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Article

Anti-Parasite and Cytotoxic Activities of Chloro and Bromo L-Tyrosine Derivatives

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A series of twenty-one L-tyrosine derivatives with modifications in the halogenation pattern of the aromatic ring and different degree of methylations on the amine and phenolic hydroxyl groups were synthesized. The structures of all the intermediates and target compounds were confirmed unambiguous by spectroscopy analysis. Additionally, all compounds were evaluated against *Plasmodium falciparum* and *Leishmania panamensis* parasites between 20-702 μg mL^{-1}. The cytotoxic evaluation was done to determine the selectivity index for each compound. Six compounds had the lower EC_{50} (effective concentration 50) against *L. panamensis*. One of these compounds was the most active with an EC_{50} at 24.13 μg mL^{-1} (76.07 μM). All derivatives showed no significant activity against *P. falciparum* and no compound has *in vitro* antifungal activity at 500 μg mL^{-1}.

Keywords: *L. panamensis*, *P. falciparum*, cytotoxic, L-tyrosine

Introduction

Diseases caused by protozoan parasites affect about one billion people, with a notable incidence in tropical countries. Diseases such as malaria and leishmaniasis are more frequent in developing countries of tropical and sub-tropical regions. These diseases are endemic problems in Colombia and other Latin American countries.

Currently, malaria is still a common cause of death with approximately 445,000 cases reported in 2016 in tropical countries of Africa, Asia and America; and tragically most of the victims are children under the age of five.[1] Despite the decrease in fatal cases recorded by the World Health Organization (WHO) in recent years, an increase in the spread of mosquitoes resistant to common insecticides is observed, and more importantly, the emergence of resistance to current drugs by different strains of *Plasmodium*.[2] Given that malaria is a disease with global implications and almost half of the world population is at risk of infection, combating malaria becomes one of the priorities of WHO programs.

On the other hand, leishmaniasis disease affect more than 10 million people worldwide, principally tropical countries. Protozoan parasites of the genus *Leishmania* responsible for the infection are: *L. panamensis*, *L. braziliensis*, *L. guyanensis*, *L. mexicana* and *L. amazonensis.*[3] Currently, chemotherapies to treat cutaneous leishmaniasis are based on old drugs; unfortunately, all of these drugs have severe toxic effects on patients. Side effects are associated with high therapeutic doses used and long term treatment schemes.[4] Compounds isolated from marine organisms are considered as rich sources for drug discovery. Marine sponges of Verongida order has a great diversity of bromotyrosine compounds, it has shown anti-parasite activities against *T. cruzi*, *P. falciparum*, and *L. panamensis* protozoa.[5-8] We reported isolations and identifications of more than sixteen bromotyrosine isolated from sponges *Verongula rigida* and *Aiolochroia crassa* with antiparasitic activity, and we found

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a close relationship between the degree of bromination in the aromatic ring, the methylation in the amino group and their influence in the antiparasitic activity.[9]

Due to the emerging resistance against current drugs, it is mandatory to develop new, safer and more effective antiparasitic agents. For that reason, in the present study, the potentials of marine natural products derivatives compounds, based on bioactive L-bromotyrosines, were evaluated as new antiparasitic compounds. The syntheses of novel chloro and bromo L-tyrosine derivatives, with different grade of methylation of the amino group, lead us to realize a structure-activity relationship between the halogenations patterns in the aromatic ring of the L-tyrosine and the substitution of the amino group, looking for the best structural parameters for the development of new antiparasitic compounds.

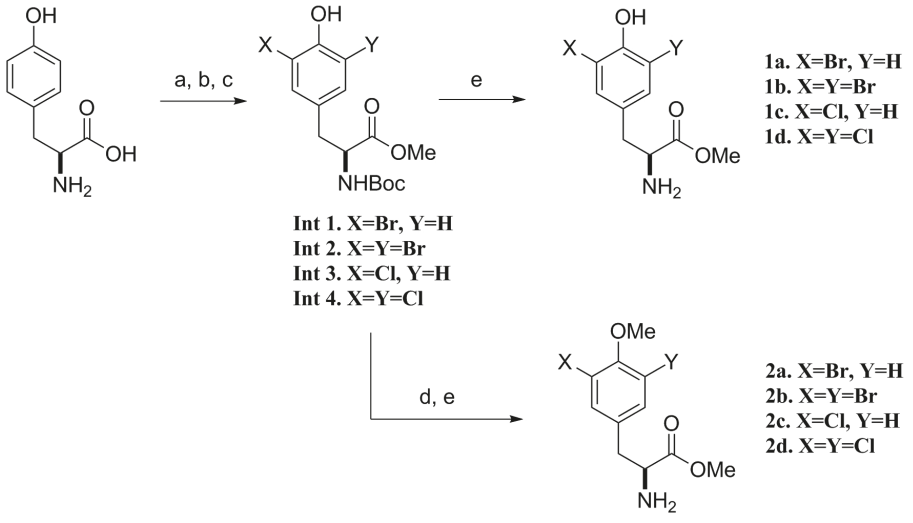
Results and Discussion

Chemistry

Synthesis of bromo and chloro *N*-substituted derivatives was made using as starting material L-tyrosine. For the synthesis of 21 products, it was necessary to synthesize initially four intermediates, which have their amino and carboxylic acids protected with a carbamate and methyl ester group, respectively. Methyl esters derivatives were formed by the reaction of L-tyrosine with $SOCl_2$/MeOH,[10] later the protection of the amino group was done by the reaction with Boc_2O/triethylamine (TEA) at room temperature.[11] The bromination and chlorination of the aromatic ring, getting mono and di halogenated compounds, were done using *N*-bromosuccinimide (NBS) and *N*-chlorosuccinimide (NCS),[12] producing intermediates (**Int1**-**Int4**). These intermediates were divided into two sets, one of them were treated with trifluoracetic acid (TFA) in dichloromethane (DCM),[13] to cleavage the amino group (**1a**-**1d**). The other intermediates were *O*-methylated with MeI in basic media,[14] and later treated with TFA in DCM (**2a-2d**) (Scheme 1).

The ^{1}H nuclear magnetic resonance (NMR) analysis of **Int1**-**Int4** shows the presence of a signal at 3.80 ppm (s, 3H) that represents the –$RCOOCH_3$ group of the methyl ester. The signal at 1.40 ppm (s, 9H) represents the three-methyl groups of the carbamate moiety. The mono halogenated compounds show in the aromatic region of the spectra three signals that corresponds to the protons at 2, 5, 6 positions in the aromatic ring, while the di halogenated compounds only show in the aromatic region of the spectra one signal, due to the symmetric ring, for two protons at 2 and 6 position.

The syntheses of primary amines were done by the hydrolysis of compounds **1a**-**1d**, **2a**-**2d** with K_2CO_3/MeOH,[15] getting compounds **4**-**11**. The syntheses of methyl ester tertiary amines were done by the reductive amination of compounds **1a**-**1d**, **2a**-**2d** with formaldehyde/$NaCNBH_3$, getting compounds **3a**-**3h**.[16] These set of compounds were divided in two, one of them were treated with K_2CO_3/MeOH to get the tertiary amines **12**-**18**. The other set was treated with MeI/acetone to get the methyl ester quaternary amines,[17] which were treated with K_2CO_3/MeOH to get the quaternary amines **19**-**24**. The synthesis of the primary, tertiary and quaternary amines are shown in Scheme 2. The ^{1}H NMR analyses of tertiary and quaternary amines show the presence of a signal at 2.88 ppm (s, 6H) for the di-methylation of the amino group in the tertiary amines, and 2.97 ppm (s, 9H) for the tri-methylation of the amino



Scheme 1. Intermediate synthesis from L-tyrosine. (a) $SOCl_2$, MEOH; (b) NBS or NCS, MeOH; (c) Boc_2O, TEA, MeOH; (d) MeI, K_2CO_3, acetone; (e) TFA, DCM.

group in the quaternary amines. *O*-Methyl derivatives were confirmed by ^{1}H NMR by the signal at 3.80 ppm (s, 3H).

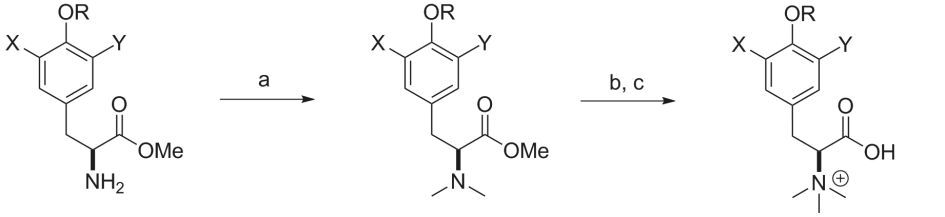
Biological activity

Compounds were tested against *P. falciparum* (NF54 chloroquine sensitive strain) and *L. panamensis* (MHOM/CO/87/UA140epir GP strain) using chloroquine and amphotericin B as positive control drugs, respectively. A cytotoxic test was done over human promonocytic cells (U-937 ATCC CRL1593.2). For every active compound, effective concentration 50 (EC_{50}) and lethal concentration 50 (LC_{50}), in μM units, were tested and selectivity index (SI) was represented as LC_{50}/EC_{50}. In Table 1, it is shown the anti-leishmanial and antiplasmodial activity of the 21 derivatives.

Compounds **12**, **16**, **18**, **19**, **20** and **22** were the most cytotoxic, while compounds **5**, **6**, **7**, **8**, **9**, **10**, **15**, **13**, **14**, **17** and **23** showed LC_{50} values higher than 200 μM, making them potentially safe against mammalian cells U-937. Compounds **4**, **5**, **6**, **10**, **21** and **24** were the most active against *L. panamensis* with values lower than 50 μg mL^{-1}. Derivative **21** were the most active with EC_{50} of 76.07 μM. Compounds **5** and **6** have the higher SI with values of 7.43 and 7.86, respectively. The twenty-one derivatives have no activity against *P. falciparum*.

In this study, it is possible to establish that the type of halogenation, in addition to the degree of methylation on the amino group, have influence in the anti-leishmanial activity of the compounds. Brominated compounds with *O*-methyl substitution in *para* and primary amine (such as compounds **8** and **9**) were less active than their correlated structure with free hydroxyl and additional amine substitutions, compounds **4** and **5**. In general, tertiary and quaternary amines derivatives are less cytotoxic than primary analogs. Mono-chloro compound **6** with *O*-methyl substitution in *para* and primary amine is the most promising derivative against *L. panamensis*.

Compounds were evaluated at a final concentration of 500 μg mL^{-1} against nineteen fungal strains of medical



1a. R=H, X=Br, Y=H
1b. R=H, X=Y=Br
1c. R=H, X=Cl, Y=H
1d. R=H, X=Y=Cl
2a. R=Me, X=Br, Y=H
2b. R=Me, X=Y=Br
2c. R=Me, X=Cl, Y=H
2d. R=Me, X=Y=Cl

3a. R=H, X=Br, Y=H
3b. R=H, X=Y=Br
3c. R=H, X=Cl, Y=H
3d. R=H, X=Y=Cl
3e. R=Me, X=Br, Y=H
3f. R=Me, X=Y=Br
3g. R=Me, X=Cl, Y=H
3h. R=Me, X=Y=Cl

19. R=H, X=Cl, Y=H
20. R=H, X=Y=Cl
21. R=Me, X=Br, Y=H
22. R=Me, X=Y=Br
23. R=Me, X=Cl, Y=H
24. R=Me, X=Y=Cl

4. R=H, X=Br, Y=H
5. R=H, X=Y=Br
6. R=H, X=Cl, Y=H
7. R=H, X=Y=Cl
8. R=Me, X=Br, Y=H
9. R=Me, X=Y=Br
10. R=Me, X=Cl, Y=H
11. R=Me, X=Y=Cl

12. R=H, X=Br, Y=H
13. R=H, X=Cl, Y=H
14. R=H, X=Y=Cl
15. R=Me, X=Br, Y=H
16. R=Me, X=Y=Br
17. R=Me, X=Cl, Y=H
18. R=Me, X=Y=Cl

Scheme 2. Synthesis of chloro and bromo L-tyrosine derivatives. (a) CH_2O, $NaCNBH_3$, MeOH; (b) MeI, acetone; (c) K_2CO_3, MeOH, THF, H_2O.

Table 1. Cytotoxicity, anti-leishmanial and antiplasmodial activities of the L-tyrosine derivatives

Compound	U-937	*L. panamensis*		*P. falciparum*	
	LC_{50}[a] / (μg mL^{-1}) [μM]	LC_{50}[b] / (μg mL^{-1}) [μM]	SI[c]	LC_{50}[b] / (μg mL^{-1}) [μM]	SI[c]
4	> 40 [153.79]	30.84 ± 1.06 [118.57 ± 4.07]	> 1.29	> 20 [76.89]	> 0.97
5	265.50 ± 7.5 [783.23 ± 22.12]	35.73 ± 5.22 [105.40 ± 15.39]	7.43	> 20 [59.00]	7.51
6	242.23 ± 8.3 [1123.35 ± 38.49]	30.83 ± 1.42 [142.97 ± 6.58]	7.86	> 20 [92.20]	8.32
7	> 200 [799.77]	281.01 ± 46.20 [1123.72 ± 184.74]	> 0.71	270.53 ± 5.71 [1081.81 ± 22.83]	> 4.59
12	> 10 [34.70]	69.09 ± 8.57 [239.78 ± 29.74]	> 0.14	> 20 [69.41]	< 0.5
13	176.8 ± 26.8 [725.54 ± 109.98]	101.9 ± 13.10 [418.17 ± 53.75]	1.75	89.35 ± 9.70 [366.66 ± 39.80]	1.97
14	143.2 ± 15.97 [514.86 ± 57.41]	185.34 ± 36.86 [666.37 ± 132.52]	0.77	> 20 [71.90]	< 7.16
19	38.53 ± 1.15 [148.92 ± 4.44]	157.96 ± 24.35 [610.54 ± 94.11]	0.24	> 20 [77.30]	< 1.92
20	20.49 ± 1.69 [69.89 ± 5.76]	75.67 ± 8.57 [258.11 ± 29.23]	0.27	> 20 [68.22]	> 1.01
8	164.03 ± 12.19 [598.40 ± 44.47]	82.25 ± 15.46 [300.06 ± 56.40]	1.99	> 20 [72.96]	4.08
9	358.00 ± 5.6 [1014.13 ± 15.86]	138.59 ± 36.23 [392.59 ± 102.63]	2.58	> 20 [56.65]	12.07
10	200.85 ± 10.0 [874.55 ± 43.54]	35.39 ± 1.74 [154.09 ± 7.57]	5.67	> 20 [87.08]	5.85
11	> 40 [151.45]	158.42 ± 34.99 [599.84 ± 132.48]	> 0.25	> 20 [75.72]	0.73
15	270.23 ± 7.40 [894.32 ± 24.49]	73.40 ± 11.20 [242.91 ± 37.06]	3.68	> 20 [66.19]	8.60
16	27.90 ± 1.69 [73.21 ± 4.43]	77.39 ± 10.61 [203.09 ± 27.84]	0.36	702.01 ± 140.4 [1842.25 ± 368.44]	0.04
17	> 200 [776.06]	534.31 ± 77.33 [2073.29 ± 300.06]	> 0.37	> 20 [77.60]	< 10
18	49.16 ± 4.93 [168.26 ± 16.87]	150.97 ± 7.98 [516.73 ± 27.31]	0.32	> 20 [68.45]	< 2.45
21	> 40 [126.10]	24.13 ± 2.50 [76.07 ± 7.88]	> 1.65	> 20 [63.05]	> 1.07
22	39.16 ± 1.48 [98.86 ± 3.73]	132.22 ± 17.27 [333.81 ± 43.06]	0.29	> 20 [50.49]	< 1.95
23	> 200 [733.27]	148.72 ± 17.80 [545.26 ± 65.26]	> 1.34	> 20 [73.32]	< 10
24	51.28 ± 6.85 [651.12 ± 7.56]	28.35 ± 1.40 [92.29 ± 57.95]	> 7.0	> 20 [65.11]	< 10
Amphotericin B	37.5 ± 0.9 [40.58 ± 0.97]	0.06 ± 0.01 [0.0.6 ± 0.01]	625	NA	NA
Chloroquine	152.2 ± 5.2 [475.81 ± 16.25]	NA	NA	0.84 ± 0.13 [2.62 ± 0.40]	181

[a]LC_{50}: lethal concentration 50; [b]EC_{50}: effective concentration 50; [c]SI: selectivity index = LC_{50} / EC_{50}. Data represent mean value ± standard deviation.

interest. No compound showed anti-fungal activity at this concentration.

Conclusions

The synthesis, cytotoxicity, and activity of twenty-one L-tyrosine derivatives against *L. panamnesis*, *P. falciparum* and 19 fungal strains of medical importance are reported. Compounds **4**, **5**, **6**, **10**, **21** and **24** were the most active against *L. panamensis* strain tested. Additionally, compounds **5**, **6** and **10** have the higher SI, they were 4 and 6-fold more active than cytotoxic.

Structural simplicity of the compounds, in addition to the high yields in obtaining them, is an advantage for their production to *in vivo* anti-leishmanial evaluation. This is the first synthetic report for eleven compounds: **12** to **15** and **18** to **24**. Most of the tertiary and quaternary amines were synthesized for the first time. Although none of the compounds showed anti-fungal or antiplasmodial activity, this is the first biological evaluation for compounds **8** to **20**, **23** and **24** against some fungal strains and *P. falciparum* parasite (3D7 strain).

Experimental

General

All the laboratory reagents were analytical grade (Sigma-Aldrich). Column chromatography and HPLC solvents were liquid chromatography grade (Merck). Compound purifications were made by column chromatography using Merck silica gel 60 and mixtures of hexane and ethyl acetate. Purifications of biologically tested compounds were made by HPLC Agilent 1200 with diode array detector (DAD, 254, 280 and 366 nm) and Eclipse XDB-C18 column using a mixture of MeOH and H_2O 0.1% formic acid as the mobile phase. ^1H and ^{13}C NMR were recorded on Bruker Ultrashield (300 and 75 MHz) and Ascend III HD (600 and 125 MHz) with 5 mm CryoProbe TCI, using $CDCl_3$, D_2O, and DMSO-d_6

as deuterated solvents. Signals were assigned using two-dimensional heteronuclear correlations (COSY (correlation spectroscopy) and HSQC (heteronuclear single quantum correlation)). High-resolution mass spectra (HRMS) were recorded using electrospray ionization (ESI-MS) in a UPLC-Q-Tof (ultra-performance liquid chromatography quadrupole time of flight) (Xevo-XS-QTof, Waters). The drying and cone gas was nitrogen set to flow rates of 300-30 L h^{-1}, respectively. Methanol samples solutions (1×10^{-5} M) were directly introduced into the ESI spectrometer at a flow rate of 10 µL min^{-1}. A capillary voltage of 3.5 kV was used in the positive scan mode, and the cone voltage (Uc) set to 10 V.

Derivatives synthesis

Synthesis of intermediates (**Int1**-**Int4**)

To MeOH (50 mL) at –5 °C, thionyl chloride (2190 µL, 30 mmol) was dropped slowly to maintain the temperature under 0 °C. Then L-tyrosine (5000 mg, 27.6 mmol) was added. The resulting mixture was stirred at 80 °C for 24 h. As the mixture cooled, the product was precipitated by the addition of diethyl ether (100 mL). The precipitate was collected, washed with ethyl ether (20 mL) and dried to give L-tyrosine methyl ester hydrochloride. The product was tested by thin layer chromatography (TLC) mobile phase *n*-BuOH:AcOH:H_2O (4:1:1) showing quantitative conversion. This reaction was done twice. The monobromination of L-tyrosine methyl ester (2000 mg, 8.63 mmol) was done with NBS (2000 mg, 11.21 mmol) in MeOH (20 mL) stirred at room temperature for 24 h. The solvent was removed, and the crude was partitioned with EtOAc:H_2O. The aqueous phase was recovered and evaporated to get red oil. This crude was tested by TLC mobile phase *n*-BuOH:AcOH:H_2O (4:1:1) showing the formation of mono bromine compound. This crude was not purified to the next reaction. Compounds dibrominated, monochlorinated and dichlorinated were obtained in the same way using NBS (3380 mg, 19 mmol), NCS (1500 mg, 11.22 mmol) and NCS (2535 mg, 19 mmol), respectively. The protections of the halogenated compounds were done by the reaction in MeOH (15 mL) with Boc_2O (2300 µL, 10 mmol) and TEA (2000 µL) for 24 h at room temperature. The solvent was removed, the crude was adjusted to pH = 3 and later extracted with EtOAc. The organic phase was dried with Na_2SO_4 and purified in column chromatography using as mobile phase hexane:EtOAc (3:1).

Methyl (*S*)-3-(3-bromo-4-hydroxyphenyl)-2-((*tert*-butoxycarbonyl)-amino) propanoate (**Int1**)

White solid, 1615 mg, 2 stages yield 50%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.21 (d, *J* 2.0 Hz, 1H), 6.94 (dd, *J* 8.3, 2.0 Hz, 1H), 6.87 (d, *J* 8.3 Hz, 1H), 5.07 (d, 1H), 4.51 (d, 1H), 3.71 (s, 3H), 2.97 (m, 2H), 1.41 (s, 9H).

Methyl (*S*)-3-(3,5-dibromo-4-hydroxyphenyl)-2-((*tert*-butoxycarbonyl)-amino) propanoate (**Int2**)

White solid, 2740 mg, 2 stages yield 70%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.02 (s, 2H), 5.07 (d, 1H), 4.51 (d, 1H), 3.73 (s, 3H), 3.13-2.98 (m, 1H), 2.90 (m, 1H), 1.42 (s, 9H).

Methyl (*S*)-3-(3-chloro-4-hydroxyphenyl)-2-((*tert*-butoxycarbonyl)-amino) propanoate (**Int3**)

White solid, 1565 mg, 2 stages yield 55%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.07 (s, 1H), 6.90 (s, 2H), 5.03 (s, 1H), 4.51 (s, 1H), 3.71 (s, 3H), 2.96 (s, 2H), 1.41 (s, 9H).

Methyl (*S*)-3-(3,5-dichloro-4-hydroxyphenyl)-2-((*tert*-butoxycarbonyl)-amino) propanoate (**Int4**)

White solid, 1930 mg, 2 stages yield 65%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.02 (s, 2H), 5.07 (d, 1H), 4.51 (m, 1H), 3.73 (s, 3H), 3.13-2.98 (m, 1H), 2.90 (dd, 1H), 1.42 (s, 9H).

Synthesis of compounds **1a**-**1d**

NHBoc cleavage, **Int 1** (1615 mg, 4.31 mmol), **Int 2** (2740 mg, 6.04 mmol), **Int 3** (1565 mg, 4.74 mmol) and **Int 4** (1930 mg, 5.29 mmol) in separate reactions were dissolved in DCM (3 mL) at 0 °C. After 5 min, TFA (1000 µL) was added to the mixture and left reacting for 24 h at room temperature. Later the solvents and reagents were evaporated, and the crude of the reaction was adjusted to pH = 7. The product was tested by TLC mobile phase hexane:EtOAc (3:1) showing quantitative conversion for **Int1**-**Int4** producing 1180 (**1a**), 2130 (**1b**), 1080 (**1c**) and 1385 mg (**1d**).

Synthesis of compounds **2a**-**2d**

The *O*-methylations of **Int1**-**Int4** were done by dissolving 1500 mg of each intermediate in acetone with 800 mg K_2CO_3 and 750 µL MeI. The mixture was stirred for 12 h at room temperature. The solvent was removed and purified in column chromatography using as mobile phase hexane:EtOAc (3:1). The products obtained in the last step were dissolved in DCM (3 mL) at 0 °C. After 5 min, TFA (1000 µL) was added to the mixture and left reacting for 24 h at room temperature. Later the solvents and reagents were evaporated, and the crude of the reaction was adjusted to pH = 7. The product was tested by TLC mobile phase hexane:EtOAc (3:1) showing quantitative conversion for compounds **2a**-**2d**: **2a**: 920 mg, two steps yield 80%; **2b**: 972 mg, two steps yield 80%; **2c**: 885 mg, two steps yield 80%; **2d**: 916 mg, two steps yield 80%.

Synthesis of compounds **3a**-**3h**

The *N*,*N*-dimethylation was done by a reductive amination using aqueous formaldehyde at 37% and $NaCNBH_3$. One equivalent of compounds **1a**-**1d**, **2a**-**2d** were dissolved in MeOH (15 mL), 2.2 equivalents of formaldehyde and 1.5 equivalents of $NaCNBH_3$. The reactions were stirred 18 h at room temperature. Later the solvents were evaporated, the crude reaction partitioned with EtOAc:H_2O (50:50) and purified by column chromatography using as a mobile phase EtOAc:hexane (2:1). (**3a**) **1a** (500 mg, 1.82 mmol), formaldehyde (300 µL, 4 mmol) and $NaCNBH_3$ (172 mg, 2.73 mmol); white solid, 383 mg, yield 70%; (**3b**) **1b** (500 mg, 1.41 mmol), formaldehyde (231 µL, 3.1 mmol) and $NaCNBH_3$ (133 mg, 2.11 mmol); white solid, 376 mg, yield 70%; (**3c**) **1c** (500 mg, 2.17 mmol), formaldehyde (355 µL, 4.8 mmol) and $NaCNBH_3$ (133 mg, 2.04 mmol); white solid, 390 mg, yield 70%; (**3d**) **1d** (500 mg, 1.89 mmol), formaldehyde (309 µL, 4.15 mmol) and $NaCNBH_3$ (178 mg, 2.83 mmol); white solid, 386 mg, yield 70%; (**3e**) **2a** (500 mg, 1.82 mmol), formaldehyde (300 µL, 4 mmol) and $NaCNBH_3$ (172 mg, 2.73 mmol); white solid, 383 mg, yield 70%; (**3f**) **2b** (500 mg, 1.41 mmol), formaldehyde (231 µL, 3.1 mmol) and $NaCNBH_3$ (133 mg, 2.11 mmol); white solid, 376 mg, yield 70%; (**3g**) **2c** (500 mg, 2.17 mmol), formaldehyde (355 µL, 4.8 mmol) and $NaCNBH_3$ (133 mg, 2.04 mmol); white solid, 390 mg, yield 70%; (**3h**) **2d** (500 mg, 1.89 mmol), formaldehyde (309 µL, 4.15 mmol) and $NaCNBH_3$ (178 mg, 2.83 mmol); white solid, 386 mg, yield 70%.

Primary amines synthesis

Synthesis of compounds **4**-**11**

150 mg of compounds **1a**-**1d**, **2a**-**2d** were dissolved in a mixture of MeOH:H_2O:THF (1:1:1) (5 mL) and then K_2CO_3 (100 mg) was added and the mixture was stirred 24 h at room temperature. The crude of reaction was adjusted to pH = 6. The compounds were tested by TLC mobile phase *n*-BuOH:AcOH:H_2O (4:1:1) showing quantitative conversion. The final products (**4**-**11**) were purified with reversed phase (RP)-HPLC using as a mobile phase a mixture of MeOH (0.01% formic acid (F.A.)) and H_2O (0.01% F.A.).

(*S*)-2-Amino-3-(3-bromo-4-hydroxyphenyl)-propanoic acid (**4**)

White solid, 140 mg, yield 100; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.32 (d, *J* 1.9 Hz, 1H), 7.00 (dd, *J* 8.4, 1.9 Hz, 1H), 6.83 (d, *J* 8.3 Hz, 1H), 4.05 (m, 1H), 3.00 (m, 2H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 172.62, 153.82, 135.45, 131.59, 128.96, 118.34, 111.24, 55.50, 35.94; $[\alpha]_D^{25}$ –12.2; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_9H_{11}NO_3Br$ $[M + H]^+$ 259.9922, found 259.9915.

(*S*)-2-Amino-3-(3,5-dibromo-4-hydroxyphenyl)-propanoic acid (**5**)

White solid, 144 mg, yield 100%; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.32 (s, 2H), 3.71-3.60 (m, 1H), 2.99 (dd, 1H), 2.83 (dd, 1H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 174.18, 150.75, 134.56, 131.54, 112.56, 56.74, 36.12; $[\alpha]_D^{25}$ –15.5; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_9H_{10}NO_3Br_2$ $[M + H]^+$ 337.9043, found 337.9027.

(*S*)-2-Amino-3-(3-chloro-4-hydroxyphenyl)-propanoic acid (**6**)

White solid, 140 mg, yield 100%; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.16 (d, *J* 2.0 Hz, 1H), 6.95 (dd, *J* 8.4, 2.1 Hz, 1H), 6.83 (d, *J* 8.3 Hz, 1H), 3.70 (dd, 1H), 3.00 (dd, 1H), 2.85 (dd, 1H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 174.51, 152.46, 132.33, 130.78, 129.64, 121.77, 118.57, 57.21, 36.66; $[\alpha]_D^{25}$ –10.1; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_9H_{11}NO_3Cl$ $[M + H]^+$ 216.0427, found 216.0438.

(*S*)-2-Amino-3-(3,5-dichloro-4-hydroxyphenyl)-propanoic acid (**7**)

White solid, 142 mg, yield 100%; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.12 (s, 2H), 3.70 (m, 1H), 3.01 (dd, 1H), 2.84 (dd, 1H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 174.48, 148.81, 131.00, 129.87, 123.73, 57.01, 36.54; $[\alpha]_D^{25}$ –13.6; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_9H_{10}NO_3Cl_2$ $[M + H]^+$ 250.0038, found 250.0065.

(*S*)-2-Amino-3-(3-bromo-4-methoxyphenyl)-propanoic acid (**8**)

White solid, 142 mg; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.56 (d, *J* 2.1 Hz, 1H), 7.29 (dd, *J* 8.5, 2.1 Hz, 1H), 7.11 (d, *J* 8.5 Hz, 1H), 4.06-3.84 (m, 4H), 3.23 (dd, 1H), 3.07 (dd, 1H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 164.63, 146.03, 125.30, 121.43, 120.57, 104.57, 102.60, 47.52, 26.64; $[\alpha]_D^{25}$ –8.4; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_{10}H_{13}NO_3Br$ $[M + H]^+$ 274.0079, found 274.0096.

(*S*)-2-Amino-3-(3,5-dibromo-4-methoxyphenyl)-propanoic acid (**9**)

White solid, 144 mg; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.59 (s, 2H), 3.97-3.80 (m, 4H), 3.23 (dd, 1H), 3.07 (dd, 1H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 164.20, 144.24, 126.41, 125.17, 109.32, 52.11, 47.14, 26.65; $[\alpha]_D^{25}$ –11.1; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_{10}H_{12}NO_3Br_2$ $[M + H]^+$ 351.9184, found 351.9227.

(*S*)-2-Amino-3-(3-chloro-4-methoxyphenyl)-propanoic acid (**10**)

White solid, 141 mg; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.20 (s, 1H), 7.06 (d, *J* 8.5 Hz, 1H), 6.96 (d, *J* 8.4 Hz, 1H), 3.73 (s, 4H), 3.02 (dd, 1H), 2.87 (dd, 1H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 174.76, 155.19, 132.35, 130.89, 130.23, 123.11, 114.77, 57.69, 57.28, 36.71; $[\alpha]_D^{25}$ –9.4; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_{10}H_{13}NO_3Cl$ $[M + H]^+$ 230.0584, found 230.0594.

(*S*)-2-Amino-3-(3,5-dichloro-4-methoxyphenyl)-propanoic acid (**11**)

White solid, 142 mg; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.20 (s, 2H), 3.74 (s, 4H), 3.02 (dd, 1H), 2.88 (dd, 1H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 174.36, 152.09, 135.25, 131.48, 130.39, 56.98, 36.82; $[\alpha]_D^{25}$ –12.2; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_{10}H_{12}NO_3Cl_2$ $[M + H]^+$ 264.0194, found 264.0199.

Tertiary amines

Synthesis of compounds **12**-**18**

150 mg of compounds **3a**-**3h** (except **3b**) were dissolved in a mixture of MeOH:H_2O:THF (1:1:1) (5 mL) and then K_2CO_3 (100 mg) was added and the mixture was stirred 24 h at room temperature. The crude of reaction was adjusted to pH = 6. The compounds were tested by TLC mobile phase *n*-BuOH:AcOH:H_2O (4:1:1) showing quantitative conversion. The final products (**12**-**18**) were purified with RP-HPLC using as a mobile phase a mixture of MeOH (0.01% F.A.) and H_2O (0.01% F.A.).

(*S*)-3-(3-Bromo-4-hydroxyphenyl)-2-(dimethylamino)-propanoic acid (**12**)

White solid, 108 mg, yield 75%; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.34 (d, *J* 1.8 Hz, 1H), 7.01 (dd, *J* 8.4, 1.8 Hz, 1H), 6.81 (d, *J* 8.3 Hz, 1H), 3.91 (m, 1H), 3.22-3.07 (m, 1H), 2.92 (dd, 1H), 2.80 (s, 6H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 171.41, 153.65, 135.30, 131.45, 129.14, 118.20, 111.05, 71.00, 42.91, 33.52; $[\alpha]_D^{25}$ 60.3; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_{11}H_{15}NO_3Br$ $[M + H]^+$ 288.0235, found 288.0243.

(*S*)-3-(3-Chloro-4-hydroxyphenyl)-2-(dimethylamino)-propanoic acid (**13**)

White solid, 106 mg, yield 75%; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.16 (s, 1H), 7.03 (d, 1H), 6.90 (dd, 1H), 6.81 (d, 1H), 3.81 (m, 1H), 3.11 (dd, 1H), 2.89 (dd, 1H), 2.78 (s, 6H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 171.60, 152.45, 131.90, 130.68, 128.70, 121.51, 118.30, 71.60, 43.56, 33.76; $[\alpha]_D^{25}$ 54.9; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_{11}H_{15}NO_3Cl$ $[M + H]^+$ 244.0740, found 244.0760.

(*S*)-3-(3,5-Dichloro-4-hydroxyphenyl)-2-(dimethylamino)-propanoic acid (**14**)

White solid, 106 mg, yield 75%; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.14 (s, 2H), 4.02 (m, 1H), 3.17 (dd, 1H), 2.94 (dd, 1H), 2.82 (s, 6H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 170.64, 149.23, 131.31, 129.16, 123.18, 69.83, 43.59, 33.52; $[\alpha]_D^{25}$ 59.1; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_{11}H_{14}NO_3Cl_2$ $[M + H]^+$ 278.0351, found 278.0352.

(*S*)-3-(3-Bromo-4-methoxyphenyl)-2-(dimethylamino)-propanoic acid (**15**)

White solid, 121 mg, yield 85%; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.56 (d, *J* 2.0 Hz, 1H), 7.30 (dd, *J* 8.5, 2.1 Hz, 1H), 7.08 (d, *J* 8.5 Hz, 1H), 3.90 (s, 3H), 3.78 (dd, 1H), 3.25 (dd, 1H), 3.10 (dd, 1H), 2.93 (s, 6H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 163.05, 145.86, 125.11, 121.23, 120.89, 104.37, 102.46, 63.38, 47.57, 24.00; $[\alpha]_D^{25}$ 53.8; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_{12}H_{17}NO_3Br$ $[M + H]^+$ 302.0392, found 302.0398.

(*S*)-3-(3,5-Dibromo-4-methoxyphenyl)-2-(dimethylamino)-propanoic acid (**16**)

White solid, 123 mg, yield 85%; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.57 (s, 2H), 3.90 (s, 2H), 3.82 (dd, 1H), 3.28 (d, 1H), 3.06 (dd, 1H), 2.95 (s, 6H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 163.23, 157.07, 125.80, 124.46, 108.54, 63.16, 51.93, 23.48; $[\alpha]_D^{25}$ 61.0; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_{12}H_{16}NO_3Br_2$ $[M + H]^+$ 379.9497, found 379.9490.

(*S*)-3-(3-Chloro-4-methoxyphenyl)-2-(dimethylamino)-propanoic acid (**17**)

White solid, 120 mg, yield 85%; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.20 (s, 1H), 7.05 (s, 1H), 6.94 (s, 1H), 3.72 (s, 3H), 3.64-3.48 (m, 1H), 3.07 (dd, 1H), 2.94-2.80 (m, 1H), 2.73 (s, 6H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 173.04, 154.61, 132.43, 130.40, 130.32, 122.66, 114.58, 73.75, 57.65, 40.25, 34.56; $[\alpha]_D^{25}$ 49.8; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_{12}H_{17}NO_3Cl$ $[M + H]^+$ 258.0897, found 258.0898.

(*S*)-3-(3,5-Dichloro-4-methoxyphenyl)-2-(dimethylamino)-propanoic acid (**18**)

White solid, 121 mg, yield 85%; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.20 (s, 2H), 3.73 (s, 3H), 3.59 (dd, 1H), 3.09 (dd, 1H), 2.86 (dd, 1H), 2.74 (s, 6H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 172.59, 151.90, 135.35, 131.32, 130.21, 73.03, 62.46, 34.21; $[\alpha]_D^{25}$ 53.5; ESI-MS *m/z* 292.6 $[M]^+$.

Quaternary amines

Synthesis of compounds **19**-**24**

The quaternization of amine group was done using 150 mg of **3c**-**3h**, methyl iodide (350 μL) and acetone (3 mL) at room temperature by 18 h. The solid were filtered, washed with acetone and dried. Later the solids were dissolved in a mixture of MeOH:H_2O:THF (1:1:1) (5 mL) and then K_2CO_3 (100 mg) was added and the mixture was stirred 24 h at room temperature. The crude of reaction was adjusted to pH = 6. The compounds were tested by TLC mobile phase *n*-BuOH:AcOH:H_2O (4:1:1) showing quantitative conversion. The final products were purified with RP-HPLC using as a mobile phase a mixture of MeOH (0.01% F.A.) and H_2O (0.01% F.A.).

(*S*)-1-Carboxy-2-(3-chloro-4-hydroxyphenyl)-*N,N,N*-trimethylethan-1-aminium (**19**)

White solid, 170 mg, yield 100%; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.17 (d, *J* 1.9 Hz, 1H), 6.97 (dd, *J* 8.4, 1.9 Hz, 1H), 6.84 (d, *J* 8.4 Hz, 1H), 4.04 (m, 1H), 3.23 (m, 1H), 3.18 (s, 9H), 2.94 (m, 1H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 169.9, 152.5, 132.3, 130.9, 127.4, 121.5, 118.4, 77.6, 53.7, 32.4; $[\alpha]_D^{25}$ 47.2; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_{12}H_{17}NO_3Cl$ $[M]^+$ 258.0901, found 258.0897.

(*S*)-1-Carboxy-2-(3,5-dichloro-4-hydroxyphenyl)-*N,N,N*-trimethylethan-1-aminium (**20**)

White solid, 172 mg, yield 100%; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.15 (s, 2H), 3.97 (m, 1H), 3.23 (dd, 1H), 3.14 (s, 9H), 2.95 (dd, 1H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 169.9, 140.0, 131.0, 128.4, 123.9, 77.7, 53.9, 32.4; $[\alpha]_D^{25}$ 53.1; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_{12}H_{16}NO_3Cl_2$ $[M]^+$ 292.0508, found 292.0507.

(*S*)-1-Carboxy-2-(3-bromo-4-methoxyphenyl)-*N,N,N*-trimethylethan-1-aminium (**21**)

White solid, 173 mg, yield 100%; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.51 (d, *J* 2.0 Hz, 1H), 7.26 (dd, *J* 8.5, 2.1 Hz, 1H), 7.04 (d, *J* 8.5 Hz, 1H), 3.87 (s, 3H), 3.80 (m, 1H), 3.28 (s, 9H), 3.24 (dd, 1H), 3.08 (dd, 1H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 161.8, 146.4, 125.5, 121.6, 120.2, 104.6, 102.6, 71.5, 47.8, 43.7, 23.2; $[\alpha]_D^{25}$ 53.7; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_{13}H_{19}NO_3Br$ $[M]^+$ 316.0547, found 316.0458.

(*S*)-1-Carboxy-2-(3,5-dibromo-4-methoxyphenyl)-*N,N,N*-trimethylethan-1-aminium (**22**)

White solid, 168 mg, yield 100%; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.59 (s, 1H), 3.93 (s, 3H), 3.85 (m, 1H), 3.33 (s, 10H), 3.11 (dd, 1H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 170.9, 153.8, 135.6, 135.1, 119.0, 80.7, 62.3, 53.5, 32.8; $[\alpha]_D^{25}$ 59.3; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_{13}H_{19}NO_3Br_2$ $[M]^+$ 393.9653, found 393.9701.

(*S*)-1-Carboxy-2-(3-chloro-4-methoxyphenyl)-*N,N,N*-trimethylethan-1-aminium (**23**)

White solid, 173 mg, yield 100%; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.21 (s, 1H), 7.06 (d, *J* 8.5 Hz, 1H), 6.95 (d, *J* 8.5 Hz, 1H), 3.71 (s, 4H), 3.13 (s, 10H), 2.93 (dd, 1H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 171.5, 155.2, 132.4, 131.0, 129.5, 123.0, 114.7, 80.8, 57.8, 53.7, 33.0; $[\alpha]_D^{25}$ 51.9; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_{13}H_{19}NO_3Cl$ $[M]^+$ 272.1053, found 272.1057.

(*S*)-1-Carboxy-2-(3,5-dichloro-4-methoxyphenyl)-*N,N,N*-trimethylethan-1-aminium (**24**)

White solid, 170 mg, yield 100%; ^{1}H NMR (300 MHz, D_2O, DMSO-d_6) δ 7.15 (s, 2H), 3.70 (s, 3H), 3.61 (m, 1H), 3.09 (s, 10H), 2.90 (dd, 1H); ^{13}C NMR (75 MHz, D_2O, DMSO-d_6) δ 171.0, 151.9, 134.5, 131.3, 130.1, 80.7, 62.3, 53.5, 33.0; $[\alpha]_D^{25}$ 54.4; ESI-Q-Tof-HRMS, *m/z*: calcd. for $C_{13}H_{19}NO_3Cl_2$ $[M]^+$ 292.0507, found 292.0519.

Compounds were stored at room temperature and prior to the biological evaluations, they were solubilized in 0.5% DMSO and then, diluted to the appropriate concentration in culture media.

Biological activity assays

The compounds were subjected to *in vitro* evaluation for their cytotoxicity, anti-leishmanial, antiplasmodial and anti-fungal activity.

Anti-leishmanial activity assay

The anti-leishmanial activity of compounds was determined according to the ability of the compound to reduce the infection by *L. panamensis* parasites. For this, the anti-leishmanial activity was tested on intracellular amastigotes of *L. panamensis* transfected with the green fluorescent protein gene (MHOM/CO/87/UA140-EpiR-GFP strain). Briefly, U-937 human cells at a density of 3×10^5 cells mL^{-1} in Roswell Park Memorial Institute medium (RPMI)-1640 and 0.1 μg mL^{-1} of PMA (phorbol 12-myristate 13-acetate) were dispensed on 24-wells microplate and then infected with stationary phase growing *L. panamensis* promastigotes in a 15:1 parasites *per* cell ratio. Plates were incubated at 34 °C and 5% CO_2 for 3 h and then the cells were washed twice with phosphate buffer solution (PBS) to eliminate not internalized parasites. Fresh RPMI-1640 was added to each well (1 mL) and plates

were incubated again to complete infection. After 24 h of infection, the RPMI-1640 medium was replaced by fresh culture medium containing each compound at four serial dilutions (50, 12.5, 3.125 and 0.78 μg mL^{-1}) and plates were then incubated at 37 °C and 5% CO_2 during 72 h, then, cells were removed from the bottom plate with a trypsin/ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) (250 mg) solution. The cells were centrifuged at 1100 rpm during 10 min at 4 °C, the supernatant was discarded, and cells were washed with 1 mL of cold PBS and centrifuged at 1100 rpm for 10 min at 4 °C. Cells were washed two times employing PBS, as previously, and after the last wash, the supernatant was discarded, and cells were suspended in 500 μL of PBS. Cells were analyzed by flow cytometry employing a flow cytometer (Cytomics FC 500MPL) reading at 488 (exciting) and 525 nm (emitting) over an argon laser and counting 10,000 events. Infected cells were determined according to the events for green fluorescence (parasites). Infected cells exposed to control drugs (amphotericin B) were used as a control for anti-leishmanial activity (positive control), while infected cells incubated in absence of any compound or drug were used as a control for infection (negative control). Nonspecific fluorescence was corrected by subtracting fluorescence of unstained cells. All determinations were performed in triplicate in at least two independent experiments.[18,19]

Antiplasmodial activity assay

Antiplasmodial activity was evaluated *in vitro* on asynchronic cultures of *P. falciparum* (3D7 strain), maintained in standard culture conditions. The effect of each compound over the growth of the parasites was determined by quantification of parasite death, based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant. Briefly, unsynchronized *P. falciparum* cultures were adjusted to 0.5% parasitemia and 1% hematocrit in RPMI medium enriched with 3% lipid-rich bovine serum albumin (Albumax II). Then, in each well of a 96-wells plate, 100 μL of parasite suspension were dispensed and subsequently exposed against 100 μL of four serial dilutions of compounds (100, 25, 6.25 and 1.56 μg mL^{-1}). Dilutions were prepared from a stock solution of 1000 μg mL^{-1}. Chloroquine (CQ) was used as positive antiplasmodial drug control. Parasites unexposed to any compound were used as a control of both growth and viability (negative control). Plates were incubated for 48 h at 37 °C in N_2 (90%), CO_2 (5%) and O_2 (5%) atmosphere. After incubation, plates were harvested, and parasites were subjected to three 20-min freeze-thaw cycles. Meanwhile, 100 μL of Malstat reagent (400 μL Triton X-100 in 80 mL deionized water, 4 g L-lactate, 1.32 g Tris buffer and 0.022 g acetylpyridine adenine dinucleotide in 200 mL deionized water; pH 9.0) and 25 μL of NBT/PES solution (0.16 g nitroblue tetrazolium salt and 0.08 g phenazine ethosulfate in 100 mL deionized water) were added to each well of a second flat-bottomed 96-well microtiter plate. After freeze-thaw cycles, the culture in each of the wells of the first plate was resuspended by pipetting and 15 μL of each well was taken and added to the corresponding well of the second plate (containing Malstat and NBT/PES reagents). After an hour of incubation in the dark, color development of the LDH reaction was monitored colorimetrically in a spectrofluorometer (Varioskan, Thermo) reading at 650 nm. The intensity of color in each experimental condition was registered as optical densities (O.D.). Non-specific absorbance was corrected by subtracting O.D. of the blank. Determinations were done in triplicate in at least two independent experiments.[20]

Antifungal activity

Antifungal activity was evaluated against 19 fungal strains of medical importance as *Candida tropicalis* (ATCC 750), *C. tropicalis* (ATCC 200956), *C. lusitaniae* (ATCC 2819), *C. albicans* (ATCC 10231), *C. krusei* (ATCC 6258), *C. glabrata* (ATCC 90030), *Cryptococcus neoformans* (ATCC 90112), and *C. gattii* (ATCC 10865). In addition, the following clinical isolates and strains of filamentous fungi were included: *Sporothrix schenckii* (UdeA 7027), *S. globosa* (UdeA 0002), *Fusarium oxysporum* (UdeA 8038), *F. graminearum* (UdeA 6003), *F. proliferatum* (Micoteca da Universidade do Minho (MUM) 16144), *F. verticillioides* (MUM 16143), *Neoscytalidium dimidiatum* (UdeA 8023), *Aspergillus flavus* (ATCC 204304), *A. fumigatus* (ATCC 204305), *Trichophyton interdigitale* (ATCC 24198) and *Fusarium oxysporum* (ATCC 48112). *In vitro* anti-fungal activity of derivatives was determined following the Anti-Fungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST) reference procedure for yeasts and for filamentous.[21] The anti-fungal tests were carried out in RPMI buffered at pH = 7 with 3-morpholinopropanesulfonic acid (MOPS). Compounds were evaluated at a final concentration of 500 μg mL^{-1}. One hundred microliters of each concentration diluted twice were dispensed into 96-well microtitration plates and the same volume of each inoculum at 1.5×10^5 UFC mL^{-1} were added. Minimal inhibitory concentration (MIC) was defined as the lowest dilution that resulted in total inhibition of visible growth after 24 or 48 h incubation at 35 °C of *Candida* spp. and *Cryptococcus* spp., respectively. The MICs for filamentous fungi were determined after 24 h

of incubation at 28 °C, except for *Sporothrix* spp. and *T. interdigitale* (ATCC 24198), which were incubated for five days. The anti-fungals itraconazole and amphotericin B (Sigma-Aldrich Co.) were included as a positive control at a concentration range of 16 to 1 μg mL^{-1} against *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019), *A. flavus* (ATCC 204304) and *A. fumigatus* (ATCC 204305).

Cytotoxic activity in human macrophages

Cytotoxic activity of compounds was assessed based on the viability of U-937 cells determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. Shortly, 100,000 cells mL^{-1} in complete RPMI-1640 and the corresponding concentration of each compound (200, 50, 12.5, 3.125 μg mL^{-1}) were incubated at 37 °C, 5% CO_2 during 72 h. The effect of each concentration of each compound was determined by adding 10 μL $well^{-1}$ of MTT solution (0.5 mg mL^{-1}) and incubating at 37 °C for 3 h. The reaction was stopped with 100 μL of 50% isopropanol solution with 10% sodium dodecyl sulfate (SDS) and incubation for 30 min. Cell viability was determined based on the quantity of formazan produced, which was measured with a Bio-Rad ELISA reader set at 570 nm. As a viability control, cultured cells in the absence of extracts were used. Doxorubicin was used as a cytotoxicity control. Nonspecific absorbance was corrected by subtracting absorbance (O.D.) of the blank. Determinations were done in triplicate in at least two independent experiments.[18]

Statistical analysis

Cytotoxicity was determined according to viability and mortality percentages obtained for each experimental condition (synthesized compounds, amphotericin B, benznidazole, chloroquine and culture medium). Results were expressed as LC_{50} that corresponds to the concentration necessary to kill 50% of cells, calculated by Probit analysis (parametric method of linear regression that permits doses-response analysis).[22] Initially, viability percentages were calculated by equation 1, where the O.D. of control well corresponds to 100% of viability. In turn, mortality percentage corresponds to 100% – %viability.

$$\%\text{Viability} = \frac{\text{O.D. exposed cells}}{\text{O.D. control cells}} \times 100 \quad (1)$$

Then, the percentage of cell growth inhibition was calculated using the equation 2:

$$\%\text{Cell growth inhibition} = 100\% - \%\text{Viability} \quad (2)$$

The cytotoxicity was graded as high, moderate or low according to LC_{50} values, as follows: high cytotoxicity when LC_{50} < 100 μg mL^{-1}; moderate cytotoxicity when LC_{50} is in 100-200 μg mL^{-1} range and low cytotoxicity when LC_{50} > 200 μg mL^{-1}.

The antiplasmodial activity of each evaluated compound was evidenced by the reduction of the absorbance (O.D.). Indeed, the viability percentage was calculated by equation 3:

$$\%\text{Viability} = \frac{\text{(O.D. of parasites exposed)} - \text{(O.D. culture medium)}}{\text{(O.D. of parasites unexposed)} - \text{(O.D. culture medium)}} \quad (3)$$

Then, the inhibition growing percentage was calculated according to the following equation 4:

$$\%\text{Inhibition} = 100 - \%\text{Viability} \quad (4)$$

Anti-leishmanial activity was determined according to reduction of the median fluorescence intensity (MFI) of infected cells percentages obtained for each experimental condition by the cytometer. The number of parasites for each concentration of tested compound was calculated by equation 5, where the MFI in infected cells in the control well corresponds to 100% of infection.

$$\%\text{Infection} = \frac{\text{MIF compounds exposed infected cells}}{\text{MIF compounds non-infected cells}} \times 100 \quad (5)$$

Then, inhibition percentage was calculated with equation 6:

$$\%\text{Inhibition} = 100 - \%\text{Infection} \quad (6)$$

The anti-leishmanial and antiplasmodial activity was graded as high, moderate or low according to the EC_{50} values, as follows: high activity when EC_{50} < 25 μg mL^{-1}; moderate activity when EC_{50} is in the 25-50 μg mL^{-1} range and low activity when EC_{50} > 50 μg mL^{-1}.

The selectivity index (SI) was calculated by dividing the cytotoxic activity and the antileishmanial or antitrypanosomal activity ($SI = LC_{50}/EC_{50}$).

Supplementary Information

Supplementary data (^{1}H NMR, ^{13}C NMR and ESI-Qtof-HRMS spectra) are available free of charge at http://jbcs.sbq.org.br as PDF file.

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