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Isolation, characterization and biological evaluation of datura lactones as potential immunomodulators

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Abstract—Phytochemical investigation of *Datura quercifolia* (Solanaceae) plant yielded a new datura lactone, 1β,5α,12α-trihydroxy-6α,7α,24α,25α-diepoxy-20*S*,22*R* with-2-enolide (**1**), along with two known compounds, **2** and **3**. The structure of **1** was established on the basis of spectral analysis, as well as by its chemical transformation into known datura lactones. These compounds have been evaluated for immunomodulatory activity by observing their effect on antibody production, T-cell and B-cell activation, and cytokine production from splenocytes. Compound **2** was found to be the most promising immunostimulator in the present study.

1. Introduction

Our previous phytochemical investigations of *Datura quercifolia* plant led to the isolation and characterization of several datura lactones,[1] which are of withanolide skeleton. These withanolides are ergostane-derived compounds generally having a δ lactone ring in the side chain, isolated from several species of Solanaceae.[2] These compounds have shown significant antitumour,[3] cytotoxic,[4] anti-inflammatory,[5] antibacterial,[6] hepatoprotective,[7] sedative,[8] cytostatic,[9] and immunosuppressive[10] activities. However, there is little information in the literature on the activity of withanolides from plant sources other than *Withania somnifera*. Datura lactones differ from other withanolides in having a rare epoxide functionality in the lactone ring. Keeping in view the structural features of datura lactones and an increase in the demand for withanolides on account of their multifaceted pharmacological and medicinal applications, we re-investigated the chemical constituents of *Datura quercifolia* plant and evaluated the compounds for immunomodulatory activity. Immunomodulation denotes any change in the immune response and may involve induction, expression, amplification, or inhibition of any part or phase response. Stimulation of immune response is required in certain patients, whereas suppression of the immune response is needed in other conditions.[11] Novel immunomodulating agents are used for the treatment of various conditions, such as infections, organ transplantation, cancer, rheumatoid arthritis, etc.[12–14] Natural products and their derivatives represent a major breakthrough in these immunological disorders.[15] Herein, we report the isolation and characterization of a new compound, **1**, along with two known compounds, **2** and **3**, from a *D. quercifolia* plant and determined their influence on various aspects of the immune system, such as antibody production, T-cell and B-cell activation, and cytokine production, from splenocytes. From our data, it was found that these compounds exerted a dose-dependent effect on humoral and cell-mediated immune responses. Compound **1** was found to be an immunosuppresser at lower doses, while **2** and **3** were immunostimulators. Compound **2** was found to be the most promising immunostimulator in the present study.

2. Results and discussion

2.1. Chemistry

The leaves of *D. quercifolia* were extracted with benzene and methanol. Column chromatography of benzene extract yielded **2** and **3**, while that of MeOH extract

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yielded a mixture of **1** and **3**, which was separated further by repeated column chromatography to give pure **1** and **3**. Among them, compound **1** proved to be a new compound, whereas **2** and **3** were reported earlier by our group from the present institute. Methanol extract of the residue on chromatographic separation in benzene: ethyl acetate (50:50, v/v) yielded a colourless silky crystalline solid (**1**), m.p. 250–252 °C, $[\alpha]_D$ +35.4 (*c* 0.50, $CHCl_3$), formula $C_{28}H_{40}O_7$, according to the elemental analysis and *m/z* 511.3 (M+Na). This molecular formula indicated nine degrees of unsaturation; two olefin signals, one carbonyl group and two epoxides accounted for four of these degrees, and **1** therefore was pentacyclic. The IR spectra exhibited bands at 1738.1 cm^{-1} (six-membered lactone ring) and 3508.4 cm^{-1} (–OH group/s). ^1H NMR (Table 1) showed characteristic proton signals for five methyl groups of the withanolide nucleus at δ 1.44 (3H, s, 28-CH_3), 1.42 (3H, s, 27-CH_3), 1.02 (d, 3H, J = 4.59 Hz, 21-CH_3), and 0.77 (6H, s, 18- and 19-CH_3). Signals at δ 5.74 (dd, 1H, J = 10.13, 2.77 Hz, H-2) and 5.91 (dq, 1H, J = 10.13, 4.23, 2.5 Hz, H-3) were due to olefinic protons; signals at δ 2.94 (d, 1H, J = 4 Hz, H-6), 3.30 (dd, 1H, J = 4 Hz, H-7) were due to the protons in the epoxy ring, and signals at δ 3.42 (m, 1H, H-1) and 4.03 (bs, 1H, H-12) were due to the protons attached to the carbon with equatorial and axial –OH groups, respectively. The lone signal at δ 4.18 (m, 1H, H-22) was due to the resonance of δ-H of the lactone ring. It was interesting to note that 19-methyl was located upfield at 0.77, which has rarely been observed with other datura lactones or withanolides. This observation has clearly indicated the absence of carbonyl at the C-1 position. Acetylation under mild conditions (Ac_2O/pyridine at rt) yielded a diacetate, δ 2.11 (s, 2× $OCOCH_3$), indicating two –OH groups. Upon catalytic hydrogenation, the compound absorbed one mole of hydrogen to give a dihydro derivative, which was identified by the disappearance of two signals in ^1H NMR at δ 5.74 and 5.91. ESI of the molecule showed a peak at *m/z* at 511.3 (M+Na). Finally, the structure of the compound and its stereochemistry were determined by its chemical transformation into known datura lactones. Jone's oxidation of **1** at 0 °C yielded **3**, while prolonged oxidation at room temperature yielded **2**. It was interesting to note that 19-CH_3 shifted downfield to δ 1.18 and δ 1.26 after oxidation to **3** and **2**, respectively, indicating the presence of hydroxyl at C-1. Since on controlled oxidation **1** gave **2** and **3** of known structures, it was therefore clear that the stereochemistry at C-5 and beyond is the same as that in **3**. The extra hydroxyl is located at C-1 and in view of the chemical shift of C-1, in spite of H at δ 3.42 being allylic to the Δ^2 bond, it became clear that C-1, H is axial (α) rather than equatorial beyond any shadow of doubt and hence the –OH group at C-1 is equatorial with a β-configuration. From the above discussion, the structure of **1** was assigned, as shown in Figure 1.

Table 1. ^1H and ^{13}C NMR of **1** in $CDCl_3$

Position	^1H, δ (J, Hz)	^{13}C, δ
1	3.42 (bs)	70.99
2	5.74 (dd, 1H, J = 10.13, 2.77 Hz)	129.65
3	5.91 (dq, 1H, J = 10.13, 4.23, 2.5 Hz)	124.80
4	2.29 (t, 2H)	28.24
5	—	72.48
6	2.94 (d, 1H, J = 4 Hz)	57.51
7	3.30 (dd, 1H, J = 4Hz)	57.07
8	1.4–1.8 (m)	35.48
9	1.4–1.8 (m)	39.36
10	—	47.34
11	1.4–1.8 (m)	28.48
12	4.05 (br, S, 1H)	72.07
13	—	47.34
14	1.4–1.8 (m)	35.71
15	1.4–1.8 (m)	22.91
16	1.4–1.8 (m)	26.65
17	1.4–1.8 (m)	43.99
18	0.77 (3H, s)	12.43
19	0.77 (3H, s)	12.02
20	1.4–1.8 (m)	36.11
21	1.02 (d, 3H, J = 4.59 Hz)	17.94
22	4.18 (m, 1H)	76.69
23	1.4–1.8 (m)	35.48
24	—	62.74
25	—	59.27
26	—	170.15
27	1.42 (s, 3H)	13.66
28	1.44 (s, 3H)	14.66

2.2. Biological activity

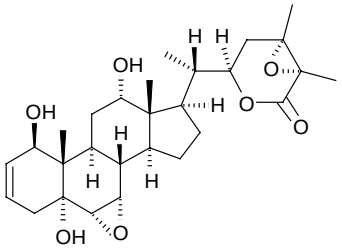
To test the immunomodulatory effect of these compounds, we used many assays to observe the influence on T-cell and B-cell activation with reference to antibody titre, DTH reaction and T-cell subtypes, CD4 and CD8. Levamisole and betamethasone were used as standards in this study.

2.3. Effect on antibody titre

The compounds were tested for any possible role of B-cell activation by determining IgM and IgG titres. Results given in Table 2 indicate that all the three compounds showed a dose-related increase or decrease of titre. Levamisole, used as a standard drug, showed a 26.6% and 45.2% increase in primary and secondary antibody synthesis, respectively, at a dose of 2.5 mg/kg. Compound **1** showed maximum suppression at a dose of 0.001 mg/kg. Compounds **2** and **3** produced a dose-related increase in the primary and secondary antibody synthesis. The maximum effect of **2** was observed at 1 mg/kg (93.3%) and 0.01 mg/kg (93.5%) for primary and secondary titres, while for **3**, the maximum effect was observed at 10 mg/kg, 72.7% and 86.2%, respectively.

2.4. Delayed type hypersensitivity response

The effect of **1**, **2**, and **3** on SRBC induced delayed type hypersensitivity (DTH) reaction was assessed in mice following various doses. The results are given in Table 3. The effect was compared to that of an equivalent dose of betamethasone (BMS) as a positive control. Out of the three compounds evaluated, **2** induced a significantly higher DTH response (69.03%) at a dose of 0.1 mg/kg p.o. The values are higher than that observed with BMS.



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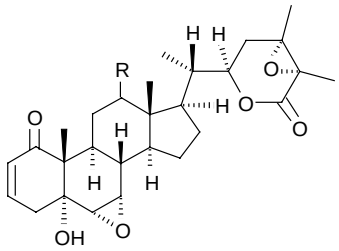

2. R = =O

3. R = ·····OH

Figure 1. Structures of **1**, **2**, and **3** datura lactones.

Table 2. Effect of **1**, **2**, and **3** on SRBC-induced antibody synthesis in mice

Compound	Treatment dose (mg/kg po)	Primary antibody (IgM) titre Mean ± SE	% change	Secondary antibody (IgG) titre Mean ± SE	% change
Control SRBC		6.0 ± 0.20		6.2 ± 0.21[b]	
1	0.001	4.4 ± 0.12[a]	26.6 ↓	4.2 ± 0.15	32.3 ↓
1	0.01	5.0 ± 0.04[a]	16.6 ↓	5.0 ± 0.16	19.3 ↓
1	0.1	5.4 ± 0.11	10.0 ↓	6.6 ± 0.12[b]	06.4 ↑
1	1	6.2 ± 0.24	3.30 ↑	6.8 ± 0.24	09.7 ↑
2	0.001	9.6 ± 0.25[a]	60.0 ↑	10.6 ± 0.33[a]	70.9 ↑
2	0.01	9.2 ± 0.34	53.3 ↑	12.0 ± 0.31[a]	93.5 ↑
2	0.1	10.2 ± 0.29	70.0 ↑	11.2 ± 0.42	80.6 ↑
2	1	11.6 ± 0.24[b]	93.3 ↑	9.6 ± 0.35[c]	54.8 ↑
2	3	9.5 ± 0.29[b]	58.3 ↑	7.0 ± 0.25[c]	12.9 ↑
2	10	5.4 ± 0.18[b]	10.0 ↓	6.4 ± 0.25	03.2 ↑
3	0.1	6.5 ± 0.26	8.30 ↑	7.2 ± 0.26	16.1 ↑
3	1	7.3 ± 0.24[c]	10.6 ↑	8.6 ± 0.31[a]	48.2 ↑
3	3	9.4 ± 0.35	42.4 ↑	9.2 ± 0.35[a]	58.6 ↑
3	10	11.4 ± 0.40[b]	72.7 ↑	10.8 ± 0.30	86.2 ↑
Levamisole	2.5	7.6 ± 0.19	26.6 ↑	9.0 ± 0.21[b]	45.2 ↑

$n = 6$.
Values are means ± SE.
[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$; (↓) reduction; (↑) stimulation.

Table 3. Effect of **1**, **2** and **3** on SRBC-induced delayed type hypersensitivity

Compound	Treatment dose (mg/kg po)	DTH response Mean ± SE	% change compared to betamethasone
Control		1.76 ± 0.06	
1	0.001	1.80 ± 0.04	16.13 ↑
1	0.01	2.01 ± 0.03	29.67 ↑
1	0.1	2.18 ± 0.05[a]	40.64 ↑
1	1	1.93 ± 0.05[a]	24.61 ↑
2	0.001	2.04 ± 0.09[a]	31.60 ↑
2	0.01	2.23 ± 0.02[b]	43.87 ↑
2	0.1	2.62 ± 0.03[b]	69.03 ↑
2	1	2.36 ± 0.12[b]	52.25 ↑
2	3	2.08 ± 0.10[a]	34.19 ↑
2	10	1.86 ± 0.05[b]	20.00 ↑
3	0.01	1.58 ± 0.05[b]	1.93 ↑
3	0.1	1.64 ± 0.10[b]	5.81 ↑
3	1	1.86 ± 0.10[b]	20.00 ↑
3	3	2.46 ± 0.14[b]	58.71 ↑
3	10	2.80 ± 0.06[a]	80.64 ↑
Betamethasone	0.01	1.55 ± 0.04	

$n = 6$.
[a] $P < 0.05$; [b] $P < 0.01$.

Since **2** was found to be the most promising stimulator against both humoral and cell-mediated immune responses, it was further studied for the activation of spleen T-cell subtypes, CD4 and CD8, using a flow cytometer and selective release of cytokines, IL-2 and TNF-α by stimulated mouse spleen cells using ELISA.

2.5. Effect of 2 on spleen T-cell subtyping

A spleen single cell suspension (10^6 cell/ml) was studied for $CD4^+/CD8^+$ T-cell subtypes by anti-mouse CD4 and CD8 monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) and phycoerthyrin (PE) using the flow cytometer. By multiplying differential ratios of each CD4 and CD8 subtype with the total spleen cell contents, their total amounts in spleen were calculated (Table 4). Maximum effect of **2** was obtained at 0.1 mg/kg po dose, 32.2% $CD4^+$ and 12.6% $CD8^+$ T cells. The control values were 20.70% of $CD4^+$ and 13.3% of $CD8^+$ T cells which show a significant increase in the $CD4^+$ T cell count. Levamisole, a standard T-cell stimulator at 2.5 mg/kg oral dose, stimulated both $CD4^+$ and $CD8^+$ T cells, showing 30.8% of $CD4^+$ and 18.3% of $CD8^+$ T cells.

Table 4. Effect of different doses of **2** on spleen T-cell subtypes

Dose of **2** (mg/kg)	$CD4^+$ T-cell (%)	$CD8^+$ T-cell (%)	CD4/CD8 ratio	Spleen $CD4^+$ content ($\times 10^7$)	Spleen $CD8^+$ content ($\times 10^7$)
Control (vehicle)	20.7 ± 0.90	13.3 ± 0.34	1.56 ± 0.09	2.70 ± 0.12	1.47 ± 0.04
Levamisole (2.5 mg/kg)	30.8 ± 1.30[a]	18.3 ± 0.53[a]	1.68 ± 0.06[a]	1.48 ± 0.10[a]	1.38 ± 0.10[a]
0.1	32.2 ± 0.59	12.6 ± 0.38	2.55 ± 0.07	1.55 ± 0.06	0.95 ± 0.03
1	27.2 ± 0.59	13.6 ± 0.38	2.00 ± 0.07	1.31 ± 0.06	1.02 ± 0.03
2.5	20.3 ± 0.63	15.4 ± 0.69	1.32 ± 0.07	1.02 ± 0.07	0.86 ± 0.04
3	19.7 ± 0.63	13.6 ± 0.69	1.44 ± 0.06	0.90 ± 0.02	1.02 ± 0.07
10	15.1 ± 1.93[b]	25.8 ± 0.35[b]	0.58 ± 0.09	0.72 ± 0.02	1.94 ± 0.06

$n = 6$.
[a] $P < 0.01$; [b] $P < 0.05$.

2.6. Effect of 2 on IL-2 and TNF-α

The effect of **2** was tested on the release of selected cytokines including IL-2 and TNF-α by stimulated mouse spleen cells. Results shown in Figure 2 indicate that compound **2** stimulated IL-2 and TNFα release in a dose-related manner. A dose as low as 0.01 mg/kg for TNF-α and IL-2 production was found to be the most effective.

The results obtained in the present study show that **1**, **2**, and **3** display an immunosuppressive or immunopotentiating activity depending on the dosage selection in relation to the antigen. A number of assays were used to investigate the immunomodulating effects. The effects of compounds **2** and **3** are clearly exerting an immunostimulatory activity on the immune system and could be suitable immunomodulatory lead compounds for future research. The only difference between **2** and **3** is the dose concentration. Compound **2** at lower doses (1 mg/kg) stimulated antibody titre in comparison to **3**, which stimulated the same at higher doses (10 mg/kg). A possible structural feature, which influences the activity, is the 12-carbonyl in **2**. In contrast, **1** has shown immunosuppressive effect on the antibody titre. The activity differences between these three compounds are clearly due to the presence or absence of functional groups (>C=O or –OH) at positions 1 and 12, which possibly interact with the immune competent T-helper cells. The results on cell mediated and humoral immune response have suggested that **2** is the most potent compound in comparison to **1** and **3**. SAR, detailed immunological studies and evaluation of more potent modified analogues are continuing.

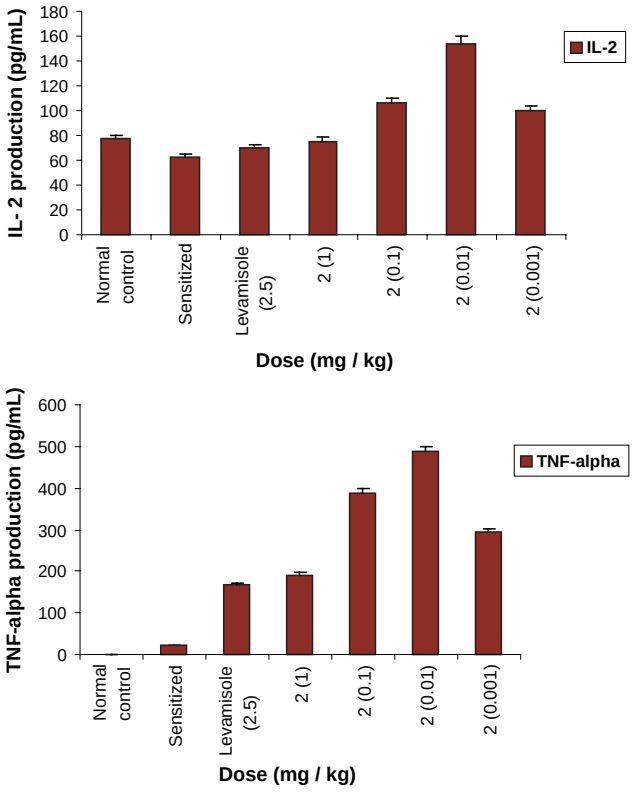

Figure 2. Effect of **2** on **IL-2** and **TNF-α** on cytokine production. Each bar represents the mean value of triplicate readings ±SE. Mouse spleen cells (2×10^6 cells/ml) were stimulated with and without (control) 2.5 μg/well Con-A in the presence of **2** for 48 h. Cell supernatant was collected to see the effect of **2** on the production of **IL-2** and **TNF-α**, measured by commercial kits (Quantikine, R&D SYSTEMS).

3. Experimental

All reagents and solvents were of reagent grade. Melting points were measured with a Digital Melting point Apparatus Electrothermal IA 19100. ^{1}H NMR and ^{13}C NMR were recorded on a Brucker DPX200 spectrometer. Chemical shifts are given in δ units, relative to the TMS signal as an internal reference. IR were recorded on a Bruker Vector 22 spectrometer as KBr pellets, with absorption given in cm^{-1}. MS were obtained by using electrospray ionization (ESI). Optical rotation was measured with a JASCO P-1020 polarimeter and elemental analyses were performed on a Carlo Erba CHNS-O-EA1108 instrument.

4. Plant material

Leaves of *D. quercifolia* were collected from the experimental fields of RRL, Srinagar, India, in the month of July 2004.

5. Extraction and isolation

Crushed and air-dried leaves of *D. quercifolia* (3 kg) were extracted with cold benzene. The extract was concentrated and allowed to stand at 0 °C for 30 h, when a light green crystalline substance was obtained. This compound was purified by passing it through a silica

gel (60–120 mesh) column. Elution with $CHCl_3$:EtOAc (3:2, v/v) yielded **2** and **3**. Their structures were confirmed by comparing their physicochemical data with the existing literature.[1] The marc of the crude leaves was then extracted with MeOH. The extract was concentrated on a rotatory evaporator and subjected to column chromatography on silica gel (60–120 mesh). Elution with $CHCl_3$:EtOAc (3:2, v/v) yielded a mixture of **1** and **3**, which was rechromatographed to give pure **1** and **3**. Yields of compounds **1**, **2** and **3** were found to be 0.001%, 0.34% and 0. 87%, respectively.

5.1. 1β,5α,12α-Trihydroxy-6α,7α,24α,25α-diepoxy-20*S*,22*R* with-2-enolide (1)

White amorphous solid; m.p. 250–252 °C; $[\alpha]_D$ +35.4 (*c* 0.50, $CHCl_3$); IR (KBr): 3508.1, 1738.4, 1685.9, 1391.8, 1304.9, 1143.5, 905.6 cm^{-1}; ^1H and ^{13}C NMR data in Table 1; *m*/*z* 511.3 (M+Na); Anal. Calcd for $C_{28}H_{40}O_7$: C, 68.83; H, 8.24. Found C, 69.08; H, 8.43.

5.2. Oxidation of 1 to 2 and 3

To an ice-cold solution of **1** (100 mg) in dry acetone (200 ml) was added Jone's reagent dropwise until a brown colour appeared. The mixture was stirred at 0 °C until the reaction was complete (monitored by TLC). It was then poured into ice-cold water and extracted with $CHCl_3$. The $CHCl_3$ extract was washed with 35% aq $NaHCO_3$ and H_2O. The extract was dried, evaporated and crystallized from methanol to give a crystalline solid, which resembled the natural compound **3** in all physicochemical properties. However, when the oxidation was carried out for 24 h with continuous stirring at rt, **2** was formed as a major product.

6. Preparation of test material

For in vivo studies in Balb/c mice, **1**, **2** and **3** were suspended in 1% (w/v) gum acacia, while for in vitro studies a stock solution of test materials in 10% dimethylsulfoxide was prepared. In the cell culture supernatant <0.1% did not interfere with the test system.

7. Serum SRBC antibody titre

Balb/c mice were immunized by injecting 200 μL of 5×10^9 SRBC/mL ip on day 0, and the blood samples were collected on day +7 (before challenge) for primary antibody titre and on day +14 (7 days after challenge) for secondary antibody titre. Haemagglutination antibody titres were determined following the microtitration technique described by Nelson and Mildenhall.[16]

8. Delayed type hypersensitivity response (DTH)

The method of Doherty[17] was followed. Test material was administered 2 h after SRBC injection and once daily on consecutive days. Six days later, the thickness of the left hind footpad was measured with a spheromicrometer and was considered as control. The mice were then challenged by injecting 200 μL of 5×10^9 SRBC/mL intradermally into the left hind footpad. The foot thickness was measured again after 24 h.

9. Spleen T-cell subtyping

T-cell sub-typing was performed, as described in Ref. 18. Briefly, a splenocyte single cell suspension in RPMI-1640 (10^6 cell/ml) was prepared and after counting viable cells by a trypan-blue dye exclusion method, spleen cellularity was obtained. The $CD4^+/CD8^-$ and $CD4^-/CD8^+$ T-cell subtypes were measured using a flow cytometer and anti-mouse CD4 and CD8 monoclonal antibodies conjugated with fluorescein-isothiocyanate and phycoerythrin. By multiplying differential ratios of each CD4 and CD8 subtype with the total spleen cell contents, their total amounts in spleen were calculated.

10. Cytokine production from splenocytes

Cytokines from mouse splenocytes were assayed using the cytokine kits (Quantikine R&D SYSTEMS).[19]

11. Statistical analysis

All the data are presented as means ± SE. Statistical analysis for all the results was compared using Student's *t* test.

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