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Antiprotozoal and antimicrobial activities of O-alkylated and formylated acylphloroglucinols

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Abstract—In the present article, we examined the antileishmanial, antimalarial, antibacterial, and antifungal activities of several newly synthesized O-alkylated phloroglucinol compounds (**11**–**19**) which are analogues of the naturally occurring antimalarial compound **1**. Analogues **12** and **16** exhibited antileishmanial activity against, *Leishmania donovani* promastigotes with IC_{50}s of 5.3 and 4.2 μg/mL, respectively. Naturally occurring monomeric formylated acylphloroglucinol compounds, grandinol (**2**), jensenone (**3**), and their analogues (**29**–**37**), were also synthesized and evaluated for antileishmanial, antimalarial, antibacterial, and antifungal activities. Amongst these, both grandinol and jensenone showed mild to moderate antibacterial, antifungal, and antileishmanial activities. Jensenone (**3**) was effective against *Candida albicans* with an IC_{50} of 5.5 μg/mL but was ineffective against *Cryptococcus neoformans* and methicillin-resistant *Staphylococcus aureus*. Among the analogues, **34** was the most active against *C. albicans* and *C. neoformans* with IC_{50}s of 2.0 and 2.5 μg/mL, respectively, and was fungicidal toward *Candida albicans*.

1. Introduction

Parasitic diseases such as leishmaniasis and malaria have a high mortality rate having a significant impact in developing countries and affecting several hundred millions of people worldwide. Leishmaniasis is a tropical disease caused by protozoal parasites of the genus *Leishmania* and it remains a significant health issue in large part due to the lack of effective and affordable drugs and increasing resistance against existing drugs.[1] Present drugs are either associated with side effects or are too expensive for use by poor populations. Malaria is one of the most important parasitic diseases in the world and is a major global health problem affecting over one hundred countries with disease prevalence escalating at an alarming rate, particularly in the last two decades. Rapid development of resistance by *Plasmodium falciparum* to the conventional drugs such as chloroquine necessitates the search for new antimalarials.[2] Similarly, an increasing number of multidrug-resistant microbial pathogens have become a serious problem particularly during the last decade and provide the impetus for the search and discovery of novel antibacterial and antifungal agents active against these pathogens.[3]

Naturally occurring phloroglucinol compounds have shown diverse range of biological activities including cancer chemopreventive, antimalarial, antibacterial, HIV-RTase inhibitory, antifouling, etc.[4] The O-prenylated phloroglucinol derivative **1** isolated from the light petroleum ether extract of the aerial parts of *Hypericum calycinum* has shown in vitro antimalarial activity with an IC_{50} of 0.88 μg/mL against *P. falciparum*, while its O-geranylated analogue showed antiviral and antimicrobial activities.[5,6] As well, in a TLC bioassay, **1** demonstrated antifungal activity against the plant pathogen *Cladosporium cucumerinum*.[5] The formylated acylphloroglucinol compound grandinol (**2**), isolated from leaves of *Eucalyptus grandis*, has shown germination inhibitory activity, Epstein-Barr Virus inhibitory activity, and antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus* (IC_{50}s 5 and 25 μg/mL, respectively).[7] Another related acylphloroglucinol compound

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jensenone (**3**), isolated from *Eucalyptus jensenii*, has shown antifeedant activity.[8] Grandinol and jensenone are assumed to be precursors of biologically active phloroglucinols such as sideroxylonals, grandinal, euglobals, and macrocarpals.[4] The acylated phloroglucinol compound, 1,3-diacetyl-2,4,6-trihydroxy benzene (**4**), isolated from the bacterium *Pseudomonas fluorescens*, demonstrated antibacterial activity against *Streptococcus pyogenes*, *Streptococcus faecaelis*, *Streptococcus mutans*, *Streptococcus sanguis*, *S. aureus*, *Clostridium welchii*, and *Lactobacillus casei*.[9] Another phloroacetophenone analogue, xanthoxylin (**5**), obtained from *Xanthoxylum piperitum* exhibited antifungal activity against *C. albicans* and two nonhuman pathogenic fungi, *Bortrytis cinera* and *Phomopsis perniciosa*, with ED_{50}s of 45 and 410 μM on spore germination, respectively, and a complete inhibition of germination of *B. cinera* was observed at 550 μM.[10] Multifidol (**6**) and its glycoside **7** containing 2-methylbutyryl functionality isolated from 20% methanol fraction of the latex of *Jatropha multifida* possessed immunomodulatory activity.[11] Sessiliflorene (**8**) isolated from the hexane extract of *Melicope sessiliflora* showed inhibitory activity against the herpes simplex virus.[12] Similarly caespitin (**9**) and caespitate (**10**) isolated from *Helichrysum caespitium* were found to possess antibacterial activity.[13] Caespitate (**10**), containing an acylated prenyl group on the phloroglucinol nucleus, exhibited cytotoxic activity as well as activity against pathogenic bacteria and fungi including *Mycobacterium tuberculosis*.[13] Acylphloroglucinols have also been proposed as lead compounds for the treatment of degenerative diseases because of their antioxidant properties.[14] The structures of bioactive monomeric phloroglucinol compounds are shown in Figure 1.

Based on the interesting biological activity profile of monomeric acylphloroglucinols, and as a part of our program to explore the naturally occurring phloroglucinol compounds and their analogues for biological potential,[15–17] we have earlier found that phloroglucinol-monoterpene adducts possessed good antileishmanial activity.[16] In this paper, we report antiprotozoal and antimicrobial activities of monomeric phloroglucinol compounds. We have synthesized several new O-alkylated phloroglucinol compounds, **11**–**19**, based on the antimalarial activity of the naturally occurring O-prenylated analogue **1**. Naturally occurring formylated acylphloroglucinols, grandinol (**2**) and jensenone (**3**), of *Eucalyptus* spp. along with several other synthetic analogues **29**–**37** were synthesized and evaluated for antimicrobial and antiprotozoal activities.

2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis of O-alkylated acylphloroglucinols. A number of *O*-alkyl phloroglucinols were designed and synthesized based on the interesting biological activity profile of the naturally occurring antimalarial O-prenylated phloroglucinol derivative, **1**. Compounds were designed by replacing in compound **1**, the prenyl (with allyl and isopentyl), methyl (with formyl, acetyl, and isovaleryl), and the isovaleryl side chain (with an acetyl or isopentyl group) (Table 2).

As the key reaction involved in synthesis of O-alkylated phloroglucinols is O-alkylation, conditions were optimized by varying bases, solvents, and temperature. The reported mono-allylation of phloroglucinol via Zn catalyzed Williamson synthesis[18] however, did not produce satisfactory yield with various alkyl halides. Several other conditions were attempted as shown in Table 1. In most of the cases, results were unsatisfactory, and although the expected product was formed in most of these cases, yields were quite low (2–20%); the expected product was obtained in 60–65% yield only with the use of sodium hydride as a base and DMF as a solvent at room temperature. It is reported that these reactions give C-alkyl-

Table 1. Conditions for O-alkylation of phloroglucinol (**20**)

Reagent/solvent	Reaction condition	Yields[a] (%)
K_2CO_3, acetone	rt, 10 h	15–20
K_2CO_3, acetone	50 °C, 6 h	20–30
NaH, DMF	rt, 10 h	60–65
NaH, DMF	60 °C, 5 h	5–10
K_2CO_3, DMF	rt, 10 h	30
NaH, THF	rt, 10 h	0
K_2CO_3, DMF	rt, 10 h	5–10
K_2CO_3, DMF	60 °C, 10 h	5–10

[a] Isolated yields.

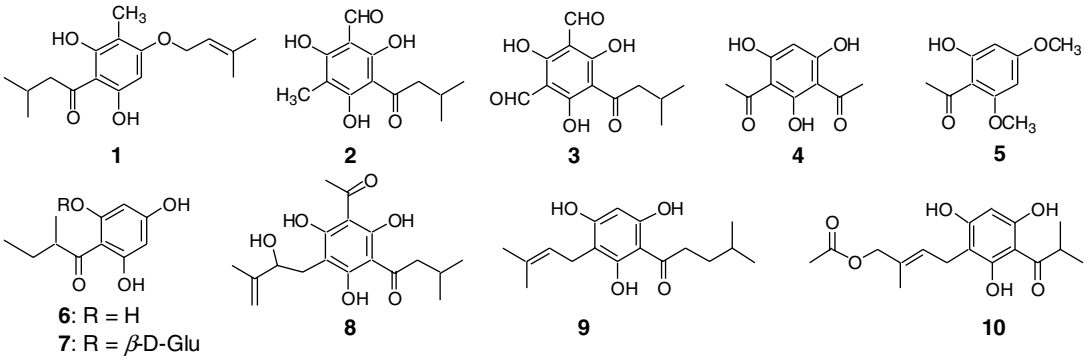


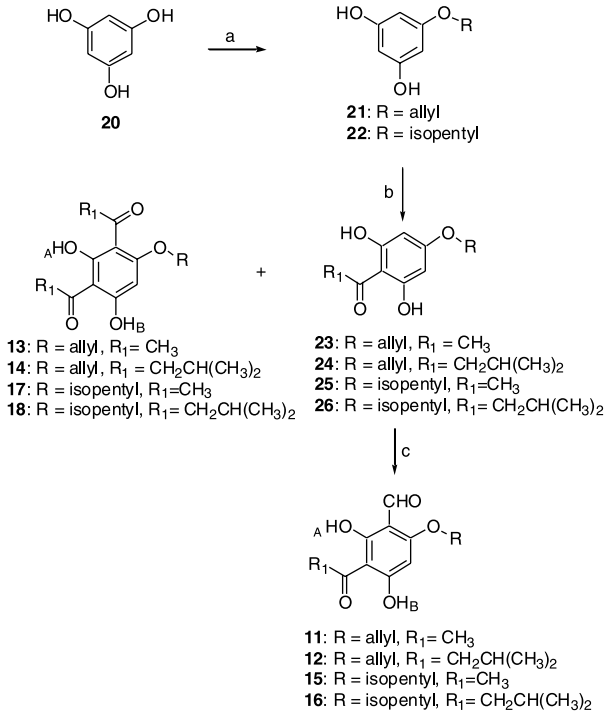
Figure 1. Bioactive monomeric acylphloroglucinols.

ated products via Claisen rearrangement at higher temperature.[19]

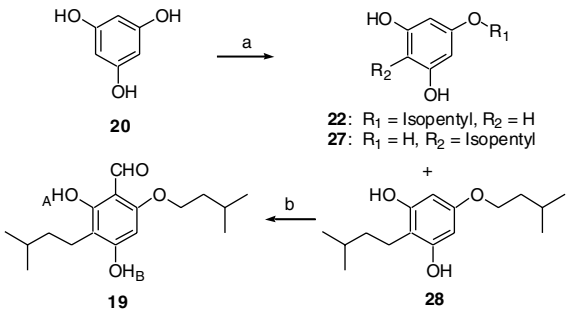
The rate of alkylation of phenolate ions is strongly dependent on the solvent; polar aprotic solvents such as dimethylformamide, dimethylsulfoxide, hexamethyl phosphoric triamide (HMPA), etc., enhance the reactivity of phenolate ions leading to O-alkylation. In contrast, polar protic solvents such as water and alcohols result in formation of C-alkylated product predominantly via direct C-alkylation. If O-allylation reaction in polar aprotic solvents such as DMF is performed at higher temperatures, C-alkylated product is formed as a result of Claisen rearrangement.[19]

Compounds, **11**–**18**, have been synthesized by the strategy shown in Scheme 1.

Treatment of phloroglucinol (**20**) with alkyl halide in the presence of sodium hydride in DMF resulted in the formation of O-alkylated phloroglucinols (**21**–**22**) in 60% yield. O-Alkylated phloroglucinols (**21** and **22**) upon Friedel-Crafts acylation with acyl chloride in the presence of titanium chloride resulted in the formation of mono- (**23**–**26**) and diacylated (**13**–**14** and **17**–**18**) phloroglucinols in 35–40% yield. Monoacyl phloroglucinols (**23**–**26**) further upon Vilsmeier–Haack formylation resulted in the formation of formylated acylphloroglucinols (**11**–**12** and **15**–**16**) in 35–40% yield. Compound **16** has a structural similarity to that of naturally occurring prenylated phloroglucinol compound **1**, differing by presence of *O*-isopentyl (reduced prenyl) and formyl (in place of methyl) functionalities.



Scheme 1. Reagents and conditions: (a) RX, NaH, DMF, 0 °C, 10 h, 60–65%; (b) RCOCl, $TiCl_4$, DCM, 0 °C, 30 min, 35–40%; (c) $POCl_3$, DMF, EtOAc, rt, 1 h, 35–40%.



Scheme 2. Reagents and conditions: (a) $(CH_3)_2CHCH_2CH_2I$, KOH/MeOH:H_2O (4:1), 70 °C, 3 h, 60%; (b) $POCl_3$, DMF, EtOAc, rt, 1 h, 70%.

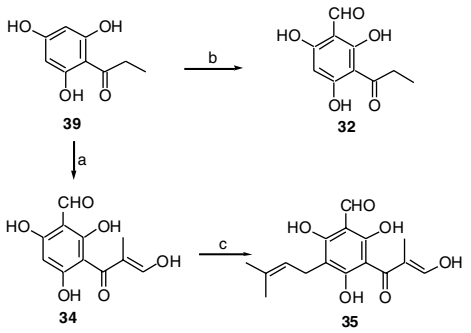
Compound **19**, which possesses a long alkyl side chain in place of the acyl group, has been synthesized as shown in Scheme 2. Treatment of phloroglucinol (**20**) with isoamyl iodide in the presence of methanolic potassium hydroxide resulted in formation of O-alkylated, **22**, C-alkylated, **27**, and O/C-dialkylated phloroglucinol compound, **28**. Compound **28** further upon Vilsmeier–Haack formylation yielded compound **19** in 70% yield.

A typical pattern of chemical shift values for aromatic hydroxyls was observed in the ^1H NMR spectrum of analogues **11**–**18**. ^1H NMR chemical shift for OH_A was downfield in analogues with diacyl functionality compared with those possessing monoacyl functionality. Hydroxyl protons, OH_A and OH_B, appeared downfield in analogues with isovaleryl (**12**, **14**, **16**, and **18**) compared with analogues with acetyl (**11**, **13**, **15**, and **17**) functionality. The location of aromatic hydroxyls, OH_A and OH_B was confirmed by 2-D NMR studies viz. HMQC and HMBC. This observation may be useful in structure elucidation of structurally similar natural unknown O-alkylated acyl phloroglucinols.

2.1.2. Synthesis of formylated acylphloroglucinols. Naturally occurring formylated phloroglucinol compounds, grandinol (**2**) and jensenone (**3**), have been synthesized along with several of their analogues **29**–**37** from commercially available phloroglucinol (**20**) as described earlier.[15,16] Naturally occurring diformyl phloroglucinol jensenone (**3**) has been synthesized by duff's formylation of phloroisovalerophenone.[15] Compound **34** has been synthesized by treatment of phloropropiophenone with five equivalents each of dimethylformamide and phosphorous oxychloride. Compound **34** upon treatment with prenyl bromide in the presence of sodium hydride in dioxane, resulted in formation of C-prenylated phloroglucinol compound **35** (Scheme 3).

The newly synthesized **34** has structural similarity with sessiliflorene (**8**), which possesses antiviral activity against the herpes simplex virus while analogue **35** is similar to caespitin (**9**) and caespitate (**10**), which are reported to possess antibacterial, antimycobacterial, and cytotoxic activities.

All synthesized compounds were characterized by NMR, MS, UV, and IR spectral data. All known acylphloroglucinols were characterized by comparison of



Scheme 3. Reagents and conditions: (a) $POCl_3$/DMF (5 equiv each), EtOAc, rt, 4 h, 40%; (b) $POCl_3$/DMF (1 equiv each), EtOAc, rt, 30 min, 70%; (c) $(CH_3)_2C{=}CH{-}CH_2Br$, NaH, dioxane, 60 °C, 60%.

their spectroscopic data with literature values.[7,8,15,16] The ^{13}C NMR data and C, H, analyses have been taken for all the final compounds.

2.2. Biological evaluation

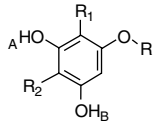
All synthesized compounds were evaluated for in vitro antileishmanial, antimalarial, antibacterial, antifungal, and cytotoxic activities. Antileishmanial activity against *Leishmania donovani* promastigotes was determined by an Alamar Blue™ assay.[20] Most of the O-alkylated acyl phloroglucinols showed moderate to mild activities with IC_{50} values ranging from 4.2 to 40 μg/mL as depicted in Table 2. The most potent antileishmanial activity was exhibited by analogues **12** and **16** (IC_{50} 5.3 and 4.2 μg/mL, respectively), which are structurally related to the naturally occurring antimalarial compound **1**. Analogue **12** differs from **1** by the presence of allyl and formyl functionalities in place of prenyl and methyl moieties, respectively. Similarly, **16** possesses isopentyl and formyl replacing prenyl and methyl of **1**. The IC_{50} of **12** is comparable to that of the drug standard pentamidine. Another interesting finding is that O-alkylated phloroglucinols bearing formyl/isovaleryl moieties (**12** and **16**; IC_{50}s 5.3 and 4.2 μg/mL, respectively) are the most active against *L. donovani* promastigotes as compared to compounds bearing formyl/acetyl (**11** and **15**), acetyl/acetyl (**13** and **17**) or isovaleryl/isovaleryl (**14** and **18**) functionalities. Similar results were obtained in our previous work on phloroglucinol-terpene adducts where natural as well as unnatural euglobals with formyl and isovaleryl moieties possessed potent antileishmanial activity.[16] Replacement of the isovaleryl (**16**) functionality by isopentyl (**19**) also resulted in a decrease in antileishmanial activity. Among, formylated acylphloroglucinols (Table 3), naturally occurring grandinol (**2**), jensenone (**3**), and 1,3-diacetyl-2,4,6-trihydroxy benzene (**4**) along with analogues **31**, **34**, and **35** exhibited mild antileishmanial activities, while the remaining analogues were inactive.

None of these compounds (O-alkylated or formylated) showed in vitro antimalarial activity against chloroquine sensitive (D_6) and chloroquine-resistant (W_2) strains of *P. falciparum*.[21]

The antibacterial activity was tested against methicillin-resistant *S. aureus* and *Mycobacterium intracellulare*. Only naturally occurring formylated phloroglucinol compounds, grandinol (**2**) and jensenone (**3**), exhibited antibacterial activity against methicillin-resistant *S. aureus*, while the rest of the analogues were inactive. Ciprofloxacin was included as positive control for comparison (Table 3).

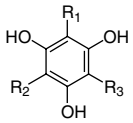
The antifungal activities were evaluated against a panel of pathogenic fungi (*C. albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*) associated with oppor-

Table 2. In vitro antileishmanial activity of O-alkylated acylphloroglucinols (**11**–**19**)[c]

Compound				Antileishmanial activity against *Leishmania donovani*		Cytotoxicity IC_{50}[a,b]	
	R	R_1	R_2	IC_{50}[a,b]	IC_{90}[a,b]	Vero cells	LLC-PK_1 cells
11	$-CH_2CH{=}CH_2$	CHO	$COCH_3$	na	na	nc	nc
12	$-CH_2CH{=}CH_2$	CHO	$COCH_2CH(CH_3)_2$	5.3	31	17.0	9.0
13	$-CH_2CH{=}CH_2$	$COCH_3$	$COCH_3$	na	na	nc	nc
14	$-CH_2CH{=}CH_2$	$COCH_2CH(CH_3)_2$	$COCH_2CH(CH_3)_2$	14	36	nc	nc
15	$-CH_2CH_2CH(CH_3)_2$	CHO	$COCH_3$	7.8	>40	nc	nc
16	$-CH_2CH_2CH(CH_3)_2$	CHO	$COCH_2CH(CH_3)_2$	4.2	>40	nc	nc
17	$-CH_2CH_2CH(CH_3)_2$	$COCH_3$	$COCH_3$	40	>40	nc	nc
18	$-CH_2CH_2CH(CH_3)_2$	$COCH_2CH(CH_3)_2$	$COCH_2CH(CH_3)_2$	24	>40	nc	nc
19	$-CH_2CH_2CH(CH_3)_2$	CHO	$CH_2CH_2CH(CH_3)_2$	10	36	4.6	11.5
Pentamidine				1.5	4.9	nt	nt
Amphoterecin B				0.32	0.9	nt	nt
Doxorubicin				nt	nt	7.5	0.65

[a] IC_{50} and IC_{90}, the concentration that affords 50% and 90% inhibition of leishmanial growth.
[b] Values expressed in terms of μg/mL; nc, not cytotoxic up to 23.8μg/mL; na, not active at 40 μg/mL.
[c] None of the compounds were active against bacteria/fungi; nt, not tested.

Table 3. In vitro antibacterial, antifungal, antileishmanial, and antimalarial activities of formylated acylphloroglucinols (**2**–**3** and **29**–**37**)

Entry				IC_{50}[a,c,d]						
	R_1	R_2	R_3	Methicillin-resistant *Staphylococcus aureus*	*Candida albicans*	*Cryptococcus neoformans*	*Leishmania donovani* (IC_{50}[a]/IC_{90}[b,c])	*Plasmodium falciparum* D_6/W_2	Cytotoxicity against Vero cells	Cytotoxicity against LLC-PK_1 cells
2	$COCH_2CH(CH_3)_2$	CHO	CH_3	8	10	10	21/39	na	8.0	7.0
29	$COCH_2CH_3$	CHO	CH_3	na	5.0	20	na	na	nc*	nc*
30	$COCH_3$	CHO	CH_3	na	15	na	na	na	nc*	nc*
31	$COCH_2CH(CH_3)_2$	CHO	H	na	na	na	27/40	na	14	9.5
32	$COCH_2CH_3$	CHO	H	na	na	15	na	na	18.0	11.0
33	$COCH_3$	CHO	H	na	na	na	na	na	17.5	10.0
4	$COCH_3$	$COCH_3$	H	na	na	na	27/>40	na	12.0	11.0
34	$COC(CH_3)=CHOH$	CHO	H	na	2.0	2.5	29/>40	na	nc	nc
35	$COC(CH_3)=CHOH$	CHO	$CH_2CH=C(CH_3)_2$	na	na	na	16/>40	na	19	14.5
3	$COCH_2CH(CH_3)_2$	CHO	CHO	20	5.5	na	19/35	na	nc*	nc*
36	$CH_2CH_2CH(CH_3)_2$	CHO	CHO	na	na	na	na	na	nc*	nc*
37	CH_3	CHO	CHO	na	na	na	na	na	nc*	nc*
Ciprofloxacin				0.10	nt	nt	nt	nt	nt	nt
Amphoterecin B				nt	0.3	0.7	0.32/ 0.9	nt	nt	nt
Pentamidine				nt	nt	nt	1.5/ 4.9	nt	nt	nt
Artemisinin				nt	nt	nt	nt	14.0/ 7.5	nt	nt
Chloroquine				nt	nt	nt	nt	13.5/ 140	nt	nt
Doxorubicin				nt	nt	nt	nt	nt	7.5	0.65

nc*, not cytotoxic up to 4.76 μg/mL; na, not active at 20 μg/mL (for MRS, *Candida* and *Cryptococcus*); nt, not tested.

[a] IC_{50}, the concentration that affords 50% inhibition of bacterial/fungal/leishmanial growth.

[b] IC_{90}, the concentration that affords 90% inhibition of leishmanial growth.

[c] Values expressed as μg/mL.

[d] None of the compounds showed activity against *Mycobacterium intracellulare* and *Aspergillus fumigatus*; nc, not cytotoxic up to 23.8 μg/mL.

tunistic infections. Amphoterecin B was included as a standard drug for comparison. None of the O-alkylated acylphloroglucinols showed any antifungal activity toward these pathogens.

Among the formylated acylphloroglucinols, jensenone (**3**) exhibited antifungal activity with an IC_{50} value of 5.5 μg/mL against *C. albicans*, while it was inactive against *C. neoformans* and *A. fumigatus*. However, grandinol (**2**) exhibited comparatively weaker antifungal activity with IC_{50} value of 10 μg/mL against *C. albicans* and *C. neoformans*. Among synthetic analogues, compound **34** showed improved antifungal activity against *C. albicans* and *C. neoformans* with an IC_{50} of 2.0 and 2.5 μg/mL, respectively. Compounds **29** and **30** were moderately active against *C. albicans*. None was active against *Aspergillus*.

None of the acylphloroglucinols showed any toxicity toward mammalian kidney fibroblasts (Vero) and kidney epithelial (LLC-PK_1) cells[22] up to a concentration of 23.8 μg/mL, however, some of the formylated acylphloroglucinols were moderately toxic with IC_{50}s in the range of 7–19 μg/mL.

3. Conclusion

O-Alkylated acylphloroglucinols bearing formyl/isovaleryl functionality exhibited antileishmanial activity. Amongst formylated acylphloroglucinols, naturally occurring grandinol (**2**) and jensenone (**3**) exhibited antileishmanial, antibacterial, and antifungal activities. Antileishmanial activity of grandinol, jensenone, and O-alkylated phloroglucinols is reported for the first time. Antimicrobial and antileishmanial properties associated with acylphloroglucinol compounds seem to be dependent on the length of the acyl side chain. Decrease in acyl chain length or replacement of acyl with alkyl resulted in loss of activity.

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) autosampler/direct injection (EI/CI) or LCMS (APCI/ESI). All chromatographic purifications were performed with silica gel (60–120 mesh), whereas all TLC (silica gel) development was performed on silica gel coated (Merck Kiesel $60F_{254}$, 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 O-alkylation: synthesis of 21 and 22

To a suspension of sodium hydride (2 mmol, previously washed with hexane to remove fats) in anhydrous DMF (25 mL), a solution of phloroglucinol (**20**, 1.0 mmol) in anhydrous DMF (30 mL) was added at 0 °C and stirred at room temperature for 2 h. This solution was cooled again at 0 °C and alkyl halide (1.2 mmol) was added slowly and the resulting mixture was stirred at room temperature for 8–10 h. Reaction mixture was diluted with water; extracted with ethyl acetate; washed with brine solution and finally dried over anhydrous sodium sulfate. Solvent evaporation on vacuo rotavapor resulted in crude product, which was purified by silica gel column chromatography using hexane/EtOAc (70:30) to give O-alkylated phloroglucinols, **21** and **22**.

4.1.1. 1-(2-Propenyloxy)-3,5-dihydroxybenzene (21). Yield: 60%; Yellow oil. IR (neat): 3351, 2958, 1608, 1506, 1472, 1367, 1270, 1154, 1061 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.01 (s, 3H, 3× –Ar-*H*), 5.95 (m, 1H, –C*H*=CH_2), 5.36 (d, J = 17.0 Hz, 1H, –CH=CH_2), 5.25 (d, J = 10.4 Hz, 1H, –CH=CH_2), 4.43 (d, J = 5.2 Hz, 2H, –OCH_2); CIMS: m/z 166.9 $[M+1]^+$.

4.1.2. 1-(3-Methyl-butyloxy)-3,5-dihydroxybenzene (22). Yield: 45%; dark brown viscous oil. IR (neat): 3356, 2957, 1607, 1471, 1506, 1471, 1366, 1153, 1050 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.99 (d, J = 1.7 Hz, 2H, 2 × Ar-*H*), 5.94 (d, J = 1.6 Hz, 1H, Ar-*H*), 3.83 (t, J = 6.4 Hz, 2H, –OCH_2–), 1.74 (sept, 1H, –C*H*–), 1.57 (q, J = 6.7, 13.4 Hz, 2H, –O$CH_2C H_2$–), 0.90 (d, J = 6.3 Hz, 6H, 2× –CH_3); CIMS: m/z 196.9 $[M+1]^+$.

4.2. Synthesis of 1-(3-methyl-butyloxy)-4-(3-methyl-butyl)-3,5-dihydroxybenzene (28)

A mixture of phloroglucinol (**20**, 10 g, 1 mmol), potassium hydroxide (6.6 g, 1.5 mmol) and isoamyl iodide (3 mL, 2 mmol) in methanol/water, 4:1 (100 mL) was refluxed for 4 h. Reaction mixture was acidified with dilute HCl; brine solution was added and extracted with ethyl acetate. Ethyl acetate layer was washed with brine solution and dried over anhydrous sodium sulfate. Solvent evaporation on vacuo rotavapor resulted in crude product which was purified by silica gel column chromatography using hexane/EtOAc (70:30) to afford 1-(3-methyl-butyloxy)-3,5-dihydroxybenzene (**22**) (2 g, 20%), 1-isopentyl 2,4,6-trihydroxy benzene (**27**, 3.5 g, 35%) and 1-(3-methyl-butyloxy)-4-(3-methylbutyl)-3,5-dihydroxy benzene (**28**) (300 mg, 5%).

4.2.1. 1-Isopentyl-2,4,6-trihydroxybenzene (27). Yield: 35%; brown sticky solid. IR (neat): 3269, 2957, 2870, 1615, 1470, 1412, 1384, 1282, 1202, 1146, 1112, 1025, 1013 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD): δ 5.83 (s, 2H, 2 × Ar-*H*), 2.48 (t, J = 6.0 Hz, 2H, Ar-CH_2), 1.55 (m, 1H, –$CH_2C H$–), 1.34 (m, 2H, –CH_2CH–), 0.93 (d, J = 6.6 Hz, 6H, 2× –CH_3); CIMS: m/z 196.9 $[M+1]^+$.

4.2.2. 1-(3-Methyl-butyloxy)-4-(3-methylbutyl)-3,5-dihydroxybenzene (28). Yield: 5%; light brown solid; mp 147–149 °C. IR (KBr): 3410, 3328, 2952, 2871, 2376, 1628, 1609, 1532, 1442, 1406, 1366, 1206, 1153, 1112, 1018 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$+CD_3OD, 4:1): δ 5.97 (s, 2H, 2 × Ar-*H*), 3.89 (t, J = 6.3 Hz, 2H, –OCH_2–), 2.53 (t, J = 8.1 Hz, 2H, Ar-CH_2–), 1.78 (m, 2H, 2× –C*H*–), 1.62 (m, 2H, –OCH_2CH_2–CH), 1.40 (m, 2H, ArCH_2-CH_2–), 0.94 (d, J = 6.6 Hz, 6H, 2× –CH_3), 0.90 (d, J = 6.7 Hz, 6H, 2× –CH_3); CIMS: 266.9 $[M+1]^+$.

4.3. General method for Friedel-Crafts acylation: synthesis of 23–26, 13–14 and 17–18

To the solution of O-alkylated phloroglucinols (**21** and **22**, 1 mmol) in DCM, titanium chloride and acyl chloride (3 mmol) was added at 0 °C and reaction mixture was stirred at room temperature for 30 min. The excess titanium chloride was quenched with methanol (5 mL) and solvent was removed from the reaction mixture under reduced pressure. The crude reaction mixture was purified by silica gel column chromatography using hexane/EtOAc (50:50) as eluent to yield mono- (**23**–**26**) and di- (**13**–**14** and **17**–**18**) acylphloroglucinols.

4.3.1. 1-(2-Propenyloxy)-4-acetyl-3,5-dihydroxybenzene (23). Yield: 35%; off white solid; mp 145–147 °C. IR (KBr): 3184, 2915, 1645, 1587, 1446, 1372, 1289, 1181, 1090, 1073, 1027 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 6.01 (m, 1H, –C*H*=CH_2), 5.94 (s, 2H, 2 × Ar-*H*), 5.39 (d, J = 17.3 Hz, 1H, –CH=CH_2), 5.31 (d, J = 10.7 Hz, 1H, –CH=CH_2), 4.51 (d, J = 5.3 Hz, 2H, –OCH_2), 2.70 (s, 3H, CH_3); CIMS: *m*/*z* 208.9 $[M+1]^+$.

4.3.2. 1-(2-Propenyloxy)-4-(3-methyl-butyryl)-3,5-dihydroxybenzene (24). Yield: 20%; light brown viscous oil. IR (neat): 3447, 2960, 2873, 1625, 1583, 1460, 1412, 1368, 1298, 1195, 1129, 1045 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 6.01 (m, 1H, –C*H*=CH_2), 5.95 (s, 2H, 2× Ar-*H*), 5.41 (d, J = 17.2 Hz, 1H, –CH=CH_2), 5.30 (d, J = 10.9 Hz, 1H, –CH=CH_2), 4.49 (d, J = 5.2 Hz, 2H, –OCH_2), 2.94 (d, J = 6.8 Hz, 2H, –COCH_2), 2.25 (m, 1H, –C*H*), 0.98 (d, J = 6.4 Hz, 6H, 2× –CH_3); CIMS: *m*/*z* 250.8 $[M+1]^+$.

4.3.3. 1-(3-Methyl-butyloxy)-4-(acetyl)-3,5-dihydroxybenzene (25). Yield: 40%; dark brown viscous oil. IR (neat): 3374, 2956, 2863, 2361, 1619, 1588, 1429, 1367, 1286, 1194, 1128, 1050 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 5.91 (s, 2H, 2× Ar-*H*); 3.93 (t, J = 6.8 Hz, 2H, –OCH_2), 2.66 (s, 3H, –COCH_3), 1.77 (m, 1H, –C*H*), 1.63 (m, 2H, –OCH_2CH_2), 0.98 (d, J = 6.8 Hz, 6H, 2× CH_3); CIMS: *m*/*z* 239 $[M+1]^+$.

4.3.4. 1-(3-Methyl-butyloxy)-4-(3-methyl-butyryl)-3,5-dihydroxybenzene (26). Yield: 35%; off white solid; mp 168–172 °C. IR (KBr): 3352, 2959, 2871, 1634, 1602, 1583, 1525, 1465, 1432, 1368, 1300, 1257, 1206, 1180, 1084, 1037, 1005 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 5.92 (s, 2H, 2× Ar-*H*), 3.98 (t, J = 6.7 Hz, 2H, –OCH_2), 2.97 (d, J = 6.7 Hz, 2H, –COCH_2), 2.24 (m, 1H, –C*H*), 1.80 (m, 1H, –C*H*), 1.65 (m, 2H, –CH_2), 0.98 (d, J = 6.4 Hz, 6H, 2× –CH_3), 0.94 (d, J = 6.3 Hz, 6H, 2× –CH_3); CIMS: *m*/*z* 281 $[M+1]^+$.

4.3.5. 1-(2-Propenyloxy)-2,4-diacetyl-3,5-dihydroxybenzene (13). Yield: 6%; white solid; mp 114–116 °C; UV (MeOH): λ_{max} ($\log\varepsilon$) 267 nm (4.65). IR (neat): 3435, 3092, 1616 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 16.26 (s, 1H, OH_A), 14.80 (s, 1H, OH_B), 6.05 (m, 1H, C*H*=CH_2), 5.90 (s, 1H, Ar-*H*), 5.45 (d, J = 17.0 Hz, 1H, –CH=CH_2), 5.38 (d, J = 10.4 Hz, 1H, –CH=CH_2), 4.64 (d, J = 5.7 Hz, 2H, –OCH_2–), 2.72 (s, 3H, –COCH_3), 2.69 (s, 3H, –COCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 204.4, 203.3, 171.9, 171.7, 166.0, 131.3, 119.7, 105.1, 104.3, 92.2, 70.2, 33.1, 33.0; CIMS: *m*/*z* 250.9 $[M+1]^+$; analysis for $C_{13}H_{14}O_5$ (250.2), calcd, C, 62.39; H, 5.64; found, C, 62.24; H, 5.79.

4.3.6. 1-(2-Propenyloxy)-2,4-di-(3-methyl-butyryl)-3,5-dihydroxybenzene (14). Yield: 10%; yellow sticky solid; UV (MeOH): λ_{max} ($\log\varepsilon$) 276 nm (4.29). IR (neat): 2959, 1767, 1621, 1465, 1412, 1368, 1292, 1195, 1148, 1122 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 16.50 (s, 1H, OH_A), 14.94 (s, 1H, OH_B), 6.09 (m, 1H, C*H*=CH_2), 5.85 (s, 1H, Ar-*H*), 5.49 (d, J = 17.3 Hz, 1H, CH=CH_2), 5.41 (d, J = 10.4 Hz, 1H, CH=CH_2), 4.61 (d, J = 5.5 Hz, 2H, –OCH_2–), 2.98 (d, J = 5.7 Hz, 2H, –COCH_2), 2.87 (d, J = 5.95 Hz, 2H, –COCH_2), 2.23 (m, 2H, 2× –C*H*–), 0.99 (d, J = 5.2 Hz, 6H, 2× –CH_3), 0.96 (d, J = 5.9 Hz, 6H, 2× –CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 207.3, 206.4, 172.5, 172.3, 166.1, 131.9, 120.4, 105.8, 104.8, 92.9, 70.7, 53.6, 53.5, 26.0, 25.4, 23.3, 23.2; CIMS: *m*/*z* 334.9 $[M+1]^+$; analysis for $C_{19}H_{26}O_5$ (334.4), calcd, C, 68.24; H, 7.84; found, C, 68.35; H, 7.91.

4.3.7. 1-(3-Methyl-butyloxy)-2,4-diacetyl-3,5-dihydroxybenzene (17). Yield: 12%; yellow solid; mp 69–71 °C; UV (MeOH): λ_{max} ($\log\varepsilon$) 280 nm (4.14). IR (KBr): 2956, 2870, 1622, 1588, 1367, 1291, 1200, 1126 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 16.27 (s, 1H, OH_A), 14.79 (s, 1H, OH_B), 5.89 (s, 1H, Ar-*H*), 4.10 (t, J = 6.4 Hz, 2H, –OCH_2–), 2.70 (s, 3H, –COCH_3), 2.63 (s, 3H, –COCH_3), 1.86–1.73 (m, 3H, –CH_2–C*H*–), 0.99 (d, J = 6.1 Hz, 6H, 2× –CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 204.9, 203.8, 172.6, 172.3, 167.2, 105.5, 104.8, 92.4, 68.7, 38.0, 33.5, 25.7, 23.0; CIMS: *m*/*z* 281 $[M+1]^+$; analysis for $C_{15}H_{20}O_5$ (280.3), calcd, C, 64.27; H, 7.19; found, C, 64.13; H, 7.08.

4.3.8. 1-(3-Methyl-butyloxy)-2,4-di-(3-methyl-butyryl)-3,5-dihydroxybenzene (18). Yield: 17%; yellow solid; mp 65–67 °C; UV (MeOH): λ_{max} ($\log\varepsilon$) 271 nm (4.24). IR (KBr): 3020, 2400, 1618, 1585, 1420 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 16.48 (s, 1H, OH_A), 14.96 (s, 1H, OH_B), 5.90 (s, 1H, Ar-*H*), 4.09 (t, J = 6.5 Hz, 2H, –OCH_2–), 3.00 (d, J = 5.1 Hz, 2H, –COCH_2–), 2.91 (d, J = 5.5 Hz, 2H, –COCH_2–), 2.25 (m, 3H, 3× –C*H*–), 0.98 (d, J = 6.0 Hz, 18H, 6× –CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 207.3, 206.2, 172.6, 172.3, 166.8, 105.6, 104.8, 92.5, 68.5, 53.5, 38.1, 25.7, 25.4, 23.3, 22.9; CIMS: *m*/*z* 365 $[M+1]^+$; analysis for $C_{21}H_{32}O_5$ (364.5), calcd, C, 69.20; H, 8.85; found, C, 69.11; H, 8.77.

4.4. General method for Vilsmeier–Haack formylation: synthesis of 11, 12, 15, 16 and 19

To the solution of **23**–**26** and **28** (1 mmol) in ethyl acetate (15 mL) were added dimethyl formamide (1 mmol) and phosphoryl chloride (1.1 mmol) at room temperature. Reaction mixture was further stirred for 30 min. Reaction mixture was diluted with water; extracted with ethyl acetate; washed with brine solution and finally dried over Na_2SO_4. The crude product was purified by column chromatography over silica gel using hexane/EtOAc (70:30) as eluent to afford formylated acylphloroglucinols (**11**–**12**, **15**–**16** and **19**).

4.4.1. 1-(2-Propenyloxy)-4-acetyl-2-formyl-3,5-dihydroxybenzene (11). Yield: 30%; cream white solid; mp 102–105 °C; UV (MeOH): λ_{max} ($\log\varepsilon$) 269 nm (4.43). IR (KBr): 3705, 3563, 3092, 2929, 2592, 1741, 1612, 1277 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 14.89 (s, 1H, OH_B), 14.53 (s, 1H, OH_A), 10.07 (s, 1H, C*H*O), 6.04 (m, 1H, –C*H*=CH_2), 5.91 (s, 1H, Ar-*H*), 5.43 (d, J = 17.4 Hz, 1H, –CH=CH_2), 5.37 (d, J = 10.5 Hz, 1H, –CH=CH_2), 4.62 (d, J = 5.1 Hz, 2H, –OCH_2–), 2.71 (s, 3H, –COCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 203.8, 191.7, 173.8, 169.8, 166.1, 131.1, 119.1, 104.3, 92.4, 69.6, 32.7; EIMS: *m*/*z* 236 $[M]^+$; analysis for $C_{12}H_{12}O_5$ (236.2), calcd, C, 61.01; H, 5.12; found, C, 61.07; H, 5.01.

4.4.2. 1-(2-Propenyloxy)-4-(3-methyl-butyryl)-2-formyl-3,5-dihydroxybenzene (12). Yield: 40%; light violet solid; mp 96–98 °C; UV (MeOH): λ_{max} ($\log\varepsilon$) 270 nm (4.54). IR (KBr): 3402, 3084, 2959, 2928, 2872, 1627, 1579 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 15.11 (s, 1H, OH_B), 14.61 (s, 1H, OH_A), 10.07 (s, 1H, C*H*O), 6.02 (m, 1H, –C*H*=CH_2), 5.91 (s, 1H, Ar-*H*), 5.43 (d, J = 17.2 Hz, 1H, –CH=CH_2), 5.37 (d, J = 10.2 Hz, 1H, –CH=CH_2), 4.62 (d, J = 5.6 Hz, 2H, –OCH_2–), 2.99 (d, J = 6.7 Hz, 2H, –COCH_2–), 2.25 (m, 1H, –C*H*–), 0.99 (d, J = 6.3 Hz, 6H, 2× –CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 206.3, 191.7, 174.2, 169.7, 165.9, 131.2, 119.1, 104.4, 92.5, 69.6, 52.7, 24.9, 22.7; EIMS: 277 $[M-1]^+$; analysis for $C_{15}H_{18}O_5$ (278.3), calcd, C, 64.74; H, 6.52; found, C, 64.70; H, 6.48.

4.4.3. 1-(3-Methyl-butyloxy)-4-acetyl-2-formyl-3,5-dihydroxybenzene (15). Yield: 30%; white solid; mp 130–132 °C; UV (MeOH): λ_{max} ($\log\varepsilon$) 269 nm (4.36). IR (KBr): 3402, 3077, 2956, 2870, 1616, 1576 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 14.88 (s, 1H, OH_B), 14.52 (s, 1H, OH_A), 10.02 (s, 1H, C*H*O), 5.91 (s, 1H, Ar-*H*), 4.10 (t, J = 6.7 Hz, 2H, –OCH_2–), 2.70 (s, 3H, –COCH_3), 1.86 (m, 1H, –C*H*–), 1.74 (m, 2H, –OCH$_2$CH_2–), 0.98 (d, J = 7 Hz, 6H, 2× –CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 203.8, 191.7, 173.9, 169.8, 167.0, 104.3, 92.0, 67.7, 37.2, 32.6, 25.1, 22.4; CIMS: *m*/*z* 266.9 $[M+1]^+$; analysis for $C_{14}H_{18}O_5$ (266.3), calcd, C, 63.15; H, 6.81; found, C, 63.10; H, 6.71.

4.4.4. 1-(3-Methyl-butyloxy)-4-(3-methyl-butyryl)-2-formyl-3,5-dihydroxybenzene (16). Yield: 40%; off white solid; mp 85–87 °C; UV (MeOH): λ_{max} ($\log\varepsilon$) 271 nm (4.55). IR (KBr): 3077, 2958, 2863, 1621, 1494 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 15.11 (s, 1H, OH_B), 14.60 (s, 1H, OH_A), 10.05 (s, 1H, C*H*O), 5.91 (s, 1H, Ar-*H*), 4.09 (t, J = 6.1 Hz, 2H, –OCH_2–), 2.99 (d, J = 6.6 Hz, 2H, –COCH_2–), 2.25 (m, 1H, –C*H*–), 1.79 (m, 1H, –C*H*–), 1.73 (m, 2H, –OCH$_2$CH_2–), 0.99 (d, J = 4.0 Hz, 6H, 2× –CH_3), 0.97 (d, J = 3.7 Hz, 6H, 2× –CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 206.2, 191.7, 174.3, 169.7, 166.5, 104.4, 104.2, 92.5, 67.7, 52.6, 37.3, 25.1, 24.9, 22.7, 22.4; CIMS: *m*/*z* 308.8 $[M+1]^+$; analysis for $C_{17}H_{24}O_5$ (308.3), calcd, C, 66.21; H, 7.84; found, C, 66.13; H, 7.87.

4.4.5. 1-(3-Methyl-butyloxy)-2-formyl-4-(3-methylbutyl)-3,5-dihydroxybenzene (19). Yield: 70%; cream solid; mp 166–168 °C; UV (MeOH): λ_{max} ($\log\varepsilon$) 279 nm (4.19). IR (KBr): 3175, 2957, 2872, 1650, 1592, 1505, 1470, 1443, 1385, 1322, 1286, 1265, 1210, 1146, 1124, 1082 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 12.64 (s, 1H, OH_A), 10.11 (s, 1H, C*H*O), 5.87 (s, 1H, Ar-*H*), 5.48 (br s, 1H, OH_B), 3.99 (t, J = 6.4 Hz, 2H, –OCH_2), 2.53 (t, J = 8.0 Hz, 2H, Ar-CH_2), 1.82 (m, 2H, 2× –C*H*), 1.71 (m, 4H, 2× –CH_2), 0.97 (d, J = 4.0 Hz, 6H, 2× –CH_3), 0.95 (d, J = 4.2 Hz, 6H, 2× –CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 191.5, 164.2, 163.2, 161.4, 108.7, 105.3, 90.6, 66.6, 37.9, 37.5, 28.8, 25.0, 22.4, 22.3, 19.6; CIMS: *m*/*z* 294.9 $[M+1]^+$; analysis for $C_{17}H_{26}O_4$ (294.4), calcd, C, 69.36; H, 8.90; found, C, 69.18; H, 9.02.

4.5. Synthesis of 2,4,6-trihydroxy-3-(3-hydroxy-2-methyl-acryloyl)-benzaldehyde (34)

To a solution of phloropropiophenone (1.0 g, 5.49 mmol) in ethyl acetate (10 mL), dimethylformamide (2.12 mL, 27.45 mmol) and phosphoryl chloride (2.56 mL, 27.45 mmol) was added and reaction mixture were stirred at room temperature for 3 h. Water was added to the reaction mixture and extracted with ethyl acetate. Solvent was evaporated under reduced pressure and crude product was purified by silica gel column chromatography using hexane/EtOAc (70:30) as eluent to afford **34**. Yield: 60%; yellow solid; mp 149–151 °C; UV ($CHCl_3$): λ_{max} ($\log\varepsilon$) 276 nm (4.00). IR (KBr): 3076, 2927, 1645, 1455, 1320, 1252, 1187, 1081 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz): δ 14.22 (s, 1H, O*H*), 12.37 (s, 1H, O*H*), 10.34 (s, 1H, C*H*O), 7.68 (s, 1H, –C*H*OH), 6.28 (s, 1H, Ar-*H*), 2.00 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$): δ 192.8, 182.6, 167.9, 167.4, 162.3, 152.4, 119.7, 106.5, 104.1, 94.5, 10.1; EIMS: *m*/*z* 220 ($M-H_2O$), 192, 163, 124, 69; analysis for $C_{11}H_{10}O_6$ (238.2), calcd, C, 55.47; H, 4.23; found, C, 55.41; H, 4.16.

4.6. Synthesis of 2,4,6-trihydroxy-3-(3-hydroxy-2-methyl-acryloyl)-5-(3-methyl-but-2-enyl)-benzaldehyde (35)

The mixture of **34** (0.2 g, 0.84 mmol) and sodium hydride (0.06 g, 2.52 mmol) in dioxane (10 mL) was heated at 60 °C for 30 min. Prenyl bromide (0.145 mL, 1.26 mmol) was added and reaction mixture was stirred further at 60 °C for 2 h. Reaction mixture was allowed to cool, filtered, and solvent evaporated under reduced pressure and crude product was purified by silica gel

column chromatography using hexane/EtOAc (80:20) as eluent to afford **35**. Yield: 39%; white solid; mp 118–120 °C; UV ($CHCl_3$): λ_{max} ($\log\varepsilon$) 282 nm (4.03). IR (KBr): 3564, 2925, 1645, 1444, 1297, 1270, 1188, 1100 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz): δ 14.14 (s, 1H, O*H*), 12.61 (s, 1H, O*H*), 10.36 (s, 1H, C*H*O), 7.74 (s, 1H, =C*H*OH), 5.15 (t, J = 5.1 Hz, 1H, –CH_2–C*H*=C), 3.36 (d, J = 7.0 Hz, 2H, ArCH_2-CH), 1.78 (s, 3H, CH_3), 1.67 (s, 3H, CH_3), 1.58 (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 193.2, 183.5, 166.6, 165.3, 160.2, 153.0, 133.3, 121.5, 119.9, 107.4, 106.7, 104.2, 26.3, 21.1, 18.3, 10.6; EIMS: *m*/*z* 288 [M−H_2O], 274, 245, 233, 193, 69; analysis for $C_{16}H_{18}O_6$ (306.3), calcd, C, 62.74; H, 5.92; found, C, 62.60; H, 5.80.

4.7. Assay for in vitro antileishmanial activity

Antileishmanial activity of the compounds was tested in vitro against a culture of *L. donovani* promastigotes. They were 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.[20] Standard fluorescence was measured on a Fluostar Galaxy plate reader (BMG Lab Technologies) at excitation wavelength of 544 nm and emission wavelength of 590 nm. Pentamidine and Amphoterecin B were used as the standard antileishmanial agents. IC_{50} and IC_{90} values were computed from dose-response curves generated by plotting percent growth versus drug concentration.

4.8. 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 96well 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.[21] 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 is 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. DMSO (0.25%) was used as vehicle control.

4.9. 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.[23] Susceptibility testing against *M. intracellulare* was done using the modified Alamar Blue procedure of Franzblau et al.[24] 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}.

4.10. Cytotoxicity assay

The in vitro cytotoxicity was determined against mammalian kidney fibroblasts (VERO) and kidney epithelial ($LLC\text{-}PK_1$) cells. The assay was performed in 96-well tissue culture-treated plates as described earlier.[22] Briefly, cells were seeded to the wells of the plate (25,000 cells/well) and incubated for 24 h. Samples 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 logarithmic graphs of growth inhibition versus concentration. Doxorubicin was used as a positive control, while DMSO was used as vehicle control.

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