MedChemComm

RESEARCH ARTICLE

View Article Online
View Journal | View Issue

Cite this: *Med. Chem. Commun.*, 2017, **8**, 211

T- and B-cell immunosuppressive activity of novel α-santonin analogs with humoral and cellular immune response in Balb/c mice†‡

Nisar A. Dangroo,[a] Jasvinder Singh,[bc] Nidhi Gupta,[a] Shashank Singh,[bc] Anapurna Kaul,[b] Mohmmed A. Khuroo[d] and Payare L. Sangwan*[ac]

Received 17th September 2016,
Accepted 31st October 2016

DOI: 10.1039/c6md00527f

www.rsc.org/medchemcomm

In continuation of our endeavours to synthesize immunosuppressive agents from α-santonin, we report herein the design and synthesis of a new series of α-santonin derived O-aryl/aliphatic ether, ester and amide analogs and the evaluation of their immunosuppressive activities. The *in vitro* studies led to several analogs with significant immunosuppressive effects by inhibiting ConA and LPS stimulated T- and B-cell proliferation in a dose dependent manner. The more significant compounds **4d**, **4e**, **4f**, **4h**, **6a** and **6b** displayed potent inhibitory activity on the mitogen-induced T- and B-cell proliferation in comparison to α-santonin **1**. Compound **4e** displayed stupendous *in vitro* immunosuppressive effects with ~80% suppression of B and ~75% suppression of T lymphocyte proliferation, respectively. The *in vivo* investigation on BALB/c mice revealed that non-cytotoxic compound **4e** suppresses both humoral and cellular immunity.

1. Introduction

Natural products (NPs) have proved to be promising sources of leads for drug discovery, and in fact, one-third of the top-selling drugs in the world are either NPs or NP-derived.[1,2] In the field of immunology, the discovery of cyclosporin A from the fungus *Cylindrocarpon lucidum* revolutionised organ transplantation.[3] Since then, NPs have played a dominant role in the development of most efficient immunosuppressive drugs, including cortisol, tacrolimus, rapamycin, rituximab, everolimus, as well as a number of NPs and their analogs, which are under clinical trials.[4] The T lymphocytes undoubtedly play a key role in the initiation of an immune response in transplant rejection and other autoimmune diseases. T-cell proliferation results from activation by antigen-presenting cells (APCs) in combination with the major histocompatibility complex class II and B7 complex. This mechanism results in the activation of calcineurin, which leads to the production of interleukin-2 (IL2). Autocrine stimulus by interleukin-2 results in T-cell proliferation.[5] Immunosuppressive drugs, for instance cyclosporin A, inhibit the abnormal activation and proliferation of T lymphocytes and the immune system associated with organ transplantation and other cell-mediated autoimmune diseases.[6] Side effects associated with these drugs, along with their high cost, have led the need for the investigation of alternative drugs, especially those belonging to the traditional system of medicine, with better safety profiles.

The plant *Artemisia laciniata* (asteraceae family) is usually found at altitudes from 2700 to 6300 m in Himalayan regions and, in India, it is widely distributed in Jammu and Kashmir, Leh, Ladakh, and Uttar Pradesh.[7] The plant has been traditionally used to treat jaundice, gall bladder, high fever and blood purification.[8] α-Santonin, a major chemical constituent of the plant, possesses several biological activities, including anticancer, antifungal and anti-inflammatory activities.[9–11] We have explored the immunosuppressive activity of α-santonin derived triazoles in our previous communications.[12,13] These findings indicate that α-santonin symbolizes an ideal scaffold for the synthesis of diverse analogues for exploration of their structure activity relationship (SAR). Acetyl α-desmotroposantonin **2** exhibited more potent anti-proliferation effects against lymphocytes compared to the parent molecule,[12] and this indicated that compound **2** could be a useful scaffold for the synthesis of new chemical entities (NCEs) to understand SAR for the immunosuppressive activity. Therefore, in continuation of our research interest for the design and synthesis of potent NCEs through the structural modification of NPs,[12–15] compound **2** was used as a starting material, and a series of new α-santonin derived O-aryl/

[a] *Bioorganic Chemistry Division, Canal Road, Jammu-180001, India*
[b] *Cancer Pharmacology Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu-180001, India*
[c] *Academy of Scientific and Innovative Research (AcSIR), CSIR-IIIM Campus, Jammu, India. E-mail: plsangwan@iiim.ac.in; Fax: +91 191 2586333; Tel: +91 191 2585006-13 Extn. 371*
[d] *University of Kashmir, Hazaratbal Road, Srinagar, India*

† The authors declare no competing interests.

‡ Electronic supplementary information (ESI) available: Spectral data (^{1}H NMR and ^{13}C NMR) and HRESIMS of all compounds are provided in the supporting information. See DOI: 10.1039/c6md00527f

aliphatic ether, ester and amide analogs were synthesized for the evaluation of the effect of carbon chain length on immunosuppressive activity in the present study. Several analogs displayed potent immunosuppressive activity and compound **4e**, showing stupendous *in vitro* activity, was further investigated for *in vivo* activity in BALB/c mice models for cellular immune response and sheep red blood cell (SRBC) induced humoral antibody production. Both *in vitro* and *in vivo* results demonstrate the immunosuppressive activity of compound **4e**.

2. Results and discussion

2.1. Chemistry

α-Santonin **1** was isolated from the dichloromethane–methanol extract of the aerial parts of *Artemisia laciniata.* Compounds **2** and **3** were synthesised as reported in our previous communication.[12] Briefly, NP santonin **1** was subjected to the dienone phenone type reaction using Ac_2O/H_2SO_4 to get acetyl α-desmotroposantonin **2**, which on deacetylation in NH_3:MeOH, furnished α-desmotroposantonin **3**. The ester analogs **4a–e** were prepared by treating **3** with the appropriate anhydride in dry dichloromethane (DCM) in the presence of pyridine at room temperature, while analogs **4f–h** were synthesized by treatment of **3** with the appropriate acid chloride in dry dichloromethane in good to excellent yields (Scheme 1).

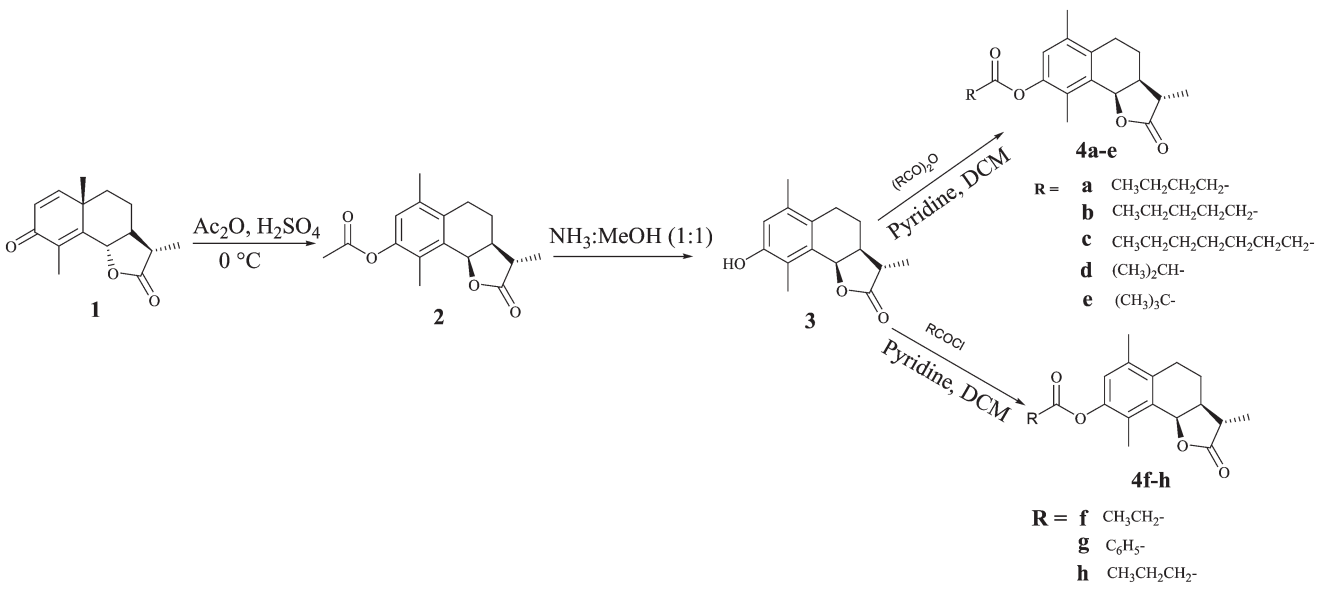
Compound **5** was prepared by reacting **3** with succinic anhydride in dry DCM, by employing different bases like $NaHCO_3$, K_2CO_3, pyridine, Et_3N, dimethyl amino pyridine (DMAP) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). However, the best results were obtained in DMAP. Compound **5**, on reaction with $SOCl_2$ in dry DCM under reflux resulted in the *in situ* generation of the acid chloride, followed by the addition of the appropriate amine, afforded compounds **6a–c** (Scheme 2).

Ether analogs were synthesised by the treatment of **3** with different alkyl halides in DCM in the presence of pyridine at room temperature to afford **7a–g** (Scheme 3). All the analogs were characterized by ^{1}H NMR, ^{13}C NMR and HRESIMS.

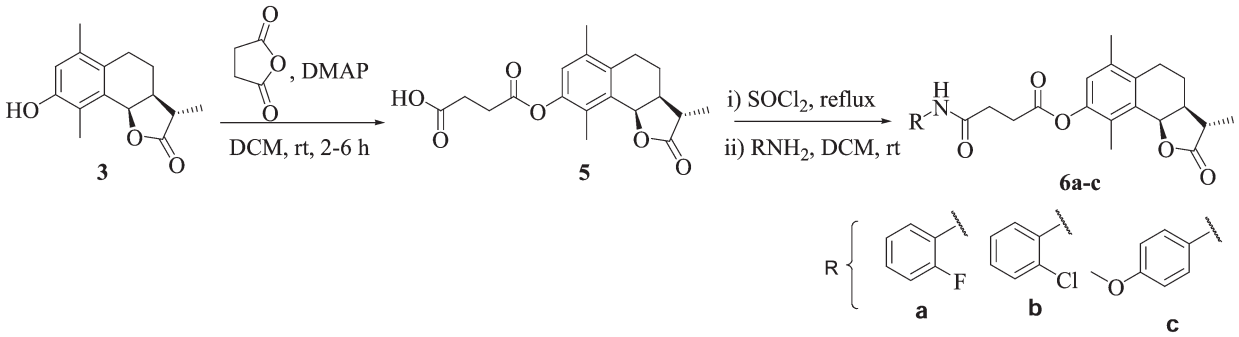
2.2. Biological activity

2.2.1. *In vitro* effect on lymphocyte proliferation. All synthesized compounds, along with α-santonin **1**, were screened for their ability to inhibit lymphocyte proliferation at three different (1, 10, 100 μM) concentrations (Table 1). Lipopolysaccharide (LPS) and concanavalin A (Con A) were used to selectively stimulate B and T lymphocytes, respectively. The proliferation of B- and T-cells was determined by measuring the absorbance using a microplate reader. Several analogs displayed improved anti-proliferation activity compared to parent molecule **1** in a dose dependent manner.

Compound **2**, with aromatized ring A, displayed better inhibition against both the mitogens than the parent compound **1**. Compound **4c** displayed 57% inhibition against LPS induced B-cell proliferation at 100 μM in the ester series (**4a–h**), while **4b** and **4h** were found to be more active against Con A and displayed 39–78% and 28–78% suppression of T-cells in the tested dose range, respectively. Non-cytotoxic compound **4d** showed potent and uniform suppression of T-cell proliferation, with 53% inhibition at 1 μM and finally attaining ~73% inhibition at 100 μM. Compound **4e** was found to be non-toxic and proved most potent among the ester analogs, with ~80% suppression of B-cells and 75% inhibition of T lymphocyte proliferation. Compound **4f** displayed a uniform suppression and reached up to 69% against LPS induced B-cell and 41% against Con A induced T-cell proliferation at 100 μM, but was cytotoxic. Compound **4g** was non cytotoxic, but could not prove potency, while cytotoxic compound **5** displayed ~61% inhibition of T-cells at 100 μM.



Scheme 1 Synthetic route for compounds **4a–h**.

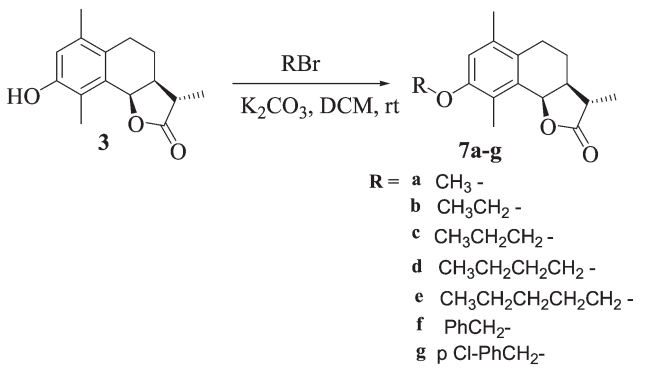


Scheme 2 Synthetic route for compounds **6a–c**.

Amide analogs (**6a–c**) were found to be non-cytotoxic and displayed uniform inhibition against both the mitogens, and among them, **6a** showed potent inhibition, reaching up to 77% at 100 μM concentration, against LPS induced B lymphocytes, and 66% against Con-A induced T-cell proliferation. Compound **6b** showed 76% suppression of B lymphocytes and 58% suppression of T lymphocytes at 100 μM concentration. Among ethers, compound **7g** displayed 74% inhibition of B and 66% inhibition of T lymphocytes without cytotoxicity at 10 μM, but the compound was found to be a stimulant, as well as cytotoxic at higher doses. The rest of the ether analogs displayed less than 50% inhibition along with cytotoxicity and were found less active compared to ester and amide derivatives.

It was evident from the cell proliferation results that ester and amide analogs display better inhibition activity, compared to ether analogs, which indicates that carbonyl groups present in these derivatives play an important role. Among the ester analogs, as the carbon chain length increases, the activity increases in compounds containing chains of up to four carbons (**4h**), and then decreases rapidly as the carbon chain increases. Further, isobutyrate (**4d**) and trimethyl acetate (**4e**) analogs proved more potent compared to straight chain analogs. The compound **6a**, bearing an *ortho*-F group in the amide moiety, exhibited potent activity among the amide analogs.

2.2.2. *In vivo* effect of compound 4e on DTH reaction and humoral immunity. The compound **4e**, with potent immunosuppressive properties, and as a representative of these derivatives, was further evaluated for *in vivo* experiments, *i.e.*, humoral and cell mediated immune response[16] on immune suppressed Balb/c mice at three different doses. Balb/c mice were immunized with antigen SRBC. Levamisole, as an immunostimulant, and cyclophosphamide and cyclosporine as immunosuppressants were used as standard controls for B- and T-cells, respectively, for the study.[17] The DTH reaction is a cell mediated pathologic response involved with T-cell activation and the production of many cytokines.[18] Treatment with **4e** suppressed the DTH reaction, as reflected by the decreased footpad thickness, compared to the control group, and down regulated the cell mediated immune response by 30% at 12.5 mg kg^{-1} and 35% at a conc. of 25 mg kg^{-1}. In humoral immunity, the antibody response to SRBC was observed by the hemagglutination titre. Compound **4e** showed 28% suppression at a dose of 6.25 mg kg^{-1} and 41% at 25 mg kg^{-1} per oral dose respectively, which was increased by about 1.5 fold (Fig. 1). The findings suggest that **4e** is able to suppress the humoral response.



Scheme 3 Synthetic route for compounds **7a–g**.

3. Conclusion

In summary, a series of new α-santonin derived O-aryl/aliphatic ether, ester and amide derivatives were synthesised. All the compounds were investigated for their *in vitro* immunosuppressive effect on T- and B-lymphocytes of BALB/c mice. Many analogs showed strong inhibition against Con-A and LPS stimulated T-cell and B-cell proliferation in a dose dependent manner. The screening data revealed that the presence of carbonyl groups and appropriate aliphatic side chains play important factors toward contributing to the high immunosuppressive potency in these analogs. More interestingly, compounds **4c**, **4d**, **4e** and **4h** exhibited potent *in vitro* activity and the compound **4e** revealed the most immunosuppressive effects. Further, compound **4e** was investigated for *in vivo* delayed-type hypersensitivity (DTH) reaction and antibody production in BALB/c mice models. The *in vitro* and *in vivo* immunosuppressive activity suggested that compound **4e** suppressed both humoral and cell mediated immunity.

4. Experimental

4.1. Chemistry

All the reagents and solvents for executing the present work were obtained from Sigma Aldrich. Dry DCM was prepared by

Table 1 *In vitro* lymphocyte proliferation activity of compound **1** and its analogs at three different concentrations (1, 10 and 100 μM)

Compounds	Conc. (μM)	Cytotoxicity	LPS mean ± SE	LPS-induced B-cell proliferation rate (%)	Con-A mean ± SE	ConA-induced T-cell proliferation rate (%)
Blank	–		0.06 ± 0.00	–	0.04 ± 0.00	–
Control	–		1.13 ± 0.12	–	0.60 ± 0.01	–
1	100	–	0.49 ± 0.35	33.25↓	0.21 ± 0.02	8.16↓
	10	–	0.50 ± 0.02	27.12↓	1.10 ± 0.45	3.55↑
	1	–	1.23 ± 0.76	19.00↓	0.80 ± 0.03	35.36↓
2	100	+	0.38 ± 0.04	**67.99↓**	0.64 ± 0.19	**63.00↓**
	10	–	0.73 ± 0.25	**44.27↓**	0.30 ± 0.09	**45.24↓**
	1	–	0.50 ± 0.04	**51.83↓**	0.16 ± 0.01	**39.72↓**
3	100	+	0.50 ± 0.02	38.25↑	0.21 ± 0.00	58.13↑
	10	+	0.91 ± 0.02	24.87↓	0.25 ± 0.00	27.35↓
	1	+	0.89 ± 0.02	23.28↓	0.32 ± 0.08	22.05↓
4a	100	+	0.90 ± 0.12	6.25↑	0.91 ± 0.16	28.16↑
	10	–	0.49 ± 0.04	48.95↓	0.50 ± 0.31	29.57↓
	1	–	0.94 ± 0.18	2.08↓	0.15 ± 0.00	43.23↓
4b	100	+	1.03 ± 0.10	7.29↑	1.25 ± 0.07	**78.87↓**
	10	+	0.71 ± 0.01	26.04↓	1.70 ± 0.29	**46.05↓**
	1	–	0.74 ± 0.33	22.91↓	0.19 ± 0.01	**39.43↓**
4c	100	+	1.51 ± 0.38	57.29↓	0.74 ± 0.14	4.22↓
	10	–	1.47 ± 0.36	53.12↑	1.49 ± 0.53	43.12↓
	1	–	0.60 ± 0.27	37.5↓	0.21 ± 0.00	50.42↓
4d	100	–	1.46 ± 0.06	**52.08↓**	1.23 ± 0.09	**73.23↓**
	10	–	1.16 ± 0.34	**33.33↓**	0.16 ± 0.00	**61.46↓**
	1	–	0.64 ± 0.30	**20.83↑**	0.14 ± 0.01	**53.28↓**
4e	100	–	0.22 ± 0.00	**80.53↓**	0.15 ± 0.01	**75.00↓**
	10	–	0.38 ± 0.13	**66.00↓**	0.25 ± 0.01	**58.11↓**
	1	–	0.58 ± 0.33	**48.67↓**	0.39 ± 0.15	**35.11↓**
4f	100	+	0.34 ± 0.15	**69.00↓**	0.35 ± 0.16	**41.00↓**
	10	+	0.64 ± 0.08	**43.00↓**	0.40 ± 0.18	**33.00↓**
	1	+	0.90 ± 0.18	**20.00↓**	0.53 ± 0.19	**11.66↓**
4g	100	–	0.79 ± 0.07	17.70↓	0.88 ± 0.16	23.94↑
	10	–	0.54 ± 0.02	43.75↓	0.43 ± 0.20	39.43↓
	1	–	0.78 ± 0.01	18.75↓	0.20 ± 0.01	71.83↓
4h	100	–	0.29 ± 0.14	**69.79↓**	0.15 ± 0.00	**71.87↓**
	10	–	0.62 ± 015	**35.41↓**	0.37 ± 0.16	**38.00↓**
	1	–	0.84 ± 0.04	**12.50↓**	0.57 ± 0.28	**28.50↓**
5	100	+	1.01 ± 0.11	5.20↓	1.29 ± 0.08	61.69↓
	10	+	0.64 ± 0.06	42.70↓	0.21 ± 0.00	53.42↓
	1	–	0.55 ± 0.02	33.33↓	0.21 ± 0.01	29.42↓
6a	100	–	0.25 ± 0.01	**77.87↓**	0.20 ± 0.12	**66.66↓**
	10	–	0.63 ± 0.36	**44.00↓**	0.30 ± 0.10	**41.66↓**
	1	–	0.89 ± 0.24	**21.00↓**	0.50 ± 0.12	**16.66↓**
6b	100	–	0.36 ± 0.00	**69.00↓**	0.21 ± 0.11	**56.00↓**
	10	–	0.41 ± 0.13	**51.00↓**	0.29 ± 0.32	**45.00↓**
	1	–	0.63 ± 0.27	**27.69↓**	0.49 ± 0.21	**13.00↓**
6c	100	+	0.26 ± 0.00	**68.00↓**	0.25 ± 0.01	**58.00↓**
	10	–	0.54 ± 0.17	**52.00↓**	0.39 ± 0.07	**35.00↓**
	1	–	0.93 ± 0.17	**17.69↓**	0.59 ± 0.20	**1.00↓**
7a	100	+	0.89 ± 0.13	21.00↓	1.30 ± 0.29	16.12↓
	10	+	0.91 ± 0.02	19.10↓	1.34 ± 0.15	23.25↓
	1	+	0.65 ± 0.21	42.00↓	0.90 ± 0.32	50.00↓
7b	100	+	0.61 ± 0.03	46.00↓	0.70 ± 0.16	16.00↑
	10	+	1.15 ± 0.31	1.00↓	0.57 ± 0.16	5.00↓
	1	–	0.33 ± 0.11	35.00↓	0.77 ± 0.05	28.00↓
7c	100	+	0.64 ± 0.08	43.00↓	0.82 ± 0.19	36.00↑
	10	+	0.34 ± 0.15	49.00↓	0.35 ± 0.16	41.00↓
	1	–	0.37 ± 0.18	47.00↓	0.40 ± 0.18	33.00↓
7d	100	–	1.11 ± 0.05	39.01↓	1.28 ± 0.16	33.82↓
	10	–	1.25 ± 0.10	33.31↓	0.85 ± 0.07	51.00↓
	1	–	1.38 ± 0.09	25.17↓	0.77 ± 0.14	32.05↓
7e	100	+	1.70 ± 0.05	9.59↓	0.96 ± 0.13	29.41↓
	10	+	1.23 ± 0.05	32.41↓	0.91 ± 0.09	33.08↓
	1	+	1.32 ± 0.09	29.47↓	1.25 ± 0.18	11.08↓
7g	100	+	1.46 ± 0.27	29.00↑	0.24 ± 0.01	16.00↑
	10	–	**0.29 ± 0.05**	**74.00↓**	**1.30 ± 0.57**	**60.00↓**
	1	–	**0.52 ± 0.28**	**53.00↓**	**0.25 ± 0.00**	**58.00↓**
7f	100	+	1.45 ± 0.14	28.00↓	0.93 ± 0.04	45.00↓

Table 1 *(continued)*

Compounds	Conc. (μM)	Cytotoxicity	LPS mean ± SE	LPS-induced B-cell proliferation rate (%)	Con-A mean ± SE	ConA-induced T-cell proliferation rate (%)
	10	−	0.71 ± 0.47	37.00↓	0.40 ± 0.11	33.00↓
	1	−	0.71 ± 0.47	37.00↓	0.51 ± 0.28	15.00↓

↑ indicates immune stimulant agents and ↓ indicates immunosuppressive agents. + indicates cytotoxic and − indicates non-cytotoxic. Results are shown as means of three readings and standard error by Student's '*t*' test (±S.E.) for three separate experiments, conducted in triplicate at three different 1, 10 and 100 μM concentrations.
The numbers with bold values represent those compounds that have displayed potent immunosuppressive activity.

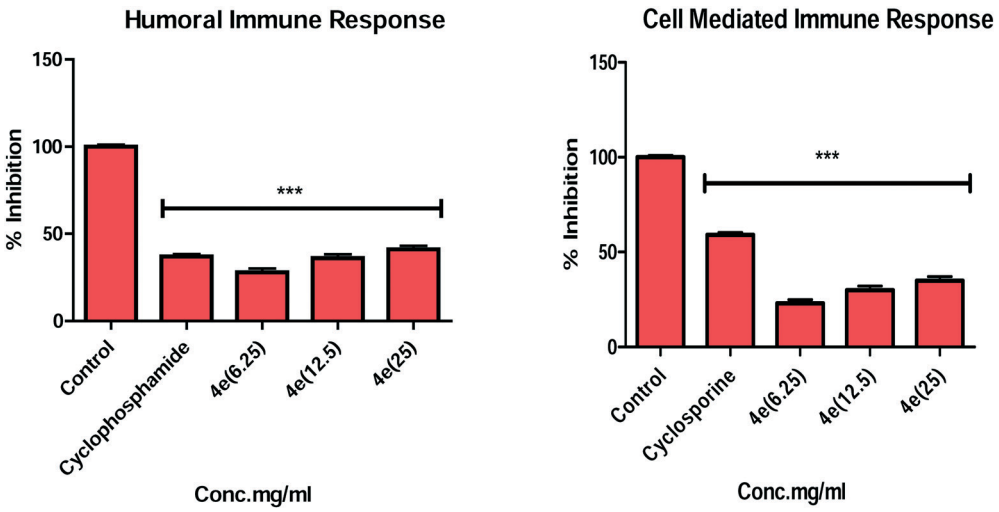


Fig. 1 Effect of compound **4e** on humoral immunity and DTH response. Data are expressed as means ± SE of five observations.

refluxing freshly distilled DCM over CaH_2 (5% w/v) for a few hours and then re-distilling it. Dry DCM was stored over 4 Å molecular sieves for future use. Pyridine was dried over KOH for one week, then distilled and stored over 4 Å molecular sieves. All the chemical reactions were monitored by TLC on 0.25 mm silica gel 60 F254 plates (E. Merck) using 2% ceric ammonium sulphate solution as a spraying reagent for the detection of the spots. Purification of compounds was carried out either by crystallization or column chromatography using silica gel 60–120 mesh as the stationary phase. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker DPX 400 and DPX 500 instruments using $CDCl_3$ or CD_3OD as the solvents, with TMS as the internal standard. The chemical shifts were expressed in δ ppm and coupling constants in Hertz. High resolution mass spectra (HRESIMS) were recorded on an Agilent Technologies 6540 instrument.

4.1.1. Isolation of α-santonin (1). α-Santonin (**1**) for the present work was isolated on a preparative scale from dichloromethane–methanol (1 : 1) extract of the aerial parts of *Artemisia laciniata* by column chromatography over silica gel 60–120 mesh, and further purified by crystallization.[12]

4.1.2. Preparation of α-desmotroposantonin acetate (2). The compound **2** was prepared as described in our previous report.[12] Briefly, the mixture of α-santonin (5.0 g, 20 mmol), acetic anhydride (25 mL) at 0 °C and H_2SO_4 (3 mL, 55.8 mmol) was added drop wise and allowed to react, and upon purification, it furnished a white crystalline product (**2**) in 92% yield, mp 139 °C. HRESIMS *m*/*z* calcd for $C_{17}H_{20}O_4$ $[M + H]^+$ 289.1434, found 289.1451.

4.1.3. Preparation of α-desmotroposantonin (3). The compound was prepared by treating compound **2** with a 1 : 1 mixture of NH_3 : MeOH to furnish α-desmotroposantonin **3** with 98% yield, mp: 197 °C, HRESIMS *m*/*z* calcd for $C_{15}H_{18}O_3$ $[M + H]^+$ 247.13287, found 247.13195.

4.1.4. General procedure for the synthesis of ester analogs (4a–e). The ester analogs **4a–e** were prepared by treating **3** (0.40 mmol) with the appropriate anhydride (0.44 mmol) in dry dichloromethane (DCM) in the presence of dry pyridine at room temperature. Upon completion, the reaction mixture was extracted with ethyl acetate and water. The organic layer was concentrated and rota evaporated to furnish the corresponding desired products in excellent yields (75–86%).

4.1.4.1. α-Desmotroposantonin pentanoate (4a). White solid; mp: 128 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.84 (s, 1H, Ar–*H*), 5.58 (d, J = 6.4 Hz, 1H, ArC*H*O–), 2.71–2.78 (m, 1H, –CH*CH*CH_3), 2.55–2.61 (m, 2H, Ar*C*$H_2$$CH_2$–), 2.51–2.60 (m, 2H, –$CH_2CH_2$CO), 2.36–2.49 (m, 1H, –CH*CH*CH_2–), 2.21 and 2.19 (s, 3H each, 2× Ar–CH_3), 1.93–1.96 and 1.69–1.75 (m, 1H

each, –CH_2CH_2CH–), 1.93–1.96 (m, 2H, –CH_2CH_2CO), 1.42–1.45 (m, 2H, –$CH_3CH_2CH_2$), 1.40 (d, J = 7.0 Hz, 3H, CH_3CH–), 0.98 (t, J = 7.4 Hz, 3H, CH_3CH_2–); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.37, 172.25, 147.45, 134.78, 133.87, 131.27, 129.05, 123.93, 75.51, 41.68, 40.40, 34.23, 31.35, 24.76, 24.02, 22.34, 19.53, 14.45, 13.91, 12.09. HRESIMS m/z calcd for $C_{20}H_{26}O_4$ $[M + H]^+$ 331.1904, found 331.1904.

*4.1.4.2. α-Desmotroposantonin hexanoate (**4b**).* White solid; mp: 132 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.84 (s, 1H, Ar–*H*), 5.58 (d, J = 6.4 Hz, 1H, ArC*H*O–), 2.71–2.78 (m, 1H, –CH*CH*CH_3), 2.51–2.59 (m, 2H each, ArCH_2CH_2– and –OCOCH_2CH_2–), 2.39–2.43 (m, 1H, –CH*CH*CH_2–), 2.21 and 2.20 (s, 3H each, 2× Ar–CH_3), 1.91–1.96 and 1.72–1.75 (m, 1H each, –CH_2CH_2CH–), 1.71–1.73 (m, 2H, $CH_3CH_2CH_2$–), 1.40 (d, J = 7.0 Hz, 3H, CH_3CH–), 0.90–0.95 (t, J = 7.4 Hz, 3H, –CH_2CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.37, 172.25, 147.45, 134.78, 133.87, 131.27, 129.05, 123.93, 75.51, 41.68, 40.40, 34.23, 31.35, 24.76, 24.02, 23.38, 22.34, 19.53, 14.45, 13.91, 12.09. HRESIMS m/z calcd for $C_{21}H_{28}O_4$ $[M + H]^+$ 345.2060, found 345.2077.

*4.1.4.3. α-Desmotroposantonin octanoate (**4c**).* White solid; mp: 135 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.85 (s, 1H, Ar–*H*), 5.58 (d, J = 6.4 Hz, 1H, ArC*H*O–), 2.71–2.77 (m, 1H, –CH*CH*CH_3), 2.50–2.59 (m, 2H each, ArCH_2CH_2– and –OCOCH_2CH_2–), 2.39–2.44 (m, 1H, –CH*CH*CH_2–), 2.21 and 2.20 (s, 3H each, 2× Ar–CH_3), 1.91–1.97 and 1.72–1.75 (m, 1H each, –CH_2CH_2CH–), 1.40 (d, J = 7.0 Hz, 3H, CH_3CH–), 1.22–1.46 (m, 10H, –$CH_2(CH_2)_5CH_3$), 0.90 (t, J = 7.4 Hz, 3H, –CH_2CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.37, 172.37, 147.50, 134.73, 133.87, 131.27, 129.06, 123.93, 75.47, 41.68, 40.40, 34.26, 31.65, 29.14, 28.91, 25.06, 24.01, 23.36, 22.59, 19.53, 14.45, 14.05, 12.09. HRESIMS m/z calcd for $C_{23}H_{32}O_4$ $[M + H]^+$ 373.2373, found 373.2406.

*4.1.4.4. α-Desmotroposantonin isobutyrate (**4d**).* White solid; mp: 121 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.83 (s, 1H, Ar–*H*), 5.58 (d, J = 6.4 Hz, 1H, ArC*H*O–), 2.81–2.89 (m, 1H, –CO*CH*$(CH_3)_2$), 2.71–2.78 (m, 1H, –CH*CH*CH_3), 2.55–2.61 (m, 2H, ArCH_2CH_2–), 2.36–2.49 (m, 1H, –CH*CH*CH_2–), 2.20 and 2.17 (s, 3H each, 2× Ar–CH_3), 1.93–1.96 and 1.69–1.75 (m, 1H each, –CH_2CH_2CH–), 1.39 (d, J = 7.0 Hz, 3H, CH_3CH–), 1.31 (d, J = 6.4 Hz, 6H, CH_3CHCH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.37, 175.25, 147.45, 134.78, 133.87, 131.27, 129.05, 123.24, 75.51, 41.68, 40.40, 34.15, 23.97, 23.40, 19.51, 19.10, 19.03, 14.47, 12.0, HRESIMS m/z calcd for $C_{19}H_{24}O_4$ $[M + H]^+$ 317.1747, found 317.1759.

*4.1.4.5. α-Desmotroposantonin trimethyl acetate (**4e**).* White solid; mp: 127 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.94 (s, 1H, Ar–*H*), 5.59 (d, J = 6.4 Hz, 1H, ArC*H*O–), 2.70–2.78 (m, 1H, –CH*CH*CH_3), 2.55–2.61 (m, 2H, ArCH_2CH_2–), 2.51–2.60 (m, 2H, –CH_2CH_2CO), 2.36–2.49 (m, 1H, –CH*CH*CH_2–), 2.26 and 2.22 (s, 3H each, 2× Ar–CH_3), 1.93–1.96 and 1.69–1.75 (m, 1H each, –CH_2CH_2CH–), 1.93–1.96 (m, 2H, –CH_2CH_2CO), 1.42–1.45 (m, 2H, –$CH_3CH_2CH_2$), 1.55 (s, 9H, $C(CH_3)_3$), 1.40 (d, J = 7.0 Hz, 3H, CH_3CH–); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.37, 167.73, 147.69, 134.78, 134.05, 131.20, 129.20, 123.93, 75.46, 41.68, 40.35, 38.04, 27.68 (3× CH_3), 23.76, 23.27, 19.17, 13.91, 13.75. HRESIMS m/z calcd for $C_{20}H_{26}O_4$ $[M + H]^+$ 331.1904, found 331.1887.

4.1.5. General procedure for the synthesis of ester analogs (4f–h). The ester analogs (**4f–h**) were prepared by treating **3** (0.40 mmol) with the appropriate acid chloride (0.45 mmol) in the presence of pyridine at room temperature to afford the desired products in excellent yields (70–79%). Acid chlorides were prepared by refluxing the appropriate acid with $SOCl_2$ in dry dichloromethane (DCM) for 1 h and drying under vacuum.

*4.1.5.1. α-Desmotroposantonin propanoate (**4f**).* White solid; mp: 132 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.84 (s, 1H, Ar–*H*), 5.58 (d, J = 6.4 Hz, 1H, ArC*H*O–), 2.71–2.78 (m, 1H, –CH*CH*CH_3), 2.51–2.59 (m, 2H, ArCH_2CH_2–), 2.55–2.61 (m, 2H, –OCOCH_2CH_3), 2.39–2.43 (m, 1H, –CH*CH*CH_2–), 2.22 and 2.20 (s, 3H each, 2× Ar–CH_3), 1.93–1.96 and 1.72–1.75 (m, 1H each, –CH_2CH_2CH–), 1.40 (d, J = 7.0 Hz, 3H, CH_3CH–), 1.30–1.35 (t, J = 7.4 Hz, 3H, –CH_2CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.37, 173.25, 147.45, 134.78, 133.87, 131.27, 129.05, 123.93, 75.51, 41.68, 40.40, 27.21, 24.01, 23.38, 19.53, 14.45, 12.09, 9.29. HRESIMS m/z calcd for $C_{18}H_{22}O_4$ $[M + H]^+$ 303.1591, found 303.1596.

*4.1.5.2. α-Desmotroposantonin benzoate (**4g**).* White solid; mp: 142 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.21–8.23 (m, 2H, 2× Ar^1–*H*), 7.60–7.67 (m, 1H, Ar^1–*H*), 7.46–7.55 (m, 2H, 2× Ar^1–*H*), 6.99 (s, 1H, Ar–*H*), 5.62 (d, J = 6.4 Hz, 1H, ArC*H*O–), 2.74–2.81 (m, 1H, –CH*CH*CH_3), 2.53–2.61 (m, 2H, ArCH_2CH_2–), 2.42–2.48 (m, 1H, –CH*CH*CH_2–), 2.27 and 2.25 (s, 3H each, 2× Ar–CH_3), 1.93–1.99 and 1.74–1.81 (m, 1H each, –CH_2CH_2CH–), 1.40 (d, J = 7.0 Hz, 3H, CH_3CH–); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.37, 165.25, 147.45, 134.78, 134.14, 133.87, 131.27, 130.17, 129.31(2× *C*H), 129.05, 128 (2× *C*H), 124.03, 75.51, 41.68, 40.40, 24.01, 23.38, 19.53, 14.45, 12.09. HRESIMS m/z calcd for $C_{22}H_{22}O_4$ $[M + H]^+$ 351.1591, found 351.1597.

*4.1.5.3. α-Desmotroposantonin butanoate (**4h**).* White solid; mp: 144 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.84 (s, 1H, Ar–*H*), 5.58 (d, J = 6.4 Hz, 1H, ArC*H*O–), 2.70–2.77 (m, 1H, –CH*CH*CH_3), 2.51–2.59 (m, 2H each, ArCH_2CH_2– and –OCOCH_2CH_2–), 2.39–2.43 (m, 1H, –CH*CH*CH_2–), 2.21 and 2.20 (s, 3H each, 2× Ar–CH_3), 1.91–1.96 and 1.72–1.75 (m, 1H each, –CH_2CH_2CH–), 1.71–1.73 (m, 2H, $CH_3CH_2CH_2$–), 1.40 (d, J = 7.0 Hz, 3H, CH_3CH–), 1.08 (t, J = 7.4 Hz, 3H, –CH_2CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.37, 172.25, 147.45, 134.78, 133.87, 131.27, 129.05, 123.93, 75.51, 41.68, 40.40, 36.11, 24.01, 23.38, 19.53, 18.60, 14.45, 13.76, 12.09. HRESIMS m/z calcd for $C_{19}H_{24}O_4$ $[M + H]^+$ 317.1747, found 317.1779.

4.1.6. Preparation of compound 4-α-desmotroposantonin-4-oxobutanoic acid (5). To a solution of **3** (2.0 mmol) in dry DCM, succinic anhydride (2.4 mmol) and DMAP (2.0 mmol) were added and stirred at room temperature till completion of the reaction. Ice cold water was added to the reaction mixture for precipitation of the crude product, which was finally washed with cold DCM to furnish the desired product **5** as a white solid; mp: 123 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.87 (s, 1H, Ar–*H*), 5.62 (d, J = 6.4 Hz, 1H, ArC*H*O–), 2.71–2.78 (m, 1H, –CH*CH*CH_3), 2.55–2.61 (m, 2H, ArCH_2CH_2–), 2.51(s, 4H, –COCH_2CH_2CO), 2.36–2.49 (m, 1H,

–CH*CH*CH_2–), 2.25 and 2.23 (s, 3H each, 2× Ar–CH_3), 1.93–1.96 and 1.69–1.75 (m, 1H each, –CH_2CH_2CH–), 1.40 (d, J = 7.0 Hz, 3H, CH_3CH–); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.37, 172.25, 168.52, 147.45, 134.78, 133.87, 131.27, 129.05, 121.24, 73.51, 39.68, 37.40, 27.21, 26.51, 21.20, 20.53, 16.87, 11.77, 9.36 HRESIMS m/z calcd for $C_{19}H_{22}O_6$ $[M + H]^+$ 347.1589, found 347.1524.

4.1.7. General procedure for the synthesis of (6a–c). A mixture of **5** (1.44 mmol) and thionyl chloride (85 mmol) in dry dichloromethane (DCM) (20 mL) was refluxed for 1 h. Excess thionyl chloride and DCM were removed in vacuum. The crude product (acyl chloride) was dissolved in dry DCM (20 mL) and added drop wise with to the appropriate aniline (2.16 mmol) in dry pyridine at room temperature. After the addition was complete, stirring was continued for another half an hour and upon purification, afforded the desired products **6a–c**.

*4.1.7.1. 4-α-Desmotroposantoninyl-(2-fluorophenyl)-4-oxobutanmide (**6a**).* White solid; mp: 123 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02 (s, 1H, N*H*), 7.27 (m, 4H, 4× Ar^1–*H*), 6.90 (s, 1H, Ar–*H*), 5.65 (d, J = 6.4 Hz, 1H, ArC*H*O–), 3.01–3.04 (m, 2H, –NHCOC$H_2$$CH_2$), 2.93–2.97 (m, 2H, –$CH_2CH_2$COO), 2.73–2.79 (m, 1H, –CH*CH*CH_3), 2.59–2.65 (m, 2H, Ar$CH_2$$CH_2$–), 2.36–2.49 (m, 1H, –CH*CH*CH_2–), 2.25 and 2.24 (s, 3H each, 2× Ar–CH_3), 1.91–1.95 and 1.63–1.71 (m, 1H each, –CH_2CH_2CH–), 1.41 (d, J = 6.4 Hz, 3H, CH_3CH–); ^{13}C NMR (100 MHz, $CDCl_3$): δ 180.56, 171.87, 169.52, 147.30, 134.94, 134.22, 130.96, 129.88, 128.92, 124.78, 123.82, 121.24, 117.55, 115.12, 114.96, 75.99, 41.76, 40.61, 31.45, 28.50, 23.89, 23.26, 19.29, 14.19, 11.86. HRESIMS m/z calcd for $C_{25}H_{26}FNO_5$ $[M + H]^+$ 440.1868, found 440.1808.

*4.1.7.2. 4-α-Desmotroposantoninyl-(2-chlorophenyl)-4-oxobutanmide (**6b**).* White solid; mp: 142 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.35 (s, 1H, N*H*), 7.36 (d, J = 8.2 Hz, 1H, Ar^1–*H*), 7.26–7.29 (m, 2H, 2× Ar^1–*H*), 7.03–7.06 (m, 1H, Ar^1–*H*), 6.88 (s, 1H, Ar–*H*), 5.57 (d, J = 6.4 Hz, 1H, ArC*H*O–), 3.04–3.07 (m, 2H, –NHCOC$H_2$$CH_2$), 2.94–2.97 (m, 2H, –$CH_2CH_2$COO), 2.71–2.78 (m, 1H, –CH*CH*CH_3), 2.55–2.61 (m, 2H, Ar$CH_2$$CH_2$–), 2.36–2.49 (m, 1H, –CH*CH*CH_2–), 2.25 and 2.23 (s, 3H each, 2× Ar–CH_3), 1.93–1.96 and 1.69–1.75 (m, 1H each, –CH_2CH_2CH–), 1.40 (d, J = 6.4 Hz, 3H, CH_3CH–); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.45, 171.44, 169.52, 147.45, 134.86, 134.46, 134.16, 131.27, 130.82 129.88, 129.07, 127.72, 124.78, 123.82, 121.24, 75.51, 41.62, 40.44, 32.18, 29.32, 24.02, 23.37, 19.56, 14.49, 12.17. HRESIMS m/z calcd for $C_{25}H_{26}ClNO_5$ $[M + H]^+$ 456.1572, found 456.1589.

*4.1.7.3. 4-α-Desmotroposantoninyl-(4-methoxyphenyl)-4-oxobutanmide (**6c**).* White solid; mp: 147 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.05 (s, 1H, N*H*), 7.37 (d, J = 8.4 Hz, 2× Ar^1–*H*), 6.86 (s, 1H, Ar–*H*), 6.83 (d, J = 8.4 Hz, 2× Ar^1–*H*), 5.55 (d, J = 6.0 Hz, 1H, ArC*H*O–), 3.78 (s, 3H, OCH_3), 3.01–3.04 (m, 2H, –NHCOC$H_2$$CH_2$), 2.71–2.75 (m, 2H, –$CH_2CH_2$COO), 2.65–2.70 (m, 1H, –CH*CH*CH_3), 2.50–2.61 (m, 2H, Ar$CH_2$$CH_2$–), 2.39–2.41 (m, 1H, –CH*CH*CH_2–), 2.19 and 2.18 (s, 3H each, 2× Ar–CH_3), 1.91–1.95 and 1.69–1.74 (m, 1H each, –CH_2CH_2CH–), 1.38 (d, J = 6.4 Hz, 3H, CH_3CH–); ^{13}C NMR (125 MHz, $CDCl_3$) δ 179.56, 171.93, 169.30, 156.34, 147.34, 134.92, 134.18, 131.25, 130.95, 129.04, 123.82, 121.77 (2× CH), 114.07 (2× CH), 75.51, 55.48, 41.61, 40.47, 31.74, 29.46, 24.02, 23.36, 19.56, 14.50, 12.18. HRESIMS m/z calcd for $C_{26}H_{29}NO_6$ $[M + H]^+$ 452.2093, found 452.2068.

4.1.8. General procedure for the synthesis of ether analogs (7a–g). The ether analogs **7a–g** were prepared by treating **3** (0.40 mmol) with the appropriate alkyl halide (0.44 mmol) in DCM in the presence of pyridine (0.40 mmol) at room temperature. Upon completion, the reaction mixture was extracted with ethyl acetate and water. The organic layer was concentrated under vacuum to afford the desired ether derivatives in excellent yields (90–95%).

*4.1.8.1. α-Desmotroposantonin methyl ether (**7a**).* White solid; mp: 141 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.75 (s, 1H, Ar–*H*), 5.63 (d, J = 6.4 Hz, 1H, ArC*H*O–), 3.81 (s, 3H, OCH_3), 2.70–2.75 (m, 1H, –CH*CH*CH_3), 2.55–2.61 (m, 2H, Ar$CH_2$$CH_2$–), 2.37–2.49 (m, 1H, –CH*CH*CH_2–), 2.25 and 2.23 (s, 3H each, 2× Ar–CH_3), 1.85–1.93 and 1.69–1.75 (m, 1H each, –CH_2CH_2CH–), 1.38 (d, J = 7.0 Hz, 3H, CH_3CH–); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.55, 155.67, 134.12, 130.74, 127.43, 125.58, 113.35, 75.83, 55.8, 41.86, 40.54, 23.70, 23.65, 20.04, 14.53, 11.52. HRESIMS m/z calcd for $C_{16}H_{20}O_3$ $[M + H]^+$ 261.1485, found 261.1491.

*4.1.8.2. α-Desmotroposantonin ethyl ether (**7b**).* White solid; mp: 113 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.71 (s, 1H, Ar–*H*), 5.60 (d, J = 6.4 Hz, 1H, ArC*H*O–), 3.99 (q, 2H, J = 5.6 Hz, –O$CH_2$$CH_3$), 2.66–2.71 (m, 1H, –CH*CH*CH_3), 2.47–2.54 (m, 2H, Ar$CH_2$$CH_2$–), 2.37–2.39 (m, 1H, –CH*CH*CH_2–), 2.27 and 2.21 (s, 3H each, 2× Ar–CH_3), 1.87–1.89 and 1.68–1.71 (m, 1H each, –CH_2CH_2CH–), 1.36–1.41 (m, 3H each, CH_3CH–OC$H_2$$CH_3$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.74, 155.20, 134.00, 130.71, 127.79, 125.97, 114.76, 75.90, 64.23, 41.87, 40.59, 23.73, 23.70, 19.99, 15.08, 14.53, 11.57. HRESIMS m/z calcd for $C_{17}H_{22}O_3$ $[M + H]^+$ 275.1642, found 275.1638.

*4.1.8.3. α-Desmotroposantonin propyl ether (**7c**).* White solid; mp: 128 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.72 (s, 1H, Ar–*H*), 5.60 (d, J = 6.4 Hz, 1H, ArC*H*O–), 3.89 (t, 2H, J = 6.4 Hz, O$CH_2$$CH_2$–), 2.66–2.72(m, 1H, –CH*CH*CH_3), 2.45–2.55 (m, 2H, Ar$CH_2$$CH_2$–), 2.36–2.42 (m, 1H, –CH*CH*CH_2–), 2.27 and 2.21 (s, 3H each, 2× Ar–CH_3), 1.87–1.92 and 1.68–1.75 (m, 1H each, –CH_2CH_2CH–), 1.80 (q, 2H, J = 6.0 Hz, –$CH_2$$CH_3$), 1.38 (d, J = 7.6 Hz, 3H, CH_3CH–), 1.04 (t, J = 7.2 Hz, 3H, C$H_3$$CH_2$–); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.77, 155.30, 134.02, 130.69, 127.67, 125.86, 114.57, 75.91, 70.13, 41.85, 40.59, 23.73, 23.69, 22.82, 19.98, 14.52, 11.57, 10.73. HRESIMS m/z calcd for $C_{18}H_{24}O_3$ $[M + H]^+$ 289.1789, found 289.1812.

*4.1.8.4. α-Desmotroposantonin butyl ether (**7d**).* White solid; mp: 118 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.72 (s, 1H, Ar–*H*), 5.61 (d, J = 6.4 Hz, 1H, ArC*H*O–), 3.93 (t, J = 7.8 Hz, 2H, –CH_2CH_2O), 2.67–2.72 (m, 1H, –CH*CH*CH_3), 2.46–2.57 (m, 2H, Ar$CH_2$$CH_2$–), 2.36–2.42 (m, 1H, –CH*CH*CH_2–), 2.27 and 2.22 (s, 3H each, 2× Ar–CH_3), 1.86–1.94 and 1.67–1.75 (m, 1H each, –CH_2CH_2CH–), 1.76–1.82

(m, 2H, –$CH_2CH_2CH_2O$), 1.42–1.45 (m, 2H, $CH_3CH_2CH_2$–), 1.38 (d, J = 7.0 Hz, 3H, CH_3CH–), 0.97 (t, J = 7.8 Hz, 3H, CH_3CH_2–); ^{13}C NMR (125 MHz, $CDCl_3$): δ 179.70, 155.49, 133.96, 130.69, 127.77, 125.75, 114.65, 75.90, 68.26, 41.86, 40.49, 31.36, 23.55, 23.52, 19.93, 19.31, 14.43, 13.71, 11.45. HRESIMS m/z calcd for $C_{19}H_{26}O_3$ $[M + H]^+$ 303.1955, found 303.1981.

4.1.8.5. α-Desmotroposantonin pentyl ether (7e). White solid; mp: 131 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.72 (s, 1H, Ar–*H*), 5.62 (d, J = 6.4 Hz, 1H, ArC*H*O–), 3.81 (t, J = 7.8 Hz, 2H, –CH_2CH_2O), 2.67–2.75 (m, 1H, –CH*CH*CH_3), 2.52–2.57 (m, 2H, ArCH_2CH_2–), 2.37–2.42 (m, 1H, –CH*CH*CH_2–), 2.28 and 2.22 (s, 3H each, 2× Ar–CH_3), 1.85–1.90 and 1.67–1.75 (m, 1H each, –CH_2CH_2CH–), 1.76–1.82 (m, 2H, –$CH_2CH_2CH_2O$), 1.42–1.45 (m, 2H, –$CH_2CH_2CH_2O$), 1.38 (d, J = 7.0 Hz, 3H, CH_3CH–), 0.93 (t, J = 7.8 Hz, 3H, $CH_3CH_2CH_2$–); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.74, 155.35, 134.00, 130.69, 127.63, 125.90, 114.54, 75.79, 68.60, 41.87, 40.55, 29.17, 28.36, 23.73, 232.69, 22.47, 20.00, 14.53, 14.10, 11.56. HRESIMS m/z calcd for $C_{20}H_{28}O_3$ $[M + H]^+$ 317.2011, found 317.2087.

4.1.8.6. α-Desmotroposantonin benzyl ether (7f). White solid; mp: 124 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.30–7.45 (m, 5H, 5× Ar^1–*H*), 6.80 (s, 1H, Ar–*H*), 5.62 (d, J = 6.4 Hz, 1H, ArC*H*O–), 5.05 (s, 2H, Ar^1CH_2O), 2.70–2.75 (m, 1H, –CH *CH*CH_3), 2.55–2.61 (m, 2H, ArCH_2CH_2–), 2.37–2.49 (m, 1H, –CH*CH*CH_2–), 2.25 and 2.21 (s, 3H each, 2× Ar–CH_3), 1.93–1.96 and 1.69–1.75 (m, 1H each, –CH_2CH_2CH–), 1.40 (d, J = 7.0 Hz, 3H, CH_3CH–); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.93, 137.57, 134.09, 130.90, 130.74, 128.68 (2× CH), 128.26, 127.84, 127.14 (2× CH), 126.90, 114.83, 75.74, 70.05, 41.85, 40.64, 23.77, 23.73, 20.07, 14.57, 11.83. HRESIMS m/z calcd for $C_{22}H_{24}O_3$ $[M + H]^+$ 337.1798, found 337.1807.

4.1.8.7. α-Desmotroposantonin p-chlorobenzyl ether (7g). White solid; mp: 139 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.33–7.38 (m, 4H, 4× Ar^1–*H*), 6.76 (s, 1H, Ar–*H*), 5.63 (d, J = 6.4 Hz, 1H, ArC*H*O–), 5.01 (s, 2H, Ar^1CH_2O), 2.70–2.75 (m, 1H, –CH *CH*CH_3), 2.55–2.61 (m, 2H, ArCH_2CH_2–), 2.37–2.49 (m, 1H, –CH*CH*CH_2–), 2.25 and 2.21 (s, 3H each, 2× Ar–CH_3), 1.93–1.96 and 1.69–1.75 (m, 1H each, –CH_2CH_2CH–), 1.40 (d, J = 7.0 Hz, 3H, CH_3CH–); ^{13}C NMR (100 MHz, $CDCl_3$): δ 179.37, 154.73, 136.02, 134.20, 133.51, 130.99, 129.20, 128.72 (2× *C*H), 128.48 (2× *C*H), 126.08, 114.86, 75.80, 69.70, 41.83, 40.36, 23.73, 23.68, 20.04, 14.54, 11.79 HRESIMS m/z calcd for $C_{22}H_{23}ClO_3$ $[M + H]^+$ 371.1408, found 371.1412.

4.2. Biology

4.2.1. Animals, Mitogens and reagents. *Animals*: female Balb/c mice, 10–12 weeks old and weighing 20–24 g, were obtained from the animal house at the Indian Institute of Integrative Medicine (IIIM), Jammu, and were taken in groups of six and employed for study. These animals were maintained in standard sized polycarbonate cages with controlled conditions of temperature of 26 ± 2 °C, humidity 50–60% and 12 h light/dark cycle. The animals were fed on pellet food (Ashirwad Industries India Ltd.), and autoclaved water *ad libitum*. The study was carried out on normal mice as per OECD guidelines no. 423.[18] The experimental protocols and animals used in the experiments were approved by the Institutional Animal Ethics Committee. The approval no. is CPCSEA: 99/65/758/8/15.

Mitogens and reagents: concanavalin-A (Con-A), lipopolysaccharide (LPS) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were procured from Sigma Aldrich. RPMI (Roswell Park Memorial Institute) 1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL, Life Technologies (USA).

4.2.2. Determination of the *in vitro* cytotoxic effect of different samples by MTT assay. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, reported in 1983 by Mosmann,[19] is a colorimetric assay used to determine the ability of viable cells to convert soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye into an insoluble blue coloured formazan precipitate, which dissolves with the addition of a solvent (*i.e.* DMSO), resulting in the liberation of the crystals that are solubilized. The number of surviving cells is directly proportional to the level of the formazan formed. The colour can then be quantified using a simple colorimetric assay. The results can be read on a multiwall scanning spectrophotometer (ELISA reader) at the wavelength of 540 nm.

4.2.3. Methodology. Balb/c mice weighing about 18–22 g were sacrificed and their spleens were removed under aseptic conditions. A single cell suspension was prepared in 5 mL of RPMI. The cell suspension was centrifuged at 1200 rpm for 10 min and the supernatant was discarded. RBCs were lysed by tris-ammonium chloride treatment. The cells were centrifuged twice at 1200 rpm for 10 min, supernatant discarded and re-suspended in RPMI. The viability of cells was checked with trypan blue. A 1×10^6 cells per mL suspension was prepared and 100 μL of the cell suspension was seeded into a 96-well flat-bottomed plate. The cells were stimulated by using 50 μL of standard mitogens (Con-A = 10 μg mL^{-1} and LPS = 10 μg mL^{-1}) to induce T-cells or B-cells, proliferation respectively. Different concentrations of test materials were added according to the experimental setup. 50 μL of different concentrations (1, 10 and 100 μM) of different compounds CRPMI were added to each well. The plates were incubated for 48 h at 37 °C in a humidified 5% CO_2 incubator. After 48 h incubation, plates were taken out from the CO_2 incubator and 10 μL of MTT dye was added to each well and placed on a shaker for 5 min to thoroughly mix the MTT into the media. Plates were then incubated for 4 h in a CO_2 incubator (37 °C, 5% CO_2 and 90% relative humidity) to allow the MTT to be metabolized. The plates were centrifuged at 2000 rpm for 10 min. Cell-free supernatants were then removed and resolved with 100 μL per well DMSO. The optical density was measured at 540 nm on a microplate reader. The means of the optical density of plates were calculated and the percentage of each value *versus* the control was evaluated.

4.2.4. Antigen. Sheep red blood cells (SRBC) were used as a source of T dependent antigen. For this purpose, the blood was withdrawn from a healthy sheep in Alsever's solution,

SRBC used for immunization were prepared in pyrogen free normal saline.[20,21]

4.2.5. Humoral immune response. Six animals were put in each group and immune system of each animal was suppressed by cyclophosphamide and then was immunized by injecting 200 μL of 1×10^7 SRBC mL^{-1} intraperitoneal (i.p.) on day 1 followed by oral drugging for six consecutive days. Blood was collected on day 7th for primary antibody titre. Haemagglutination antibody titre were determined following the modified micro titration technique described by Nelson and Mildenhall.[17]

4.2.6. Delayed type hypersensitivity (DTH). Compound **4e** 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 (pitch, 0.01 mm) and was considered as a control. The mice were then challenged by injecting 20 μL of 5×10^9 SRBC mL^{-1} intradermally into the left hind footpad. The footpad thickness was measured again after 24 and 48 h.[21,22]

Acknowledgements

Council of Scientific and Industrial Research (CSIR), New Delhi is well acknowledged for funding support under 12th FYP Project BSC-0108. One of the authors (NAD) is greatly thankful to the CSIR-UGC, New Delhi for the award of senior research fellowship. The authors are also thankful to the staff of instrumentation division of the institute for recording the spectroscopic (NMR, HRESIMS) data.

References

1 D. J. Newman and G. M. Cragg, *J. Nat. Prod.*, 2016, **79**, 629–661.
2 G. M. Cragg, P. G. Grothaus and D. J. Newman, *J. Nat. Prod.*, 2014, **77**, 703–723.
3 H. Hackstein and A. W. Thomson, *Nat. Rev. Immunol.*, 2004, **4**, 24–35.
4 J. Mann, *Nat. Prod. Rep.*, 2001, **18**, 417–430.
5 J. A. Kobashigawa and J. K. Patel, *Nat. Clin. Pract. Cardiovasc. Med.*, 2006, **3**, 203–212.
6 F. Issa, A. Schiopu and K. J. Wood, *Expert Rev. Clin. Immunol.*, 2010, **6**, 155–169.
7 P. Weyerstahl, H. Marschall-Weyerstahl, M. Schröder and V. K. Kaul, *J. Essent. Oil Res.*, 1992, **4**, 107–112.
8 S. Fahad and A. Bano, *Pak. J. Bot.*, 2012, **44**, 165–170.
9 J. Khazir, P. P. Singh, D. M. Reddy, I. Hyder, S. Shafi, S. D. Sawant, G. Chashoo, A. Mahajan, M. S. Alam, A. K. Saxena, S. Aravinda, B. D. Gupta and H. M. S. Kumar, *Eur. J. Med. Chem.*, 2013, **63**, 279–289.
10 B. Singh, J. Srivastava, R. Khosa and U. Singh, *Folia Microbiol.*, 2001, **46**, 137–142.
11 G. Blay, L. Cardona, B. Garcia and J. R. Pedro, *Stud. Nat. Prod. Chem.*, 2000, **24**, 53–129.
12 N. A. Dangroo, J. Singh, A. A. Dar, N. Gupta, P. K. Chinthakindi, A. Kaul, M. A. Khuroo and P. L. Sangwan, *Eur. J. Med. Chem.*, 2016, **120**, 160–169.
13 P. K. Chinthakindi, P. L. Sangwan, S. Farooq, R. R. Aleti, A. Kaul, A. K. Saxena, Y. L. N. Murthy, R. A. Vishwakarma and S. Koul, *Eur. J. Med. Chem.*, 2013, **60**, 365–375.
14 R. Majeed, P. L. Sangwan, P. K. Chinthakindi, I. Khan, N. A. Dangroo, N. Thota, A. Hamid, P. R. Sharma, A. K. Saxena and S. Koul, *Eur. J. Med. Chem.*, 2013, **63**, 782–792.
15 I. Khan, S. K. Guru, S. K. Rath, P. K. Chinthakindi, B. Singh, S. Koul, S. Bhushan and P. L. Sangwan, *Eur. J. Med. Chem.*, 2016, **108**, 104–116.
16 N. Doherty, *Agents Actions*, 1981, **11**, 237–242.
17 D. S. Nelson and P. Mildenhall, *Aust. J. Exp. Biol. Med. Sci.*, 1968, **46**, 33–49.
18 M. Jonsson, M. Jestoi, A. V. Nathanail, U.-M. Kokkonen, M. Anttila, P. Koivisto, P. Karhunen and K. Peltonen, *Food Chem. Toxicol.*, 2013, **53**, 27–32.
19 T. Mosmann, *J. Immunol. Methods*, 1983, **65**, 55–63.
20 K. Kobayashi, K. Kaneda and T. Kasama, *Microsc. Res. Tech.*, 2001, **53**, 241–245.
21 F. Malik, J. Singh, A. Khajuria, K. A. Suri, N. K. Satti, S. Singh, M. K. Kaul, A. Kumar, A. Bhatia and G. N. Qazi, *Life Sci.*, 2007, **80**, 1525–1538.
22 A. Gupta, A. Khajuria, J. Singh, K. L. Bedi, N. K. Satti, P. Dutt, K. A. Suri, O. P. Suri and G. N. Qazi, *Int. Immunopharmacol.*, 2006, **6**, 1543–1549.