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Antiparasitic activities of new lawsone Mannich bases

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Abstract

A series of new lawsone Mannich bases derived from salicylaldehydes or nitrofurfural were prepared and tested for their activities against *Leishmania major*, *Toxoplasma gondii*, and *Trypanosoma brucei brucei* parasites. The hydrochloride salts **5a** and **6a** of the Mannich bases **2a** and **3a**, derived from unsubstituted salicylaldehyde and long-chained alkyl amines, were selectively and strongly active against *T. gondii* cells and appear to be new promising drug candidates against this parasite. Compound **6a** showed an even higher activity against *T. gondii* than the known lawsone Mannich base **1b**. Compound **4a**, derived from salicylaldehyde and 2-methylaminopyridine, was also distinctly active against *T. gondii* cells. The derivatives **3a** (salicyl derivative), **3b** (3,5-dichloro-2-hydroxyphenyl derivative), and **3d** (5-nitrofuranyl derivative) as well as the hydrochlorides **6a** and **6b** were also efficacious against *T. b. brucei* cells with compounds **3a** and **3b** being more selective for *T. b. brucei* over Vero cells when compared with the known control compound **1b**. The derivatives **5a**, **5c**, **6a**, and **6c** proved to be up to five times more active than **1b** against *L. major* promastigotes and up to four times more efficacious against *L. major* amastigotes.

KEYWORDS

antiparasitic drugs, lawsone, Mannich base, neglected tropical diseases, salicyl derivatives

1 | INTRODUCTION

Parasitic diseases still pose an enormous world-wide medical challenge.[1] Neglected tropical diseases (NTDs) are particularly perilous both for locals and travelers in many tropical and subtropical countries.[2] Due to climate change, further regions of the world are likely to get affected by these parasitic diseases in the future.[2] New drugs against NTDs are few and far between because most pharma companies feel little inclination to develop more efficacious drugs for diseases mainly affecting people that cannot afford them.[3] Against this backdrop, it is gratifying that quite a few drug candidates for the treatment of various NTDs, derived from cheap and renewable natural products, have emerged lately.[3] Another aspect that needs to be addressed is the predisposition of immune-compromised people to infections such as *Toxoplasma gondii*.[4]

Lawsone (**1a**) is a natural 2-hydroxy-1,4-naphthoquinone readily available from the henna plant (*Lawsonia inermis*), which is applied for the management of skin diseases in South Asia as a component of the local Ayurveda and Unani traditional medicine (Figure 1).[5,6] Lawsone is a useful starting material for the preparation of various quinone derivatives with proven bioactivity such as lapachol or atovaquone.[1,2] The chemical modification of lawsone has led to compounds with activity against tumor cells and fungi.[7,8] Lawsone Mannich bases have shown particularly promising activities against cancer cells and parasites including *Trypanosoma brucei brucei* and *Entamoeba histolytica*.[9–13] We have previously found that longer alkyl chains attached to the naphthoquinone scaffold as in compound **1b** led to enhanced bioactivities (Figure 1).[13] Further, lawsone derivatives were reported to act against *T. gondii*, *Trypanosoma cruzi*, *Leishmania donovani*, and *Plasmodium falciparum* parasites.[14–18] In
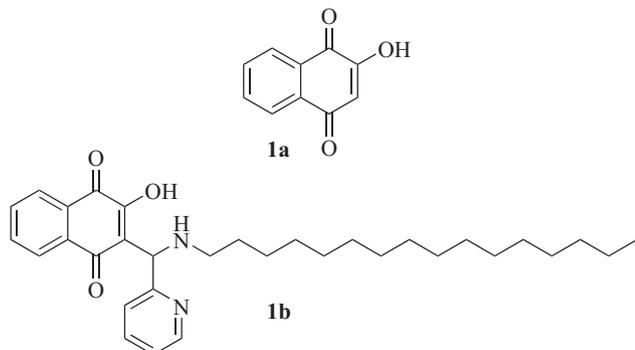
FIGURE 1 Structures of lawsone (**1a**) and its antiparasitic Mannich base **1b**

the current study, we prepared new lawsone Mannich bases with long-chain appendages and tested their activities against the cutaneous leishmaniasis causing parasite *L. major*, against the Nagana cattle-disease causing parasite *T. b. brucei* (both kinetoplastid parasites), and against toxoplasmosis causing *T. gondii* parasites (an apicomplexan parasite).
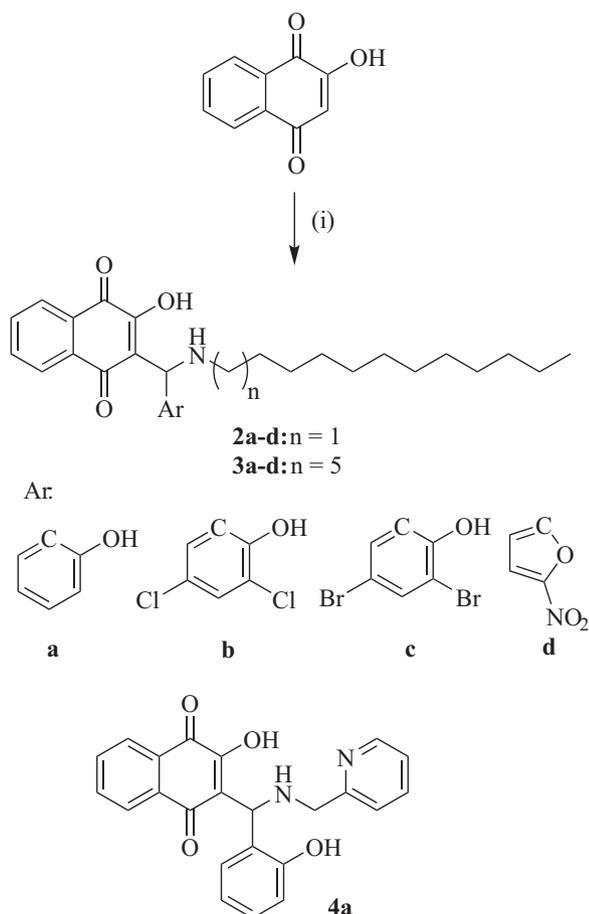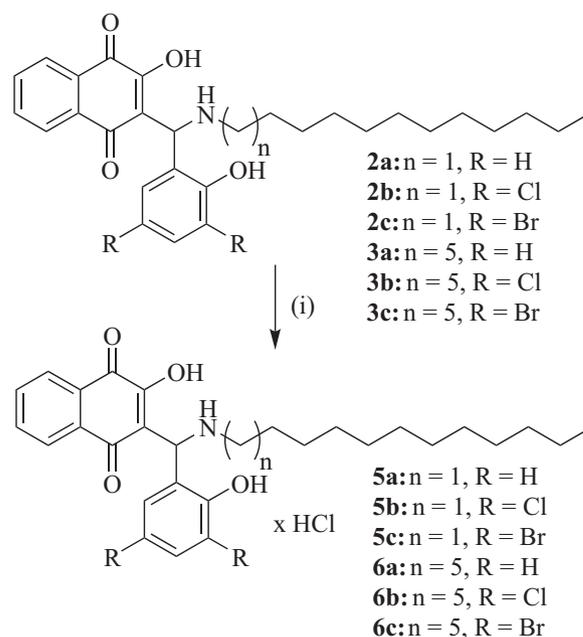
2 | RESULTS AND DISCUSSION

Compounds **2a–d**, **3a–d**, and **4a** were prepared in moderate yields by Mannich reactions of lawsone, the respective aryl aldehyde and the respective amine (Scheme 1).[19] They were obtained as racemic orange to orange-red solids. Compounds **2a–c** and **3a–c** were converted to the hydrochloride salts **5a–c** and **6a–c** by reaction with acetyl chloride in ethyl alcohol (EtOH; Scheme 2).

The new lawsone Mannich bases were tested on three protozoal parasites including *T. gondii* against which the related lawsone derivative atovaquone had already shown reasonable activity.[20] Our hydrochlorides **5a** and **6a** proved highly active against *T. gondii* tachyzoites, surpassing even the effect of the known positive control **1b** (Table 1). Both **5a** and **6a** also displayed some selectivity for the *T. gondii* parasite when compared with nonmalignant Vero cells with selectivity index (SI) values of 2.38 for **5a** and 3.12 for **6a**. The new compounds **4a**, **5c**, and **6c** also exhibited good activities against *T. gondii* in the range of that of **1b** with some selectivity in the case of **5c** (SI = 1.91) and **6c** (SI = 3.38).

The new compounds were also tested for trypanocidal activity against the bloodstream-form *T. b. brucei* parasites using the Alamar Blue (AB) assay (Table 2). Compounds **3a**, **3b**, and **3d**, carrying a long hexadecyl chain, reached IC_{50} values in the low one-digit micromolar concentration range (IC_{50} = 1.17–1.39 µM) and so performed better than their dodecyl analogs **2a**, **2b**, and **2d**. It is possible that **3a**, **3b**, and **3d** share similar modes of action with the approved antileishmanial drug miltefosine, which is a hexadecylphosphocholine derivative.[21,22] Interestingly, **3a**, **3b**, and **3d** showed only a weak activity against *T. gondii* and Vero cells and the selectivity of **3a** and **3b** for *T. b. brucei* was distinctly higher when compared with that of the known **1b**.

SCHEME 1 Synthesis of compounds **2a–d**, **3a–d**, and **4a**. Reagents and conditions: (i) Aryl aldehyde, dodecylamine for **2a–d**, hexadecylamine for **3a–d**, 2-aminomethylpyridine for **4a**, EtOH, r.t., 30 min to 1 hr, 30–64%

SCHEME 2 Synthesis of compounds **5a–c** and **6a–c**. Reagents and conditions: (i) AcCl, EtOH, 50°C, 1 hr

TABLE 1 Inhibitory concentrations IC_{50} (μM) of test compounds **1b** (positive control), **2a–d**, **3a–d**, **4a**, **5a–c**, and **6a–c** when applied to cells of the Vero (African green monkey kidney epithelial) cell line and effective concentrations of EC_{50} when applied to cells of *Toxoplasma gondii*

Compound	EC_{50} (*T. gondii*)	IC_{50} (Vero)	SI (Vero/*T. gondii*)[a]
1b	4.50	17.4	3.86
2a	126.7	96.2	0.76
2b	15.8	164.1	10.4
2c	10.3	20.3	1.97
2d	40.0	38.3	0.96
3a	45.3	318.3	7.03
3b	51.4	251.5	4.89
3c	9.73	24.2	2.48
3d	64.1	31.7	0.49
4a	4.48	3.73	0.83
5a	3.60	8.56	2.38
5b	–	<0.1	–
5c	4.71	8.98	1.91
6a	1.56	4.86	3.12
6b	–	<0.1	–
6c	4.76	16.1	3.38

Note: Values are the means of at least three independent experiments (standard deviation ± 15%). They were derived from concentration–response curves obtained by measuring the percentage of vital cells relative to untreated controls after 72 hr.
[a]Selectivity index (SI; IC_{50}/EC_{50}) calculated from the corresponding IC_{50} values from the Vero cells and the EC_{50} values against *T. gondii*.

TABLE 2 Inhibitory concentrations IC_{50} (μM) of test compounds **1b** (positive control), **2a–d**, **3a–d**, **4a**, **5a–c**, and **6a–c** when applied to *Trypanosoma brucei brucei* cells[a]

Compound	IC_{50} (*T. b. brucei*)	SI (Vero/*T. b. brucei*)[b]
1b	0.30[c]	57.9
2a	3.19	30.2
2b	>10	–
2c	>10	–
2d	6.11	6.27
3a	1.39	229.0
3b	1.17	215.0
3c	>10	–
3d	1.26	25.1
4a	5.44	0.69
5a	3.25	2.63
5b	>10	–
5c	>10	–
6a	1.66	2.93
6b	0.96	–
6c	4.42	3.64

[a]Values are the means of at least three independent experiments (standard deviation ± 15%). They were derived from concentration–response curves obtained by measuring the percentage of vital cells relative to untreated controls after 72 hr.
[b]Selectivity index (SI) calculated from the corresponding IC_{50} values from the Vero cells (Table 1) and the IC_{50} values against *T. b. brucei*.
[c]Value is taken from Ahmed et al.[11]

Finally, the new compounds were tested against *L. major* promastigotes and amastigotes (Table 3). The hydrochlorides **5c**, **6a**, and **6c** were active against the promastigotes with IC_{50} values ranging from 5.04 to 6.54 μM, and against the amastigotes with IC_{50} values between 4.06 and 4.71 μM. The dibromo derivative **6c** showed SI values of 3.19 for promastigotes and 3.97 for amastigotes, each over Vero cells. The highest activity against the amastigotes was found for **5a** (IC_{50} = 3.62 μM), which showed a distinctly weaker activity against the promastigotes. With the exception of the dichloro derivatives **2b** and **3b**, all new compounds were more active against the amastigotes than the promastigotes. The combination of the lawsone pharmacophore with hydrophobic side-chains (as in atovaquone) generates amphiphilic conjugates with potentially enhanced cell membrane penetration to the effect of an increased cytotoxic activity. Such a hybrid molecule strategy can indeed lead to compounds with high activity against *L. major*.[23] Atovaquone, for instance, showed a reasonable activity against visceral leishmaniasis.[24] The observation of a higher activity of most of the new lawsone Mannich bases against *L. major* amastigotes compared to promastigotes supports earlier studies with similar drugs and drug candidates. The approved antileishmaniasis drug miltefosine proved toxic to *L. major* amastigotes without negatively affecting the host immune system.[25] Ketotifen and cromolyn sodium also performed better against *L. major* amastigotes than promastigotes.[26] Another study described the potent activity of the CM11 peptide against *L. major* amastigotes.[27]

3 | CONCLUSIONS

A series of new lawsone Mannich bases derived from (halo-)salicylaldehydes and long-chained alkyl amines (C_{12} and C_{16} chains) were prepared in reasonable yields and were tested against the pathogenic parasites *L. major*, *T. gondii*, and *T. b. brucei*. New compounds with inhibitory activities against *T. gondii* (**5a** and **6a**) exceeding that of the known 2-pyridyl lawsone Mannich base **1b** were identified. Compound **6a** is a particularly promising new drug candidate for the treatment of toxoplasmosis, warranting further in-depth tests. In addition, we observed a distinctly higher selectivity of compounds **3a** and **3b** for *T. b. brucei* cells when compared to the known Mannich base **1b**. Improved selectivity of **3a** and **3b** might lead to less severe side effects in future tests with *Trypanosoma* animal models. The most striking data were obtained from tests against *L. major* with distinctly improved activities of the new compounds **5a**, **5c**, **6a**, and **6c** when compared with the known compound **1b**. A topical application of these new lawsone Mannich

TABLE 3 Effective concentrations EC_{50} (μM) of test compounds **1b** (positive control), **2a–d**, **3a–d**, **4a**, **5a**, **5c**, **6a**, and **6c** when applied to promastigotes and amastigotes of *Leishmania major*[a]

Compound	EC_{50} (*L. major* promastigotes)	EC_{50} (*L. major* amastigotes)	SI (Vero/promastigotes)[b]	SI (Vero/amastigotes)[b]
1b	25.8	12.3	0.67	1.41
2a	101.8	61.7	0.95	1.56
2b	34.9	58.2	4.70	2.82
2c	13.2	11.6	1.54	1.75
2d	58.0	34.6	0.66	1.11
3a	>192.4	32.3	–	9.86
3b	29.9	69.7	8.41	3.61
3c	15.4	7.82	1.57	3.10
3d	60.5	23.0	0.52	1.38
4a	9.83	5.43	0.38	0.69
5a	10.2	3.62	0.84	2.37
5b	>200	–	–	–
5c	6.54	4.71	1.37	1.91
6a	5.57	4.16	0.87	1.17
6b	>200	–	–	–
6c	5.04	4.06	3.19	3.97

[a]Values are the means of at least three independent experiments (standard deviation ± 15%). They were derived from concentration–response curves obtained by measuring the percentage of vital cells relative to untreated controls after 72 hr.
[b]Selectivity index (SI; IC_{50}/EC_{50}) calculated from the corresponding IC_{50} values from the Vero cells (Table 1) and the EC_{50} values against *L. major*.

bases for the in vivo treatment of cutaneous leishmaniasis caused by *L. major* parasites appears especially promising.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General

All starting compounds were purchased from Aldrich. The known compound **1b** was prepared according to a literature procedure.[13] The following instruments were used: melting points (uncorrected), Gallenkamp; IR spectra, Perkin–Elmer Spectrum One FT-IR spectrophotometer with attenuated total reflection (ATR) sampling unit; nuclear magnetic resonance (NMR) spectra, Bruker Avance 300 spectrometer; chemical shifts are given in parts per million (δ) downfield from tetramethylsilane as internal standard; mass spectra, Varian MAT 311 A (EI), UPLC/Orbitrap (ESI); microanalyses, Perkin–Elmer 2400 CHN elemental analyzer. All tested compounds are >95% pure by elemental analysis.

The compound codes together with some biological activity data are provided as Supporting Information.

4.1.2 | Compound characterization

3-[(Dodecylamino)(2-hydroxyphenyl)methyl]-2-hydroxy-1,4-naphthoquinone (2a)
2-Hydroxy-1,4-naphthoquinone (435 mg, 2.5 mmol) was suspended in EtOH (15 ml), dodecylamine (510 mg, 2.75 mmol) was added and the resulting solution was stirred at room temperature for 5 min. Salicylaldehyde (314 μl, 3.0 mmol) was added and the reaction mixture was stirred at room temperature for 1 hr. The formed precipitate was collected, washed with EtOH and dried in vacuum. Yield: 740 mg (1.60 mmol, 64%); orange-red solid of mp 201°C; ν_{max} (ATR)/cm^{-1} 3,068, 2,923, 2,852, 2,732, 2,606, 1,679, 1,633, 1,590, 1,515, 1,456, 1,365, 1,274, 1,226, 1,154, 1,100, 1,042, 973, 897, 849, 829, 808, 793, 757, 737, 720, 695, 666, and 627; ^{1}H NMR (300 MHz, CDCl$_3$); δ 0.84 (3H, t, *J* = 6.7 Hz), 1.1–1.3 (16H, m), 1.3–1.4 (2H, m), 1.7–1.9 (2H, m), 3.0–3.1 (2H, m), 5.87 (1H, s), 6.6–6.8 (2H, m), 6.9–7.0 (1H, m), 7.3–7.4 (2H, m), 7.4–7.5 (1H, m), 7.6–7.7 (1H, m), 7.8–7.9 (1H, m), 9.6–10.0 (1H, br s); ^{13}C NMR (75.5 MHz, CDCl$_3$); δ 14.1, 22.7, 26.6, 29.0, 29.3, 29.4, 29.5, 29.6, 31.9, 46.8, 55.4, 59.5, 112.7, 117.1, 118.3, 120.2, 123.1, 125.6, 126.2, 127.2, 129.5, 130.9, 131.5, 132.0, 133.9, 154.8, 164.4, 170.1, 183.1, and 184.5; *m/z* (ESI, %) 465.4 (53) [M$^+$], 464.3 (100) [M$^+$], 279.0 (53), 261.0 (98), and 186.2 (98). Anal calcd. C$_{29}$H$_{37}$NO$_4$: C, 75.13; H, 8.04; N, 3.02; Found: C, 75.03; H, 7.98; N, 2.95%.

3-[(Dodecylamino)(3,5-dichloro-2-hydroxyphenyl)methyl]-2-hydroxy-1,4-naphthoquinone (2b)
2-Hydroxy-1,4-naphthoquinone (435 mg, 2.5 mmol) and dodecylamine (510 mg, 2.75 mmol) were dissolved in hot EtOH (10 ml) and the resulting solution was stirred for 5 min. 3,5-Dichloro-2-hydroxybenzaldehyde (571 mg, 3.0 mmol) was added and the reaction mixture was slowly cooled down and stirred at room temperature for 30 min. The formed precipitate was collected, washed with EtOH and dried in vacuum. Yield: 822 mg (1.54 mmol, 62%); orange solid of mp

188–189°C; ^{1}H NMR (300 MHz, CDCl$_3$/dimethyl sulfoxide [DMSO]-d_6); δ 0.85 (3H, t, J = 6.5 Hz), 1.0–1.2 (18H, m), 2.5–2.7 (2H, m), 2.7–2.9 (2H, m), 5.58 (1H, s), 7.05 (1H, s), 7.27 (1H, s), 7.3–7.5 (2H, m), and 7.7–7.9 (2H, m); ^{13}C NMR (75.5 MHz, CDCl$_3$/DMSO-d_6); δ 13.7, 22.3, 26.1, 28.6, 28.9, 29.1, 29.2, 31.5, 47.0, 55.0, 110.3, 123.7, 124.0, 125.1, 125.3, 126.1, 127.4, 128.8, 131.0, 131.3, 132.1, 133.6, 149.7, 171.2, 181.9, and 183.5; m/z (%) 357 (22), 346 (100), 289 (64), 255 (52), 105 (98), 77 (42), and 41 (59). Anal calcd. C$_{29}$H$_{35}$Cl$_2$NO$_4$: C, 65.41; H, 6.62; N, 2.63; Found: C, 65.31; H, 6.55; N, 2.70%.

3-[(Dodecylamino)(3,5-dibromo-2-hydroxyphenyl)methyl]-2-hydroxy-1,4-naphthoquinone (2c)

2-Hydroxy-1,4-naphthoquinone (435 mg, 2.5 mmol) and dodecylamine (510 mg, 2.75 mmol) were dissolved in hot EtOH (10 ml) and the resulting solution was stirred for 5 min. 3,5-Dibromo-2-hydroxybenz-aldehyde (840 mg, 3.0 mmol) was added and the reaction mixture was slowly cooled down and stirred at room temperature for 1 hr. The formed precipitate was collected, washed with EtOH and dried in vacuum. Yield: 850 mg (1.37 mmol, 55%); orange solid of mp 212–214°C; ν_{max} (ATR)/cm^{-1} 3,177, 3,070, 2,922, 282, 2,551, 1,679, 1,588, 1,497, 1,466, 1,417, 1,372, 1,322, 1,271, 1,230, 1,149, 1,092, 1,052, 988, 917, 881, 864, 840, 821, 795, 746, 733, 712, 691, 665, 629, and 603; ^{1}H NMR (300 MHz, CDCl$_3$); δ 0.8–0.9 (3H, m), 1.1–1.3 (16H, m), 1.4–1.5 (2H, m), 1.7–1.9 (2H, m), 3.0–3.1 (2H, m), 5.87 (1H, s), 7.3–7.5 (2H, m), 7.5–7.6 (2H, m), 7.7–7.8 (1H, m), and 7.9–8.0 (1H, m); ^{13}C NMR (75.5 MHz, CDCl$_3$); δ 14.1, 22.7, 26.4, 26.7, 29.0, 29.3, 29.5, 29.6, 31.9, 47.2, 55.5, 111.9, 125.9, 126.6, 129.1, 130.7, 131.9, 132.6, 133.7, 134.4, 135.0, 138.0, 151.2, 162.9, 170.1, 178.1, and 179.8; m/z (%) 584 (14), 419 (23), 174 (25), 105 (33), and 44 (100). Anal calcd. C$_{29}$H$_{35}$Br$_2$NO$_4$: C, 56.05; H, 5.68; N, 2.25; Found: C, 56.11; H, 5.60; N, 2.22%.

3-[(Dodecylamino)(5-nitrofuran-2-yl)methyl]-2-hydroxy-1,4-naphthoquinone (2d)

2-Hydroxy-1,4-naphthoquinone (435 mg, 2.5 mmol) and dodecyla-mine (510 mg, 2.75 mmol) were dissolved in hot EtOH (10 ml) and the resulting solution was stirred for 5 min. 5-Nitrofuranyl-2-carboxal-dehyde (423 mg, 3.0 mmol) was added and the reaction mixture was slowly cooled down and stirred at room temperature for 30 min. The formed precipitate was collected, washed with EtOH and dried in vacuum. Yield: 371 mg (0.77 mmol, 31%); orange-red solid of mp 168–169°C; ν_{max} (ATR)/cm^{-1} 2,923, 2,853, 1,675, 1,623, 1,589, 1,527, 1,495, 1,470, 1,370, 1,353, 1,321, 1,158, 1,017, 969, 873, 810, 774, 733, 719, 693, and 663; ^{1}H NMR (300 MHz, DMSO-d_6); δ 0.85 (3H, t, J = 6.5 Hz), 1.1–1.3 (18H, m), 1.5–1.7 (2H, m), 2.8–2.9 (2H, m), 5.73 (1H, s), 6.91 (1H, d, J = 3.8 Hz), 7.6–8.0 (5H, m), 9.1–9.3 (1H, br s); ^{13}C NMR (75.5 MHz, DMSO-d_6); δ 13.9, 22.0, 25.2, 25.7, 26.7, 28.3, 28.4, 28.6, 28.7, 28.8, 28.9, 30.0, 31.2, 45.2, 51.4, 60.8, 106.6, 112.8, 114.0, 114.2, 114.8, 115.6, 118.9, 125.3, 125.5, 125.7, 130.9, 132.1, 132.9, 133.8, 133.9, 134.6, 149.5, 152.5, 160.8, 164.7, 171.2, 178.2, and 183.9; m/z (%) 291 (4), 262 (15), 209 (9), 195 (100), 154 (30), 108 (15), 79 (30), 55 (34), and 41 (46). Anal calcd.

C$_{27}$H$_{34}$N$_2$O$_6$: C, 67.20; H, 7.10; N, 5.81; Found: C, 67.03; H, 7.01; N, 5.72%.

3-[(Hexadecylamino)(2-hydroxyphenyl)methyl]-2-hydroxy-1,4-naphthoquinone (3a)

2-Hydroxy-1,4-naphthoquinone (435 mg, 2.5 mmol) was suspended in EtOH (15 ml), dodecylamine (664 mg, 2.75 mmol) was added and the resulting solution was stirred at room temperature for 5 min. Salicylaldehyde (314 µl, 3.0 mmol) was added and the reaction mixture was stirred at room temperature for 1 hr. The formed precipitate was collected, washed with EtOH and dried in vacuum. Yield: 636 mg (1.22 mmol, 49%); orange-red solid of mp 208–209°C; ν_{max} (ATR)/cm^{-1} 3,069, 2,922, 2,851, 2,738, 1,679, 1,590, 1,516, 1,456, 1,363, 1,274, 1,233, 1,155, 1,099, 1,041, 973, 883, 849, 828, 793, 756, 737, 720, 695, 666, and 626; ^{1}H NMR (300 MHz, CDCl$_3$); δ 0.85 (3H, t, J = 6.7 Hz), 1.0–1.2 (24H, m), 1.3–1.4 (2H, m), 1.8–1.9 (2H, m), 3.0–3.1 (2H, m), 5.86 (1H, s), 6.6–6.8 (2H, m), 6.9–7.0 (1H, m), 7.3–7.4 (2H, m), 7.4–7.5 (1H, m), 7.6–7.7 (1H, m), and 7.8–7.9 (1H, m); ^{13}C NMR (75.5 MHz, CDCl$_3$); δ 14.0, 22.7, 26.5, 29.0, 29.3, 29.5, 29.6, 29.7, 31.9, 46.7, 55.4, 112.8, 118.5, 120.3, 123.2, 125.6, 126.2, 127.2, 129.5, 130.8, 131.5, 133.9, 134.0, 154.8, 170.1, 183.0, and 184.5; m/z (ESI, %) 521.5 (35) [M$^+$], 520.4 (93) [M$^+$], 261.0 (58), and 242.3 (100). Anal calcd. C$_{33}$H$_{45}$NO$_4$: C, 76.26; H, 8.73; N, 2.70; Found: C, 76.13; H, 8.66; N, 2.74%.

3-[(Hexadecylamino)(3,5-dichloro-2-hydroxyphenyl)methyl]-2-hydroxy-1,4-naphthoquinone (3b)

2-Hydroxy-1,4-naphthoquinone (435 mg, 2.5 mmol) and hexadecyla-mine (664 mg, 2.75 mmol) were dissolved in hot EtOH (10 ml) and the resulting solution was stirred for 5 min. 3,5-Dichloro-2-hydroxybenz-aldehyde (571 mg, 3.0 mmol) was added and the reaction mixture was slowly cooled down and stirred at room temperature for 1 hr. The formed precipitate was collected, washed with EtOH and dried in vacuum. Yield: 812 mg (1.38 mmol, 55%); orange-red solid of mp 181–182°C; ν_{max} (ATR)/cm^{-1} 2,922, 2,852, 1,677, 1,590, 1,506, 1,467, 1,368, 1,274, 1,234, 1,168, 1,095, 1,055, 992, 864, 826, 735, 719, 699, 666, and 611; ^{1}H NMR (300 MHz, CDCl$_3$); δ 0.85 (3H, t, J = 6.5 Hz), 1.0–1.3 (26H, m), 1.7–1.9 (2H, m), 3.0–3.2 (2H, m), 5.91 (1H, s), 7.0–7.1 (1H, m), 7.3–7.8 (4H, m), 7.9–8.0 (1H, m), and 9.7–10.0 (1H, br s); ^{13}C NMR (75.5 MHz, CDCl$_3$); δ 14.6, 22.7, 26.4, 26.6, 28.9, 29.3, 29.5, 29.7, 31.9, 47.1, 55.3, 111.2, 124.7, 125.9, 126.5, 128.8, 129.5, 130.7, 131.8, 132.5, 133.7, 134.4, 149.7, 163.0, 170.1, 183.2, and 184.4; m/z (%) 413 (72), 244 (76), 174 (93), 105 (100), 55 (51), and 43 (73). Anal calcd. C$_{33}$H$_{43}$Cl$_2$NO$_4$: C, 67.34; H, 7.36; N, 2.38; Found: C, 67.22; H, 7.28; N, 2.30%.

3-[(Hexadecylamino)(3,5-dibromo-2-hydroxyphenyl)methyl]-2-hydroxy-1,4-naphthoquinone (3c)

2-Hydroxy-1,4-naphthoquinone (435 mg, 2.5 mmol) and hexadecyla-mine (664 mg, 2.75 mmol) were dissolved in hot EtOH (10 ml) and the resulting solution was stirred for 5 min. 3,5-Dibromo-2-hydroxybenz-aldehyde (840 mg, 3.0 mmol) was added and the reaction mixture was slowly cooled down and stirred at room temperature for 1 hr.

The formed precipitate was collected, washed with EtOH and dried in vacuum. Yield: 837 mg (1.24 mmol, 50%); orange solid of mp 202–203°C; ν_{max} (ATR)/cm^{-1} 3,039, 2,921, 2,852, 2,551, 1,676, 1,589, 1,506, 1,466, 1,435, 1,417, 1,367, 1,303, 1,272, 1,234, 1,149, 1,093, 1,054, 991, 916, 864, 837, 823, 796, 748, 735, 713, 690, 666, 656, 629, and 600; ^{1}H NMR (300 MHz, CDCl$_3$); δ 0.8–0.9 (3H, m), 1.1–1.5 (24H, m), 1.7–1.9 (2H, m), 3.0–3.2 (2H, m), 5.85 (1H, s), and 7.4–8.1 (6H, m); ^{13}C NMR (75.5 MHz, DMSO-d_6); δ 13.9, 22.0, 25.3, 26.0, 28.3, 28.4, 28.6, 28.8, 28.9, 31.2, 46.2, 53.7, 103.7, 110.2, 110.6, 110.8, 112.5, 117.1, 125.3, 125.7, 125.9, 128.5, 129.0, 131.0, 131.2, 131.6, 133.1, 133.6, 133.9, 134.0, 138.0, 151.5, 165.2, 171.4, 180.2, and 183.2; m/z (%) 639 (26), 503 (27), 436 (87), 420 (33), 334 (47), 105 (100), 55 (43), and 44 (68). Anal calcd. C$_{33}$H$_{43}$Br$_2$NO$_4$: C, 58.50; H, 6.40; N, 2.07; Found: C, 58.43; H, 6.37; N, 2.13%.

3-[(Hexadecylamino)(5-nitrofuran-2-yl)methyl]-2-hydroxy-1,4-naphthoquinone (3d)

2-Hydroxy-1,4-naphthoquinone (435 mg, 2.5 mmol), and hexadecylamine (664 mg, 2.75 mmol) were dissolved in hot EtOH (10 ml) and the resulting solution was stirred for 5 min. 5-Nitrofuranyl-2-carboxaldehyde (423 mg, 3.0 mmol) was added and the reaction mixture was slowly cooled down and stirred at room temperature for 30 min. The formed precipitate was collected, washed with EtOH and dried in vacuum. Yield: 400 mg (0.74 mmol, 30%); orange-red solid of mp 152–154°C; ν_{max} (ATR)/cm^{-1} 2,921, 2,852, 1,676, 1,621, 1,589, 1,528, 1,496, 1,469, 1,371, 1,353, 1,320, 1,273, 1,241, 1,158, 1,017, 968, 873, 828, 810, 775, 734, 720, 693, 680, and 662; ^{1}H NMR (300 MHz, DMSO-d_6); δ 0.85 (3H, t, J = 6.5 Hz), 1.1–1.3 (26H, m), 1.5–1.7 (2H, m), 2.8–2.9 (2H, m), 5.73 (1H, s), 6.91 (1H, d, J = 4.4 Hz), 7.6–8.0 (5H, m), and 9.2–9.4 (1H, br s); ^{13}C NMR (75.5 MHz, DMSO-d_6); δ 13.9, 22.1, 25.2, 25.8, 28.4, 28.5, 28.7, 28.9, 31.3, 45.2, 51.4, 106.6, 112.8, 114.2, 115.6, 125.2, 125.3, 125.6, 125.8, 130.9, 131.6, 132.1, 133.8, 133.9, 134.6, 155.0, 171.2, 178.2, and 183.9; m/z (%) 347 (8), 318 (23), 195 (100), and 154 (17). Anal calcd. C$_{31}$H$_{42}$N$_2$O$_6$: C, 69.12; H, 7.86; N, 5.20; Found: C, 68.99; H, 7.79; N, 5.14%.

3-[Pyridine-2-methylamino)(2-hydroxyphenyl)methyl]-2-hydroxy-1,4-naphthoquinone (4a)

2-Hydroxy-1,4-naphthoquinone (435 mg, 2.5 mmol) was suspended in EtOH (10 ml), 2-aminomethylpyridine (281 µl, 2.75 mmol) was added and the resulting solution was stirred at room temperature for 5 min. Salicylaldehyde (314 µl, 3.0 mmol) was added and the reaction mixture was stirred at room temperature for 2 hr. The formed precipitate was collected, washed with EtOH and dried in vacuum. Yield: 412 mg (1.07 mmol, 43%); orange solid of mp 187–189°C; ν_{max} (ATR)/cm^{-1} 3,057, 2,769, 2,611, 1,672, 1,608, 1,590, 1,537, 1,475, 1,455, 1,356, 1,335, 1,273, 1,253, 1,239, 1,220, 1,190, 1,154, 1,097, 1,035, 997, 959, 937, 904, 870, 826, 797, 749, 735, 719, 697, 679, 662, 636, and 612; ^{1}H NMR (300 MHz, DMSO-d_6); δ 4.1–4.3 (2H, m), 5.84 (1H, s), 6.7–6.9 (2H, m), 7.1–7.2 (1H, m), 7.3–7.5 (3H, m), 7.6–7.7 (1H, m), 7.7–8.0 (5H, m), 8.5–8.6 (1H, m), and 9.6–10.0 (2H, br s); ^{13}C NMR (75.5 MHz, DMSO-d_6); δ 42.7, 49.7, 54.3, 63.8, 92.4, 110.2, 115.9, 116.5, 117.0, 118.6, 119.0, 122.5, 122.7, 123.1, 123.3,

125.0, 125.8, 126.9, 127.5, 128.6, 129.3, 130.2, 130.9, 131.6, 132.9, 133.1, 133.7, 134.8, 137.0, 137.2, 148.9, 155.5, 167.4, 171.4, 179.3, and 184.0; m/z (ESI, %) 387.2 (95) [M$^+$], 372.2 (38), 264.1 (100), and 109.1 (98). Anal calcd. C$_{23}$H$_{18}$N$_2$O$_4$: C, 71.49; H, 4.70; N, 7.25; Found: C, 71.20; H, 4.63; N, 7.08%.

3-[(Dodecylamino)(2-hydroxyphenyl)methyl]-2-hydroxy-1,4-naphtho-quinone × HCl (5a)

2a (231 mg, 0.5 mmol) was dissolved in ethanol (20 ml) upon heating and treated with acetyl chloride (53 µl, 0.75 mmol). The resulting yellow solution was stirred at 50°C for 1 hr. The solvent was evaporated and the yellow solid residue was dried in vacuum. Yield: 250 mg (0.5 mmol, 100%); yellow-orange solid of mp 144–146°C; ν_{max} (ATR)/cm^{-1} 3,071, 2,922, 2,853, 2,729, 1,683, 1,640, 1,593, 1,506, 1,457, 1,362, 1,268, 1,218, 1,157, 1,093, 1,046, 953, 881, 796, 755, 722, 696, and 658; ^{1}H NMR (300 MHz, CDCl$_3$); δ 0.84 (3H, t, J = 6.7 Hz), 1.0–1.2 (18H, m), 1.7–1.9 (2H, m), 2.9–3.1 (2H, m), 6.00 (1H, s), 6.7–6.8 (1H, m), 7.0–7.1 (1H, m), 7.2–7.4 (2H, m), 7.5–7.6 (1H, m), 7.7–7.8 (1H, m), 7.8–7.9 (1H, m), 8.0–8.1 (1H, m), 8.6–8.7 (1H, br s), and 10.1–10.3 (1H, br s); ^{13}C NMR (75.5 MHz, CDCl$_3$); δ 14.1, 22.7, 26.3, 26.6, 29.0, 29.3, 29.5, 29.6, 31.9, 46.8, 54.6, 116.2, 117.7, 118.7, 120.5, 126.7, 127.0, 129.5, 130.9, 132.2, 133.5, 135.3, 155.3, 155.7, 180.2, and 185.1; m/z (%) 426 (57), 402 (20), 286 (19), 261 (100), and 247 (42). Anal calcd. C$_{29}$H$_{38}$ClNO$_4$: C, 69.65; H, 7.66; N, 2.80; Found: C, 69.60; H, 7.59; N, 2.75%.

3-[(Dodecylamino)(3,5-dichloro-2-hydroxyphenyl)methyl]-2-hydroxy-1,4-naphthoquinone × HCl (5b)

2b (266 mg, 0.5 mmol) was dissolved in ethanol (25 ml) upon heating and treated with acetyl chloride (53 µl, 0.75 mmol). The resulting yellow solution was stirred at 50°C for 1 hr. The solvent was evaporated and the yellow solid residue was dried in vacuum. Yield: 284 mg (0.5 mmol, 100%); yellow-orange solid of mp 151–153°C; ν_{max} (ATR)/cm^{-1} 2,921, 2,852, 1,679, 1,644, 1,590, 1,520, 1,466, 1,414, 1,365, 1,305, 1,275, 1,218, 1,160, 1,095, 1,046, 861, 794, 754, 725, 700, 665, 652, and 635; ^{1}H NMR (300 MHz, CDCl$_3$); δ 0.7–0.8 (3H, m), 1.1–1.3 (18H, m), 1.8–1.9 (2H, m), 3.0–3.1 (2H, m), 6.05 (1H, s), 7.19 (1H, s), 7.44 (1H, s), 7.5–7.7 (2H, m), 7.8–7.9 (1H, m), and 8.0–8.1 (1H, m); ^{13}C NMR (75.5 MHz, CDCl$_3$); δ 14.1, 22.6, 26.1, 26.6, 29.0, 29.3, 29.4, 29.5, 29.6, 31.9, 47.5, 53.9, 114.7, 124.2, 124.4, 126.0, 126.7, 126.9, 128.0, 130.4, 132.3, 133.4, 135.1, 149.4, 180.5, and 184.6; m/z (%) 495 (88), 355 (34), 330 (100), 239 (21), 174 (43), and 105 (43). Anal calcd. C$_{29}$H$_{36}$Cl$_3$NO$_4$: C, 61.22; H, 6.38; N, 2.46; Found: C, 61.13; H, 6.33; N, 2.42%.

3-[(Dodecylamino)(3,5-dibromo-2-hydroxyphenyl)methyl]-2-hydroxy-1,4-naphthoquinone × HCl (5c)

2c (310 mg, 0.5 mmol) was dissolved in ethanol (25 ml) upon heating and treated with acetyl chloride (53 µl, 0.75 mmol). The resulting yellow solution was stirred at 50°C for 1 hr. The solvent was evaporated and the yellow solid residue was dried in vacuum. Yield: 320 mg (0.49 mmol, 98%); yellow-orange solid of mp 115–117°C; ν_{max} (ATR)/cm^{-1} 2,922, 2,852, 1,679, 1,645, 1,590, 1,516, 1,459,

1,364, 1,275, 1,218, 1,143, 1,093, 1,046, 865, 794, 723, 689, 657, 625, 610, and 601; ^{1}H NMR (300 MHz, CDCl$_3$); δ 0.7–0.9 (3H, m), 1.1–1.3 (18H, m), 1.8–1.9 (2H, m), 3.0–3.1 (2H, m), 6.03 (1H, s), 7.47 (1H, s), 7.5–7.6 (2H, m), 7.6–7.7 (1H, m), 7.8–7.9 (1H, m), and 8.0–8.1 (1H, m); ^{13}C NMR (75.5 MHz, DMSO-d_6); δ 13.9, 22.0, 25.2, 25.8, 28.4, 28.5, 28.6, 28.7, 28.8, 28.9, 31.2, 46.2, 52.7, 110.7, 111.0, 111.9, 112.7, 125.4, 125.8, 128.0, 129.9, 130.9, 131.9, 132.3, 133.0, 133.2, 134.0, 134.3, 134.4, 138.2, 140.5, 151.3, 167.1, 181.4, 182.1, and 192.9; m/z (%) 600 (12), 584 (57), 517 (59), 444 (18), 419 (100), 333 (26), 174 (67), 105 (48), 43 (40), and 36 (11). Anal calcd. C$_{29}$H$_{36}$Br$_2$ClNO$_4$: C, 52.95; H, 5.52; N, 2.13; Found: C, 53.01; H, 5.60; N, 2.09%.

3-[(Hexadecylamino)(2-hydroxyphenyl)methyl]-2-hydroxy-1,4-naphthoquinone × HCl (6a)

3a (260 mg, 0.5 mmol) was dissolved in ethanol (20 ml) upon heating and treated with acetyl chloride (53 µl, 0.75 mmol). The resulting yellow solution was stirred at 50°C for 1 hr. The solvent was evaporated and the yellow solid residue was dried in vacuum. Yield: 278 mg (0.5 mmol, 100%); yellow-orange solid of mp 142–144°C; ν_{max} (ATR)/cm^{-1} 3,070, 2,922, 2,852, 1,678, 1,646, 1,594, 1,507, 1,457, 1,355, 1,274, 1,218, 1,158, 1,097, 1,044, 1,028, 947, 862, 837, 794, 753, 724, 696, and 655; ^{1}H NMR (300 MHz, CDCl$_3$); δ 0.85 (3H, t, J = 6.7 Hz), 1.0–1.3 (26H, m), 1.6–1.8 (2H, m), 2.9–3.1 (2H, m), 6.00 (1H, s), 6.7–6.8 (1H, m), 7.0–7.1 (1H, m), 7.2–7.4 (2H, m), 7.5–7.6 (1H, m), 7.7–7.8 (1H, m), 7.8–7.9 (1H, m), 8.0–8.1 (1H, m), 8.6–8.7 (1H, br s), and 10.2–10.3 (1H, br s); ^{13}C NMR (75.5 MHz, CDCl$_3$); δ 14.1, 22.7, 26.3, 26.7, 29.0, 29.3, 29.5, 29.6, 29.7, 31.9, 46.8, 54.7, 116.2, 117.7, 118.6, 120.5, 124.8, 126.5, 126.7, 127.0, 129.4, 129.6, 130.9, 132.2, 133.5, 135.3, 155.3, 180.2, and 185.1; m/z (%) 500 (25), 482 (100), 287 (18), 262 (97), 233 (26), and 206 (29). Anal calcd. C$_{33}$H$_{46}$ClNO$_4$: C, 71.26; H, 8.34; N, 2.52; Found: C, 71.16; H, 8.28; N, 2.48%.

3-[(Hexadecylamino)(3,5-dichloro-2-hydroxyphenyl)methyl]-2-hydroxy-1,4-naphthoquinone × HCl (6b)

3b (294 mg, 0.5 mmol) was dissolved in ethanol (25 ml) upon heating and treated with acetyl chloride (53 µl, 0.75 mmol). The resulting yellow solution was stirred at 50°C for 1 hr. The solvent was evaporated and the residue was recrystallized from CH$_2$Cl$_2$/n-hexane. Yield: 246 mg (0.39 mmol, 79%); yellow-orange solid of mp 171–173°C; ν_{max} (ATR)/cm^{-1} 2,918, 2,850, 1,683, 1,668, 1,615, 1,592, 1,574, 1,546, 1,519, 1,470, 1,440, 1,379, 1,362, 1,277, 1,245, 1,218, 1,173, 1,142, 1,046, 990, 937, 883, 860, 818, 756, 743, 732, 720, 702, 639, 624, and 611; ^{1}H NMR (300 MHz, CDCl$_3$); δ 0.85 (3H, t, J = 6.7 Hz), 1.1–1.3 (26H, m), 1.7–1.9 (2H, m), 2.9–3.1 (2H, m), 6.09 (1H, s), 7.20 (1H, s), 7.32 (1H, s), 7.6–7.8 (2H, m), 7.7–7.8 (1H, m), 8.0–8.1 (1H, m), and 8.2–8.3 (1H, m); ^{13}C NMR (75.5 MHz, CDCl$_3$); δ 14.1, 22.7, 26.1, 26.6, 26.7, 27.6, 29.0, 29.4, 29.5, 29.6, 29.7, 31.9, 40.2, 47.4, 53.6, 114.7, 117.7, 124.1, 124.3, 126.0, 126.7, 126.9, 127.9, 129.8, 130.4, 131.1, 132.3, 133.3, 133.4, 135.1, 135.3, 136.5, 149.4, 180.7, and 184.7; m/z (%) 500 (25), 482 (100), 287 (18), 262 (97), 233 (26), and 206 (29). m/z (%) 567 (6), 551 (100), 471 (8), 395

(17), 355 (30), 332 (26), 174 (34), 105 (32), and 43 (17). Anal calcd. C$_{33}$H$_{44}$Cl$_3$NO$_4$: C, 63.41; H, 7.10; N, 2.24; Found: C, 63.33; H, 7.05; N, 2.19%.

3-[(Hexadecylamino)(3,5-dibromo-2-hydroxyphenyl)methyl]-2-hydroxy-1,4-naphthoquinone × HCl (6c)

3c (338 mg, 0.5 mmol) was dissolved in ethanol (25 ml) upon heating and treated with acetyl chloride (53 µl, 0.75 mmol). The resulting yellow solution was stirred at 50°C for 1 hr. The solvent was evaporated and the residue was recrystallized from CH$_2$Cl$_2$/n-hexane. Yield: 318 mg (0.46 mmol, 92%); yellow-orange solid of mp 120–122°C; ν_{max} (ATR)/cm^{-1} 3,373, 3,192, 2,922, 2,851, 2,636, 1,685, 1,616, 1,589, 1,574, 1,544, 1,465, 1,440, 1,405, 1,378, 1,360, 1,275, 1,239, 1,219, 1,158, 1,133, 1,096, 1,042, 988, 922, 886, 860, 835, 817, 799, 751, 731, 705, 695, 637, and 624; ^{1}H NMR (300 MHz, CDCl$_3$); δ 0.85 (3H, t, J = 6.7 Hz), 1.1–1.3 (26H, m), 1.8–2.0 (2H, m), 3.0–3.1 (2H, m), 6.01 (1H, s), 7.55 (1H, s), 7.6–7.8 (3H, m), 7.9–8.0 (1H, m), and 8.0–8.1 (1H, m); ^{13}C NMR (75.5 MHz, CDCl$_3$); δ 14.1, 22.7, 26.1, 26.6, 29.0, 29.2, 29.4, 29.5, 29.6, 29.7, 31.9, 40.2, 47.5, 53.9, 113.7, 114.7, 115.0, 124.3, 126.5, 126.8, 127.1, 129.7, 131.8, 132.2, 133.1, 133.7, 135.2, 136.1, 142.0, 150.7, 157.1, 180.2, and 184.8; m/z (%) 640 (100), 444 (21), 419 (16), 174 (24), 105 (17), 57 (13), and 43 (19). Anal calcd. C$_{33}$H$_{44}$Br$_2$ClNO$_4$: C, 55.51; H, 6.21; N, 1.96; Found: C, 55.46; H, 6.13; N, 1.92%.

4.2 | Biological assays

4.2.1 | *Leishmania major* cell isolation, culture conditions, and assays

Promastigotes of *L. major* were isolated from a Saudi male patient in February 2016 and maintained at 26°C in Schneider's Drosophila medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) and antibiotics in a tissue culture flask with weekly transfers. Promastigotes were cryopreserved in liquid nitrogen at concentrations of 3×10^6 parasites/ml. The virulence of *L. major* parasites was maintained by passing in female BALB/c mice by injecting hind footpads with 1×10^6 stationary-phase promastigotes. After 8 weeks, *L. major* amastigotes were isolated from mice. Isolated amastigotes were transformed to promastigote forms by culturing at 26°C in Schneider's medium supplemented with 10% FBS and antibiotics. For infection, amastigote-derived promastigotes with less than five in vitro passages were used. Male and female BALB/c mice were obtained from Pharmaceutical College, King Saud University, Kingdom of Saudi Arabia, and maintained in specific pathogen-free facilities.

To evaluate the activity of test compounds against *L. major* promastigotes, promastigotes from logarithmic-phase cultured in phenol red-free Rosewell Park Memorial Institute (RPMI)-1640 medium (Invitrogen) with 10% FBS were suspended on 96-wells plates to yield 10^6 cells/ml (200 µl/well) after hemocytometer counting. Compounds were added to obtain the final concentrations (200, 40, 8, 1.6, etc. µg/ml). Negative control wells containing cultures with DMSO (1%) and without compound and positive

control wells containing cultures with decreasing concentration of amphotericin B (reference compound, 200, 40, 8, 1.6, etc. µg/ml) were used. Plates were incubated at 26°C for 24, 48, and 72 hr to evaluate the antiproliferative effect. The number of viable promastigotes were assessed by the colorimetric method using tetrazolium dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, MTT). It measures the reduction of the MTT component into an insoluble formazan product. This colored product was solubilized by adding detergent solution to lyse the cells. The samples were analyzed by using an enzyme-linked immunosorbent assay reader at 570 nm. Obtained EC_{50} values resulted from three independent experiments.

To evaluate the activity of test compounds against amastigotes in macrophages, peritoneal macrophages from female BALB/c (6–8 weeks of age) were collected by aspiration, then 5×10^4 cells/well were seeded on 96-wells plates in phenol red-free RPMI-1640 medium with 10% FBS, for 4 hr at 37°C in 4% CO_2 atmosphere to promote cell adhesion. The medium was discarded and washed with phosphate-buffered saline (PBS). Two hundred microliters of solution containing *L. major* promastigotes (at the ratio of 10 promastigotes/1 macrophage in phenol red-free RPMI-1640 medium with 10% FBS) was added per well. Plates were incubated for 24 hr at 37°C in humidified 5% CO_2 atmosphere to allow infection and amastigote differentiation. Then, the infected macrophages were washed three times with PBS to remove the free promastigotes and overlaid with fresh phenol red-free RPMI-1640 medium containing compounds at final concentrations (200, 40, 8, 1.6, etc. µg/ml) were added and cells were incubated at 37°C in humidified 5% CO_2 atmosphere for 72 hr. Negative control containing cultures with DMSO (1%) and without compounds and positive control wells containing cultures with decreasing concentration of amphotericin B (reference compound, 200, 40, 8, 1.6, etc. µg/ml) were used. The percentage of infected macrophages was evaluated microscopically after removing medium, washing, fixation, and Giemsa staining. Obtained EC_{50} values resulted from three independent experiments.

4.2.2 | *T. gondii* cell line, culture conditions, and assay

Serial passages of the Vero cell line (ATCC®, CCL81™) were used for the cultivation of *T. gondii* tachyzoites of the RH strain (a gift from Dr. Saeed El-Ashram, State Key Laboratory for Agrobiotechnology, China Agricultural University, Beijing, China). Vero cells were cultured by using complete RPMI-1640 medium with heat-inactivated 10% FBS in a humidified 5% CO_2 atmosphere at 37°C. 96-Well plates (5×10^3 cells/well in 200 µl RPMI-1640 medium with 10% FBS) were used for the cultivation of the Vero cells and then incubated at 37°C and 5% CO_2 for 1 day, followed by removal of medium and washing the cells with PBS. Then, RPMI-1640 medium with 2% FBS containing tachyzoites (RH strain) of *T. gondii* at a ratio of 5:1 (parasite/Vero cells) was added. After incubation at 37°C and 5% CO_2 for 6 hr, the cells were washed with PBS and then treated as described below.

Control: RPMI-1640 medium only. Experimental: Medium + compounds (dissolved in DMSO; 50 µg/ml, 25, 12.5, etc.). After incubation at 37°C and 5% CO_2 for 72 hr, the cells were stained with 1% toluidine blue after washing with PBS and fixation in 10% formalin. The cells were examined under an inverted photomicroscope to determine the infection index (number of cells infected from 200 cells tested) of *T. gondii*. The following equation was used for the calculation of the observed inhibition (in %):

$$\text{Inhibition } (\%) = (I_{\text{Control}} - I_{\text{Experimental}})/(I_{\text{Control}}) \times 100,$$

where I_{Control} refers to the infection index of untreated cells and $I_{\text{Experimental}}$ refers to the infection index of cells treated with test compounds.

Then, effects of test compounds on parasite growth were expressed as EC_{50} (effective concentration at 50%) values.

4.2.3 | *Trypanosoma* cell line and culture conditions

T. b. brucei bloodstream-form cell strain Lister 427 was maintained in HMI-9 medium, pH 7.5, supplemented with 10% FBS in a humidified 5% CO_2 atmosphere at 37°C.[28]

4.2.4 | Alamar blue (AB) assay

The AB assay was used to identify viable cells after treatment with drug candidates.[29–32] This assay is based on the irreversible reaction of the blue dye resazurin and NADH to pink resofurin in intact cells. *T. b. brucei* cells (8,000/well) were seeded on 96-well microplates, treated with the test compounds (dissolved in DMSO) and incubated for 72 hr (5% CO_2, 95% humidity, 37°C). Ten microliters of the AB reagent (500 µM resazurin sodium salt in PBS) was added and incubated for further 4 hr at 37°C. The fluorescence (extinction at 544 nm, emission at 590 nm) was measured on an Omega Fluostar (BMG Labtech) fluorescence plate reader.

4.2.5 | In vitro cytotoxicity assay

Tetrazolium salt colorimetric assay (MTT) was carried out for cytotoxicity evaluation of compounds. Briefly, Vero cells were cultured in 96-well plates (5×10^3 cells/well per 200 µl) for 24 hr in RPMI-1640 medium with 10% FBS and 5% CO_2 at 37°C. The cells were washed with PBS and treated with test compounds for 72 hr at varying concentrations (50 µg/ml, 25, 12.5, etc.) in a medium with 10% FBS. As negative control, cells were treated with a medium containing 10% FBS. The cells were left for incubation with the test compounds for 72 hr. Thereafter, the supernatant was removed and 100 µl RPMI-1640 medium containing 10 µl MTT (5 mg/ml) was added and incubated for 4 hr. After that, the supernatant was removed and 200 ml DMSO was added to dissolve the formazan. A FLUOstar OPTIMA spectrophotometer was used for colorimetric analysis ($\lambda = 540$ nm). Cytotoxic effects were expressed by IC_{50} values (concentration that caused a 50% reduction in viable cells).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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