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S-Euglobals: Biomimetic synthesis, antileishmanial, antimalarial, and antimicrobial activities☆

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Abstract—Several new euglobal analogues (named as *S*-euglobals) were synthesized from phloroglucinol via a biomimetic three-component reaction involving Knoevenagel condensation followed by [4+2]-Diels–Alder cycloaddition with monoterpene. Newly synthesized euglobal analogues involve monoterpenes that have not yet been encountered in natural euglobals. *S*-Euglobals along with previously synthesized robustadial A and B were evaluated for in vitro antileishmanial, antimalarial, antimicrobial, and cytotoxic activities. Out of 16, nine analogues were found to exhibit antileishmanial activity against *Leishmania donovani* promastigotes. Analogue **7** was the most potent with IC_{50} of 2.4 μg/mL and IC_{90} of 8 μg/mL, followed by analogues **8** and **11** (IC_{50} 5.5 and 9.5 μg/mL). Antileishmanial activity of robustadial A (**5**) and B (**6**) was moderate with IC_{50} of 20 and 16 μg/mL, respectively. Robustadial A and B and *S*-euglobal **8** exhibited weak antimalarial activity against *Plasmodium falciparum* (IC_{50} of 2.7–4.76 μg/mL). Few of the euglobal analogues showed antibacterial activity against methicillin-resistant *Staphylococcus aureus*. Amongst these, analogue **11** was the most potent with IC_{50} of 1.0 μg/mL and MIC of 5.0 μg/mL. Most of the compounds were not cytotoxic up to 25 μg/mL in a panel of cell lines consisting of both cancer (SK-MEL, KB, BT-549, and SK-OV-3) as well as non-cancer kidney (Vero and LLC-PK11) cells.

1. Introduction

Euglobals are formyl–isovaleryl or diformyl phloroglucinol–monoterpene or –sesquiterpene adducts occurring widely amongst *Eucalyptus* species and are potent inhibitors of Epstein–Barr virus activation. Biogenetically these are proposed to be formed by Diels–Alder type cycloaddition of *O*-quinone methides derived from oxidation of phloroglucinol derivatives jensenone or grandinol with various mono- or sesquiterpenes.[1]

As we have discussed earlier,[2] a number of terpene adducts occur in *Eucalyptus* species but phloroglucinol-terpene adducts isolated so far contain only nine terpenes as terpene component, of which eight are monoterpenes (α-pinene, β-pinene, sabinene, α-phellandrene, β-phellandrene, terpinolene, α-terpinene, γ-terpinene) and one is a sesquiterpene (bicyclogermacrene). Terpenes of *Eucalyptus* species that have not yet been encountered in natural euglobals include camphene, 3-carene, 2-carene, myrtenol, α-terpineol, limonene, nopol, and citronellol. The possibility of phloroglucinol-terpene adducts involving these terpenes being discovered at some stage from *Eucalyptus* cannot be ruled out. In previous work, we have synthesized α-pinene, β-pinene, 3-carene and camphene phloroglucinol-terpene adducts for evaluation of their antimicrobial, antileishmanial, and antimalarial activities. These adducts, not reported in the literature, were named as *S*-euglobals (*S*-for synthetic).[2]

We also found that α-pinene adducts, euglobal G1–G2 (**1–2**) and 3-carene adducts (**3–4**, *S*-euglobals, Fig. 1), demonstrated potent antileishmanial activity against *Leishmania donovani* with IC_{50} values in the range of 2.8–3.6 μg/mL. The following structural requirements for antileishmanial activity were identified: (a) presence

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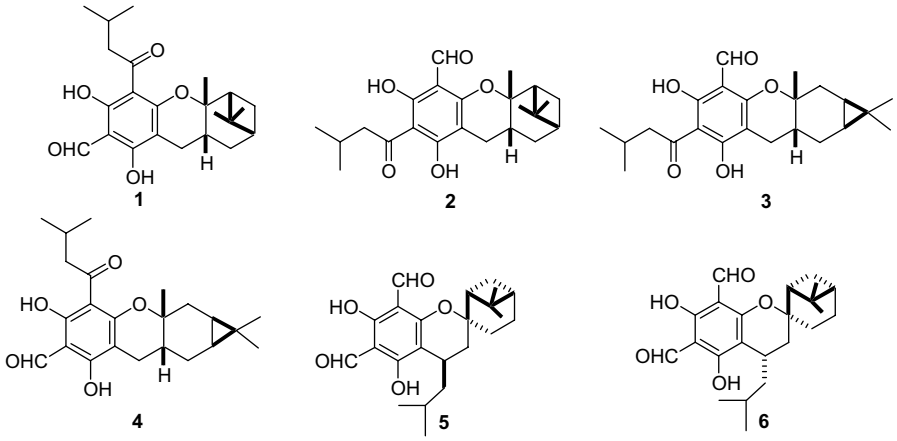


Figure 1. Antileishmanial euglobal G1–G2 (**1–2**) and *S*-euglobals (**3–4**) and antimalarial robustadial A–B (**5–6**).

of isovaleryl functionality, (b) formyl group β- to the pyran oxygen, and (c) terpenoid moiety attached in linear fashion resulting in formation of xanthan skeleton.[2]

Based on the interesting biological activity results of our previous work on euglobals[2] and as a part of our continuing program to synthesize naturally occurring phloroglucinol compounds and their analogues to explore their biological potential,[2–5] we have further designed and synthesized several *S*-euglobals by introducing different naturally occurring monoterpenes such as 2-carene, myrtenol, and nopol in order to further characterize the structural features required for their antileishmanial activity. Analogues with diisovaleryl functionality on the aromatic ring were also designed and synthesized in order to study the effect of two isovaleryl moieties on antileishmanial activity. Naturally occurring antimalarial diastereomeric robustadial A and B were also evaluated for antimicrobial, antileishmanial and antimalarial activities.

2. Results and discussion

2.1. Synthesis of *S*-euglobals

Several new phloroglucinol-terpene adducts (**7–18** and **19–22**, *S*-euglobals) were synthesized by a biomimetic approach with an overall yield of 50–70% and characterized by NMR, MS, IR, and UV spectroscopic data. The point of attachment of the terpene moiety onto the pyran ring has been determined by DEPT analysis and location of formyl and acyl functionalities was established by extensive 2D-NMR spectral data, viz. HSQC and HMBC.

We have earlier reported a two-step biomimetic synthesis of robustadial A (**5**) and B (**6**) from phloroglucinol (**23**) by a key biomimetic three-component reaction. The strategy involves in situ generation of *O*-quinone methide via Knoevenagel condensation and subsequent [4+2]-Diels–Alder cycloaddition with β-pinene.[4] This reaction scheme gave better yield compared to DDQ mediated synthesis[2] of euglobals and was therefore adopted for the synthesis of *S*-euglobals.[4]

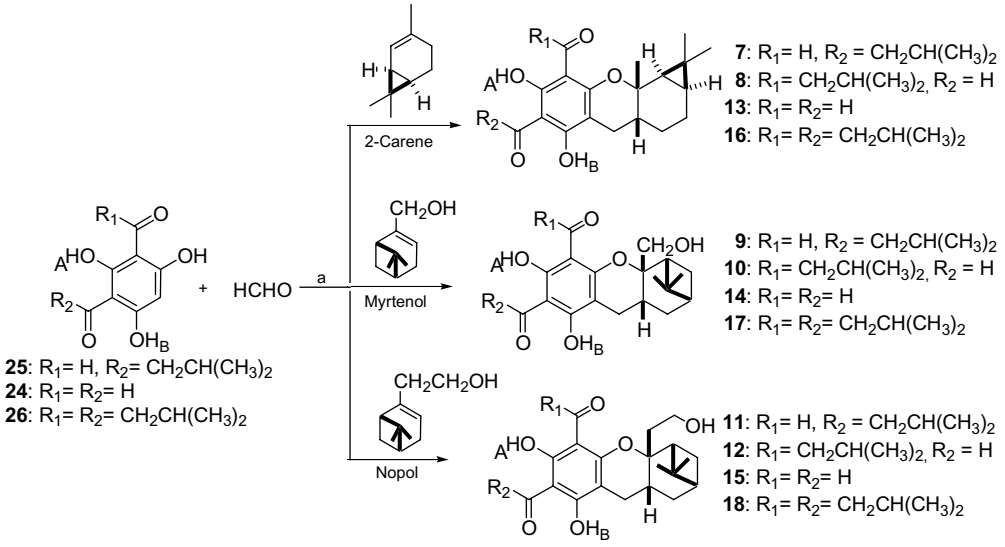
In order to synthesize *S*-euglobals using a similar synthetic strategy, the key precursors, **24–26** were required. These precursors were synthesized from phloroglucinol as reported earlier.[2–4]

Treatment of **25** with formaldehyde and different terpenes including (+)-2-carene, (*1R*)-(−)-myrtenol, and (*1R*)-(−)-nopol in the presence of sodium acetate in acetic acid resulted in the formation of respective pairs of regioisomers, **7** and **8** from 2-carene, **9** and **10** from myrtenol, and **11** and **12** from nopol in 65–70% yield. Similarly, treatment of **24** and **26** with formaldehyde and different terpenes in the presence of sodium acetate in acetic acid resulted in the formation of desired cycloadducts, **13–15** and **16–18**, respectively, in 60–65% yield as shown in Scheme 1.

The pairs of regioisomers (**7–12**) were separated by preparative RP-HPLC using methanol:water:acetic acid (100:5:3) as a mobile phase with the flow rate of 4 mL/min. The separated isomers were characterized by NMR, MS, IR, and UV. The typical pattern of chemical shifts of aromatic hydroxyls and formyl in ^{1}H NMR and elution behaviour in HPLC was observed for all pairs of regioisomers as depicted in Table 1.

With the diacylated euglobals, ^{1}H NMR chemical shift for OH_A hydroxyls were downfield (δ 16.25–16.45) when compared with those possessing monoacyl functionality (δ 13.10–13.30 or 14.30–14.50) or diformyl functionality (δ 13.64–13.18). The location of aromatic hydroxyls, OH_A and OH_B, was confirmed by 2D-NMR studies viz. HMQC and HMBC.

The point of attachment of the terpene moiety to the chroman ring has been identified on the basis of ^{1}H NMR chemical shifts. In case of three-component reaction between 2,4-diisovaleryl phloroglucinol (**26**), formaldehyde and myrtenol, two possibilities (structures **17a** and **17b**, Fig. 2) arise with respect to the orientation of terpene moiety. Absence of a triplet at ~δ4.0 (for CH at C_{4a}) ruled out the possibility of **17b**. The structure **17a** was confirmed by DEPT experiments (Fig. 2) that showed C_{4a} as a singlet at δ 91.1 indicating quaternary



Scheme 1. Reagent and condition: (a) AcOH, NaOAc, 60 °C, 2 h, 50–70%.

Table 1. Parameters to distinguish pair of regioisomers

Entry	HPLC*	^{1}H NMR (δ values)[b]		
	tR[a]	CHO	OH_A	OH_B
7/8	10.71/8.51	10.05/10.19	14.48/13.26	15.44/15.39
9/10	4.70/4.12	9.95/10.20	14.30/13.13	15.36/15.34
11/12	5.12/4.38	9.92/10.21	14.32/13.15	15.38/15.35

[a] Retention time in minutes.
[b] ^{1}H NMR chemical shift values for formyl and hydroxyl groups (OH_A and OH_B) on aromatic ring.
* RP-HPLC, C_{18} (Luna, 5 μm, 250 mm × 4.6 mm), MeOH:H_2O:acetic acid—100:5:3 at 1.7 mL/min

nature of this oxycarbon while in **17b**, this oxycarbon would afford a doublet.

Newly synthesized euglobal analogues, **7–12**, contain 2-carene, myrtenol, and nopol monoterpenes which have not yet been encountered in natural euglobals.

Diisovaleryl euglobal analogues using monoterpenes, α-pinene, β-pinene, 3-carene, and camphene, were also synthesized. Formyl–isovaleryl and diformyl euglobal analogues using these terpenes have been synthesized and evaluated earlier.[2] Treatment of 2,4-diisovaleryl phloroglucinol (**26**) with formaldehyde and different terpenes viz. (*1S*)-(−)-α-pinene, (−)-β-pinene at 60 °C for 2 h resulted in the formation of desired cycloadducts, **19–22**, in 65–70% yield as depicted in Scheme 2.

2.2. Biological evaluation

All synthesized compounds were screened for in vitro antileishmanial, antimalarial, antimicrobial, and cytotoxic activities. Activity against *L. donovani* promastigotes was determined by the Alamar Blue™ assay.[6] Nine *S*-euglobal analogues along with robustadial A (**5**) and B (**6**) showed antileishmanial activities (Table 2).

The antileishmanial activities of robustadial A and B (IC_{50} 20 and 16 μg/mL, respectively) were not as potent as some of the newly synthesized euglobals (e.g., **7**, **8** and **11**, IC_{50} 2.4, 5.5 and 9.5 μg/mL, respectively). Amongst the pair of regioisomers, the regioisomers having formyl moiety located at β- position to pyran oxygen (**7**, **9** and **11**) were more potent compared with their respective

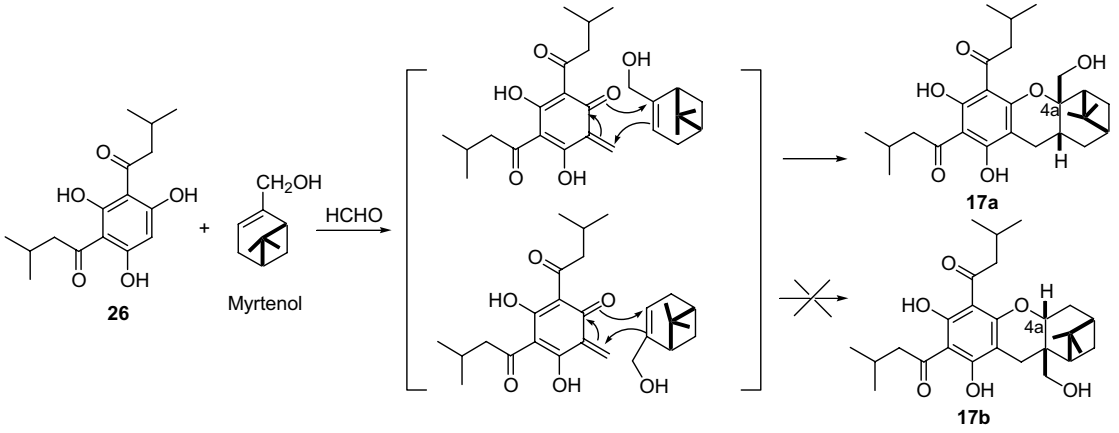
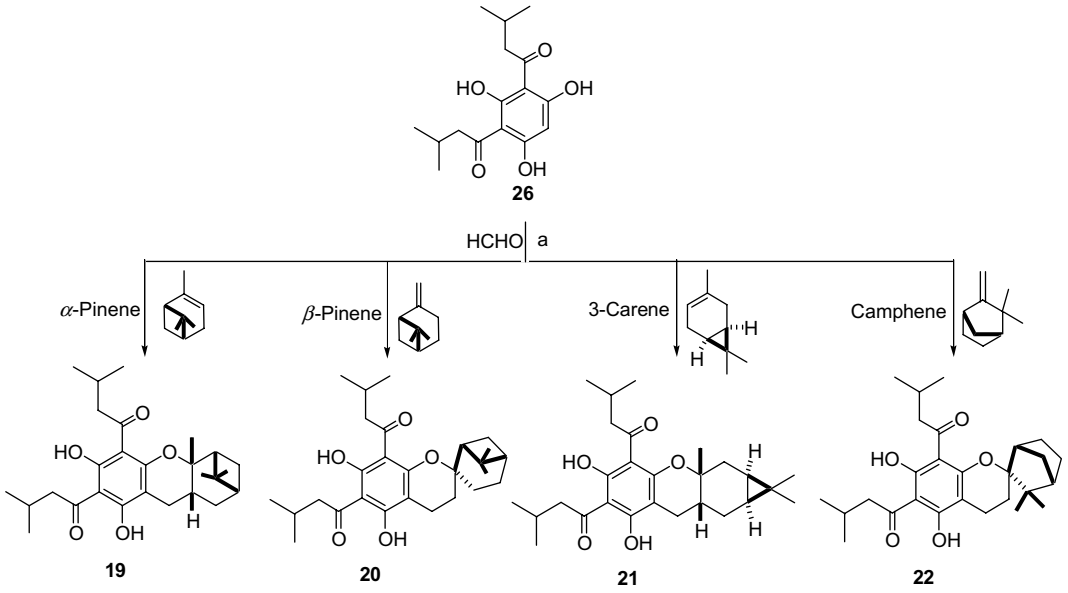


Figure 2. Three-component reaction between 2,4-diisovaleryl phloroglucinol (**26**), formaldehyde, and myrtenol.



Scheme 2. Reagent and condition: (a) AcOH, NaOAc, 60 °C, 2 h, 65–70%.

Table 2. In vitro antileishmanial activity of *S*-euglobals (**7–22**) and robustadial A and B (**5–6**)

Entry	*L. donovani*	
	IC_{50} (μg/mL)	IC_{90} (μg/mL)
5	20	39
6	16	16
7	2.4	8
8	5.5	28
9	18	39
10	30	NA
11	9.5	33
12	32	NA
13	20	40
14	NA	NA
15	22	NA
16	NA	NA
17	24	NA
18	NA	NA
19	NA	NA
20	NA	NA
21	NA	NA
22	NA	NA
Pentamidine	1.2	6
Amphotericin B	0.19	0.36

IC_{50}, the concentration that affords 50% inhibition of leishmanial growth; IC_{90}, the concentration that affords 90% inhibition of leishmanial growth; NA, not active.

regioisomers with isovaleryl moiety *β*- to the pyran ring oxygen (**8**, **10**, and **12**). 2-Carene adduct **7** was the most potent euglobal analogue with IC_{50} of 2.4 μg/mL and IC_{90} of 8 μg/mL. Antileishmanial activity of **7** was comparable to that of standard drug pentamidine. The presence of a hydroxyl moiety on the terpenoid portion of the euglobal (**9** and **11**) resulted in decrease or loss of activity. The antileishmanial activity of previously reported euglobal G2 (α-pinene adduct, **2**; IC_{50} 3.6 μg/mL)[2] was decreased with the replacement of bridgehead methyl with hydroxymethyl (myrtenol adduct, **9**; IC_{50} 18 μg/mL) or hydroxy ethyl (nopol adduct, **11**; IC_{50} 9.5 μg/mL). The analogues with diformyl or diisovaleryl moieties on aromatic ring also lost the antileishmanial activity.

In summary, out of total of 38 analogues synthesized using 7 monoterpenes (including our earlier work), 3-carene (**3**)[2] and 2-carene adduct (**7**) with formyl and isovaleryl moieties at *β*- and *δ*- to the pyran oxygen, respectively, were the most active. The comparative activity of 2-carene and 3-carene adducts by varying substitution on aromatic ring is depicted in Table 3.

Cytotoxicity was tested by Neutral Red assay in a panel of four cancer cell lines (SK-MEL: human malignant melanoma; KB: human epidermal carcinoma; BT-549: human breast ductal carcinoma and SK-OV-3: human ovary carcinoma) and two non-cancer kidney cell lines (Vero: monkey kidney fibroblasts and LLC-PK11: pig kidney epithelial cells).[7] Most of the compounds were not cytotoxic up to 25 μg/mL (Table 4). Doxorubicin was used as reference standard for toxicity.

Antimalarial activity was evaluated against chloroquine sensitive (D6) and chloroquine resistant (W2) strains of

Table 3. Comparison of antileishmanial activity of carene adducts

Substitution of aromatic ring/terpene	Antileishmanial activity (IC_{50}/IC_{90} μg/mL)	
	3-Carene	2-Carene
Formyl/isovaleryl	2.8/7.8 (**3**)[a]	2.4/ 8.0 (**7**)
Isovaleryl/formyl	6.2/27 (**4**)[a]	5.5/28 (**8**)
Formyl/formyl	19/36 (*)[a]	20/40 (**13**)
Isovaleryl/isovaleryl	NA/NA (**21**)	NA/NA (**16**)

[a] Earlier work.[2]

* Structure not shown in this article.

Table 4. In vitro cytotoxicity of *S*-euglobals (**7–22**) and robustadial A and B (**5**, **6**) in cancer and non-cancer cells

Entry	Cytotoxicity (IC_{50} μg/mL)					
	Cancer cells				Non-cancer cells	
	SK-MEL	KB	BT-549	SK-OV-3	Vero	LLC-PK11
5	>25	NC	19	>25	19	17
6	NC	NC	25	NC	NC	NC
7	NC	NC	>25	>25	NC	>25
8	25	NC	NC	NC	NC	NC
9	NC	NC	23	NC	NC	NC
10	NC	NC	22.5	NC	NC	NC
11	25	20	18.5	25	20	25
12	NC	>25	>25	>25	>25	>25
13	NC	20	>25	NC	NC	NC
14	25	NC	25	NC	NC	NC
15	NC	NC	NC	NC	NC	NC
16	NC	NC	NC	NC	NC	NC
17	NC	NC	25	NC	>25	NC
18	NC	NC	>25	NC	NC	NC
19	NC	NC	NC	NC	NC	NC
20	NC	NC	NC	NC	NC	NC
21	NC	NC	NC	NC	NC	NC
22	NC	NC	NC	NC	NC	NC
Doxorubicin	0.6	0.9	0.6	0.75	7.5	0.5

NC, not cytotoxic up to 25 μg/mL.

Table 5. In vitro antimalarial activities of robustadial A–B (**5–6**) and *S*-euglobal **8**

Entry	*Plasmodium falciparum*			
	D6 clone		W2 clone	
	IC_{50}[a] (μg/mL)	S.I.[b]	IC_{50}[a] (μg/mL)	S.I.[b]
5	4.76	4.0	2.80	6.8
6	4.50	>5.6	4.76	>5.3
8	3.4	>7.4	2.7	>9.3
Chloroquine	13.5	—	115	—
Artemisinin	14.0	—	7.5	—

[a] IC_{50}, the concentration that affords 50% inhibition of plasmodial growth.
[b] S.I., selectivity index = IC_{50} vero cells/IC_{50} *P. falciparum*.

Table 6. In vitro antibacterial activities of *S*-euglobals

Entry	Methicillin-resistant *S. aureus*	
	IC_{50}[a] (μg/mL)	MIC[b] (μg/mL)
9	2.5	NA
10	10	NA
11	1.0	5.0
12	5.0	NA
Ciprofloxacin	0.08	0.25

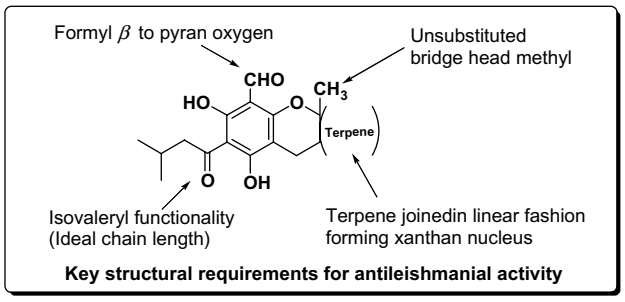
[a] IC_{50}, the concentration that affords 50% inhibition of bacterial growth.
[b] MIC, minimum inhibitory concentration (the lowest concentration that allows no detectable growth).

Plasmodium falciparum in an in vitro assay based on the determination of plasmodial LDH activity.[8] Robustadial A (**5**) and B (**6**) and one of the regioisomers of 2-carene adduct **8** showed weak antimalarial activities in comparison to standard drugs chloroquine and artemisinin as shown in Table 5. However, selectivity index of euglobal analogue **8** was slightly higher than robustadials **5** and **6** towards *Plasmodium* cells (Table 5).

The antibacterial activity was evaluated against methicillin-resistant *Staphylococcus aureus* and *Mycobacterium intracellulare*.[9,10] Four analogues (**9–12**) showed mild to moderate antibacterial activity against methicillin-resistant *S. aureus* with IC_{50} ranging from 1.0 to 5.0 μg/mL. Ciprofloxacin was included as positive control (Table 6).

The antifungal activities were evaluated against a panel of pathogenic fungi (*Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus)* associated with opportunistic infections.[9] None of the analogues exhibited any activity against these pathogens. Amphotericin B was included as a standard drug.

From the present work as well as that reported in our previous paper,[2] following structure–activity relationships could be defined for antileishmanial activity of *S*-euglobals and euglobals: (a) compounds with xanthan skeleton were more active than those with spiro skeleton, (b) variation in location of formyl and isovaleryl moiety has effect on activity, analogues with formyl located β- to pyran oxygen (**3**, **7**, **9**, and **11**) showed better activity compared with respective analogues having isovaleryl β- to pyran oxygen (**4**, **8**, **10**, and **12**), (c) compounds with formyl/isovaleryl moiety (**7**, **8**) were more active than compounds with formyl/formyl (**13**) or isovaleryl/isovaleryl (**16**) moiety, (d) decrease in acyl chain length also resulted in decrease or loss of activity (**13** was less active than **7** or **8**), (e) substitution of bridgehead methyl with hydroxyl or hydroxymethyl also resulted in decrease of activity, compounds **7** and **8** were more active than **9–12**.



3. Conclusions

In conclusion several *S*-euglobals were designed and synthesized by varying terpenoid moiety and substitution on aromatic ring. *S*-Euglobals and robustadials possessed in vitro antileishmanial activity against *L. donovani* promastigotes. Amongst these, 2-carene adduct, **7**, showed most potent antileishmanial activity with IC_{50} and IC_{90} of 2.4 and 8.0 μg/mL, respectively. Few of the euglobal analogues showed antibacterial activity against methicillin-resistant *S. aureus*. Analogue **11** was the most active with IC_{50} of 1.0 μg/mL and MIC of 5.0 μg/mL.

Out of a total of 38 euglobal analogues from our study, 2-carene adduct, **7** and 3-carene adduct, **3** were found to possess most potent antileishmanial activities with IC_{50} values of 2.4 and 2.8 μg/mL, respectively. The key structural features for antileishmanial activity have been identified.

From this work, euglobals have emerged as a promising new class of antileishmanial compounds.

4. Experimental

Melting points were recorded on capillary melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on 300 MHz Bruker FT-NMR (Avance DPX300) spectrometer using tetramethylsilane as internal standard and the chemical shifts are reported in δ units. Mass spectra were recorded on either GCMS (Shimadzu QP 5000 spectrometer) auto sampler/direct injection (EI/CI) or LCMS (APCI/ESI). Domestic microwave oven (Whirlpool, Sweden, Model: MT-243) was used to carry out microwave heated reactions. All chromatographic purifications were performed with silica gel (60–120 mesh), whereas all TLC (silica gel) development was performed on silica gel coated (Merck Keiselgel 60F_{254}, 0.2 mm thickness) sheets. All chemicals were purchased from Sigma–Aldrich, SD Fine Chemicals, Lancaster, and CDH. Solvents used for the chemical synthesis purchased from commercial sources were of analytical grade and were used without further purification unless otherwise stated.

4.1. General method for synthesis of euglobal analogues 7–18 and 19–22

A mixture of disubstituted phloroglucinol, **24–26** (1.09 mmol), formaldehyde (2.18 mmol), monoterpene (3.27 mmol), and sodium acetate (10 mg) in acetic acid (5 mL) was heated in a domestic microwave oven (1000 W, 4 min). On cooling, reaction mixture was diluted with ethyl acetate and the resultant mixture was washed with water and brine solution and finally dried over sodium sulfate. The pairs of regioisomers were formed in reactions of **25** and these were separated by semi-preparative HPLC on Princeton SPHER-100, C_{18} (100 A, 5 μm, 250 mm × 10.0 mm) column using methanol:water:acetic acid = 100:5:3 as the mobile phase with a flow rate of 4.0 mL/min. Reaction of **24** and **26** with formaldehyde and monoterpenes resulted in formation of single product and these were purified by silica gel (#60–120) column chromatography (10% EtOAc in hexane).

4.1.1. 5,7-Dihydroxy-1,1,9a-trimethyl-6-(3-methyl-butanoyl)-1,1a,2,3,3a,4,9a,9b-octahydro-9-oxa-cyclopropa[*a*]-anthracene-8-carbaldehyde (7). Yield: 35%; brown oil; UV (MeOH): λ_{max} (log ε) 284 nm (4.14); IR (Neat): ν_{max} 2956, 2868, 1618, 1453, 1415, 1375, 1313, 1190, 1136, 1039 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 15.44 (s, 1H, OH_B), 14.48 (s, 1H, OH_A), 10.05 (s, 1H, C*H*O), 2.99 (d, J = 6.6 Hz, 2H), 2.80 (dd, J = 5.5, 16.7 Hz, 1H), 2.70 (d, J = 16.4 Hz, 1H), 2.27 (m, 1H), 2.05–1.84 (m, 2H), 1.75–1.56 (m, 2H), 1.31 (m, 1H), 1.24 (s, 3H), 1.02 (s, 6H), 0.99 (d, J = 6.5 Hz, 6H), 0.54 (t, J = 8.3 Hz, 1H), 0.07 (d, J = 3.7 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 206.3, 191.9, 172.2, 168.3, 162.3, 104.1, 103.4, 99.3, 79.5, 52.7, 34.6, 30.0, 29.1, 25.2, 25.1, 24.1, 24.0, 22.8, 19.5, 17.1, 15.5, 15.3; CIMS: *m*/*z* 387 $[M+1]^+$, 251 [M-$C_{10}H_{16}$]; analysis for $C_{23}H_{30}O_5$ (386.2), calcd, C, 71.48; H, 7.82; found, C, 71.35; H, 7.78.

4.1.2. 5,7-Dihydroxy-1,1,9a-trimethyl-8-(3-methyl-butanoyl)-1,1a,2,3,3a,4,9a,9b-octahydro-9-oxa-cyclopropa[*a*]-anthracene-6-carbaldehyde (8). Yield: 37%; brown oil; UV (MeOH): λ_{max} (log ε) 282 nm (4.13); IR (Neat): ν_{max} 2956, 2930, 2873, 1618, 1454, 1408, 1311, 1190, 1140, 1075 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 15.39 (s, 1H, OH_B), 13.26 (s, 1H, OH_A), 10.20 (s, 1H, C*H*O), 3.06 (dd, J = 6.1, 15.5 Hz, 2H), 2.84–2.65 (m, 2H), 2.25 (m, 1H), 2.06–1.86 (m, 2H), 1.74–1.65 (m, 1H), 1.58 (m, 1H), 1.34 (m, 1H), 1.27 (s, 3H), 1.03 (s, 3H), 1.02 (s, 3H), 1.00 (d, J = 5.8 Hz, 6H), 0.57 (t, J = 8.3 Hz, 1H), 0.07 (dd, 3.7, 5.1 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 205.9, 192.4, 170.0, 167.9, 163.5, 104.5, 104.4, 98.5, 79.5, 53.1, 34.8, 29.5, 29.0, 25.3, 25.1, 24.0, 23.8, 23.0, 22.5, 19.2, 17.1, 15.3, 15.2; CIMS: *m*/*z* 387 $[M+1]^+$, 251 [M-$C_{10}H_{16}$]; analysis for $C_{23}H_{30}O_5$ (386.2), calcd, C, 71.48; H, 7.82; found, C, 71.36; H, 7.89.

4.1.3. 6,8-Dihydroxy-3,3-dimethyl-4a-hydroxymethyl-7-(3-methyl-butanoyl)-2,3,4,4a,9,9a-hexahydro-2,4-methano-1*H*-xanthene-5-carboxaldehyde (9). Yield: 30%; brown oil; UV (MeOH): λ_{max} (log ε) 278 nm (4.19); IR (Neat): ν_{max} 2956, 2930, 1618, 1459, 1418, 1316, 1190, 1139 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 15.36 (s, 1H, OH_B), 14.30 (s, 1H, OH_A), 9.95 (s, 1H, C*H*O), 3.78 (d, J = 11.9 Hz, 1H), 3.71 (d, J = 11.9 Hz, 1H), 2.99 (d, J = 6.5 Hz, 2H), 2.77 (m, 2H), 2.55 (dd, J = 5.8, 15.2 Hz, 1H), 2.42 (t, J = 5.5 Hz, 1H), 2.30–

2.13 (m, 3H), 1.89 (m, 2H), 1.29 (s, 3H), 1.08 (s, 3H), 0.99 (d, $J = 6.6$ Hz, 6H), 0.86 (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 207.0, 191.9, 172.0, 168.7, 164.5, 104.4, 102.0, 90.0, 70.3, 53.3, 50.1, 41.1, 40.8, 35.2, 30.2, 29.4, 28.3, 27.4, 25.5, 23.4, 23.3, 22.3; CIMS: *m/z* 403 $[M+1]^+$, 251 [M-$C_{10}H_{16}O$]; analysis for $C_{23}H_{30}O_6$ (402.2), calcd, C, 68.64; H, 7.51; found, C, 68.53; H, 7.45.

4.1.4. 6,8-Dihydroxy-3,3-dimethyl-4a-hydroxymethyl-5-(3-methyl-butanoyl)-2,3,4,4a,9,9a-hexahydro-2,4-methano-1*H*-xanthene-7-carboxaldehyde (10). Yield: 28%; brown oil; UV (MeOH): λ_{max} ($\log\varepsilon$) 282 nm (4.29); IR (Neat): ν_{max} 2956, 2925, 1618, 1452, 1415, 1373, 1313, 1190, 1139 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 15.34 (s, 1H, OH_B), 13.13 (s, 1H, OH_A), 10.21 (s, 1H, C*H*O), 3.81 (d, $J = 11.9$ Hz, 1H), 3.74 (d, $J = 11.9$ Hz, 1H), 2.97 (dd, $J = 5.6$, 15.9 Hz, 2H), 2.82–2.62 (m, 2H), 2.52–2.42 (m, 2H), 2.30–2.20 (m, 3H), 1.90 (m, 2H), 1.30 (s, 3H), 1.08 (s, 3H), 0.98 (d, $J = 6.2$ Hz, 6H), 0.86 (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 206.0, 193.2, 170.7, 167.5, 166.1, 105.5, 104.9, 101.6, 91.7, 70.4, 53.2, 50.6, 40.8, 34.8, 30.2, 29.5, 28.3, 27.2, 25.1, 23.5, 23.1, 22.2; CIMS: *m/z* 403 $[M+1]^+$, 251[M-$C_{10}H_{16}O$]; analysis for $C_{23}H_{30}O_6$ (402.2), calcd, C, 68.64; H, 7.51; found, C, 68.50; H, 7.60.

4.1.5. 6,8-Dihydroxy-3,3-dimethyl-4a-hydroxyethyl-7-(3-methyl-butanoyl)-2,3,4,4a,9,9a-hexahydro-2,4-methano-1*H*-xanthene-5-carboxaldehyde (11). Yield: 32%; brown oil; UV (MeOH): λ_{max} ($\log\varepsilon$) 281 nm (4.38); IR (Neat): ν_{max} 2955, 2930, 1623, 1444, 1377, 1293, 1191, 1116 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 15.38 (s, 1H, OH_B), 14.32 (s, 1H, OH_A), 9.92 (s, 1H, C*H*O), 3.91 (m, 2H), 3.03 (dd, $J = 5.4$, 11.7 Hz, 2H), 2.80–2.68 (m, 2H), 2.48–2.40 (m, 2H), 2.32–1.92 (m, 5H), 1.89 (m, 2H), 1.69 (m, 1H), 1.33 (m, 1H), 1.30 (s, 3H), 1.11 (s, 3H), 1.00 (d, $J = 6.4$ Hz, 3H), 0.98 (d, $J = 6.4$ Hz, 3H), 0.77 (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 207.0, 191.8, 172.1, 168.8, 166.9, 104.3, 101.4, 89.5, 59.0, 53.3, 52.9, 44.8, 41.0, 40.8, 35.3, 32.3, 28.6, 27.9, 25.5, 23.3, 21.1; CIMS: *m/z* 417 $[M+1]^+$, 251 [M-$C_{11}H_{18}O$]; analysis for $C_{24}H_{32}O_6$ (416.2), calcd, C, 69.21; H, 7.74; found, C, 69.16; H, 7.84.

4.1.6. 6,8-Dihydroxy-3,3-dimethyl-4a-hydroxyethyl-5-(3-methyl-butanoyl)-2,3,4,4a,9,9a-hexahydro-2,4-methano-1*H*-xanthene-7-carboxaldehyde (12). Yield: 30%; yellow oil; UV (MeOH): λ_{max} ($\log\varepsilon$) 278 nm (4.25); IR (Neat): ν_{max} 3352, 2960, 2928, 1624, 1445, 1379, 1293, 1192, 1123 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 15.35 (s, 1H, OH_B), 13.15 (s, 1H, OH_A), 10.22 (s, 1H, C*H*O), 3.95 (m, 2H), 2.97 (dd, $J = 6.4$, 17.1 Hz, 2H), 2.89–2.61 (m, 2H), 2.46 (t, $J = 5.6$ Hz, 1H), 2.33–2.12 (m, 2H), 2.08–1.92 (m, 2H), 1.89–1.72 (m, 2H), 1.70–1.65 (m, 1H), 1.31–1.26 (m, 1H), 1.30 (s, 3H), 1.12 (s, 3H), 0.98 (d, $J = 6.4$ Hz, 6H), 0.77 (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 206.5, 193.1, 170.8, 170.6, 168.4, 104.8, 104.2, 101.2, 91.2, 59.1, 53.9, 53.1, 45.1, 41.1, 40.7, 34.8, 32.3, 28.5, 27.6, 24.7, 23.4, 23.2, 20.9; CIMS: *m/z* 417 $[M+1]^+$, 251 [M-$C_{11}H_{18}O$]; analysis for $C_{24}H_{32}O_6$ (416.2), calcd, C, 69.21; H, 7.74; found, C, 69.12; H, 7.88.

4.1.7. 5,7-Dihydroxy-1,1,9a-trimethyl-1,1a,2,3,3a,4,9a,9b-octahydro-9-oxa-cyclopropa[a]anthracene-6,8-dicarbaldehyde (13). Yield: 62%; yellow oil; UV (MeOH): λ_{max} ($\log\varepsilon$) 280 nm (4.26); IR (Neat): ν_{max} 3445, 2923, 1635, 1445, 1388, 1305, 1180, 1060 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 13.64 and 13.46 (s, 1H each, 2× O*H*), 10.33 and 10.24 (s, 1H each, 2× C*H*O), 3.00 (dd, $J = 5.6$, 16.6 Hz, 1H), 2.87 (m, 1H), 2.27–2.02 (m, 2H), 1.85–1.66 (m, 2H), 1.48 (m, 1H), 1.44 (s, 3H), 1.15 (s, 3H), 1.12 (s, 3H), 0.76 (t, $J = 8.6$ Hz, 1H), 0.27 (dd, $J = 3.7$, 9.2 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 192.8, 192.7, 170.2, 168.7, 164.6, 104.7, 104.3, 99.4, 80.5, 35.1, 30.4, 29.7, 25.8, 24.6, 24.0, 20.0, 17.6, 16.0, 15.8; CIMS: *m/z* 331 $[M+1]^+$, 195 [M-$C_{10}H_{16}$]; analysis for $C_{19}H_{22}O_5$ (330.1), calcd, C, 69.07; H, 6.71; found, C, 68.91; H, 6.56.

4.1.8. 6,8-Dihydroxy-3,3-dimethyl-4a-hydroxymethyl-2,3,4,4a,9,9a-hexahydro-2,4-methano-1*H*-xanthene-5,7-dicarboxaldehyde (14). Yield: 63%; yellow oil; UV (MeOH): λ_{max} ($\log\varepsilon$) 278 nm (4.30); IR (Neat): ν_{max} 2923, 2853, 2391, 1635, 1445, 1383, 1305, 1180, 1065 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 13.30 and 13.18 (s, 1H each, 2× O*H*), 10.15 and 9.97 (s, 1H each, 2× C*H*O), 3.82 (d, $J = 12.0$ Hz, 1H), 3.73 (d, $J = 12.0$ Hz, 1H), 2.75–2.69 (m, 1H), 2.57 (dd, $J = 5.8$, 15.2 Hz, 1H), 2.39 (t, $J = 5.5$ Hz, 1H), 2.36–2.02 (m, 3H), 1.92–1.87 (m, 2H), 1.29 (s, 3H), 1.08 (s, 3H), 0.86 (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 192.3, 169.5, 168.2, 166.3, 104.6, 101.6, 90.8, 70.3, 50.2, 41.1, 35.2, 30.2, 29.2, 28.3, 27.5, 23.4, 22.0; CIMS: *m/z* 347 $[M+1]^+$, 195 [M-$C_{10}H_{16}O$]; analysis for $C_{19}H_{22}O_6$ (346.1), calcd, C, 65.88; H, 6.40; found, C, 65.72; H, 6.52.

4.1.9. 6,8-Dihydroxy-3,3-dimethyl-4a-hydroxyethyl-2,3,4,4a,9,9a-hexahydro-2,4-methano-1*H*-xanthene-5,7-dicarboxaldehyde (15). Yield: 55%; yellow oil; UV (MeOH): λ_{max} ($\log\varepsilon$) 281 nm (4.18); IR (Neat): ν_{max} 3467, 2930, 2863, 1628, 1445, 1372, 1305, 1180, 1065 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 13.45 and 13.26 (s, 1H each, 2× O*H*), 10.14 and 10.02 (s, 1H each, 2× C*H*O), 4.31 (t, $J = 6.9$ Hz, 2H), 2.96 (m, 1H), 2.69–2.65 (m, 1H), 2.45–2.37 (m, 2H), 2.24–1.98 (m, 4H), 1.92–1.88 (m, 2H), 1.61 (br s, 1H), 1.31 (s, 3H), 1.11 (s, 3H), 0.88 (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 192.1, 169.3, 168.7, 166.1, 104.5, 101.5, 90.6, 60.3, 50.0, 44.1, 40.9, 35.1, 29.7, 28.1, 27.3, 23.2, 23.2, 21.8; CIMS: *m/z* 361 $[M+1]^+$, 195 [M-$C_{11}H_{18}O$]; analysis for $C_{20}H_{24}O_6$ (360.2), calcd, C, 66.65; H, 6.71; found, C, 66.53; H, 6.84.

4.1.10. 5,7-Dihydroxy-1,1,9a-trimethyl-6,8-di-(3-methyl-butanoyl)-1,1a,2,3,3a,4,9a,9b-octahydro-9-oxa-cyclopropa[*a*]anthracene (16). Yield: 65%; yellow oil; UV (MeOH): λ_{max} ($\log\varepsilon$) 283 nm (4.28); IR (Neat): ν_{max} 3379, 2960, 2916, 1614, 1446, 1368, 1304, 1197, 1170, 1131, 1059 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 16.33 (s, 1H, OH_A), 15.37 (s, 1H, OH_B), 3.13 (dd, $J = 6.2$, 15.2 Hz, 2H), 3.02 (d, $J = 6.7$ Hz, 2H), 2.84–2.70 (m, 2H), 2.27 (m, 2H), 2.06–1.87 (m, 2H), 1.70–1.64 (m, 1H), 1.56 (m, 1H), 1.33 (m, 1H), 1.25 (s, 3H), 1.02 (s, 6H), 0.99 (d, $J = 3.6$ Hz, 6H), 0.97 (d,

J = 4.7 Hz, 6H), 0.88 (m, 1H), 0.55 (t, J = 8.3 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 207.3, 206.3, 170.7, 162.4, 104.9, 99.4, 79.4, 53.7, 53.6, 35.4, 30.7, 29.5, 25.8, 25.7, 25.6, 24.9, 24.7, 23.6, 23.3, 23.2, 23.1, 19.8, 17.6, 16.3, 15.9; CIMS: m/z 443 $[M+1]^+$, 307 $[M-C_{10}H_{16}]$; analysis for $C_{27}H_{38}O_5$ (442.3), calcd, C, 73.27; H, 8.65; found, C, 73.19; H, 8.74.

4.1.11. 6,8-Dihydroxy-3,3-dimethyl-4a-hydroxymethyl-5,7-di-(3-methyl-butanoyl)-2,3,4,4a,9,9a-hexahydro-2,4-methano-1*H*-xanthene (17). Yield: 65%; yellow oil; UV (MeOH): λ_{max} (log ε) 282 nm (4.30); IR (Neat): ν_{max} 3374, 2960, 2917, 1614, 1447, 1367, 1301, 1196, 1167, 1060 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 16.28 (s, 1H, OH_A), 15.24 (s, 1H, OH_B), 3.84 (d, J = 11.9 Hz, 1H), 3.72 (d, J = 11.9 Hz, 1H), 3.03 (d, J = 4.1 Hz, 2H), 3.00 (d, J = 4.0 Hz, 2H), 2.80–2.69 (m, 3H), 2.50–2.42 (m, 2H), 2.32–2.12 (m, 3H), 1.89 (m, 2H), 1.30 (s, 3H), 1.08 (s, 3H), 1.00 (d, J = 6.6 Hz, 6H), 0.98 (d, J = 4.0 Hz, 6H), 0.85 (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 207.5, 206.0, 170.8, 169.9, 164.5, 105.5, 104.6, 102.0, 91.1, 70.4, 53.7, 53.2, 50.5, 40.8, 34.8, 29.7, 28.3, 27.2, 25.5, 25.3, 23.6, 23.4, 23.1, 22.6; CIMS: m/z 459 $[M+1]^+$, 307 $[M-C_{10}H_{16}O]$; analysis for $C_{27}H_{38}O_6$ (458.3), calcd, C, 70.71; H, 8.35; found, C, 70.79; H, 8.27.

4.1.12. 6,8-Dihydroxy-3,3-dimethyl-4a-hydroxyethyl-5,7-di-(3-methyl-butanoyl)-2,3,4,4a,9,9a-hexahydro-2,4-methano-1*H*-xanthene (18). Yield: 64%; yellow oil; UV (MeOH): λ_{max} (log ε) 281 nm (4.21); IR (Neat): ν_{max} 3380, 2917, 2872, 1613, 1445, 1366, 1302, 1195, 1168, 1060 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 16.30 (s, 1H, OH_A), 15.25 (s, 1H, OH_B), 3.97 (t, J = 7.0 Hz, 2H), 3.01 (m, 4H), 2.94–2.72 (m, 2H), 2.45 (t, J = 5.3 Hz, 1H), 2.38–2.18 (m, 4H), 2.16–1.98 (m, 2H), 1.88 (m, 1H), 1.62 (m, 2H), 1.34 (m, 1H), 1.30 (s, 3H), 1.12 (s, 3H), 1.00 (d, J = 6.3 Hz, 6H), 0.98 (d, J = 5.8 Hz, 6H), 0.86 (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 207.5, 205.9, 170.9, 169.9, 165.3, 105.3, 104.5, 101.5, 90.5, 59.2, 53.9, 53.7, 53.0, 45.2, 41.0, 40.7, 34.9, 32.5, 28.6, 27.6, 25.5, 24.9, 23.5, 23.4, 23.2, 21.4; CIMS: m/z 473 $[M+1]^+$, 307 $[M-C_{11}H_{18}O]$; analysis for $C_{28}H_{40}O_6$ (472.3), calcd, C, 71.16; H, 8.53; found, C, 71.08; H, 8.61.

4.1.13. 5,7-Di-(3-methyl-butanoyl)-2,3,4,4a,9,9a-hexahydro-6,8-dihydroxy-3,3,4a-trimethyl-2,4-methano-1*H*-xanthene (19). Yield: 65%; yellow oil; UV (MeOH): λ_{max} (log ε) 281 nm (4.30); IR (Neat): ν_{max} 2955, 2925, 2863, 1614, 1415, 1368, 1298, 1193, 1167, 1125 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 16.41 (s, 1H, OH_A), 15.25 (s, 1H, OH_B), 3.03 (d, J = 6.3 Hz, 2H), 2.98 (d, J = 5.4 Hz, 2H), 2.75 (dd, J = 2.7, 15.1 Hz, 1H), 2.60 (dd, J = 7.7, 15.1 Hz, 2H), 2.40 (dd, J = 5.6, 15.0 Hz, 1H), 2.30–2.21 (m, 2H), 2.12 (m, 2H), 1.89 (m, 1H), 1.55 (m, 1H), 1.49 (s, 3H), 1.31 (s, 3H), 1.09 (s, 3H), 1.00 (d, J = 6.4 Hz, 6H), 0.98 (d, J = 7.1 Hz, 6H), 0.85 (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 207.3, 206.2, 171.1, 169.8, 165.0, 105.1, 104.3, 101.4, 89.0, 55.9, 53.6, 53.2, 40.9, 34.3, 33.0, 29.7, 28.7, 28.1, 25.6, 25.5, 23.8, 23.6, 23.4, 23.3, 23.2, 23.0, 20.9; CIMS: m/z 443 $[M+1]^+$, 307 $[M-C_{10}H_{16}]$; analysis for $C_{27}H_{38}O_5$ (442.3), calcd, C, 73.27; H, 8.65; found, C, 73.19; H, 8.56.

4.1.14. 6,8-Di-(3-methyl-butanoyl)-3,4-dihydro-5,7-dihydroxy-6′,6′-dimethyl-*spiro*-2*H*-1-benzopyran-2,2′-bicyclo-[3.1.1]-heptane (20). Yield: 65%; yellow oil; UV (MeOH): λ_{max} (log ε) 278 nm (4.20); IR (Neat): ν_{max} 3437, 2955, 2361, 1615, 1414, 1369, 1193, 1162, 1119 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 16.29, (s, 1H, OH_A), 15.27 (s, 1H, OH_B), 3.00 (d, J = 6.0 Hz, 2H), 2.96 (d, J = 6.5 Hz, 2H), 2.55 (t, J = 6.7 Hz, 2H), 2.29–2.21 (m, 2H), 2.20–2.15 (m, 2H), 2.08–1.96 (m, 5H), 1.92–1.85 (m, 1H), 1.65 (m, 2H), 1.29 (s, 3H), 1.02 (s, 3H), 0.98 (d, J = 6.4 Hz, 12H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 207.3, 206.2, 170.6, 169.7, 162.2, 105.1, 104.7, 101.3, 86.5, 53.6, 53.5, 49.9, 41.1, 38.8, 32.1, 29.2, 28.1, 27.9, 25.6, 25.4, 23.9, 23.4, 23.3, 16.3; CIMS: m/z 443 $[M+1]^+$, 307 $[M-C_{10}H_{16}]$; analysis for $C_{27}H_{38}O_5$ (442.3), calcd, C, 73.27; H, 8.65; found, C, 73.21; H, 8.53.

4.1.15. 5,7-Dihydroxy-1,1,2a-trimethyl-4,6-di-(3-methyl-butanoyl)-1,1a,2,2a,8,8a,9,9a-octahydro-3-oxa-cyclopropa[*b*]anthracene (21). Yield: 65%; yellow oil; UV (MeOH): λ_{max} (log ε) 282 nm (4.25); IR (Neat): ν_{max} 2957, 2930, 1611, 1434, 1377, 1301, 1194, 1162, 1121 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 16.33 (s, 1H, OH_A), 15.33 (s, 1H, OH_B), 3.01 (d, J = 6.7 Hz, 2H), 2.97 (d, J = 7.0 Hz, 2H), 2.63 (dd, J = 5.6, 16.8 Hz, 1H), 2.38–2.23 (m, 5H), 1.75 (dd, J = 7.3, 14.4 Hz, 1H), 1.55 (m, 1H), 1.42 (dd, J = 3.8, 15.7 Hz, 1H), 1.23 (s, 3H), 1.03 (s, 3H), 1.02 (s, 3H), 0.99 (d, J = 7.2 Hz, 12H), 0.69 (m, 1H), 0.66 (m, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 207.2, 206.5, 171.0, 170.7, 162.4, 105.0, 98.9, 78.0, 53.6, 53.5, 32.6, 31.7, 29.2, 26.0, 25.5, 25.3, 24.1, 23.5, 23.4, 23.3, 19.6, 17.3, 15.8; CIMS: m/z 443 $[M+1]^+$, 307 $[M-C_{10}H_{16}]$; analysis for $C_{27}H_{38}O_5$ (442.3), calcd, C, 73.27; H, 8.65; found, C, 73.22; H, 8.49.

4.1.16. 3,4-Dihydro-5,7-dihydroxy-3′,3′-dimethyl-6,8-Di-(3-methyl-butanoyl)-*spiro*-2*H*-1-benzopyran-2,2′-bicyclo-[2.2.1]heptane (22). Yield: 65%; yellow oil; UV (MeOH): λ_{max} (log ε) 279 nm (4.29); IR (Neat): ν_{max} 2955, 1613, 1444, 1408, 1295, 1192, 1116, 1070 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 16.28, (s, 1H, OH_A), 15.23 (s, 1H, OH_B), 3.03 (d, J = 3.9 Hz, 2H), 3.00 (d, J = 6.8 Hz, 2H), 2.61 (m, 1H), 2.45 (m, 1H), 2.32–2.22 (m, 3H), 2.08–1.96 (m, 2H), 1.87 (m, 2H), 1.63 (m, 1H), 1.50–1.38 (m, 2H), 1.23 (m, 2H), 1.10 (s, 3H), 1.05 (s, 3H), 0.98 (d, J = 4.2 Hz, 12H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 207.3, 205.9, 170.3, 169.6, 162.9, 105.4, 104.7, 102.1, 92.8, 53.6, 53.3, 50.7, 47.2, 46.1, 35.5, 27.7, 25.6, 24.9, 24.1, 23.6, 23.3, 23.2, 23.0, 17.6; CIMS: m/z 443 $[M+1]^+$, 307 $[M-C_{10}H_{16}]$; analysis for $C_{27}H_{38}O_5$ (442.3), calcd, C, 73.27; H, 8.65; found, C, 73.34; H, 8.46.

4.2. Assay for in vitro antileishmanial activity

Antileishmanial activity of the compounds was tested in vitro against a culture of *L. donovani* promastigotes grown in RPMI 1640 medium supplemented with 10%

fetal calf serum (Gibco Chem. Co.) at 26 °C. A 3-day-old culture was diluted to 5×10^5 promastigotes/mL. Drug dilutions were prepared directly in cell suspension in 96-well plates. Plates were incubated at 26 °C for 48 h and growth of leishmania promastigotes was determined by Alamar Blue assay as described earlier.[6] Fluorescence was measured on a Fluostar Galaxy plate reader (BMG Lab Technologies) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Pentamidine and amphotericin B were used as the standard antileishmanial agents. IC_{50} and IC_{90} values were computed from dose curves generated by plotting percent growth versus drug concentration.

4.3. Assay for in vitro antimalarial activity

The assay is based on the determination of plasmodial LDH activity. For the assay, a suspension of red blood cells infected with D6 or W2 strains of *P. falciparum* (200 μL, with 2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 μg/mL Amikacin) was added to the wells of a 96-well plate containing 10 μL of test samples diluted in medium at various concentrations. The plate was placed in a modular incubation chamber (Billups-Rothenberg, CA) flushed with a gas mixture of 90% N_2, 5% O_2, and 5% CO_2 and incubated at 37 °C, for 72 h. Parasitic LDH activity was determined by using Malstat™ reagent (Flow Inc., Portland, OR) according to the procedure of Makler and Hinrichs.[8] Briefly, 20 μL of the incubation mixture was mixed with 100 μL of the Malstat™ reagent and incubated at room temperature for 30 min. Twenty microliters of a 1:1 mixture of NBT/PES (Sigma, St. Louis, MO) was then added and the plate was further incubated in the dark for 1 h. The reaction was then stopped by the addition of 100 μL of a 5% acetic acid solution. The plate was read at 650 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont). IC_{50} values were computed from the dose–response curves. Artemisinin and chloroquine were included in each assay as the drug controls. Percent growth was plotted versus test concentration to obtain IC_{50} values.

4.4. Assay for in vitro antimicrobial activity

Susceptibility testing against *C. albicans*, *C. neoformans*, methicillin-resistant *S. aureus* (MRS), and *A. fumigatus* was performed using a modified version of the NCCLS methods.[9] Susceptibility testing against *M. intracellulare* was done using the modified Alamar Blue procedure of Franzblau et al.[10] Samples (dissolved in DMSO) were serially diluted using 0.9% saline and transferred in duplicate to 96-well microplates. Microbial inocula were prepared after comparison of the absorbance at 630 nm of cell suspensions to the 0.5 McFarland standard and diluting the suspensions in broth to afford recommended inocula. Microbial inocula were added to the diluted samples to achieve a final volume of 200 μL. Growth, solvent, and media controls were included in each assay. Plates were read at either 630 nm or 544ex/590em (*M. intracellulare*) prior to and after incubation. Percent growth was plotted versus test concentration to afford the IC_{50}.

4.5. Cytotoxicity assay

The in vitro cytotoxicity was determined against a panel of cell lines consisting of SK-MEL (human malignant, melanoma), KB (human epidermal carcinoma), BT-549 (human breast carcinoma), SK-OV-3 (human ovary carcinoma), Vero (monkey kidney fibroblasts), and LLC-PK11 (pig kidney epithelial cells). The assay was performed in 96-well tissue culture-treated plates as described earlier.[7] Briefly, cells were seeded to the wells of 96-well plate (25,000 cells/well) and incubated for 24 h. Samples at different concentrations were added and plates were again incubated for 48 h. The number of viable cells was determined by Neutral Red assay. IC_{50} values were determined from dose curves of percent growth versus test concentrations. Doxorubicin was used as a positive control.

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