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Avaliação *In Vitro* de Flavonas Sintéticas contra o *Trypanosoma cruzi*

Resumo: A doença de Chagas é uma infeção causada pelo protozoário parasita *Trypanosoma cruzi* e afeta cerca de 8 milhões de pessoas em 21 países da América Latina. O tratamento dessa doença ainda se baseia no uso de benzonidazol ou nifurtimox, que apresentam baixas taxas de cura na fase crônica e frequentemente apresentam muitos efeitos colaterais indesejáveis. Aqui, descrevemos a síntese de flavonas e a avaliação de sua atividade tripanocida. As flavonas foram testadas *in vitro* contra o *T. cruzi* e dentre os 13 compostos testados, 6 destes demonstraram alguma atividade tripanocida modesta *in vitro*. Observaram-se melhorias na atividade anti *T. cruzi* para flavonas portadoras de substituintes nitro ou metóxi. Notavelmente, foram mantidas citotoxicidades muito baixas com grupos metoxila, o que sugere que esse grupo funcional favorece compostos tripanocidas mais seletivos. Além disso, a modificação estrutural na posição 3 do anel diidropirona forneceu a flavona mais ativa, o que sugere que a introdução de diferentes funcionalidades nessa posição poderia gerar novos compostos promissores com propriedades tripanocidas.

Palavras-chave: Doença de Chagas; flavonas; *Trypanosoma cruzi*; in vitro.

Abstract

Chagas disease is caused by infection of the parasite protozoan *Trypanosoma cruzi* and affects about 8 million people in 21 countries in Latin America. Treatment of this disease is still based on the use of benznidazole or nifurtimox, which both present low cure rates in the chronic phase and often have many undesirable side effects. Herein, we describe the synthesis of flavones and evaluation of their trypanocidal activity. The flavones were tested to *in vitro* against *T. cruzi* and amongst the 13 compounds tested, 6 of these demonstrated some modest trypanocidal activity *in vitro*. Enhancements in anti *T. cruzi* activity were noted for flavones bearing either nitro or methoxy substituents. Moreover, very low cytotoxicities were maintained for flavones with methoxy groups which suggests that this functional group favors more selective trypanocidal compounds. Finally, structural modification at position 3 of the dihydropyrone ring provided the most active flavone, which suggests that the introduction of different functionalities at this position could yield promising new compounds with trypanocidal properties.

Keywords: Chagas disease; Flavones; *Trypanosoma cruzi*; Trypanocidal.

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In Vitro Evaluation of Synthetic Flavones Against *Trypanosoma cruzi*

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1. Introduction

Neglected Tropical Diseases affect people living on low incomes in mainly developing countries, causing economic and health problems in many communities. Chagas disease, also called American trypanossomiasis, is an infectious disease caused by the protozoan parasite *Trypanosoma cruzi (T. cruzi),* through direct contact with contaminated feces of triatomine bugs.[1,2] Approximately 6 to 7 million people are infected with Chagas disease worldwide, mostly in Latin America.[3,4,5] Currently, only two medicines are employed for the treatment of Chagas disease: nifurtimox (NFX) or benznidazole (BZ), both of which cause undesirable side effects and present low cure rates in the chronic phase of disease.[6, 7] Given the negative economic and social impact caused by Chagas disease, the search for new drugs has become increasingly necessary to treat this disease. Flavones isolated from plants have been isolated and tested for their anti *T. cruzi* activity. For example, flavone and 7-methoxyflavone isolated from the leaves of *Conchocarphus heterophyllus*[8] and flavone-C-diglycoside isoswertisin-α-L-rhamnoside isolated from the leaves of *Peperomia obtusifolia* (Piperaceae)[9] were assayed against *T. cruzi* and all displayed weak trypanocidal activity. In comparison, the flavone-C-glycoside, isoorientin isolated from Turkish *Ajuga laxmannii* (Lamiaceae) was significantly more potent against *T. cruzi in vitro* (Figure 1).[10]

Generally, flavones exhibit very low toxicity and moderate trypanocidal activity, becoming an interesting template for designing more potent and selective derivatives. In this regard, we have prepared and tested synthetic flavones against *T. cruzi,* evaluated their cytotoxic effect against L929 cells and determined the selectivity index.

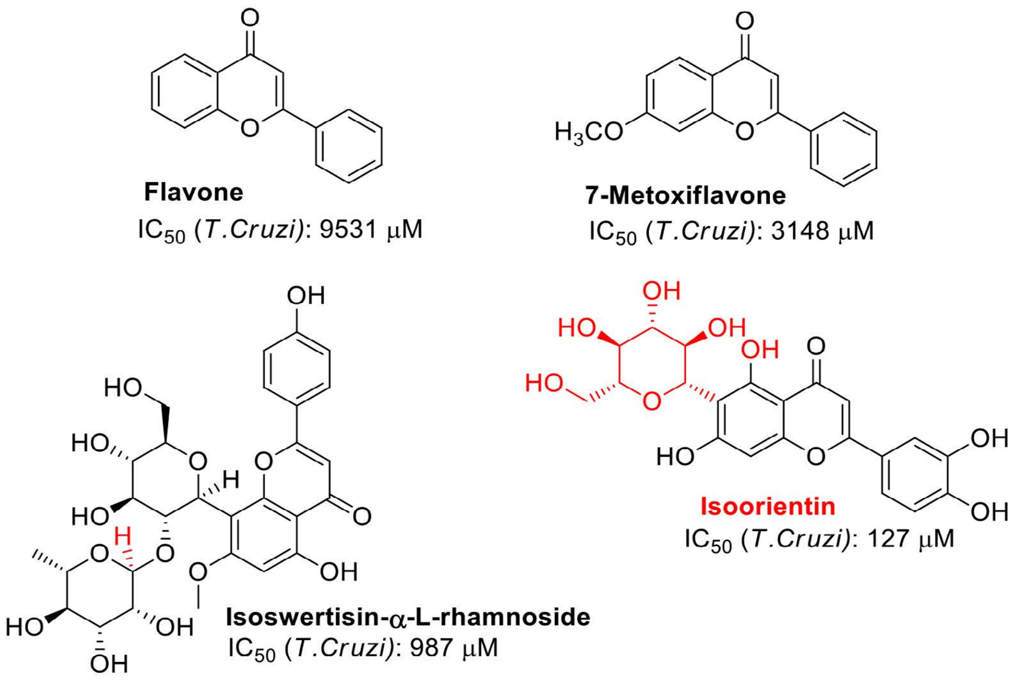


Figure 1. Trypanocidal Flavones

2. Results and Discussion

The method of choice for the synthesis of a range of structurally diverse flavones is based on a straightforward procedure described by Wheeler in 1952 and involves the dehydrative cyclization of certain 1,3-diaryl diketones.[11] The synthesis of the target flavones began with the esterification of 2-hydroxyacetophenones with substituted benzoyl chlorides to provide the corresponding esters **1** (Scheme 1). The esters undergo a Baker-Venkataraman rearrangement in the presence of KOH to afford 1,3-diketones **2**. The 1,3-diaryl-1,3-propanediones **2** have proven to be versatile building blocks for the synthesis of trypanocidal diazepines,[12] isoxazoles[13] and flavanones.[14] Compounds **2** when isolated by precipitation and filtration, were immediately subjected to a condensation reaction under refluxing acetic acid to provide flavones **3a-k** (Scheme 1).

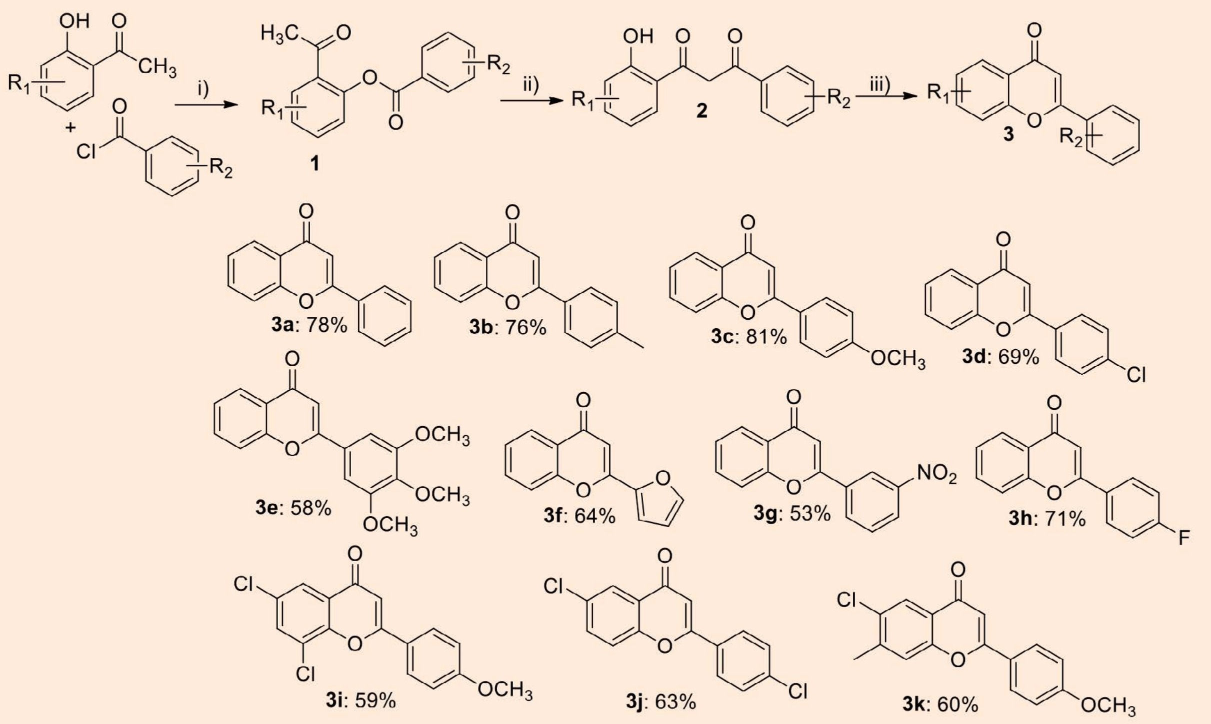
All data for flavones **3a-k** were in complete accordance with literature values (see experimental section).

When employing 2-hydroxyacetophenone and isophthaloyl chloride in the aforementioned synthetic route, the “diflavone” **3l** (Scheme 2) was prepared following a slightly modified procedure described previously by our group.[15]

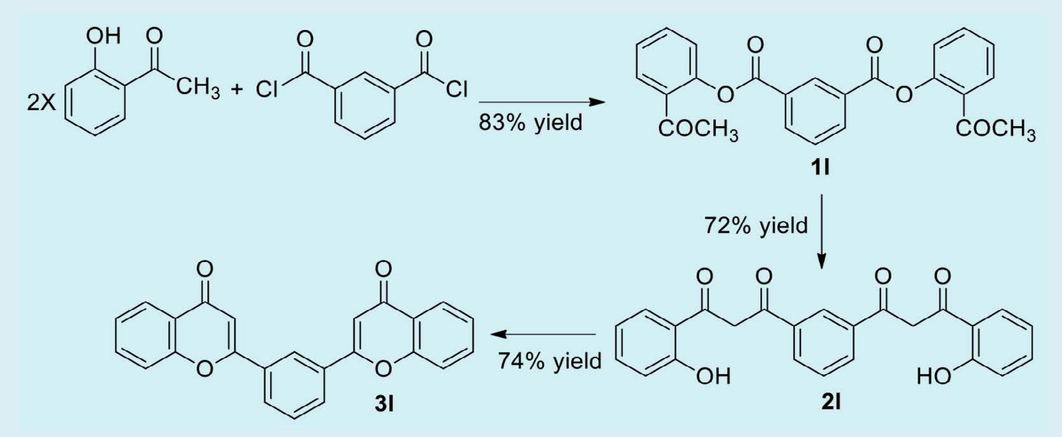
Finally, in order to assess how the removal of the olefinic hydrogen would impact the trypanocidal activity, flavone **3d** was brominated at the position 3 of the dihydropyrone ring to afford the corresponding flavone **3m** in good yield (Scheme 3).

Thus, with the target compounds in hand, *in vitro* bioassays using trypomastigote and amastigote forms of Tulahuen strain *T. cruzi* were carried out. Once the final products were purified and fully characterized, we carried out *in vitro* bioassays against trypomastigote and intracellular amastigote of β-galactosidase transfected Tulahuen strain of *T. cruzi.* We have opted for an *in vitro* methodology that simultaneously evaluates trypomastigote forms that are initially present in the blood after entering through the bite wound and intracellular amastigotes forms present in the vertebrate host during the acute and chronic phases of the disease.[16] This approach is in accordance with the guidelines proposed by the Fiocruz Program for Research and Technological Development on Chagas Disease and the Drugs for Neglected Diseases Initiative (DNDi).[17] Benznidazole was used as a positive control against *T. cruzi* and cytotoxicity was determined in mammalian L929 cells (Table 1).

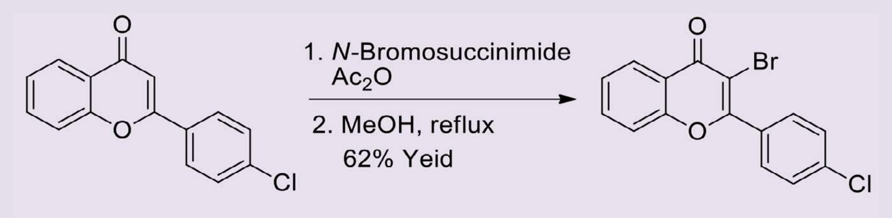
Amongst the 13 compounds tested, only six of these presented measurable trypanocidal activity and none of the flavone derivatives were



Scheme 1. Synthetic route for the preparation of flavones: i) pyridine, rt, 1 h; ii) pyridine, KOH, 50°C, 1 h; iii) AcOH, H_2SO_4, reflux, 1 h

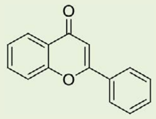
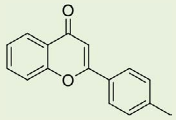
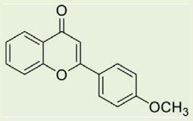
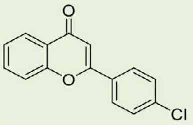
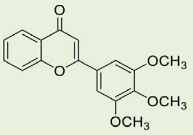
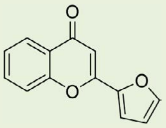
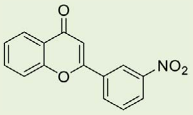
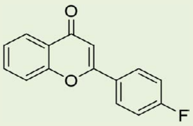
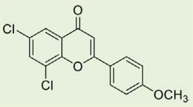
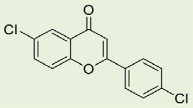
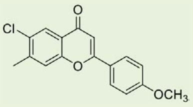
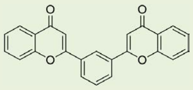
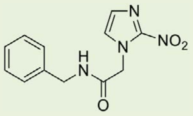


Scheme 2. Synthesis of Flavone **3l**



Scheme 3. Synthesis of Flavone **3m**

Table 1. *In vitro* trypanocidal activity, cytotoxicity and selectivity index of bioactive flavones

Compd No.	Flavone	Trypanocide IC_{50}(μM)	Cytotoxicity CC_{50}(μM)	SI	Log P	TPSA (Å^2)
3a		Inactive	>300	-	3.74	30.21
3b		Inactive	>300	-	4.19	30.21
3c		383.7	>300	-	3.79	39.45
3d		Inactive	>300	-	4.42	30.21
3e		211.8	>300	-	3.37	57.91
3f		Inactive	>300	-	2.88	43.35
3g		97.2	122.5	1.3	3.67	76.03
3h		Inactive	>300	-	3.90	30.21
3i		312.4	>300	-	5.05	39.45
3j		Inactive	>300	-	5.07	30.21
3k		356.1	>300	-	4.82	39.45
3l		Inactive	>300	-	5.51	60.42
3m		39.0	60.0	1.5	5.15	30.21
Benz		3.8	2381	625	-	-

more active than reference drug benznidazole. As expected, almost all of the flavones presented very low cytotoxicity and their cytotoxicity could not be quantified. Initially, compound **3a** was evaluated for trypanocidal activity and this result used for comparison in order to assess Structure Activity Relationships (SAR). Although the anti *T. cruzi* activity for flavone **3a** had been reported to be 9531 µM,[8] the present *in vitro* assay was not capable of determining the IC_{50} for such a weakly active compound. The same was also true for flavones **3b**, **3d**, **3f**, **3h**, **3j** and **3l**. From these results, we can conclude that the introduction of a methyl group and halogens on the pendant benzenoid ring or substitution of this moiety for a furan were not conducive to increasing trypanocidal activity. In contrast, the inclusion of methoxy substituents did allow for measurable trypanocidal activities to be uncovered. Furthermore, flavones **3c**, **3e**, **3i** and **3k** were essentially non-toxic which suggests that the methoxy group favors more selective trypanocidal compounds. The improvements in anti *T. cruzi* activity provided by methoxy substituents has been highlighted in studies on flavanones and chalcones.[14, 18] Furthermore, in the case of tricyclic coumarins, a 7 fold increase anti *T. cruzi* activity was observed with the introduction of methoxy substituents.[19] Although flavone **3g** bearing a nitro group was significantly the most active flavone, unfortunately the cytotoxicity and poor selectivity index render flavone **3g** an undesirable candidate for further *in vivo* studies. Finally, a remarkable improvement in trypanocidal activity was noted for brominated flavone **3m** suggesting that modifications at position 3 of the dihydropyrone ring greatly enhances the trypanocidal properties of flavones. Once again, the cytotoxicity was unfavorable for further investigation *in vivo;* nevertheless, these preliminary results suggest that the modification at this position with other functionalities could yield active and selective trypanocidal compounds.

The potential of compounds **3a-m** as candidates for new drugs was also evaluated by using Linpinski parameters, which considers the physicochemical drug descriptors of the molecular properties for the synthesized compounds as calculated by Molinspiration software. The partition coefficient (LogP: octanol/water partition coefficient) describes the equilibrium distribution between two liquid phases such as octanol and water. The total polar surface area (TPSA) is a measure of the extent of the molecules exposed polar area. The results show that compounds **3c, 3e, 3g, 3K** satisfy Linpinski's rule of five with no violations.[20] Flavones **3**j, **3i**, **3l** and **3m** violate the rule by presenting lipophilicity (Log P) greater than 5.0, which could cause problems with oral bioavailability. Flavones **3c, 3e, 3g, 3K, 3i** and **3m** showed TPSA values lower than 90 $Å^2$, values ranging from 30.21 - 76.03 $Å^2$, indicating that these compounds would have a good permeability in the plasma cell membrane and across the blood brain barrier.

3. Conclusion

In conclusion, flavones **3c**, **3e**, **3g**, **3i**, **3k** and **3m** showed moderate trypanocidal activity but none of the tested flavones were more active than reference compound benznidazole. However, this study showed some important aspects with regard to how structural modifications could favour the development of lead compounds for the treatment of Chagas disease. The results indicated that the presence of the methoxy group is beneficial for anti *T. cruzi* activity since flavones bearing this substituent were moderately active and essentially non-toxic. Moreover, structural modification with a halogen at position 3 of the dihydropyrone ring provided the most active flavone, which suggests that the introduction of different functionalities at this position could yield promising new compounds with trypanocidal properties.

4. Experimental Section

All commercial reagents were used as received. Anhydrous solvents were purchased from Sigma Aldrich. Flash column chromatography was performed using silica gel 200-400 Mesh. TLC analyses were performed using silica gel plates, using ultraviolet light (254 nm), phosphomolybdic acid or vanillin solution for visualization. Melting points are uncorrected and were recorded on a Buchi B-540 apparatus. For NMR data, the chemical shifts are reported in δ (ppm) referenced to residual solvent protons and ^{13}C signals in deuterated chloroform. Coupling constants (*J*) are expressed in Hertz (*Hz*). Infrared spectra were obtained on a Thermo Scientific Nicolet 380 FT-IR apparatus (600–4000 cm^{-1}, Nicolet Instrument Corp., Madison, WI, USA) using attenuated total reflection (ATR). High Resolution Mass Spectra were obtained on a Shimadzu HPLC-ESI-IT-TOF. SMILES notations of the flavone derivatives were inputted into an online software and subjected to molecular properties prediction by Molinspiration software (software version v2015.01).

4.1. Characterization data

Flavones were prepared according to literature methods.[11]

2-phenyl-4H-chromen-4-one (3a): Product obtained as a white solid in 78%. m.p.: 95–97 °C (Lit.[21] mp. 95 – 96 °C); ^{1}H-NMR (300 MHz, $CDCl_3$): *δ* 6.83 (s, 1 H), 7.42 (t, *J* = 8.0 Hz, 1H), 7.50–7.59 (m, 4H), 7.67−7.74 (m, 1H), 7.91−7.95 (m, 2H), 8.21 (dd, *J* = 1.7 Hz, 8.0 Hz, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 107.5, 118.1, 123.9, 125.2, 125.7, 126.3, 129.1, 131.6, 131.7, 133.8, 156.3, 163.4, 178.52; HRMS (ESI-TOF) *m/z* [M + H] Calculated for $C_{15}H_{11}O_2^+$: 223.0754. Found: 223.0749.

2-(*p*-tolyl)-4H-chromen-4-one (**3b**): Product obtained as a white solid in 76%. m.p.: 109–110 °C (Lit.[22] mp. 110 – 112 °C); ^{1}H-NMR (300 MHz, $CDCl_3$): *δ* 2.43 (s, 3H), δ 6.87 (s, 1H), 7.31 (d, *J* = 8.0 Hz, 2H), 7.42 (t, *J* = 8.0 Hz, 1H), 7.55 (d, *J* = 8.0 Hz, 1H), 7.67 -7.72 (m, 1H), 7.81 (d, *J* = 8.3 Hz, 2H), 8.21 (dd, *J* = 1.5 Hz, 8.0 Hz, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 21.5, 106.8, 118.0, 123.8, 125.1, 125.6, 126.2, 128.8, 129.7, 133.7, 142.3, 156.2, 163.6, 178.45; HRMS (ESI-TOF) *m/z* [M + H] Calculated for $C_{16}H_{13}O_2^+$: 237.2775. Found: 237.1779.

2-(4-methoxyphenyl)-4H-chromen-4-one (3c): Product obtained as a white solid in 81%. m.p.: 156–157 °C (Lit.[23] mp. 157 – 159 °C); ^{1}H-NMR (300 MHz, $CDCl_3$): *δ* 3.90 (s, 3H), 6.75 (s, 1H), 7.02 (d, *J* = 8.9 Hz, 2H), 7.40 (t, *J* = 7.3 Hz, 1H), 7.54 (d, *J* = 8.3 Hz, 1H), 7.67-7.69 (m, 1H), 7.88 (d, *J* = 8.9 Hz, 2H), 8.23 (dd, *J* = 1.4 Hz, 7.9 Hz, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 55.5, 106.2, 114.5, 117.9, 123.9, 124.0, 125.1, 125.6, 127.9, 133.6, 156.2, 162.4, 163.4, 178.3; HRMS (ESI-TOF) *m/z* [M + H] Calculated for $C_{16}H_{13}O_3^+$: 253.0859. Found: 253.0851.

2-(4-chlorophenyl)-4H-chromen-4-one (3d): Product obtained as a white solid in 69%. m.p.: 186–187 °C (Lit.[24] mp. 187 – 188 °C); ^{1}H-NMR (300 MHz, $CDCl_3$): *δ* 6.78 (s, 1H), 7.38 – 7.56 (m, 4H) 7.69 -7.72 (m, 1H), 7.83 (d, *J* = 8.7 Hz, 2H), 8.19 (dd, *J* = 1.6 Hz, 7.9 Hz, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 107.6, 118.0, 123.8, 125.4, 125.7, 127.5, 129.3, 130.2, 133.9, 137.9, 156.2, 162.2, 178.2; HRMS (ESI-TOF) *m/z* [M + H] Calculated for $C_{15}H_{10}ClO_2^+$: 257.0364. Found: 257.0371.

2-(3,4,5-trimethoxyphenyl)-4H-chromen-4-one (3e): Product obtained as a white solid in 58%. m.p.: 172–174 °C (Lit.[25] mp. 172 – 174 °C); ^{1}H-NMR (300 MHz, $CDCl_3$): *δ* 3.92 (s, 3H), 3.95 (s, 6H), 6.78 (s, 1H), 7.12 (s, 2H), 7.42 (t, *J* = 7.7 Hz, 1H), 7.56 (d, *J* = 8.4 Hz, 1H), 7.70 (t, *J* = 8.2 Hz, 1H), 8.20 (d, *J* = 7.9 Hz, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 56.3, 61.0, 103.6, 107.3, 118.1, 123.8, 125.3, 125.6, 126.9, 133.8, 141.1, 153.5, 156.2, 163.3, 178.4; HRMS (ESI-TOF) *m/z* [M + H] Calculated for $C_{18}H_{17}O_5^+$: 313.1071. Found: 313.1073.

2-(furan-2-yl)-4H-chromen-4-one (3f): Product obtained as a white solid in 64%. m.p.: 130–131 °C (Lit.[26] mp. 134 – 135 °C); ^{1}H-NMR (400 MHz, $CDCl_3$): *δ* 6.59 – 7.71 (m, 1H), 6.75 (s, 1H), 7.13 (d, *J* = 3.4 Hz 1H), 7.39 (t, *J* = 7.8 Hz, 1H), 7.47 (d, *J* = 8.2 Hz, 1H), 7.62 – 7.70 (m, 2H), 8.20 (dd, *J* = 1.4 Hz, 7.9 Hz, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 105.3, 112.6, 113.4, 117.8, 124.0, 125.2, 125.7, 133.8, 145.9, 146.3, 155.3, 155.7, 177.8; HRMS (ESI-TOF) *m/z* [M + H] Calculated for $C_{13}H_9O_3^+$: 213.0546. Found: 213.0553.

2-(3-nitrophenyl)-4H-chromen-4-one (3g): Product obtained as a white solid in 53%. m.p.: 195–196 °C (Lit.[27] mp. 196 – 197 °C); ^{1}H-NMR (300 MHz, $CDCl_3$): *δ* 6.90 (s, 1H), 7.46 (t, *J* = 8 Hz, 1H), 7.62 (d, *J* = 8.3 Hz 1H), 7.72 - 7.78 (m, 2H), 8.21 (dd, *J* = 1.5 Hz, 8.0 Hz, 2H), 8.38 (dd, *J* = 2 Hz, 8.2 Hz, 1H), 8.80 (t, *J* = 2 Hz, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 108.8, 118.2, 121.2, 123.8, 125.7, 125.8, 125.9, 130.3, 131.8, 133.6, 134.3, 148.7, 156.1, 160.5; HRMS (ESI-TOF) *m/z* [M + H] Calculated for $C_{15}H_{10}NO_4^+$: 268.0604. Found: 268.0601.

2-(4-fluorophenyl)-4H-chromen-4-one (3h): Product obtained as a white solid in 71%. m.p.: 147–148 °C (Lit.[28] mp. 145 – 148 °C); ^{1}H-NMR (300 MHz, $CDCl_3$): *δ* 6.77 (s, 1H), 7.18 - 7.26 (m, 2H), 7.42 (t, *J* = 7.8 Hz, 1H), 7.54 (d, *J* = 8.4 Hz 1H), 7.69 – 7.72 (m, 1H), 7.90 - 7.95 (m, 2H), 8.20 (dd, *J* = 1.5 Hz, 8.0 Hz, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 108.8, 118.2 (C-F, d, *J* = 22.9 Hz), 121.2, 123.8, 125.7, 125.8, 125.9 (C-F, d, *J* = 3.1 Hz), 130.3 , (C-F, d, *J* = 8.8 Hz), 131.8, 133.6, 134.3, 148.7, 156.1, 160.5 (C-F, d, *J* = 250.6 Hz), 178.0; HRMS (ESI-TOF) *m/z* [M + H] Calculated for $C_{15}H_{10}FO_2^+$: 241.0659. Found: 241.0665.

6,8-dichloro-2-(4-methoxyphenyl)-4H-chromen-4-one (3i): Product obtained as a white solid in 59%. m.p.: 183–185 °C (Lit.[29] mp. 182 – 184 °C); ^{1}H-NMR (300 MHz, $CDCl_3$): δ 3.92 (s, 3H), 6.78 (s, 1H), 7.05 (d, *J* = 8.0 Hz, 2H), 7.73 (d, *J* = 4.0 Hz, 1H), 7.94 (d, *J* = 8.0 Hz, 2H), 8.10 (d, *J* = 4 Hz, 1 H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 55.6, 105.7, 114.7, 123.1, 123.8, 124.3, 125.7, 128.2, 130.7, 133.5, 150.4, 162.9, 163.5, 176.3; HRMS (ESI-TOF) *m/z* [M + H] Calculated for $C_{16}H_{11}Cl_2O_3^+$: 321.0080. Found: 321.0084.

6-chloro-2-(4-chlorophenyl)-4H-chromen-4-one (3j): Product obtained as a white solid in 63%. m.p.: 226–227 °C (Lit.[23] mp. 226 – 227 °C); ^{1}H-NMR (300 MHz, $CDCl_3$): δ 6.83 (s, 1H), 7.53 – 7.57 (m, 3H), 7.67 (dd, *J* = 2.6 Hz, 8.9 Hz, 1H), 7.87 (d, *J* = 8.7 Hz, 2H), 8.82 (d, *J* = 2,6 Hz, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 107.6, 119.8, 124.9, 125.2, 127.6, 129.5, 129.8, 131.4, 134.1, 138.2, 154.5, 162.5, 177.0; HRMS (ESI-TOF) *m/z* [M + H] Calculated for $C_{15}H_9Cl_2O_2^+$: 290.9974. Found: 290.9969.

6-chloro-2-(4-methoxyphenyl)-7-methyl-4H-chromen-4-one (3k): Product obtained as a white solid in 60%. m.p.: 225–227 °C (Lit.[30] mp. 227 – 227 °C); ^{1}H-NMR (300 MHz, $CDCl_3$): δ 2.54 (s, 3H), 3.92 (s, 3H), 6.74 (s, 1H), 7.04 (d, *J* = 8.9 Hz, 2H), 7.47 (s, 1H), 7.87 (d, *J* = 8.9 Hz, 2H), 8.19 (s, 1 H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 20.9, 55.5, 105.9, 114.5, 119.8, 123.0, 123.8, 125.4, 128.0, 131.7, 142.7, 154.4, 162.5, 163.5, 177.2; HRMS (ESI-TOF) *m/z* [M + H] Calculated for $C_{17}H_{14}ClO_3^+$: 301.0626. Found: 301.0619.

2,2’-(1,3-phenylene)bis(4H-chromen-4-one) (3l): Product obtained as a white solid in 74%. m.p.: 248–250 °C (Lit.[31] mp. 249 – 250 °C); ^{1}H-NMR (300 MHz, $CDCl_3$): δ 6.98 (s, 2H), 7.50 (t, *J* = 8.0 Hz, 2H), 7.67 (d, *J* = 8.4 Hz, 1H), 7.73 – 7.81 (m, 4H), 8.11 (dd, J = 2 Hz, 8.0 Hz, 2H), 8.28 (dd, J = 2Hz, 8.0 Hz, 2H), 8,54 (m, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 108.3, 118.2, 123.9 124.0, 125.6, 125.8, 129.1, 129.9, 132.9, 134.2, 156.3, 162.2, 178.4; HRMS (ESI-TOF) *m/z* [M + H] Calculated for $C_{24}H_{15}O_4^+$: 367.0965. Found: 367.0970.

Flavone **3d** was converted to flavone **3m** following the method described by Bird and co-workers [32]. 3-bromo-2-(4-chlorophenyl)-4H-chromen-4-one (**3m**): Product obtained as a white solid in 62%. m.p.: 175–176 °C (Lit.[22] mp. 178 – 179 °C); ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.46 – 7.52 (m, 4H), 7.70 – 7.74 (m, 1H), 7.80 – 7.83 (m, 2H), 8.26 (dd, J = 1.6Hz, 8.0Hz, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 109.4, 117.8, 121.7 125.9, 126.5, 128.7, 130.7, 131.2, 134.3, 137.4, 155.5, 160.8, 172.9; HRMS (ESI-TOF) *m/z* [M + H] Calculated for $C_{15}H_9BrClO_2^+$: 334.9496. Found: 334.9489.

4.2. Anti-*Trypanosoma cruzi* activity assay (amastigotes and trypomastigotes)

The *in vitro* anti-*T. cruzi* activity was evaluated on L929 cells (mouse fibroblasts) infected with Tulahuen strain of the parasite expressing the *Escherichia coli β*-galactosidase as reporter gene. Briefly, for the bioassay, 4,000 L929 cells were added to each well of a 96-well microtiter plate. After an overnight incubation, 40,000 trypomastigotes were added to the cells and incubated for 2 h. Then the medium containing extracelullar parasites was replaced with 200 µl of fresh medium and the plate was incubated for an additional 48 h to establish the infection. For IC_{50} determination, the cells were exposed to each synthesized compound at serial decreasing dilutions and the plate was incubated for 96 h. After this period, 50 µl of 500 µM chlorophenol red beta-*D*-galactopyranoside (CPRG) in 0.5% Nonidet P40 was added to each well, and the plate was incubated for 16 to 20 h, after which the absorbance at 570 nm was measured. Controls with uninfected cells, untreated infected cells, infected cells treated with benznidazole at 3.8 µM (positive control) or DMSO 1% were used. The results were expressed as the percentage of *T. cruzi* growth inhibition in compound-tested cells as compared to the infected cells and untreated cells. The IC_{50} values were calculated by linear interpolation. Quadruplicates were run in the same plate, and the experiments were repeated at least once.

4.3. *In vitro* cytotoxic test of trypanocidal compounds

The active compounds were tested *in vitro* for determination of cellular toxicity against uninfected L-929 cells using the alamarBlue® dye. The cells were exposed to compounds at increasing concentrations starting at IC_{50} value for *T. cruzi*. After 96 h of incubation with the tested compounds, the alamarBlue® was added and the absorbance at 570 and 600 nm measured after 4-6 h. The cell viability was expressed as the percentage of difference in the reduction between treated and untreated cells. Quadruplicates were run in the same plate, and the experiments were repeated at least once. CC_{50} values were calculated by linear interpolation and the selectivity index (SI) was determined based on the ratio of the CC_{50} value in the host cell divided by the IC_{50} value of the parasite.

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