Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 17 (2007) 1608–1612

Immunomodulatory effects of two sapogenins 1 and 2 isolated from *Luffa cylindrica* in Balb/C mice

Anamika Khajuria,[a,*] Amit Gupta,[a] Saraswati Garai[b] and Basanti Purinima Wakhloo[b]

[a]*Division of Pharmacology, Regional Research Laboratory (CSIR), Jammu, Tawi 180001, India*
[b]*Division of Natural Products Chemistry, Regional Research Laboratory (CSIR), Jammu 180001, India*

Received 26 June 2006; revised 4 December 2006; accepted 26 December 2006
Available online 4 January 2007

Abstract—Two Triterpenoids (sapogenins **1** and **2**) isolated from *Luffa cylindrica* were subjected to immunomodulatory activity in male Balb/c mice. Mice were treated with three doses of sapogenins **1** and **2** (10, 30 and 100 mg/kg) and levamisole (2.5 mg/kg) used as a standard reference drug for 15 days. Immune responses to T-dependent antigen SRBCs were observed using parameters like HA, PFC, DTH, lymphocyte proliferation and phagocytosis. As regards these parameters, sapogenins **1** and **2** elicited a significant increase in the HA, PFC and DTH response at dose 10 mg/kg ($P < 0.01$) and 100 mg/kg ($P < 0.001$), respectively. Sapogenins **1** and **2** also showed significant dose-dependent decrease and increase in lymphocyte proliferation assay and phagocytic activity of macrophages. Overall, sapogenins **1** and **2** showed dose relative immunostimulatory effect on in vivo immune functions in mice.

Triterpenoids are a diverse and ubiquitous group of C_{30} pentacyclic compounds[1] that are derived biosynthetically from squalene cyclization.[2] Triterpenoids are a large class of isoprenoidal natural products present in higher plants. They are often stored in the parts of plants beneath the soil surface as glycosides and saponins.They exhibit a wide range of both structural diversity and biological activity, and therefore are regarded as important and promising sources for medicinal compounds. There have been more than 90 different carbon skeletons reported from plant sources. Large number of plants and their isolated constituents have been shown to potentiate immune responses. Besides source of medicine, some plants are used as dietary constituents in India and other Asian countries. *Luffa cylindrica* family (cucurbitaceae) is one such plant whose seeds and fruit are used not only as food but also as an ingredient in traditional medicine. In the present communication, we report the immunomodulatory effects of two triterpenoids sapogenins **1** and **2** isolated from *L. cylindrica* as immunomodulatory agents in mice.

Luffa cylindrica (cucurbitaceae) grows throughout the tropics. It is used as a vegetable either prepared like squash or eaten raw like cucumbers.[3] The seeds contain saponins. Isolation and structure elucidation of two sapogenins **1** and **2** are reported here. The methanolic extract of the defatted powdered seeds of *L. cylindrica* was hydrolysed with aqueous MeOH–HCl under reflux for 4 h. The acid hydrolysate was separated into acidic and neutral fractions by treatment with a saturated solution of $NaHCO_3$. The acidic fraction on chromatographic separation over silica gel column yielded two sapogenins **1** and **2**. The two sapogenols were characterized by ESIMS, 1D NMR and also 2D NMR especially HSQC, HMBC and ^1H–^1H COSY techniques.

The less polar sapogenin **1** which analyzed for mf $C_{30}H_{48}O_3$; mp 308–310 °C; $[\alpha]_D$ +77 ° − +83 ° (c 0.8, $CHCl_3$) showed Positive Liebermann–Buchard test suggesting the presence of a triterpene skeleton. In the IR spectra of **1** there were absorption bands at 3400 (–OH), 2900(–CO–OH) cm^{-1} and carbonyl groups at 1705 cm^{-1}. Sapogenin **1** showed the molecular ion peak at *m/z* 455 ascribable to $[M-H]^-$ in its mass spectrum. It also showed Retro-Diels-Alder fragment typical of Δ^{12}-oleanene or ursane triterpenes. Thus, a peak at *m/z* 248 was attributed to the RDA fragment ion, involving ring D/E containing a carboxyl group. Its ^1H NMR spectrum showed signals of seven tertiary methyl groups at δ 0.91, 0.97, 1.02, 1.03, 1.04, 1.26 and 1.30. The ^1H NMR spectrum exhibited a signal integrating for ^1H at δ 5.8

* Corresponding author. Fax: +91 548607/546383; e-mail: anamikakhajuria@yahoo.com

0960-894X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2006.12.091

reminiscent of presence of 12:13 double bond, which was also supported by the HSQC spectrum. The HSQC spectrum showed cross peaks between the signals at δ 5.8 (t, $J = 4.0$ Hz) and the corresponding carbon peak at δ 122.1, δ 3.47 (dd, $J = 5.5, 10.5$ Hz) and δ 78.5. The carbon and the proton signals of **1** in the NMR spectra were assigned by extensive techniques including HSQC, HMBC and ^{1}H–^{1}H COSY (Table 1). The ^{13}C NMR spectrum showed signals of six C–C bonded saturated quaternary carbons at δ 38.9, 39.3, 38.4, 41.7, 47.6 and 30.5 and a free carboxylic acid carbon at δ 179.7. The number and chemical shifts of the tertiary methyl groups and quaternary carbons suggested that the sapogenin **1** is an oleanane-type triterpene. Thus, the structure of sapogenin **1** may be suggested as 3β-hydroxy-oleanane-12: 13-ene-28-oic-acid, oleanolic acid (**1**).

The more polar sapogenin **2** which analyzed for mf $C_{30}H_{48}O_4$; mp 300–305 °C; $[\alpha]_D$ +80° (c 1.64, $CHCl_3$) displayed in its MS the molecular ion peak at *m/z* 471 assignable to $[M-H]^-$. The difference of 16 mass units between the two sapogenins **1** and **2** indicated the presence of two hydroxyl groups in the latter. The IR spectrum revealed the presence of hydroxyl group (3400 cm^{-1}), free carboxylic acid group (1709 cm^{-1}) and double bond (1640 cm^{-1}). The ^{1}H NMR spectrum showed the signals of two hydroxy methine groups at δ 3.49 (dd, $J = 5.3$, 10.6 Hz) and 5.28 (br s) and ^{13}C NMR showed the signals at δ 77.7 and 74.3.

The ^{1}H NMR spectrum of sapogenin **2** showed the presence of one trisubstituted double bond. The ^{13}C NMR and DEPT data (Table 2) suggested the presence of C 12:13 double bond, two secondary hydroxyl groups, an acid carbonyl function, seven methyls, nine methylenes, three methines and six quaternary carbons. Thus, the structure of sapogenin **2** was defined as 3β,16α-dihydroxy-oleanane-12: 13-ene-28-oic-acid, echinocystic acid (**2**).

Table 1. ^{1}H NMR chemical shifts of oleanolic acid (**1**) and echinocystic acid (**2**)) (in C_5D_5N)

	1	**2**
1	α 1.54 (ddd, $J = 2.4, 12.3, 12.3$ Hz) β 1.72 (overlapped)	α 0.93 (m) β 1.60 (m)
2	α 1.80 (m) β 2.13 (ddd, $J = 3.3, 3.4, 5.5, 1.34$ Hz)	α 1.78 (ddd, $J = 2.2, 2.3, 1.45$ Hz) β 2.31 (dddd, $J = 2.3, 4.9, 5.3, 13.5$ Hz)
3	3.47 (dd, $J = 5.5, 10.5$ Hz)	3.49 (dd, $J = 5.3, 10.6$ Hz)
4	—	—
5	0.87 (dd, $J = 3.1, 11.5$ Hz)	0.91 (m)
6	α 1.39 (ddd, $J = 3.0, 2.8, 12.2$ Hz) β 1.60 (ddd, $J = 2.4, 9.3, 12.7$ Hz)	1.67 (m)
7	α 1.57 (ddd, $J = 2.4, 6.0, 6.0$ Hz) β 1.87 (m)	α 1.44 (m) β 1.67 (m)
8	—	—
9	1.70 (overlapped)	1.78 (dd, $J = 2.3, 1.45$ Hz)
10	—	—
11	α 1.98 (dd, $J = 3.0, 7.6$ Hz) β 2.21 (dd, $J = 3.1, 12.8$ Hz)	2.05 (dd, $J = 3.4, 8.5$ Hz)
12	5.8 (d, $J = 4.0$ Hz)	5.7 (d, $J = 3.9$ Hz)
13	—	—
14	—	—
15	α 2.23 (ddd, $J = 2.5, 3.7, 13.3$ Hz) β1.55 (m)	α 1.78 (dd, $J = 2.3, 14.5$ Hz) β 2.47 (m)
16	α 2.16 (ddd, $J = 2.5, 2.7, 12.5$ Hz) β 1.96 (ddd, $J = 2.5, 12.5, 13.2$ Hz)	5.28 (br s)
17	—	—
18	3.33 (dd, $J = 2.8, 10.2$ Hz)	3.67 (dd, $J = 3.9, 14.2$ Hz)
19	α 1.31 (dd, $J = 2.2, 6.3$ Hz) β 1.86 (m)	α 1.44 (m) β 2.99 (overlapped)
20	—	—
21	α 1.71 (overlapped) β 0.89 (ddd, $J = 3.1, 11.5, 11.5$ Hz)	α 1.38 (ddd, $J = 3.2, 12.7, 13.5$ Hz) β 2.57 (ddd, $J = 4.5, 4.6, 12.7$ Hz)
22	α 1.97 (ddd, $J = 3.1, 7.6, 7.6$ Hz) β 1.85 (m)	α 2.31 (ddd, $J = 4.9, 5.0, 13.5$ Hz) β 2.47 (m)
23	1.26 (s)	1.24 (s)
24	0.91 (s)	1.08 (s)
25	1.03 (s)	0.95 (s)
26	1.04 (s)	1.04 (s)
27	1.30 (s)	1.86 (s)
28	—	—
29	0.97 (s)	1.07 (s)
30	1.02 (s)	1.20 (s)

Assignments aided by ^{1}H–^{1}H COSY, HSQC and HMBC experiments.

Table 2. Chemical shifts [δc (±0.1)] of oleanolic acid (**1**) and echinocystic acid (**2**) (in C_5D_5N)

Carbon no.	(**1**)	(**2**)
1	38.9	38.6
2	27.6	27.7
3	77.6	77.7
4	38.9	39.1
5	55.3	55.5
6	18.3	18.5
7	32.8	33.6
8	39.3	39.6
9	49.2	47.1
10	38.4	37.0
11	23.2	23.4
12	122.1	122.1
13	144.3	144.7
14	41.7	41.7
15	27.8	35.7
16	23.3	74.3
17	47.6	48.5
18	41.3	41.1
19	46.2	47.1
20	30.5	30.6
21	36.9	36.8
22	32.7	32.9
23	28.3	28.3
24	16.0	16.2
25	15.0	15.3
26	16.9	17.1
27	25.7	26.9
28	179.7	179.5
29	33.7	33.5
30	23.3	24.4

We investigated the effect of two sapogenins **1** and **2** on antibody production.[4] As shown in Figure 1 treatments with two sapogenins **1** and **2** enhanced humoral responses to SRBC with dose-dependent decrease and increase in antibody production. The potentiating effect of sapogenin **1** was maximum at 10 mg/kg and further decreased at higher dose of 100 mg/kg. The sapogenin **2** showed moderate effect at dose 100 mg/kg. This was reflected in decrease and increase in HA after sapogenins **1** and **2** treatment observed on day 7 and 15 following antigen priming. The mode of antibody production was compared with animals treated with reference drug levamisole which showed significant increases in antibody production at $P < 0.05$.

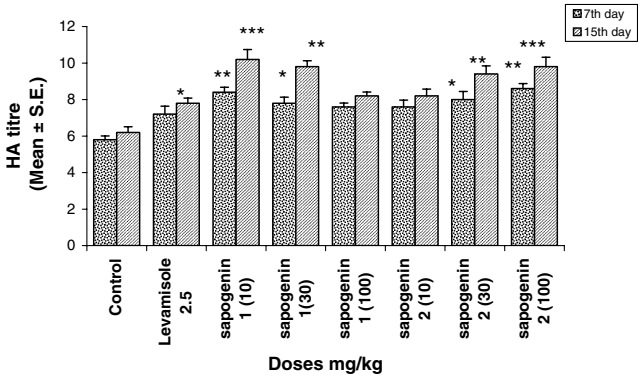

Figure 1. Effect of sapogenins **1** and **2** on SRBC-induced humoral response in mice. Data are expressed as means ± SE of five observations. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ as compared to control determined by one-way Anova (Bonferroni correction multiple comparison test).

The immunostimulatory effect of sapogenins **1** and **2** in vivo was investigated using T-dependent antigen sensitization and specific IgM antibody production,[5] which require the participation of antibody secreting B cells and accessory cells such as macrophages. The number of PFCs/10^6 cells 7 days after immunization with SRBC antigen is shown in Figure 2. Moreover, the administration of sapogenins **1** and **2** at 10, 30 and 100 mg/kg body weight per oral decreased and increased the number of PFCs in comparison to control. The maximum number of antibody secreting B cells was observed at 10 and 100 mg/kg in sapogenins **1** and **2**. The augmentation of

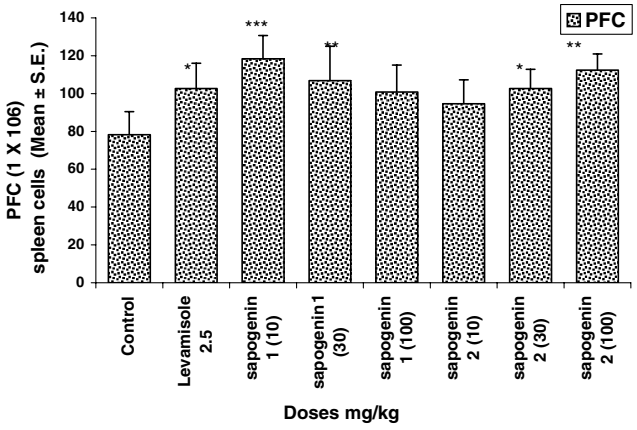

Figure 2. Effect of sapogenins **1** and **2** on SRBC-induced plaque forming assay in mice. Data are expressed as means ± SE of five observations. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ as compared to control determined by one-way Anova (Bonferroni correction multiple comparison test).

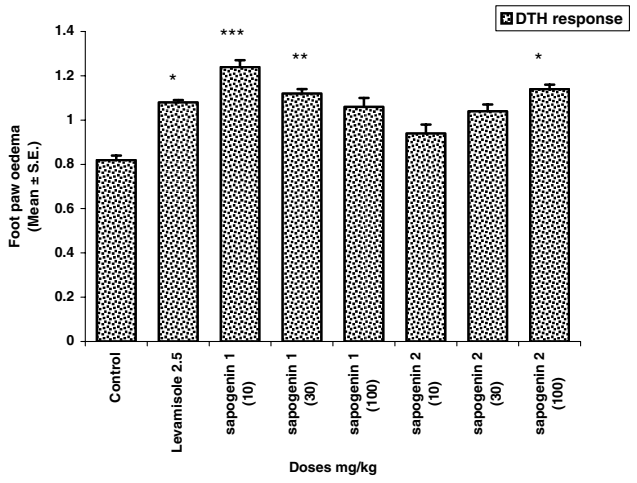

Figure 3. Effect of sapogenins **1** and **2** on SRBC-induced DTH response in mice. Data are expressed as means ± SE of five observations. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ as compared to control determined by one-way Anova (Bonferroni correction multiple comparison test).

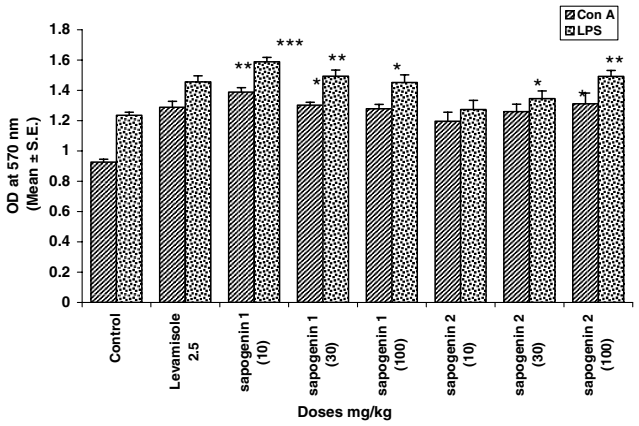

Figure 4. Effect of sapogenins **1** and **2** on lymphocyte proliferation assay. Data are expressed as means ± SE of five observations. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ as compared to control determined by one-way Anova (Bonferroni correction multiple comparison test).

the humoral response to SRBC by sapogenins **1** and **2**, as evidenced by decrease and increase in the number of HA and PFC in mice, also indicated the enhanced responsiveness of macrophages and T- and B-lymphocyte subsets, involved in antibody synthesis[6] in view of the pivotal role played by macrophages in coordinating the presentation of antigen to T-cell and B-cells. The stimulatory effect of sapogenins **1** and **2** at 10, 30 and 100 mg/kg body weight per oral decreased and increased the DTH[7] response in comparison to control as shown in Figure 3. A DTH reaction is an expression of cell-mediated immunity and plays a role in many inflammatory disorders.

Lymphocyte proliferation[8] was observed by the reduction of MTT. The results of in vivo studies of splenic lymphocyte transformation to Con A and LPS are shown in Figure 4 and showed that proliferative response reaches maximum stimulation at 10 and 100 mg/kg dose in sapogenins **1** and **2**. This reflected a dose dependent decrease and increase in proliferative response. Sapogenins **1** and **2** were found to enhance the blastogenic responsiveness of murine splenocytes to Con A and LPS. The significant effect was observed in sapogenin **1** at 10 mg/kg while sapogenin **2** at 100 mg/kg.

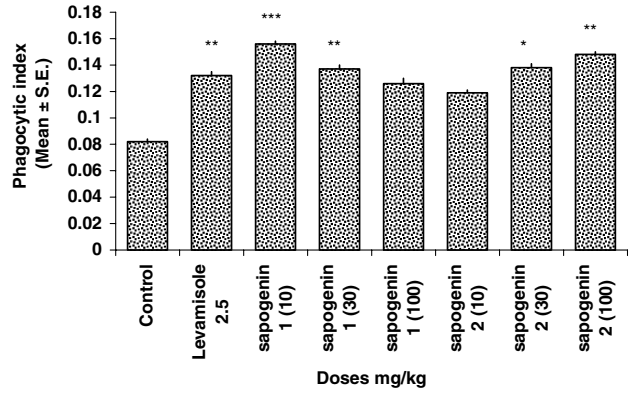

Figure 5. Effect of sapogenins **1** and **2** on macrophage phagocytosis. Data are expressed as means ± SE of five observations. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ as compared to control determined by one-way Anova (Bonferroni correction multiple comparison test).

The effects of sapogenins **1** and **2** on macrophage functions[9] are presented in Figure 5. Sapogenins **1** and **2** at a dose of 10 and 100 mg/kg body weight showed significant increase in phagocytic index ($P < 0.01$) compared with control animals. Sapogenins **1** and **2** showed stimulatory effects on macrophages. Phagocytosis and killing of invading microorganisms by macrophages constitute body's primary line of defence against infections.[10] Macrophages are an integral part of the immune system, acting as phagocytic, microbicidal and tumoricidal effector cells. Through interaction with lymphocytes, macrophages play an important role in the initiation and regulation of immune response.[11]

Sapogenins **1** and **2** were investigated for immunomodulatory activity using parameters like HA, PFC, DTH, lymphocyte proliferation assay and phagocytosis. The results revealed that sapogenins **1** and **2** had obvious immunomodulatory potential. Taken together, the results of this study indicate sapogenins **1** and **2** have immunostimulating properties; increasing both humoral and cell-mediated immune responses.

The biological relevance of these changes is under investigation, using a series of host resistance studies. These results provide more evidence in support of using sapogenins **1** and **2** as immunomodulating drug. We have described the immunomodulatory activity of the compounds sapogenins **1** and **2** isolated from *L. cylindrica* and demonstrated that sapogenins **1** and **2** showed immunomodulatory profiles.

In conclusion, the result obtained in the present study has shown the dose-dependent immunostimulatory effect within normal range for fine tuning of immune system and their therapeutic potential studies on mechanism responsible for immunomodulation are under progress.

Acknowledgment

The authors thank Dr. Surjeet Singh for interpreting the data.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.12.091.

References and notes

1. Connolly, J. D.; Hill, R. A. *Nat. Prod. Rep.* **2000**, *19*, 494, and previous reviews in this series.
2. Prestwich, G. D.; Abe, I.; Zheng, Y. F.; Robustell, B. J.; Tongyun, D. *Pure Appl. Chem.* **1999**, *71*, 1127.

3. Sastri, B. N. Wealth of India: A Dictionary of Indian Raw Materials and Industrial Products. *CSIR Research* **1962**, *6*, 179.
4. On the fifth day after immunization with antigen sheep red blood cell (SRBC), blood was collected from orbital plexus of each mouse for serum preparation. Serial twofold dilution of serum was made in 50 μl PBS (pH 7.2) in 96-well microtitre plates and mixed with 50 μl of 1% SRBC suspension in PBS. After mixing, plates were kept at room temperature for 2 h. The value of antibody titre was assigned to the highest serum dilution showing visible haemagglutination.
5. The animals were immunized on the fifth day of immunization with SRBC. The spleen was removed, cleaned free of extraneous tissues, and a single cell suspension of 10^6 cells/ml was prepared from it in RPMI-1640 medium. For PFC assay, the SRBCs were prepared at a cell density of 5×10^8 cells/ml in PBS. One millilitre of SRBC in medium along with 0.5 ml of diluted guinea pig complement (1:10 diluted with normal saline) was added to 1 ml of spleen cell suspension. Cunningham chambers were prepared using glass slide, coverslips and double-sided tape. The chambers were sealed with xylene and incubated at 37 °C for 1 h. The plaques were counted under a light microscope (Olympus BX50) and expressed as PFC per 10^6 spleen cells.
6. Benacerraf, B. *J. Immunol.* **1978**, *120*, 1809.
7. Mice each were immunized by injecting 20 μl of 5×10^9 SRBC subcutaneously (sc) into the right hind footpad. The day of sensitization was designated as day 0. Seven days later the animals were challenged by injecting the same amount of SRBC intradermally (id) into the left hind footpad. The thickness of the left hind footpad was measured at 0 and 24 h after the challenge. The difference between two values was taken as the measure of DTH and mean percent change in foot thickness as compared to control group was determined.
8. Splenic lymphocyte proliferative responses to the T-cell mitogens, concanavalin A (Con A), as well as to B cell mitogen, lipopolysaccharide (LPS, *Escherichia coli*), were determined. A single cell suspension of spleens from immunized mice was prepared under sterile conditions in 5–10 ml RPMI-1640 medium. After initial wash, the contaminating RBCs were removed by suspending the cell pellet in distilled water for 20 s followed by an equal volume of $2 \times$ RPMI-1640 medium. Cells were then washed again at 1000 rpm for 10 min at 4 °C and suspended in RPMI-1640 medium containing 5% foetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin and 100 mM/l sodium pyruvate (hereafter referred to as complete medium). The cell viability was determined by Trypan exclusion test and final concentration of viable cells adjusted to 2×10^6 cells/ml. One hundred microliters (100 μl) of this cell suspension of treated mouses sapogenins **1** and **2** (10, 30 and 100 mg/kg) was pipetted in triplicate into each well of a 96-well flat-bottomed titration plates and added 50 μl of Con A (2.5 μg/ml) to stimulate T-cell mitogenesis or LPS (10 μg/ml) to stimulate B cell mitogenesis. The plates were incubated at 37 °C in 95% humidity at 5% CO_2 in a CO_2 incubator for 4–7 days. The proliferation was calculated based on MTT assay. MTT in PBS (10 μl of a 5 mg/ml solution) was added to 100 μl of cultures 4–6 h before measurement. Plates were centrifuged for 5 min at 200*g* and supernatant removed. One hundred microliters (100 μl) of DMSO was added to each well and the plates placed on plate shaker for 15 min to dissolve the crystals. Absorbance was recorded at 570 nm.
9. Sapogenins **1** and **2** were administered 15 days prior to injection of carbon particles. On day 16, mice were injected with 0.1 ml of carbon suspension, iv through the tail vein. Blood samples (25 μl) were collected from the orbital plexus of individual animals immediately before and at 3, 6, 9 and 12 min after the injection of carbon suspension, lysed with 2 ml of 0.1% glacial acetic acid[8] and the absorbance was measured spectrophotometrically at 675 nm. The rate of carbon clearance, termed as phagocytic index, was calculated as the slope of time–concentration curve.
10. Atal, C. K.; Sharma, M. L. *J. Ethnopharmacol.* **1986**, *18*, 133.
11. VanFurt, R. *Immunobiology* **1982**, *161*, 178.