Full Paper

Leishmanicidal Evaluation of Novel Synthetic Chromenes

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In the present paper, twelve chromenes were synthesized by coupling of 2,2,8,8-tetramethyl-8*H*-pyrano[2,3-*f*]chroman-4-one **1** with various aryl and benzylmagnesium chlorides. The synthetic compounds were examined for *in-vitro* activity against *Leishmania major*, and some of them displayed efficient anti-leishmanial activity. Among the compounds tested, compounds **9** (4-(2-chloro-benzylidene)-2,2,8,8-tetramethyl-3,4-dihydro-2*H*,8*H*-pyrano[2,3-*f*]chromene **9a** and 4-(2-chloro-benzyl)-2,2,8,8-tetramethyl-2*H*,8*H*-pyrano[2,3-*f*]chromene **9b**) were the most active with an inhibitory activity of 73.4%.

Keywords: Chromene / Leishmanicidal activity / Synthesis

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Introduction

Protozoal parasitic diseases continue to pose a serious problem for the public health in the world. Among them, *Leishmania* spp. are causative agents of mortality and morbidity in the human known as leishmaniasis, and it was estimated that worldwide about twelve million people are affected, with approximately 350 million individuals at risk. During the last ten year, there has been this global burden and extensive epidemic forms of the disease due to human migration, particularly problematic is its coincidence with HIV and the capacity of *Leishmania* to infect specialized immune cells [1, 2]. *Leishmania* spp. are obligate intracellular parasites that proliferate and develop in a virulent metacyclic stage into an invertebrate vector which can infect diverse vertebrate hosts. The two distinct developmental stages of *Leishmania* are recognized as promastigotes and amastigotes. The first form which is found within the midgut of sandflies has an elongated shape and long flagellum, but upon the bite of an infected sandfly, in the mammalian hosts they differentiate to the amastigote stage, which lack flagella and is an obligate intracellular pathogen infecting hematopoietic cells [3, 4]. Unfortunately, there are no effective vaccines against the parasite yet. Thus, control of the disease relies primarily on chemical treatment. There are over 17 species of *Leishmania* known to be infective to humans. The species have been characterized on the basis of biochemical and molecular differences and these differences provide an explanation for variation in the clinical responses and species' sensitivity to the pentavalent antimonials, sodium stibogluconate and meglumine antimonate (Glucantime), in the treatment of leishmaniasis over the past 50 years. Generally, the antileishmanial drugs presently on the market possess severe side effects, are expensive, need long-term treatment, and do not provide complete eradication of the disease. In addition, resistance to current drugs develops rapidly and a large-scale resistance to pentavalent antimonials has been reported in parts of the world, for example in India and Sudan, so they are quickly becoming obsolete [2, 5, 6]. Therefore, new efficacious and less toxic chemical agents for human are urgently needed.

There has been a revival of drug research and development regarding neglected parasitic diseases compared to

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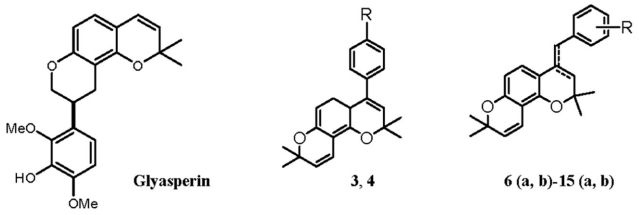
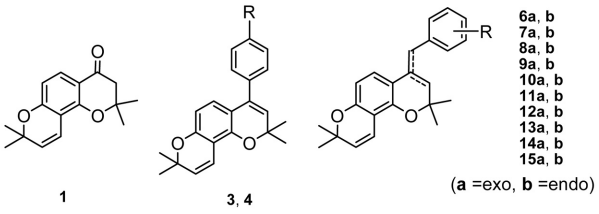


Figure 1. Structure of synthetic leishmanicidal chromenes and Glyasperin.

the last 15 years, and novel chemical classes are being pursued for the treatment. Natural products are known as the chief source and are often identified as lead structures; the derivatives of such compounds may result in highly effective agents with reduced toxicity and side effects [7, 8].

Recently, Glyasperin, a chromene isolated from *Smirnovia iranica*, has been reported to be highly effective against the *Leishmania* parasite (Fig. 1). Chromenes are structurally simple compounds belonging to a large class of molecules known as benzopyranes, and the chromane-4-one moiety is an integral part of many natural products [9]. These compounds and related derivatives have diverse biological activities, including antitumor, antibacterial, and leishmanicidal potency [10] that make them attractive agents for screening novel therapeutic drugs as do further backbone substitutions [11, 12]. In order to find new drugs with more potent activity against *Leishmania* parasite, we have been engaged in a program to investigate some new synthetic compounds [13–16]. Moreover, the biological potency of heterocyclic chromenes has been widely documented and considerable efforts have been made to explore new routes for the synthesis of therapeutically more efficient heterocyclic chromenes. In an investigation to find new anti-leishmanial agents which are structurally related to 2,2-dimethyl-2*H*-chromenes, a series of synthetic chromenes was prepared by a condensation reaction of chromene **1** and Grignard reagents [17, 18]. The prepared compounds, namely compounds **1**, **3**, **4**, and **6**–**15** were tested against *Leishmania major* promastigotes. Some of the derivatives were moderately active against *L. major*, whereas the others displayed good activity against the parasite (Table 1).

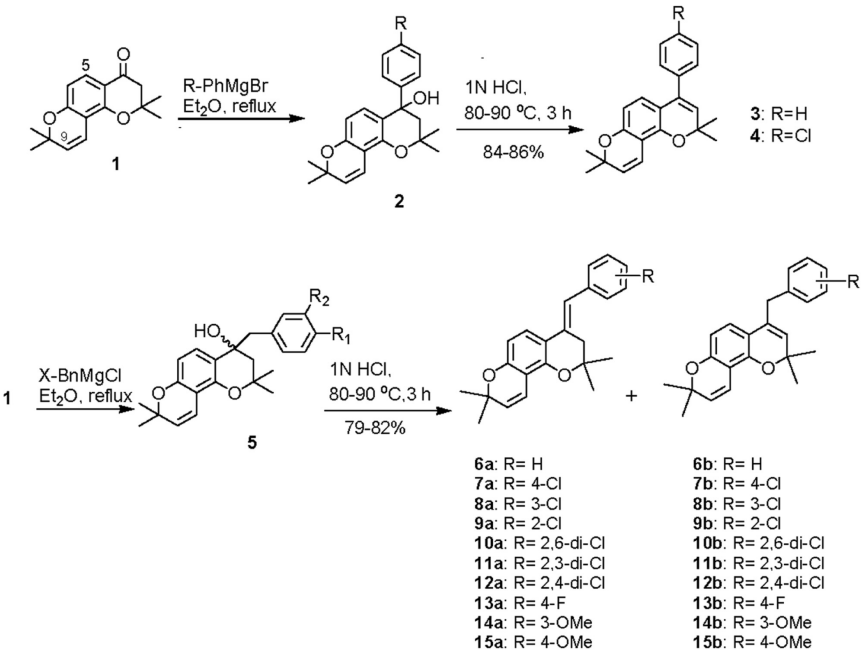
Table 1. Camparison of the inhibitory effect of the compounds on the viability of *L. major* promastigotes under *in-vitro* conditions.



Compound	R	Inhibition % after 72 h
1	–	49.4
3	h	38.0
4	4-Cl	53.2
6a, b	H	38.0
7a, b	4-Cl	41.8
8a, b	3-Cl	48.1
9a, b	2-Cl	73.4
10a, b	2,6-diCl	65.8
11a, b	2,3-diCl	29.1
12a, b	2,4-diCl	60.8
13a, b	4-F	59.5
14a, b	3-OMe	36.7
15a, b	4-OMe	30.4
Glucantime		67.2

Chemistry

Compound **1** (Scheme 1) was prepared by thermal cyclization of 2,2-dimethyl-7-(2-methylbut-3-yn-2-yloxy)-chroman-4-one in *N*,*N*-dimethyl-aniline at 180°C [19–21]. Compounds **3**, **4**, and **6**(**a**, **b**) to **15**(**a**, **b**) were synthesized by the reaction of compound **1** with appropriate Grignard reagents and an subsequent acidic dehydration reaction in refluxing 1 N HCl [22] (Scheme 1). Compounds **6**(**a**, **b**) to **15**(**a**, **b**) were a mixture of two regio-isomers and their structures were established by ^{1}H-NMR analysis (compounds **3**, **4**, **6**(**a**, **b**) to **8**(**a**, **b**); they were reported previously [23]). In addition, exo-isomers (**9b** to **15b**) were a mixture of *E*- and *Z*-isomers. It should be noted that none of these isomers could be separated by conventional methods [24]. Differentiation between exo- and endo-isomers as well as the *E*- and *Z*-form of exo-isomers were based on the ^{1}H-NMR, ^{13}C-NMR, and NOESY experiments. In ^{1}H-NMR, the observation of a doublet at 2.27 to 2.72 ppm (3-CH_2 of compound **9a** to **15a**) and a singlet at 3.63 to 3.93 ppm (benzylic CH_2 of compound **9a** to **15a**) showed the existence of both exo- and endo-isomers in the ratio of the 46 : 54 and 75 : 25, respectively. Moreover, the exo-product appeared to be a mixture of *E*- and *Z*-steroisomers which were assigned based on the anisotropic effect of the substituted phenyl ring on H_5 of the *Z*-isomer. In the *E*-isomer, H_5 of compound **10a** appeared as a doublet at 7.47 (J = 8.5 Hz) and in the *Z*-isomer the H_5 appeared at 7.45 ppm (J = 8.5 Hz). However, in most compounds the *Z*-isomers were a minor and an undetectable product. The configuration of the *E*-isomer as a major product was confirmed through 2D-NOESY NMR spectroscopy and the assignment of stereochemistry of com-



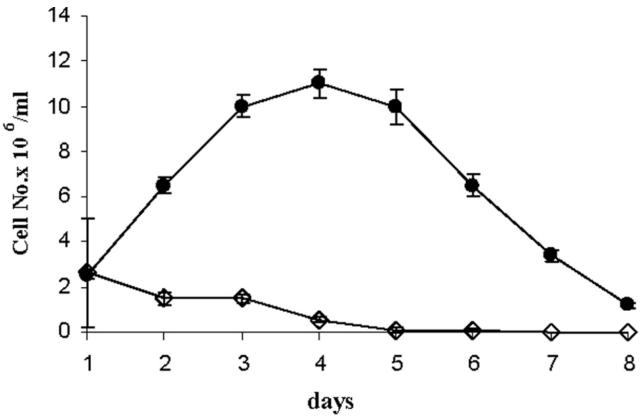
Scheme 1. Synthesis of compounds **3**, **4**, **6**(**a**, **b**)-**15**(**a**, **b**).

pound **10a** is consisted with the observed strong cross-peak between H_5 and vinylic-H (Ar-CH=C) and the relatively weak cross-peak between H_3 and the *ortho*-H phenyl ring. In addition, in ^{13}C-NMR the 3-CH_2 of exo and vinylic CH_2 of the endo-isomers appeared as two separate signals at 32.7 – 37.3 and 37.1 – 37.9 ppm. The vinylic CH (exo-isomer) and olefinic CH (endo-3-CH) appeared as two separate singlets at 117.2 – 120.5 ppm and 123.2 – 123.6 ppm, respectively. The Dept-135 and Dept-90 experiments confirmed the mentioned CH_2 and CH groups.

Results and discussion

Inhibitory effects of the synthesized compounds in a concentration of 10 μg/mL were determined by counting the number of treated promastigotes remaining alive in the cultures, and by analyzing the growth inhibition of the parasit as described above. The growth kinetics of *L. major* for a period of 8 days is shown in Fig. 2. The chromenes exhibited a clear time-dependent inhibitory effect on *L. major* (Fig. 3). Five compounds (**4**, **9**, **10**, **12**, and **13**) were shown to be effective after 72 h incubation, with a growth inhibition > 50% (Table 1).

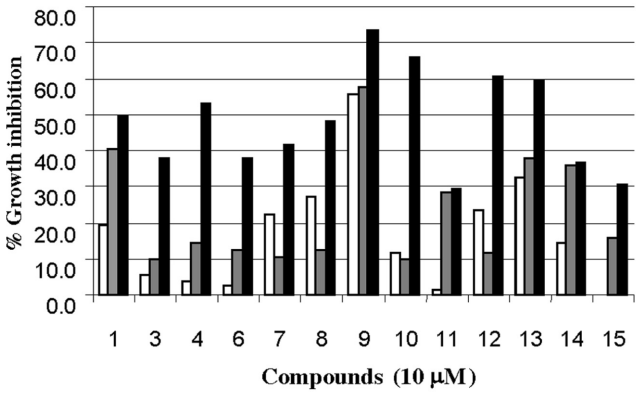
The chromanone intermediate **1** was moderately active (Inh.% = 49.4). The target compounds **3** and **6** having no substitution in phenyl ring had the same inhibitory activity (Inh % = 38.0). Insertion of a Cl in the 4-position of phenyl ring in compound **3**, increased the inhibitory effect to 53.2% (compound **4**). As can be deduced from the



Promastigotes were seeded at approximately 2×10^6 cells/mL and during 8 days. The cell concentrations were determined daily by Neubauer`s chamber. (●) The growth phases of untreated cells, (◇) Promastigote growth rate after treatment with Glucantime as a standard. Each time point represents the mean value of three replicates with the standard deviation.

Figure 2. The growth curve of *L. major.*

data in Table 1, substitution of a chlorine atom in phenyl ring of compound **6** enhanced the biological activity of the compounds in the following order: 2-Cl > 3-Cl > 4-Cl. In fact, chromene **9** having 2-chloro substitution was the most active compound showing 73.4% inhibitory activity. Addition of the second Cl in the 3-position of the phenyl ring in compound **9** (namely compound **11**) resulted in a large decrease in inhibitory activity (Inh. % = 29.1). However, compounds **10** and **12** having 2,6- and 2,4-dichloro substitutions showed 65.8% and 60.8% inhibi-



The parasites were cultured for 24 h (white bar), 48 h (grey bar), and 72 h (black bar) in the presence of the compounds as described in Experimental, Section 4. The results are given as the percentage of inhibition (mean ± SD).

Figure 3. The effects of the synthetic agents on the *in vitro* growth rate of *L. major* promastigotes.

tory activity, respectively. Replacement of Cl in compound **7** with F increased the inhibitory action to 59.5%. In contrast, substitution with a methoxy group, compare compounds **14** and **15**, decreased the inhibitory percentage to 36.7 and 30.4, respectively.

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The authors have declared no conflict of interest.

Experimental

General

All chemicals and reagents were obtained from Merck Chemical Company (Darmstadt, Germany) and Sigma-Aldrich Chemical Company (Steinhein, Germany). ^{1}H-NMR spectra were measured using a Bruker 500 spectrometer (Brucker, Rheinstetten, Germany) and chemical shifts are expressed as δ (ppm) with tetramethylsilane as internal standard. The IR spectra were taken using a Nicolet FT-IR Magna 550 spectrographs (KBr disks) (Nicolet, Madison, WI, USA). MS spectra were obtained with a Finnigan MAT TSQ-70 spectrometer (Finnigan Mat, Bremen, Germany). The purity of a compound was confirmed by TLC using different mobile phases. The results of elemental analyses (C, H, N) were within ± 0.4% of theoretical values for C, H, and N.

Chemistry

*4-(2-Chloro-benzylidene)-2,2,8,8-tetramethyl-3,4-dihydro-2H,8H-pyrano[2,3-f] chroman 9a and 4-(2-Chloro-benzyl)-2,2,8,8-tetramethyl- 2H,8H- pyrano[2,3-f]chromene **9b***

To a stirred solution of **1** (0.3 g, 1.2 mmol) in dry ether (3 mL) was added 2-chlorobenzylmagnesium chloride (1.25 mL, 1 mmol, 0.8 M) under argon. The resulting mixture was stirred and refluxed for 18 hours. After cooling, the organic phase was washed with 1 N HCl (3 × 5 mL). The solvent was evaporated under reduce pressure to give an oil. To this oily residue was added 2 N HCl (15 mL) and the mixture was refluxed for 12 hours. After cooling, it was extracted with ethyl acetate (3 × 20 mL). The organic layer was washed with 1 N NaOH and saturated aqueous $NaHCO_3$ and was dried with Na_2SO_4. After filtration, the solvent was evaporated under reduce pressure. The residue was purified by flash column chromatography (silica gel = 25 g, hexane / EtOAc = 5 : 1) to give **9a**, **b** (0.33 g, 0.92 mmol, 79%) as a colorless oil as mixture of exo- and endo-isomers (48 : 52).

***9a** (exo-isomer)*

^{1}H-NMR ($CDCl_3$) δ [ppm]: 1.27 (s, 6H, 2-CH_3), 1.45 (s, 6H, 8-CH_3), 2.56 (d, 2H, *J* = 1.2 Hz, 3-CH_2), 5.54 (d, 1H, *J* = 9.9 Hz, H_9), 6.68 (d, 1H, *J* = 8.4 Hz, H_6), 6.69 (d, 1H, *J* = 9.9 Hz, H_{10}), 7.01 (s, 1H, 2-Cl-Ph-CH=C), 7.16 – 7.40 (m, 4H, Ar), 7.46 (d, 1H, *J* = 8.4 Hz, H_5).

***9b** (endo-isomer)*

^{1}H-NMR ($CDCl_3$) δ [ppm]: 1.40 (s, 6H, 2-CH_3), 1.41 (s, 6H, 8-CH_3), 3.76 (s, 2H, 2-ClPh-CH_2), 5.08 (s, 1H, 3-CH), 5.57 (d, 1H, *J* = 9.9 Hz, H_9), 6.31 (d, 1H, *J* = 8.4 Hz, H_6), 6.71 (d, 1H, *J* = 9.9 Hz, H_{10}), 6.86 (d, 1H, *J* = 8.4 Hz, H_5), 7.16 – 7.40 (m, 4H, Ar).

***9a/9b** (exo-/endo-isomers)*

^{13}C-NMR ($CDCl_3$) δ [ppm]: 27.8 (q), 27.9 (q), 28.0 (q), 35.0 (t), 37.4 (t), 75.7 (s), 76.0 (s), 76.1 (s), 108.3 (d), 109.1 (d), 110.1 (s), 110.2 (s), 114.3 (s), 115.2 (s), 116.9 (d), 117.0 (d), 118.4 (d), 123.4 (d), 124.6 (d), 126.1 (d), 126.8 (d), 127.7 (d), 127.8 (d), 127.9 (d), 128.2 (d), 128.5 (d), 129.0 (d), 129.4 (d), 130.5 (d), 131.1 (d), 131.3 (s), 131.9 (s), 134.4 (s), 134.5 (s), 135.9 (s), 136.7 (s), 148.9 (s), 149.3 (s), 153.7 (s), 154.2 (s). IR (KBr) [cm^{-1}]: 2975 (s), 2930 (sh), 1632 (s), 1610 (sh), 1472 (m), 1250 (w), 1170 (w), 1114 (s), 1055 (s), 815 (s). MS *m/z* [%]: 368 [M^+ + 2] (13), 366 [M^+] (40), 354 (18), 251 (22), 339 (87), 336 (100), 163 (13). Anal. Calcd. for $C_{23}H_{23}ClO_2$: C, 75.30; H, 6.32. Found: C, 75.28; H, 6.36.

The other compounds **10**(**a**, **b**) to **15**(**a**, **b**) were prepared by the same method. In addition, analytical data of compounds **3**, **4**, **6**(**a**, **b**) to **8**(**a**, **b**) were reported previously [19].

*4-(2,6-di-Chloro-benzylidene)-2,2,8,8-tetramethyl-3,4-dihydro-2H,8H-pyrano[2,3-f]chroman (**10a**-exo) and 4-(2,6-di-Chloro-benzyl)-2,2,8,8- tetramethyl-2H,8H-pyrano[2,3-f]chromene (**10b**-endo)*

A colorless oil (69%) as mixture of exo- (*E* and *Z*, 97 : 3) and endo-isomers (63 : 37).

***10a** (exo-isomer)*

^{1}H-NMR ($CDCl_3$) δ [ppm]: $1.25_{(Z)}$ and $1.26_{(E)}$ (2s, 6H, 2-CH_3), 1.46 (s, 6H, 8-CH_3), 2.27 (d, 2H, *J* = 1.4 Hz, 3-CH_2), 5.56 (d, 1H, *J* = 9.9 Hz, H_9), 6.44 (d, 1H, *J* =8.4 Hz, H_6), 6.67 (d, 1H, *J* = 9.9 Hz, H_{10}), 6.71 (s, 1H, 2,6-di-Cl-Ph-CH=C), 7.07 – 7.34 (m, 3H, Ar), $7.45_{(Z)}$ (d, 1H, *J* = 8.5 Hz, H_5), $7.47_{(E)}$ (d, 1H, *J* = 8.5 Hz, H_5).

***10b** (endo-isomer)*

^{1}H-NMR ($CDCl_3$) δ [ppm]: 1.31 (s, 6H, 2-CH_3), 1.43 (s, 6H, 8-CH_3), 3.93 (s, 2H, 2,6-di-Cl-Ph-CH_2), 5.07 (s, 1H, 3-CH), 5.58 (d, 1H, *J* = 9.9 Hz, H_9), 6.42 (d, 1H, *J* = 8.5 Hz, H_6), 6.70 (d, 1H, *J* = 9.9 Hz, H_{10}), 7.12 (d, 1H, *J* = 8.5 Hz, H_5), 7.07 – 7.34 (m, 3H, Ar).

10a/10b *(exo-/endo-isomers)*

^{13}C-NMR ($CDCl_3$) δ [ppm]: 26.6 (q), 27.9 (q), 28.0 (q), 32.7 (t), 38.0 (t), 75.8 (s), 76.0 (s), 76.0 (s), 76.5 (s), 108.1 (d), 108.6 (d), 110.0 (s), 110.2 (s), 113.6 (s), 115.5 (s), 116.9 (d), 117.0 (d), 122.4 (d), 122.9 (d), 124.5 (d), 127.3 (d), 127.8 (d), 127.9 (d), 128.1 (d), 128.2 (d), 128.6 (d), 129.0 (d), 131.7 (s), 131.9 (s), 133.3 (s), 134.8 (s), 135.2 (s), 135.7 (s), 136.4 (s), 148.7 (s), 149.3 (s), 153.7 (s), 154.3 (s). IR (KBr) [cm^{-1}]: 2980 (s), 2930 (sh), 1638 (vs), 1625 (sh), 1410 (sh), 1435 (s), 1365 (m), 1207 (m), 1118 (vs), 1054 (m), 777 (m). MS m/z [%]: 404 [M^+ + 4] (4), 403 [M^+ + 3] (7), 402 [M^+ + 2] (26), 401 [M^+ + 1] (10), 400 [M^+] (40), 386 (100), 323 (10), 239 (11), 149 (14). Anal. Calcd. for $C_{23}H_{22}Cl_2O_2$: C, 68.83; H, 5.53. Found: C, 68.73; H, 5.45.

*4-(2,3-di-Chloro-benzylidene)-2,2,8,8-tetramethyl-3,4-dihydro-2H,8H-pyrano[2,3-f]chroman (**11a**) and 4-(2,3-di-Chloro-benzyl)-2,2,8,8-tetramethyl-2H,8H- pyrano[2,3-f]chromene (**11b**)*

A colorless oil (67%) as mixture of exo- and endo-isomers (46 : 54).

11a *(exo-isomer)*

^{1}H-NMR ($CDCl_3$) δ [ppm]: 1.28 (s, 6H, 2-CH_3), 1.45 (s, 6H, 8-CH_3), 2.53 (d, 2H, *J* = 1.4 Hz, 3-CH_2), 5.52 (d, 1H, *J* = 9.8 Hz, H_9), 6.45 (d, 1H, *J* = 8.4 Hz, H_6), 6.69 (d, 1H, *J* = 9.8 Hz, H_{10}), 6.97 (s, 1H, 2,3-di-Cl-Ph-CH=C), 7.10 – 7.36 (m, 3H, Ar), 7.45 (d, 1H, *J* = 8.6 Hz, H_5).

11b *(endo-isomer)*

^{1}H-NMR ($CDCl_3$) δ [ppm]: 1.39 (s, 6H, 2-CH_3), 1.40 (s, 6H, 8-CH_3), 3.79 (s, 2H, 2,3-di-Cl-Ph-CH_2), 5.10 (s, 1H, 3-CH), 5.58 (d, 1H, *J* = 9.8 Hz, H_9), 6.28 (d, 1H, *J* = 8.4 Hz, H_6), 6.71 (d, 1H, *J* = 9.8 Hz, H_{10}), 6.80 (d, 1H, *J* = 8.4 Hz, H_5), 7.10 – 7.36 (m, 3H, Ar).

11a/11b *(exo-/endo-isomers)*

^{13}C-NMR ($CDCl_3$) δ [ppm]: 26.7 (q), 27.9 (q), 28.0 (q), 36.0 (t), 37.4 (t), 75.7 (s), 76.0 (s), 76.1 (s), 76.5 (s), 108.4 (d), 109.1 (d), 110.1 (s), 110.3 (s), 114.0 (s), 114.9 (s), 116.8 (d), 116.9 (d), 118.3 (d), 123.3 (d), 124.6 (d), 126.5 (d), 126.6 (d), 127.0 (d), 127.2 (d), 128.5 (d), 128.6 (d), 129.1 (d), 129.2 (d), 129.4 (d), 132.5 (s), 132.6 (s), 132.7 (s), 133.0 (s), 133.2 (s), 138.2 (s), 139.2 (s), 148.9 (s), 149.4 (s), 153.8 (s), 154.4 (s). IR (KBr) cm^{-1}: 2974 (vs), 1634 (s), 1576 (s), 1480 (s), 1450 (sh), 1368 (m), 1271 (m), 1213 (s), 1174 (s), 1150 (s), 1112 (vs), 1058 (s), 942 (w), 894 (w), 812 (w), 725 (m). MS m/z (%): 404 [M^+ + 4] (5), 403 [M^+ + 3] (8), 402 [M^+ + 2] (32), 401 [M^+ + 1] (13), 400 [M^+] (50), 386 (100), 380 (12), 223 (10), 184 (16), 141 (13), 99 (31), 61 (50). Anal. Calcd. for $C_{23}H_{22}Cl_2O_2$: C, 68.83; H, 5.53. Found: C, 68.76; H, 5.58.

*4-(2,4-di-Chloro-benzylidene)-2,2,8,8-tetramethyl-3,4-dihydro-2H,8H-pyrano[2,3-f]chroman (**12a**) and 4-(2,4-di-Chloro-benzyl)-2,2,8,8-tetramethyl- 2H,8H- pyrano[2,3-f]chromene (**12b**)*

A colorless oil (64%) as mixture of exo-and endo-isomers (56 : 44).

12a *(exo-isomer)*

^{1}H-NMR ($CDCl_3$) δ [ppm]: 1.28 (s, 6H, 2-CH_3), 1.48 (s, 6H, 8-CH_3), 2.52 (d, 2H, *J* = 1.3 Hz, 3-CH_2), 5.54 (d, 1H, *J* = 9.8 Hz, H_9), 6.45 (d, 1H, *J* = 8.4 Hz, H_6), 6.68 (d, 1H, *J* = 9.8 Hz, H_{10}), 6.92 (s, 1H, 2,4-di-Cl-Ph-CH=C), 7.08 – 7.42 (m, 3H, Ar), 7.43 (d, 1H, *J* = 8.4 Hz, H_5).

12b *(endo-isomer)*

^{1}H-NMR ($CDCl_3$) δ [ppm]: 1.37 (s, 6H, 2-CH_3), 1.41 (s, 6H, 8-CH_3), 3.71 (s, 2H, 2,4-di-Cl-Ph-CH_2), 5.08 (s, 1H, 3-CH), 5.58 (d, 1H, *J* = 9.8 Hz, H_9), 6.28 (d, 1H, *J* = 8.4 Hz, H_6), 6.70 (d, 1H, *J* = 9.8 Hz, H_{10}), 6.80 (d, 1H, *J* = 8.4 Hz, H_5), 7.08 – 7.42 (m, 3H, Ar).

12a/12b *(exo-/endo-isomers)*

^{13}C-NMR ($CDCl_3$) δ [ppm]: 27.2 (q), 27.9 (q), 28.0 (q), 34.5 (t), 37.4 (t), 75.6 (s), 76.1 (s), 76.5 (s), 108.4 (d), 109.2 (d), 110.2 (s), 110.4 (s), 114.0 (s), 114.9 (s), 116.8 (d), 116.9 (d), 117.2 (d), 123.2 (d), 124.6 (d), 126.4 (d), 126.5 (d), 127.2 (d), 127.9 (d), 128.2 (d), 128.5 (d), 129.0 (d), 129.1 (d), 129.3 (d), 131.3 (d), 131.7 (d), 132.7 (s), 132.8 (s), 134.5 (s), 134.9 (s), 135.2 (s), 135.4 (s), 135.9 (s), 136.5 (s), 148.8 (s), 149.3 (s), 153.8 (s), 154.4 (s). IR (KBr) [cm^{-1}]: 2971 (s), 2894 (m), 1629 (vs), 1571 (s), 1475 (s), 1430 (m), 1369 (s), 1272 (m), 1180 (s), 1108 (s), 1150 (s), 1059 (m), 942 (m), 894 (w), 813 (s), 721 (s). MS m/z [%]: 404 [M^+ + 4] (6), 403 [M^+ + 3] (10), 402 [M^+ + 2] (40), 401 [M^+ + 1] (16), 400 [M^+] (60), 386 (100), 382 (24), 240 (10), 224 (13), 169 (15), 161 (27), 150 (22), 142 (15), 79 (18). Anal. Calcd. for $C_{23}H_{22}Cl_2O_2$: C, 68.83; H, 5.53. Found: C, 68.73; H, 5.48.

*4-(4-Fluorobenzylidene)-2,2,8,8-tetramethyl-3,4-dihydro-2H,8H-pyrano[2,3-f] chroman (**13a**) and 4-(4-Fluoro benzyl)-2,2,8,8-tetramethyl-2H,8H-pyrano[2,3-f]chromene (**13b**)*

A colorless oil (70%) as mixture of exo- and endo-isomers (53 : 47).

13a *(exo-isomer)*

^{1}H-NMR ($CDCl_3$) δ [ppm]: 1.28 (s, 6H, 2-CH_3), 1.45 (s, 6H, 8-CH_3), 2.66 (d, 2H, *J* = 1.4 Hz, 3-CH_2), 5.55 (d, 1H, *J* = 9.9 Hz, H_9), 6.28 (d, 1H, *J* = 8.4 Hz, H_6), 6.70 (d, 1H, *J* = 9.9 Hz, H_{10}), 6.98 (s, 1H, 4-F-Ph-CH=C), 6.99 – 7.24 (m, 4H, Ar), 7.40 (d, 1H, *J* = 8.5 Hz, H_5).

13b *(endo-isomer)*

^{1}H-NMR ($CDCl_3$) δ [ppm]: 1.40 (s, 6H, 2-CH_3), 1.41 (s, 6H, 8-CH_3), 3.63 (s, 2H, 4-F-Ph-CH_2), 5.16 (s, 1H, 3-CH), 5.57 (d, 1H, *J* = 9.9 Hz, H_9), 6.42 (d, 1H, *J* = 8.4 Hz, H_6), 6.70 (d, 1H, *J* = 9.9 Hz, H_{10}), 6.85 (d, 1H, *J* = 8.5 Hz, H_5), 6.99 – 7.24 (m, 4H, Ar).

13a/13b *(exo-/endo-isomers)*

^{13}C-NMR ($CDCl_3$) δ [ppm]: 26.7 (q), 27.9 (q), 28.1 (q), 37.0 (t), 37.1 (t), 75.4 (s), 75.9 (s), 76.0 (s), 76.4 (s), 108.2 (d), 109.0 (d), 110.1 (s), 110.2 (s), 114.5 (s), 115.0 (d), 115.1 (d), 115.2 (d), 115.3 (d), 116.8 (d), 117.0 (d), 120.0 (d), 123.4 (d), 124.1 (d), 126.2 (d), 128.5 (d), 128.9 (d), 130.1 (d), 130.2 (d), 130.5 (s), 130.7 (d), 130.8 (d), 133.5 (s), 133.6 (s), 134.5 (s), 134.6 (s), 149.1 (s), 153.6 (s), 153.9 (s), 160.4 (s), 160.5 (s), 162.3 (s), 162.4 (s). IR (KBr) [cm^{-1}]: 2974 (s), 2930 (m), 1592 (vs), 1506 (vs), 1471 (s), 1366 (s), 1270 (m), 1215 (s), 1114 (s), 1054 (s), 818 (m). MS m/z [%]: 351 [M^+ + 1] (24), 350 [M^+] (40), 285 (25), 267 (100), 174 (10), 108 (28), 68 (41), 66 (57). Anal. Calcd. for $C_{23}H_{23}FO_2$: C, 78.83; H, 6.62. Found: C, 78.78; H, 6.68.

*4-(3-Methoxy-benzylidene)-2,2,8,8-tetramethyl-3,4-dihydro-2H,8H-pyrano[2,3-f]chroman (**14a**) and 4-(3-Methoxy-benzyl)-2,2,8,8-tetramethyl-2H,8H- pyrano[2,3-f]chromene (**14b**)*

A colorless oil (70%) as mixture of exo- and endo-isomers (54 : 46).

***14a** (exo-isomer)*

^{1}H-NMR ($CDCl_3$) δ [ppm]: 1.30 (s, 6H, 2-CH_3), 1.45 (s, 6H, 8-CH_3), 2.72 (d, 2H, *J* = 1.3 Hz, 3-CH_2), 3.85 (2s, 3H, 3-MeOPh), 5.57 (d, 1H, *J* = 10.0 Hz, H_9), 6.44 (d, 1H, *J* = 8.4 Hz, H_6), 6.71 (d, 1H, *J* = 10.0 Hz, H_{10}), 6.80–7.30 (m, 4H, Ar), 7.01 (s, 1H, 3-MeO-Ph-CH=C), 7.44 (d, 1H, *J* = 8.4 Hz, H_5).

***14b** (endo-isomer)*

^{1}H-NMR ($CDCl_3$) δ [ppm]: 1.42 (s, 6H, 2-CH_3), 1.43 (s, 6H, 8-CH_3), 3.66 (s, 2H, 3-MeO-Ph-CH_2), 3.79 (s, 3H, 3-MeOPh), 5.23 (s, 1H, 3-CH), 5.58 (d, 1H, *J* = 10.0 Hz, H_9), 6.29 (d, 1H, *J* = 8.4 Hz, H_6), 6.71 (d, 1H, *J* = 10.0 Hz, H_{10}), 6.80–7.30 (m, 4H, Ar), 6.90 (d, 1H, *J* = 8.4 Hz, H_5).

***14a/14b** (exo-/endo-isomers)*

^{13}C-NMR ($CDCl_3$) δ [ppm]: 26.8 (q), 27.9 (q), 28.1 (q), 37.3 (t), 37.9 (t), 55.1 (q), 55.2 (q), 75.5 (s), 76.0 (s), 76.1 (s), 76.4 (s), 108.2 (d), 109.0 (d), 110.1 (s), 110.2 (s), 111.5 (s), 111.6 (d), 114.5 (d), 114.6 (s), 115.1 (d), 115.4 (s), 116.9 (d), 117.1 (d), 120.8 (d), 120.9 (d), 121.2 (d), 122.0 (d), 123.6 (d), 124.2 (d), 126.3 (d), 128.5 (d), 128.9 (d), 129.1 (d), 129.3 (d), 129.4 (d), 130.5 (s), 130.9 (s), 139.1 (s), 140.7 (s), 143.4 (s), 148.9 (s), 149.2 (s), 153.5 (s), 153.9 (s), 159.4 (s), 159.7 (s). IR (KBr) [cm^{-1}]: 2981 (s), 2932 (s), 1648 (s), 1591 (vs), 1460 (s), 1370 (m), 1277 (s), 1220 (w), 1161 (s), 1108 (vs), 1054 (s), 777 (m), 727 (s). MS m/z [%]: 363 [M^+ + 1] (18), 362 [M^+] (63), 346 (100), 240 (14), 165 (16), 120 (54), 77 (14). Anal. Calcd. for $C_{24}H_{26}O_3$: C, 79.53; H, 7.23. Found: C, 79.50; H, 7.24.

*4-(4-Methoxy-benzylidene)-2,2,8,8-tetramethyl-3,4-dihydro-2H,8H-pyrano[2,3-f]chroman (**15a**) and 4-(4-Methoxy-benzyl)-2,2,8,8-tetramethyl-2H,8H- pyrano[2,3-f]chromene (**15b**)*

A colorless oil (72%) as mixture of exo- and endo-isomers (75 : 25).

***15a** (exo-isomer)*

^{1}H-NMR ($CDCl_3$) δ [ppm]: 1.30 (s, 6H, 2-CH_3), 1.46 (s, 6H, 8-CH_3), 2.72 (d, 2H, *J* = 1.5 Hz, 3-CH_2), 3.81 (2 s, 3H, 4-MeOPh), 5.58 (d, 1H, *J* = 10.0 Hz, H_9), 6.43 (d, 1H, *J* = 8.4 Hz, H_6), 6.72 (d, 1H, *J* = 10.0 Hz, H_{10}), 6.84 (d, 2H, *J* = 8.4 Hz, Ar), 6.99 (s, 1H, 4-MeO-Ph-CH=C), 7.12 (d, 2H, *J* = 8.4 Hz, Ar), 7.43 (d, 1H, *J*= 8.4 Hz, H_5).

***15b** (endo-isomer)*

^{1}H-NMR ($CDCl_3$) δ [ppm]: 1.41 (s, 6H, 2-CH_3), 1.43 (s, 6H, 8-CH_3), 3.68 (s, 2H, 4-MeO-Ph-CH_2), 3.85 (s, 3H, 4-MeOPh), 5.23 (s, 1H, 3-CH), 5.59 (d, 1H, *J* = 10.0 Hz, H_9), 6.29 (d, 1H, *J* = 8.4 Hz, H_6), 6.71 (d, 1H, *J* = 10.0 Hz, H_{10}), 6.93 (d, 2H, *J* = 8.5 Hz, Ar), 7.17 (d, 1H, *J* = 8.5 Hz, H_5), 7.25 (d, 2H, *J* = 8.5 Hz, Ar).

***15a/15b** (exo-/endo-isomers)*

^{13}C-NMR ($CDCl_3$) δ [ppm]: 26.7 (q), 27.9 (q), 28.1 (q), 36.9 (t), 37.3 (t), 55.2 (q), 55.3 (q), 75.4 (s), 76.0 (s), 76.5 (s), 108.2 (d), 109.0 (d), 110.1 (s), 113.64 (d), 113.7 (s), 113.8 (d), 114.9 (s), 116.9(d), 117.2 (d), 120.5 (d), 123.6 (d), 124.1 (d), 126.0 (d), 128.4 (d), 128.9 (d), 129.1 (d), 129.5 (d), 129.7 (d), 130.2 (s), 130.6 (s), 130.5 (s), 133.7 (s), 134.0 (s), 149.0 (s), 153.5 (s), 153.7 (s), 157.8 (s), 158.1 (s). IR (KBr) [cm^{-1}]: 2965 (m), 2931 (m), 2830 (m), 1607 (s), 1509 (vs), 1456 (s), 1370 (m), 1260 (vs), 1174 (s), 1116 (s), 1035 (s), 829 (s), 723 (w). MS m/z [%]: 363 [M^+ + 1] (23), 362 [M^+] (85), 348 (100), 243 (28), 164 (14), 120 (100), 75 (57). Anal. Calcd. for $C_{24}H_{26}O_3$: C, 79.53; H, 7.23. Found: C, 79.56; H, 7.25.

Biological activity

The strain of *L. major* used in this study was the vaccine strain (MRHO/IR/75/ER) obtained from Pasteur Institute, Tehran, Iran. The infectivity of the parasites was maintained by regular passage in susceptible BALB/C mice. The promastigote form of the parasite was grown in NNN-blood agar medium at 25°C. The stationary parasite inoculation was 2×10^6 cells/mL. For the experiments described here, the stationary-phase promastigotes were washed with phosphate-buffered saline (PBS) and recultured in RPMI 1640 medium, pH ~ 7.2, at 2×10^6 cells/mL density, supplemented with 10% of heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin) and 100 μg/mL streptomycin [3, 4].

Analysis of L. major growth kinetics

At first, the growth curve of the parasite was determined by daily counting, using a Neubauer's chamber and light microscopy. 2×10^6 cells/mL log-phase promastigotes were inoculated into 5 mL fresh RPMI 1640 medium in T25 culture flasks, besides, the current anti-leishmanial drug Glucantime was used as a reference. Assessment of growth by cell counting (Neubauer Chamber) was determined at 24-hours intervals for 8 days. The values obtained were used to determine the relative growth rate and the results were expressed as the mean values of at least three experiments.

In-vitro L. major promastigote culture and compound toxicity assays

The *in-vitro* growth inhibition of the chromenes was evaluated by direct counting under a light microscope. Briefly, promastigotes in the logarithmic growth phase were incubated at an average of 2×10^6 cells/mL in RPMI 1640 medium supplemented with 10% FBS as described above, in the presence of chromene compounds dissolved in dimethylsulphoxide (DMSO), and incubated for 24, 48, and 72 h at 25°C. Promastigotes in the control groups were incubated with DMSO alone or with the antimonial drug Glucantime. Finally, the number of promastigotes was recorded for at least three independent experiments and the results are expressed as the mean percentage reduction of parasite numbers compared with the untreated controls. The percentage of growth inhibition was calculated as (1 − (Cell number of drug-treated culture / Cell number of control culture)) × 100.

Growth curve of L. major

A general procedure was used in the current study to identify three different growth phases of promastigotes. The growth kinetics for a period of 8 days is shown in Fig. 3. The logarithmic

phase of the promastigotes lasted until day 3, thereafter, the parasites gradually entered the stationery phase. The maximum cell concentration of 1.1×10^7/mL was achieved after 96 hours. Besides, we assessed the growth curve of the promastigotes treated with Glucantime which showed gradually reduction during 5 days. The determinations of the growth inhibition achieved by the drug were made when the parasites were still in the log phase, prior to entering the stationary phase.

Statistical analysis

The Student *t* test, with significance at $P < 0.05$ was used to compare compound susceptibilities of the parasite at logarithmic phase.

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