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Immuno-stimulating properties of diosgenyl saponins isolated from *Paris polyphylla*

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Abstract—The effects of three diosgenyl saponins isolated from *Paris polyphylla* on the immuno-stimulating activity in relation to phagocytosis, respiratory burst, and nitric oxide production in mouse macrophage cells RAW 264.7 have been investigated. Our results showed that all three diosgenyl saponins significantly enhanced phagocytic activity that increased with the concentration of saponins to reach a maximum, and then tended to decrease with higher concentrations. Saponins with sugar moiety directly induced respiratory burst response in RAW 264.7 cells that increased with the concentrations and reached a maximum, then decreased with higher concentrations after 2-h incubations, however, diosgenin had no PMA-triggered respiratory burst response. Treatment of RAW 264.7 cells with saponins with sugar moiety for 24-h caused a significant increase in the production of nitric oxide, while diosgenin had no effect at all. Consequently, relationship between molecular structures of three diosgenyl saponins and their immunomodulatory activities was discussed, and a possible mechanism of immuno-stimulating function of diosgenyl saponins was accordingly explored.

Macrophages play a significant role in the regulation of immunological reactions through various functions including phagocytic elimination of foreign or denatured substances and secretion of cytokines and reactive oxygen species (ROS).[1] Stimulation of macrophages leads to phagocytosis, respiratory burst, and nitric oxide (NO) production. Macrophage phagocytosis is an essential cell defense mechanism against foreign, non-self materials and has been used as an important non-specific immunological parameter to evaluate the immune function.[2] It is generally accepted that phagocytes are able to generate superoxide anion and its reactive derivations during a period of intense oxygen consumption called the respiratory burst.[3] Respiratory burst that plays an important role in microbicidal activity can be considered as functional tests for the evaluation of immune potential at a cellular and organismal level.[4] In macrophages, nitric oxide (NO) is synthesized from L-arginine in the presence of nitric oxide synthase (iNOS), which is involved in various biological processes including inflammation and immunoregulation.[5]

Plant of *Paris polyphylla* as a famous folk medicinal herb in south of China has been used not only as an anti-cancer, anti-biotic, and anti-inflammatory drug but also to treat snake bite, parotitis, mastitis, chronic bronchitis, injuries from fractures as well as to stop bleeding.[6] Diosgenyl saponins are one of the most abundant steroid saponins, with diosgenin as the steroidal sapogenin, and exert a large variety of biological functions, such as anti-fungal, anti-bacterial, and anti-cancer.[7] In the present study, three diosgenyl saponins (exhibited in Fig. 1), diosgenin 3-*O*-α-L-rhamnopyranosyl(1 → 4)-α-L-rhamnopyranosyl (1 → 4)-[α-L-rhamnopyranosyl(1 → 2)-β-D-glucopyranoside (**1**), diosgenin 3-*O*-α-L-rhamnopyranosyl(1 → 2)-α-L-arabino furanosyl-β-D-glucopyranoside (**2**) and diosgenin (**3**), were isolated from the rhizomes of *Paris polyphylla*. The

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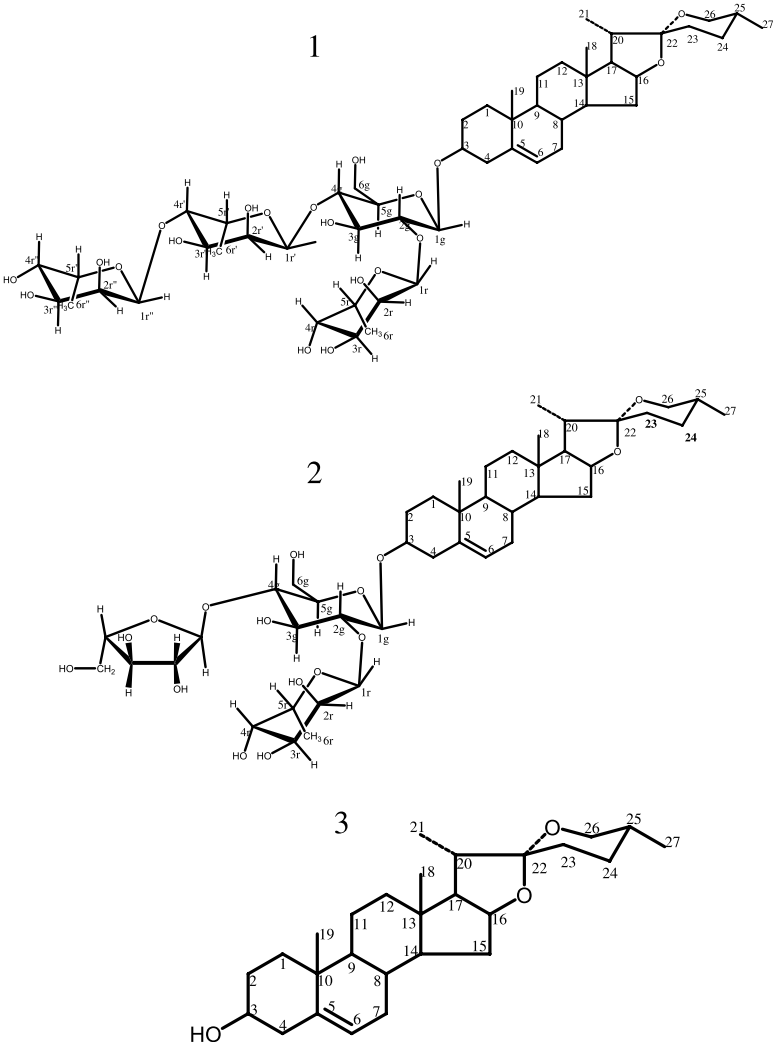

Figure 1. Molecular structures of three diosgenyl saponins.

most indispensable non-specific parameters of immunomodulatory activity about phagocytosis, respiratory burst, and NO production of the three diosgenyl saponins, and the effects of the glucoside moiety of diosgenyl saponins on their immunomodulatory activities have not been reported to date, although **1** and **2** showed immunomodulatory effects on proliferative response of mouse lymphocytes to concanavalin-A and augmentation of mouse granulocyte/macrophage colony forming cells in mouse fibroblast cell L929.[9] We investigated the effects of these three saponins on RAW 264.7 cells' immuno-stimulating activities in relation to phagocytosis, respiratory burst, and NO production. Consequently, relationship between molecular structures of three diosgenyl saponins and their immunomodulatory activities was discussed, and a possible mechanism of immuno-stimulating function of diosgenyl saponins was accordingly explored.

Air-dried tubers of *Paris polyphylla* (3.5 kg) were extracted with 95% ethanol 3 times under reflux for 3 h. After evaporation of ethanol in vacuo, the residue (720 g) was suspended in water and then extracted successively with petroleum ether, chloroform, and *n*-BuOH. The *n*-BuOH fraction (480 g) was subjected to pass over D-101 highly porous polymer, using an EtOH–H_2O gradient system (0–100%). The fraction (220 g) eluted by 70% EtOH was subjected to silica gel column chromatography with a $CHCl_3$–MeOH–H_2O solvent system. Finally, it gives three fractions. The second fraction (150 g) was further chromatographed on Sephadex LH-20 and RP-18 silica gel yielding saponins **1** (25 g) and **2** (32 g). Fractionation of the $CHCl_3$ fraction (32 g) using chromatography (silica gel) eluting with *n*-hexane–EtOAc of increasing polarity afforded compound **3** (2.3 g). Their structures were identified by comparison of ESI-MS and NMR data with those in the previous reports.[8]

One of the most distinguished features of macrophage activation would be an increase in phagocytic activity. Effects of three steroid saponins on phagocytic activity were examined after culturing RAW264.7cells with desired compounds for 24 h. Thus in the experiment, cultured cells were allowed to phagocytize neutral red dye for 30 min, washed, and then the amount of phagocy-

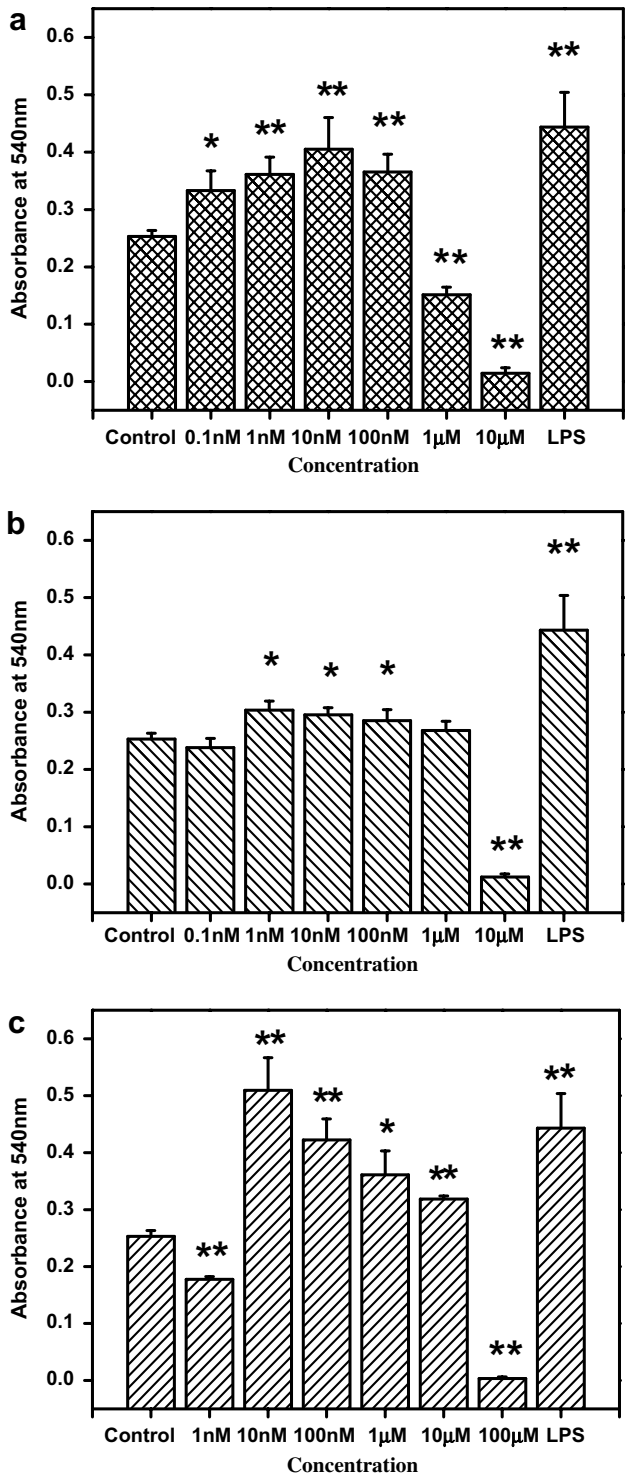

Figure 2. Augmentation of phagocytotic function by three diosgenyl saponins in RAW 264.7 cells. Data are represented as means ± SD of triplicate samples. Significant differences with control group (macrophages incubated in the absence of compounds) are designated as $^{*}P < 0.05$, $^{**}P < 0.01$. This experiment is representative of three-independent experiments.

tized dye was analyzed by spectrophotometry at 540 nm. As shown in Figure 2, all the diosgenyl saponins significantly enhanced phagocytic activity that increased with the concentration of saponins to reach a maximum, and then tended to decrease with higher saponin concentrations. In this case, more dye is phagocytized in **3** treated cells than in **1** and **2** treated cells at the concentration which induced the maximum absorbance.

Superoxide anion production in diosgenyl saponin-stimulated RAW 264.7 cells and PMA-triggered macrophages was measured spectrophotometrically using the NBT test. After 2-h incubation, **1** and **2** directly induced respiratory burst response in RAW 264.7 cells that increased with the concentrations and reached a maximum at concentration 10 nM, then decreased with higher concentrations. The NBT reduction induced by **1**, at a concentration of 10 nM, was significantly higher ($P < 0.01$) than the reduction induced by **2**. However, there was almost no obvious increase in NBT reduction induced by **3**, which indicates that the superoxide generation simulated by **3** did not occur (shown in Fig. 3).

When cells were preincubated with three saponins for 2, 6 or 12 h, respectively, then triggered by PMA, the respiratory burst was similar to or even lower than that of control cells without the saponin added. There were no significant differences in the NBT responses to any of the groups of saponins (data not shown).

To investigate the effects of three diosgenyl saponins on NO production, we measured the accumulation of nitrite, a stable end product of NO, in the culture media using Griess reagents. As shown in Figure 4, treatment of the cells with **1** and **2** caused a significant increase in the production of NO, while **3** had no effect on NO production in RAW 264.7 cell at all. The NO production had increased dose-dependently for **1** and **2** at concentrations of 0.1–100 nM, and reached the maximum response at 100 nM, then the response tended to decrease with higher concentrations.

The present study demonstrated that three diosgenyl saponins isolated from *Paris polyphylla*, a plant that has been used in cancer treatment by folk people in China, exerted immuno-stimulating activities on mouse macrophage RAW 264.7 cells. Although all of three diosgenyl saponins significantly enhanced phagocytic activity, but only **1** and **2** directly induced respiratory burst response and increased NO production in RAW 264.7 cells, while **3** had no effect on respiratory burst and NO production.

Phagocytosis represents the final and most indispensable step of the immunological defense system,[10] since phagocytes act as regulatory and effecter cells in the immune system, so the enhancement of phagocyte function is expected to be applicable for therapy of microbial infections and cancer.[11] The capacity of RAW264.7 cells for uptaking neutral red is comparatively illustrated in Figure 2. It is clearly indicated that the phagocytotic function of RAW 264.7 was significantly increased by all of three diosgenyl saponins. However, the phagocytic activity is inhibited by **1**, **2** at 10 μM, which can be explained as the cytotoxic activity of saponins at higher concentrations (cell death was observed under light microscope). There are a few reports showing that **2**

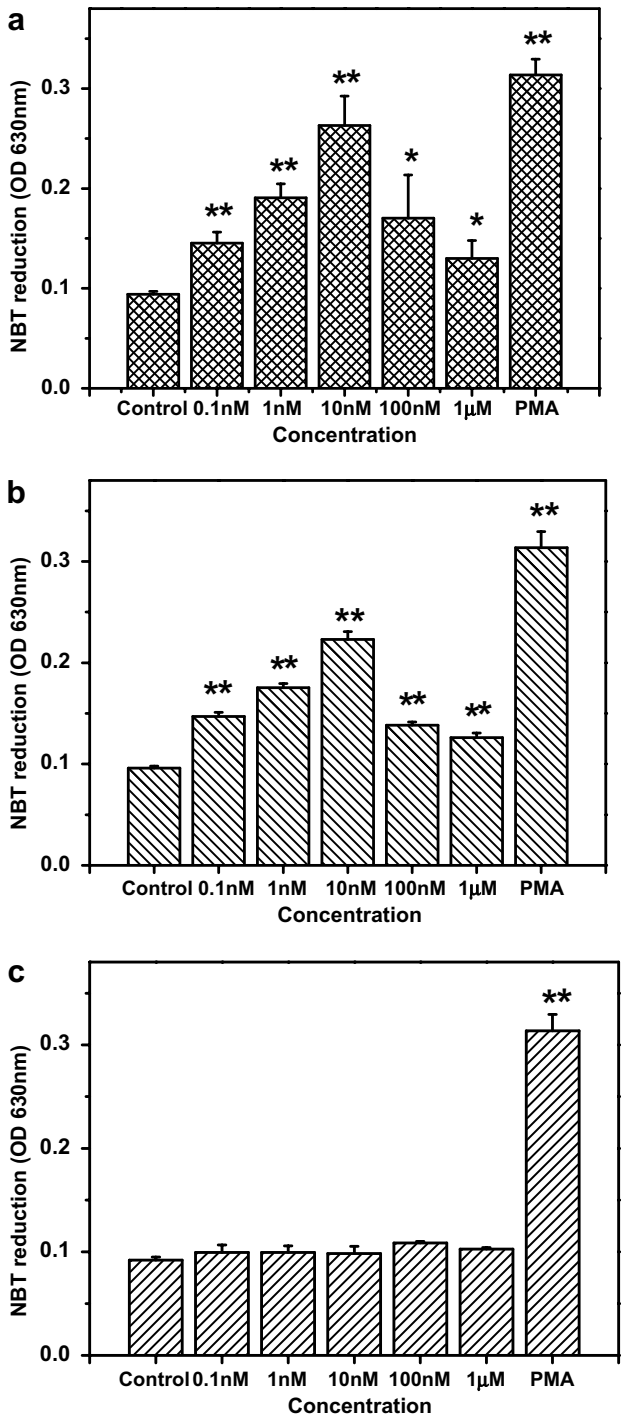

Figure 3. Effects of three steroid saponins on the respiratory burst activity of RAW 264.7 (preincubated with saponins and NBT for 2 h). Data are represented as means ± SD of triplicate samples. Significant differences with control group (macrophages incubated in the absence of compounds) are designated as $^{*}P < 0.05$, $^{**}P < 0.01$. This experiment is representative of three-independent experiments.

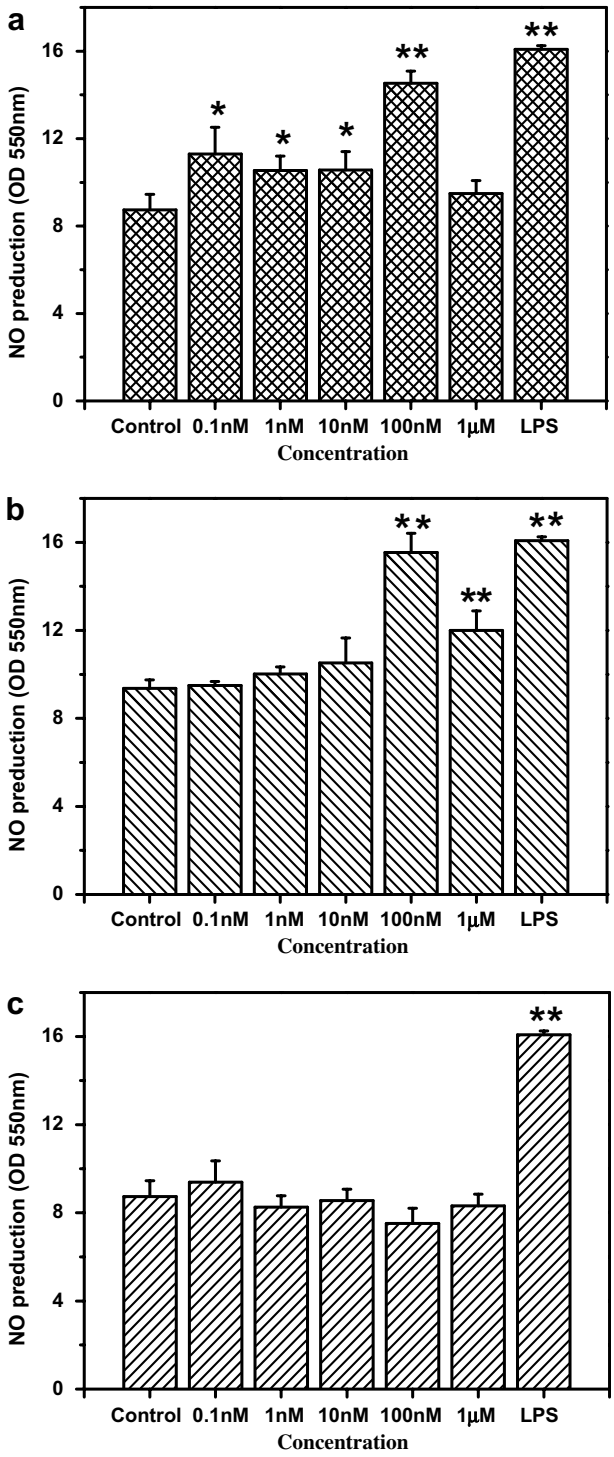

Figure 4. Effects of three steroid saponins on the nitric oxide production of RAW 264.7 cells. Supernatants were collected after 24-h incubation. Nitrite was measured using Griess reagent. Data are represented as means ± SD of triplicate samples. Significant differences with control group (macrophages incubated in the absence of compounds) are designated as $^{*}P < 0.05$, $^{**}P < 0.01$. This experiment is representative of three-independent experiments.

induced cytotoxic effects in HepG2 (IC_{50} = 7 μM), R-HepG2 (IC_{50} = 5 μM),[12] and HL-60 cells (IC_{50} = 3.3 μg/ml).[13] Although there was no direct report on the cytotoxic effect of **1**, a recent study demonstrated that diosgenin 3-*O*-α-L-rhamnopyranosyl (1 → 2)-α-L-rhamnopyranosyl (1 → 4)-β-D-glucopyranoside (dioscin), which can be considered an analogue of **1**, inhibited growth of human leukemia cell HL-60 (IC_{50} = 7.5 μM), human cervical cancer line HeLa (IC_{50} = 4.5 μM), human mammary cancer cell line

MDA-MB-435 (IC_{50} = 2.6 μM), and human lung cancer line H14 (IC_{50} = 0.8 μM) in a dose-dependent manner.[14] **1** can be considered to attach an α-L-rhamnopyranosyl to C-4 of the rhamnopyranosyl moiety of dioscin, and showed similar influence on the cytotoxic activity to **2** in our experiment that phagocytic activity is inhibited similarly **1** and **2** incubated at 10 μM. This result agrees with those findings in which diosgenin β-D-glucoside showed no cytotoxic activity against HL-60 cells, and the attachment of an α-L-rhamnopyranosyl group at C-2 of the glucosyl moiety led to the appearance of considerable activity, further addition of an α-L-rhamnopyranosyl, an α-L-arabinofuranosyl or a β-D-glucosyl to C-4 of the inner glucosyl moiety either gave no influence on the activity or slightly increased the activity.[13] As shown in Figures 2 and 3 at 100 μM inhibited the phagocytosis and enhanced phagocytotic function of RAW 264.7 at 10 μM–1 nM, which is in relationship to the cytotoxity. The result was consistent with reported finding about **3** showing no cytotoxity against the 1547 human osteosarcoma cell line at 40 μM by using LDH test.[15]

Macrophages generate large quantities of ROS, including superoxide anion and nitric oxide, in response to a variety of membrane stimulants, by a coordinated sequence of biochemical reactions known as the oxidative respiratory burst. In the beginning of this process, the membrane-bound enzyme complex, a multi-component NADPH oxidase, assembles after binding of the cell to a foreign particle and reduces molecular oxygen to the superoxide anion, subsequently leading to the production of numerous other reactive compounds.[16] The superoxide anion is the first product released from the respiratory burst, and plays an important role in microbicidal activity,[4] and therefore can be considered as functional tests for the evaluation of immune potential at a cellular and organismal level.

Because the cytotoxic activities of the saponins appeared at higher concentration, we chose lower concentration at 0.1 nM–1 μM for **1** and **2**, and 1 nM–10 μM for **3** to study unaffected the effects of three diosgenyl saponins on superoxide anion and NO production of RAW 264.7 cells. Two sets of experiments, direct stimulatory and priming effects, were carried out to evaluate the effect of three diosgenyl saponins on the respiratory burst of RAW 264.7 cells. In the present study, we showed that **1** and **2** are very good stimulators of the respiratory burst of RAW 264.7, but **3** had no effect on it. Main difference in the structure among three diosgenyl saponins is whether there is a sugar group bonded, that is to say, the sugar moiety is a key functional group that has diverse pharmacological activity. Polysaccharides are known immune stimulants of which glucans have recently received considerable attention. Glucans or other biological response modifiers, because of their structural identity with the conserved 'pathogen-associated molecular patten,' activate the immune system by binding to specific receptors (patten recognition receptors) of the innate immune system and stimulate the phagocytic, cytotoxic, and anti-microbial activities by the synthesis and release of cytokines, chemokines, and reactive oxygen and nitrogen intermediates.[17] Perhaps the possible mechanism of **1** and **2** stimulates macrophages to produce respiratory burst through the induction of NADPH oxidase expression.

On the other hand, our results also indicated that, when PMA was added, cells that were pre-incubated with three diosgenyl saponins showed a respiratory burst which was similar to the control. However, the respiratory burst of RAW 264.7 treated with **1** or **2** increased with the concentrations of saponins. The present result agrees with those findings in which in the absence of PMA, the respiratory burst of the fish phagocytes preincubated with the glucans for several hours increased with the concentration of glucans, however, when PMA was added, cells which were preincubated with high doses of glucans showed a respiratory burst that was similar to or lower than the control.[18] Similarly, our explanation could be that cells pre-incubated with **1** or **2** for several hours become exhausted and cannot respond to PMA stimulation as control cells do.

NO is involved in various biological processes including inflammation and immunoregulation.[19] We demonstrated that treatment with **1** and **2** significantly induced NO production in the mouse macrophage line RAW 264.7, but **3** did not. Hongxiang Sun[20] had reported that the number, the length, and position of sugar side chains, and various types of glucosyl group in the structure of protopanaxatriol-type saponins could not only affect their haemolytic activities and adjuvant potentials, but also have significant effects on the nature of the immune responses. Since many reports[22] have demonstrated that polysaccharide-stimulated NO production of the murine macrophage results from the expression of an induction NO synthase, which catalyzes the production of large amounts of NO from L-arginine and molecular oxygen,[22] perhaps the possible mechanism of **1** and **2** stimulates macrophages to produce NO through the induction of iNOS gene expression. Since NO is related to cytolytic function of macrophages against a variety of tumors,[24] the increased synthesis of NO might interfere with the growth of tumors. This was further evidenced by the experiments using the inhibitors of NO production.[25]

Reactive oxygen and nitrogen species produced by macrophages are considered important in pathogen killing, particularly simultaneous generation of both nitric oxide (NO) and superoxide $O_2^{\cdot -}$ can lead to the formation of peroxynitrite ($ONOO^-$), which is a powerful anti-microbial and anti-cancerous agent.[25] Generally speaking, macrophages produce and release reactive oxygen species and nitric oxide in response to phagocytosis or stimulation with various agents.[26] In our experiments, **1** and **2** exhibited significant enhancement of phagocytosis, respiratory burst, and NO production in RAW 264.7 cells, but **3** only showed great augmentation of phagocytotic function. The result agrees with the finding in which Astragalus radix elevated phagocytosis activity of phagocytic cells in tilapia, but had no effect on respiratory burst activities.[27] The similar tendency in the respiratory burst and nitric oxide production was

observed in our experiments. Above all, it could be concluded that the presence of glucoside moieties of diosgenyl saponins is essential for the activation of immunological reactions, especially during the period of oxygen consumption such as in the process including inflammation and microbicidal activity, although diosgenin (**3**) could only stimulate the macrophage phagocytiosis including elimination of foreign or denatured substances.

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