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Glycolipids and other constituents from *Desmodium gangeticum* with antileishmanial and immunomodulatory activities☆

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Abstract—Nineteen compounds of various classes, such as flavonoid glycosides, pterocarpanoids, lipids, glycolipids, and alkaloids, were isolated and identified from the *Desmodium gangeticum* whole plant. Aminoglucosyl glycerolipid (**8**) is reported here for the first time. Its structure has been elucidated by spectroscopic and degradation studies. This novel compound exhibited in vitro antileishmanial and immunomodulatory activities, as it enhanced nitric oxide (NO) production and provided resistance against infection established in peritoneal macrophages by the protozoan parasite *Leishmania donovani*. Another known compound, glycosphingolipid (cerebroside) (**7**) was found to possess significant in vitro antileishmanial and immunomodulatory activities against the same parasite. Other compounds were found to be inactive.

1. Introduction

Desmodium gangeticum DC (Leguminosae, suborder Papilionaceae) is a small perennial shrub growing throughout India. The plant is of great therapeutic value in treating typhoid, piles, inflammation, asthma, bronchitis, and dysentery.[1] The aqueous extracts of this species are reported to have strong antiwrithing activity and moderate central nervous system (CNS) depressant activity.[2] Total alkaloids of this species possess smooth muscle stimulant, anticholinesterase, CNS stimulant, and antileishmanial activities.[3] Chemical investigations have revealed that this plant contains alkaloids such as tryptamines, phenethylamines, and their N-oxides;[4] pterocarpanoids such as gangetin, gangetinin, desmodin, and desmocarpin;[5] phospholipids,[6] sterols,[7] and flavone glycosides, 4′,5,7-trihydroxy-8-prenylflavone-4′-*O*-α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranoside,[8] and 8-C-prenyl-5,7,5′-trimethoxy-3′,4′-methylenedioxy flavone[9] have also been reported from this plant.

Visceral leishmaniasis (VL) or Kala-azar is the most dreaded disease amongst various forms of leishmaniasis complexes. It is caused by *Leishmania donovani*, an obligate intracellular protozoan parasite belonging to the family Trypanosomatidae. In our earlier studies, the chloroform and *n*-butanol fractions of the ethanolic extract of this plant have been reported to exert significant chemoprophylactic, as well as leishmanicidal activities, against experimental visceral leishmaniasis in hamsters.[10] In view of this aspect, we decided to perform activity-guided isolation of pure compounds from the bioactive fractions and to evaluate them for the antileishmanial and immunostimulant activities. Our efforts resulted in the isolation of nineteen compounds out of which, we have subjected eight compounds, **7**, **8**, **9**, **14**, **15**, **17**, **18**, and **19**, to undergo determination of their antileishmanial and immunomodulatory activities by in vitro assays.

2. Results and discussion

Repeated-column chromatography of chloroform-soluble fraction of the ethanolic extract of *Desmodium gangeticum* whole plant afforded 10 compounds, **1**–**10**, while the *n*-butanol fraction afforded nine compounds, **11**–**19** (Fig. 1).

Keywords: *Desmodium gangeticum*; Glycolipids; Flavonoids; Alkaloids; *Leishmania donovani*; Immunomodulation; Antileishmanial.
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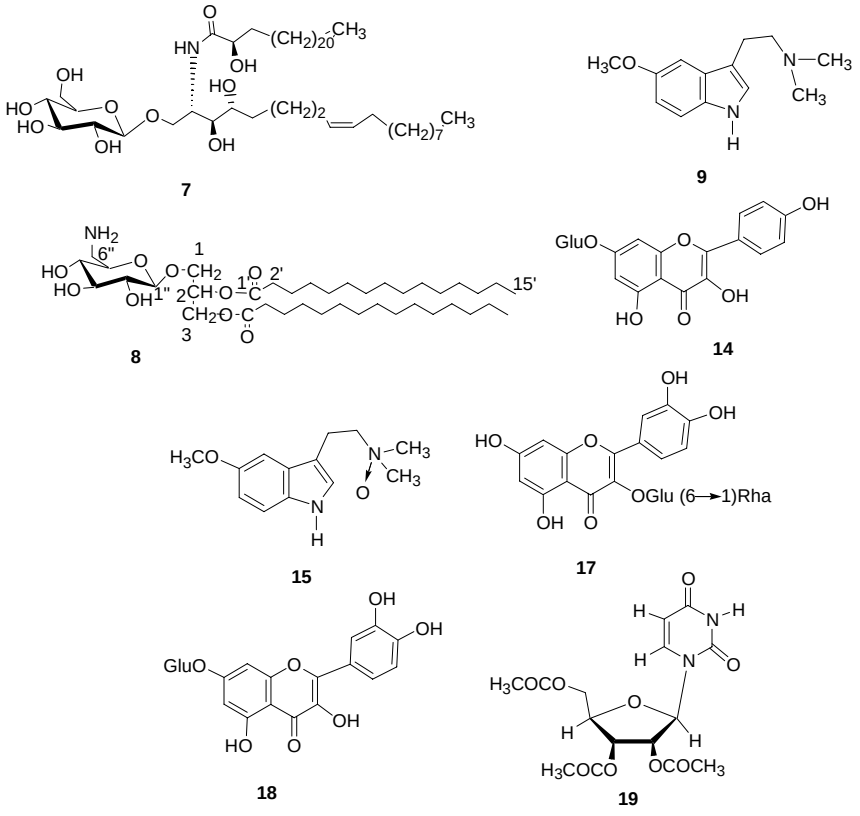

Figure 1. Isolated compounds tested for the activity.

Powdered whole plant (6.5 kg) was extracted with ethanol by percolation, afforded extract (425 g). Ethanolic extract (400 g) was fractionated into $CHCl_3$ (122.8 g) and *n*-butanol (75.2 g) soluble fractions. The $CHCl_3$ fraction was subjected to column chromatography over flash silica gel (230–400 mesh), eluting with a gradient of hexane/$CHCl_3$ (1:0 to 0:1) and then eluting with a gradient of $CHCl_3$/MeOH (1:0 to 8:2) to afford nine fractions (F-1 to F-9). Flash CC of F-2 using hexane/$CHCl_3$ (9:1) afforded **1** (520 mg), **2** (350 mg), and **3** (150 mg); similar purification of F-3 using $CHCl_3$/MeOH (98:2) as eluent yielded **4** (700 mg), while CC of F-4 afforded **5** (150 mg). CC purification of F-5, performed with a gradient of $CHCl_3$/MeOH (98:2 to 95:5), afforded **6** (20 mg). Successive purification of F-6 by reverse-phase flash chromatography, eluted with a gradient of MeOH/H_2O (1:9 to 4:6) afforded **7** (100 mg). The fraction F-7 was purified by reverse-phase flash chromatography, eluted with a gradient of MeOH/H_2O (1:9 to 4:6), to afford **8** (60 mg). The repeated-column chromatography of fraction F-8, eluted with a gradient of $CHCl_3$/MeOH (95:5 to 85:15), affording the compounds **9** (40 mg) and **10** (24 mg).

The *n*-butanol fraction was subjected to column chromatography over flash silica gel (230–400 mesh), eluting with a gradient of $CHCl_3$/MeOH (1:0 to 8:3) to give fractions F-14 to F-23. Flash CC of F-14 using a gradient elution of $CHCl_3$/MeOH (97:3 to 9:1) afforded compound **11** (700 mg). Flash CC of F-15 using a gradient elution of $CHCl_3$/MeOH (1:0 to 9:1), afforded two compounds **12** (15 mg) and **13** (50 mg). Similar purification of F-16 by flash CC with a gradient elution of $CHCl_3$/MeOH (1:0 to 8:3) afforded three compounds **14** (150 mg), **15** (85 mg), and **16** (280 mg). Similarly, compounds **17** (400 mg) and **18** (250 mg) were obtained by repeated-column chromatography of fractions F-17 and F-18 by gradient elution with $CHCl_3$/MeOH (93:7 to 85:15). Repeated efforts to obtain compound **19** from fraction F-19 were not successful; therefore, the fraction F-23 was acetylated using pyridine and acetic anhydride under standard conditions, which was purified further by flash CC with a constant elution of $CHCl_3$/MeOH (98:2), to afford compound **19** (320 mg) as its acetate.

Compound **8** revealed strong absorption peaks at 3416 cm^{-1} for hydroxyl and amino groups and at 1737 cm^{-1} for the ester carbonyl group in the IR spectrum. FAB-MS spectrum showed a molecular ion peak $[M+H]^+$ at *m/z* 702, while its elemental analysis (found (%): C, 67.1; H, 10.9; N, 2.2; required (%): C, 66.7; H, 10.7; N, 2.0.) indicated the molecular formula to be $C_{39}H_{75}NO_9$. The ^1H NMR spectrum (Table 1) exhibited two terminal methyl signals at δ 0.84 (6H, t, $J = 6.3$ Hz), a broad signal at δ 1.23 (44H, br s) due to methylene protons, a signal at δ 2.26 (4H, m) due to two methylene protons linked to the ester functional group, signal at δ 1.49 (4H, m) due to methylene protons adjacent to the terminal methyl group while protons of the 6-amino 6-deoxy glucopyranosyl moiety appeared in the region δ 2.56–4.55 and protons of the glycerol unit at 4.14 (1H, dd, $J = 7.2$, 11.2 Hz), 4.34 (1H, d, $J = 12.0$ Hz), 5.14 (1H, br s), 3.44 (1H, dd, $J = 10.4$, 4.7 Hz), 3.89 (1H, dd, $J = 9.6$, 5.7 Hz), as determined by the ^1H–^1H COSY

Table 1. ^{1}H and ^{13}C data of compound **8** in DMSO-d_6 (200 MHz)

Position	δ_H (J in Hz)	δ_C
1a	4.14 (1H, dd, 7.2, 11.7)	62.7
1b	4.34 (1H, d, 12.0)	—
2	5.14 (1H, br s)	69.7
3a	3.44 (1H, dd, 10.4, 4.7)	64.6
3b	3.89 (1H, dd, 9.6, 5.7)	—
1″	4.55 (1H, br s)	98.3
2″	3.18 (1H, br s)	71.6
3″	3.34 (1H, dd, 9.1, 9.6)	72.9
4″	2.92 (1H, m),	74.1
5″	3.77 (1H, br s)	68.4
6″a	2.56 (1H, d, 6.9)	54.3
6″b	2.88 (1H, m)	—
1′	—	172.3, 172.5
2′	2.26 (2 × 2H, m)	33.4, 33.3
3′-13′	1.23 (2 × 22H, br s)	29.8–31.1
14′	1.49 (2 × 2H, m)	22.1
15′	0.84 (2 × 3H, t, 6.3)	13.8

spectrum. The ^{13}C NMR spectrum (Table 1) showed two quaternary carbons at δ 172.3 and 172.5 for ester carbonyl functions, two methyl carbons at δ 13.8, twenty-six methylene carbons in the region δ 22.13–33.45, three carbon signals characteristic of glycerol moiety at δ 62.7 (CH_2–O), 69.7 (CH–O), and 64.6 (CH_2–O), a peculiar methylene carbon at δ 54.3 attached to the amino group,[11] and an anomeric carbon at δ 98.3. The β-linkage of sugar with glycerol unit was determined by the appearance of an anomeric proton at δ 4.55 as doublet with 7.5 Hz coupling. The compound gave positive Fiegel's test for glycosides but the sugar could not be identified as any of the normal pyranose sugars by hydrolysis.

Absence of the CH_2-6″ signal in ^{13}C NMR around δ 61 instead of the appearance of a CH_2 signal at δ 54.3, indicated the attachment of an amino group at CH_2-6″ in the sugar unit. Further, when the compound was hydrolyzed in MeOH and NaOMe,[12] it gave pentadecanoic acid (CH_3–$(CH_2)_{13}$–COOH), as analyzed by FAB-MS and compared with an authentic sample, indicating the length the of fatty acid side chain attached to the glycerol unit at C-2 and C-3. Presence of 6-amino, 6-deoxy glucose was determined in the aqueous fraction by comparing the TLC with the standard sample.[13] Therefore, compound **8** was identified as an aminoglucosyl glycerolipid (pentadecanoic acid-3-(6-aminomethyl-3,4,5-trihydroxy-tetrahydro-pyran-2-yloxy)-2-pentadecanoyloxy-propyl ester), a novel compound that has been reported for the first time from a plant. This compound was found to possess good antileishmanial and immunomodulatory activities.

The 18 known compounds, *trans*-5-hexadecenoic acid (**1**),[14] 1-tritriacontanol (**2**),[15] 1-heptadecanol(**3**),[16] β-sitosterol(**4**),[17] β-amyrone (**5**),[18] gangetin (**6**),[5] glycosphingolipid (**7**),[19] 5-methoxy *N*,*N*-dimethyl tryptamine (**9**),[4] 8-*C*-prenyl-5,7,5′-trimethoxy 3′,4′-methylene dioxy flavone(**10**),[8] salicylic acid(**11**),[20] 5-*O*-methyl genistein 7-*O*-β-D-glucopyranoside(**12**),[21] 3,4-dihydroxy benzoic acid (**13**),[20] kaempferol 7-*O*-β-D-glucopyranoside (**14**),[22] 5-methoxy *N*,*N*-dimethyl tryptamine N_b-oxide (**15**),[4] 3-*O*-β-D-glucopyranoside-β-sitosterol (**16**),[23] rutin (**17**),[24] quercetin 7-*O*-β-D-glucopyranoside (**18**),[25] and uridine triacetate (**19**),[26] were characterized by comparing their spectroscopic data with those reported in the literature.

2.1. In vitro antileishmanial and immunomodulatory activities of compounds

Peritoneal macrophages were isolated from hamsters and grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum in 16-well chamber slides. Macrophages were incubated with compounds at a conc of 100, 50, 25, and 10 μg/ml. After 48 h, macrophages were infected with *L. donovani*, as described earlier.[10] Culture supernatants were collected for nitrite estimation by Griess reaction.

Chamber slides were fixed in methanol, stained with Giemsa, and examined under a microscope for calculating the number of macrophages infected with *L. donovani*.[27] For nitrite estimation, 100 μl of cell supernatant was mixed with an equal volume of a mixture of 1% sulfanilamide and 0.1% naphthylethylenediamine in 2.5% H_3PO_4 and incubated at room temperature for 10 min. The absorbance was read at 550 nm and the nitrite levels were measured from a standard curve of $NaNO_2$.

Compound **8** induced significant production of NO as measured in the form of nitrite (12.4 ± 2.4 μM), whereas compound **7** resulted in lesser (8.5 ± 1.8 μM) stimulation of NO production. Maximum NO production was shown by LPS (23.9 ± 5.2 μM) followed by compound **8**. Compound **8** showed maximum activity at 100 μg/ml, whereas at other doses it failed to show significant activity. Other six compounds failed to induce nitrite production (Fig. 2).

As compared to an untreated group where 88.5 ± 5.3% macrophages were infected, only 48.5 ± 6.1% were found to be infected in the compound **8**-treated group (72.0 ± 5.0%). Compound **7** showed moderate activity, as it exerted 53.4 ± 4.4% inhibition of parasite multiplication while other compounds were found to be inactive. Miltefosine, a known antileishmanial drug, clears all the amastigotes from macrophages (Table 2).

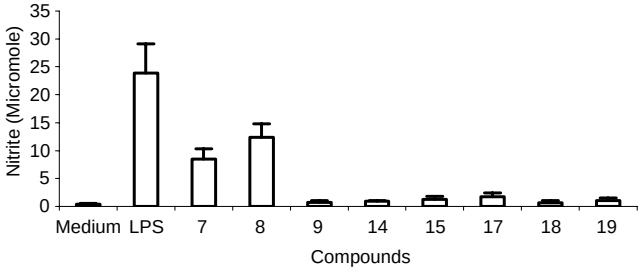

Figure 2. Effect of pure compounds of *Desmodium gangeticum* on the production of nitric oxide by peritoneal macrophages of hamsters. Data (means ± SD) are representative of three separate experiments.

Table 2. In vitro antileishmanial activity of pure compounds of *Desmodium gangeticum*

Compounds	Concentration (μg/ml)	% Infected macrophages	% Inhibition
7	100	71.7 ± 4.7	53.4 ± 4.4
8	100	48.5 ± 6.1	72 ± 5
9	100	88.4 ± 7.4	NI[a]
14	100	87.5 ± 6.5	NI
15	100	87.4 ± 5.9	NI
17	100	88.3 ± 8.3	NI
18	100	88.6 ± 5.9	NI
19	100	85.3 ± 7.4	NI
Miltefosine	10	0	100
Untreated control	—	88.5 ± 5.2	—

Data (means ± SD) represent results of three independent experiments.
[a] NI = no inhibition.

The *n*-butanol and chloroform fractions of crude extract of this plant had already been reported to exert significant activity in vivo.[10] Several plants are also reported to promote nitric oxide release from macrophages.[28] This prompted us to investigate further the action of its pure compounds on macrophages, which are immunocompetent cells,[29] by measuring the capacity of compounds to promote the production of NO, as well as to induce intracellular killing of amastigotes.[30] Compound **8** possesses significant in vitro immunostimulant activity since it is able to induce NO production in macrophages. NO is an effector molecule that exhibits leishmanicidal activity by killing amastigotes. Compounds **7** and **8** also imparted resistance to macrophages against the establishment of *Leishmania* infection. NO is involved in several inflammatory diseases and is cytotoxic or cytostatic in a wide range of infections caused by parasitic fungi, protozoa, helminthes, mycobacteria, and viruses. [31]

In conclusion, our study demonstrates the isolation and characterization of two biologically active glycolipids, which are possibly responsible for immunostimulatory activity of *Desmodium gangeticum* through their capacity to promote the production of NO and enhancing intracellular killing of parasites in peritoneal macrophages.

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