SHORT REPORT

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Isolation of Phytochemicals from *Cordia rothii* (Boraginaceae) and Evaluation of their Immunomodulatory Properties

Sadiqa Firdous[1], Kehkashan Khan[1], Sadia Zikr-Ur-Rehman[2], Zulfiqar Ali[3], Samreen Soomro[4], Viqar U. Ahmad[2], Munawwer Rasheed[1,5], Muhammed A. Mesaik[*4] and Shaheen Faizi[*2]

[1]*Department of Chemistry, University of Karachi, Karachi-75270, Pakistan*
[2]*H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan*
[3]*National Center for Natural Product Research, School of Pharmacy, University of Mississippi, MS-38677, USA*
[4]*Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan*
[5]*Centre of Excellence in Marine Biology, University of Karachi, Karachi-75270, Pakistan*

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Abstract: *n*-Hexane, EtOAc, and BuOH fractions obtained from methanol extract of leaves of *Cordia rothii* Roem and Schult, yielded 1-octacosanol (**1**), *β*-sitosterol (**2**), stigmast-5-en-3-*O*-*β*-D-glucoside (**3**), (2*S*,1′*S*,2′*S*,3′*R*,7′*Z*)-*N*-1′-(*O*-*β*-D-glucopyranosyl)methyl-2′,3′-dihydroxy-heptadec-7′-enyl-2-hydroxytetracosane-amide (**4**), methyl 2-hydroxy-3-(4′-hydroxy)-phenyl propionate (**5**), (2*R*)-(4-hydroxyphenyl)lactic acid (**6**), syringaresinol mono-*β*-D-glucoside (**7**), 6-hydroxy-3-oxo-*α*-ionol 9-*O*-*β*-D-glucopyranoside (**8**), staphylionoside D (**9**), and 3-(3′,5′-dimethoxy-4′-*O*-*β*-D-glucopyranosyl-phenyl)-prop-2*E*-en-1-ol (**10**). To the best of our knowledge, all compounds except **2** are reported for the first time from this source. Norterpenoid, lignan, cerebroside, and megastigmane are also reported for the first time. Immunomodulatory activity was evaluated using oxidative burst; phytohaemagglutinin (PHA) stimulated T-cell proliferation and nitric oxide (NO) assays. EtOAc fraction exhibited significant inhibitory activity against ROS with IC_{50} value of 29.4 ± 2.8 µg/mL. *n*-Hexane fraction exhibited very strong suppressive effect on PHA stimulated T-cell proliferation with IC_{50} value of <3.12 µg/mL. Some of the sub-fractions also affected the adaptive immune system with >85% inhibition. Compound **7** exhibited significant inhibitory activity (IC_{50} = 11.3 and 18.0 on PMN and whole blood respectively) on zymosan activated ROS generation. Eight sub-fractions showed potent inhibitory activity on the NO with >60% inhibition. These results suggest that compounds from *C. rothii* could be potential inhibitor for the mediators involved in innate and adaptive immune responses, and potential anti-inflammatory agents.

Keywords: *Cordia rothii*; Natural constituents; Immunomodulatory activity.

1. Plant Material

Leaves of *C. rothii* were collected in December 2007 from the local nursery (Karachi, Pakistan). Prof. Dr. Surayya Khatoon identified the species and voucher specimen number 85853 was deposited at the Herbarium, Department of Botany, University of Karachi, Karachi, Pakistan.

[*] Corresponding author: E- Mail: shaheenfaizi@hotmail.com, Phone: 021-34824912, mmesaik@hotmail.com, Phone:021-34819018

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2. Previous Studies

Genus *Cordia* (Boraginaceae) has been reported for various biological activities [1-6] and constituents [1-2]. Earlier only *Cordia curassavica* [7, 8], *Cordia superba* Cham., and *Cordia rufescens* A. DC [9] were subjected to immunomodulatory assay. This is the first report on the immunomodulatory studies of *C. rothii* (syn. gondani [10]). It is reputed for cardiotonic activity [11].

3. Present Study

Methanol extract (CRM) of the leaves of *C. rothii* was fractionated sequentially into *n*-hexane (CRMH), EtOAc (CRME) and BuOH (CRMB) fractions, and has resulted in isolation and identification of 1-octacosanol (**1**) [12], *β*-sitosterol (**2**) [13], stigmast-5-en-3-*O*-*β*-D-glucoside (**3**) [14], (2*S*,1′*S*,2′*S*,3′*R*,7′*Z*)-*N*-1′-(*O*-*β*-D-glucopyranosyl)methyl-2′,3′-dihydroxy-heptadec-7′-enyl-2-hydroxy-tetracosaneamide (**4**) [15], methyl 2-hydroxy-3-(4′-hydroxy)-phenyl-propionate (**5**) [16], (2*R*)-(4-hydroxyphenyl)lactic acid (**6**) [17], syringaresinol mono-*β*-D-glucoside (**7**) [18], 6-hydroxy-3-oxo-*α*-ionol 9-*O*-*β*-D-glucopyranoside (**8**) [19,20], staphylionoside D (**9**) [21], and 3-(3′,5′-dimethoxy-4′-*O*-*β*-D-glucopyranosyl-phenyl)-prop-2*E*-en-1-ol (**10**) [22] (Figure 1).

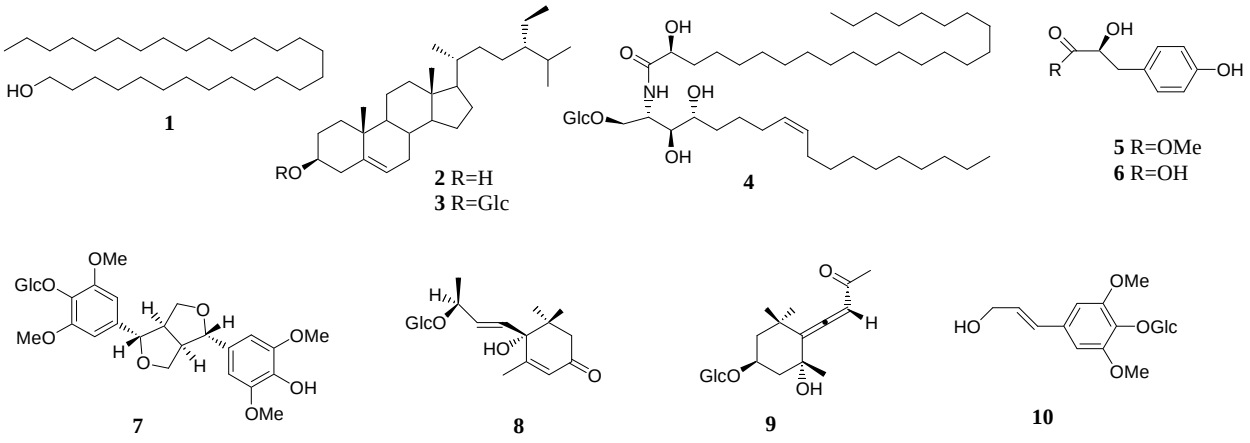

Figure 1. Chemical constituents **1-10** isolated from *C. rothii*

Bioactivities: Immunomodulatory properties of crude extracts, fractions and pure compounds isolated from CRMH, CRME and CRMB were evaluated using oxidative burst, PHA stimulated T-cell proliferation and nitric oxide (NO) assays. In preliminary chemiluminescence assay screening, CRM showed no inhibitory activity against reactive oxygen species (ROS) production at >100 µg/mL. CRME showed significant inhibitory activity while CRMH and CRMB were moderate at the initial screening doses, 25, 100, and 200 µg/mL (Table 1) (Figure 2, **S: 21**; Supplementary data).

Table 1. Effect of fractions on ROS production determined by chemiluminescence technique

Sample (code)	IC_{50} ± SD (µg/mL)
Methanol extract (CRM)	>100
n-Hexane fraction (CRMH)	62.4 ± 7.0
EtOAc fraction (CRME)	29.4 ± 2.8
BuOH fraction (CRMB)	75.4 ± 11.5
Control (Ibuprofen)	11.8 ± 1.2

CRMH also exhibited very strong suppressive effect on phytohaemagglutinin (PHA) activated T-cell proliferation stimulated by thymidine incorporation method (IC_{50} <3.12 µg/mL) (Figure 3, **S:22**; Supplementary data).

The effect of sub-fractions and pure compounds on ROS production was observed (Table 2 and 3). Almost all the sub-fractions exerted low to moderate inhibitory effect on ROS production with <50% inhibition. Moreover, pure compound **7** exerted significant inhibitions of ROS production on whole blood phagocytes and neutrophils ROS production with IC_{50} of 18.0 and 11.3 µg/mL respectively (Table 3).

Table 2. Effect of sub-fractions on oxidative burst on whole blood phagocytes and on NO production by mouse macrophages J774.2. Results are expressed as mean % inhibition of three determinations.

Sub-fractions	Inhibition oxidative burst on whole blood phagocytes (ROS) 20 µg/mL	%Inhibition nitric oxide (NO) 25 µg/mL	Sub-fractions	Inhibition oxidative burst on whole blood phagocytes (ROS) 20 µg/mL	%Inhibition nitric oxide (NO) 25 µg/mL
CRMHM	43.7	53.37	BA2	28.6	61.36
CRMHH	23.9	45.89	CRMEE	35.7	19.13
BH	36.4	42.83	C2	34.6	73.62
BCT	33.9	49.82	C6	30.8	65.14
BD	47.3	61.22	CRMEA	20.9	28.68
BCH	21.9	60.5	D1	49.1	59.15
BE1	29.3	73.49	D2	41.8	59.79
BE2	15.8	85.02	D3	43.3	63.11
BA1	19.4	52.68	D4	47.1	49.43

Table 3. IC_{50} of compounds against ROS production in whole blood and neutrophils, and effect of pure compounds at 25 µg/mL on NO production by mouse macrophages J774.2. Results are expressed as mean ± SD of three determinations.

Compound No.	ROS IC_{50} Whole blood (µg/mL)	ROS IC_{50} Neutrophils (µg/mL)	% Inhibition NO	Compound No.	ROS IC_{50} Whole blood (µg/mL)	ROS IC_{50} Neutrophils (µg/mL)	% Inhibition NO
1	>100	>50	16.27	**8**	>100	>50	-15.62
2	>100	>50	-3.06	**9**	>100	>50	12.31
3	>100	>50	-11.52	**10**	>100	>50	26.49
4	>100	>50	6.66	Ibuprofen*	11.2 ± 1.8	1.2 ± 0.1	-
5	>100	>50	43.09	N^G-Methyl	-	-	65.65
6	>100	>50	9.29	L-arginine			
7	18.0 ± 1.3	11.3 ± 0.0	13.86	acetate**			

*Control, **standard Inhibitor of nitric oxide synthase used as control

Among 18 different sub-fractions, the BD, BCH, BE1, BE2, BA2, C2, C6, and D3 (Table 2) showed significant inhibition on NO production. The inhibitory effect ranges between 60 to 80%, with sub-fraction BE2 scoring maximum inhibitory effect. However, the pure compounds did not show any significant activity on NO production, except in case of **5** where moderate inhibition (43.09%) was observed (Table 3).

Almost all sub-fractions produce significant immunomodulatory effect on T-cell proliferation with % inhibition greater than 85 at concentration of 200 µg/mL. Interestingly fraction D-4, obtained from CRMB showed variation (Table 4)**.** Pure compounds did not show any anti-proliferative effect in similar experiments, however, compound **7** moderately inhibit the cell proliferation with IC_{50} of 14.6 µg/mL (Table 5).

Table 4. Effect of sub-fractions (200 µg/mL) on PHA induced T-cell proliferation using human peripheral blood T lymphocytes. Results are expressed as mean % inhibition of three readings.

Sub-fractions	% Inhibition	Sub-fractions	% Inhibition	Sub-fractions	% Inhibition
CRMHM	99.7	BE1	99.6	C6	99.6
CRMHH	99.8	BE2	99.6	CRMEA	99.5
BH	99.7	BA1	99.7	D1	87.5
BCT	97.0	BA2	99.7	D2	88.8
BD	99.6	CRMEE	95.1	D3	93.4
BCH	99.7	C2	99.8	D4	-24.5

Table 5. Effect of pure compounds (12.5 to 50 µg/mL) from plant on T-cell proliferation. IC_{50} values are expressed as mean of three determinations.

Compound No.	T-cell proliferation IC_{50} (µg/mL)	Compound No.	T-cell proliferation IC_{50} (µg/mL)
1	>50	**6**	>50
2	>50	**7**	14.6 ± 0.3
3	>50	**8**	>50
4	>50	**9**	>50
5	27.5 ± 1.9	**10**	>50

Cytotoxicity of compounds **5** and **7**, which were found to be active as immunomodulating agents, was studied at three different concentrations (0.5, 5 and 25 µg/mL) on 3T3 NIH mouse fibroblast cell line. The IC_{50} was found to be > 25 µg/mL.

It is worth mentioning that compound **5** was reported as a very important regulator of normal and malignant cell growth [23] and aglycone of compound **7** significantly reduced NO production in LPS-stimulated BV-2 microglia cells [24]. Moreover, cytotoxicity of compounds **2** [25], **3** [26], **4** [27], **9** [28], and **10** [29] is also reported in literature.

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