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Article

Preparation of Derivatives of Betulinic Acid, Steviol and Isosteviol and Evaluation of Antitrypanosomal and Antimalarial Activities

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Derivatives of steviol, isosteviol and betulinic acid were prepared and tested *in vitro* against *Plasmodium falciparum* W2 (chloroquine-resistant) and *Trypanosoma cruzi*. The best results against *P. falciparum* were found for the betulinic acid derivatives 3-[(2,4-dinitrophenyl)hydrazono]lup-20(29)-en-28-oic acid and 3-hydroxy-20-[(2,4-dinitrophenyl)hydrazono]-29-norlupan-28-oic acid (values concentration in that the growth of the parasites is 50% inhibited, IC_{50} = 9.96, 6.45 and 21.06 μg mL^{-1}, respectively). Isosteviol was inactive but its 4-nitro- and 2,4-dinitrophenylhydrazone showed moderate activity (IC_{50} = 17.50-22.58 μg mL^{-1}). The derivatives of betulinic acid were more selective to the parasite when compared with hepatoblastoma HepG2 A16 cells. The results showed that nitrophenylhydrazone derivatives of lupane and beyerane terpenes are promising prototypes of antimalarial agents. Among the compounds tested against *T. cruzi* the oxime of isosteviol was active against both epimastigote and trypomastigote forms of the parasite.

Keywords: betulinic acid, steviol, isosteviol, *Trypanosoma cruzi*, *Plasmodium falciparum*

Introduction

Malaria is a disease of worldwide importance caused by the parasite *Plasmodium falciparum*. A report from the World Health Organization estimates that 207 million cases and 607,000 deaths occurred in 2012.[1] In Brazil more than 267,000 cases were reported in 2011.[2] Moreover, the resistance of *P. falciparum* to drugs, like the classical cloroquine and also the newer artemisinin and derivatives, has increased.[3] The rise of resistant strains of the parasite motivates the search for new and more effective drugs.

Natural products have also been an important source of antimalarial drugs. Atovaquone (**1a**), quinine (**1b**), artemisinin (**1c**, Figure 1) and semi-synthetic derivatives have been used for the treatment of the disease.[4] Many others have also been described as active *in vitro* against the parasite and could become lead compounds with new mechanisms of action in order to overcome resistance.[5] Triterpene-rich plant extracts containing betulinic acid (**2a**, Figure 2) have been reported as antiplasmodial. Initial evidences of the activity of betulinic acid indicated a moderately action against *P. falciparum* (NF 54) with IC_{50} values (concentration in that the growth of the parasites is 50% inhibited) = 10.5 μg mL^{-1}.[6] Other studies have also reported the antimalarial potential of that compound and its derivatives.[7-20] A few tetracyclic diterpenes have also shown antimalarial properties. One example is a norpimaran lactone (**1d**, Figure 1) obtained from manool,[21] and another is kaurenoic acid (**3a**, Figure 3) described as weakly active.[22] Lactone kaurane diterpenoids (**1e**, Figure 1), isolated from *Parinari capensis*, have shown strong *in vitro* antimalarial activity, but they were highly toxic to human kidney epithelial cells.[23]

Chagas disease is another serious chronic illness caused by *Trypanosoma cruzi*, with most infected people living in Latin America countries. It is estimated that 7-8 million people are infected worldwide with Chagas disease.[24] Benznidazole has been used to treat Chagas disease in both acute and initial chronic phases.[25] However, this drug presents serious side effects is not totally effective development of resistance.[26] Therefore the search for alternative drugs is necessary.

Kaurenes (e.g., steviol, **3c**, Figure 3) and beyerenes (e.g., isosteviol, **4a**, Scheme 1) are tetracyclic diterpenes

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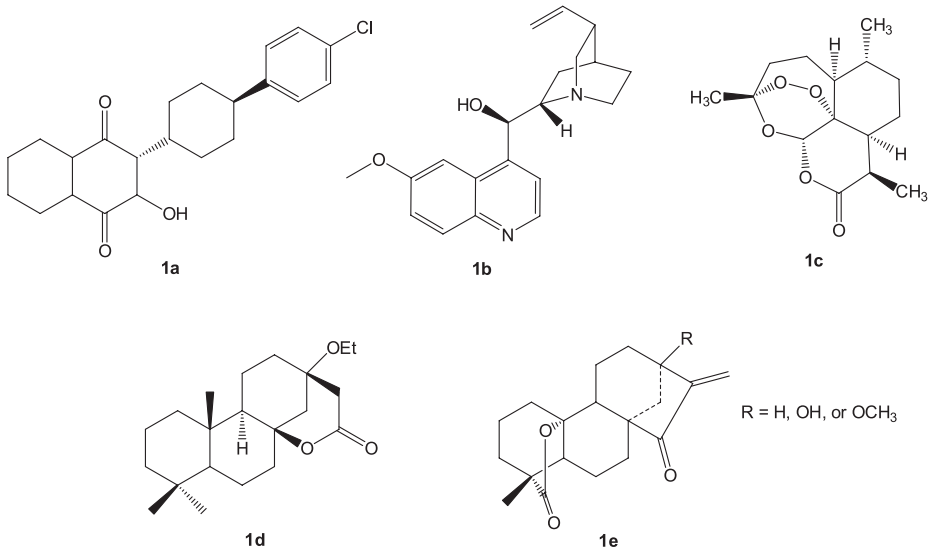


Figure 1. Structures of antimalarial natural products.

with similar carbon backbones, except for rings C and D due to the inversion of stereochemistry at C-8 and C-13. Some kaurenes have been described as active against *T. cruzi*. One report describes significant *in vitro* and *in vivo* activities of the natural compounds kaurenol and kaurenoic acid.[27] Derivatives of kaurenoic acid were prepared and methyl ent-16*Z*-oxime-17-norkauran-19-oate showed improved activity.[28] Another natural ent-9a-hydroxy-15b-*E*-cinnamoyloxy-16-kauren-19-oic acid was also active *in vitro*.[29] Thiosemicarbazones derivatives of kaurenoic acid were also prepared and some showed increased activity; the *o*-nitrobenzaldehyde-thiosemicarbazone derivative was the most active compound with IC_{50} of 2.0 mM.[30]

In this report we describe the preparation and evaluation of the antimalarial and antitrypanosomal activities of known and new derivatives of betulinic acid and some kaurene and beyerene tetracyclic diterpenoids.

Results and Discussion

Chemistry

The structures of all compounds prepared are shown in Figures 1, 2 and 3. The known compounds were obtained as previously reported (see Experimental section). The *p*-methoxyphenacyl esters of steviol and

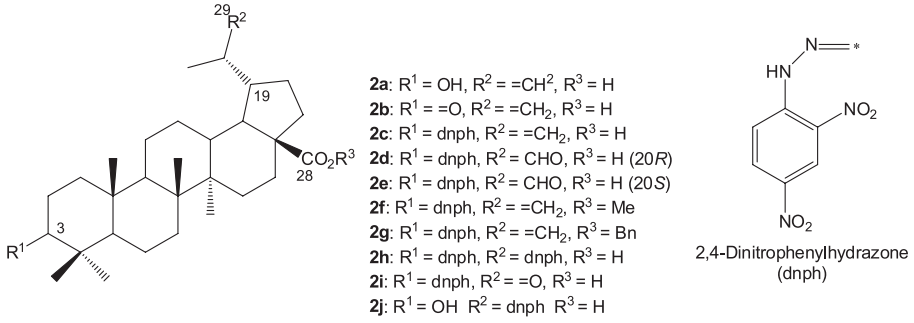


Figure 2. Structures of betulinic acid (**2a**) and derivatives.

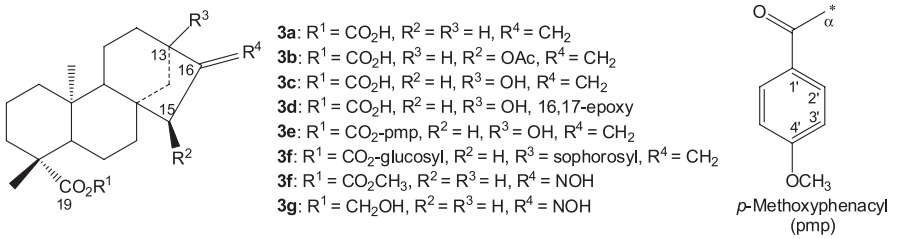
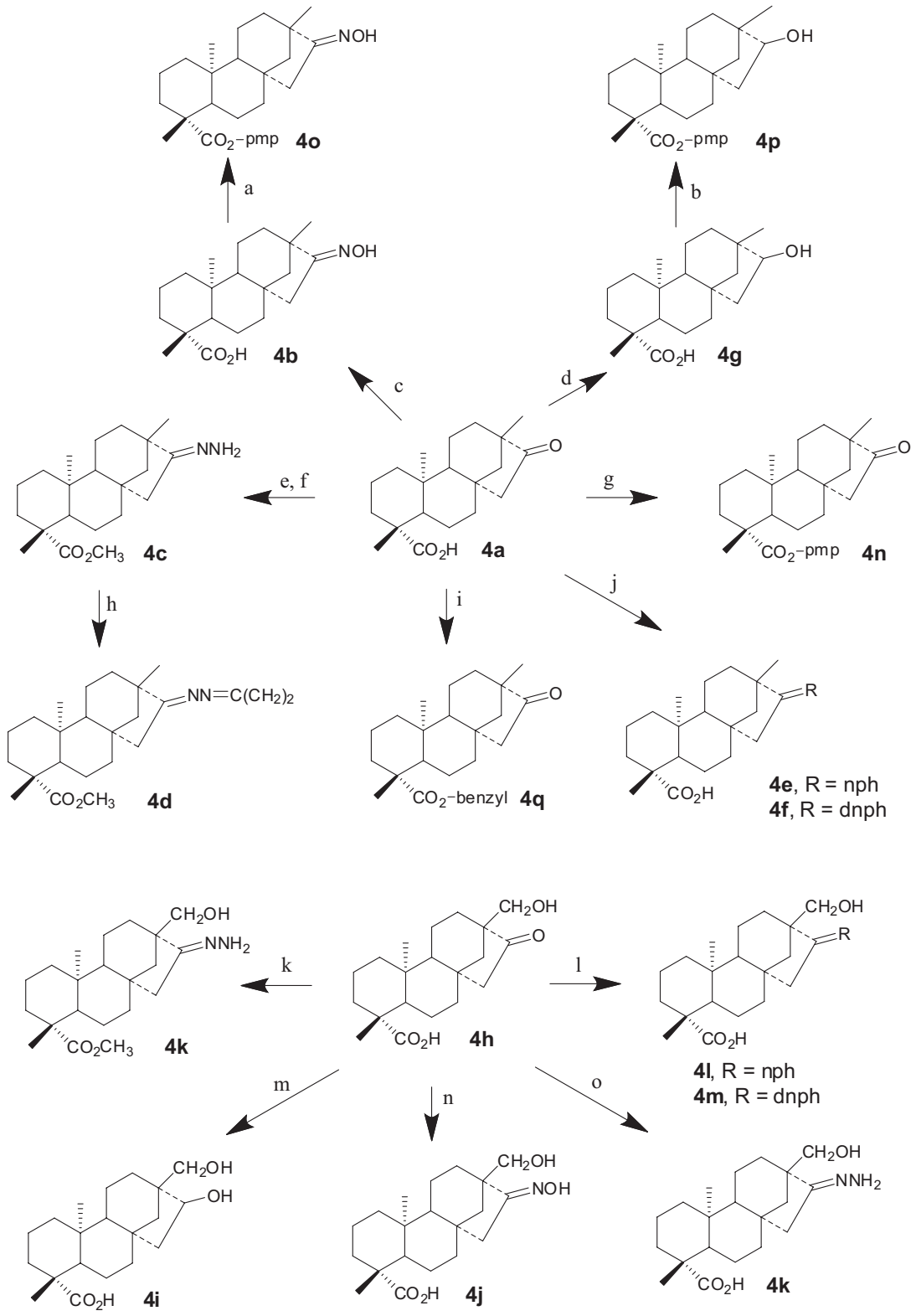


Figure 3. Structures of steviol (**3c**) and derivatives.



Scheme 1. Preparation of isosteviol derivatives. (a, b) *p*-methoxyphenacyl bromide, acetone, triethylamine, microwave, 4 min, (**4o** 78%, **4p** 75%); (c) hydroxylamine chloride, sodium acetate, water, EtOH, room temperature (rt), 24 h (78%); (d) $NaBH_4$, EtOH, rt, 1 h, 80%; (e) CH_2N_2, ethyl ether, MeOH; (f) hydrazine hydrate, MeOH, reflux, 8 h, 83%; (g) *p*-methoxyphenacyl bromide, acetone, triethylamine, microwave, 4 min, 81%; (h) acetone, reflux, 2 h, 83%; (i) benzyl chloride, acetone, K_2CO_3, reflux, 2 h, 83%; (j, l) nitrophenylhydrazine (or 2,4-dinitrophenylhydrazine), EtOH, H_2O, H_2SO_4, rt, 12 h (**4e** 51%, **4f** 70%, **4l** 49%, **4m** 57%); (k) hydrazine hydrate, MeOH, reflux, 2 h, 86%; (o) *p*-methoxyphenacyl bromide, acetone, triethylamine, microwave, 4 min, 78%; (m) $NaBH_4$, EtOH, rt, 1 h, 80%; (n) hydroxylamine chloride, sodium acetate, water, EtOH, rt, 24 h (68%); (o) hydrazine hydrate, MeOH, reflux, 8 h, 86%; pmp = *p*-methoxyphenacyl; nph = 4-nitrophenylhydrazone; dnph = 2,4-dinitrophenylhydrazone.

isosteviol were obtained by reaction with 2-bromo-4'-methoxyacetophenone with microwave irradiation, which allowed a short reaction time (ca. 5 minutes). The ^{13}C nuclear magnetic resonance (NMR) data showed the characteristic signals for the methoxyphenacyl moiety e.g., the ketone (ca. 190 ppm) and methoxy (ca. 65 ppm) groups.

The hydrazones were obtained in the usual fashion by reaction of the carbonyl group (C16) with selected hydrazines. The ^{13}C NMR data showed the characteristic signal of C=N group at 162-174 ppm. The other spectroscopic data mass spectroscopy, infrared (MS, IR) were also compatible with respective structures.

Bioassays

Anti *P. falciparum*

The results of growth inhibition of the test compounds on the chloroquine-resistant strain *P. falciparum* W2 are shown on Table 1 (the inactive and slightly active were omitted). The activity of betulinic acid (**2a**) was confirmed and most of the modifications in its molecular structure did not increased activity significantly. Oxidation at C-3 to betulonic acid (**2b**) drastically reduced the activity. Attachment of a 2,4-dinitrophenylhydrazone (dnph) moiety at either C-3 (**2c**) or C-29 (**2j**) increased activity two and three fold relative to parent compound, respectively (Table 1). However, the derivative containing the same substituent at both positions (**2h**) was less active than betulinic acid (**2a**). Esterification at C-28 with either methyl (**2f**) or benzyl (**2g**) or modifications at the isopropenyl group at C-19 (**2d**, **2e**, **2i**) decreased the activity of the 2,4-dnph derivative (**2c**). Therefore, a free carboxyl at C-28, hydroxyl at C-3 and an isopropenyl at C-19 are important for the activity of these 2,4-dnph derivatives of betulinic acid.

Table 1. IC_{50} of the most active compounds against *P. falciparum* W2

Compound	IC_{50}[a] / (μg mL^{-1})	Classification
2a	17.7 ± 1.3	moderately active
2c	9.96 ± 1.20	active
2j	6.45 ± 1.47	active
4e	17.50 ± 4.83	moderately active
4f	21.06 ± 3.15	moderately active
4l	22.58 ± 4.92	moderately active
4m	18.80 ± 6.00	moderately active
Chloroquine	0.085 ± 0.0201	very active

[a]IC_{50}: concentration in that the growth of the parasites is 50% inhibited.

An important aspect of drug candidates is their selectivity. Therefore, the most active compounds, **2c** and **2j**, were tested against HepG2 A16 cells, considered as hepatic cytotoxicity markers.[31] The results (Table 2) showed they were non-toxic, and the selectivity index indicated that both derivatives are at least 100 times more selective against *P. falciparum*.

Table 2. CC_{50} (μg mL^{-1}) of **2c** and **2j** against HepG2 A16 cells and respective selectivity indexes (SI)

Compound	CC_{50}[a] / (μg mL^{-1})	Selectivity index (SI)
2c	> 1000	100.40
2j	> 1000	155.03
Chloroquine	193.59	2,277.52

[a]CC_{50}: cytotoxic concentration that inhibit 50% the cell growth.

Some kaurenes, like kaurenoic acid (**3a**), have been described as weakly antiplasmodial,[22] whereas some kaurene lactones (Figure 1) were very active but highly toxic against human epithelial kidney cells.[23] Xylopic acid (**3b**), on the other hand, has been described as antimicrobial.[32] Our results showed that kaurenoic acid (**3a**), xylopic acid (**3b**) and steviol (**3c**) were inactive (data not shown). The results of the beyerene compounds showed that all modifications of the inactive isosteviol (**4a**) led to moderately active derivatives. The most actives were 4-nitrophenyl (**4e**, **4l**) or 2,4-dinitrophenylhydrazone (**4f**, **4m**) derivatives. The other hydrazones were inactive. Comparing the results for the two classes of diterpenes, those with beyerene backbone displayed the best activities.

Anti *T. cruzi*

Some compounds were also tested against epimastigotes and trypomastigotes forms of *T. cruzi*. The results (Table 3) shows that most compounds were inactive to the parasite. The oxime of isosteviol (**4b**), however, showed significant activity against both forms of the parasite. The same compound, on the other hand, did not show activity against *P. falciparum*, indicating species selectivity.

Similar oximes have shown activity *in vitro* against *T. cruzi*. The oximes **3f** and **3g**, obtained from kaurenoic acid (**3a**) showed significant activity.[28] Oximes of tetracyclic diterpenoids are, therefore, important leads for development of anti-malarial compounds. Interestingly, the same compound was inactive against *P. falciparum*.

Conclusions

Our results showed that the antiplasmodial activity of tetracyclic diterpenes and triterpenes may be increased by structural modifications. A total of 33 compounds (10 derivatives of betulinic acid, 6 kauranes and 17 beyeranes) were prepared and tested *in vitro* against *P. falciparum*. The addition of a nitrophenylhydrazone moiety increased

Table 3. *In vitro* biological activity of isosteviol derivatives on *T. cruzi* epimastigotes, trypomastigotes (IC_{50}) and Vero cells (CC_{50}) and corresponding selective index (SI)

Compound	IC_{50}, 24 h / µM	CC_{50}, 24 h / µM	Selective index (SI)
On epimastigotes			
3a	> 700	ND	
3d	> 1400	ND	
3e	> 2300	ND	
4a	> 500	ND	
4b	167.9	135.44	0.81
4f	> 400	ND	
4g	> 18,000	ND	
4n	> 400	ND	
4q	> 600	ND	
Benznidazole	45	3954.3	87.87
On cell-derived Trypomastigotes			
4b	106.08	135.44	1.28
Benznidazole	61.8	3924.3	63.9

ND: not done; IC_{50}: concentration in that the growth of the parasites is 50% inhibited; CC_{50}: cytotoxic concentration that inhibit 50% the cell growth.

significantly the activity. Some beyerene derivatives were moderately actives and the best results were obtained for the derivatives of betulinic acid, which were also very selective. The activity of the oxime of isosteviol (**4b**) against *T. cruzi*, and its inactivity against *P. falciparum*, indicates that appropriate modification of these compounds may result in species selective activities. These classes of terpenes, therefore, are promising models for the development of new drugs for the treatment of malaria and Chagas disease.

Experimental

General experimental details

IR and NMR spectra were obtained with a Bio-Rad (FTS3500GX) and Bruker (Avance DRX400 or DPX 200) spectrometers, respectively. Mass spectra electrospray ionisation (ESI) were acquired with a Thermo Fisher Scientific Inc. (LTQ XL Linear Ion Trap). Planar centrifugal chromatography was performed with a Chromatotron (Harrison Research) model 7924T, using silica-gel PF254 (Merck, art. No.7749). Thin-layer chromatography (TLC) for reaction monitoring was performed on silica plates from Merck (art. No. 5554). Solvents were distilled before use.

Isolation of betulinic acid and steviol

Betulinic acid and derivatives (**2a**-**2j**) were prepared as previously described.[33] Kaurenoic acid (**3a**) was isolated from *Annona glabra*[34] and xylopic acid (**3b**) was isolated from *Xylopia frutescens*.[35] Steviol (**3c**) was obtained from stevioside (**3f**) by reaction with $NaIO_4$ followed by treatment with base.[36] Isosteviol (**4a**)[37] and its derivatives 16-hydroxyisosteviol (**4g**),[38] isosteviol dnph (**4f**),[39] and isosteviol oxime (**4b**)[40] were prepared as described previously.

General procedure for the preparation of *p*-methoxyphenacyl esters

Steviol or isosteviol derivative (0.31 mmol) was added to a mixture of 2-bromo-4'-methoxyacetophenone (225 mg, 0.98 mmol) and triethylamine (20 mg mL^{-1} in acetone) and the mixture was irradiated in a microwave oven.[41] After reaction completion (ca. 4 minutes) acetic acid (40 µL) was added and the mixture was irradiated again for 1 minute. The mixture was then chromatographed on a Chromatotron using silica rotors (1 mm), and elution was made with appropriate mixtures of acetone:*n*-hexane. The product obtained was then characterized by spectroscopic methods.

Steviol *p*-methoxyphenacyl ester (**3e**)

White solid; yield 78%; IR (KBr) ν / cm^{-1} 3490, 2986, 1728, 1689, 1602, 1145; ^{1}H NMR (200 MHz, $CDCl_3$) 7.90 (d, 2H, *J* 8.9 Hz, H2', H6'), δ 6.95 (d, 2H, *J* 8.9 Hz, H3', H5'), δ 5.27 (dd, 2H, *J* 16.1 Hz, αH), δ 4.90 (d, 2H, *J* 32.2 Hz, H17), δ 3.87 (s, 3H, OCH_3), δ 2.13 (d, 1H, *J* 10.6 Hz, H14), δ 1.32 (s, 3H, H18), δ 0.90 (s, 3H, H20); ^{13}C NMR (50 MHz, $CDCl_3$) δ 191.0 (C=O), δ 176.95 (C19), δ 163.92 (C4), δ 156.19 (C16), δ 130.07 (C6), δ 130.07 (C2), δ 127.44 (C1), δ 113.99 (C3), δ 113.99 (C5), δ 102.87 (C17), δ 80.25 (C13), δ 65.17 (α C), δ 56.98 (C5), δ 55.51 (OCH_3), δ 53.76 (C9), δ 47.40 (C14), δ 46.96 (C15), δ 44.07 (C4), δ 41.66 (C8), δ 41.34 (C7), δ 40.66 (C1), δ 39.37 (C10), δ 39.20 (C12), δ 38.05 (C3), δ 28.99 (C18), δ 21.88 (C6), δ 20.42 (C11), δ 19.11 (C2), δ 15.68 (C20); ESI-MS *m/z* calcd. for $C_{29}H_{38}O_5$: 489.27; found: 489.46 $[M + Na]^+$.

General procedure for the preparation of hydrazones

A solution of 4-nitrophenylhydrazine or 2,4-dinitrophenylhydrazine (1.0 mmol) in concentrated H_2SO_4 (1 mL), water (1.5 mL) ethanol (1.5 mL) was added to the solution **4a** or **4h** (0.5 mmol) in ethanol (10 mL) and the reaction mixture stirred for 12 h at 25 °C. Water (30 mL) was then added and the product was recovered with EtOAc. The organic extract was dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure. The residue was fractionated on silica rotors

(1 mm, Chromatotron), and elution was made with suitable mixtures of acetone:*n*-hexane.

Isosteviol isopropylhydrazone (**4d**)

Isosteviol 16-hydrazone methyl ester (100 mg, 0.3 mmol) was refluxed with excess of acetone for 2 h. A light yellow crystalline compound was isolated (83%). IR (KBr) ν / cm^{-1} 1721, 1658, 1452; ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.63 (s, 3H, OCH_3), δ 2.68 (dd, 1H, *J* 18.4, 3.2 Hz, H15), δ 2.01 (s, 3H, H1'), δ 1.83 (s, 3H, H3'), δ 1.17 (s, 3H, H17), δ 1.12 (s, 3H, H18), δ 0.69 (s, 3H, H20); ^{13}C NMR (50 MHz, $CDCl_3$) δ 177.9 (C19), δ 174.3 (C16), δ 159.2 (C2'), δ 57.2 (C5), δ 56.0 (C14), δ 55.0 (C9), δ 51.2 (OCH_3), δ 44.2 (C13), δ 43.7 (C4), δ 41.1 (C15), δ 40.6 (C8), δ 39.9 (C1), δ 39.4 (C7), δ 39.0 (C12), δ 38.0 (C10), δ 37.9 (C3), δ 28.8 (C18), δ 24.9 (C17), δ 22.2 (C3'), δ 21.69 (C6), δ 20.5 (C11), δ 18.95 (C2), δ 17.6 (C1'), δ 13.25 (C20); ESI-MS *m/z* calcd. for $C_{24}H_{39}N_2O_2$: 387.57; found: 387.36 $[M + H]^+$.

Isosteviol 4-nitrophenylhydrazone (**4e**)

Yellow crystalline solid; yield 56%; IR (KBr) ν / cm^{-1} 3319, 1693, 1595, 1322; ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.15 (d, 2H, *J* 9.1 Hz, H3', H5'), δ 7.0 (d, 2H, *J* 9.1 Hz, H2', H6'), δ 2.96 (d, 1H, *J* 17.0 Hz, H15), δ 1.3 (s, 3H, H17), δ 1.19 (s, 3H, H18), δ 0.89 (s, 3H, H20); ^{13}C NMR (50 MHz, $CDCl_3$,) δ 184.0 (C19), δ 163.5 (C16), δ 150.5 (C4'), δ 139.6 (C1'), δ 126.1 (C3', C5'), δ 111.4 (C2', C6'), δ 56.9 (C5), δ 55.9 (C14), δ 54.8 (C9), δ 44.7 (C13), δ 43.7 (C4), δ 41.4 (C8), δ 41.1 (C15), δ 39.7 (C1), δ 39.4 (C7), δ 38.3 (C10), δ 37.6 (C3), δ 36.6 (C12), δ 29.0 (C18), δ 22.2 (C17), δ 21.6 (C6), δ 20.6 (C11), δ 18.8 (C2), δ 13.6 (C20); ESI-MS *m/z* calcd. for $C_{26}H_{34}N_3O_4$: 452.26; found: 452.33 $[M - H]^-$.

17-Hydroxyisosteviol hydrazone (**4k**)

Hydrazine hydrate (2 mL) was added into solution of 17-hydroxyisosteviol (100 mg, 0.3 mmol), in methanol (10 mL) and the mixture was refluxed for 8 hours. The solvent and excess of hydrazine were removed under reduced pressure and the product was recrystallized from methanol (90 mg, 86%); IR (KBr) ν / cm^{-1} 3412, 3221, 1698, 1175; ^{1}H NMR (200 MHz, CD_3OD) δ 3.57 (s, 2H, H17), δ 2.86 (d, 1H, *J* 17.5 Hz, H12), δ 1.18 (s, 3H, H18), δ 0.96 (s, 3H, H20); ^{13}C NMR (50 MHz, CD_3OD), δ 182.9 (C19), δ 165.3 (C16), δ 66.1 (C17), δ 57.4 (C5), δ 55.6 (C9), δ 51.0 (C14), δ 49.1 (C13), δ 44.1 (C4), δ 41.2 (C15), δ 40.8 (C8), δ 40.2 (C1), δ 38.6 (C3), δ 38.0 (C10), δ 36.8 (C7), δ 34.2 (C12), δ 28.8 (C18), δ 21.9 (C6), δ 19.6 (C11), δ 19.2 (C2), δ 12.9 (C20); ESI-MS *m/z* calcd. for $C_{20}H_{33}N_2O_3$: 349.24; found: 349.30 $[M + H]^+$.

17-Hydroxyisosteviol, 4-nitrophenylhydrazone (**4l**)

Yellow solid; yield 56%; IR (KBr) ν / cm^{-1} 3315, 1692, 1618, 1337; ^{1}H NMR (200 MHz, acetone-d_6) 9.1 (s, 1H, NH), 8.09 (d, 2H, *J* 9.36 Hz, H3', H5'), δ 7.15 (d, 2H, *J* 9.33 Hz, H2', H6'), δ 3.6 (d, 1H, *J* 7.4 Hz, H17), 3.7 (d, 1H, *J* 7.4 Hz, H17) δ 2.97 (dd, 1H, *J* 18.23, 2.86 Hz, H15), δ 1.21 (s, 3H, H18), 0.87 (s, 3H, H20); ^{13}CNMR (50 MHz, acetone-d_6) δ 178.1 (C19), δ 162.7 (C16), δ 151.5 (C4'), δ 138.8 (C1'), δ 125.6 (C3', C5'), δ 111.1 (C2', C6'), δ 65.9 (C17), δ 56.7 (C5), δ 55.3 (C9), δ 50.7 (C14), δ 50.0 (C13), δ 43.2 (C4), δ 41.1 (C8), δ 40.9 (C15), δ 39.7 (C1), δ 38.0 (C10), δ 37.9 (C3), δ 37.8 (C7), δ 34.5 (C12), δ 28.4 (C18), δ 21.7 (C6), δ 19.8 (C11), δ 18.8 (C2), δ 13.1 (C20); ESI-MS *m/z* calcd. for $C_{26}H_{33}N_3O_5$: 468.26; found: 468.37 $[M - H]^-$.

17-Hydroxyisosteviol-2,4-dinitrophenylhydrazone (**4m**)

Yellow solid; yield 55%; IR (KBr) ν / cm^{-1} 3446, 3315, 1692, 1618, 1337; ^{1}H NMR (200 MHz, acetone-d_6) δ 10.7 (s, 1H, NH), δ 8.99 (d, 1H, *J* 2.5 Hz, H3'), δ 8.19 (dd, 1H, *J* 9.6, 2.51 Hz, H5'), δ 7.7 (d, 1H, *J* 9.6 Hz, H6'), δ 3.78 (dd, 1H, *J* 19.2, 11.4 Hz, H17), δ 2.96 (dd, 1H, *J* 18.2, 2.86 Hz, H15), δ 1.3 (s, 3H, H18), δ 0.94 (s, 3H, H20); ^{13}C NMR (50 MHz, acetone-d_6) δ 183.7 (C19), δ 170.90 (C16), δ 144.5 (C4'), δ 137.7 (C2'), δ 129.8 (C3'), δ 128.8 (C1'), δ 123.3 (C5'), δ 115.8 (C6'), δ 66.3 (C17), δ 56.8 (C5), δ 55.4 (C9), δ 50.6 (C13), δ 50.5 (C14), δ 43.6 (C4), δ 41.5 (C8), δ 40.6 (C15), δ 39.6 (C1), δ 38.3 (C10), δ 37.9 (C3), δ 37.6 (C7), δ 34.3 (C12), δ 28.9 (C18), δ 21.53 (C6), δ 19.7 (C11), δ 18.7 (C2), δ 12.9 (C20); ESI-MS *m/z* calcd. for $C_{26}H_{33}N_4O_7$: 513.24; found: 513.24 $[M - H]^-$.

Isosteviol *p*-methoxyphenacyl ester (**4n**)

White solid; yield 81%; IR (KBr) ν / cm^{-1} 2953, 1733, 1694, 1598, 1144; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.89 (d, 2H, *J* 9.0 Hz, H2', H6'), δ 6.94 (d, 2H, *J* 9.0 Hz, H3', H5'), δ 5.2 (d, 1H, *J* 16.0 Hz, αH), 5.3 (d, 1H, *J* 16.0 Hz, αH), δ 3.87 (s, 3H, OCH_3), δ 2.65 (dd, 1H, *J* 18.7, 3.65 Hz, H15), δ 1.33 (s, 3H, H17), δ 0.97 (s, 3H, H18), δ 0.77 (s, 3H, H20); ^{13}C NMR (50 MHz, $CDCl_3$) δ 190.93 (C=O), δ 176.80 (C19), δ 163.95 (C4'), δ 130.05 (C2', C6'), δ 127.44 (C1'), δ 114.00 (C3', C5'), δ 65.17 (αC), δ 57.14 (C5), δ 55.50 (OCH_3), δ 54.75 (C9), δ 54.30 (C14), δ 48.69 (C13), δ 48.48 (C15), δ 44.07 (C4), δ 41.53 (C7), δ 39.82 (C1), δ 39.46 (C8), δ 38.09 (C10), δ 38.01 (C12), δ 37.30 (C3), δ 29.10 (C18), δ 21.63 (C6), δ 20.32 (C11), δ 19.84 (C17), δ 18.93 (C2), δ 13.55 (C20); ESI-MS *m/z* calcd. for $C_{29}H_{38}O_5Na$: 466.27; found: 466.59 $[M + Na]^+$.

Isosteviol oxime *p*-methoxyphenacyl ester (**4o**)

Amorphous white solid; yield 78%; IR (KBr) ν / cm^{-1}

3446, 2949, 1723, 1690, 1599, 1159; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.89 (d, 2H, *J* 8.9 Hz, H2', H6'), δ 6.95 (d, 1H, *J* 8.91 Hz, H3', H5'), δ 5.23 (d, 1H, *J* 16.0 Hz, α H), δ 3.87 (s, 3H, OCH_3), δ 2.98 (dd, 1H, *J* 18.8, 3.00 Hz, H15), δ 1.33 (s, 3H, H17), δ 1.09 (s, 3H, H18), δ 0.82 (s, 3H, H20); ^{13}C NMR (50 MHz, $CDCl_3$) δ 190.93 (C=O), δ 177.00 (C19), δ 170.45 (C16), δ 163.92 (C4'), δ 130.06 (C2', C6'), δ 127.44 (C1'), δ 113.99 (C3', C5'), δ 65.16 (αC), δ 57.14 (C5), δ 56.28 (C14), δ 55.49 (OCH_3), δ 54.90 (C9), δ 44.06 (C13), δ 43.72 (C4), δ 40.90 (C15), δ 40.63(C8), δ 39.92 (C1), δ 39.45 (C7), δ 38.13 (C10), δ 38.06 (C3), δ 36.78 (C12), δ 29.04 (C18), δ 22.12 (C17), δ 21.67 (C6), δ 20.42 (C11), δ 18.95 (C2), δ 13.55 (C20); ESI-MS *m/z* calcd. for $C_{29}H_{39}NO_5$: 481.28; found: 481.45 $[M]^+$.

16-Hydroxyisosteviol *p*-methoxyphenacyl ester (**4p**)

Amorphous white solid; yield 75%; IR (KBr) ν / cm^{-1} 3556, 2958, 1723, 1690, 1603, 1159; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.90 (d, 2H, *J* 8.9 Hz, H2', H6'), δ 6.95 (d, 1H, *J* 8.9 Hz, H3', H5'), δ 5.2 (d, 1H, *J* 16.1 Hz, αH), δ 5.3 (d, 1H, *J* 16.1 Hz, αH), δ 3.87 (s, 3H, OCH_3), δ 1.31 (s, 3H, H17), δ 0.91 (s, 3H, H18), δ 0.80 (s, 3H, H20); ^{13}C NMR (50 MHz, $CDCl_3$) δ 191.08 (C=O), δ 177.03 (C19), δ 163.88 (C4'), δ 130.04 (C2', C6'), δ 127.50 (C1'), δ 113.95 (C3', C5'), δ 80.52 (C16), δ 65.17 (αC), δ 57.22 (C5), δ 55.85 (C9), δ 55.47 (OCH_3), δ 55.30 (C14), δ 44.04 (C4), δ 42.84 (C15), δ 42.07 (C13), δ 41.99 (C8), δ 41.75 (C7), δ 39.91 (C1), δ 38.11 (C3), δ 38.09 (C10), δ 33.71 (C12), δ 29.12 (C18), δ 24.88 (C17), δ 21.69 (C6), δ 20.45 (C11), δ 18.95 (C2), δ 13.47 (C20); ESI-MS *m/z* calcd. for $C_{29}H_{40}O_5Na$: 491.29; found: 491.35 $[M + Na]^+$.

Isosteviol benzyl ester (**4q**)

To the solution of isosteviol (318 mg, 1 mmol), in acetone (40 mL), potassium carbonate (200 mg, 1.45 mmol), and benzyl chloride (2 mL), were added and the mixture was refluxed for two hours. After filtration the solvent and excess benzyl chloride were removed under reduced pressure. A white crystalline solid was recovered (340 mg, 83%); IR (KBr) ν / cm^{-1} 1738, 1720, 1454, 1147, 694; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.34 (s, 5H, H'-H6'), 5.08 (dd, 1H, *J* 18.1, 12.4 Hz, Hα), δ 2.55 (dd, 1H, *J* 18.1, 12.4 Hz, H15), δ 1.21 (s, 3H, H17), 0.96 (s, 3H, H18), δ 0.6 (s, 3H, H20); C^{13} NMR (50 MHz, $CDCl_3$) δ 176.9 (C19), δ 135.9 (C1'), δ 128.4 (C3', C5'), δ 128.35 (C2', C6'), δ 128.1 (C4'), δ 66.1 (αC), δ 57.1 (C5), δ 54.6 (C9), δ 54.2 (C14), δ 48.6 (C13), δ 48.3 (C15), δ 43.8 (C4), δ 41.4 (C7), δ 39.7 (C1), δ 39.4 (C8), δ 37.9 (C10, C12), δ 37.3 (C3), δ 28.9 (C18), δ 21.7 (C6), δ 20.3 (C11), δ 19.8 (C17), δ 18.89 (C2), δ 13.3 (C20); ESI-MS *m/z* calcd. for $C_{27}H_{37}O_3$: 409.27; found: 409.30 $[M + H]^+$.

Antitrypanosomal bioassay

Vero cells (ATCC CCL-81) were maintained at 37 °C in a humidified 5% CO_2 atmosphere. The cells were grown in 75 cm^2 culture flasks with RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 1% *L*-glutamine, 1% penicillin and 10 mg mL^{-1} streptomycin. For the cytotoxicity bioassays, 4-day-old confluent Vero cells monolayers were washed with phosphate buffered saline (PBS, pH 7.2) and detached from the substrate by treatment with 0.25% trypsin + 0.1% EDTA for 5 minutes at 37 °C. The cells were then resuspended in the same medium, centrifuged for 2 minutes at 100 g and the cell pellet was collected.

Culture epimastigote forms of *Trypanosoma cruzi* clone Dm28c were maintained in LIT (liver infusion-tryptose) medium at 28 °C with serial passagesat every three days (mid-log phase of growth).[42] For the experiments, parasites obtained from 72-hour cultures were inoculated into fresh LIT medium and then added to 96-well plates at a concentration of 5×10^7 cells *per* well.

To obtain cell-derived trypomastigote forms, Vero cell cultures were infected with trypomastigote forms at a 10:1 parasite:host cell ratio. After four hours of interaction, the host cell monolayers were washed with PBS to remove non-internalized parasites. The cultures were kept for 96 hours at 37 °C in RPMI/2.5% FCS in a 5% CO_2 humidified atmosphere. After that period, trypomastigotes released to the supernatant were collected and washed with PBS by centrifugation at 3000 g. The purified trypomastigotes were transferred to RPMI-1640 medium at a concentration of 5×10^7 cells mL^{-1} and then added to 96-well plates at 100 µL *per* well.

For the assays steviol and isosteviol derivatives were diluted in dimethylsulfoxide (DMSO) and added to 96 well plates containing parasites (epimastigotes or trypomastigotes), at final concentrations ranging from 10 to 600 µM. The 50% inhibitory concentration after 24 hours of incubation (IC_{50} *per* 24 h) was determined with the cell viability marker MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], according to standard protocols.[43] The assays were quantified at 550 nm using the ELISA reader BIOTEXEL-800 (Biotek, Winooski, VT, USA). All experiments were performed in biological and technical triplicate. The CompuSyn software was used to calculate the IC_{50} *per* 24 h. Controls were grown in medium containing 0.5% DMSO, without addition of derivates.

For the cytotoxicity assays Vero cells were seeded at a concentration of 2×10^4 cells *per* well in 96-well plates, containing RPMI-1640 medium supplemented with 10% FCS, and maintained at 37 °C and 5% CO_2 atmosphere.

After 24 hours of cultivation, oxime of isosteviol (10 to 75 μM) or benznidazole (3.8 to 4000 μM) were added. The cytotoxic concentration (CC_{50} *per* 24 h) was determined after 24 h of incubation, using the MTT enzymatic assay as described above. 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.

Antimalarial bioassay

The antimalarial activity was evaluated by [^{3}H]-hypoxanthine incorporation[44] and lactate dehydrogenase[45] assays. The compounds were diluted in DMSO (50 mg mL^{-1}) and serial dilutions (1.56-50 μg mL^{-1}) were prepared in RPMI-1640 medium. The assay was carried out in 96-well plates using suspension of erythrocytes infected with *P. falciparum* W2, chloroquine-resistant (ring-stage parasites synchronized in sorbitol, 2% hematocrit, 1% parasitemia). Controls with infected or non-infected erythrocytes were used. The results were analyzed as sigmoidal dose-response curves and the IC_{50} values (concentration in that the growth of the parasites is 50% inhibited) of three independent experiments were obtained. The results were classified in accordance to a standard criteria: very active (IC_{50} < 1 μg mL^{-1}), active (IC_{50} 1-15 μg mL^{-1}), moderately active (IC_{50} 15.1-25 μg mL^{-1}), slightly active (IC_{50} 25.1-50 μg mL^{-1}) and inactive (IC_{50} > 50 μg mL^{-1}).

The cytotoxicity of the most active compounds, **2c** and **2j**, was evaluated against human hepathoblastoma HepG2 A16 cell line[46] using MTT assay.[47] The results were expressed as CC_{50} values (cytotoxic concentration that inhibit 50% the cell growth) of three independent experiments. Selectivity index (SI) was calculated by the CC_{50} value for HepG2 A16 cells divided by IC_{50} value for *P. falciparum*.

Supplementary Information

Spectroscopic spectra of compounds and further bioassays data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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