15-17, 21**). The melting range values corresponding to these compounds were between 2 to 3°C, which indicates a relatively high degree of purity and are in accordance with the values described in the literature (see Experimental Section).

From the chalcones and dbas obtained, the synthesis of thiosemicarbazones in good yields (71-83 %) was possible by reactions with the desired thiosemicarbazide in ethanol under acid catalysis (Scheme 1), providing the chalcones (**10-14**) and dibenzalacetones (**18-20** and **22**) derivatives.

3.2. Spectroscopic analysis

The characterization of all compounds was performed by FTIR and multinuclear NMR (^1H and ^{13}C) spectroscopies and by comparison of values of melting points with those from literature for the compounds previously described. The IR spectra analysis of chalcones (**5-9**) and dbas (**15-17, 21**) showed the main bands of absorption related to the desired products. All of them showed asymmetric intense stretching bands for typical unsaturated carbonyl group (C=O) absorptions at 1660 cm^{-1} or close to this region. The band referring to the olefin C=C double bond was found close to 1500 cm^{-1}, while the stretches associated with the aromatic ring (C=C) showed two absorptions between 1480 and 1402 cm^{-1}. The formation of the products was also verified by the analysis of the ^1H NMR spectra, from which it's possible to observe a doublet (*J* 15.7 Hz) related to the β-H in the 7.40 to 7.80 ppm region, belonging to the *trans* isomer of the olefin. In the ^{13}C NMR spectra, we highlight the signal at around 190.0 ppm which is attributed to carbonyl

Table 1
IC_{50} and CC_{50} values of the trypanocidal activity and cytotoxicity of the chalcone derived thiosemicarbazones **10**-**14**.

Compound	IC_{50} (μM)	$CC_{50\ (LLC\text{-}MK2)}$ (μM)	SI
10	12.25	39.89	3.25
11	46.42	45.12	0.97
12	31.96	65.17	2.03
13	30.40	80.49	2.64
14	40.59	36.03	0.88
BZN	5.64	204.00	36.00

carbon. In the range of 122.5 to 143.3 ppm the aromatic carbon signals and α and β carbonyl signals are observed.

The structures of thiosemicarbazones were also confirmed by NMR and FTIR techniques. By analyzing their IR spectra, we could observe the expected stretching bands associated with the groups N-H and C=N in the region of 3400 and 1630 cm^{-1}, respectively. In general, for the ^1H NMR spectra, the structures of thiosemicarbazones were confirmed by observing the presence of a wide integrated singlet peak with chemical shift close to 6.4 ppm, referring to the hydrogen atom from the NH group, as well as another singlet in the region of 8.6 ppm referring to the hydrogen atoms from the amine group (NH_2). Likewise, the signals arising in their ^{13}C NMR spectra at around 152.0 ppm and 187 ppm can be attributed to the carbon atoms from the C=N and C=S groups, respectively, which confirm the expected structures.

3.3. Evaluation of the antichagasic activity

To evaluate the potential of these compounds as therapeutic agents for Chagas disease treatment, preliminary biological tests were performed on the intracellular form of Trypanosoma cruzi and determined their cytotoxicity using the LLC-MK2 (Macaca mulatta) cell line. The data obtained are shown in Table 1.

From the data shown in Table 1, it is possible to notice that thiosemicarbazone **10** showed a remarkable IC_{50} value when compared to benznidazole (standard drug used to treat the disease). When comparing compounds **10** and **11**, structural analogues substituted at the same position by different halogen atoms, it is observed that compound containing the more electronegative halogen atom, **10**, is about three times more effective than compound **11**. Similarly, the replacement of the chlorine atom by a methoxy group, compound **12**, did not improve the activity. Furthermore, by increasing the number of substitutions by methoxy groups, compounds **13** and **14**, the activity was kept or decreased when compared to the monosubstituted **12**. Regarding the CC_{50} values, it is possible to verify that the peripheral groups of the thiosemicarbazones play a role on the cytotoxicity. Therefore, although the compounds studied here showed low selectivity indexes, it was possible to verify that the activity and cytotoxicity may be tuned by changes in their structure.

4. Conclusions

In this work, 18 compounds were synthesized, from which 9 are thiosemicarbazones, and all of them were characterized by FT-IR and $^1H/^{13}C$ NMR analysis. The products were obtained with global yields (two steps) that varied from 70 to 78%. The thiosemicarbazones derived from chalcones **10**-**14** showed activity against the protozoan *Trypanosoma cruzi*, the etiological agent of CD, being compound **10** the most effective (IC_{50} = 12.25 μM). Furthermore, it was observed that the cytotoxicity on LLC-MK2 cells is dependent on the substituents on the thiosemicarbazone moiety. These preliminary results show that the investigated compounds present potential as basis for the development of new drugs for Chagas disease treatment. However, more modifications in their structure by including other withdrawing and donating groups in the aromatic rings or their coordination to metal ions must be performed in order to establish a better structure-activity relationship of this class of compounds in order to fine tune their antiparasitc activity, which is an ongoing subject.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare the following financial interests/personal relationships which may be considered as potential competing interests

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.molstruc.2021.130014.

CRediT authorship contribution statement

Aline Alves da Silva: Visualization, Investigation, Data curation, Writing - original draft. **Pedro Ivo da Silva Maia:** Writing - review & editing, Data curation, Writing - original draft. **Carla Duque Lopes:** Data curation, Writing - original draft, Validation. **Sergio de Albuquerque:** Data curation, Writing - original draft, Validation. **Marcelo Siqueira Valle:** Conceptualization, Methodology, Visualization, Investigation, Writing - review & editing, Supervision, Data curation, Writing - original draft.

References

[1] D.H. Molyneux, L. Savioli, D. Engels, Neglected tropical diseases: progress towards addressing the chronic pandemic, Lancet 389 (10066) (2017) 312–325, doi:10.1016/S0140-6736(16)30171-4.
[2] WHO, "What is Chagas disease?," *Who*, 2020. [Online]. Available: http://www.who.int/chagas/disease/en/. [Accessed: 26-Jul-2020].
[3] M. Castillo-Riquelme, Chagas disease in non-endemic countries, Lancet Glob. Heal. 5 (4) (2017) e379–e380, doi:10.1016/S2214-109X(17)30090-6.
[4] J.A. Urbina, Recent clinical trials for the etiological treatment of chronic chagas disease: advances, challenges and perspectives, J. Eukaryot. Microbiol. 62 (1) (2015) 149–156, doi:10.1111/jeu.12184.
[5] I. Molina, et al., Randomized trial of posaconazole and benznidazole for chronic Chagas' disease, N. Engl. J. Med. 370 (20) (2014) 1899–1908, doi:10.1056/NEJMoa1313122.
[6] F. Villalta, G. Rachakonda, Advances in preclinical approaches to Chagas disease drug discovery, Expert Opin. Drug Discov. 14 (11) (2019) 1161–1174, doi:10.1080/17460441.2019.1652593.
[7] R. Viotti, et al., Towards a paradigm shift in the treatment of chronic chagas disease, Antimicrob. Agents Chemother. 58 (2) (2014) 635–639, doi:10.1128/AAC.01662-13.
[8] J. Bermudez, C. Davies, A. Simonazzi, J. Pablo Real, S. Palma, Current drug therapy and pharmaceutical challenges for Chagas disease, Acta Trop. 156 (2016) 1–16, doi:10.1016/j.actatropica.2015.12.017.
[9] J.A. Urbina, Specific chemotherapy of chagas disease: relevance, current limitations and new approaches, Acta Trop. 115 (1–2) (2010) 55–68, doi:10.1016/j.actatropica.2009.10.023.
[10] C.A. Morillo, et al., Benznidazole and posaconazole in eliminating parasites in asymptomatic T. Cruzi carriers: the Stop-Chagas trial, J. Am. Coll. Cardiol. 69 (8) (2017) 939–947, doi:10.1016/j.jacc.2016.12.023.
[11] L. Feun, et al., Phase I and pharmacokinetic study of 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP) using a single intravenous dose schedule, Cancer Chemother. Pharmacol. 50 (3) (2002) 223–229, doi:10.1007/s00280-002-0480-0.
[12] N.C. Kasuga, et al., Synthesis, structural characterization and antimicrobial activities of 12 zinc(II) complexes with four thiosemicarbazone and two semicarbazone ligands, J. Inorg. Biochem. 96 (2–3) (2003) 298–310, doi:10.1016/S0162-0134(03)00156-9.
[13] Y. Teitz, D. Ronen, A. Vansover, T. Stematsky, J.L. Riggs, Inhibition of human immunodeficiency virus by N-methylisatin-β4′:4′-diethylthiosemicarbazone and N-allylisatin-β-4′:4′-diallythiosemicarbazone, Antiviral Res. 24 (4) (1994) 305–314, doi:10.1016/0166-3542(94)90077-9.
[14] D.X. West, et al., Thiosemicarbazone complexes of copper(II): structural and biological studies, Coord. Chem. Rev. 123 (1–2) (1993) 49–71, doi:10.1016/0010-8545(93)85052-6.
[15] N. Bharti, K. Husain, M.T.G. Garza, D.E. Cruz-vega, B.D. Mata-cardenas, A. Azam, Synthesis and in vitro antiprotozoal activity of thiosemicarbazone derivatives, Bioorg. Med. Chem. Lett. 12 (2002) 3475–3478.

[16] D.G. Vital, et al., Application of bioisosterism in design of the semicarbazone derivatives as cruzain inhibitors: a theoretical and experimental study, J. Biomol. Struct. Dyn. 35 (6) (2017) 1244–1259, doi:10.1080/07391102.2016.1176603.
[17] L. Blau, et al., Design, synthesis and biological evaluation of new aryl thiosemicarbazone as antichagasic candidates, Eur. J. Med. Chem. 67 (2013) 142–151, doi:10.1016/j.ejmech.2013.04.022.
[18] C.D. Lopes, et al., Organometallic Gold(III) complex [Au(Hdamp)(L14)]+ (L1 = SNS-donating thiosemicarbazone) as a candidate to new formulations against Chagas disease, ACS Infect. Dis. 5 (10) (2019) 1698–1707, doi:10.1021/acsinfecdis.8b00284.
[19] R. Arif, et al., Facile synthesis of chalcone derivatives as antibacterial agents: synthesis, DNA binding, molecular docking, DFT and antioxidant studies, J. Mol. Struct. 1208 (2020) 127905, doi:10.1016/j.molstruc.2020.127905.
[20] M. Mustafa, Y.A. Mostafa, A facile synthesis, drug-likeness, and in silico molecular docking of certain new azidosulfonamide–chalcones and their in vitro antimicrobial activity, Monatshefte fur Chemie 151 (3) (2020) 417–427, doi:10.1007/s00706-020-02568-8.
[21] A.M. Jawad, M.N.M. Salih, T.A. Helal, Review on chalcone (preparation, reactions, medical and bio applications), Int. J. Chem. Synth. Chem. React. 5 (1) (2019) 16–27.
[22] R.P. Tenório, A.J.S. Góes, J.G. De Lima, A.R. De Faria, A.J. Alves, T.M. De Aquino, Tiossemicarbazonas: Métodos de obtenção, aplicações sintéticas e importância biológica, Quim. Nova 28 (6) (2005) 1030–1037, doi:10.1590/S0100-40422005000600018.
[23] S.R. Sarda et al., "Solvent-free NaOH-Al 2 O 3 supported synthesis of Ar," vol. 1, no. 2, pp. 265–269, 2009.
[24] A. Stroba, et al., 3,5-Diphenylpent-2-enoic acids as allosteric activators of the protein kinase PDK1: structure-activity relationships and thermodynamic characterization of binding as paradigms for PIF-binding pocket-targeting compounds, J. Med. Chem. 52 (15) (2009) 4683–4693, doi:10.1021/jm9001499.
[25] T.P. Acids, et al., Synthesis of some benzalacetophenones and their imino derivatives, J. Cryst. Growth 310 (10) (2008) 4702–4708, doi:10.1055/sos-sd-039-00764.
[26] V.D. Silva, B.U. Stambuk, M.da G. Nascimento, Efficient chemoselective biohydrogenation of 1,3-diaryl-2-propen-1-ones catalyzed by Saccharomyces cerevisiae yeasts in biphasic system, J. Mol. Catal. B Enzym. 63 (3–4) (2010) 157–163, doi:10.1016/j.molcatb.2010.01.010.
[27] S. Ducki, et al., Combretastatin-like chalcones as inhibitors of microtubule polymerization. Part 1: synthesis and biological evaluation of antivascular activity, Bioorganic Med. Chem. 17 (22) (2009) 7698–7710, doi:10.1016/j.bmc.2009.09.039.
[28] H.J. Ravindra, et al., Synthesis, crystal growth and characterization of a phase matchable nonlinear optical single crystal: p-chloro dibenzylideneacetone, J. Cryst. Growth 310 (10) (2008) 2543–2549, doi:10.1016/j.jcrysgro.2007.12.066.
[29] H. Ohori, et al., Synthesis and biolgical analysis of new curcumin analogues bearing an enhanced potential for the medicinal treatment of cancer, Mol. Cancer Ther. 5 (10) (2006) 2563–2571, doi:10.1158/1535-7163.MCT-06-0174.
[30] T.P. Acids, Studies the xvii, 1931 no. 232.